Université de Montréal

Monocyte and T cell plasticity in Crohn's disease and ulcerative colitis

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Thèse présentée en vue de l'obtention du grade de Doctorat en Philosophie

en Microbiologie et Immunologie

Septembre 2019

Université de Montréal

Unité académique : Département de microbiologie, infectiologie et immunologie, Faculté de Médecine

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Cette thèse intitulée

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"Je suis de ceux qui pensent que la science est d'une grande beauté. Un scientifique dans son laboratoire est non seulement un technicien : il est aussi un enfant placé devant des phénomènes naturels qui l'impressionnent comme des contes de fées." -Marie Curie

Abstract

Crohn's disease (CD) and ulcerative colitis (UC), the two forms of inflammatory bowel diseases (IBD), are associated with dysregulated immune response in the intestinal tissue. It is mediated by mononuclear phagocytes (MNPs) that dialogue via the cytokine they produce with innate and adaptive immune cells. In mice, MNPs are stratified into conventional dendritic cells (DCs), macrophages (Mϕ) and monocyte-derived cells that regroup tissue monocyte-derived DCs, monocyte-derived Mϕ and monocytes-like cells. However, the phenotypic, molecular and functional diversity of MNPs and their plasticity remain to be elucidated in IBD patients. Therapies in IBD employ antibodies that block IL12 and IL23, thus control Th17 pathogenicity and plasticity and decrease intestinal inflammation. However, no cure exist nowadays for the treatment of IBD. In-depth study of T cell plasticity and the tissue where it occurs remain to be investigated.

In the first chapter, we revealed the existence of two distinct $CD14^+$ MNP subsets in colon of UC patients. Only, $CD163^-CD64^+$ inflammatory monocyte-like cells (P3) but not anti-inflammatory $CD163^+CD64^+$ Mϕ (P4) accumulate in inflamed UC colon. Our findings further established a link between monocyte-like $CD14^{hi}CD172\alpha^+$ $CD163^-$ MNPs, IL12, IL1β and the detection of colonic memory Th17 cells that produce IFNγ and IL8, which might all contribute to UC pathogenesis. Two $CD14^+$ MNP subsets, resembling their counterparts in UC mucosa at the functional and molecular level, were also detected in CD colon.

In contrast, in the second chapter, we provide evidence that $Slan^+$ monocyte subset may contribute to CD but not UC immunopathogenesis. Frequency, phenotype, and function of $Slan^+$ cells were examined in blood, colon, and mesenteric lymph nodes (MLN) of patients with IBD. We showed that pro-inflammatory $CD14^{hi}CD172\alpha^+Slan^+$ cells are a distinguishing feature between CD and UC, as they only accumulate in MLNs and colonic mucosa of CD patients.

In the third chapter, we showed that MLNs of CD and UC, tissues that were hard to access for research use, can also be distinguished by frequencies of $CXCR3^-CCR6^+$ Th17 effector memory T cells (TEM) and their molecular profile. Our data further suggested that Th17 plasticity is

taking place in MLN, before T cell homing to gut tissues. This investigation has clear implications in furthering our understanding of the disease.

Finally, it has been demonstrated that monocytes are continuously recruited into murine gut mucosa and progressively differentiate into macrophages under homeostatic conditions, a maturation process interrupted in the context of inflammation. However, the environmental cues that regulate tissue inflammatory monocyte "waterfall" remain to be investigated in humans. In the fourth chapter we recapitulated *in vitro* human monocyte differentiation cascade, from $CD163^-$ inflammatory monocyte-like cells (P3) towards anti-inflammatory $CD163^+$ macrophages (P4) and showed their molecular similarities to tissue $CD14^+$ MNPs. Manipulating this pathway might open therapeutic avenues to restore tissue homeostasis.

In conclusion, a better understanding of MNP subsets, function and plasticity in IBD pathogenesis would help identify novel therapeutic targets and shed light for the development of personalized treatments.

Key words: Macrophages, Monocytes, Th17 cells, Plasticity, Slan cells, Crohn's disease, Ulcerative colitis, Culture, Pathogenicity.

Résumé

La maladie de Crohn (Crohn's disease; CD) et la colite ulcéreuse (Ulcerative colitis ;CU) représentent deux formes distinctes de maladies inflammatoires chroniques de l'intestin (MICI), qui sont associées à une réponse immunitaire aberrante des tissus intestinaux à la flore intestinale. Les phagocytes mononucléés (MNPs) qui dialoguent, via les cytokines qu'ils produisent, avec les cellules immunitaires innées et adaptatives sont impliqués dans l'induction, la perpétuation et le maintien de la réponse inflammatoire des MICI. Chez la souris, les MNPs sont stratifiées en cellules dendritiques conventionnelles (cDCs), macrophages (MΦ) et cellules dérivées de monocytes, une entité qui regroupe dans le tissu des cellules dendritiques dérivées de monocytes (Mo-DC), des Mϕ dérivés de monocytes et des « monocyte-like ». Toutefois, la diversité phénotypique, moléculaire et fonctionnelle des monocytes et des MNPs ainsi que la plasticité des monocytes restent à élucider dans les MICI. Les anticorps bloquant les cytokines IL12 et IL23 contrôlent la pathogénicité et la plasticité des cellules Th17, réduisant l'inflammation intestinale chez les patients atteints de MII. Cependant, il n'existe à ce jour aucun traitement curatif. L'étude approfondie de la plasticité des cellules T et du site tissulaire où elle pourrait se produire ne sont toujours pas clarifiés.

Dans le premier chapitre, nous avons révélé l'existence de deux sous-populations distinctes de $CD14^{+}$MNPs dans le colon de patients atteints de CU. Les cellules de type « inflammatory monocyte-like » $CD14^{+}CD163^{-}CD64^{+}$ (P3) à l'opposé des $CD14^{+}CD163^{+}CD64^{+}$ Mϕ (P4) s'accumulent dans le côlon inflammatoire. Nos résultats ont de plus établi un lien entre les P3 MNPs, l'IL12, l'IL1β et la détection de cellules Th17 mémoires produisant de l'IFNγ et de l'IL8, qui contribueraient collectivement à la pathogenèse de la CU. De plus, deux sous-

populations $CD14^+$ MNPs similaires sur le plan fonctionnel et moléculaire a ceux trouvés en CU, ont été détectées dans le côlon de patients atteints de CD.

En revanche, dans le deuxième chapitre, nous fournissons des évidences que la sous-population monocytaire $Slan^+$ pourrait contribuer à l'immunopathogenèse de la CD, mais pas à celle de la CU. La fréquence, le phénotype et la fonction des cellules $Slan^+$ ont été examinés dans le sang, les ganglions mésentériques (MLN) et le côlon de patients atteints de MICI. Nous proposons que les cellules pro-inflammatoires $CD14^{hi}CD172\alpha^+Slan^+$ discriminent les tissus de CD et CU. En effet, elles ne s'accumulent que dans les MLNs et la muqueuse colique des patients atteints de CD.

Dans le troisième chapitre, nous avons montré que les MLNs de CD et de CU, qui sont des tissus difficiles d'accès pour leur étude fonctionnelle en recherche, peuvent également être distingués par la distribution et le profil moléculaire des cellules T mémoire effectrices $CXCR3^-$ $CCR6^+$ ($Th17T_{EM}$). Nos données suggèrent également que la plasticité de Th17 se produit dans les MLNs avant leur migration vers l'intestin. Cette étude pourrait avoir des implications pour améliorer notre compréhension de la maladie.

Enfin, il a été démontré qu'à l'homéostasie chez la souris, les monocytes sont continuellement recrutés dans la muqueuse intestinale où ils se différencient progressivement en Mϕ anti-inflammatoires. Ce processus de maturation est interrompu dans le contexte d'une inflammation. Les signaux environnementaux qui régulent la « cascade » de maturation d'un monocyte classique tissulaire demeurent inconnus chez l'homme. Dans le quatrième chapitre, nous avons récapitulé in *vitro* la cascade de différenciation des monocytes humains de «$CD163^-$ P3-like» en «$CD163^+$P4-like» et avons montré leurs similitudes moléculaires avec les $CD14^+$

MNP tissulaires. La manipulation de cette voie de différentiation pourrait ouvrir des pistes thérapeutiques pour restaurer l'homéostasie intestinale dans les MICI.

En conclusion, une meilleure compréhension des sous-populations de MNPs, leurs fonction et plasticité dans la pathogenèse des MICI aidera à identifier des nouvelles cibles thérapeutiques et contribuera à augmenter les connaissances pour la mise au point de traitements personnalisés.

Mots-clés: Macrophages, Monocytes, Th17 cells, Plasticité, Slan^{+} cells, Crohn's disease, Ulcerative colitis, Culture, Pathogenicité.

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List of acronyms

AhR	Aryl Hydrocarbon Receptor
APC	Antigen Presenting Cell
BBB	Blood-Brain Barrier
CCL	C-C Chemokine Ligand
CCR	C-C Chemokine Receptor
CD	Crohn's Disease
CD-	Cluster Of Differentiation
cDC	Classical Dendritic Cell
CDP	Common Dendritic Cell Precursors
CLP	Common Lymphocyte Precursors
cMoP	Common Monocyte Precursors
CMP	Common Myeloid Precursors
CSF1	Macrophage Colony-Stimulating Factor
CSF1R	Macrophage Colony-Stimulating Factor Receptor
CSF2	Granulocyte-Macrophage Colony-Stimulating Factor
CSN	Culture Supernatant
CTLA-4	Cytotoxic T-Lymphocyte-Associated Protein-4
CX3CL1	Chemokine (C-X3-C Motif) Ligand 1
CX3CR1	Chemokine (C-X3-C Motif) Receptor 1
CXCL	Chemokine (C-X-C Motif) Ligand
CXCR	Chemokine (C-X-C Motif) Receptor
DC	Dendritic Cells
DNA	Deoxyribonucleic Acid
DSS	Dextran Sulfate Sodium
EAE	Experimental Autoimmune Encephalomyelitis
EMP	Erythro-Myeloid Progenitor
FcRn	Neonatal Fc Receptor,
FcγR	Fcγ Receptor
GALT	Gut-Associated Lymphoid Tissue
GFP	Green Fluoresenct protein
GI	Gastrointestinal
GM-CSF	Granulocyte-Macrophage Colony-Stimulating Factor
GMP	Granulocyte-Macrophage Precursor
GPR15	Orphan G Protein Coupled Receptor15
GPR15L	Orphan G Protein Coupled Receptor15 Ligand
GWAS	Genome Wide Association Studies
H2S	Hydrogen Sulfide

HEV	High Endothelial Venule
HGF	Hepatocyte Growth Factor
HSC	Hematopoietic Stem Cells
IBD	Inflammatory Bowel Disease
ICAM	Intercellular Adhesion Molecule
ICOS	Inducible T Cell Co-Stimulator
Ig	Immunoglobulin
IL	Interleukin
IL23R	Interleukin 23 receptor
ILC	Innate Lymphoid Cells
ILF	Isolate Lymphoid Follicle
Infl mo-like	Inflammatory Monocyte-like
iNOS	Inducible Nitric Oxide Synthase
IRF4	Interferon Regulatory Factor 4
IRF5	Interferon Regulatory Factor 5
IRF8	Interferon Regulatory Factor 8
IRGM	Immunity-Related GTPase Family M Protein
KLR2	Kruppel-like factor 2
LFA-1	Lymphocyte Function-Associated Antigen 1 (CD11a/CD18)
LN	Lymph Node
LP	Lamina Propria
LPMC	Lamina Propria Mononuclear Cells
LPS	Lipopolysaccharide
M1	Classically Activated Macrophages
M2	Alternative Activated Macrophages
mAb	Monoclonal Antibody
MAC-1	Macrophage-1 Antigen (CD11b/CD18)
Mad-CAM1	Mucosal Vascular Addressin Cell Adhesion Molecule 1
MALT	Mucosa Associated Lymphoid Tissue
M-CSF	Macrophage Colony-Stimulating Factor
MDP	Muramyl Dipeptide
MF	Macrophage
MHCII	Major Histocompatibility Complex Class II
miRNA	Micro RNA
MLN	Mesenteric Lymph Node
MLR	Mixed Lymphocyte Reaction
MNPs	Mononuclear Phagocytes

MoDC	Monocyte-Derived Dendritic Cell
MPP	Multipotent Progenitors
MPS	Mononuclear Phagocyte System
mRNA	Messenger RNA
MS	Multiple Sclerosis
NLR	Nod Like Receptor
NLT	Non-Lymphoid Tissue
NO	Nitric Oxide
NOD2	Nucleotide-Binding Oligomerization Domain Containing Protein 2
pDCs	Plasmacytoid Dendritic Cells
PGE2	Prostaglandin E2
PP	Peyer's Patches
PRR	Pattern Recognition Receptor
PSGL-1	P-Selection Gylcoprotein Ligand 1
pTreg	Peripheral Regulatory T Cell
RA	Retinoic Acid
RAG	Recombination activating gene 1
REG3	Regenerating islet-derived protein 3 gamma
RGC-32	Gene to complement 32 protein
RNA	Ribonucleic Acid
ROI	Reactive Oxygen Intermediate
RORγ	RAR-Related Orphan Receptor Gamma
S1P	Sphingosine 1-Phosphate
S1PR1	Sphingosine 1-Phosphate Receptor Type1
SCFA	Short Chain Fatty Acids
scRNAseq	Single cell RNA sequencing
SFB	Segmented Filamentous Bacteria
SLE	Systemic Lupus Erythematosus
SLO	Secondary Lymphoid Organ
SNPS	Single Nucleotide Polymorphism
SS	Steady State
T reg	Regulatory T Cell
TCM	Central Memory T Cell
TCR	T Cell Receptor
TED	Transepithelial Dendrite
Teff	Effector T Cells
TEM	Effector Memory T Cell
TFH	Follicular Helper T Cell
TFR	T Follicular Regulatory

TGFβ	Transforming growth Factor β
TH	Helper T Cell
Th1*	Non Classic Th1
TLR	Toll-Like Receptor
TNBS	2,4,6-Trinitrobenzene sulfonic acid
TNF	Tumor Necrosis Factor
TNFR2	Tumor necrosis factor receptor 2
TREM2	Triggering receptor expressed on myeloid cells 2
TRM	Tissue Resident Memory
TSCM	Stem Memory T Cells
tTreg	Thymus-generated Treg
UC	Ulcerative Colitis
VCAM-1	Vascular Cell Adhesionmolecule-1
VLA-4	Very Late Antigen-4

Chapter 1 :

Introduction

1.1 Inflammatory Bowel Disease (IBD)

Inflammatory bowel disease (IBD) is a worldwide health problem as its incidence is constantly increasing (de Souza and Fiocchi, 2016; Kaplan, 2015; Malik, 2015). It encompasses a spectrum of disabling, chronic, gastrointestinal (GI) inflammatory disorders including, Crohn's disease (CD) and ulcerative colitis (UC).

1.1.1 CLINICAL DESCRIPTION : CD VERSUS UC

CD and UC are relapsing inflammatory disorders with distinct clinical and pathological features (Zhang and Li, 2014). First, CD involvement extends to the entire GI tract from mouth to anus, but most commonly affect the terminal ileum and colon (Baumgart and Sandborn, 2012). It is characterized by patchy and discontinuous inflammation that penetrates the intestinal wall. Histological appearance include thickened submucosa with granulomas and deep fissuring ulcerations. Macroscopically, "creeping fat" and mesenteric fat hypertrophy are characteristics of CD, but not UC (Bertin et al., 2010). Inflammation in UC is continuous and involves the colon and rectal mucosa (Ordas et al., 2012). It is superficial and restricted to the mucosal and submucosal layers. Histologically, UC presents with distorted crypt architecture and crypt granulomas.

CD and UC are debilitating conditions. Patients suffer from a variety of symptoms related to gut inflammation, including abdominal pain, diarrhea, vomiting, weight loss, fatigue, fever and rectal bleeding. Also, CD patients might present with complications, such as fistulas between the GI tract and the skin, anus or vagina, or intestinal blockage due to strictures. There is still no cure for IBD, but symptoms are controlled via immunosuppressants or anti-inflammatory

steroids that decrease inflammation, changes in diet that reduce environmental triggers, and surgery in extreme cases to remove injured sections of the GI tract (Baumgart and Sandborn, 2007). Another complication faced by IBD patients is the significantly higher risk of developing colorectal cancer (Herszenyi et al., 2015). The constant inflammation may lead to non-neoplastic inflammatory epithelium that progresses to dysplasia, leading to carcinoma. In addition, long use of immunosuppressive therapies contribute to cancer development (Yashiro, 2015).

1.1.2 EPIDEMIOLOGY AND ECONOMIC BURDEN

Since the middle of the twentieth century, IBD has become a growing problem in industrialized countries including Canada. The incidence of CD and UC steadily increased in North America, Europe, Australia and New Zealand, to reach a plateau in the twenty-first century. It is presently up to 0.5% of the general population in the Western world (Molodecky et al., 2012). The last few decades observed a rapid increase in IBD incidence rate in countries that are adopting a more western lifestyle (Loftus, 2004). In fact, emergence of IBD has been documented in recently industrialized countries in Asia, Middle East and South America; although the incidence is still significantly lower than in Western countries (Kaplan, 2015). Hence, IBD is a global concern.

The direct and indirect financial burden for managing IBD in the Western world is considerable. For instance in Canada, 200,000 people are estimated to have IBD and the direct medical cost is over 1.2 billion Canadian dollars yearly, including ambulatory care, hospitalizations, surgery and pharmaceuticals (Rocchi et al., 2012). The treatment of IBD has drastically evolved in the

last two decades with the introduction of biologics that decreased the need for surgeries (Frolkis et al., 2013). Unfortunately, the increased use of biologic agents considerably augmented health care costs, as the annual cost per patient is estimated at more than 25,000 United States dollars (Marchetti and Liberato, 2014). Furthermore, an indirect cost is due to loss in work productivity, which adds on the cost of managing IBD: it is estimated at 1.6 billion Canadian dollars annually (Rocchi et al., 2012). Finally, the reduced quality of life for patients and their families comes at an unmeasurable cost. This growing global IBD burden needs to be met with equivalent studies to better understand and manage both the cause and disease progression.

1.1.3 CAUSES OF IBD

The exact cause of IBD is not completely clear. It is suggested to involve an abnormal immune-mediated inflammatory response against host microbiome in genetically susceptible individuals (de Souza and Fiocchi, 2016; Zhang and Li, 2014).

The progress seen in the understanding of IBD genetics relays two key messages regarding genetics and its role in IBD pathogenesis (Zhang and Li, 2014). On the one hand, the increasing number of susceptibility genes identified in IBD supports a critical role of genetics in IBD development. On the other hand, susceptibility genes account for about 25% of IBD heritability (Zuk et al., 2012), reinforcing the idea that IBD is a polygenetic and complex disease.

These complex, multi-factorial interactions have been the subject of a multitude of studies over the past 20 years, and will be briefly discussed below.

1.1.3.1 *Genetics*

The contribution of genetics to IBD came from early epidemiological studies that demonstrated familial clustering in CD and UC, as they noted high sibling risk ratios. Furthermore, twin studies that compared disease concordance rates between monozygotic and dizygotic twins that share the same environment have supported the role of genetics in IBD (Halme et al., 2006). Linkage studies tried to identify the specific gene responsible for IBD. The genetic risk, however, is not a single locus, but spread through the genome as with other complex disorders (de Lange and Barrett, 2015); thus, more in-depth genetic studies were needed.

The last two decades have seen technological improvements in RNA/DNA sequencing and analysis, which lead to major advancement in our knowledge of the genetic contributions to IBD (McGovern et al., 2015). Indeed, genome-wide association studies (GWAS) have identified single nucleotide polymorphisms (SNPs) in IBD. The number of IBD-gene loci are currently at more than 200, most are shared between CD and UC (Jostins et al., 2012; Liu et al., 2015).

The first CD susceptibility gene identified was *NOD2* (nucleotide-binding oligomerization domain-containing protein 2) (Ogura et al., 2001). *NOD2*, a member of the NOD-like receptor (NLR) family, codes for the intracellular receptor of muramyl dipeptide (MDP), a motif found in peptidoglycan of Gram-positive and Gram-negative bacteria that is crucial for bacterial recognition (Inohara et al., 2003). Pathogenicity of NOD2 in CD stems in part from impairment of bacterial clearance that results in increased inflammation. Moreover, NOD2 is a negative regulator of TLR2 stimulation; defects in NOD2 lead to increased Th1 response (Watanabe et

al., 2004), and suppression of transcription of the anti-inflammatory cytokine IL10 (Noguchi et al., 2009). These are only a few of the many effects on innate and adaptive immunity observed with NOD2 defects (de Souza and Fiocchi, 2016).

The role of autophagy in IBD pathogenesis has been confirmed by the identification of *ATG16L1* and *IRGM* variants in IBD patients (Hampe et al., 2007; McCarroll et al., 2008). Autophagy is implicated in housekeeping duties via the removal of damaged organelles and misfolded proteins that help maintain cellular homeostasis. Dysregulation in the unfolded protein response leads to endoplasmic reticulum stress that has also been linked to IBD pathogenesis (Kaser and Blumberg, 2011). In addition, autophagy is essential for host defense against intracellular pathogens (Deretic and Levine, 2009). Noteworthy, *ATG16L1* and *IRGM* have been shown to act together in CD development (Hoefkens et al., 2013). Furthermore, stimulation of NOD2 in epithelial cells activates ATG16L1-dependent autophagy and bacterial killing, a mechanism impaired in CD patients (Homer et al., 2010). Hence, gene variant interactions are implicated in IBD and fit perfectly in its complexity.

GWAS also permitted the identification of another significant association between CD, UC, and the *IL23R* gene (Duerr et al., 2006). The latter codes for the receptor of the pro-inflammatory cytokine IL23, implicated in the generation and maintenance of T helper cell 17 (Th17). Noteworthy, one of the *IL23R* variant accords a two to three-fold protection against IBD development (Duerr et al., 2006), that is higher in CD than UC (Jostins et al., 2012). This loss-of-function allele causes a decrease in the number of IL17-producing $CD4^+$ and $CD8^+$ T cells (Sarin et al., 2011), in addition to a decrease in IL17 production and STAT3

phosphorylation (Di Meglio et al., 2011; Pidasheva et al., 2011). Other gain-of-function *IL23R* variants have been described in IBD (Duerr et al., 2006; Kim et al., 2011b; Newman et al., 2009). Furthermore, variants in *IL12B*, encoding the p40 subunit of IL12 and IL23, have been associated with CD and UC (Zhang and Li, 2014). The Th17-IL23 axis plays an important role in the pathogenesis of IBD and will be further discussed in this thesis.

1.1.3.2 ***Environment***

Traditionally, IBD affected people living in the Western world. This notion was challenged by epidemiological studies that ruled out ancestry and ethnicity as risk factors of IBD, and instead implicated the environment that we live in. Indeed, people of all ethnic groups, living in the Western world for many generations, have been affected by IBD (Afzali and Cross, 2016). Furthermore, in Western countries, first generation descendants of immigrants from developing countries with a low prevalence of IBD, have similar risks as the base population for IBD development. In fact, pediatric patients with IBD in western countries are culturally diverse (Afzali and Cross, 2016; Benchimol et al., 2015a; Benchimol et al., 2015b). This suggests that as developing countries adapt a western mode of life, rates of IBD will escalate similar to western countries (Kaplan, 2015).

Multiple environmental factors are involved in IBD pathogenesis including diet, smoking and antibiotic use (Zhang and Li, 2014). As discussed earlier, adaptation of a westernized diet, high in meat, saturated fat, and carbohydrates but low in vegetables, has been associated with an increased incidence of IBD (Malik, 2015). Vitamin D deficiency has also been associated with an increased risk for IBD (Leslie et al., 2008). Interestingly, smoking has been shown to have

protective effect in UC, but not CD, patients by decreasing relapse rate (Lakatos et al., 2007). Finally, the use of antibiotics has been linked with pediatric IBD development; actually, children diagnosed with IBD were more likely to have taken antibiotics in the first year of life compared to controls (Shaw et al., 2010). Thus, environmental factors affect IBD development.

1.1.3.3 *Microbiota*

The healthy human gut microbiota is composed of four major phyla: Firmicutes and Bacteroidetes phyla dominate, followed by Proteobacteria and Actinobacteria phyla that are rare.

The link between IBD pathogenesis and microbial dysbiosis has been well established (Somineni and Kugathasan, 2019). First, the gut flora in CD and UC have a significantly reduced biodiversity and richness in fecal microbiome when compared to healthy controls (Frank et al., 2007; Gevers et al., 2014; Joossens et al., 2011), mainly due to a decline in Firmicutes diversity (Matsuoka and Kanai, 2015). Second, a shift in the equilibrium between commensal and pathogenic microbial populations has been seen in IBD. While Firmicutes and Bacteroidetes phyla predominate in healthy intestine, they are under-represented in CD and UC. An increase in enterobacteria and Proteobacteria have been reported in IBD patients (Martinez et al., 2008). Interestingly, healthy family relatives of IBD patients showed the same alterations in their microbiota and have a higher risk than the general population of developing IBD (Joossens et al., 2011; Varela et al., 2013). Noteworthy, dysbiosis in IBD is not restricted to bacterial populations only, it includes viruses, bacteriophages and fungi (Somineni and Kugathasan, 2019).

IBD patients have a faulty epithelial barrier leading to augmented intestinal permeability to commensal and pathogenic bacterial populations in the intestinal lumen (Martini et al., 2017). The first physical barrier encountered by intestinal microorganisms is the mucous layer, secreted by goblet cells to cover the epithelium. The mucus layer is normally composed of two layers. The outer layer, with its loose composition, favors bacterial growth, whereas the inner layer is tightly adherent and sterile. In IBD, however, the inner adherent mucosal layer has a marked increase in bacterial populations (Johansson et al., 2008; Ott et al., 2004). The second line of defense against bacterial invasion is the intestinal epithelial layer, composed of enterocytes and specialized epithelial cells (goblet cells and Paneth cells). Epithelial cells secrete various anti-microbial peptides to ensure protection from invading pathogenic microbes. Patients with CD have a dysfunctional expression of anti-microbial peptides (Wehkamp et al., 2003).

Microbial dysbiosis leads to disturbance in functional processes that contribute to IBD establishment. For example, IBD patients have lower frequencies of butyrate-producing bacteria, belonging to the Firmicutes phylum. The lower levels of the short chain fatty acid (SFCA) butyrate have been well documented in IBD (Sokol et al., 2008; Sokol et al., 2009; Takaishi et al., 2008). Butyrate plays an important role in intestinal homeostasis (Somineni and Kugathasan, 2019) through inhibition of NFκB activation. Also, it maintains epithelial health by serving as an energy source for colonic epithelial cells and increasing the expression of tight junction proteins, thus ensuring intestinal epithelial impermeability. On the contrary, higher frequencies of sulfate-reducing bacteria have been observed in IBD patients, leading to higher levels of hydrogen sulfide (H_2S). The latter has been shown to cause DNA damage and reduce disulfide bridges of the mucus layer compromising the integrity of the mucosal barrier and

making it more permeable to bacterial growth (Ijssennagger et al., 2015). Collectively, these data suggest that gut microbiome manipulation could be a therapeutic avenue in IBD patients.

1.1.3.4 *Immune cells*

The study of IBD pathogenesis examines mucosal immunity. It is established that an erroneous innate and adaptive immune response to the commensal flora contributes to intestinal inflammation in IBD patients (de Souza and Fiocchi, 2016). Recent, immunological studies examining the role of mucosal innate immune cells implicate them in maintenance of epithelial barrier integrity, microbial sensing, phagocytosis, and autophagy.

The innate immune response is the first line of defense against invading pathogens. It is not specific to antigens, and thus quick, allowing the body to respond within minutes or hours to stimuli. The innate immune response implicates epithelial cells, granulocytes (basophils, neutrophils, eosinophils, and mast cell), mononuclear phagocytes (monocytes, macrophages and dendritic cells (DCs)), innate lymphoid cells (ILC1, ILC2 and ILC3) and natural killer (NK) cells. Innate cells are specialized in recognition of microbial antigens via their cell surface receptors known as pattern recognition receptors (PRRs) and include toll-like receptors (TLRs), as well as cytoplasmic receptors known as NOD-like receptors (NLRs) (Abreu et al., 2005). Studies demonstrated abnormal behavior of cells mediating innate immunity upon stimulation of TLRs and NOD proteins in individuals with IBD (Abreu et al., 2005). On the other hand, adaptive immunity is specific for the antigen and takes several days to establish; it involves B and T cells (de Souza and Fiocchi, 2016).

Humoral homeostasis is disturbed in IBD patients (Uzzan et al., 2016). More specifically, an impaired production of the non-inflammatory immunoglobulin A (IgA) has been noted. IgA antibodies have a role in maintaining intestinal homeostasis by coating commensal bacteria that translocate across the epithelial barrier. Furthermore, IBD patients have an increased production of pro-inflammatory IgG antibodies, and their B cells have a higher secretion of the pro-inflammatory cytokine IL8/CXCL8 (Brandtzaeg et al., 2006; Noronha et al., 2009). Finally, a higher frequency of plasma cells is well-documented in the intestinal lamina propria of IBD patients (Uzzan et al., 2016).

Work in the last two decades has focused primarily on the role of T cell-mediated immune responses in IBD pathogenesis, which led to the view that CD is driven by a helper type 1 (Th1) response, based on elevated levels of IL12 and IFNγ, whereas UC by a helper type 2 (Th2) response due to the enhanced production of IL5 and IL13 (Zhang and Li, 2014). The discovery of IL17-producing Th17 cells required the revision of the Th1-Th2 paradigm to include the interplay between Th1, Th2, Th17, and also regulatory T cells (T_{reg}) (Ueno et al., 2018).

In this thesis work we will specifically explore Th cells, mononuclear phagocytic cells, their interactions, and also the potential role they play in IBD pathogenesis.

1.2 Monocyte's journey from the bone marrow to the blood

Monocytes along with macrophages and dendritic cells (DCs) make up the mononuclear phagocyte system (MPS) (Guilliams et al., 2018). In humans, CD14 and CD16 expression in the HLA-DR^{+} population identifies the classical CD14^{+}CD16^{-}, non-classical CD14^{low}CD16^{+} and intermediate CD14^{+}CD16^{+} monocyte populations. The mice equivalent of classical and non-classical monocyte subsets have been identified as Ly6C^{high}CX3CR1^{int} and Ly6C^{low}CX3CR1^{high}, respectively. Thus far, no murine counterpart has been established for the human intermediate phenotype; a potential equivalent was suggested by Mildner *et al.* as Ly6C^{int}MHCII^{+} (Mildner et al., 2017).

Although this thesis focuses on human IBD, most of the data presented in this section stems from murine studies, reflecting its abundance in the literature. Furthermore, it is more feasible to investigate cell ontogeny in murine models. Human studies will be specifically mentioned.

1.2.1 GENERATION OF MONOCYTES IN THE BONE MARROW

After weaning, monocytes develop in the bone marrow starting from the oligopotent hematopoietic stem cell (HSC) in a process known as monopoiesis. In this hierarchical model, progenitor cells gradually become more restricted and ultimately give rise to various types of circulating cells, a phenomenon studied mainly in mice (**Figure 1-1A**) (Ginhoux and Jung, 2014; Guilliams et al., 2018).

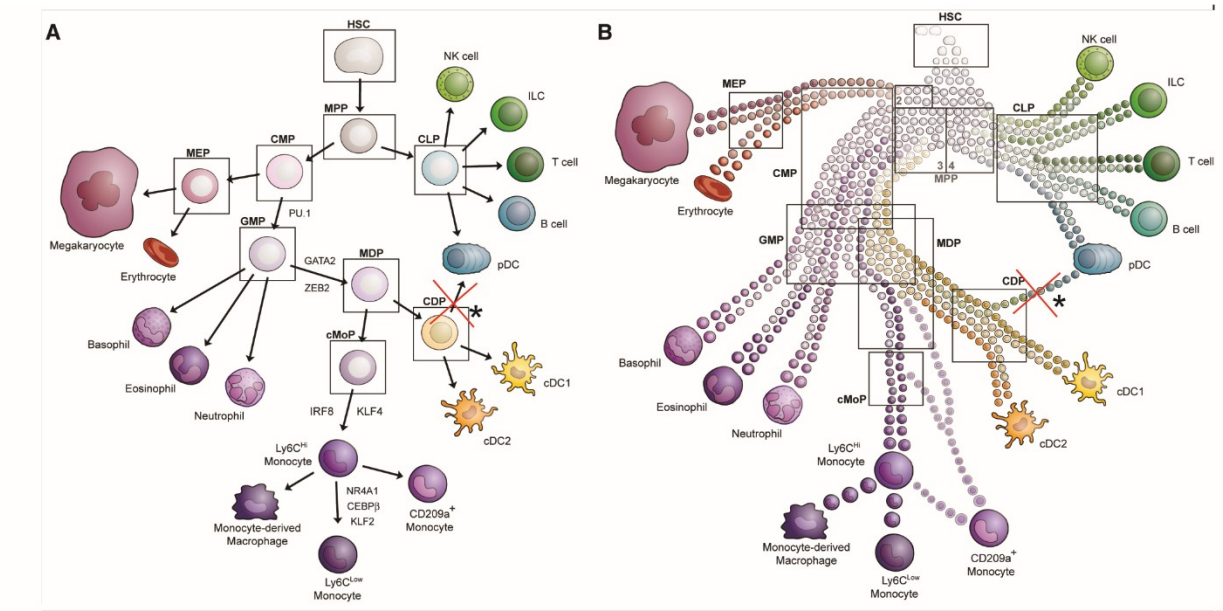


Figure 1-1: Development of murine monocytes under physiological conditions.
(A) The classical hierarchical model of hematopoiesis in the murine bone marrow. (B) The updated model of hematopoiesis proved by single-cell RNA sequencing technologies. In this model, HSC multiply and fill the multi-potent progenitor (MPP) pool, with subsets primed to a cellular lineage. Downstream precursor populations are a heterogenous mix of committed cells. The rectangles correspond to the precursors represented in panel A.
** Dress et al. recently showed that pDCs do not develop from the CDPs (Dress et al., 2019).*
(Adapted from (Guilliams et al., 2018), authorization code: 4550280688908).

In this classic tree-model, the multi-potent progenitors (MPP) sub-divide into the myeloerythroid or lymphoid lineage through the common myeloid or lymphoid progenitor (CMP or CLP), respectively. The CMP differentiates into the granulocyte and macrophage progenitor (GMP) that generates the granulocytic lineage - which includes neutrophils, eosinophils and basophils - or further divides into the bipotent monocyte-macrophage/dendritic cell precursors (MDP). The later splits into either the common DC precursor (CDP), or the common monocyte progenitor (cMoP) that gives rise to Ly6C^{hi} and Ly6C^{lo} monocytes in *in vitro* cultures and adoptive transfer models (Hettinger et al., 2013). Studies of cMoP biology,

expressing Ly6C in mice and CD14 in humans, revealed its high proliferative abilities (Chong et al., 2016; Hettinger et al., 2013; Kawamura et al., 2017). cMoP transition to a CXCR4-expressing pre-monocyte stage before differentiating in the bone marrow, within 24 hours, into mature $Ly6C^{+}CXCR4^{-}CCR2^{+}$ monocytes (Chong et al., 2016).

Conventional dendritic cell (cDC) populations, cDC1 and cDC2, arise from a distinct precursor, the CDP (Murphy et al., 2016). B, T, and NK cells as well as ILCs arise from a separate oligopotent precursor, the CLP, early in the differentiation process (Rothenberg, 2014). Plasmacytoid dendritic cells (pDCs) were originally thought to have a myeloid or lymphoid developmental pathway, arising from a B cell precursor (Shortman et al., 2013). However, Dress *et al.* recently demonstrated using a combination of transcriptomic studies, as well as *in vivo* and *in vitro* assays, that pDCs develop from the lymphoid progenitor, independent of the cDC lineage (Dress et al., 2019).

Recent single cell RNA sequencing studies on both human and mice bone marrow cells made it clear that the tree-like hierarchical structure for hematopoiesis is an oversimplification. First, the circles, defined by expression of a few markers, do not represent homogenous populations. In fact, heterogeneity within the "oligopotent" HSC compartment has been observed, with cells pre-committed to the lymphoid, myeloid and megakaryocyte lineage (Dykstra et al., 2007; Notta et al., 2016; Sanjuan-Pla et al., 2013; Velten et al., 2017). Second, the tree implies limited potential transitions between various circles, an underestimation of the differentiation *in vivo* (Laurenti and Gottgens, 2018). Indeed, unipotent progenitors can either emerge through intermediate bipotent populations (classical model) or directly emerge from HSC (Naik et al.,

2013; Velten et al., 2017). Moreover, monocyte transcriptional profile was already expressed in some CMPs and GMPs (Paul et al., 2015). Thus, a new model was proposed that is mapped over the classical tree-model (**Figure 1-1B**). It highlights the heterogeneity within the precursor populations, the early lineage commitment, as well as the diversity in routes taken (Guilliams et al., 2018; Laurenti and Gottgens, 2018)

Monocyte lineage commitment is ensured by growth and transcriptional factors. Indeed, deletion of PU.1, IRF8 and KLF4 (Alder et al., 2008; Feinberg et al., 2007; Kurotaki et al., 2013) reduces the frequency of Ly6C^{hi} monocytes. Some studies also report lower levels of non-classical monocytes, as it is believed that they arise, in part, from Ly6C^{+} monocytes (Sunderkotter et al., 2004). In humans, an autosomal recessive IRF8 mutation was associated with anti-mycobacterial immunity as the infant lacked circulating monocytes and DCs (Hambleton et al., 2011). Moreover, a human GATA2 deficiency is associated with a progressive loss of monocytes and DCs (Bigley et al., 2011; Collin et al., 2015; Dickinson et al., 2011). On the other hand, Bach2 negatively regulates monocyte development as *Bach2*$^{-/-}$ mice have higher Ly6C^{+} counts (Kurotaki et al., 2018).

1.2.2 MONOCYTE SUBSETS IN THE BLOOD

Like other leukocyte populations, circulating monocytes are heterogeneous at the phenotypic and functional level.

Flow cytometry development allowed the identification of FcγRIII/CD16-expressing monocyte subsets (Passlick et al., 1989), starting a new chapter in understanding monocyte biology in

humans. A novel monocyte nomenclature was proposed based on the combination of CD14 and CD16 expression on HLA-DR$^+$ cells: "classical" CD14$^+$CD16$^-$ monocytes, representing 80–90% of the circulating monocyte pool, and the remaining 10–20% distributed between CD14$^+$CD16$^+$ intermediate and CD14^{lo}CD16$^+$ "non-classical" monocytes (Ziegler-Heitbrock and Hofer, 2013).

Monocyte classification in mice evolved with the generation of CX3CR1-GFP mice (Jung et al., 2000). It allowed the identification of two CD11b$^+$CD115$^+$ monocyte populations: Ly6C^{hi}CX3CR1^{int}CCR2$^+$CD62L$^+$CD43^{lo} and Ly6C^{low}CX3CR1^{hi}CCR2^{lo}CD62L$^-$CD43$^+$ (Geissmann et al., 2003; Jakubzick et al., 2013; Jung et al., 2000; Palframan et al., 2001). As was pointed out earlier, Ly6C^{hi} monocytes associated with human "classical" CD14$^+$CD16$^-$, and Ly6C^{low} with "non-classical" CD14^{low}CD16$^+$ monocytes. However, genetic and phenotypic differences are noted. For instance, HLA-DR expression is used for monocyte identification in humans, while MHCII expression is seen only on a fraction of murine monocytes. Moreover, murine, but not human, monocytes express PPARγ signature and have a differential expression of phagocytic genes (Cros et al., 2010; Ingersoll et al., 2010). Finally, classical to non-classical monocyte ratio is different in humans and mice; whereas classical monocytes are favored in humans, both subsets are equally represented in mice (Guilliams et al., 2018).

Classical monocytes have been shown to transition to non-classical monocytes in mice (Sunderkotter et al., 2004; Tacke et al., 2006; Thomas et al., 2016; Varol et al., 2007; Yona et al., 2013), and recently in humans (Patel et al., 2017). Deuterium labeling analysis in humans recapitulated what was seen in mice studies, and revealed a sequential labeling of circulating

monocyte subpopulations (Patel et al., 2017). First, classical monocytes develop from precursors in the bone marrow; they enter the circulation where they either differentiate into intermediate monocytes, extravasate into tissues or die. Analogously, intermediate monocytes differentiate into either non-classical monocytes or die (**Figure 1-2**). The relationship between intermediate and non-classical monocyte subsets in human was further suggested by their proximity at the molecular level (Wong et al., 2011). Plus, a time course study in patients with skin infection observed that an upsurge in $CD14^{+}CD16^{+}$ intermediate monocytes was followed by an increase in the frequency of $CD14^{lo}CD16^{+}$ non-classical monocytes (Ziegler-Heitbrock and Hofer, 2013).

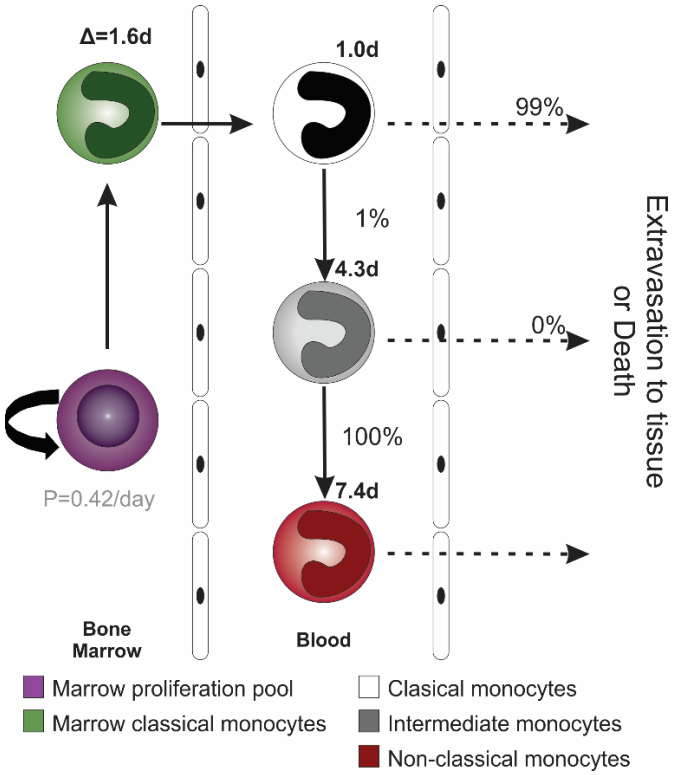


Figure 1-2: The sequential model of monocyte kinetics at steady state in humans.
Patel et al. demonstrated, using deuterium labeling and a mathematical model, the sequential development of the various monocyte subsets. Progenitor cells in the bone marrow (purple) proliferate and differentiate into classical monocytes. They stay in the bone marrow for 1.6

days, before entering the circulation. 99% of circulating classical monocytes ($CD14^+CD16^-$) will leave the circulation (due to death or migration into tissues), and 1% will differentiate into intermediate monocytes ($CD14^+CD16^+$) that in turn will mature into non-classical monocytes ($CD14^{lo}CD16^+$). Black bold text represents the lifespan, in days, of each subset. (Adapted from (Patel et al., 2017); authorization code: 4601400556234).

The human intermediate monocyte population is less characterized. While classical and non-classical monocyte subsets were homogenous, the intermediate population ($CD14^+CD16^+$ in humans, $Ly6C^{int}$ in mice) was heterogenous as demonstrated by unbiased single cell RNA sequencing studies (Mildner et al., 2017; Villani et al., 2017). Two populations formed the murine $Ly6C^{int}$ monocytes: $CD209a^+MHCII^+Ly6C^{hi\text{-}int}$ and the "real" intermediate population that had a transitional transcriptional profile between $Ly6C^{hi}$ and $Ly6C^{lo}$ populations (Mildner et al., 2017). Under pathological conditions $CD209a^+MHCII^+Ly6C^{hi\text{-}int}$ population develop into monocyte-derived cells (Menezes et al., 2016). Similarly, Villani *et al.*, showed heterogeneity within the $CD14^+CD16^+$ monocyte compartment (Villani et al., 2017), identified by higher HLA expression (Guilliams et al., 2018). A fraction of the $CD14^+CD16^+$ gate spread across the classical and non-classical monocyte clusters. The remaining cells were found in two clusters; Mono3 expressing cell cycle and differentiation genes, and Mono4 with cytotoxic gene signature (Villani et al., 2017). The equivalent to the Mono4 subset was absent in mice. New correlation studies are required to find equivalents of the newly identified monocyte populations in mice and humans.

Thus, in the circulation, classical monocytes' function is limited to their conversion into non-classical monocytes

1.2.2.1 *Monocyte function in the vasculature*

Murine and human non-classical monocytes display "patrolling" function at steady state as they crawl on the luminal surface of vascular endothelium (Auffray et al., 2007; Carlin et al., 2013). Moreover, they phagocytose necrotic endothelial cellular debris (Carlin et al., 2013). Thus, an endothelial surface sensing and scanning function is implied for $Ly6C^-$ monocytes. Upon TLR7 stimulation, the latter secrete inflammatory cytokines and chemokines to recruit neutrophils for triggering endothelial necrosis (Carlin et al., 2013). In humans, $CD14^{lo}CD16^+$ monocytes have a similar TLR7/TLR8-dependent patrolling activity (Cros et al., 2010). Noteworthy, non-classical monocytes respond poorly to bacterial lipopolysaccharide (LPS) (Cros et al., 2010).

Human non-classical monocytes comprise a population expressing slan, a carbohydrate modification of P-selectin glycoprotein ligand 1 (PSGL-1) (Cros et al., 2010; Hofer et al., 2015; Schakel et al., 2002). The latter is targeted by the IgM monoclonal antibody M-DC8 that was generated by Schakel et al. via immunization of Balb/c mice with mononuclear cells depleted of lymphocytes and monocytes from human blood (Schakel et al., 2002; Schakel et al., 1998). $Slan^+$ cells with a modified PSGL-1 failed to bind either P- or E- selectins (Schakel et al., 2002); this raises the question of whether slan control cell migration into specific tissues. Of note, the slan ligand remains unknown (Ahmad et al., 2019).

$Slan^+$Class II^+ cells were originally thought to delineate the major circulating DC subset in the blood of healthy individuals; the current consensus is that they cluster with non-classical monocytes (Hofer et al., 2015). At homeostasis, $slan^+$ cells are implicated in anti-viral response (Cros et al., 2010). In the context of pathology, these cells produce pro-inflammatory cytokines

that include IL1β, IL6, IL12, IL23, and TNF, and promote Th1/Th17 cell responses (Gunther et al., 2013; Hansel et al., 2011). Histologic analysis revealed the presence of slan^{+} cells in patients with psoriasis, rheumatoid arthritis, systemic lupus erythematosus (SLE), multiple sclerosis (MS), cancer and CD (Costantini et al., 2011; Hansel et al., 2011; Schakel et al., 2006; Thomas et al., 2014). However, they were not compared between CD and UC patients, nor was their function assessed in IBD. Consequently, in this thesis work, we have investigated the differential accumulation of slan^{+} cells in blood, lymphoid tissues, and colonic mucosa of CD and UC patients, and also evaluated their phenotype and function.

Circulating monocytes serve as an "emergency squad" capable of mobilizing rapidly and in great numbers from the bone marrow to site of inflammation, where they contribute to defence or resolution of inflammation. In fact, in humans, classical monocytes are held in the bone marrow for 38 hours for a post-mitotic maturation phase, forming a "reservoir" (Patel et al., 2017). Noteworthy, the spleen is a peripheral reservoir of both Ly6C^{+} and Ly6C^{-} monocyte populations (Swirski et al., 2009). Under inflammatory conditions, the reserve population is quickly released into circulation. Let us follow monocyte's journey to the intestine, where classical monocyte function in the tissue will be further discussed.

1.3 Monocyte's journey from the blood to the intestine

In contrast to non-classical monocytes, classical monocytes exert their protective function outside the vasculature, at sites of injury or infection. Hence, monocyte trafficking and migration into tissues is fundamental, followed by monocyte differentiation into macrophages or DCs (Guilliams et al., 2018).

1.3.1 MONOCYTE ARRIVAL TO THE INTESTINE

1.3.1.1 *Signals for the recruitment of monocytes*

Much of the work on monocyte trafficking has come from studies in heart and skin diseases that established different homing mechanisms for classical and non-classical monocytes in both humans and mice. $CCR2^{+}CX3CR1^{-}$ classical monocytes employ mainly CCR2/CCL2 and L-selectin/CD62L for trafficking (Ancuta et al., 2009; Geissmann et al., 2003; Weber et al., 2000). Conversely, $CX3CR1^{+}CXCR4^{+}CCR2^{-}CD62L^{-}$ non-classical monocytes undergo trans-endothelial migration upon binding to CXCL12, as well as soluble or membrane-bound CX3CL1 (neurotactin in mice, fractalkine in humans) expressed on endothelial cells and tissues (Ancuta et al., 2009; Ancuta et al., 2003; Auffray et al., 2007; Geissmann et al., 2003; Weber et al., 2000).

The role of CCR2/CCL2 axis in monocyte homing was confirmed for gut homing (Allers et al., 2014). In fact, deletion of CCR2 or its ligands, CCL2 and CCL7, result in reduced circulating monocytes in mice (Serbina and Pamer, 2006; Tsou et al., 2007). Even though the main cellular source of CCL2 in the intestine remains elusive, $CX3CR1^{+}$ resident macrophages express CCL2 and CCL7 (Takada et al., 2010; Zigmond et al., 2012), and thus, regulate their own maintenance

by recruiting CCR2^{+} monocytes to the tissue. Other molecules have also been described in monocyte trafficking to the small and large intestine and these include CCR9 (Bernardo et al., 2013; Linton et al., 2012) and integrin α4β7, respectively. They are implicated in non-classical monocyte homing to the gut to mediate intestinal healing (Schleier et al., 2019).

1.3.1.2 *Monocyte extravasation*

In response to chemokine signals, monocytes exit the circulation in a process known as diapedesis.

Monocyte diapedesis through the endothelium follows the closely regulated, multi-step cascade initially described for neutrophils involving a series of interactions with endothelial cells (Gerhardt and Ley, 2015; Kolaczkowska and Kubes, 2013). Leukocyte recruitment cascade in most tissues follows these steps: (1) tethering (2) rolling (3) adhesion (4) crawling (5) transmigration (**Figure 1-3**).

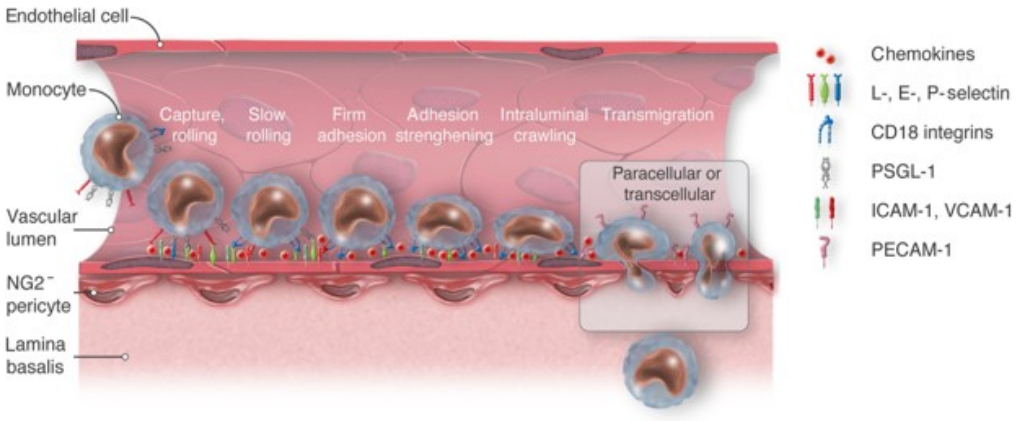


Figure 1-3: The monocyte adhesion cascade.
Activated endothelial cells express adhesion molecules (ICAM-1, VCAM-1), selectins (P and E) and chemokines that interact with monocyte ligands. A multi-step cascade for the

transendothelial migration of monocytes. Monocyte transmigration can follow either a paracellular or transcellular migration mechanism.
(PECAM-1, platelet endothelial cell adhesion molecule-1)
(Taken from (Gerhardt and Ley, 2015); authorization code: 4602550940193).

Upon receiving inflammatory signals, endothelial cells upregulate the expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), E- and P-selectins as well as CCL5 and CXCL8/IL8 (referred to as IL8 throughout this thesis) chemokines on the luminal surface of the endothelium (Gerhardt and Ley, 2015; Imhof and Aurrand-Lions, 2004). First, E- and P- selectins interact with glycoprotein ligands, more specifically the *O*-glycosylated carbohydrate ligand on PSGL-1 expressed on all monocytes, permitting a weak binding or tethering to endothelial cells and initiation of adhesion cascade. Noteworthy, PSGL-1 is more highly expressed by classical Ly6C^{+} monocytes when compared to Ly6C^{-} monocytes, which explains their higher recruitment into tissues (An et al., 2008). Second, VLA-4 on monocytes binds to VCAM-1 and mediates slow rolling on the endothelium in the direction of blood flow. It requires fast adhesive bond forming and breaking (Ramachandran et al., 2004). Third, monocytes' firm attachment to the endothelium is ensured by CCL2, IL8 and VLA-4 adhesion (Gerszten et al., 1999; Huo et al., 2001). Fourth, monocytes crawl on the intraluminal side of the endothelium, and send protrusions in order to find a site for extravasation into tissues (Carman et al., 2007; Gerhardt and Ley, 2015; Schenkel et al., 2004). This depends on the interaction of CD11a/CD18 (LFA-1) and CD11b/CD18 (MAC-1) integrins expressed on monocytes with ICAM-1 and ICAM-2 on endothelial cells (Schenkel et al., 2004). Finally, in order to exit the vasculature, monocytes must cross the endothelium and basement membrane which is an irreversible process (Gerhardt and Ley, 2015; Kolaczkowska and Kubes, 2013). Transmigration necessitates the involvement of cell adhesion molecules,

integrins and junctional proteins (CD31 (also known as PECAM1), CD99 and ECAM), as well as other endothelial cell molecules. Passage through the endothelium occurs predominantly paracellularly, i.e., between endothelial cells through the junctions and requires junctional remodeling. In 10-30% of cases, monocytes travel directly through the endothelial cells, transcellularly, by fusing vesicles in the endothelial cell cytoplasm (Carman and Springer, 2004; Ferreira et al., 2005; Hashimoto et al., 2012; Woodfin et al., 2011); consequently arriving in the tissue. Noteworthy, the process of diapedesis provokes phenotypic and genetic modifications in monocytes that could vary from one location to the other (Jakubzick et al., 2013).

1.3.2 MONOCYTE'S FATE IN STEADY STATE INTESTINE

Under homeostasis, classical monocytes traffic to tissues, including the intestine, to patrol tissues as monocyte-like cells or re-populate the anergic macrophage pool.

1.3.2.1 *Tissue-patrolling classical monocytes*

Classical monocyte function under physiological conditions remains poorly understood. As was mentioned earlier, classical monocytes convert in blood to non-classical monocytes. Another function was suggested for murine Ly6C$^+$ monocytes that enter non-lymphoid tissues without differentiating into either macrophages or DCs (Jakubzick et al., 2013). They upregulate a limited number of genes, including MHCII, co-stimulatory molecules and CCR7, induced by endothelium interaction during diapedesis, proposing a tissue surveying function. Subsequently, monocytes can exit the tissue and re-circulate through the lymph to the lymph nodes where they present antigen to T cells. Thus, classical monocytes are also classified as effector cells and labeled "tissue monocytes" (Jakubzick et al., 2013). Classical monocytes can

enter the lymph node directly from blood via the high endothelial venules (HEV) in a CD62L-dependent manner (Cheong et al., 2010; Leon and Ardavin, 2008).

1.3.2.2 *Intestinal macrophages*

Macrophages are stationary cells abundant in the various layers of the small and large intestine (**Figure 1-4**) (Mowat et al., 2017). The primary population is found in the lamina propria, generally directly below the epithelial layer. Other macrophage populations are found in the muscularis externa and serosa, where they connect with the enteric nervous system (Joeris et al., 2017). These macrophages are distinct from those in the lamina propria, and not the focus of this thesis as we do not capture them with our tissue processing protocol which mainly allows the isolation of lamina propria cells.

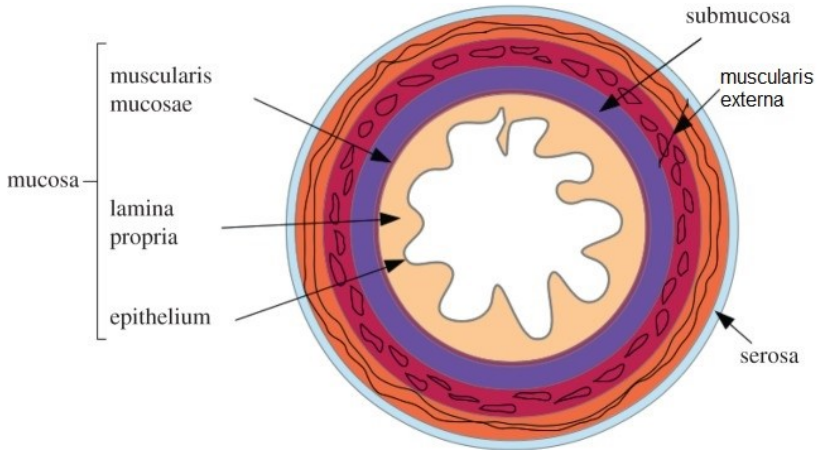


Figure 1-4: The different layers of the intestinal wall.
The intestinal wall is composed of multiple layers. The innermost layer is the mucosa that includes epithelium, lamina propria and muscularis mucosae. Next, the submucosa is formed by dense irregular connective tissue with the smooth muscle layer named muscularis propria/externa underneath and the outermost layer called serosa.
(Taken from from (Balbi and Ciarletta, 2013); authorization code: 11821288).

In murine lamina propria, mature macrophages are large cells with abundant vacuoles and lysosomes. They express high levels of MHCII and CD11c, also found on DCs, leading to ambiguity in discerning between the two cell types (Bain et al., 2013; Cerovic et al., 2014; Rivollier et al., 2012; Tamoutounour et al., 2012; Zigmond et al., 2012). Better macrophage identification relies on the expression of F4/80, CX3CR1, high affinity IgG receptor CD64 as well as molecules associated with phagocytosis and uptake of apoptotic cells such as CD163 and CD206 (Bain et al., 2013; Schridde et al., 2017; Smith et al., 2011; Weber et al., 2011; Zigmond et al., 2014a). Similarly, human intestinal macrophages express many of these markers (Bain et al., 2013). However, unlike in mice, CX3CR1 and MHCII cannot be used to identify macrophages in humans (Chakarov et al., 2019).

1.3.2.2.1 Ontogeny of tissue macrophages

It was long believed that bone marrow-derived monocytes were the sole precursors of tissue-resident macrophages and continuously replenish the pool. *In vitro* monocyte differentiation studies as well as adoptive transfer experiments of labeled monocytes supported this concept. However, irradiation/transplantation and parabiotic mice experiments revealed that Langerhans cells, microglia and alveolar macrophages develop independently of bone marrow monocytes (Ginhoux and Guilliams, 2016).

The "niche model" explains the developmental origin of macrophages (**Figure 1-5**), by taking into consideration three variables: niche accessibility, availability, as well as precursor plasticity (Ginhoux and Guilliams, 2016; Guilliams and Scott, 2017). It suggests that all macrophage precursors – yolk sac macrophages, fetal liver monocytes and bone marrow

monocytes - compete for a limited number of niches in various tissues. Although some precursors are better at colonizing a specific niche, it does not constitute the key factor that controls the cellular origin of macrophages, but rather depends on niche accessibility and availability. During early embryogenesis, niches are still not completely formed; they are created as the organs develop and receive the first wave of embryonic precursors (yolk sac-derived macrophages). Microglia and Langerhans cells originate from yolk sac-derived macrophages. This is not determined by the precursor, as yolk sac-derived macrophages are not the sole progenitors; but rather by niche availability before the generation of fetal liver as well as bone marrow monocytes (Daneman et al., 2010) (**Figure 1-5A**). During late embryogenesis and as the organs grow, extra niches are made making place for a second wave of embryonic precursors, the fetal liver-derived monocytes (Ginhoux and Guilliams, 2016; Guilliams and Scott, 2017). As the niche in the lung opens near birth, fetal liver monocytes arrive and differentiate into alveolar macrophages. This niche closes in adult mice by the formation of lung epithelial barrier thus preventing bone marrow monocyte access. Further growth during the neonatal phase justifies hematopoietic stem cell (HSC)-derived monocyte engraftment in neonate animals. As the liver continues to grow till the fourth week of murine life, a few Kupffer cells originate from HSC-derived monocytes, while others arise from the two embryonic precursors (**Figure 1-5**) (Fausto et al., 1995; Hoeffel et al., 2015; Scott et al., 2016b). Noteworthy, Kupffer cells are endowed with self-maintenance capacities, thus adult monocytes barely contribute to the Kupffer cell pool. In contrast, the intestinal macrophage niche is always accessible as macrophages continuously perish; so, they are derived mainly from bone marrow monocytes (**Figure 1-5**).

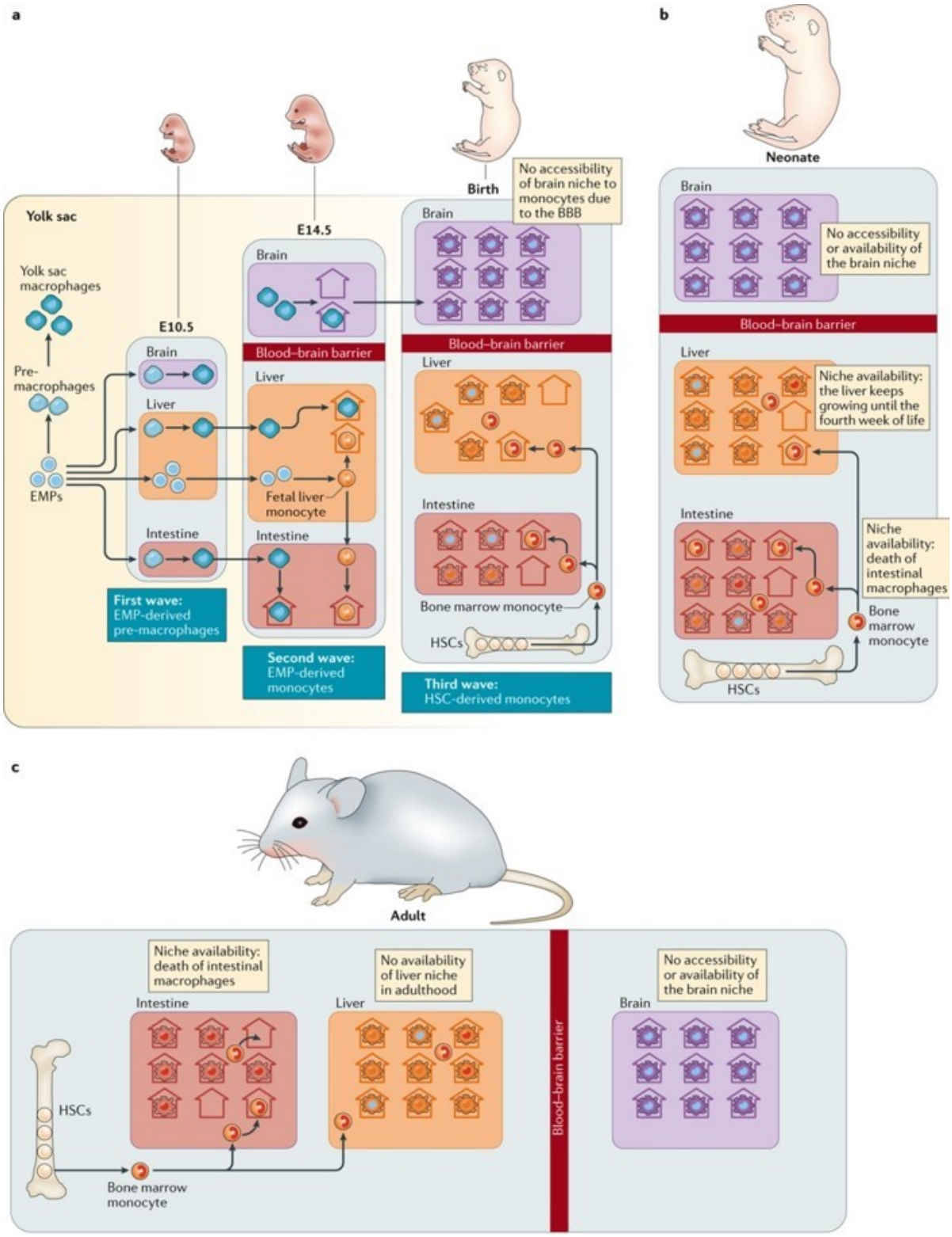


Figure 1-5: The niche model explains the developmental origin of macrophages.
(A) Between embryonic day 8.5 (E8.5) and E10.5, erythro-myeloid progenitors (EMPs) in the yolk sac develop into pre-macrophages. The latter seed the embryo head and differentiate

into yolk sac macrophages starting from E10.5. The liver, on the other hand, is seeded by both EMPs and pre-macrophages, that develop into yolk sac macrophages. At E14.5, the EMPs in the fetal liver generate fetal liver monocytes, that fill the available and accessible niches in the liver and intestine along with yolk sac macrophages. However, in the brain, the blood–brain barrier (BBB) blocks the entry of fetal liver monocytes; thus, only yolk sac macrophages, already present in the tissue, seed it. (A and B) With growth, the number of available niches augment and get filled by: (1) proliferation of macrophage populations already found in the tissue or (2) for open niches, recruitment and differentiation of fetal liver or bone marrow monocytes based on timing (prenatally or neonatally, respectively). (B and C) In the intestine, niches become constantly available through macrophage death, and get repopulated by bone marrow-derived monocytes, from birth and throughout life (B and C). (HSC: haematopoietic stem cells). (C) In the adult mice at homeostasis, the brain and liver macrophage niches are occupied and self-sustain with no or minimal contribution from bone marrow monocytes, respectively.
(Adapted from (Guilliams and Scott, 2017); authorization code: 4603700140005).

Multiple studies reinforce the concept that intestinal macrophage replenishment relies mainly on classical monocyte recruitment. First, intestinal macrophages have been shown to have a short half-life of 3-6 weeks (Bain et al., 2014; Jaensson et al., 2008). Second, intestinal macrophages do not proliferate (Bain et al., 2013; Smythies et al., 2006). Third, adoptive transfer experiments of monocytes into mice with conditional depletion of DCs and macrophages established the $Ly6C^{+}$ monocytic origin of $CX3CR1^{hi}$ macrophages (Bogunovic et al., 2009; Varol et al., 2007; Varol et al., 2009). Fourth, disruption of CCL2-CCR2 axis in mice lead to significantly lower quantities of colonic macrophages (Bain et al., 2013; Kinnebrew et al., 2012; Takada et al., 2010). In wild type (WT):$CCR2^{-/-}$ mixed bone marrow chimeric mice, $CD64^{+}CX3CR1^{hi}$ intestinal macrophages were derived solely from WT bone marrow (Tamoutounour et al., 2012). The CCR2-CCL2 axis is not only implicated in bone marrow egress of monocytes, but also in monocyte entry into the intestinal lamina propria as was discussed earlier. In fact, CCR2 expressing $Ly6C^{+}$ monocytes failed to enter gut mucosa in WT:$CCL2^{-/-}$ parabiotic mice (Takada et al., 2010). Noteworthy, $CCR2^{-/-}$ and $CCL2^{-/-}$ mice are

not completely devoid of intestinal macrophages (Dyer et al., 2019; Takada et al., 2010). This suggests the involvement of other recruiting mechanisms, or the existence of embryonically-derived macrophages in the intestine. Finally, Bain *et al*. demonstrated that $CD14^+$ monocytes differentiate into macrophages, by downregulation of CD14 and CD11c and upregulation of CD209 and CD163 expression, in healthy intestine of humans (Bain et al., 2013).

Importantly, non-classical $Ly6C^-$ monocytes do not contribute to the intestinal macrophage pool. In fact, they did not migrate into tissues in adoptive transfer experiments (Bain et al., 2013; Varol et al., 2007; Varol et al., 2009). In addition, the number of intestinal macrophages remain stable in CX3CR1-deficient mice that have fewer non-classical monocytes (Hadis et al., 2011; Kim et al., 2011a; Landsman et al., 2009)

Similar to the intestine, dermis, heart and pancreas rely on monocyte input for macrophage maintenance (Calderon et al., 2015; Epelman et al., 2014; Molawi et al., 2014; Tamoutounour et al., 2013) (**Figure 1-6**). In these organs, embryonically-derived as well as monocyte-derived macrophages are unable to self-maintain, leaving niches continuously available for replenishment by circulating monocytes (Guilliams and Scott, 2017; Sieweke and Allen, 2013; Tamoutounour et al., 2013). The failure of macrophage self-renewal remains to be fully elucidated; possible mechanisms involve the microbiota in intestine but not skin (Bain et al., 2014; Tamoutounour et al., 2013). In the heart, mechanical stress participated in continuous recruitment of monocytes (Lambrecht and Guilliams, 2014).

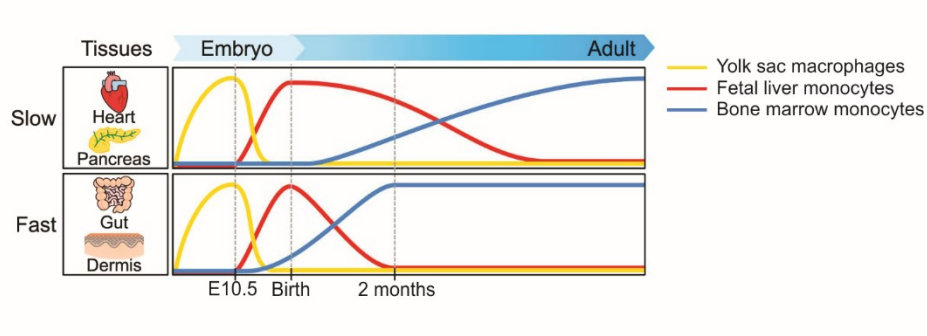


Figure 1-6: Bone marrow monocytes contribute to the resident macrophage pool in heart, pancreas, gut and dermis.

Yolk sac macrophages and fetal liver monocytes do not contribute to the tissue-resident macrophage population in the heart, pancreas, gut and dermis, after birth. These open tissue niches require bone marrow-derived monocytes to maintain the macrophage pool. This phenomenon follows different kinetics in different tissues.

(Adapted from (Ginhoux and Guilliams, 2016); authorization code: 4620311400757).

In conclusion, the resident macrophage pool in the lamina propria of intestine relies mainly on constant recruitment of classical monocytes in a CCR2-dependent manner.

1.3.2.2.2 Maintenance of intestinal homeostasis

Macrophages play a crucial role in the maintenance of intestinal homeostasis (**Figure 1-7**).

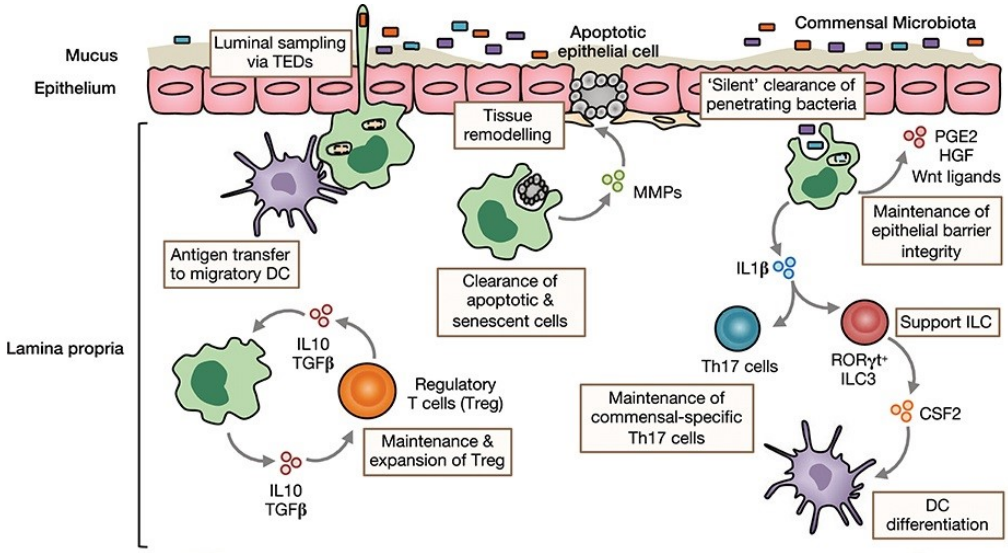


Figure 1-7: The various functions of lamina propria macrophages in the homeostatic intestine.
Intestinal lamina propria macrophages are involved in maintaining epithelial integrity, bacterial sampling and clearance, and aid innate and adaptive immune cells in their function. (MMP: matrix metaloproteinases)
(Taken from (Bain and Schridde, 2018); open access).

The primary function of all macrophages is phagocytosis to clear apoptotic and senescent cells. The intestinal epithelium is highly regenerative with rapidly dividing and dying cells; so, the high phagocytic activity of intestinal macrophages is perfectly suited for the housekeeping of this tissue. Intestinal macrophages express *Cd206, Mertk, Gas6, CD36, Axl, Itgav* and *Itgb5* genes associated with phagocytosis (Kumawat et al., 2018; Schridde et al., 2017). The dimerization of αv and β5 produces a receptor for the uptake of apoptotic cells, known as efferocytosis (Kumawat et al., 2018). Indeed, macrophages expressing cytokeratin, an epithelial cell antigen, have been found in the intestine at steady state (Nagashima et al., 1996). Furthermore, intestinal macrophages secrete tissue remodeling metalloproteinases and TNF (Battegay et al., 1995; Pender et al., 2000), and secrete mediators that stimulate intestinal epithelial cell proliferation in intestinal crypts, such as prostaglandin E2 (PGE2), Wnt signaling ligands and hepatocyte growth factor (HGF) (Chng et al., 2016; Cosin-Roger et al., 2016; D'Angelo et al., 2013; Ortiz-Masia et al., 2014). Thereby, intestinal macrophages regulate epithelial barrier integrity.

Intestinal macrophages are typically located below the epithelium and are more numerous in the colon relative to the small intestine (Mowat and Agace, 2014), thus they are perfectly localized to uptake and eliminate microbes invading the epithelium. Indeed, murine studies showed that $CX3CR1^{+}$ macrophages sample luminal bacteria by extending processes across the

epithelium, known as transepithelial dendrites (TED) without disturbing tight junctions, in a CX3CL1-dependent manner (Chieppa et al., 2006; Kim et al., 2011a; Niess et al., 2005). Also, CCR2-dependent macrophages have recently been shown to induce, through IL1β secretion, a Th17 cell response to segmentated filamentous bacteria (SFB) at steady state (Panea et al., 2015; Shaw et al., 2012). The bactericidal function of macrophages in the intestine at steady state does not lead to an overt inflammatory response in neither humans nor mice (Bain et al., 2013; Bernardo et al., 2018; Bujko et al., 2018b; Kumawat et al., 2018); this is due to TGFβ exposure during differentiation, which activates SMAD signaling and inhibits NF-κB signaling (Smythies et al., 2010). Indeed, bacteria ingestion by intestinal macrophages does not lead to respiratory burst, nor generation of nitric oxide (Roberts et al., 2001; Rugtveit et al., 1995). Also, TLR stimulation does not enhance the secretion of inflammatory cytokines (IL1, IL6, TNF), despite normal expression of pattern recognition receptors (such as TLRs) (Smythies et al., 2005).

Intestinal macrophages are implicated in the survival and maintenance of both adaptive and innate immune cells. First, macrophage-derived IL1β sustains Th17 cells in the mucosa (Shaw et al., 2012). Lamina propria macrophages have also been indirectly implicated in the induction of naïve T cell priming. In fact, lamina propria $CX3CR1^{+}$ macrophages pass the captured luminal antigen (orally administered protein or bacteria) to migratory $CD103^{+}$ cDC, thus indirectly contributing to priming of mucosal immunity (Mazzini et al., 2014; Rossini et al., 2014). Also, in response to the microbiota, lamina propria macrophages secrete IL1β that pushes the production of CSF-2 from ILC3, which drives cDC development (Mortha et al., 2014). Moreover, macrophages regulate their own replenishment via the secretion of monocyte-

recruiting chemokines (CCL2, CCL7 and CXCL16) (Takada et al., 2010; Zigmond et al., 2012). Finally, through the production of IL10, intestinal macrophages facilitate expansion of T_{reg} primed in the mesenteric lymph nodes (MLN) and involved in oral tolerance (Hadis et al., 2011). Although macrophages in the colonic lamina propria constitutively produce large amounts of IL10 (Bain et al., 2013), it is not the key factor in maintaining homeostasis. In fact, deletion of IL10R, but not IL10 itself, in macrophages causes spontaneous colitis in murine models (Zigmond et al., 2014a). Accordingly, maintenance of tolerance to microbiota seems to require IL10R signaling on macrophages, and IL10 produced by $CD4^+$ T_{reg} (Imbratta et al., 2019; Shouval et al., 2016; Zigmond et al., 2014a).

1.3.2.2.3 Monocyte differentiation into macrophages

Upon their arrival to the lamina propria, $Ly6C^{hi}$ monocytes go through a differentiation course that results in mature macrophage generation (**Figure 1-8**) (Bain et al., 2013; Tamoutounour et al., 2012). This process known as the "monocyte waterfall" (Tamoutounour et al., 2012) refers to the sequential differentiation of monocyte at the phenotypical, molecular, morphological and functional level (Bain et al., 2013; Kumawat et al., 2018; Tamoutounour et al., 2012). It involves short-lived $CXC3CR1^{int}$ intermediaries that first acquire MHCII, before losing Ly6C expression, and finally upregulating CX3CR1 expression to become $CX3CR1^{hi}Ly6C^{-}MHCII^{hi}$ mature macrophages (Bain et al., 2013; Smythies et al., 2010; Tamoutounour et al., 2012). Within 5-6 days, monocytes display the phenotype ($F4/80^{hi}CD64^{+}MHCII^{+}CD11c^{+}CX3CR1^{hi}$) and functional properties of resident intestinal macrophages by acquiring scavenger receptors, enhancing phagocytic activities, increasing IL10 production, and developing hypo-responsiveness to TLR stimulation (Bain et al., 2013; Smythies et al., 2010; Tamoutounour et

al., 2012). A similar process can be suggested in normal human intestine with the presence of $CD14^{hi}$ monocytes, intermediate populations, and $CD14^{lo}CD163^{+}CD206^{+}$ macrophages (Bain et al., 2013; Scott et al., 2016a).

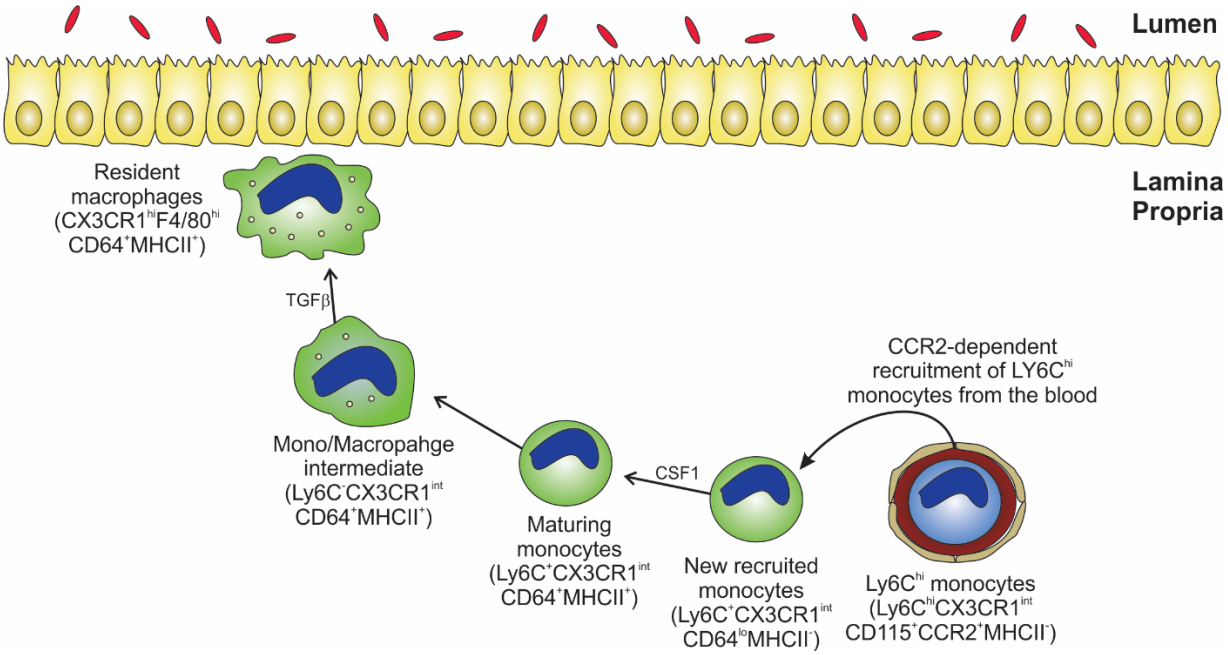


Figure 1-8: Monocyte "waterfall" in steady state intestine.
$Ly6C^{hi}$ monocytes are continuously recruited to the adult intestine at steady state in a CCR2-dependent manner. Upon arrival to the mucosa they differentiate through a series of intermediaries to finally develop into mature macrophages. It is guided by factors specific to the intestinal microenvironment, with the last step requiring TGFβ.

This developmental process is determined by multiple environmental factors specific to the intestinal mucosa. CSF1 is implicated in differentiation and survival of intestinal macrophage development. Indeed, $Csf1^{op/op}$ mice have reduced numbers of intestinal macrophages, despite normal level of monocytes in the bone marrow (Dai et al., 2004). Additionally, in WT:$CSF1R^{-/-}$ mixed bone marrow chimeric experiments macrophages are derived largely from WT (Ryan et al., 2001), and anti-CSF1R antibody treatments depleted intestinal macrophages (MacDonald

et al., 2010). Furthermore, IFNγR signaling has been associated with MHCII upregulation on macrophages, a key feature in the intestine (Steimle et al., 1994) Moreover, global transcriptomic and epigenetic analysis revealed that TGFβR plays an indispensable role in macrophage maturation in the intestine (Schridde et al., 2017). Furthermore, TGFβ has been described to increase CX3CR1 expression on microglia (Chen et al., 2002), and *Klf10* deletion in mice, a transcription factor crucial for TGFβR signaling, results in decreased numbers of CX3CR1^{hi} intestinal macrophages. Moreover, a unique feature of intestinal macrophages is RUNX3 expression, involved in the regulation of TGFβ signaling (Lavin et al., 2014). TGFβ does not play a role in the first stages of monocyte maturation in the tissue, rather in the final stages (Joeris et al., 2017).

The IL10-IL10R axis plays an important role in conditioning intestinal macrophage behavior. Disruption of the IL10-IL10R axis, either in myeloid cells or globally, results in a heightened response to TLR stimulation in murine colonic macrophages, such as increased expression of iNOS, IL23 and IL12, leading to the development of spontaneous colitis (Girard-Madoux et al., 2016; Kuhn et al., 1993; Shouval et al., 2016; Takeda et al., 1999; Zigmond et al., 2014a). In humans, non-functional mutations in IL10 or IL10R signaling lead to very early onset IBD (Glocker et al., 2009). In fact, macrophages from IBD patients with IL10RA and IL10RB polymorphisms respond to *in vitro* stimulation with LPS and adenosine triphosphate via the secretion of IL1β (Shouval et al., 2016).

The acquisition of CX3CR1^{high} expression by murine intestinal macrophages as well as their localization below the CX3CL1-producing epithelial cells implies a role of CX3CL1-CX3CR1

axis in macrophage differentiation. CX3CR1 is crucial for TED formation and thus sampling of luminal antigens (Chieppa et al., 2006; Kim et al., 2011a). Also, IL10 production is reduced in CX3CR1-deficient macrophages suggesting a role in the promotion of anti-inflammatory feature of murine intestinal macrophages. Importantly, and as quoted above, CX3CR1 is not expressed on human intestinal macrophages (Chakarov et al., 2019).

Finally, microbiota greatly influences macrophage development. First, monocyte recruitment, and thus, turnover of macrophage population is largely driven by microbiota (Bain et al., 2014; Niess et al., 2005; Shaw et al., 2018). The latter is required for IL1β and IL10 production (Kim et al., 2018; Ueda et al., 2010; Zigmond et al., 2014a). The microbiota may have a direct effect on macrophages via the secretion of short chain fatty acids or polysaccharides, as described for *Helicobacter hepaticus* (Correa-Oliveira et al., 2016; Danne and Powrie, 2018). Short chain fatty acids have various effects on macrophages, for example, butyrate suppresses *Nos2* and *Il12b* expression and increases oxidative phosphorylation (Chang et al., 2014; Scott et al., 2018), and propionate diminishes *in vitro* activation of macrophages (Ciarlo et al., 2016).

Taken together, several environmental factors control the differentiation and function of anti-inflammatory macrophages in the intestinal lamina propria at steady state.

1.3.3 MONOCYTE'S FATE IN INTESTINAL INFLAMMATION

Inflammation in the intestinal mucosa is generally caused by pathogenic microbes, injury to the protective epithelial layer leading to more contact with commensal bacteria, or defects in the

anergic macrophage populations. Under these inflammatory conditions the composition of the intestinal mononuclear phagocyte (MNP) pool changes.

1.3.3.1 *Interruption of the monocyte "waterfall"*

Various murine models of intestinal inflammation have been developed to better understand the inflammatory process; these include but are not limited to DSS-induced colitis, T cell transfer model of experimental colitis, and *H. hepaticus*-induced colitis. In these models, Ly6C^{hi} classical monocytes flood the intestine, and contrary to steady state, do not fully mature into anti-inflammatory CX3CR1^{hi} macrophages leading to an accumulation of CX3CR1^{int} populations (Arnold et al., 2016; Bain et al., 2018; Bain et al., 2013; Platt et al., 2010; Rivollier et al., 2012; Tamoutounour et al., 2012; Weber et al., 2011; Zigmond et al., 2012). The latter display pro-inflammatory characteristics: they secrete high levels of pro-inflammatory cytokines such as IL1β, IL6, IL12, IL23 and TNF, as well as reactive oxygen intermediates (ROI), and express high levels of TREM1 (Bain et al., 2013; Rivollier et al., 2012; Tamoutounour et al., 2012; Varol et al., 2009; Weber et al., 2011; Zigmond et al., 2012). During inflammation, monocyte recruitment involves CCR1 and its ligand CCL3 in addition to the classic CCR2-CCL2 axis governing its recruitment at steady state (Schulthess et al., 2012).

Similarly, inflammatory mucosa from patients with CD and UC present with an altered monocyte/macrophage compartment. Indeed, the accumulation of a unique, cytokine-producing CD14^{hi} monocyte-like population was noted in inflamed intestinal mucosa that outnumbered CD14^{lo}CD64^{+} resident macrophages (Bain et al., 2013; Grimm et al., 1995; Kamada et al., 2008; Rugtveit et al., 1997; Thiesen et al., 2014).

The reason why monocytes do not complete their maturation process in the inflamed mucosa remains elusive. Multiple factors may be involved in the interruption of the differentiation process during inflammation. For instance, the loss of anti-inflammatory signals promoting macrophage differentiation, as well as increased levels of pro-inflammatory cytokines block monocyte differentiation "waterfall." The re-establishment of the normal monocyte course in the inflamed intestine could prove to be a therapeutic route in IBD patients. In this thesis work, we aimed to better characterize $CD14^+$ MNP subsets in mucosa from IBD patients and investigate their generation and plasticity from monocytes.

1.3.3.2 *Monocyte-derived dendritic cells*

Monocytes arriving to the inflamed intestinal mucosa can also differentiate into DCs (Tang-Huau and Segura, 2019). This concept was described in peritoneal ascites from cancer patients and in synovial fluid from rheumatoid arthritis patients (Segura et al., 2013). These *in vivo* differentiated monocyte -derived DCs are referred to as "inflammatory DCs" since they develop mainly during inflammation. Although inflammatory DCs are morphologically and functionally similar to DCs, with their dendrites and ability to stimulate naïve T cells (Segura et al., 2013), transcriptional studies segregated them from cDC populations; they were found to closely resemble classical monocytes, (Goudot et al., 2017; Segura et al., 2013) thereby suggesting that inflammatory DCs are monocyte-derived.

$CD103^+CX3CR1^{int}$ DCs from murine intestine have been shown to travel to MLN, and activate, prime, and imprint naïve T cells to return to the gut but contrary to $CD103^+$ DC they induce

IL17 and IFNγ production by effector T cells (Cerovic et al., 2013). They secrete pro-inflammatory cytokines such as IL1β, IL6, IL12p70, IL23, and TNF upon *ex vivo* stimulation (Liao et al., 2017; Segura et al., 2013). Consistent with their IL23-secreting abilities, inflammatory monocyte-derived DCs from psoriatic skin, tumor ascites and synovial fluid of rheumatoid arthritis are strong inducers of Th17 polarization *ex vivo* (Segura et al., 2013; Zaba et al., 2009).

The term "inflammatory DCs" employed to describe monocyte derived-DCs can be misleading as it suggests that these cells are intrinsically inflammatory. Indeed, a DC subset identified as $CD103^{-}CCR2^{+}SIRP\alpha^{+}$ has been described in healthy intestine of humans, and is believed to have monocytic origins (Richter et al., 2018; Scott et al., 2015; Watchmaker et al., 2014). Recently, Richter *et al*. demonstrated by examining the phenotype and transcriptional profile of human non-inflammatory intestinal $SIRP\alpha^{+}$ DCs that monocytes contribute substantially to that heterogenous compartment (Richter et al., 2018). Transcriptional analysis related $CD103^{-}$ $SIRP\alpha^{+}$ DCs with circulating monocytes and functional characteristics were shared with mouse monocyte-derived $CD103^{-}CX3CR1^{int}$ inflammatory DCs (Watchmaker et al., 2014). Finally, during inflammation associated with CD, an accumulation of the $CD103^{-}SIRP\alpha^{+}$ DC population in the colon and MLN has been noted (Baba et al., 2013).

Collectively, monocytes arriving to the inflammatory intestinal mucosa are able to differentiate into inflammatory DCs, which are different from conventional DCs, *in situ*.

1.4 Monocyte differentiation in vitro

In order to better understand the function, differentiation, and plasticity of macrophages and monocyte-derived inflammatory DCs, several *in vitro* differentiation culture conditions have been developed starting from human circulating monocytes.

1.4.1 CLASSIC MONOCYTE-DERIVED DENDRITIC CELLS

Upon exposure to GM-CSF and IL4, monocytes from bone marrow of mice, or peripheral blood of humans or mice differentiate *in vitro* into DCs, referred to as monocyte-derived DCs (MoDCs) (Chow et al., 2017; Sallusto and Lanzavecchia, 1994). Indeed, the latter have dendrites and high expression of MHC and co-stimulatory molecules that are characteristic of DCs. Treatment with LPS, TNF, IFNγ or CD40L induced further maturation of MoDCs that upregulated their DC functional capacities: antigen processing and presentation, migration, and modulation of T cell responses (Leon et al., 2005). Noteworthy, mass cytometry analysis revealed multiple activation states within the *in vitro*-generated MoDCs (Helft et al., 2015; Sander et al., 2017).

In vitro MoDCs have been long considered surrogates of MoDC populations in tissue. Although it is now established that these in vitro-generated $CD14^-$ DCs do not resemble MoDCs in tissue; this *in vitro* model laid the ground for much of the current knowledge on MoDCs. For instance, they introduced the concept of DC maturation, with immature DCs requiring an extra cytokine signal to upregulate MHCII and co-stimulatory molecules, and reach their full functional capacities (Leon et al., 2005). The nature of the signal they receive confers variable functions to the DC population (Banchereau and Palucka, 2005). Furthermore, *in vitro* MoDCs were used

in anti-tumour vaccination trials (Banchereau and Palucka, 2005). Unfortunately, the tumour antigen loaded GM-CSF+IL4-derived DCs were unable to reach the draining lymph node and generate a strong adaptive response towards the tumour antigen (Guilliams and Malissen, 2015; Wimmers et al., 2014). Therefore, *in vitro* derived MoDCs helped better understand the function of MoDCs.

1.4.2 MONOCYTE-DERIVED MACROPHAGES

Macrophage polarization and plasticity takes place under distinct cytokine contexts that leads to macrophages capable of responding to their specific environmental milieu. Functional diversity is the key feature of macrophages. In this section, I will summarize what is currently known regarding macrophage polarization and the generation of classically activated macrophages (M1) and alternative activated macrophages (M2).

1.4.2.1 *M1 versus M2 macrophages*

In the 1980s, Nathan *et al.* recognized that the intracellular pathogen killing function of macrophages is promoted by IFNγ (Nathan et al., 1983). In direct contrast, IL4 induced a different gene expression program on macrophages as compared to IFNγ (Stein et al., 1992). The M1/M2 macrophage nomenclature was established by Mills *et al.* in 2000, founded on macrophage activation experiments using T lymphocytes from two mouse strains (Mills et al., 2000). In fact, upon LPS stimulation, T lymphocytes from C57BL/6J mice mainly secreted IFNγ and activated nitric oxide (NO) production from arginine by the so-called M1 macrophages, whereas T cells from Balb/c mice secreted IL4 and activated ornithine production from arginine

by the so-called M2 macrophages. It was not until 2014 that experimental guidelines for M1 and M2 *in vitro* generation were proposed for data reproducibility (Murray et al., 2014).

The classically activated M1 macrophages secrete pro-inflammatory cytokines and have potent microbicidal abilities. The classical activation is induced by IFNγ or the gram-negative bacterial component LPS or GM-CSF (CSF-2) (Chavez-Galan et al., 2015; Italiani and Boraschi, 2014; Martinez and Gordon, 2014; Orecchioni et al., 2019). M1 macrophages are characterized at the phenotypical level by expression of CD68, MHCII, and the co-stimulatory molecules CD80 and CD86. Furthermore, M1 macrophages are $IL12^{hi}IL23^{hi}IL10^{lo}$ and secrete IL1β, TNF and IL6, thus they induce efficient Th1 and Th17 responses in an IRF5-dependent manner (Hoeve et al., 2006; Krausgruber et al., 2011; Verreck et al., 2006). M1 macrophages express an intracellular protein SOCS3 and activate iNOS to generate NO from arginine; thus, they aggravate the inflammatory response (Arnold et al., 2014; Zeidler et al., 2004). However, M1 macrophages play a protective role in the context of pathogen invasion with their aptitude to phagocyte large numbers of pathogens and kill intracellular bacteria via NO synthesis, iron and nutrient restriction as well as phagosome acidification (Andrade et al., 2012; Nairz et al., 2013; Podinovskaia et al., 2013).

Alternatively activated M2 macrophages are generated upon exposure to M-CSF (CSF1) with IL4, IL10, IL13, TGFβ, helminth or fungal infections (Chavez-Galan et al., 2015; Italiani and Boraschi, 2014; Martinez and Gordon, 2014; Orecchioni et al., 2019). They play a protective role in parasite infection and are involved in tissue remodeling and repair. M2 macrophages are identified by the expression of CD200R and CD163 in combination with the expression of the

transcription factor CMAF (Barros et al., 2013). Although the mannose receptor (CD206) was previously a marker for M2, Jaguin *et al.* observed no difference in its expression between M1 and M2 macrophages (Jaguin et al., 2013). In addition, M2 polarization under M-CSF and IL4 stimulation involves RGC-32, a cell cycle regulator whose expression is increased by M2 macrophages, and could be considered a marker for this population (Zhao et al., 2015). Moreover, the production of arginase 1, upon exposure to type 2 cytokines (IL4 and IL13), is a hallmark of alternatively activated M2 macrophages (Pauleau et al., 2004). It protects the host from parasite infections such as intestinal nematode parasites (Anthony et al., 2006). The tissue repair function of M2 macrophages is ensured in part by ornithine since it promotes cell proliferation and repair via polyamines, glutamate and collagen synthesis (Morris, 2007). Finally, M2 macrophages are $IL10^{hi}TGF\beta^{hi}IL12^{lo}IL23^{lo}$, and thus, contribute to immunoregulation.

Although T cell cytokine production was employed to promote M1 and M2 macrophage polarization, it is now clear that their polarization can occur without lymphocytes (Italiani and Boraschi, 2014; Mills, 2012). In fact, microbial molecules such as LPS and inflammatory cytokines such as TNFα and IFNγ activate the M1 differentiation/functional program. Conversely, *in vitro* M2 polarization is observed in response to anti-inflammatory cytokines such as IL4, IL10, IL13 or TGFβ, as well as glucocorticoids, immune complexes or concomitant activation of Fcγ receptors and TLR (Chavez-Galan et al., 2015; El-Behi et al., 2011; Italiani and Boraschi, 2014; Martinez and Gordon, 2014; Orecchioni et al., 2019). These different stimuli lead to a variety of functional programs in macrophages (**Figure 1-9**), thus three types of M2 macrophages were described: M2a (activated with IL4 and IL13), M2b (activated with

immune complexes or simultaneous FcγR and TLR triggering), and M2c (activated by IL10, TGFβ, glucocorticoids) (Mantovani and Marchesi, 2014). In particular, M2a macrophages secrete chemokines for the recruitment of Th2 cells, basophils and eosinophils and drive a type II response. M2b macrophages recruit T_{reg} and therefore, play a role in immunoregulation. Finally, M2c are implicated more for tissue remodeling.

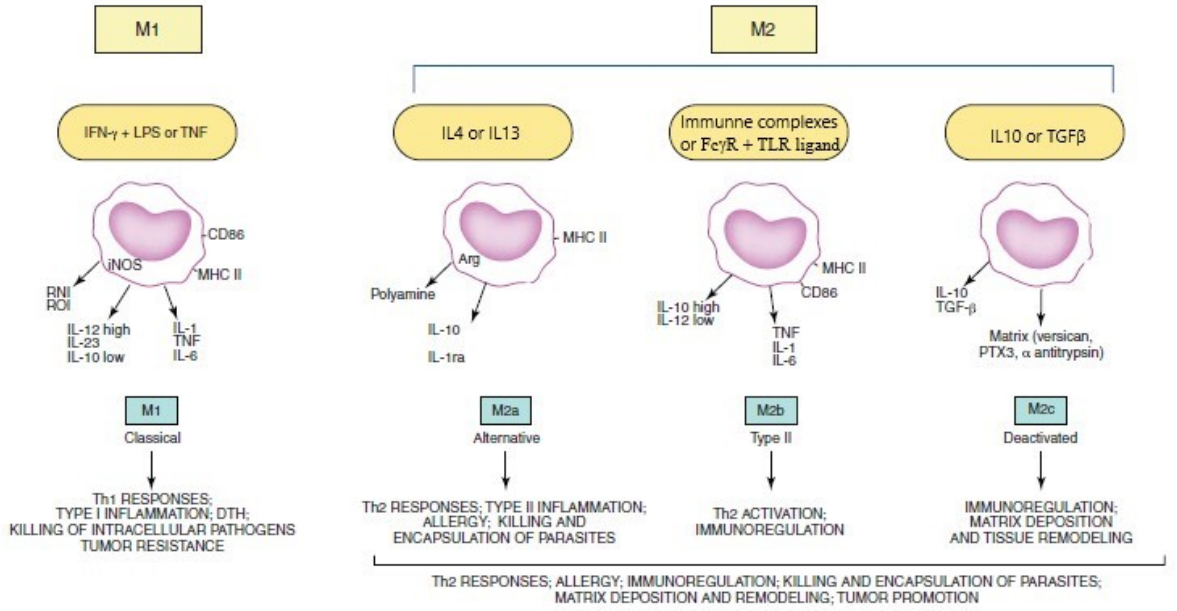


Figure 1-9: The M1 – M2 model proposed by Mantovani et al.
Montovani et al. proposed a model that dissects the in vitro differentiated M2 macrophages based on the stimuli they receive: IL4 and IL13 lead to M2a, immune complexes or TLR with FcγR ligands lead to M2b, and finally IL10 or TGFβ lead to M2c.
(DTH: delayed type hypersensitivity; MR: mannose receptor; RNI: reactive nitrogen intermediates).
(Taken from from (Mantovani and Marchesi, 2014); authorization code: 4623251232702).

Taken together, *in vitro* macrophage activation/differentiation state depends on the various stimuli they receive, including cytokines, microbial products, and other factors.

1.4.2.2 *The spectrum of macrophages*

Until very recently, macrophage complexity and plasticity were explained by sub-dividing them into two functionally discrete M1 and M2 subsets. However, studies over the last few years proved that this dichotomous notion is too simplistic since macrophages are highly heterogeneous and can respond to most changes in the tissue environment (Guilliams and van de Laar, 2015; Italiani and Boraschi, 2014; Martinez and Gordon, 2014; Nahrendorf and Swirski, 2016; Natoli and Monticelli, 2014; Xue et al., 2014). Furthermore, M1 and M2 macrophages were defined in the era where a few phenotypic markers were sufficient to properly distinguish two populations. Transcriptomic and proteomic analyses in tissues from healthy and diseased individuals revealed that the picture is far more complex, thus challenging the current nomenclature (**Figure 1-10**).

Macrophages modulate their functional phenotypes based on the changing tissue environment. Therefore, the M1/M2 discrete polarization model simplifies the continuum of the various functional states observed within tissues. Noteworthy, M1 and M2 macrophages are not ontogenically defined *in vivo* subsets, they are however the two extremes of *in vitro* derived macrophages under different culture conditions (Martinez and Gordon, 2014; Mosser and Edwards, 2008; Sica and Mantovani, 2012). In 2008, Mosser and Edwards reclassified the M1/M2 macrophages into three population based on their function in host defense, wound healing and immune regulation; a spectrum of macrophages was suggested in between (Fleming and Mosser, 2011; Mosser and Edwards, 2008) . Later, Xue *et al.* reinforced this notion by differentiating human monocyte derived cells using a combination of 28 different stimuli

leading to a wide variety of transcriptionally distinct macrophage populations (Natoli and Monticelli, 2014; Xue et al., 2014), thus portraying a high level of macrophage diversity.

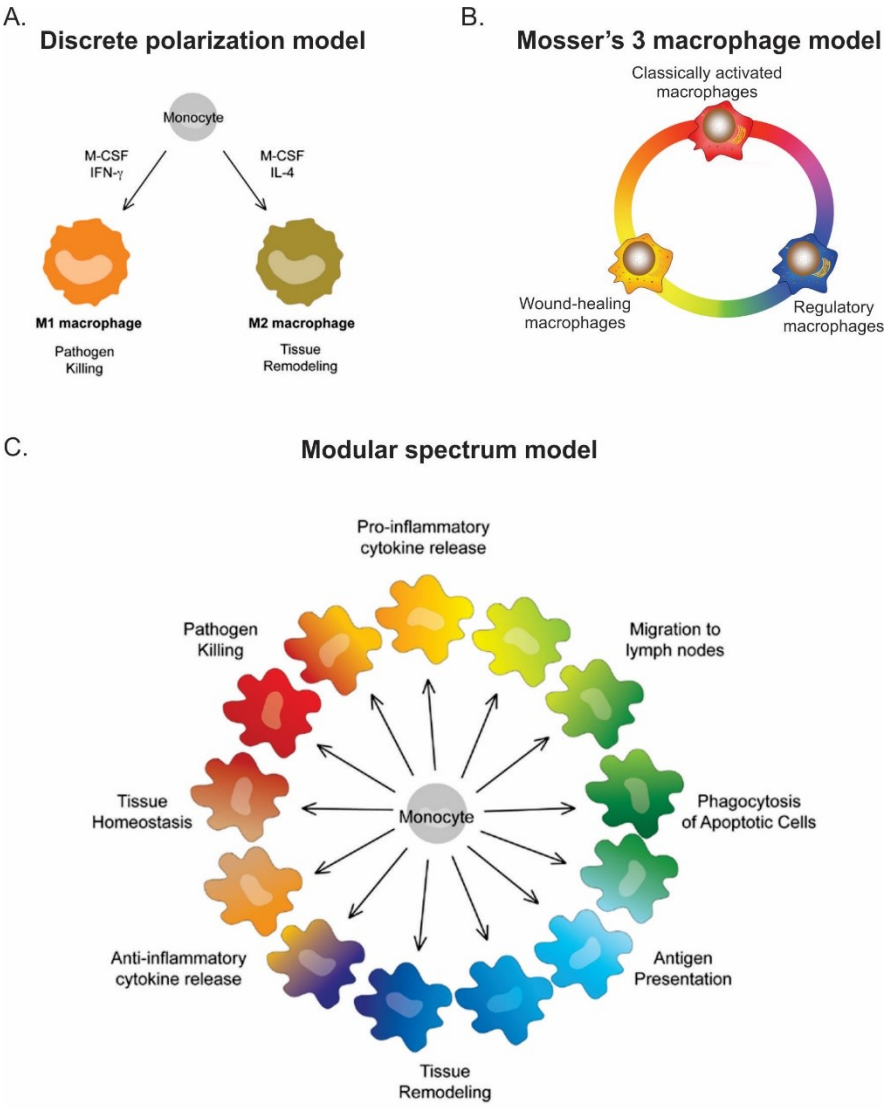


Figure 1-10: The color wheel spectrum of macrophage activation.
Replacement of the (A) polarized M1 and M2 model by (B) the color wheel of macrophage activation defined by three distinct macrophage populations (Mosser and Edwards, 2008), leading to (C) the modular spectrum model.
(Adapted from (Guilliams and van de Laar, 2015) and (Fleming and Mosser, 2011)).

Although this work advanced our understanding of macrophage activation, yet it is only the tip of the iceberg since it does not account for macrophage plasticity. The latter is a key characteristic of macrophages that has been demonstrated *in vitro* (Italiani and Boraschi, 2014); whether and how it occurs *in vivo* remains questionable, and will be further discussed in this thesis work.

1.5 Journey of antigen presenting cells from intestinal mucosa to mesenteric lymph nodes

The establishment of an antigen-specific adaptive immune response involves the migration of professional antigen presenting cells (APCs), such as DCs, and to a lesser extent, monocytes to the MLN for antigen presentation to naïve T cells.

1.5.1 INTESTINAL ANTIGEN ARRIVAL TO MLN

The antigen follows one of two mechanisms to reach the LN: it can either drain through the lymph or be carried by professional APCs.

The former mechanism requires that the antigen (of specific diameter <200nm) is present at high dose at the site of infection (Bachmann and Jennings, 2010). The antigen reaches the LN through the lymph and travels to the marginal sinus where LN resident macrophages and DCs sample it. Bacterial translocation to the LN, detected by bacterial messenger RNA (mRNA) presence in the lymphoid tissue, has been reported in patients with either CD or UC (Kiely et al., 2018; Kiernan et al., 2019; O'Brien et al., 2014). The diversity and abundance of the identified bacterial populations were analogous in the intestinal mucosa and MLN of the same patient, suggesting the gut origin of LN detected bacteria (Kiely et al., 2018). In fact, Kiernan *et al.* recently reported apparent differences in MLN microbiome of CD and UC patients reflecting further the differential microbiome composition in the mucosa (Kiernan et al., 2019).

The second mechanism involves migratory DCs that first capture the luminal antigens that could be accomplished by several mechanisms (**Figure 1-11**). In the epithelial layer overlying the

lymphoid follicles (Peyer's patches, (PP) and isolate lymphoid follicles, (ILFs)), microfold (M) cells transport microbes and particles to DCs that lie in the M cells' basolateral membrane, or the underlying sub-epithelium dome (Pabst and Mowat, 2012). Antigen access through the villus epithelium can involve the rare M cells, described outside PP, or direct transportation across enterocytes (Jang et al., 2004). The latter may also transport antigen-antibody complexes by transcytosis in a neonatal IgG receptor (FcRn)-dependent manner to $FcRN^+$ DCs below the epithelial layer (Yoshida et al., 2004). Furthermore, goblet cells have been described as an antigen conduit across the epithelial layer to the underlying $CD103^+$ DCs, a mechanism essential for oral tolerance (McDole et al., 2012). Intestinal $CD103^+$ DCs are actually equipped to extend dendrites across the epithelial layer and capture bacteria (Farache et al., 2013). $CX3CR1^+$ macrophages are also capable of extending processes to sample antigen from the lumen (Chieppa et al., 2006; Niess et al., 2005). As macrophages do not migrate to the MLN, the antigen gets transferred via connexin 43-dependent gap junction to $CD103^+$ DCs (Mazzini et al., 2014) that travel to the lymphoid tissue.

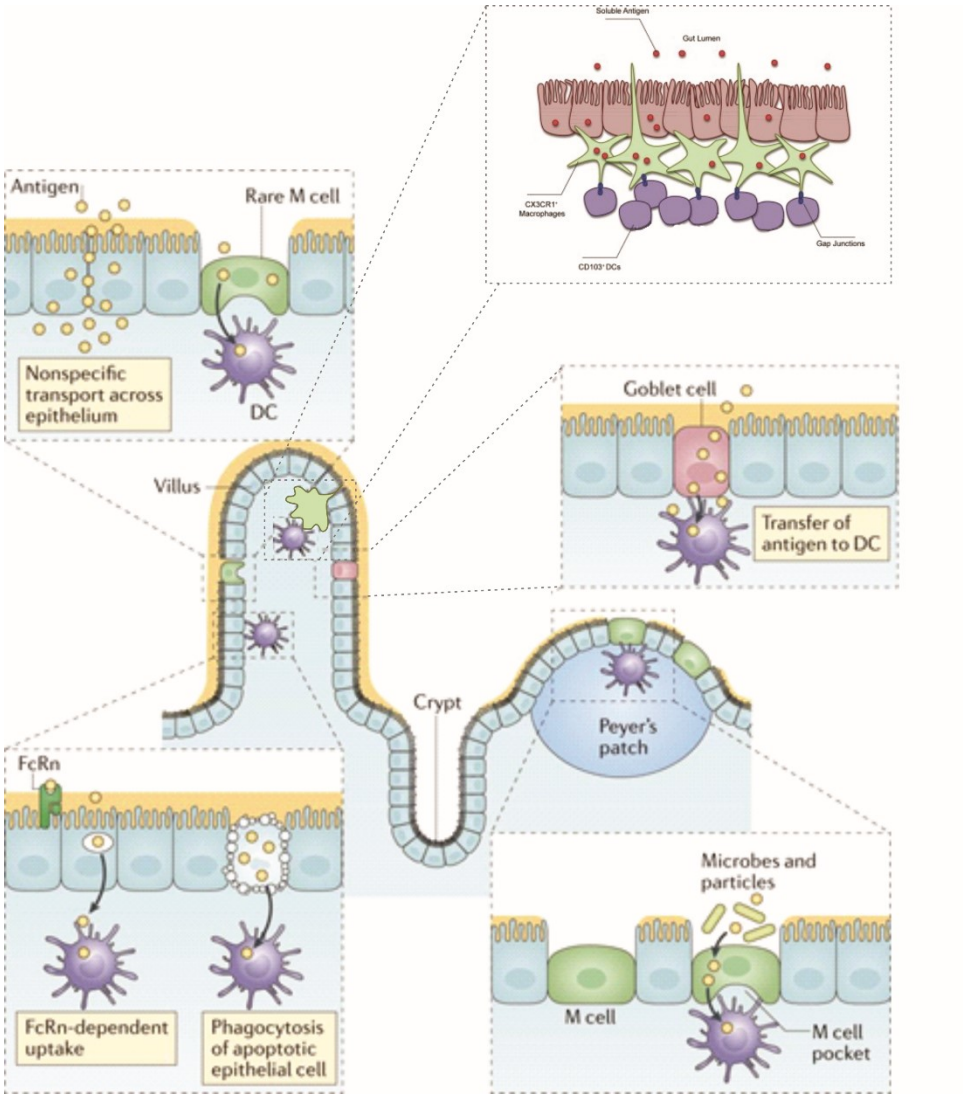


Figure 1-11: Antigen acquisition by dendritic cells in the intestine for the induction of tolerance.

DCs utilize several mechanisms to gain access to antigens in the intestinal lumen at steady state by positioning under the epithelium and sampling antigens that cross the epithelial layer (1) non-specifically, (2) by transcytosis in an FcRn-dependent manner, (3) through goblet cells. Finally, (4) CX3CR1$^+$ macrophages transfer the captured antigen to intestinal CD103$^+$ via connexin 43.

(Adapted from (Mowat, 2018); authorization code: 4624370245227 and (Mazzini et al., 2014); authorization code: 4624831142050).

The antigen-loaded migratory DC travel from the intestinal lamina propria, through the lymphatic vessels, to the MLN guided by CCR7 and sphingosine 1-phosphate (S1P). Migratory

DCs have the option of presenting the antigen to T cells in the T cell zone or transfer it to MLN-resident DCs or B cells (Allan et al., 2006).

1.5.2 PRESENTATION OF ANTIGEN BY ANTIGEN PRESENTING CELLS TO NAÏVE T CELLS AND POLARIZATION OF THE IMMUNE RESPONSE

The initiation of a T cell response involves the encounter of an activated APC displaying the proper peptide-MHC ligand with a naïve $CD4^+$ or $CD8^+$ T cell in the T cell zone of the LN. It leads to naïve T cell proliferation and differentiation into various types of effector and memory cells.

In order to guarantee an immune response against pathogens, large number of naïve T cells with unique T cell receptors (TCRs) must be confined to a limited physical space, such as the T cell zone in the LN. With age-related thymus atrophy, naïve T cell pool in humans is maintained by peripheral T cell proliferation (den Braber et al., 2012). The survival and proliferation of the naive T cell compartment in the periphery under steady state conditions is upheld by IL7 and self-peptide MHC complexes (Takada and Jameson, 2009).

$CD4^+$ naive T cell priming involves three signals: the first derives from the specific interaction of TCR with the right peptide:MHC complex. Although TCR engagement is essential for naïve T cell activation, induction of T cell proliferation and differentiation necessitates two other signals delivered by the same APC. In fact, the second signal is delivered by co-stimulatory molecules, such as CD80 and CD86 (B7 molecules), specifically expressed on the surface of APCs. They bind to CD28 and induce naïve T cell expansion. Finally, the third signal is dictated

by the cytokines secreted by APCs, and instruct the differentiation of functionally specific effector T cell (T_{eff}) subsets.

1.5.3 GENERATION OF EFFECTOR T CELLS

Following cognate antigen recognition by TCR, activated $CD4^+$ T cells differentiate into T_{reg} or effector helper T cells (Th) depending on the cytokine milieu. These cells orchestrate immune responses via the cytokines they secrete.

1.5.3.1 *The classics: Th1 and Th2 cells*

Under inflammatory conditions, naïve T cells differentiate into helper T cells, including Th1 and Th2 cells. Mosmann *et al.* reported in 1986 that naïve $CD4^+$ T cells differentiated into two functionally different cells based on their cytokine production profile, the classic Th1 and Th2 effector cells (Mosmann et al., 1986). Th1 cells play a vital role against intracellular bacteria and viruses, and have been described in autoimmune diseases. They differentiate in the presence of IL12 and IL18, which phosphorylates STAT4 and STAT1, causing T-bet upregulation, the key Th1 transcription factor. T-bet, encoded by *TBX21* gene, targets Th1 signature genes *CXCR3* and *IFNG*. In contrast, Th2 cells control, via IL5 and IL13 secretion, helminth infections, and have been associated with allergic diseases. Th2 cell differentiation is guided by IL4 and IL5 which activates STAT6, and therefore, upregulates *GATA3* transcription (Stadhouders et al., 2018). This dichotomous Th1/Th2 division is further reinforced by the fact that T-bet, IL12 and IFNγ antagonize Th2 polarization by inhibiting GATA3, whereas Th1 differentiation is repressed by the IL4-GATA3 axis (Szabo et al., 2003). This notion of mutually

exclusive, terminally differentiated Th subsets was challenged by the discovery of a hybrid Th1/Th2 cell co-expressing IL4 and IFNγ (Peine et al., 2013).

1.5.3.2 *The new Th17/T_{reg} paradigm*

The classical Th1/Th2 paradigm shifted during the last two decades to the Th17/T_{reg} paradigm, which helped shed light on T cell-mediated immune disorders including IBD.

1.5.3.2.1 Th17 cells

Identified in 2005, Th17 cells (Harrington et al., 2005; Park et al., 2005) are mainly found in mucosal tissues such as the gastrointestinal tract. They express the Th17 marker CCR6 and lack CXCR3 expression, the Th1 marker (Annunziato et al., 2007). Th17 lineage differentiation requires the transcription factor RAR-related Orphan Receptor gamma (RORγ) that is stimulated by STAT3 phosphorylation under the influence of IL1β, IL6 and TGFβ in mice. Although previously perceived as a main Th17 inducer, IL23 is only needed for Th17 expansion and maintenance (Zhou et al., 2007). The master transcription factor RORγ, along with the STAT3, IRF4, PLZF and BATF, control the expression of Th17 specific genes including *CD161, IL23R, IL17A* and *IL17F* (Singh et al., 2015; Sun et al., 2017) (Veldhoen et al., 2009; Veldhoen et al., 2008). Indeed, Th17 cells secrete IL17A, IL17F, IL21, IL22 and GM-CSF, which mediate mucosal homeostasis and protection against bacterial and fungal pathogens. Noteworthy, GM-CSF has a dual role as T cell-derived GM-CSF has been implicated in intestinal inflammation development in a colitis model (Pearson et al., 2016).

Until recently, CD was labeled a Th1 disease due to the high levels of IL12 and IFNγ, whereas UC had higher production of IL5 and IL13 and was designated a Th2 disease. However, both diseases are now considered Th17 diseases.

1.5.3.2.2 Regulatory T cells

The generation of T_{reg} involves TGFβ and the up-regulation of the transcription factor FoxP3 (Russler-Germain et al., 2017). $FoxP3^+$ T_{reg} are either generated in the thymus (tT_{reg}), or induced by TGFβ in peripheral sites (pT_{reg}). This heterogenous $CD4^+$ T cell population is endowed with suppressive functions, maintained by IL10 and surface expression of CTLA4 that prevents CD28 binding to its ligands on APCs, thus preventing T cell co-stimulation (Liu et al., 2001). Moreover, Helios expression, an Ikaros transcription factor (TF) family member, is required for the stable inhibitory activities of $FoxP3^+$ T_{reg} (Kim et al., 2015a).

1.5.3.2.3 Maintenance of intestinal homeostasis by regulatory T cells

The intestinal mucosa is constantly exposed to exogenous antigens from food and microbiota. It is crucial that the intestinal immune system hypo-respond to the foreign antigens it encounters on a daily basis, a process referred to as oral tolerance (Mowat, 2018). It is mediated in part by $CD103^+$ DCs that acquire and process intestinal antigens, present it to naïve T cells in the MLN and induce the generation of T_{regs} expressing gut homing molecules (Jaensson et al., 2008; Sun et al., 2007). The latter engage the ability of $CD103^+$ DCs to metabolize retinoic acid (RA) from dietary vitamin A. In fact, $CD103^+$ DC-derived RA, along with TGFβ, mediate naïve Treg differentiation (Sun et al., 2007). Additionally, *in vitro* RA is sufficient to upregulate CCR9 and integrin α4β7 gut-homing molecules on activated T cells, and thus, facilitate their return to the

gut tissue. Indeed, blockade of RA synthesis or its signaling inhibit gut-homing molecule induction by $CD103^+$ DCs (Iwata et al., 2004), and thus, induction of oral tolerance.

Once in the mucosa, T_{reg} cells expand and are maintained by IL10 produced by $CX3CR1^{hi}$ macrophages, thus sustaining an inhibitory environment (Hadis et al., 2011; Murai et al., 2009).

T_{regs} employ several mechanisms for suppression of the inflammatory response (Whibley et al., 2019). First, T_{regs} express CTLA-4, an inhibitory receptor that controls intestinal inflammation, more specifically the type 2 response (Ohnmacht et al., 2015; Read et al., 2000). Indeed, patients treated with anti-CTLA-4 antibody developed colitis (Gupta et al., 2015). Second, T_{regs} immunosuppressive role in the gut implicate TGFβ1 and IL10. Actually, genetic mutations in TGFβ1 and IL10 receptors (*TGFBR1*, *TGFBR2, IL10RA*, *IL10RB)* lead to the development of severe, early-onset IBD (Barnes and Powrie, 2009; Uhlig and Powrie, 2018). Third, intestinal $Helios^+GATA3^+$ T_{regs} express ST2 and amphiregulin, and thus, might play a role in tissue repair (Schiering et al., 2014; Sefik et al., 2015; Wohlfert et al., 2011). Finally, T_{regs} in the mucosa play an important role in promoting a Th17 response. Depletion of T_{regs} in mice increases susceptibility to *Citrobacter rodentium* infection, normally linked with Th17 cells (Wang et al., 2014). The exact mechanism used by T_{regs} *in vivo* remains poorly understood.

1.5.3.2.4 Maintenance of intestinal homeostasis by Th17 cells

Th17 cells in the intestinal mucosa depend on gut microbiota, more specifically by the presence of the Gram-positive commensal SFB (Gaboriau-Routhiau et al., 2009; Ivanov et al., 2009). Indeed, colonization of germ-free mice with SFB generates Th17, but not Th1, cells that home

to small and large intestine lamina propria (Ivanov et al., 2009); reciprocally, Th17 cells regulate SFB levels. Moreover, deletion of IL17R expression in enteric epithelium causes a decrease in α-defensins, NADPH oxidase 1 expression and polymeric immunoglobulin receptor required for secretory IgA transcytosis; thus, leading to SFB dysbiosis (Kumar et al., 2016). Generation of Th17 cells by SFB is mainly rendered by the recognition of microbe adhesion to the intestinal epithelial cells (Atarashi et al., 2015). Interestingly, the latter also promote intestinal IgA^+ cells (Atarashi et al., 2015). IgA is mostly secreted across the intestinal mucosal surface and play an important role in maintaining homeostasis towards the microbiota, especially in the small intestine (Macpherson et al., 2018). This is accomplished through IgA's diverse functions, such as blocking bacterial adherence and translocation across the epithelial layer, neutralizing viruses and toxins, sampling of luminal antigens, as well as removing immune complexes at the epithelial surface. IgA can also exert its function by altering bacterial gene expression and limiting their effect on the intestinal mucosa. Therefore, IgA plays an important role in preserving equilibrium with microbiota.

The protective function of Th17 cells at the barrier is achieved by IL17 and IL22, which induce the regenerating islet-derived 3 (REG3) anti-microbial proteins REG3β and REG3γ (Kolls et al., 2008). Moreover, IL17 modulates tight junction protein expression in epithelial cells. In fact, it increases claudin (Kinugasa et al., 2000) and regulates occludin expression during epithelial injury in a DSS-induced colitis model, thus reducing gut permeability (Lee et al., 2015). Moreover, IL17 mediates the expression of neutrophil recruiting signals (CXCL1 and CXCL5), important for protection against bacterial and fungal infections (Conti and Gaffen, 2015). IL17-secretion is not restricted to Th17 cells. However, in the intestine they are the main

source (Hirota et al., 2011), whereas ILC3 contribute mainly to intestinal IL22 (Ahlfors et al., 2014). The latter is essential in tissue repair, and prevention of pathogen attachment and tissue dissemination (Zheng et al., 2008). IL22 also regulates goblet cells differentiation and mucin 2 expression (Birchenough et al., 2015).

1.5.3.3 *The other Th cells and their role in IBD*

Polarized Th states including Th9, Th22 and follicular helper T (TFH) cells have also been described in IBD. Each population is characterized by a transcription factor network and a cytokine secretion pattern.

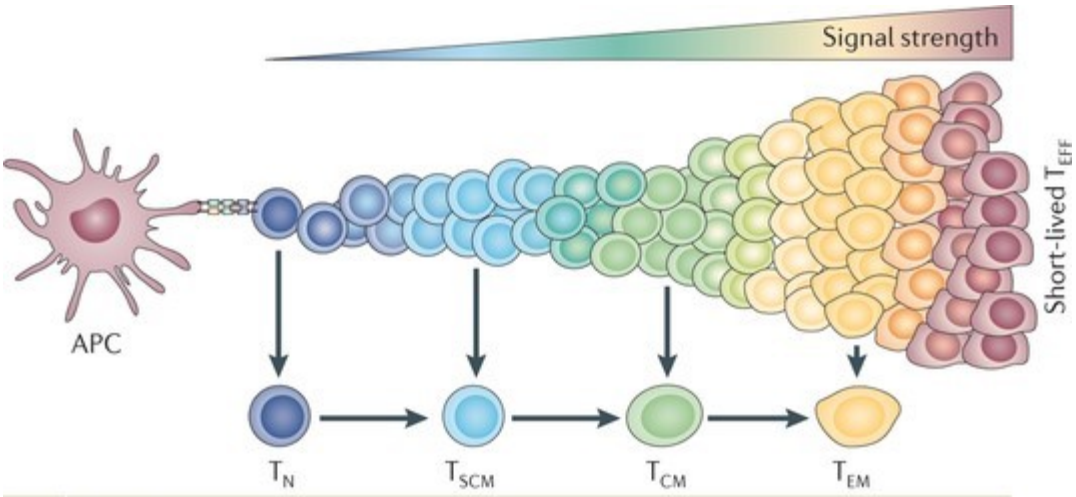
In addition to promoting T_{regs} and non-pathogenic Th17 differentiation, TGFβ plays a role along with IL4 in the development of Th9, an IL9-expressing Th subset. Although originally thought of as a Th2 population, it is now clear that the $GATA3^-$ population represents a population on its own (Dardalhon et al., 2008). In fact, TGFβ, through Sox4 transcription factor, suppressed GATA3 induction (Kuwahara et al., 2012). In addition to its protective role in helminth infections, Th9 cells have been shown to induce inflammation and exacerbate inflammatory disease such as IBD (Vyas and Goswami, 2018). In fact, it is observed that Th9 cell transfer to RAG-deficient mice aggravated gut mucosa inflammation (Gerlach et al., 2014). Furthermore, a positive correlation is noted between IL9 mRNA levels and the inflammation score in colonic biopsies from UC patients (Nalleweg et al., 2015). These data suggest a crucial role for Th9 in IBD progression.

Another Th subset described to play a role in IBD pathogenesis is Th22 cells. These $CCR6^+$ $CD4^+$ T cells are related to Th17 cells but have low expression of RORγ. Th22 development is induced by TNF and IL6, which promote STAT3 and aryl hydrocarbon receptor (AHR) expression, leading to the secretion of IL22. The latter is a member of the IL10 cytokine family. IL22 plays an important role in epithelium healing, and defence against invading pathogens at epithelial surfaces as IL22 receptor is only found on epithelial cells in the gut (Azizi et al., 2015; Basu et al., 2012). In fact, a decline in $IL22^+IL17^-$ $CD4^+$ cells was noted in the inflamed colonic mucosa of UC, but not CD, patients relative to healthy controls. This inhibition was thought to be due to an up-regulation of TGFβ expression (Leung et al., 2014). In fact, high levels of IL22 have been associated with the development of colorectal cancer (Kryczek et al., 2014)

Finally, TFH cells exist in germinal centers and help B cell proliferation, differentiation and immunoglobulin class switch. Generation of the $CXCR5^+PD\text{-}1^+$ TFH IL21-producing cells was recently shown to implicate both cDC2 and $CD14^+$ macrophages in the tonsils (Durand et al., 2019), as well as regulatory TFH (known as TFR), residing at the T-B border in the MLN (Sayin et al., 2018). It involves the transcription factor BCL6, and IRF4, TCF-1, c-MAF and STAT3 signaling molecules. TFH cells play an important role in the control of commensal microbiota through T cell dependent IgA production control (Crotty, 2014; Jogdand et al., 2016). A recent study observed an upregulation of TFH signature genes (*IL21*, *CXCR5*, *ICOS*, *PD1* and *BCL6*) in colonic tissues of CD patients, and mice with colitis when compared to normal controls (Zhang et al., 2019). Furthermore, in the intestinal lamina propria of CD patients the fraction of IL21 secreting TFH cells was higher than in UC patients and healthy controls (Sarra et al., 2010). Thus, dysregulation of TFH cells has been described in CD pathogenesis.

1.5.4 RECALLING ANTIGENS: MEMORY T CELLS

T cell antigen exposure is recalled by memory T cells that offer long-term protection against pathogens. Studies of mouse models have documented that in a primary immune response antigen-specific $CD4^+$ or $CD8^+$ naïve T cells encounter antigen-bearing APCs, become activated, undergo expansion, and differentiate, through a continuum, into short-lived T_{eff} cells (**Figure 1-12**). This differentiation process involves the up or down regulation of several chemokine receptors and adhesion molecules involved in T cell trafficking which will be explored in a later section. Effector T cells comprise the precursors of antigen-specific memory T cells. This heterogeneous population persists in different locations *in vivo* and orchestrates a protective immune response upon pathogen re-encounter. Memory T cells are endowed with key properties including tissue residence/trafficking, effector function and long-life (Jameson and Masopust, 2018).



Phenotype	T_N	T_{SCM}	T_{CM}	T_{EM}
CCR7	+++	+++	++	–
CD62L	+++	+++	++	–
CD27	+++	+++	++	+/–
CD28	++	+++	+++	+/–
CD45RA	+	+	–	+/–
CD45RO	–	–	+	+
CD122	–	++	+++	+++
CD95	–	++	+++	+++
KLRG1	–	–	+/–	++

Figure 1-12: Progressive T cell differentiation to generate short-lived T_{eff}.
During an immune response, APCs prime naïve T cells and induce T cell proliferation culminating in the generation of T_{eff}. This process depends on the strength and quality of the signal. In fact, when antigenic stimuli cease, some primed T cells enter the pool of memory cells depending on the signal strength received. Other models for memory T cell generation have been proposed as well. The table describes the phenotype of naïve and memory T cell subsets.
(KLRG1: killer cell lectin-like receptor)
(Adapted from (Gattinoni et al., 2012); authorization code: 4624961295462).

1.5.4.1 *Heterogeneity within the T cell memory compartment*

T cell subset diversity results from antigenic and environmental stimuli received during T cell priming and subsequent recall responses.

In humans, memory T cells are classically identified by CD45RO expression and lack of CD45RA. The heterogeneity of the $CD45RO^{+}CD45RA^{-}$ memory T cell population was first described in human peripheral blood by Sallusto based on the expression of a LN homing molecule CCR7. While the $CCR7^{+}$ central memory T cells (TCM) traffic through lymphoid tissues, the $CCR7^{-}$ effector memory T cells (TEM) migrate to peripheral tissues (Sallusto et al., 1999). Despite the name, effector capacity is not limited to TEM cells. Actually, effector cytokine production in response to antigen stimulation has been noted in both TCM and TEM cells, but TCM cells have higher proliferative capacity (Mahnke et al., 2013; Sallusto et al., 1999).

Human peripheral blood memory T cells have been further subdivided based on the expression of additional surface markers. Expression of the prototypic death receptor CD95 as well as CD122 defined the stem cell memory T (TSCM) in humans. This naïve-like population, which arises following antigen stimulation, is $CD45RA^{+}CD45RO^{-}$ and expresses the co-stimulatory receptors CD27 and CD28, as well as CD62L and CCR7. TSCM are endowed with high proliferative and self-renewing capacities, combined with multipotency to differentiate into TCM, TEM and T_{eff} cells, thus, defining their 'stem cell-like' properties (Gattinoni et al., 2012; Gattinoni et al., 2011).

Peripheral blood does not show the full heterogeneity within memory T cells. Murine studies established that memory $CD4^{+}$ and $CD8^{+}$ T cells populate tissues and persist after viral or antigen clearance (Masopust et al., 2001; Reinhardt et al., 2001). In fact, in human intestinal and lung explants, $CCR7^{-}$ TEM were the principal memory T cell subset, whereas tonsils

included TCM and TEM populations (Campbell et al., 2001b). A further look at memory T cells within mouse tissues recognized the existence of a new tissue-resident memory T (TRM) cell subset that resides in peripheral tissues and induces a rapid protective response *in situ* upon re-exposure to antigen. TRM are discriminated from splenic and circulating memory T cells by expression of the early activation marker CD69 that retains them in tissue as well as CD103 (Gebhardt et al., 2018).

1.5.4.2 *Models of memory T cell generation*

The pathways that govern an activated T cell fate have been extensively studied in mice. Yet the stage during which a T cell decides whether to become a long-lived memory cell or die remains very controversial. After experimentally dismissing the idea that different naïve T cell clones give rise to short-lived effector and long-lived memory cells (Gerlach et al., 2013; Stemberger et al., 2007), the field is left with two strong models (**Figure 1-13**). In the first model, T cell fate is determined early in the immune response during the first asymmetric cell division that gives rise to a daughter memory cell and a short-lived effector cell. The second model proposes that following activated T cell proliferation a "fate-decision" is made (Farber et al., 2014; Jameson and Masopust, 2018). Evidence supporting the two models are compelling but there is no definitive answer to settle the conflict.

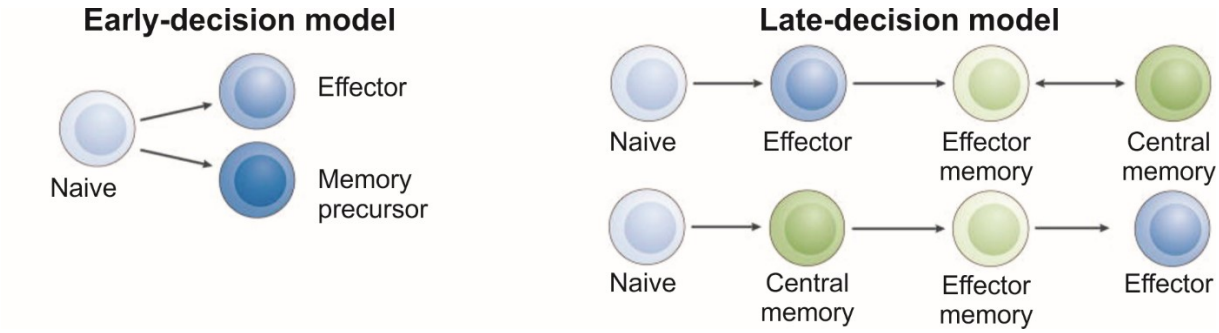


Figure 1-13: The two models recognized for memory T cell differentiation.
The "early-decision model" involves an asymmetric division generating effector or memory cells, whereas the "late-decision model" involves a linear progression.
(Adapted from (Espinosa et al., 2016); authorization code: 4625001273486).

The "early decision model" has been validated by recent single-cell gene expression studies that explored $CD8^+$ T cells isolated hours or days after viral infection, i.e., cells undergoing the first division towards effector or memory populations. Interestingly, gene expression patterns differed between cells at the first-division stage, effector and memory cells. Computational models determined that during the first cell division "pre-effector" or "pre-memory" gene-expression patterns are already present (Arsenio et al., 2014; Kakaradov et al., 2017). Arsenio *et al.* added that cells with "pre-effector" trait can still have a long-life as a population resembling TEM, whereas "pre-memory" daughter cells give rise to classic TCM and TEM cells (Arsenio et al., 2014). In contrast, a common differentiation program for effector and memory cells is supported by the fact that memory $CD8^+$ T cells possess effector-characteristics, for example transcriptional expression of granzyme B (Bannard et al., 2009). Furthermore, epigenetic studies showed that effector and memory precursor cells had similar DNA methylation patterns (Youngblood et al., 2017), thus suggesting a common differentiation program. Thus, evidence in support of both the "early-decision" and "common-differentiation"

models are remarkably resilient; the field awaits resolution of the mechanism and timing of T cell fate determination.

In this thesis work, we have examined TEM cells in the tissue and MLN of IBD patients, and their interaction with MNPs.

1.6 Journey of T cells from mesenteric lymph node to intestinal mucosa

An effective immune response depends on the organized localization and migration of memory and effector T cell subsets into different tissues, more specifically into the tissue microenvironment. The LN environment of T cells dictate the specific homing capacity of activated T cells to peripheral tissues. For instance, T cells activated in intestinal SLOs, such as PP and MLNs, have a greater ability to migrate to intestinal tissues compared to, for example, T cells activated in the skin (Agace, 2010). In this section, I will review T cell migration and trafficking to the gut, as well as Th17 cell fate in the inflamed intestinal lamina propria.

1.6.1 T CELL LOCALIZATION AND TRAFFICKING: CRUCIAL FOR AN EFFECTIVE IMMUNE RESPONSE

The key feature of immunological memory is the generation of a quick and effective immune response upon antigen re-encounter. This proves simpler in humoral immunity with the extensive tissue access by antibodies. However, T cells need to act locally and move to the infection site to control the pathogen, thus their localization and migration patterns are crucial for the generation of a fast immune response (**Figure 1-14**) (Masopust and Schenkel, 2013).

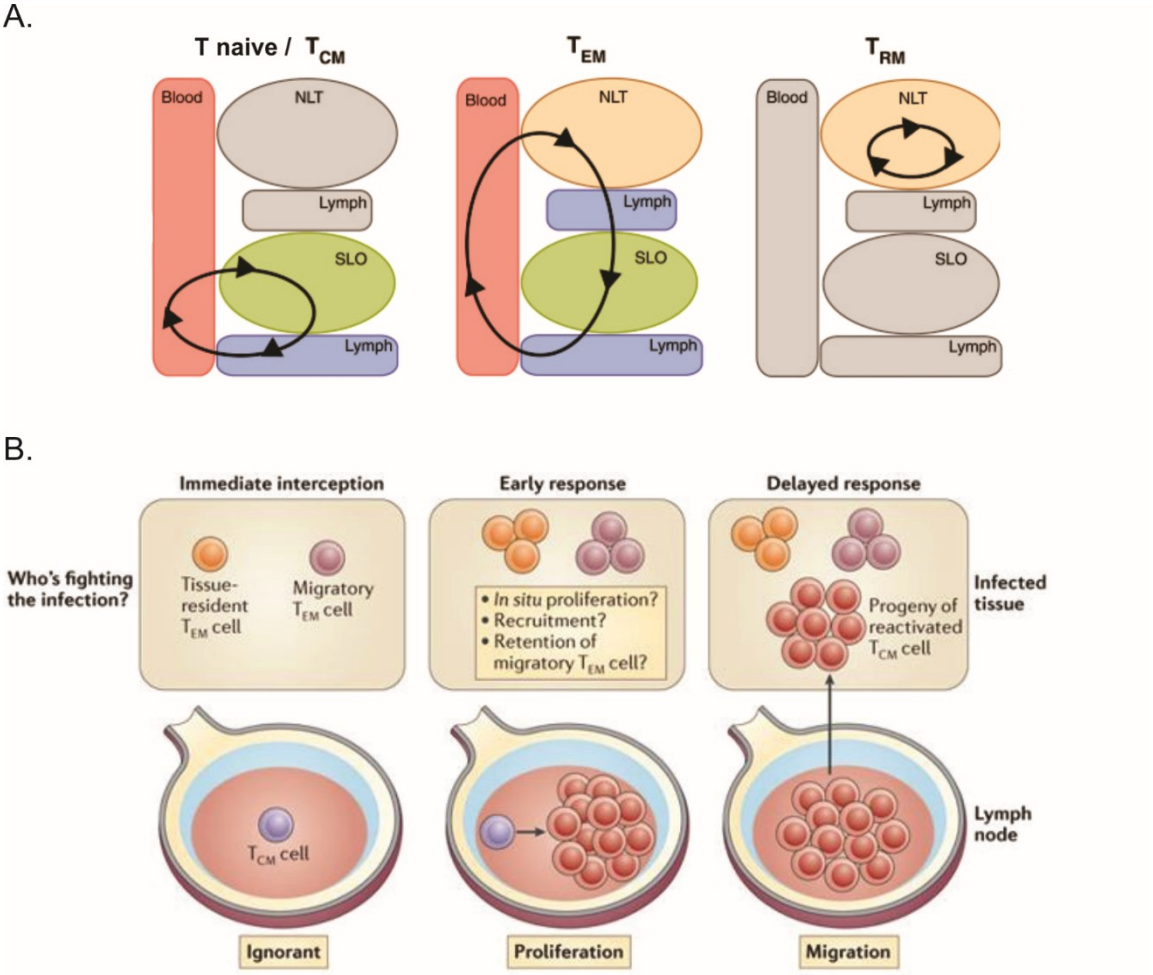


Figure 1-14: Memory T cell migration patterns and location dictate the rapidity of an immune response upon antigen re-encounter.

Since T cells need to act locally and fast to eliminate pathogens, they adapt different circulation patterns or reside in non-lymphoid tissues (NLT) to intercept the antigen quickly and initiate a response to contain it. (A) Naïve and memory T cells adopt different migration patterns. Naïve T cells and TCM focus on the surveillance of secondary lymphoid organs (SLOs) that include Peyer's patches, lymph nodes and white pulp of spleen. A rare fraction of TEM circulate through NLT and transiently pass through SLOs. Finally, and as their name imply, tissue-resident memory T cells (TRM) are retained and survey NLTs. (B) The position of TRM and TEM at the front line for antigen encounter allows them to initiate a small, in situ, antigen-specific response, while antigen-bearing cDC2 reach the TCM in SLO. Upon antigen encounter, TCM will proliferate and differentiate in SLOs, and generate the second wave of effector cells that will migrate to the site of infection. The latter process takes several days.

(Adapted from (Masopust and Schenkel, 2013); authorization code: 4625050452934 and (Rosato et al., 2017); authorization code 4625020974585).

In order to immediately intercept pathogens, T cells circulate through the body between SLOs and peripheral tissue. In fact, while TCM cells oversee the lymphoid tissues, TEM cells travel through the blood and non-lymphoid tissues inspecting for infections. Another tissue surveillance tactic involves implanting TRM cells long-term in tissues, especially at barrier sites. This allows local surveillance and an immediate response upon pathogen tissue entry (Jameson and Masopust, 2018; Masopust and Schenkel, 2013; Rosato et al., 2017).

1.6.1.1 *Naïve T cell trafficking*

Following positive and negative selection in the thymus, naïve T cells ($CD45RA^{+}CD45RO^{-}$) circulate in the lymphatic system between SLOs, which include spleen, tonsils, LN, PP, and mucosa associated lymphoid tissues (MALT), guided by lymphoid-homing receptor expression CCR7 and CD62L.

Naïve T cell trafficking has been recently extended to include non-lymphoid tissues such as the gut lamina propria (Lewis et al., 2008; Thome et al., 2016a; Thome et al., 2016b). In fact, $CD45RA^{+}CCR7^{+}$ naïve T cell frequencies reached 60% of the tissue-resident T cells in the colon of young children (0-2 years). In adults, naïve T cell proportion was lower in blood, spleen, LN and mucosal tissues (20-30% for $CD4^{+}$ and 30-40% for $CD8^{+}$ naïve T cells) (Thome et al., 2016a; Thome et al., 2016b). Almost 20% of tissue-isolated naïve T cells from adult humans, up-regulated their expression of the tissue-resident marker CD69, proposed to be necessary for their long-term maintenance, leading to their retention in LN and mucosal tissues (Thome et al., 2016a).

1.6.1.2 *Memory T cell trafficking*

TEM cells are positioned to immediately intercept pathogens at site of infections, where they immediately generate effector cells for protection (**Figure 1-14B**). In direct contrast, TCM localization in SLOs that drain the infection site allows them to induce large numbers of effector cells, a process that requires several days (Jameson and Masopust, 2018; Masopust and Schenkel, 2013; Rosato et al., 2017).

The regulation of memory cell migration pattern is largely unknown. The egress factor sphingosine 1-phosphate receptor type 1 (S1PR1) seems to play a central role for naive T cell recirculation and the ability of T_{eff} cells to exit lymphoid tissues following activation. This G-protein coupled receptor binds S1P, which is highly concentrated in blood and lymph, and facilitates the egress of lymphocytes from tissues to the circulation (Schwab and Cyster, 2007). S1PR1 expression is controlled directly at the gene level during T cell differentiation by KLF2 (Carlson et al., 2006) or indirectly via CD69 expression. CD69 binds S1PR1 and inhibits its expression on the cell surface (Shiow et al., 2006). Both mechanisms have been proven useful in stopping tissue T cells from entering the vessels (Mackay et al., 2015). Noteworthy, diminished S1PR1 expression seems necessary for residency of $CD8^+$ T cells in non-lymphoid tissues (Skon et al., 2013), and $CD4^+$ T cells in SLOs (Durand et al., 2018).

Another factor that seems to dictate whether T cells remain in lymphoid tissue is the strength of TCR stimulation. Although weak TCR-ligand interactions are enough to induce T cell proliferation and generate effector and memory cells, the strength of the interaction dictates lymphoid organ exit and immune response contraction. For instance, encounter with a low

affinity ligand moves $CD8^+$ T cells to the blood earlier and reduces T cell expansion but does not affect memory cell generation (Zehn et al., 2009).

1.6.1.3 *TRM: "Immigrants that Mediate Border Security" (Jameson and Masopust, 2018)*

$CD4^+$ and $CD8^+$ memory T cells residing in non-lymphoid tissues, the TRMs, are anatomically prepared for immediate antigen detection and infection control by their enhanced effector-like properties (Masopust et al., 2001; Reinhardt et al., 2001; Schenkel et al., 2014).

Little is known about TRM differentiation in peripheral sites. In the intestine, TRMs are not seeded by circulating memory T cells (Klonowski et al., 2004) but rather by activated T cells expressing intestinal homing molecules during the primary immune response (Masopust et al., 2010). Their retention in non-lymphoid tissues is assured by the upregulation of CD69 expression upon T cell arrival to the tissue, and thus, preventing their exit (Mackay et al., 2013; Skon et al., 2013). Noteworthy, the fidelity of CD69 in labeling TRMs in non-lymphoid tissues has been recently questioned as CD69 does not seem to distinguish re-circulating cells from TRM at steady state in the pancreas, salivary glands and thymus (Park et al., 2016; Steinert et al., 2015). CD103-E-Cadherin interaction has also been shown to play a role in maintaining T cells in tissues. CD103 integrin expression has been noted on $CD8^+$ and a fraction of $CD4^+$ TRMs (Schenkel and Masopust, 2014). The combination of CD69 and CD103 expression has also been used to identify TRMs in the gut (de Vries et al., 2019b; Senda et al., 2019). CD103 expression is fundamental to maintain TRM in intestinal epithelium (Casey et al., 2012).

Finally, TRM localization is reflected at the molecular level by the downregulation of *S1PR1* and *CCR7* that seem to enforce tissue retention (Mackay and Kallies, 2017).

In addition to their strategic localization in non-lymphoid tissues, TRMs assure fast protection upon antigen re-encounter by their frequency and enhanced effector-like properties. First, the numbers of TRM cells in non-lymphoid tissues has been recently reassessed by quantitative immunofluorescence microscopy that resulted in higher numbers in non-lymphoid tissues, including the small and large intestine, than previously thought (Steinert et al., 2015). Second, TRM cells have the capacity to amplify local immune response by sending an innate-like alarm signal leading to the recruitment and activation of DCs, classical monocytes, NK, B and T cells (Glennie et al., 2017; Glennie et al., 2015; Schenkel et al., 2014; Schenkel et al., 2013). In the intestinal mucosa, various studies demonstrated the accelerated abilities of $CD4^+$ and $CD8^+$ TRM cells in protection from bacterial and parasitic reinfections (Sheridan et al., 2014; Steinfelder et al., 2017). Although the majority of work on TRMs focused on their role in protection against pathogens, their self-regulating capacities play a role in T cell driven autoimmune diseases such as psoriasis (Park and Kupper, 2015). This is only the tip of the iceberg in our understanding of TRM biology. TRM site specific elimination could be considered in therapies for autoimmune diseases such as IBD.

1.6.2 HOMING TO THE INTESTINAL LAMINA PROPRIA: SMALL INTESTINE OR COLON?

T cell entry into peripheral mucosal tissues, such as the intestine, is accomplished by the interaction of cell adhesion receptors, found on the surface of circulating effector cells, with

their ligands expressed on the vascular endothelium. The latter expresses different adhesion receptor ligands in distinct peripheral tissues, which is considerably influenced by inflammation. Thus, the entry of a circulating effector T cell subset into a specific peripheral tissue is determined by their expression of receptors for these ligands. T cell priming and activation in the GALT upregulates integrin and chemokine receptor expression that directs them to the small intestine or colonic LP (Agace, 2010; Campbell et al., 2001a).

Small intestine homing is dictated mainly by T_{eff} cell surface expression of integrin α4β7 and CCR9. Intestinal lamina propria venules express MAdCAM-1, the receptor for integrin α4β7 (Berlin et al., 1993). Its expression is increased upon lymphocyte activation by RA-producing $CD103^+$ DCs in MLN (Agace and Persson, 2012). Adoptive cell transfer studies verified the requirement of α4β7 and MAdCAM-1 in small and large intestinal lamina propria, but not spleen, for T cell binding to small intestine microvessels (Fujimori et al., 2002). Furthermore, CCR9 and its ligand CCL25 have been shown to localize effector T cells to the small intestine (Svensson et al., 2002). In fact, CCL25 is constitutively produced by the epithelium (Briskin et al., 1997) in varying amounts throughout the length of the small intestine. In fact, its expression in the small intestine of mice is highest in the proximal part and lowest in the distal region (Stenstad et al., 2007). Thus, CCR9-independent T cell homing mechanisms to the distal small intestine exist (Stenstad et al., 2006). In the colon, CCR9 is not required for T cell homing with few T cells expressing CCR9 in the colon of mouse and human (Kunkel et al., 2000; Papadakis et al., 2001). Furthermore, colonic epithelial cells secrete little CCL25 (Kunkel et al., 2000; Papadakis et al., 2000), but they produce the chemokine CCL28. The latter binds CCR10 expressed on IgA-producing plasma cells recruited to the colon (Hieshima et al., 2004), but it

does not seem to play a role in T cell recruitment to the colon as few T cells are $CCR10^+$ (Lazarus et al., 2003).

The orphan G-protein-coupled receptor 15 (GPR15) plays a role in T cell homing to the colonic mucosa. In fact, $CD4^+$ T cells isolated from the large intestine of mice have a 40-fold higher GPR15 expression compared to $CD4^+$ T cells from the small intestine (Houston et al., 2016). Its colonic homing capacity is noted for $FoxP3^+$ T_{reg} and TEM localization in mice (Kim et al., 2013; Nguyen et al., 2015). However, in humans, only $CD4^+$ TEM are guided by GPR15 to the colon (Nguyen et al., 2015). Recent work identified GPR15L as the ligand for GPR15. This chemokine is secreted by skin and gastrointestinal epithelial cells. Expression of GPR15L is constitutive in the colon, from early development, and is slightly affected by inflammation (Ocon et al., 2017). Due to its role in TEM localization to the colon, GPR15/GPR15L axis plays a role in colitis in mice and humans. First, GPR15 induces colitis in the $CD45RB^{hi}$ T cell model requiring effector T cell homing to the colon. Furthermore, GPR15 expression was higher on Th2 cells isolated from the colon of UC patients when compared to controls. Thus, GPR15 plays a role in T_{eff}, more specifically Th2, recruitment to the colon of UC (Nguyen et al., 2015), and warrants further investigation. Noteworthy, the role of GPR15 in T_{reg} recruitment was demonstrated in mice but not humans (Adamczyk et al., 2017).

Other chemokines have also been implicated in T cell localization to the intestine, such as CCL20 and its receptor CCR6. CCL20 is normally secreted by epithelial cells coating the PP under inflammatory settings (Wang et al., 2009). The CCR6/CCL20 axis contributes to T cell recruitment to small and large intestinal mucosa only under inflammatory conditions (Oyama

et al., 2007). In fact, CCL20 expression is higher in inflamed compared to non-inflamed colonic mucosa of IBD patients (Kaser et al., 2004) and mice with DSS-induced colitis (Teramoto et al., 2005). Also, blockade of CCR6 and CCL20 attenuates DSS-induced T cell recruitment (Teramoto et al., 2005). Finally, $CCR6^+$ Th17 cells are recruited by CCL20 to the intestinal lamina propria of mice, especially in inflammation (Wang et al., 2009). These results indicate that CCR6 and CCL20 localize T cells to the small intestine and colonic mucosa during inflammation.

1.6.3 FATE OF TH17 CELLS IN IBD

The protective role of Th17 cells in maintenance of intestinal homeostasis, described earlier, seems to contradict its involvement in IBD. However, the role of Th17 in IBD development has been well established. It is unclear whether it is due to differentiation of new pro-inflammatory Th17 cells or if these cells shift to an inflammatory phenotype (Stockinger and Omenetti, 2017). Th17 cells are controlled by a unique master transcription factor that controls the transcription of only a few Th17 associated genes. Contrary to GATA3 and T-bet, RORγ expression is not maintained by a positive feedback loop, but is rather influenced by environmental signals rendering Th17 unstable and plastic (Ciofani et al., 2012).

1.6.3.1 *Non-pathogenic Th17 cells*

Th17 plasticity is not only involved in pathogenicity, it can have beneficial functions in the mucosa. First, Th17 cells, under TGFβ and AHR signaling, trans-differentiate into IL10-producing regulatory T cells, known as T regulatory type 1 (TR1)-like cells, that contribute to resolution of intestinal inflammation (Gagliani et al., 2015). The generation of $ROR\gamma^+FoxP3^+$

T cells is inhibited in germ-free conditions (Lochner et al., 2011; Yang et al., 2016). Indeed, *Clostridium* species induced colonic RORγ-expressing, over GATA3-expressing, FoxP3^{+} T_{reg} cells (Ohnmacht et al., 2015). Therefore, intestinal bacteria play a role in balancing Th17 immune responses at mucosal surface. Second, in PP plastic Th17 cells adopted a TFH profile and induced the development of IgA-producing B cells in germinal centers. Under these conditions, IL23 does not contribute to Th17 survival or plasticity. In fact, in *Il23*$^{-/-}$ mice, the intestine Th17 cells count is normal, and they equivalently convert to a TFH phenotype (Hirota et al., 2013). Accordingly, the balance between pathogenic versus beneficial Th17 cell responses depend in part on IL23 in the intestinal mucosa.

1.6.3.2 *Pathogenic Th17 cells*

First, pathogenic Th17 cells were identified in an *in vitro* model in the presence of different cytokine combinations (Ghoreschi et al., 2010; McGeachy et al., 2007). Whereas IL6 and TGFβ1 generate non-pathogenic IL10-producing Th17 cells unable to induce experimental autoimmune encephalitis (EAE) following adoptive transfer, the combination of IL6, IL1β and IL23 gave rise to EAE-inducing pathogenic Th17 cells (Lee et al., 2012). Further characterization of non-pathogenic versus pathogenic Th17 cells relied on transcriptome analysis of *in vitro* generated cells that varied in their pathogenicity signatures, echoing their differentiation conditions. In fact, a high pathogenicity score was associated with IL6+IL1β+IL23 derived IL17-secreting cells; while IL6+TGFβ1 derived IL17^{+} cells correlated with regulatory cytokines such as IL10. Interestingly and given the importance of IL23 signaling in CD development, IL23R$^{-/-}$ cells differentiated with IL6+IL1β+IL23 cytokine

combination correlated with the non-pathogenic cells, substantiating the role of IL23 pathway in pathogenicity (Gaublomme et al., 2015).

The plasticity of Th17 cells has been shown to be a common feature under pathogenic conditions. In fact, a subset of Th17 cells co-expressing IFNγ was identified in the gut of CD patients and delineated as Th17/Th1 cells (Annunziato et al., 2007); the latter could lose IL17 expression under inflammatory condition and become non-classic Th1, also known as Th1* or ex-Th17, cells (Lee et al., 2009b). The gain of IFNγ production in Th17 cells has been noted in intestinal infections with *C. rodentium* (Ahlfors et al., 2014) and *H. hepaticus* (Morrison et al., 2013) as well as in a Th17 transfer model of colitis (Harbour et al., 2015). Moreover, Th1 cells were previously associated with CD (de Souza and Fiocchi, 2016). Thus, the predisposition of Th17 cells to adopt a Th1-like profile suggests that inflammation in CD is mainly powered by the plastic Th17 cells.

IL12 and IL23 play an important role in driving Th17 plasticity. In fact, IL12 and IL23 signaling promote IFNγ while suppressing IL17 expression in a STAT4/T-bet dependent pathway (Lee et al., 2009a). Furthermore, the expression of pro-inflammatory IFNγ and GM-CSF by Th17 cells is halted in the absence of IL23 (Hirota et al., 2011). The latter mediates its function through induction of the transcription factor BLIMP1 expression that, along with Th17 transcription factors (RORγ, STAT3), enhance the expression of *IL23r*, *IL17a/f* and *Gmcsf* (Jain et al., 2016). Moreover, IL23 fuels intestinal inflammation by the emergence of $IFN\gamma^{+}Tbet^{+}$ pathogenic Th17 cells (Krausgruber et al., 2016) in parallel to restraining T_{reg} activity (Izcue et al., 2008). This is consistent with GWAS identification of *IL23R* as an IBD

gene (Duerr et al., 2006). Much of the work on Th17 shift to Th17/Th1 cells in the inflamed mucosa has been described in CD patients. Although IL12 have been shown to augment IFNγ secretion in the culture supernatant (CSN) of CD3+CD28 stimulated LPMC from CD and UC patients (Kobayashi et al., 2008), the regulation of mucosal Th17, Th17/Th1 and Th1 responses remain to be fully investigated in UC.

Exploring Th17 cell plasticity in the mucosa of UC patients is a subject of my thesis. Furthermore, no study compared Th17 cells in the MLN of CD and UC patients. Consequently, in this thesis work, we will investigate the frequencies of Th17 cells and their molecular profile in colon-draining MLN of CD and UC patients.

Given that IBD pathogenicity is mediated in part by MNPs that dialogue via the cytokine they produce with innate and adaptive immune cells. A better understanding of MNP phenotypic, molecular and functional diversity as well as plasticity will shed light on IBD pathophysiology, and would help identify novel therapeutic targets for the development of personalized treatments. Therefore, understanding the phenotypic and functional diversity of $CD14^+$MNPs and the monocyte subset ($Slan^+$ cells) in UC and CD is the first objective of our present work (**Chapters 2 and 3**). Moreover, comprehending the environmental cues that regulate "tissue inflammatory monocytes" maturation into anti-inflammatory macrophages in humans might open therapeutic avenues to restore tissue homeostasis. Thus, examining *in vitro* the plasticity of monocytes and their progressive differentiation from monocyte-like cells ($CD14^+CD64^+CD163^-$) to macrophages ($CD14^+CD64^+CD163^+$) resembling functionally and molecularly tissue $CD14^+$MNP subsets is the second objective of this thesis (**Chapter 5**).

Therapies in IBD employ antibodies that block MNP-derived IL12 and IL23, thus control Th17 pathogenicity and plasticity and decrease intestinal inflammation. Consequently, the third objective aims at studying the link between colonic $CD14^+$ MNP subset, the cytokines they produce and intestinal effector $CD4^+$ T cell profile (**Chapter 2**). Finally, we will evaluate Th17 pathogenicity and plasticity under the influence of MNP-derived cytokines in UC and CD (**Chapter 4**).

Chapter 2 :

IL-12 and mucosal CD14$^+$ monocyte-like cells induce IL-8 in colonic memory CD4$^+$ T cells of patients with ulcerative colitis but not Crohn's disease

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Author's contribution	Equal contribution with Dr. Laurence Chapuy in the design of the study (50%), conduction of the experimental work (55%), data analysis (50%) and manuscript writing (40%).
Journal	Journal of Crohn and Colitis
Status	Published June 2019
DOI	10.1093/ecco-jcc/jjz115
Key words	Ulcerative colitis, IL-8, mononuclear phagocytes, Th17 cells, Th1

2.1 Abstract

Background and Aims. $CD14^+$ mononuclear phagocytes (MNPs) and T cells infiltrate colon in ulcerative colitis (UC). We here investigated how $CD14^+$MNPs and cytokines they produce, shape colonic effector T cell profile.

Methods. Colonic or mesenteric lymph node (mLNs) $CD4^+$T cells isolated from UC or Crohn's disease (CD) were stimulated with cytokines or autologous $CD14^+$ MNPs. Cytokine expression was assessed by intracytoplasmic staining and multiplex ELISA. Unsupervised phenotypic multicolor analysis of colonic $CD14^+$ MNPs was performed using FlowSOM algorithm.

Results. Among $CD14^+CD64^+$HLA-$DR^+SIRP\alpha^+$MNPs, only the pro-inflammatory cytokine-producing $CD163^-$ subpopulation accumulated in inflamed UC colon and promoted mucosal IL-1β-dependent Th17, Th17/Th1, Th17/Th22 but not Th1 responses. Unsupervised phenotypic analysis of $CD14^+CD64^+$MNPs segregated $CD163^-$ monocyte-like cells and $CD163^+$ macrophages. Unexpectedly, IL-12, IL-1β and $CD163^-$, but not $CD163^+$, cells induced IL-8 expression in colonic $CD4^+$T cells, which co-expressed IFN-γ and/or IL-17 in UC and not CD. The $CD163^-$ monocyte-like cells increased the frequency of IL-8^+IL-$17^{+/-}$IFN-$\gamma^{+/-}$ T cells through IL-1β and IL-12. Finally, colonic IL-8^+ T cells co-expressed GM-CSF, TNF-α and IL-6 were detected *ex vivo* and, promoted by IL-12 in the mucosa and mLNs in UC only.

Conclusions. Our findings established a link between monocyte-like $CD163^-$MNPs, IL-12, IL-1β and the detection of colonic memory IL-8-producing $CD4^+$T cells, which might all contribute to UC pathogenesis.

2.2 Article

2.2.1 INTRODUCTION

Ulcerative colitis (UC) is a chronic inflammatory disease of the colon. Epithelial cells that produce mucus, the microbiota as well as innate and adaptive immune cells, all contribute to the pathophysiology of the disease (Ungaro et al., 2017). UC was first considered a Th2 disease since elevated levels of IL-5 and IL-13 were detected in the colonic mucosa of patients (Fuss et al., 2004; Heller et al., 2005). Nonetheless, the view that UC is a type 2 disease distinct from Th1-associated Crohn's disease (CD) - the other common form of inflammatory bowel diseases (IBD)- has been challenged in the last few years (Christophi et al., 2012; Neurath et al., 2002). Firstly, some studies did not note the increased IL-5 and IL-13 expression in the mucosa of UC patients (Biancheri et al., 2014), while others proposed a protective role for IL-13 in pediatric patients with UC (Rosen et al., 2017). Also, two anti-IL-13 monoclonal antibodies failed in improving the outcome of UC patients (Danese et al., 2015; Reinisch et al., 2015). Secondly, the discovery of IL-17-secreting $CD4^+$T cells (Th17) brought new insights into the pathophysiology of UC. IL-17 and IFN-γ mRNA is elevated in biopsies from UC mucosa when compared to normal controls (Bogaert et al., 2010; Granlund et al., 2013; Kobayashi et al., 2008). Furthermore, Th17 and IL-17^+IFN-γ^+CD4^+ T cells (Th17/Th1) are observed in inflamed mucosa of patients with UC (Globig et al., 2014; Kryczek et al., 2011; Li et al., 2016; Rovedatti et al., 2009). However, Th17 cell fate towards Th17/Th1 profile is much less studied in UC than CD. In inflamed CD mucosa, IL-17^+IFN-γ^+ T cells accumulate in lesional sites and participate in the mucosal inflammatory process (Annunziato et al., 2007; Globig et al., 2014; Ramesh et al., 2014). The cytokines that drive the shift from Th17 towards Th17/Th1 cells have been described in CD. $CD161^+CD4^+$ T cells isolated from patients with CD express IL-23R, and

produce IL-17 and IFN-γ under IL-23 stimulation (Kleinschek et al., 2009). Moreover, Ramesh et al showed that IL-23 increased the percentage of IFN-γ^+IL-$17^+$$CD4^+$ T cells, particularly in MDR1^+IL-$23R^+$ Th17/Th1 population isolated from the blood of healthy donors (Ramesh et al., 2014). Recombinant IL-12, that shares a common IL-12p40 chain with IL-23, increases IFN-γ expression while decreasing IL-17 in Th17 clones isolated from the mucosa of patients with CD (Annunziato et al., 2007). Although IL-12 augments IFN-γ secretion in the culture supernatant (CSN) of CD3 and CD28 stimulated lamina propria mononuclear cells (LPMC) from CD and UC patients (Kobayashi et al., 2008), the regulation of mucosal Th17, Th17/Th1 and Th1 responses remain to be fully investigated in UC.

IL-23 and IL-12 are pro-inflammatory cytokines, which are produced by mononuclear phagocytes (MNPs). Currently, MNPs are classified as dendritic cells (DCs), macrophages (Mϕ), inflammatory monocytes and/or monocyte-derived cells (Guilliams and van de Laar, 2015). In the mucosa of patients with UC, a population expressing the monocyte marker CD14 has been reported (Kamada et al., 2008); they are considered as Mϕ or monocyte-derived cells (Baba et al., 2013; Thiesen et al., 2014). Also, Magnusson et al described the accumulation of a HLA-$DR^{dim}$$CD64^+$ subset in the inflamed mucosa of UC patients compared to normal controls (Magnusson et al., 2016). These mucosal MNPs secrete pro-inflammatory cytokine including TNF-α, IL-23, IL-1β and IL-6 (Kamada et al., 2008). However, the impact of mucosal $CD14^+$ MNPs on effector memory $CD4^+$T cell response remains to be investigated in UC.

In the present study, we examined whether and how $CD14^+$ MNPs that infiltrate the mucosa of patients with UC control autologous memory colonic Th response. We showed that the pro-inflammatory $CD14^+$ monocyte-like subpopulation, that did not express CD163, accumulated in inflamed colonic UC mucosa and favored Th17, Th17/Th1 but not Th1 responses. A few

reports have shown that circulating T cells isolated from healthy adults and cord blood secrete IL-8 (Akhade and Qadri, 2015; Gasch et al., 2014; Gibbons et al., 2014). This chemokine plays a key role in UC pathogenesis (Beck et al., 2016; Bennike et al., 2015; Mitsuyama et al., 1994). Unexpectedly, our data further revealed that $CD163^-$ but not $CD163^+$ MNPs augmented IL-8 expression in colonic memory $CD4^+$ T cells in UC but not CD.

2.2.2 MATERIALS AND METHODS

2.2.2.1 *Human clinical samples*

All participants signed an informed consent form that has been approved by the Institutional Ethics Research Committee of the Centre Hospitalier de l'Université de Montréal (CHUM). This study includes 83 patients with UC (median age 42), 19 patients with CD (median age 37) and 6 patients undergoing a screening colonoscopy (non IBD) (median age 60) (**Table 2-1**). IBD patient recruitment was based on clinical, endoscopic activity and histological criteria. UC patients presented with bloody stools, diarrhea, and abdominal pain. Endoscopically, they presented a continuous inflammation, extending from the rectum to the colon. CD patients presented with diarrhea, weight loss or abdominal pain. Endoscopically, the mucosa was eroded and exhibited patchy inflammation, deep ulcers and/or strictures. Histologically, the architecture of the crypts was disturbed; the mucosa was infiltrated by mono or polynuclear cells, with or without pathognomonic granuloma in the case of CD patients. No histological data or bacteriological infections suggested a differential diagnosis. An endoscopic score (Mayo or SES-CD) was not available for most of the IBD patients since it is not performed routinely by gastroenterologists at CHUM. Disease remission was assessed on the basis of endoscopic criteria. Non-inflamed and inflamed colonic tissues (from the same patient), non IBD (control) colonic tissue and mesenteric lymph nodes (mLNs) from UC patients only, were acquired from endoscopic biopsies or surgical resections, respectively.

2.2.2.2 *Cell purification*

Intestinal mucosa, from biopsies or surgical samples, was first processed by enzymatic digestion with DNase I (Roche, Basel, Switzerland) and Collagenase D (Roche, Basel,

Switzerland) followed by mechanical digestion with gentle MACS (Miltenyi Biotec, Bergisch Gladbach, Germany) to isolate lamina propria mononuclear cells (LPMC). MLNs were digested mechanically to obtain cellular suspensions (Baba et al., 2013).

2.2.2.3 *Cell staining*

LPMC were stained using monoclonal antibodies listed in **Table 2-S1**, and analyses were performed with FCS Express 6 (De novo software) or FlowJo v10.5.3. Unsupervised analyses were performed using plugins available (*t*-SNE (*t*-Distributed Stochastic Neighbor Embedding) and FlowSOM (Flow self-organizing map) (R Core Team, 2016; Van Gassen et al., 2015), in FlowJo. The data were manually gated on single viable $CD45^{+}HLADR^{+}SIPR\alpha^{+}CD14^{+}CD64^{+}$ cells, and all gated cells were subjected to analysis. The cells were assigned to a self-organizing map, automatically segregating cells into 5 clusters and visualizing data in four ways: a) cell affiliation in 5 clusters visualized in *t*-SNE plot; b) surface markers expression level visualized by histograms; c) relative mean intensities depicted according color gradient in heatmap; d) assignment of cells to the self-organizing map with a 5x5 grid, resulting in 25 nodes, depicted as a minimal spanning tree, built to visualize similar nodes in branches. Concatenated file from 4 UC patients as well as each individual file were analyzed separately with *t*-SNE and FlowSOM algorithm. FlowSOM algorithm was run 5 times to insure reproducibility of the results.

2.2.2.4 *Cell sorting*

Human $CD4^{+}CD8^{-}CD45RA^{-}CD25^{-}$ mucosal T cells and HLA-$DR^{+}SIRP\alpha^{+}CD14^{+}CD64^{+}$ MNPs co-expressing or not CD163 were sorted at the same time to perform co-culture experiments.

HLA-DR$^+$SIRPα$^+$CD14$^+$CD64$^+$ MNPs co-expressing or not CD163 were also sorted to analyze cell morphology. MLN CD4$^+$CD45RO$^+$CD62L^{low}CD8$^-$CD45RA$^-$CD25$^-$ effector memory T cells (T_{EM}) were stratified into CCR6$^+$CXCR3$^-$ (Th17 T_{EM}) and CCR6$^-$CXCR3$^+$ (Th1 T_{EM}) and sorted for culture with either IL-1β or IL-12, and *ex vivo* PMA ionomycin stimulation. Sorting were performed using FACS Aria II cell sorter and data were analyzed using FACS Diva 6 (BD Biosciences, San Diego, CA, USA).

2.2.2.5 *In vitro MNP/T cell co-cultures*

Total CD4$^+$T cells, depleted in CD8$^+$ T cells, CD25$^+$regulatory T cells and CD45RA$^+$naïve T cells were purified from inflamed colon. T cells were stimulated with anti-CD3/CD28 coated beads (Miltenyi Biotec), and either a) cultured with or without IL-1β (10ng/ml, R&D system), IL-12 (20ng/ml, R&D system) or IL-23 (10ng/ml, R&D system) for 6 days; or b) co-cultured with autologous MNP subsets purified from inflamed colonic mucosa, at a 10:1 ratio for 6 days, in the presence of PGN (10µg/ml). For some experiments, anti-IL-1β receptor (10µg/ml), anti-IL-1β (10µg/ml) or anti-IL12p70 (10µg/ml, R&D system) mAbs were added to the co-cultures. Total CD4$^+$CD8$^-$CD45RA$^-$CD25$^-$ T cells, Th17 $T_{EM,}$ and Th1 $T_{EM,}$ purified from mLNs were co-cultured in the presence of anti-CD3/CD28 coated beads, with or without IL-1β (10ng/ml) or IL-12 (20ng/ml) for 6 days.

For all cultures: a) RPMI 1640 medium with 10% FCS, 1% Penicillin/Streptomycin was used; b) for intracytoplasmic staining, cells were re-stimulated after culture, with PMA and ionomycin for 6 hours in the presence of brefeldin A for the last 3 hours, then fixed and stained with mAbs (CD3, IL-17, IFN-γ, IL-8, IL-22, IL-6, TNF-α, GM-CSF, as listed in **Table 2-S1**);

c) IL-17, IFN-γ, IL-6, TNF-α, GM-CSF, IL-8 release were measured by multiplex assay (Eve Technologies, Calgary, AB, Canada) in the culture supernatants.

2.2.2.6 *Cytokine expression*

Ex vivo isolated LPMC were immediately stained for CD45, HLA-DR, CD172α (SIRPα), CD64 and CD163, in the absence of BrefeldinA, then fixed/permeabilized and stained for intracytoplasmic cytokine expression (IL-1β, IL-10, IL-12p40 and IL-23).

Freshly isolated LPMC were cultured with PMA and ionomycin for 4 hours, in the presence of brefeldin A, then fixed and stained for CD45, CD3, CD4, CD8 and CD25. Intra-cytoplasmic expression of Foxp3, IL-8, IL-17A, TNF-α, IFN-γ, IL-6, GM-CSF was evaluated after permeabilization. Co-expression of IL-17A, TNF-α, IFN-γ, IL-6, GM-CSF was evaluated in $CD3^{+}CD4^{+}CD8^{-}CD25^{-}Foxp3^{-}IL\text{-}8^{+}$ cells.

Freshly purified Th1 T_{EM} and Th17 T_{EM} were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin for 4 hours, in the presence of brefeldin A, then stained for intracytoplasmic IFN-γ and IL-17 expression.

2.2.2.7 *Morphology*

For morphological studies, FACS-sorted MNPs were cytospun and stained according to the Wright Stain procedure. Leica DM4000B microscope, equipped with Leica DFC300FX camera was used to visualize cells.

2.2.2.8 *Statistical analysis*

Statistical analysis was performed with GraphPad Prism version 6 (GraphPad Software, La Jolla, CA, USA). Data were checked for normality using Shapiro-Wilk test and then the appropriate test was applied as indicated in figure legends. Two-tailed Wilcoxon signed rank test (represented by *) and Mann Whitney test (represented by §) were used. Friedman test was employed followed by Dunn's test (represented by Ω). Threshold for significance was adjusted when indicated to account for test multiplicity. Kruskal-Wallis test was employed followed by Dunn's test (represented by #). Repeated measure one-way Anova was employed followed by Bonferroni test (represented by ¤). For all tests, 1 symbol means $p<0.05$; 2 symbols mean $p<0.01$; 3 symbols mean $p<0.001$. Bar graph data are shown as mean ± s.e.m.

2.2.3 RESULTS

2.2.3.1 *IL-1β promotes Th17 and Th17/Th1 responses in CD4$^+$T cells isolated from inflamed colon of UC patients.*

IL-23 and IL-12 are key cytokines in Th17/Th1-associated CD pathogenesis (Annunziato et al., 2007; Kleinschek et al., 2009; Ramesh et al., 2014). Here, we first evaluated how IL-23 and IL-12 regulated the Th17, Th17/Th1 and Th1 profile of CD4$^+$T cells isolated from inflamed colon of UC patients. As expected, IL-12 augmented the frequency of single IFN-γ-producing CD4$^+$T cells (Th1) (p<0.002) and decreased the frequency of single IL-17-producing CD4$^+$T cells (Th17) (p<0.006). However, the percentages of Th17 and double IL-17/IFN-γ-producing CD4$^+$T cells (Th17/Th1) were not modulated by IL-23 (**Figure 2-1**). As recently reported in CD (Chapuy et al., 2019a), IL-1β significantly increased Th17 (p<0.0002) and Th17/Th1 (p<0.0002), but not Th1 responses in all UC patients examined (**Figure 2-1**).

Thus, IL-1β, but not IL-23 or IL-12, promotes a Th17 and Th17/Th1 profile in mucosal CD4$^+$T cells in UC.

2.2.3.2 *HLA-DR$^+$SIRPα$^+$CD14$^+$CD64$^+$CD163$^-$ cells selectively accumulate in inflamed UC mucosa.*

CD14$^+$MNPs are a cellular source of IL-1β, IL-12 and IL-23 in inflamed gut mucosa (Kamada et al., 2008). A previous report showed that HLA-DR$^+$SIRPα$^+$ MNPs accumulate in inflamed compared to non-inflamed colonic mucosa of patients with CD (Baba et al., 2013). We here examined whether MNPs with a similar phenotype infiltrated the UC mucosa. The frequencies of HLA-DR$^+$SIRPα$^+$ MNPs were higher in inflamed relative to paired non-inflamed colonic mucosa (n=31), as well as to colon of UC patients in remission (n=8) and non-IBD control (n=6)

(p<0.0007, p<0.007, p<0.009, respectively; **Figure 2-2a**). In inflamed UC mucosa, more than 95% of HLA-DR$^+$SIRPα$^+$ MNPs expressed CD14 (**Figure 2-2b**). To assess heterogeneity of the HLA-DR$^+$SIRPα$^+$CD14$^+$ population, these cells were further stratified according to expression of CD64 (the Fc-gamma receptor 1) and scavenger receptor CD163, expressed on human gut Mϕ (Jakubzick et al., 2017; Segura and Amigorena, 2013). CD14$^+$CD64$^+$ cells were subdivided according to the intensity of CD163 expression (**Figure 2-2c**). Data revealed that CD163$^-$, but not CD163^{dim} or CD163$^+$, cells accumulated in inflamed when compared to paired non-inflamed UC colon (p<0.0001), and were detected in low frequencies in healed mucosa of UC patients in endoscopic remission and control patients (p<0.01 and p<0.008 respectively; **Figure 2-2d**). In conclusion, CD163$^-$ cells are the predominant HLA-DR$^+$SIRPα$^+$CD14$^+$CD64$^+$ MNP subpopulation that infiltrates inflamed UC mucosa.

2.2.3.3 *Mucosal CD163$^-$ and CD163$^+$ MNPs express similar amounts of pro-inflammatory cytokines but CD163$^+$ cells produce more IL-10 relative to CD163$^-$ cells.*

Next, we analyzed cytokine expression in colonic CD163$^+$ or CD163$^-$ subpopulations. The frequencies of IL-1β, IL-12p40 and IL-23-producing cells were augmented in inflamed relative to paired non-inflamed UC mucosa in CD163$^-$ (p<0.008, p<0.004, p<0.5, respectively) and CD163$^+$ cells (p<0.04; p<0.02, p<0.03) (**Figure 2-3a**). Furthermore, the amount of pro-inflammatory cytokine expression per cell (MFI) was similar in CD163$^-$ and CD163$^+$ cells in both non-inflamed and inflamed tissues (**Figure 2-3b**). Since colonic CD163$^+$ macrophages are known to produce IL-10 (Gonzalez-Dominguez et al., 2015; Ogino et al., 2013), we further examined IL-10 expression in CD163$^-$ and CD163$^+$ cells (**Figure 2-3c**). Unlike pro-inflammatory cytokine secretion, the amount of IL-10 per cell was higher in CD163$^+$ relative

to CD163$^-$ cells in inflamed and non-inflamed mucosa (p<0.002 and p<0.005) while the percentage of IL-10-producing cells was higher in CD163$^+$ when compared to CD163$^-$ cells in non-inflamed mucosa only (p<0.04) (**Figure 2-3c**).

Overall, both CD163$^-$ and CD163$^+$ cells produce IL-1β, IL-12p40 and IL-23. However, considering the relative distribution of these 2 cell subpopulations, with increased proportion of CD163$^-$ relative to CD163$^+$ cells within CD14$^+$CD64$^+$ MNPs (**Figure 2-3a**, middle panel), the former is the major contributor to pro-inflammatory cytokine production in the inflamed mucosa. In contrast, CD163$^+$ cells produce more IL-10 relative to CD163$^-$ cells.

2.2.3.4 *Mucosal CD163$^-$ but not CD163$^+$ MNPs favor autologous Th17/Th1 and Th17/Th22 responses in an IL-1β-dependent manner in inflamed UC colon.*

We next asked whether and how CD163$^-$ and CD163$^+$ MNPs from inflamed tissue regulate autologous memory Th17, Th17/Th1 and Th1 responses. To this end, we simultaneously purified CD4$^+$ T cells, CD163$^-$ and CD163$^+$ MNPs from colonic biopsies, thus excluding the intermediate CD163^{dim} cells and co-cultured the cells for 6 days (**Figure 2-S1**). Remarkably, CD163$^-$, but not CD163$^+$, MNPs favored the emergence of Th17 (p<0.0001) and Th17/Th1 (p<0.0001), but not Th1 cells (**Figure 2-4a**). Noteworthy, CD14$^+$CD64$^+$CD163$^-$ MNPs were not able to induce naïve T cell proliferation and drive their differentiation into Th effector subsets, while the minor CD14$^-$CD64$^-$CD163$^-$ cells, which are enriched in DCs (Chapuy et al., 2019a), primed naïve T cells and induced polarization towards Th1 effectors (data not shown).

We further explored the mechanisms underlying the facilitating activity of colonic CD163$^-$ cells on Th17 and Th17/Th1 responses. The frequencies of IL-17$^+$IFN-γ$^-$ and IL-17$^+$IFN-γ$^+$ T cells were decreased, by adding a monoclonal antibody (mAb) that neutralizes IL-1β function to the

CD163$^-$ plus CD4$^+$ T cells co-cultures (p<0.05 and p<0.01, respectively, **Figure 2-4b**). CD163$^-$ MNPs and IL-1β appeared to favor the selective expansion of Th17 and Th17/Th1 cells, while proliferation of Th1 cell population seems unaltered (data not shown). However, the Th17 and Th17/Th1 responses were not influenced by anti-IL-12p70 mAb that selectively blocked IL-12 (**Figure 2-4b**). Similarly, elevated frequencies of IL-17$^+$IL-22$^-$ (p<0.0001) and IL-17$^+$IL-22$^+$ (p<0.002) cells observed in CD4$^+$ T cells co-cultured with CD163$^-$ cells, were reduced when neutralizing IL-1β function (p<0.05 and p<0.01 respectively; **Figure 2-4c** and **Figure 2-4d**). These data indicate that only CD163$^-$ MNPs promote a Th17, Th17/Th1 or Th17/Th22 profile in an IL-1β-dependent manner, corroborating our observations with recombinant IL-1β-stimulated CD4$^+$ T cells cultures (**Figure 2-1**).

2.2.3.5 *Mucosal CD4$^+$ T cells produce IL-8 in UC patients.*

Next, we examined how IL-1β and IL-12 regulates the expression of pro-inflammatory cytokines in the culture supernatant of *in vitro* activated colonic CD4$^+$ T cells. In agreement with the data of intracellular IL-17 and IFN-γ expression, IL-17 production was significantly increased (p<0.004) by IL-1β, while IL-12, but not IL-23 augmented IFN-γ (p<0.004) (**Figure 2-5a**). Furthermore, IL-12 but not IL-1β increased TNF-α, GM-CSF and IL-6 secretion (p<0.04; p<0.004; p<0.02 respectively), suggesting that IL-12 drives a potential pathogenic CD4$^+$ T cell profile in colon (**Figure 2-5b**). By serendipity, the multiplex cytokine assay revealed that mucosal CD4$^+$ T cells produced IL-8 in response to IL-12 (p<0.004) and IL-1β (p<0.004) but not IL-23 (**Figure 2-5c**). We therefore verified IL-8 expression at the single cell level using intra-cytoplasmic staining and observed that both IL-12 and IL-1β augmented the frequency of IL-8$^+$CD4$^+$ T cells in UC (p<0.002 and p<0.007 respectively) (**Figure 2-5d**). Since IL-8

produced by activated Th17 clones, which were generated from IBD mucosa, promotes neutrophil migration (Pelletier et al., 2010), we explored whether IL-8 secreted by IL-1β or IL-12-activated colonic $CD4^+$T cells attracted neutrophils. Unfortunately, we did not succeed in showing IL-8-mediated chemotactic activity in the culture supernatant of activated primary mucosal $CD4^+$ T cells (data not shown). Phenotypic analysis of IL-8^+CD4^+ T cells in UC patients further showed that the majority of these cells expressed α4, while β7 and CD103 expression was barely detectable in all culture conditions (**Figure 2-S2a**). CCR6, a Th17 associated surface marker, as well as CD69, a surface marker expressed by activated T cells or tissue resident memory T cells, but not the Th1-associated surface marker CXCR3, were expressed on IL-8-producing T cells (**Figure 2-S2b**). IL-12 significantly decreased the proportion of CCR6 and CD69 positive cells in IL-8^+CD4^+ T cells (n=4, p<0.01 and p<0.04 respectively) while the percentage of IL-8^+ cells expressing CCR6 augmented in the presence of IL-1β (n=4, p<0.04).

We further examined *ex vivo* IL-8 expression in colonic $CD4^+$ T cells using freshly isolated cells from inflamed mucosa (**Figure 2-5e**). A significant proportion (40%) of the IL-8-producing cells co-expressed pro-inflammatory cytokines. Specifically, IL-8^+ cells co-produced GM-CSF (14%), TNF-α (20.2%), IL-6 (8.6%), IFN-γ (2.1%) and IL-17 (4.7%)**.** Noteworthy, IL-8 could not be detected in significant proportions in $CD4^+CD25^+Foxp3^+$ regulatory T cells (**Figure 2-S3**).

Taken together, IL-8^+CD4^+ T cells co-producing GM-CSF, TNF-α, IL-6 and IFN-γ are detected *ex vivo* in inflamed UC colon and this IL-8 pathogenic profile is further augmented by IL-12 but not IL-1β *in vitro*.

2.2.3.6 *IL-12 promotes IL-8 and IFN-γ expression whereas IL-1β favors IL-17 and IL-8 in colon of UC patients.*

We further showed that frequencies of IL-8^+IL-17^+ (p<0.002) but not IL-8^+IL-22^+ $CD4^+$ T cells were increased by IL-1β while IL-12 augmented IL-8^+IFN-γ^+ $CD4^+$ T cells (p<0.003) (**Figure 2-6a**). More precisely, IL-1β augmented the proportion IL-8^+IL-17^+IFN-γ^- and IL-8^+IL-17^+IFN-γ^+ cells (p<0.004 and p<0.009, respectively; **Figure 2-6b**). The frequency of IL-8^+IFN-γ^+IL-17^- (named hereafter Th8/Th1) cells was increased by IL-12 (p<0.0002; **Figure 2-6b**).

Taken together, IL-1β favored the emergence of IL-8^+IL-17^+IFN-$\gamma^{-/+}$ $CD4^+$ T cells whereas IL-12 promoted IL-8^+IFN-γ^+IL-$17^{-/+}$ $CD4^+$ T cells in UC colon.

2.2.3.7 *IL-12 promotes IL-8 expression in effector memory Th17 cells from mesenteric lymph nodes of UC patients.*

Since mucosal $CD4^+$T cells emigrate from mesenteric lymph nodes (mLNs) to gut tissue, we also examined the ability of IL-12 and IL-1β to regulate IL-8 expression in mLNs before their recruitment to colon. Similar to mucosal $CD4^+$ T cells, IL-1β augmented the frequencies of IL-8^+IL-17^+IFN-γ^- (p<0.05) and IL-8^+IL-17^+IFN-γ^+ $CD4^+$ T cells (p<0.05), while IL-12 increased IL-8^+ IFN-γ^+IL-17^- (p<0.03) and IL-8^+IL-17^+IFN-γ^+ $CD4^+$ T cells (p<0.05) in mLNs (**Figure 2-7a** and **Figure 2-7b**). To further examine the contribution of Th17 and Th1 cells to the increased IL-8 production in response to IL-1β or IL-12, we purified effector memory ($CD62L^{low}$) $CD4^+$ T cells according to $CCR6^+CXCR3^-$ and $CCR6^-CXCR3^+$ expression, which displayed a Th17 T_{EM} and Th1 T_{EM} cytokine profile, respectively (**Figure 2-7a**). In cultures with Only Th17 T_{EM}, but not Th1 T_{EM}, was increased IL-8 expression observed under the influence of IL-1β or IL-12 (p<0.007 and p<0.008 respectively) (**Figure 2-7c**). Finally, similar to its effect on mucosal

$CD4^+$ T cells (**Figure 2-5**), IL-12 favored a pathogenic IL-8 profile in mLN Th17 T_{EM} as shown by co-expression of pro-inflammatory cytokines (p<0.05; **Figure 2-7d**).

2.2.3.8 *Mucosal $CD163^-$, but not $CD163^+$, MNPs augment IL-8 expression in colonic $CD4^+$ T cells of UC but not CD patients.*

Finally, we examined the ability of $CD163^-$ cells to regulate IL-8 expression. $CD163^-$ cells increased the frequency of IL-8-producing $CD4^+$ T cells (p<0.0009; **Figure 2-8a**). Interestingly, the enhanced IL-8 expression appeared to be restricted to UC since it was not observed in co-cultures of autologous colonic $CD163^-$ and $CD4^+$ T cells isolated from CD patients (**Figure 2-8a**). Notably, IL-8 expression was not detected *ex vivo* in colonic $CD4^+$ T cells isolated from CD patients nor was it increased in co-cultures with autologous colonic $CD163^-$ MNPs, or in response to either IL-1β or IL-12 (**Figure 2-S4a**, **Figure 2-S4b** and **Figure 2-S4c**). Furthermore, $CD163^-$ but not $CD163^+$ cells increased the frequency of $IL\text{-}8^+IL\text{-}17^+$ and $IL\text{-}8^+IFN\text{-}\gamma^+$ $CD4^+$ T cells in UC patients (p<0.0004 and p<0.0009 respectively; **Figure 2-8b**). Also, the frequencies of $IL\text{-}8^+IL\text{-}17^+IFN\text{-}\gamma^-$ (p<0.02), $IL\text{-}8^+IL\text{-}17^+IFN\text{-}\gamma^+$ (p<0.0007) and $IL\text{-}8^+IFN\text{-}\gamma^+IL\text{-}17^-$($p$<0.02) $CD4^+$ T cells were augmented by $CD163^-$cells (**Figure 2-8c**, left panels). Finally, we explored some of the mechanisms that governed the ability of $CD163^-$ cells to increase IL-8 expression in mucosal $CD4^+$T cells in UC. When IL-1β function was neutralized, the proportion of $IL\text{-}8^+IL\text{-}17^+IFN\text{-}\gamma^-$ and $IL\text{-}8^+IL\text{-}17^+IFN\text{-}\gamma^+$ $CD4^+$T cells were not augmented by $CD163^-$ cells **(Figure 2-8c**, right panels). Combined IL-1β and IL-12 blockade significantly decreased the frequencies of $IL\text{-}8^+IFN\text{-}\gamma^+IL\text{-}17^-$($p$<0.02) and $IL\text{-}8^+IL\text{-}17^+IFN\text{-}\gamma^+$ $CD4^+$T cells (p<0.009), which were not reduced by adding anti-IL-12p70 mAb alone (**Figure 2-8c**, right panels).

Taken collectively, mucosal $CD163^-$ MNPs augment IL-8 expression by colonic $CD4^+$ T cells in UC but not CD mucosa, and further promote IL-8 and IL-17 co-expression through their secretion of IL-1β, and IL-8 and IFN-γ co-expression via IL-1β and IL-12 production.

2.2.3.9 *Unsupervised multi-color flow cytometry analysis reveals that $CD163^-$ and $CD163^+$ MNPs form distinct clusters related to monocyte-like and macrophage cell populations respectively.*

The heterogeneity of $CD14^+CD64^+$ MNPs was further assessed using multi-color FACS analysis (inflamed mucosa n=4 UC patients) (**Figure 2-9a**). Feature *t*-SNE plots of CD163, CD16, CD206 and CD209 expression identified a cluster, which was distinct from the $CD163^{-/dim}$ cluster best defined by CD11b, CCR2 expression and low FSC-cell size. Noteworthy, feature plot of CLEC5-A expression appeared to cluster with a minor fraction of CD11b-expressing cells. To evaluate which markers were driving the $CD163^-$ and $CD163^+$ cell-specific signature, the flow cytometry data was next analyzed using an unsupervised self-organizing map (FlowSOM) method (**Figure 2-9b**, **Figure 2-9c**, **Figure 2-9d** and **Figure 2-9e**). The five clusters identified using FlowSOM were overlaid in the *t*-SNE plot of concatenated $HLA\text{-}DR^+SIRP\alpha^+CD14^+CD64^+$ cells (**Figure 2-9b**). Two $CD163^-$ clusters were best identified using CD11b and CD206 expression marker with the major one (blue: 47.7%) expressing CD11b at the highest and CD206 at the lowest intensity, and vice versa for the minor one (green: 6%), relative to the other three clusters. The $CD163^{dim}$ clusters were defined as $CD209^{dim}CD206^{dim}$ (purple: 11.1%)) or $CLEC5A^{bright}$ $TREM\text{-}1^+$ (red: 3.5%) cells. Elevated relative expression of CD14, CD64, MERTK, CD209, CD206 and CD16 but low CD11b expression identified the $CD163^+$ cluster (yellow: 31.6%). Heatmap and FlowSOM minimal spanning tree, with star

charts displaying different intensities of co-expressed surface markers, further characterized $CD163^{+}$ population (yellow) that clustered apart from $CD163^{-/dim}$ populations (**Figure 2-9c** and **Figure 2-d**). Noteworthy, analysis of a second panel of surface markers that included 3 additional markers (CD169, TIM4 and CD4) and 12 common markers revealed that $CD163^{+}$ cells were further subdivided into $CD11b^{-}CD169^{+}TIM4^{+}CD4^{+}$ and $CD11b^{dim}CD169^{-}$ and $TIM4^{-}$ cells (**Figure 2-9e**).

The $CD14^{+}CD64^{+}$ MNPs were next purified at the extreme ends of the spectrum of CD163 expression, according to the gating strategy that was originally selected to quantify $CD163^{-}$ and $CD163^{+}$ subsets in the UC mucosa in **Figure 2-2d**, to assess their morphology (**Figure 2-S5**). $CD163^{-}$ cells displayed a kidney-shaped nucleus while $CD163^{+}$ cells resembled typical Mϕ with vacuoles and a large cell size, corroborating our multi-color FACS analysis.

Collectively, the $CD14^{+}CD64^{+}$ MNP subpopulation that predominates in inflamed UC mucosa is best defined as $CD163^{-}CD206^{-}CD209^{-}MERTK^{-}CLEC5\text{-}A^{-}TREM\text{-}1^{dim}HLA\text{-}DR^{dim}CCR2^{+}$ $CD11b^{bright}$ monocyte-like pro-inflammatory cells, while the minor $CD163^{+}$ cells are $CD209^{+}CD206^{+}$ $MERTK^{+}$ Mϕ.

2.2.4 DISCUSSION

The novel and unexpected finding of the present study is that IL-8-expressing T cells represented a minor $CD4^+$ T population, which was detected in inflamed colon of UC but not CD patients. As such, these cells might be implicated in the development and/or perpetuation of UC. UC shares several genetic, clinical, histological and immunologic features with CD (Ungaro et al., 2017), which are both T cell-mediated diseases (Globig et al., 2014; Li et al., 2016). Nonetheless, these two IBD represent distinct entities (Christophi et al., 2012; Haberman et al., 2014; Iboshi et al., 2014), as highlighted in our present study. Firstly, mucosal $CD4^+$ T cells isolated from UC colon did not increase their IL-17 or IFN-γ secretion in response to IL-23, while we and others reported that IL-23 increased mucosal Th17 and Th17/Th1 responses in CD (Ramesh et al., 2014). The absence of IL-23 response in UC could not be attributed to the loss of IL-23 receptor (IL-23R) on the surface of mucosal $CD4^+$T cells, since Kobayashi et al demonstrated that colonic $CD4^+$T cells express *IL23R* mRNA in both UC and CD (Chapuy et al., 2019a; Kobayashi et al., 2008). Secondly, IL-8 expression was detected *ex vivo* in colonic $CD4^+$ T cells, and augmented by IL-12, IL-1β, and $CD163^-$ but not $CD163^+$ MNPs in UC only. Thirdly, IL-12 and IL-1β differentially regulated TNF-α, GM-CSF and IL-6 production by colonic $CD4^+$ T cells in CD (Chapuy et al., 2019a) and , as shown here in UC. These three cytokines were augmented by IL-12 but not IL-1β in UC only and, by IL-1β but not IL-12 in CD only. We therefore propose that the distinct IL-8 responses observed between UC and CD likely result from disease-specific differences in T cells rather than a difference intrinsic to $CD163^-$ cell function, which might be clinically relevant with regard to UC and CD pathogenesis.

MNPs play a critical role in the maintenance of gut homeostasis, orchestrating the dialogue between innate and adaptive immunity (Grainger and Konkel, 2017). Morphology and phenotypic studies of $CD163^-$ and $CD163^+$ cells attempted to relate the nature of these two functionally distinct $CD14^+CD64^+$ subpopulations to intestinal $CD14^+$ MNPs and their murine counterparts previously identified under inflammatory or homeostatic conditions (Bain et al., 2013). Human $CD163^-$ cells displayed a monocyte-like shape, and thus could not be considered as Mϕ. These cells resemble tissue $Ly6C^+CD64^+$ inflammatory monocytes in murine colon (Grainger et al., 2013). In colitic mice and ileal CD, extravasated inflammatory monocyte-derived cells (P1), best defined as $CD11c^{-/dim}CD11b^{dim/+}CD14^+CD64^{low}$ $SIRP\alpha^+$MHC $classII^-$ cells progressively develop into mature $CD11c^{++}CD11b^+CD14^{++}CD64^{++,}$ $SIRP\alpha^+$MHC $classII^{+++}$ (P4) Mϕ, unless the maturation process is interrupted under inflammatory conditions (Bain et al., 2013).

We hypothesize that recruited HLA-$DR^{dim}CD14^+$ monocytes ($CD206^-CD209^-$MERTK$^-$ TREM-1 $^{dim}CCR2^+CD11b^{bright}CD163^-$ cells) progressively acquired CD163, MERTK, CD209, CD206 and down-regulated CD11b and CCR2. In that regard, in inflamed CD colon, we recently characterized two $CD14^+$ populations using single cell RNA profiling and demonstrated that inflammatory monocyte-like (TREM-$1^+CD206^-CD209^-CD163^-$) are distinct from TREM-1^- $CD206^+CD209^+$MERTK$^+CD163^+$ Mϕ. Like in UC, $CD163^-$ and not $CD163^+$ cells accumulate in inflamed CD colon (Chapuy et al., 2019a). Furthermore, $CD163^-$ cells might be related but still distinct from $CD14^+$CLEC5-$A^+CD209^-CD11b^+CD11c^+$ cells, which are potential drivers of chronic intestinal inflammatory response (Gonzalez-Dominguez et al., 2015). In fact, $CD163^-$ cells clustered apart from CLEC5-A^{bright}TREM-1^+ cells, which together with $CD209^{dim}CD206^{dim}$ cells might represent transitioning $CD163^{dim}$ cells. The latter were excluded

for our functional studies. Regarding $CD163^+$ cells, they displayed a Mϕ morphology and thus resemble Mϕ3 or Mϕ4 subsets that are also derived from recruited monocytes in human jejunum at homeostasis (Bujko et al., 2018a). As opposed to Mϕ1 or Mϕ2 precursors that expressed CD11c in healthy small intestine, Mϕ3 or Mϕ4 are not $CD11c^+$ (Bujko et al., 2018a). However, CD11c expression was not a discriminative surface marker between $CD163^-$ and $CD163^+$ cells in inflamed UC mucosa since it was expressed at high intensity in both subsets, corroborating the phenotype of $CD14^+$ MNPs in human inflamed colon (Bernardo et al., 2018; Gonzalez-Dominguez et al., 2015). The $CD16^+CD163^+$ Mϕ did not accumulate in inflamed UC colon or regulate IL-8 expression, Th17, Th17/Th1 responses in colonic $CD4^+$ T cells. In fact, inflammatory $CD16^+$ Mϕ do not regulate memory T cell responses in ascites of cancer patients (Segura et al., 2013). Finally, $CD163^+$ Mϕ also included a subset of $TIM4^+CD4^+$ cells, which were not yet reported in humans, but recently defined as tissue resident Mϕ in mice (De Schepper et al., 2018; Shaw and Houston, 2018). The $TIM4^+CD4^+CD163^+$ Mϕ subpopulation co-expressed CD169; $CD169^+$ Mϕ phenotype contribute to monocyte recruitment in mice (Asano et al., 2015).

Overall, we propose to refer to $CD163^-$ inflammatory monocyte-like cells as "monocytes-derived effector cells (MDEC)". Conversely, the $CD163^+$ cells might be considered anti-inflammatory, regulatory Mϕ and/or "post-inflammatory" Mϕ since these cells produced more IL-10 while expressing similar amount of pro-inflammatory cytokines as compared to MDEC ($CD163^-$ cells). Although the interpretation of these data should be taken cautiously, human colonic $CD163^-$ cells that accumulate in inflamed UC mucosa could represent a functionally distinct $CD14^+$ monocyte-like subpopulation endowed with plastic capacities.

IL-8 is a potent chemoattractant for neutrophils (Bruno et al., 2015; Fonseca-Camarillo and Yamamoto-Furusho, 2013; Mitsuyama et al., 1994), which plays an important role in the pathophysiology of UC. Increased *IL8* mRNA expression has been detected in the inflamed mucosa of IBD patients and levels of IL-8 expression correlate with endoscopic severity in UC (Mitsuyama et al., 1994). After multiple rounds of expansion and activation, mucosal Th17 clones, which were generated from IBD or colorectal cancer, secrete IL-8 that attracts neutrophils (Amicarella et al., 2017; Pelletier et al., 2010). Both studies highlight the biological relevance of IL-8 produced by mucosal Th17 cells. However, the limitation of the present study was the inability to demonstrate IL-8-induced neutrophil chemotaxis using primary colonic $CD4^+$ T cells of UC patients. Furthermore, IL-8 is abundantly expressed by a variety of cells in the gut mucosa, notably by neutrophils, endothelial and epithelial cells as well as monocytes, Mϕ, fibroblasts and possibly T cells (Beck et al., 2016; Brandt et al., 2000). We showed here that a minor colonic $CD4^+$ T population expressed IL-8 in UC, but not CD mucosa. Some studies demonstrate IL-8 production by circulating T cells isolated from healthy adults and cord blood, suggesting that T cell priming has occurred *in utero* (Akhade and Qadri, 2015; Gasch et al., 2014; Gibbons et al., 2014). However, polarizing conditions to differentiate naïve T cells into single IL-8-producing cells ("Th8") remain unknown. IL-8 itself might induce IL-8 in human $CD4^+$ T cells (Gesser et al., 1995). Furthermore, addition of flagellin, which is abundantly detected in the colon, increases the percentage of $IL\text{-}8^+$ cells in circulating TCR-stimulated $CD4^+$ T cells (Gibbons et al., 2014). In a rat model of colitis, IL-8 levels increase before the influx of neutrophils (Harada et al., 1994), supporting the concept that IL-8-producing T cells could be implicated at the early phase of disease. However, IL-8 is also detected in $CD3^+$ cells

in chronic lesions using immunofluorescence technique, arguing for a role for T cell derived-IL-8 in the perpetuation of the disease (Brandt et al., 2000).

It is unclear whether IL-8-producing T cells play a protective or pro-inflammatory role in UC mucosa. In that regard, circulating IL-8$^+$Foxp3$^+$CD25$^+$ T cells, with a dual suppressive and inflammatory phenotype, that promote pro-inflammatory cytokine production and neutrophil attraction, are also reported in UC inflamed mucosa (Kryczek et al., 2016). Since we could not detect *ex vivo* IL-8 expression in Foxp3$^+$CD25$^+$ T cells in inflamed UC mucosa, these T cells were excluded from our gating strategy that exclusively analyzed CD4$^+$CD25$^-$ T cells. A protective function might be attributed to colonic IL-8$^+$CD4$^+$ T cell population, due to their GM-CSF production. GM-CSF ameliorates colitis in mice via its effect on monocytes that led to bacterial clearance and epithelial healing (Dabritz et al., 2015). Furthermore, human circulating activated Th17 clones directly attract neutrophils through IL-8 release while Th17/Th1 clones increase neutrophil activity via GM-CSF (Pelletier et al., 2010). In contrast, both innate lymphoid cells type 3 and T cell-derived GM-CSF contribute to intestinal inflammation in experimental colitis (Pearson et al., 2016). IL-8-producing T cells that co-expressed GM-CSF, IL-6, TNF-α and IFN-γ *ex vivo*, might therefore lead to an early destruction of epithelial barrier. Our data further revealed that IL-12 biased Th17 T_{EM} towards IL-8$^+$IFN-γ$^+$ CD4$^+$ T cells that expressed TNF-α and GM-CSF in mLNs, highlighting the potential pathogenicity of IL-8$^+$ T cells in disease tissue. Noteworthy, IL-12 but not IL-1β significantly augmented IL-8$^+$IFN-γ$^-$ IL-17$^-$IL-22$^-$ CD4$^+$ T cells in UC mucosa, and concomitantly reduced the proportion of IL-22$^+$IL-8$^-$CD4$^+$ T cells, which might further contribute to epithelial cell destruction (**Figure 2-S6a**). Furthermore, IL-12, IL-1β and CD163$^-$ MNPs augmented the percentages of IL-8$^+$IFN-γ$^+$IL-17$^-$ or IL-8$^+$L-17$^+$IFNγ$^+$ T cells, irrespective of IL-22 co-expression, while the frequencies

of IL-8$^+$IL-17$^+$IFN-γ$^-$ T cells not co-expressing IL-22 were augmented only by IL-1β or CD163$^-$ MNPs (**Figure 2-S6b**). CD163$^-$ MNPs, through IL-1β production, amplified colonic inflammatory Th17 and Th17/Th1 responses in both UC and CD (Chapuy et al., 2019a), while IL-1β-dependent increased frequencies of IL-8$^+$IL-17$^+$ CD4$^+$ T cells was observed in UC only. In that regard, IL-1β correlates with IL-8 levels in UC mucosa (Mitsuyama et al., 1994) and promotes the survival of Th17 cells in murine IBD (Coccia et al., 2012).

Currently approved therapeutic approaches using anti-TNF-α or anti-IL-12p40 mAbs and ongoing clinical trials using anti-α4β7 or αEβ7 integrin mAbs in UC patients, are aimed toward impairment of cell recruitment, function and/or retention to inflamed mucosa (Feagan et al., 2013; Zundler et al., 2017). Unlike Th17, Th17/Th1 and Th9 cells (Zundler et al., 2017), colonic IL-8$^+$ T cells appeared to be α4β7 or αEβ7 negative since these cells expressed α4 but not β7 integrin, suggesting that intestinal inflammatory IL-8$^+$ T cells might utilize α1 for their tissue recruitment (Rivera-Nieves et al., 2005). Expression of CD69, but not CD103, by colonic IL-8$^+$ CD4 T cells suggested that they are not related to recently described tissue resident memory CD4$^+$ T cells in CD mucosa (Bishu et al., 2019; Zundler et al., 2019b).

In conclusion, colonic CD14$^+$CD163$^-$ MDECs producing IL-1β and IL-12p40, and their propensity to augment IL-8 expression in tissue T cells, might all be implicated in the regulation of gut inflammation in UC. Nevertheless, the potential role of colonic effector T cells producing IL-8 *in vivo* and its clinical relevance in UC but not CD warrants further investigations to better understand potentially distinct disease pathogenesis.

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2.2.6 FIGURES AND TABLES

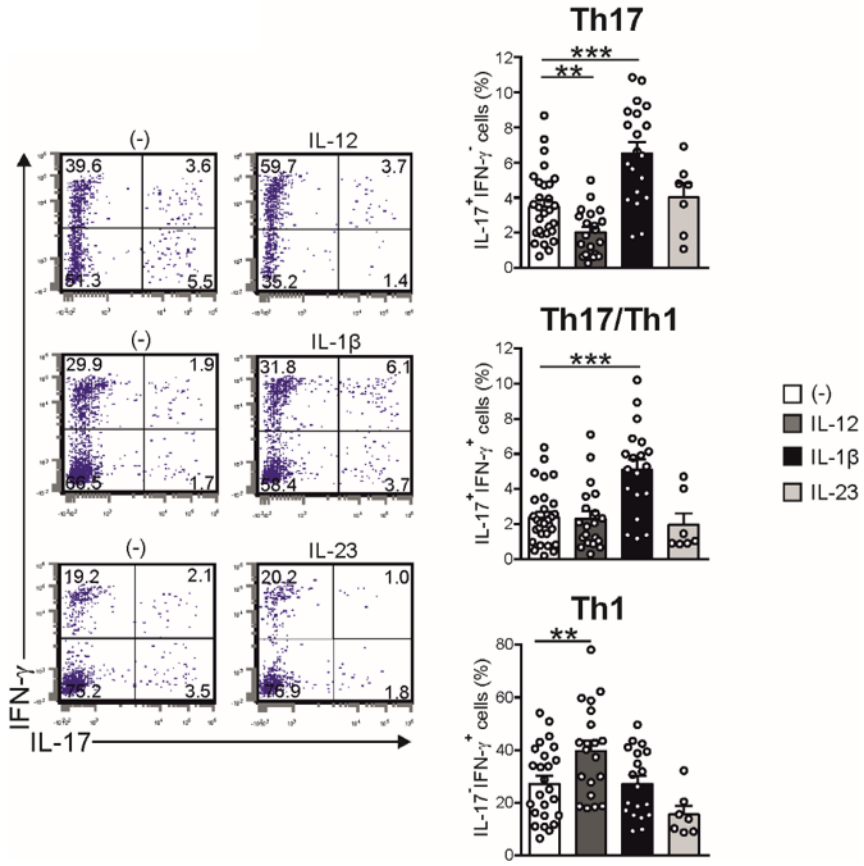

Figure 2-1: IL-1β increases Th17 and Th17/Th1 responses in UC patients.
Representative dot plots and percentages of mucosal CD4$^+$ T cells expressing IL-17 and/or IFN-γ after 6 days culture with either recombinant IL-12 (n=20), IL-1β (n=18) or IL-23 (n=7). Wilcoxon signed rank test, p<0.01 threshold for significance to account for test multiplicity.

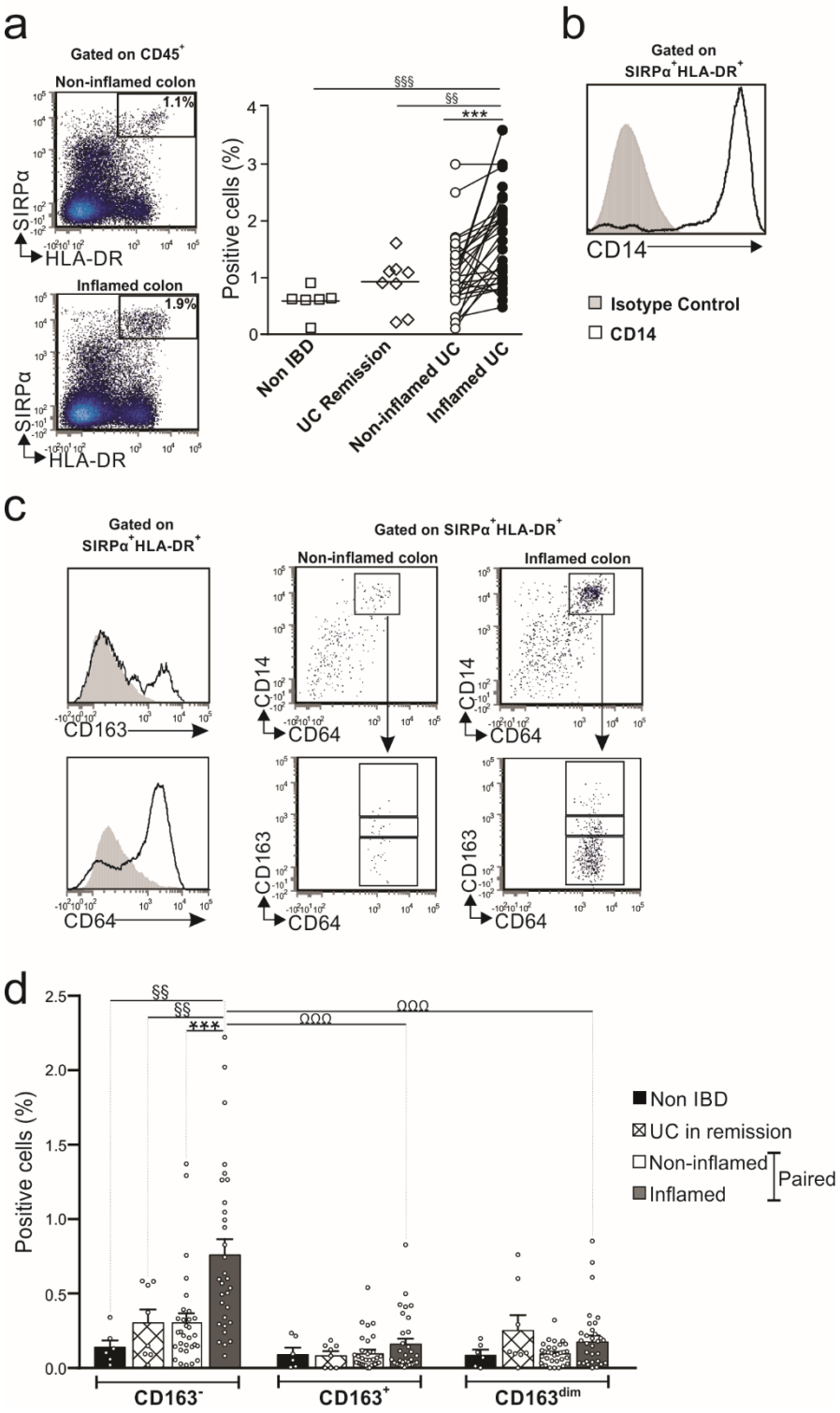

Figure 2-2: HLA-DR$^+$SIRPα$^+$CD14$^+$CD64$^+$CD163$^-$ MNPs accumulate in inflamed UC mucosa

a) *Percentage of HLA-DR$^+$SIRPα$^+$ cells among CD45$^+$ intestinal lamina propria mononuclear cells (LPMC): cell distribution in non-IBD control (n=6), UC in remission (n=8) and paired*

non-inflamed and inflamed UC patients (n=31). ***b)*** *CD14 expression on HLA-DR$^+$SIRPα$^+$ cells.* ***c)*** *HLA-DR$^+$SIRPα$^+$CD14$^+$ MNPs subdivided according to CD64 and CD163 expression.* ***d)*** *Frequencies of CD163$^-$, CD163$^+$, CD163^{dim} cells among CD45$^+$LPMC in non-IBD control (n=6), UC in remission (n=8), and paired non-inflamed and inflamed UC patients (n=31).* ***a and d****, Wilcoxon signed rank test, Mann-Whitney test and Friedman test with Dunn 's post test.* p<*0.01 threshold for significance to account for test multiplicity*.

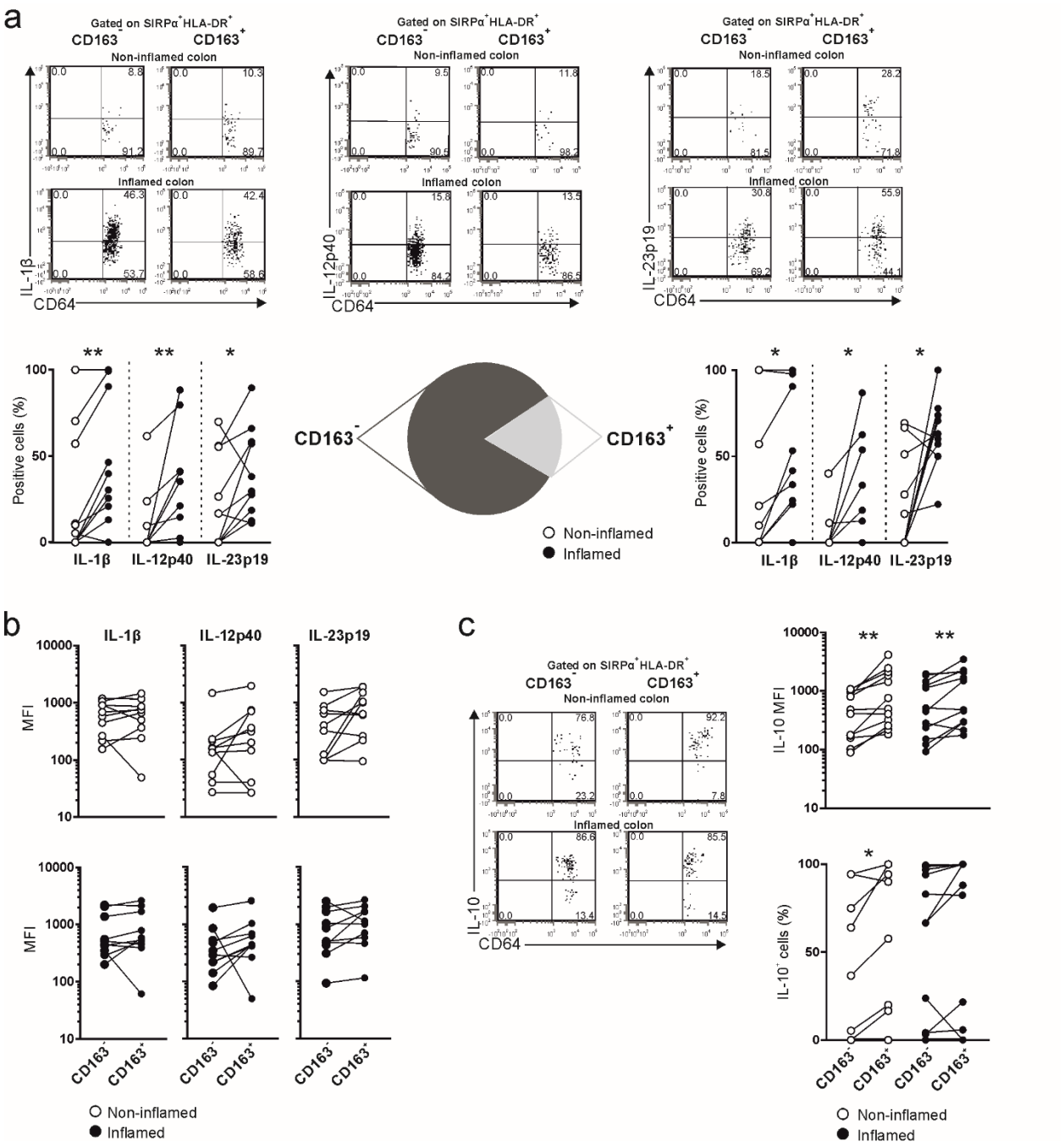

Figure 2-3: Mucosal CD163⁻ and CD163⁺ MNPs express similar amounts of pro-inflammatory cytokines but CD163⁺ cells produce more IL-10 relative to CD163⁻ cells.

a) *Frequencies and* ***b)*** *MFI of IL-1ß (n=10), IL-12p40 (n=10) and IL-23p19 (n=10) producing cells among CD163⁻ and CD163⁺ MNPs in non-inflamed and inflamed UC. Pie displays the relative frequency of CD163⁻ and CD163⁺ cells among CD14⁺CD64⁺ in inflamed UC mucosa.* ***c)*** *Frequencies and MFI of IL-10 (n=12) producing cells among CD163⁻ and CD163⁺ MNPs in non-inflamed and inflamed UC mucosa.* ***a to c****, Wilcoxon signed rank test.*

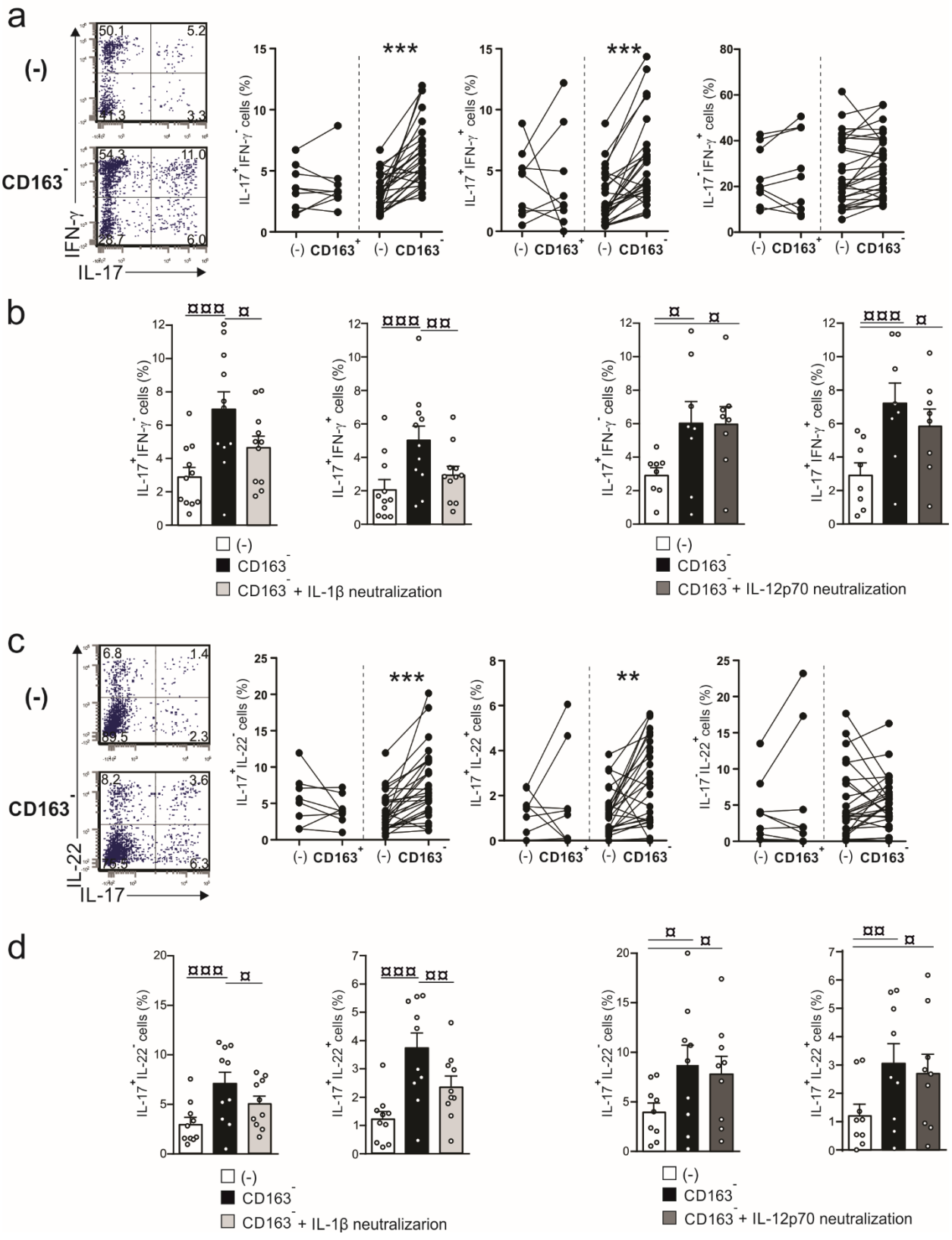
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Figure 2-4: CD163$^-$ but not CD163$^+$ MNPs promote Th17, Th17/Th1 and Th17/Th22 responses in an IL-1-dependent manner in UC patients.

CD4$^+$ T cells isolated from inflamed UC colon were co-cultured with or without autologous mucosal CD163$^+$ (n=9) or CD163$^-$ (n=27) cells, in the absence or presence of αIL-1R (n=6), αIL-1β (n=5) or αIL-12p70 mAbs (n=8), then stained for intracytoplasmic ***a and b,*** *IL-17/IFN-γ, as well as* ***c and d,*** *IL-17/IL-22 expression.* ***a and c,*** *Wilcoxon signed rank test;* ***b and d,*** *Repeated measures ANOVA with Bonferroni's multiple comparison post test*.

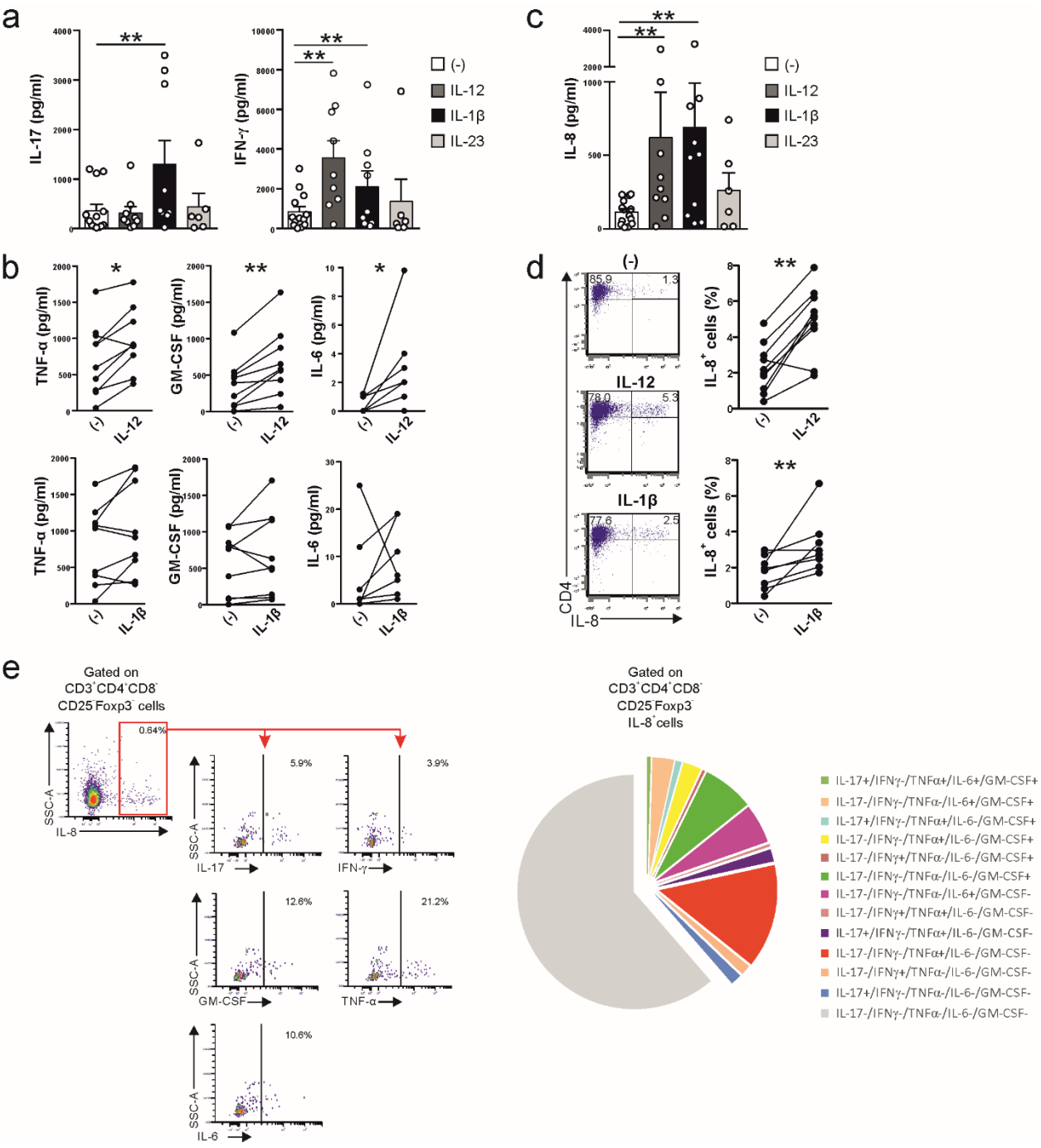

Figure 2-5: Mucosal CD4⁺ T cells produce IL-8 in UC patients.

UC mucosal CD4⁺ T cells were cultured with recombinant IL-12 (n=9), IL-1β (n=9) or IL-23 (n=4 to 6) for 6 days. ***a)*** *IL-17 and IFN-γ,* ***b)*** *TNF-a, GM-CSF, IL-6 and* ***c)*** *IL-8 secretion were measured in the culture supernatant.* ***d)*** *Percentage of IL-8⁺CD4⁺ T cells after culture of UC mucosal CD4⁺T cells with recombinant IL-12 (n=10) and IL-1β (n=8) for 6 days.* ***e)*** *Ex vivo stimulation of LPMC with PMA-ionomycin in the presence of brefeldin A for 4 hours (n=6),* ***left panel,*** *Percentage of IL-17, IFN-γ, GM-CSF, TNF-α, IL-6 positive cells among CD4⁺CD25⁻*

Foxp3$^-$IL-8$^+$ T cells; ***right panel,*** *Pie chart depicting the co-expression of IL-17, IFN-γ, GM-CSF, TNF-α and IL-6 in CD4$^+$CD25$^-$Foxp3$^-$IL-8$^+$ T cells.* ***a to d****, Wilcoxon signed rank test; for* ***a and c****, p<0.01 threshold for significance to account for test multiplicity.*

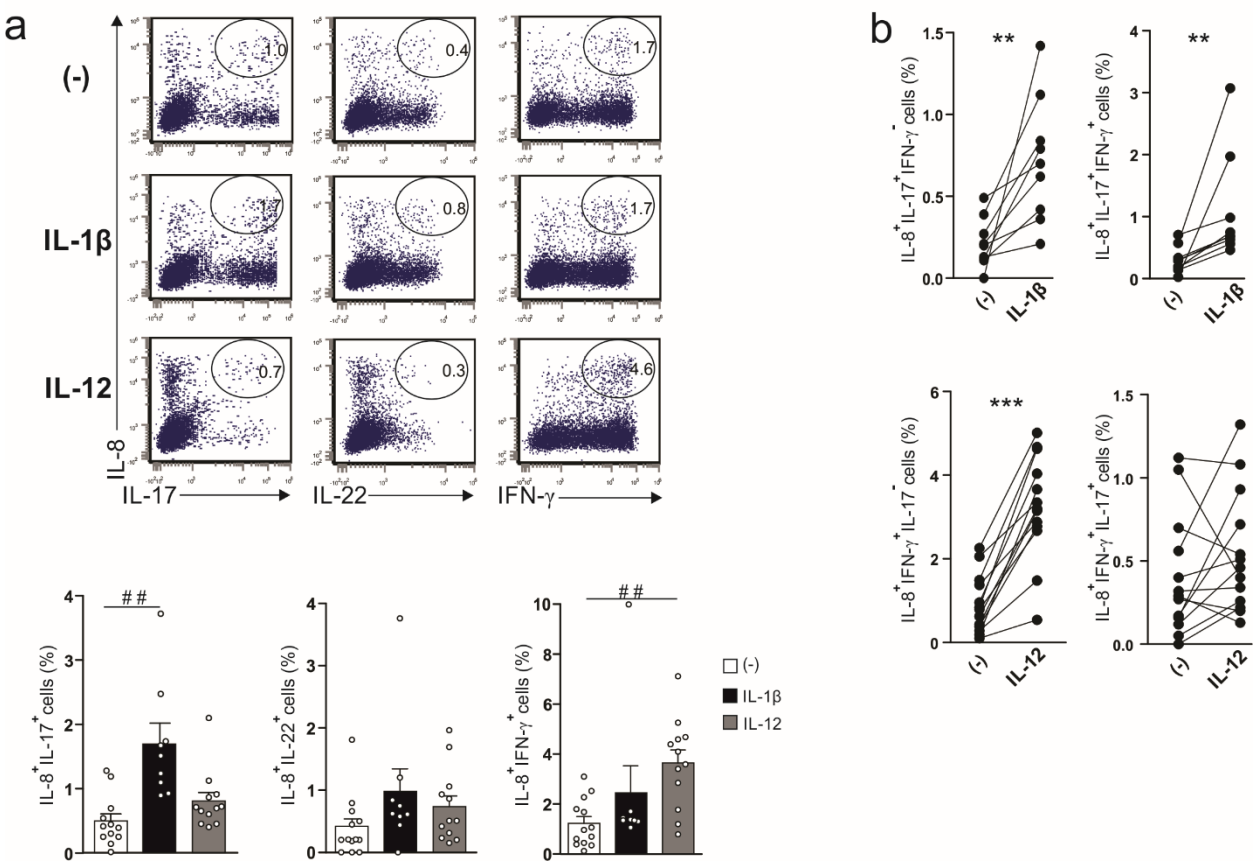

Figure 2-6: IL-12 promotes IL-8 and IFN-γ expression whereas IL-1β favors IL-17 and IL-8 in colon of UC patients.

UC mucosal CD4$^+$T cells were cultured for 6 days with recombinant IL-1β (n=9) or IL-12 (n=12), ***a)*** *Representative dot plots , percentage of IL-8$^+$IL17$^+$, IL-8$^+$IL-22$^+$, IL-8$^+$IFN-γ$^+$ T cells, and* ***b)*** *Percentages of IL-8$^{+/-}$IFN-γ$^{+/-}$IL17$^{+/-}$T cells* ***a,*** *Kruskal-Wallis test with Dunn's post test;* ***b,*** *Wilcoxon signed rank test.*

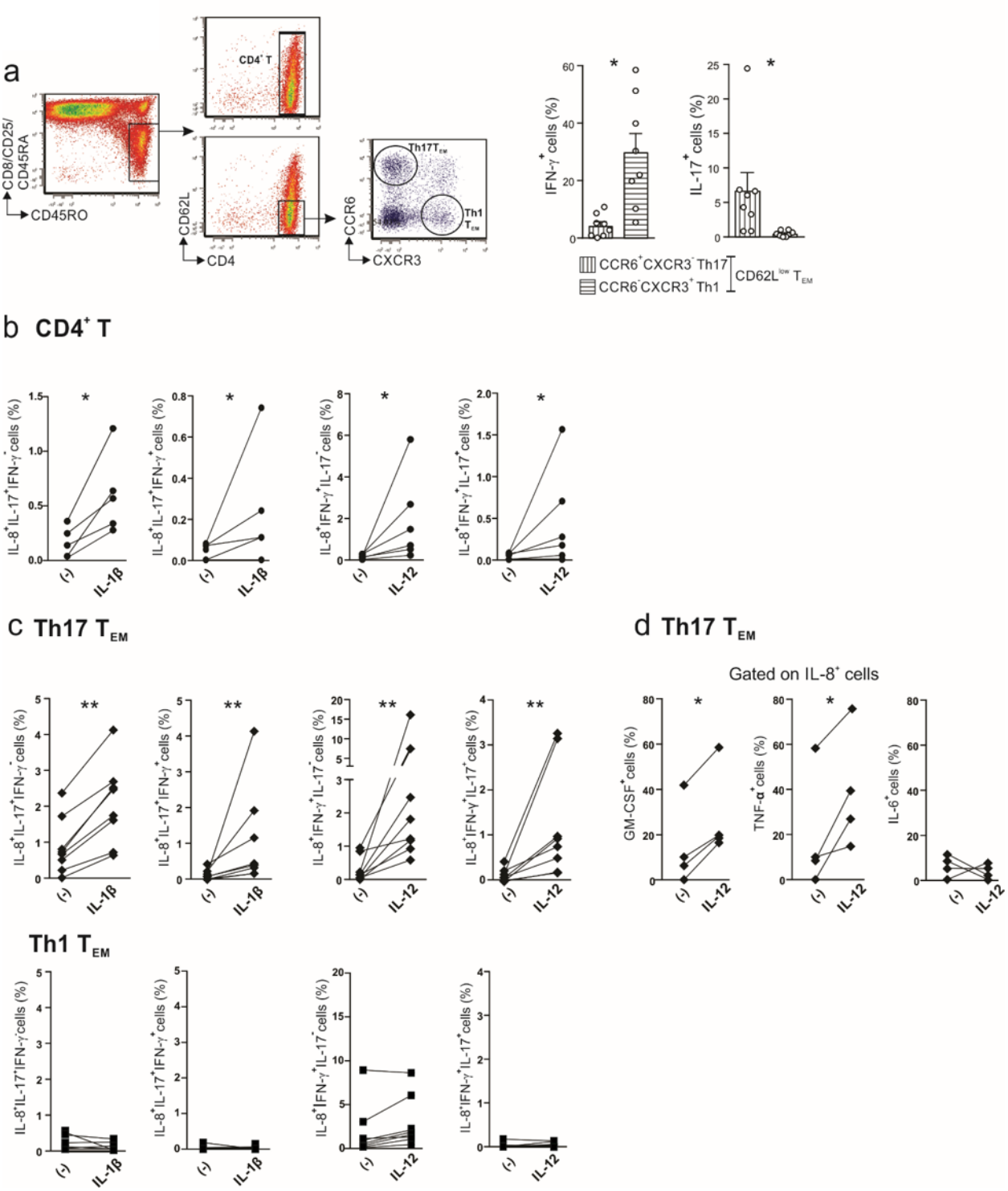

Figure 2-7: IL-1β and IL-12 favor IL-8 responses in Th17 cells isolated from mLNs of UC patients.

a) *Gating strategy for sorting CD4$^+$ T cells, Th17 T_{EM} (CD62L^{low}CCR6$^+$CXCR3$^-$) and Th1 T_{EM} (CD62L^{low}CCR6$^-$CXCR3$^+$) cells from mLNs of UC patients. Ex vivo intracytoplasmic staining for IFN-γ and IL-17 expression after PMA-ionomycin stimulation in the presence of brefeldin*

A for 4 hours. ***b-c)*** *Percentages of IL-8^{+}IL-17$^{+/-}$IFN-γ$^{+/-}$ after 6 days culture of* ***b)*** *total CD4^{+}T cells with recombinant IL-1β (n=5) or IL-12 (n=6).* ***c)*** *Th17 or Th1 T_{EM} with recombinant IL-1β (n=8) or IL-12 (n=8).* ***d)*** *Percentages of GM-CSF^{+}, TNF-α^{+} and IL-6^{+} T cells among IL-8^{+} T cells after 6 days culture of Th17 T_{EM} with recombinant IL-12 (n=4).* ***a to d,*** *Wilcoxon signed rank test.*

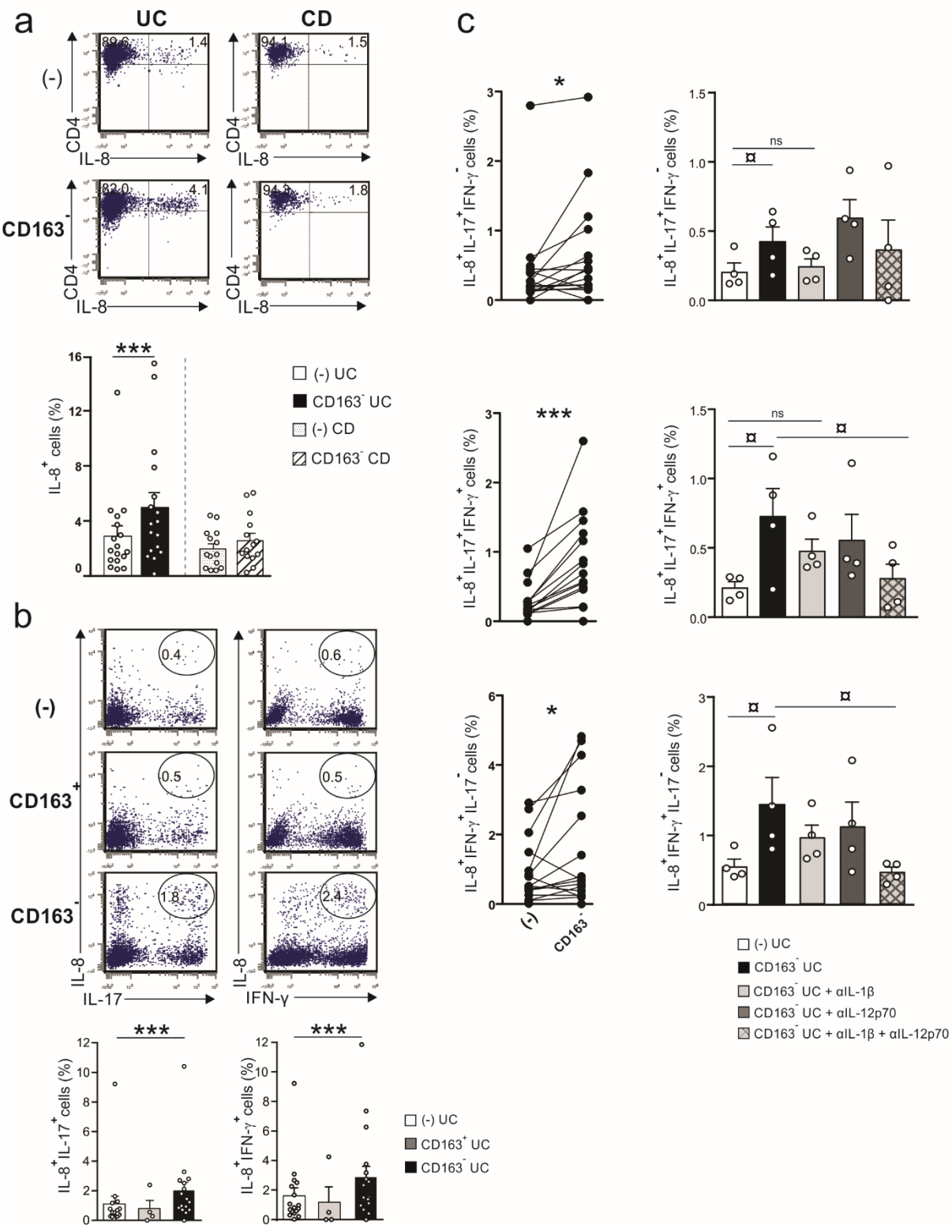

Figure 2-8: CD163⁻ MNPs increase IL-8 expression in colonic CD4⁺T cells in UC but not CD patients.

$CD4^+$ T cells isolated from inflamed UC or CD colons were co-cultured with or without ***a)*** *autologous mucosal $CD163^-$ cells (n=17 UC, n=14 CD) and stained for IL-8 intracytoplasmic expression,* ***b)*** *autologous mucosal $CD163^+$ (n=4) or $CD163^-$ cells (n=17) and stained for IL-8, IL-17 and IFN-γ intracytoplasmic expression;* ***c)*** *autologous $CD163^-$ cells (n=17), in the absence or presence of αIL-1β (n=4) and/or αIL-12p70 mAbs (n=4), and stained for IL-8, IL-17 and IFN-γ intracytoplasmic expression.* ***a, b, c (left panel),*** *Wilcoxon signed rank test;* ***for b****, $p<0.01$ threshold for significance to account for test multiplicity.* ***c (right panel),*** *Repeated measures Anova with Bonferroni's multiple comparison post test.*

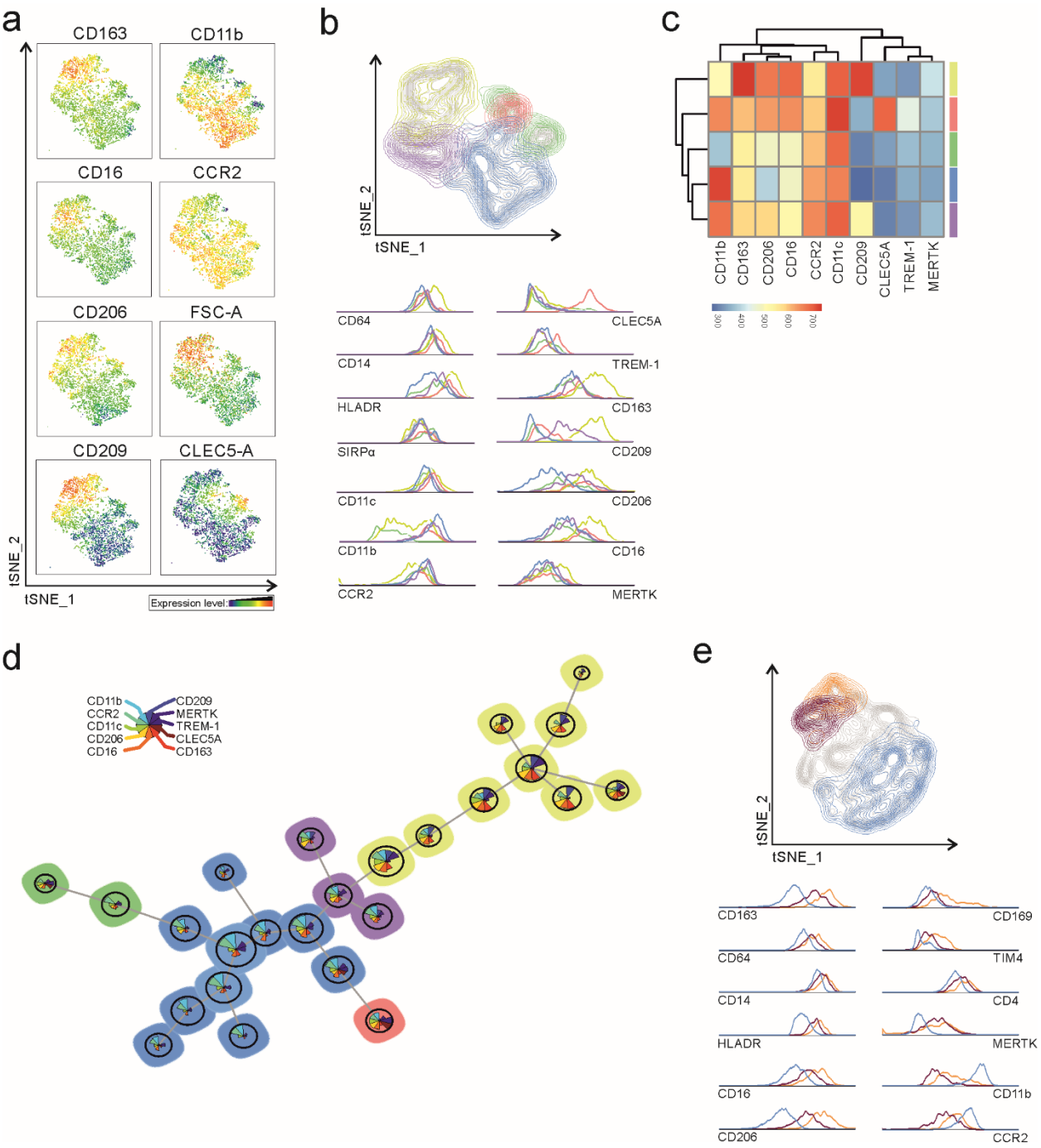

Figure 2-9: Unsupervised analysis of the phenotype of HLA-DR$^+$SIRPα$^+$CD14$^+$CD64$^+$ MNPs in inflamed UC mucosa.

LPMC from 4 UC patients were stained with a panel of 15 surface markers. Concatenated file from the 4 patients were subjected to unsupervised clustering analysis. ***a)*** *Expression feature plot of the depicted surface markers in HLADR$^+$SIRPα$^+$CD14$^+$CD64$^+$ cells, using t-SNE algorithm.* ***b to d)*** *FlowSOM analysis of HLADR$^+$SIRPα$^+$CD14$^+$CD64$^+$ cells based on expression data;* ***b)*** *affiliation of cells to 5 clusters identified in FlowSOM, indicated by color*

coding and visualized in t-SNE plot; surface marker expression levels depicted by histograms; ***c)*** *heatmap of mean surface marker expression of the 5 individual clusters;* ***d)*** *cells were clustered into 25 nodes and depicted as a minimal spanning tree, with color coding indicating the 5 identifed clusters. Circle size are proportional to the number of cells represented in each node and triangle size in star charts depicts the mean intensities of each marker for all cells assigned to the node (color legend on the left).* ***e)*** *LPMC from the same 4 UC patients were stained with another panel of 15 surface markers, only differing for CD169, TIM4 and CD4. Concatenated file of HLA-DR$^+$SIRPa$^+$CD14$^+$CD64$^+$ cells from the 4 UC patients were subjected to FlowSOM analysis. Affiliation of cells to 3 out of 5 clusters identified in FlowSOM indicated by color coding and visualized in t-SNE plot; surface marker expression levels depicted by histograms.*

Table 2-1: Patient's characteristics

	UC	CD	Non IBD
N	83	19	6
Females, n(%)	47 (56.6)	11 (63.1)	3 (50)
Age, median (range)	42 (18-80)	37 (21-80)	60 (36-76)
Age at diagnosis			
< 16	6	3	
17-40	52	11	
> 40	25	5	
Treatment			
None	15	8	
5-ASA alone	38	1	
Thiopurine or methotrexate	14	6	
TNFα inhibitor	9	4	
Corticosteroid	21	2	
Disease location – UC			
Proctitis	15		
Left side colitis	39		
Pancolitis	26		
Proximal colitis	3		
Disease location – CD			
Terminal ileum		0	
Colon		15	
Ileocolonic		4	
Upper GI tract		0	
Disease behavior			
Non stricturing - Non penetrating		15	
Stricturing		3	
Penetrating		1	
Perianal disease		3	
Diagnosis – Control			
Screening colonoscopy			6

2.2.7 SUPPLEMENTARY INFORMATION

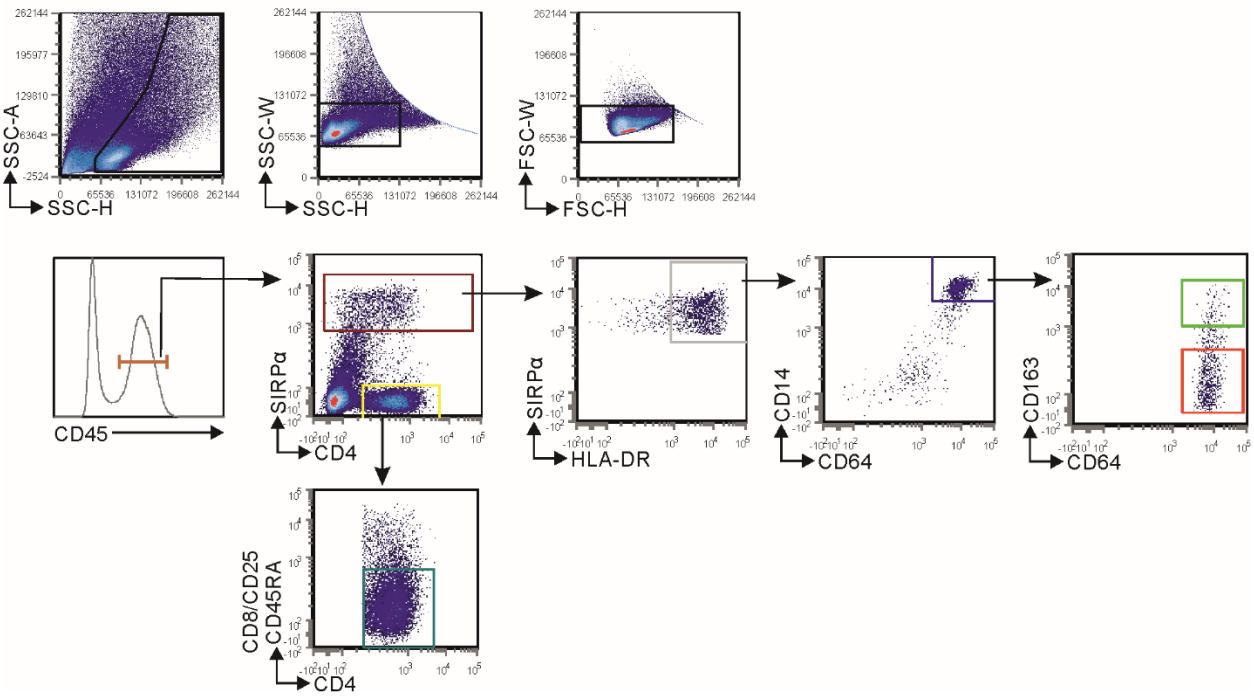

Figure 2-S1: Gating strategy for sorting CD163$^-$ and CD163$^+$ MNPs and CD4$^+$ T cells in inflamed UC mucosa.

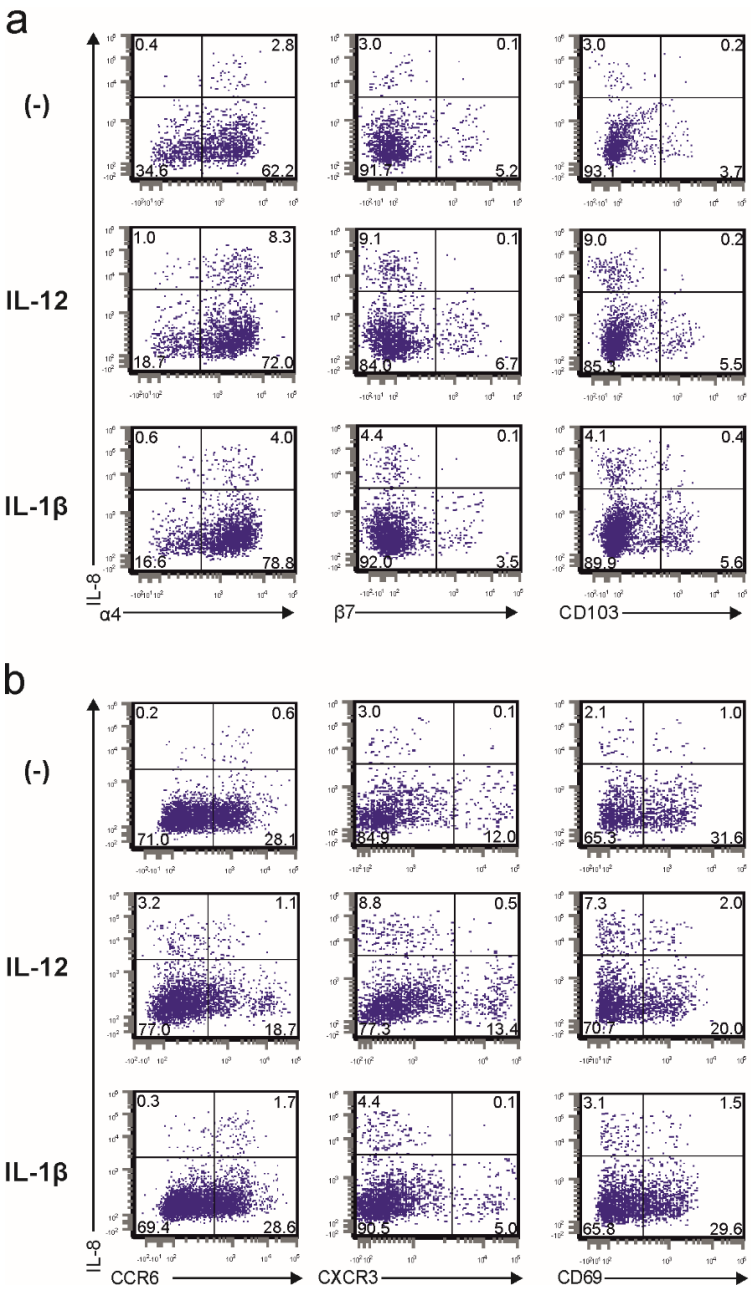

Figure 2-S2: Phenotype of IL-8-expressing CD4$^+$ T cells.

Representative dot plots of a) IL-8, α4, ß7, CD103 and b) IL-8, CCR6, CXCR3 and CD69 co-expression on UC colonic CD4$^+$ T cells after 6 days culture with or without IL-12 or IL-1ß (n=4).

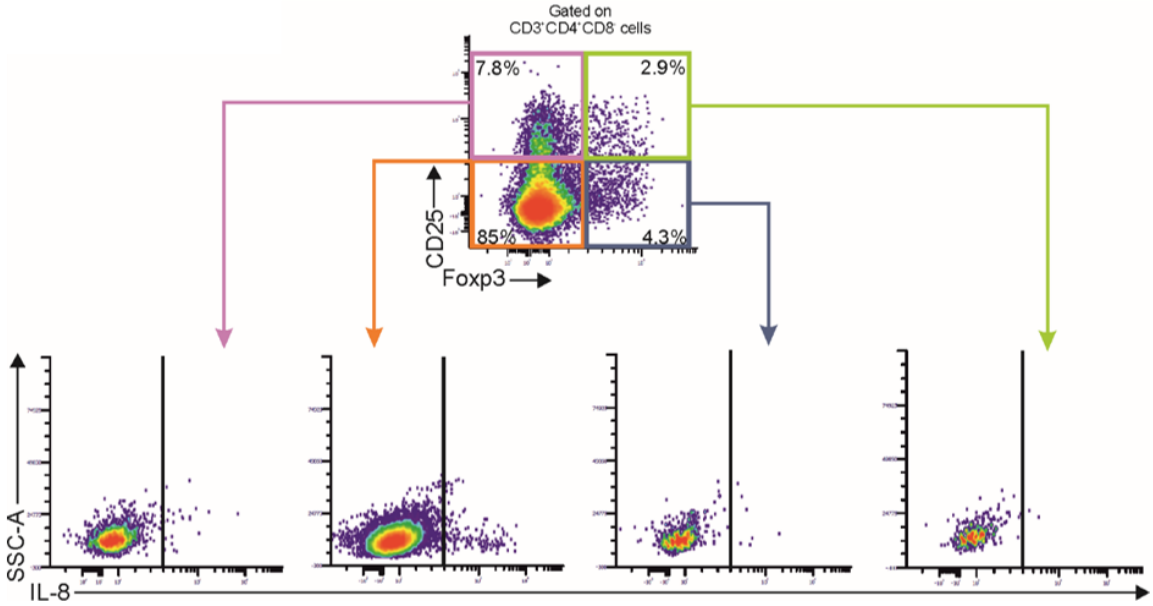

Figure 2-S3: IL-8 expression by colonic CD4^{+}CD25$^{+/-}$Foxp3$^{+/-}$ T cells.

Intra-cytoplasmic IL-8 expression in colonic CD4^{+}CD25$^{+/-}$Foxp3$^{+/-}$ T cells after stimulation with PMA-ionomycin in the presence of brefeldin A for 4 hours (representative dot plot of 6 UC patients).

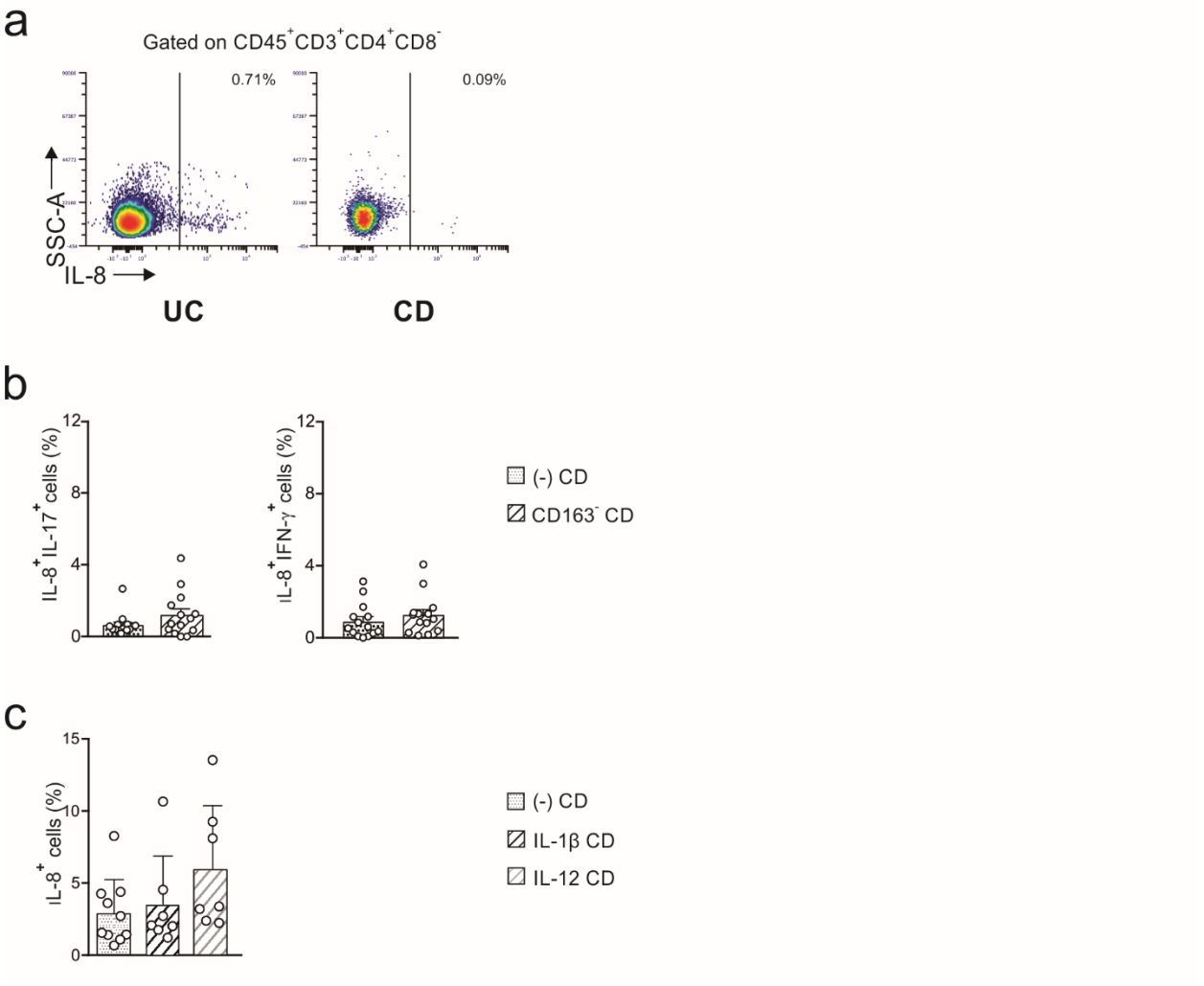

Figure 2-S4: CD163$^-$ MNPs did not increase IL-8 expression by CD4$^+$ T cells in the colon of CD patients.

a) *LPMC from inflamed UC and CD mucosa were stimulated for 4 hours with PMA-ionomycin in the presence of Brefeldin A, then stained for IL-8 expression (representative dot plot of 6 UC and 4 CD patients).* ***b)*** *CD4$^+$ T cells and CD163$^-$ cells were purified from inflamed CD colon (n=14) according to the gating strategy depicted in figure 2-S2. CD4$^+$ T cells and MNPs were co-cultured for 6 days and stained for IL-8, IL-17 and IFN-γ expression. Wilcoxon signed rank test.* ***c)*** *CD4$^+$ T cells purified from inflamed CD colon were cultured for 6 days with or without IL-1β (n=7) or IL-12 (n=7) and stained for IL-8 expression. Wilcoxon signed rank test.*

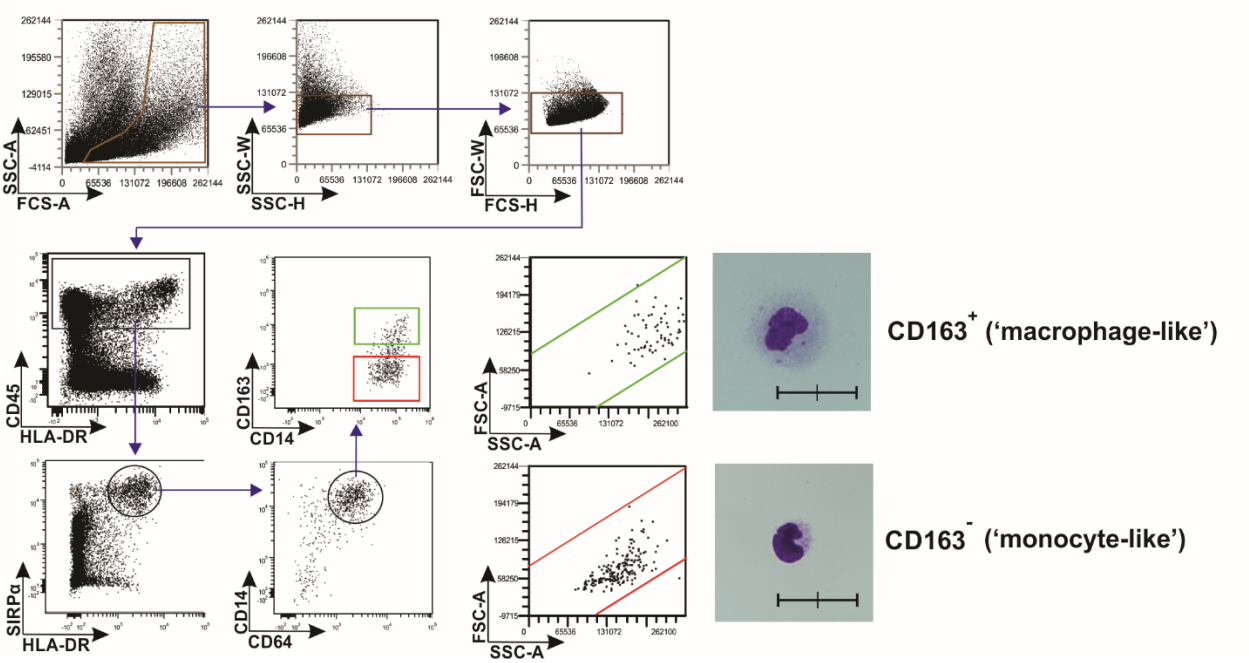

Figure 2-S5: Morphology of CD163$^-$ and CD163$^+$ cells.

Gating strategy for sorting CD163$^-$ and CD163$^+$ cells, cell size and morphology (bar=20µm).

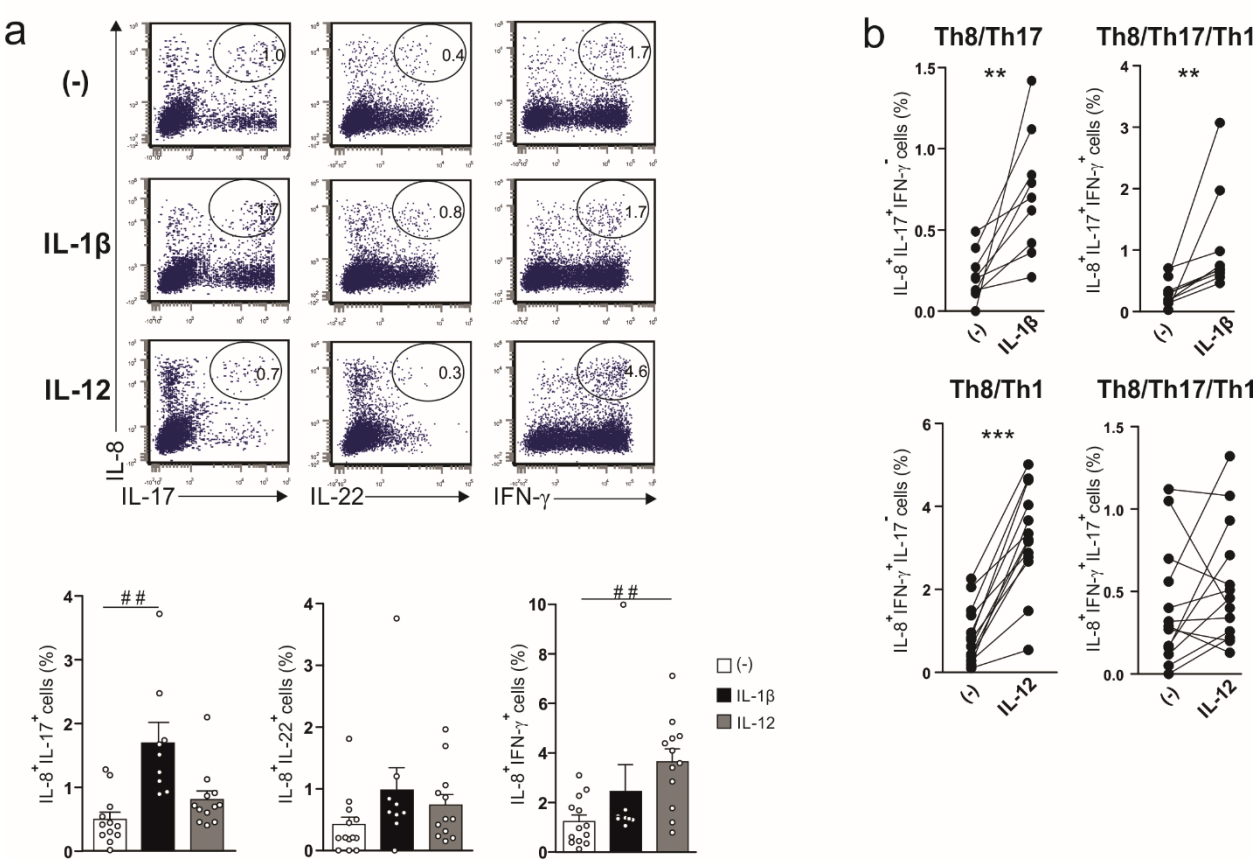

Figure 2-S6: IL-12, IL-1β and CD163$^-$ MNPs regulate frequencies of IL-8$^+$CD4$^+$ T cells co-expressing or not IL-17, IFN-γ or IL-22 while IL-12 but not IL-1β decreases IL-22$^+$IL-8$^-$ CD4$^+$ T cells in the colon of UC patients.

a *and* ***b)*** *mucosal CD4$^+$ T cells from UC patients were cultured with recombinant IL-12 (n=11), IL-1β (n=8) or CD163$^-$ cells (n=17). After 6 days culture, cells were stained for IL-8, IL-17, IFN-γ, and IL-22 intracytoplasmic expression. Percentages of IL-8$^{+/-}$IL-17$^{+/-}$IFN-γ$^{+/-}$IL-22$^{+/-}$ T cells. Wilcoxon signed rank test.*

Table 2-S1: Anti-human antibodies

Antibody	Conjugate	Clone	Company
Anti-HLADR	APC	L243	BioLegend
Anti-HLADR	AF700	L243	BioLegend
Anti-HLADR	BV510	L243	BioLegend
Anti-CD3	BV510	UCHT1	BioLegend
Anti-CD3	BV496	UCHT1	BD Biosciences
Anti-CD4	BV510	RPA-T4	BioLegend
Anti-CD4	BV785	OKT4	Biolegend
Anti-CD8a	APC	RPA-T8	BioLegend
Anti-CD8a	BV787	SK1	BD Biosciences
Anti-CD11b	BV510	D12	BD Biosciences
Anti-CD11c	BV711	B.Ly6	BD Biosciences
Anti-CD14	APC	M5E2	BioLegend
Anti-CD14	Pacific blue	HCD14	BioLegend
Anti-CD14	BUV737	M5E2	BD Biosciences
Anti-CD16	BUV496	3G8	BD Biosciences
Anti-CD25	APC	M-A251	BD Biosciences
Anti-CD25	BV510	M-A521	BD Biosciences
Anti-CD45	APC-H7	2D1	BD Biosciences
Anti-CD45RA	Alexa fluor 488	HI100	BioLegend
Anti-CD49d	PeCy7	TS217	BioLegend
Anti-CD62L	PeCy7	DREG-56	Biolegend
Anti-CD64	FITC	10.1	BioLegend
Anti-CD69	APC	IVA91	Biolegend
Anti-CD103	FITC	Be-ACT8	BD Biosciences
Anti-CD163	PerCP/Cy5.5	GHI/61	BioLegend
Anti-CD169	APC	7.239	BioLegend
Anti-CD172α	PE-Cy7	SE5A5	BioLegend
Anti-CD183 (CXCR3)	AlexaFluor 488	G025H7	BioLegend
Anti-CD196 (CCR6)	PE	G034E3	BioLegend
Anti-CD192 (CCR2)	BV650	LS132.1D9	BD Biosciences
Anti-CD206	BUV395	19.2	BD Biosciences
Anti-CD209	Pacific-Blue/BV421	DCN46	BD Biosciences
Anti-CLEC5A	PE	283834	R&D
Anti-CX$_3$CR1	PE	2A9-1	BioLegend
Anti-MERTK	APC-Cy7	590H11G1E3	BioLegend
Anti-MERTK	BV421	590H11G1E3	BioLegend

Anti-TIM-4	PE	9F4	BioLegend
Anti-TREM	APC	193015	R&D
Anti-β7	APC	Fib504	BD Biosciences
Anti-FoxP3	APC	PCH101	eBiosciences
Anti-GMCSF	PerCP/Cy5.5	BVD2-21C11	BioLegend
Anti-TNFα	Alexa Fluor-700	Mab11	Biolegend
Anti-INFγ	PerCP/Cy5.5	4S.B3	BioLegend
Anti-INFγ	AF700	4S.B3	BioLegend
Anti-INFγ	BV711	4S.B3	BioLegend
Anti-IL-1β	PE	8516	R&D
Anti-IL-6	Pacific Blue	MQ2.13A5	Biolegend
Anti-IL-8	FITC	E8N1	Biolegend
Anti-IL12p40	PE	C8.6	eBiosciences
Anti-IL-17A	Alexa 647	BL168	BioLegend
Anti-IL-17A	PE-Cy7	BL168	BioLegend

Chapter 3 :

Differential accumulation and function of pro-inflammatory 6-sulfo LacNAc dendritic cells in lymph node and colon of Crohn's versus ulcerative colitis patients

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Author's contribution	I participated in the experimental studies (40%) and analyses (50%), and the writing of the manuscript (60%).
Journal	Journal of Leukocyte Biology
Status	Published October 2015
DOI	10.1189/jlb.5A1014-509RR
Key words	CD172; CD47; cytokines; human; inflammatory bowel diseases

3.1 Abstract

Human 6-sulfo LacNac dendritic cells (Slan DCs) have been studied in patients with psoriasis, rheumatoid arthritis, cancer and autoimmune diseases. In this study we investigated the frequency, phenotype and function of Slan DCs in blood, colon as well as mesenteric lymph nodes (mLNs) of patients with inflammatory bowel disease. We first show that the frequency of circulating $CD14^{dull}$Slan DCs was reduced in Crohn's disease (CD) patients refractory to immunosuppressive drugs or TNFα blockers relative to untreated CD, ulcerative colitis (UC) and healthy subjects. In blood of CD patients, Slan DCs expressed CD172a, as detected by CD47 fusion protein binding, when compared to its lack of expression in control subjects. Next, we demonstrate that $CD172a^{+}$Slan DCs that produced IL1β and TNFα accumulated in mLNs and colons of CD patients. The $CD172a^{+}$Slan DCs up-regulated their expression of CD14 in CD tissues and the pro-inflammatory cytokines were produced in $CD14^{bright}CD172a^{+}$Slan DCs. By contrast, no difference was noted in the frequency of Slan DCs between inflamed, non-inflamed colonic mucosa of UC patients and control non-IBD donors. Finally, the percentage of cytokine-producing Slan DCs also augmented in response to TLR2 and NOD2 *in vitro* stimulation in PBMC of CD, but not UC, patients. In conclusion, we propose that pro-inflammatory $CD14^{bright}CD172a^{+}$Slan DCs are a distinguishing feature between CD and UC since these cells uniquely accumulate in mLNs and colonic mucosa of CD patients. Thus, Slan DCs may contribute to CD immunopathogenesis.

3.2 Article

3.2.1 INTRODUCTION

Inflammatory bowel diseases (IBD), which include Crohn's disease (CD) and ulcerative colitis (UC), are common chronic relapsing disorders of the gastrointestinal tract. CD and UC are distinct entities that share clinical characteristics. CD is characterized by patchy inflammatory lesions that can be transmural and affect the entire gastrointestinal tract; whereas inflammation in UC is continuous, superficial and restricted to the colon (Kaser et al., 2010).

The pathogenesis of IBD is a complex interplay between the immune system, genetic and environmental factors (MacDonald et al., 2011; Maloy and Powrie, 2011). The altered immune response to intestinal microbiota leads to chronic inflammation of gut mucosa. This abnormal immune response implicates innate immune cells, which include dendritic cells (DCs) and macrophages, T cells as well as pro-inflammatory cytokines. Studies have provided evidence that 6-sulfo LacNAc (Slan) expression on MHC ClassII^{+} cells delineates the major circulating DC subset in the blood of healthy individuals, while other reports indicated that these cells clustered with CD14^{dull} monocytes (Cros et al., 2010; Schakel et al., 2006). However, ClassII^{+}CD14$^{-/dull}$CD16^{+} Slan DCs are distinct from conventional CD1c^{+} DCs, plasmacytoid DCs and classic CD14^{bright} monocytes in blood (Schakel et al., 2002). Slan DCs are unique to humans because their counterpart in mice has not been identified. However, Cros *et al.* considered Slan DCs as part of the CD16^{+}CD14^{dull} monocyte population that display several genetic similarities to murine Ly6C^{lo}CX3CR1^{hi} monocyte population. The latter patrols lymphatic vessels and exert a protective role by attracting neutrophils and clearing debris in response to environmental insults (Cros et al., 2010). Furthermore, Slan DCs have been implicated in anti-viral responses (Cros et al., 2010). However, in context of pathology these

cells produce pro-inflammatory cytokines that include IL1β, IL6, IL12, IL23 and TNFα and promote Th1/Th17 cell responses (Gunther et al., 2013; Hansel et al., 2011). Histological analysis revealed the presence of Slan DCs in patients with psoriasis, rheumatoid arthritis, CD, systemic lupus erythematosus, multiple sclerosis and cancer (Costantini et al., 2011; de Baey et al., 2003; Hansel et al., 2011; Schakel et al., 2006; Thomas et al., 2014; Vermi et al., 2014). In the present report, we investigated the differential accumulation of Slan DCs in blood, lymphoid tissues and colonic mucosa of CD and UC patients and evaluated their phenotype and function.

3.2.2 MATERIALS AND METHODS

3.2.2.1 *Human clinical samples*

All human subjects signed informed consent forms that have been approved by the Institutional Ethics Research Committee of the Centre Hospitalier de l'Université de Montréal. Patients were recruited based on clinical, endoscopic and histological criteria (**Table 3-1**). CD patients presented with abdominal pain, diarrhea or weight loss and endoscopically with patchy inflammation, eroded mucosa, deep ulcers and/or strictures. Histologically, the architecture of the crypts was disturbed; the mucosa was infiltrated by mono- or polynuclear cells with or without pathognomonic granuloma. Transmural inflammation was confirmed on surgical samples. UC patients presented with abdominal pain, diarrhea and bleeding, with a continuous and circumferential inflammation of the mucosa. The mucosa was eroded, granular and friable. Histologically, the architecture of the crypts was disturbed, with mucus depletion and diffuse infiltrate of mono- or polynuclear cells to the mucosa and crypts. No histological or bacteriological infections suggested a differential diagnosis.

Blood was collected from healthy subjects (control; n=44) and IBD patients (CD=48 and UC=14) in tubes coated with heparin. MLNs were obtained from CD patients and control non-IBD donors (n=20) during surgical resection. Colonic samples were obtained during an endoscopy (n=13) or a surgical resection (n=10) of IBD patients. Macroscopically non-inflamed and inflamed mucosa were taken from the same IBD patient. In patients with ileo-colonic CD, only biopsies from the colon were taken for our study.

3.2.2.2 *Cell purification*

Peripheral blood cells (PBMC) were obtained by density gradient centrifugation of heparinized peripheral blood. The intestinal mucosa, from biopsies or surgical samples, was first processed by enzymatic digestion with DNase I (Roche, Basel, Switzerland) and Collagenase D (Roche, Basel, Switzerland) followed by mechanical digestion with gentle MACS (Miltenyi Biotec, Bergisch Gladbach, Germany) to isolate lamina propria mononuclear cells (LPMC). mLNs, gathered from surgical samples, were digested mechanically to get mLN cell suspensions (Baba et al., 2013).

3.2.2.3 *Flow cytometry analysis and cell sorting*

PBMC, mLN and LPMC were stained using monoclonal antibodies to CD14, CD172b (clone B4B6), CD172ab (clone SE5A5), HLA-DR, IL1β, TNFα (BioLegend, San Diego, CA, USA), CD16, CD45, (BD Biosciences, San Jose, CA, USA), IL12/23p40, IL23p19 (eBiosciences Inc., San Diego, CA, USA), Slan (Miltenyi Biotec, Bergisch Gladbach, Germany), MHC class II (ID Labs, London, ON, Canada) and Alexa Fluor 647-conjugated CD47Var-1 (Novartis, Basel, Switzerland). Isotype-matched control monoclonal antibodies (mAbs) were used. Data were analyzed with FACS Diva (BD Biosciences, San Diego, CA, USA).

3.2.2.4 *Immunohistochemistry (IHC)*

Intestinal and mLN tissue sections from CD patients and control donors were treated according to well-established FFPE (formalin-fixed paraffin embedded) methods. 4µm tissue sections were stained with the Benchmark XT autostainer (Ventana Medical System Inc., Tucson, AZ, USA). Antigen retrieval was obtained using Cell Conditioning 1 (Ventana Medical System Inc.,

Tucson, AZ, USA) for 60 minutes. Pre-diluted DD2 (clone DD2, kindly provided by Dr. K. Schäkel, University Hospital Heidelberg, Germany) antibody (1:50) was manually added to the slides and incubated at 37˚C for 20 minutes. Reactions were performed using the UltraView Alkaline Phosphatase Red detection kit (Ventana Medical System Inc., Tucson, AZ, USA). Counterstaining was achieved with hematoxylin and bluing reagent (Ventana Medical System Inc., Tucson, AZ, USA). Leica DM4000B microscope, equipped with Leica DFC300FX camera was used to visualize the tissue sections.

3.2.2.5 *Pro-inflammatory cytokine expression*

PBMC (1 x 10^6/mL) were cultured for 24 hours in the presence of muramyl dipeptide (MDP, 1µg/mL, Invivogen, San Diego, CA, USA) and Pam3Csk4 (1µg/mL, Invivogen, San Diego, CA, USA). For the last 3 hours of culture, BrefeldinA (Calbiochem, Billerica, MA, USA) was added. RPMI 1640 medium (Wisent Inc., St Bruno, QC, CA) with 10% fetal bovine serum (Wisent Inc., St Bruno, QC, CA) and 1% penicillin-streptavidin (Wisent Inc., St Bruno, QC, CA) was used for all cultures. The cells were then fixed for intracytoplasmic staining with anti-IL1β, IL23p19, IL12/23p40 and TNFα or isotype-matched control mAbs. *Ex vivo* isolated mLN cell suspensions and LPMC were immediately stained for CD45, ClassII, CD14, Slan, CD47Var-1 in the absence of BrefeldinA, then fixed/permeabilized and stained for intracytoplasmic cytokine expression.

3.2.2.6 *Statistical analysis*

Statistical analysis was performed with GraphPad Prism version 6. Paired and Unpaired Student *t* test or Mann-Whitney test or Wilcoxon signed rank tests were used. Data are shown as mean ± s.e.m.

3.2.3 RESULTS

3.2.3.1 *Frequency and phenotype of Slan DCs in the blood of IBD patients*

We first quantified Slan DCs in the blood of IBD patients and compared their frequency to that of healthy subjects (control). As expected, Class II^+ Slan DCs were characterized as *bona fide* $CD16^+$ cells, while CD14 monocytes included $CD14^+CD16^-$ and $CD14^+CD16^+$ subsets (**Figure 3-1A**). No difference was seen in the percentage of Slan DCs between control donors and untreated CD patients (**Figure 3-1B**, left panel). Remarkably, in CD patients with therapeutic failure to immunosuppressive agents and TNFα blockers, the percentage of circulating Slan DCs was significantly decreased. Notably, CD patients refractory to treatment had a moderate-to-severe disease. In untreated and refractory UC patients, the percentage of circulating Slan DCs was unchanged compared to control donors (**Figure 3-S1**). Treatment with 5-ASA had no impact on the frequency of Slan DCs in CD patients (**Figure 3-1B**, left panel). Conversely, 5-ASA, but not immunosuppressive drugs nor anti-TNFα mAb, significantly reduced the percentage of $CD14^{bright}$ monocytes in the same cohort of CD patients (**Figure 3-1B**, right panel).

Signal regulatory protein alpha and beta (SIRPαβ/CD172ab) is expressed on circulating monocytes and DCs with the exception of the minor $CD141^+$ DC population (Baba et al., 2013; Watchmaker et al., 2014). We therefore examined CD172ab expression, using an anti-CD172ab mAb (SE5A5) that recognizes the 2 isoforms of CD172, on Slan DCs in the blood of CD patients. As depicted in **Figure 3-2A** and **Figure 3-2B**, Slan DCs that were clearly defined as $CD172ab^{dull}$ clustered with the $CD14^{dull}$ cell population while, $CD172ab^{bright}Slan^-$ cells corresponded to the $CD14^{bright}$ monocyte population, confirming our previous report (Baba et al., 2013). We found that the expression of CD172ab was significantly lower on Slan DCs

compared to monocytes in both untreated and refractory cohorts of CD patients as well as control subjects (**Figure 3-2C** and data not shown). Next, we asked whether the difference in intensity of CD172ab expression observed between Slan DCs and monocytes results from their differential expression of CD172a and CD172b. To this end, we used CD47Var-1, an avidity improved CD47 fusion protein that binds CD172a but not CD172b; and anti-CD172b mAb, which selectively identifies CD172b (Baba et al., 2013). The CD172b expression was significantly lower on Slan DCs compared to the $CD14^{bright}$ monocyte population as depicted by the mean fluorescent intensity (MFI) with no difference in the level of expression between CD and control donors (**Figure 3-2D**). As previously reported, CD47Var-1 binding was up-regulated on $CD14^{bright}$ monocytes ($p=0.02$) in CD patients relative to control donors but CD47Var-1 failed to bind circulating Slan DCs in healthy donors (Baba et al., 2013). By contrast, binding to CD47Var-1, thus CD172a expression, was significantly detected on Slan DCs in CD patients ($p=0.03$) (**Figure 3-2D**).

We conclude that Slan DCs express CD172a in the blood of CD patients and decrease in frequency in CD, but not UC, patients refractory to immunosuppressive agents and TNFα blockers.

3.2.3.2 *$CD14^{bright}CD47Var\text{-}1^{+}$ Slan DCs accumulate in the mLNs of CD patients and produce IL1β and TNFα*

Vermi et al. recently reported an accumulation of Slan DCs in the metastatic tumor-draining lymph nodes of carcinoma patients (Vermi et al., 2014). However, these cells were undetectable in lymph nodes showing non-specific lymphadenitis. Although technically challenging to access, we searched for and detected, for the first time, Slan DCs by immunohistochemistry

(IHC) in inflamed mLNs of CD patients (**Figure 3-3A**). By contrast, they were rarely observed in mLNs of control non-IBD donors that included cold diverticulitis patients. In CD patients, Slan DCs had a stellate shape and were scattered in the T and B cell areas of the disorganized mLN (data not shown). Next, we quantified ClassII^{+}Slan DCs by flow cytometry in mLNs freshly collected from CD and non-IBD patients undergoing surgery (**Table 3-1**). Surgical samples were obtained from CD patients refractory to drug therapy (8 out of 10). We found a significant increase in the percentage of Slan DCs in CD when compared to control patients group, which included carcinoma as well as cold diverticulitis non-IBD donors (**Figure 3-3B**). Next, we gated on CD45^{+}ClassII^{+} cells and determined CD47Var-1 binding on Slan DCs in CD mLNs. Slan DCs were identified as CD47Var-1^{+}cells that are CD14^{bright} and CD47Var-1^{-} cells that have a low expression of CD14 (**Figure 3-3C**). The frequency of CD47Var-1^{+}Slan DCs was significantly augmented in the mLNs of CD compared to control non-IBD donors (**Figure 3-3D**). Notably, the CD14$^{dull/-}$CD47Var-1^{-}Slan^{+} population predominated in mLN from control patients (**Figure 3-S2A**). These data indicate that CD14^{bright}CD47Var-1^{+}Slan DCs accumulate in the mLN of CD patients. Finally, cytokine production was examined *ex vivo* in 6 CD versus 5 non-IBD mLNs. The frequency of IL1β and TNFα-producing Slan DCs was significantly higher in CD than in control patients (**Figure 3-3E**). Notably, the large majority of cytokine producing Slan DCs were CD14^{bright}, thus CD47Var-1^{+}, compared to the IL1β^{-} or TNFα^{-} Slan DCs that were mainly CD47Var-1^{-} CD14^{dull} cells (**Figure 3-3C** and **Figure 3-3E**).

Taken collectively, IL1β and TNFα-producing CD14^{bright}CD172a^{+} Slan DCs accumulate in mLNs of CD patients.

3.2.3.3 *CD14^{bright}CD47Var-1^{+} Slan DCs accumulate in the inflamed colonic tissue of CD, but not UC, patients and produce IL1β and TNFα.*

Next, we quantified Slan DCs in CD and UC patients by flow cytometry; inflamed and non-inflamed colonic mucosa from the same patient were compared. A significant increase in the percentage of Slan DCs was detected in inflamed compared to non-inflamed tissue of CD (n=4 biopsies, n=7surgery), but not UC (n=5 biopsies, n=1surgery), patients (**Figure 3-4A**). Since no difference in the frequency of Slan DCs was noted in colonic mucosa of UC, we focused on colonic data obtained from CD patients only. First, we confirmed the presence of Slan DCs using IHC in the inflamed colonic mucosa of CD (**Figure 3-4B**, lower panel), corroborating earlier reports (Costantini et al., 2011; de Baey et al., 2003). We showed an accumulation of Slan DCs in the apical part of villi in inflamed CD tissue. These cells were seen infiltrating the deeper layers of inflamed mucosa. In control tissue, Slan DCs were sometimes detected in isolated lymphoid follicles (data not shown) but were virtually absent from the mucosa itself (**Figure 3-4B**, upper panel).

Next, we examined the expression of CD14 and CD172a on Slan DCs in CD45^{+}ClassII^{+} cell population. Similar to our mLN data, we found two populations of Slan DCs in inflamed CD mucosa: CD14^{bright}CD47Var-1^{+} and CD14$^{-/dull}$CD47Var-1^{-} cells (**Figure 3-4C**). Note that the CD14^{bright}CD47Var-1^{+}Slan^{+} population was also detected in non-inflamed CD colons (**Figure 3-S2B**). As a consequence, the frequency of CD47Var-1^{+}Slan DCs (**Figure 3-4D**) as well as CD14^{bright}Slan DCs (**Figure 3-4E**) was significantly increased in inflamed CD colon compared to colonic mucosa of control non-IBD donors but not non-inflamed colon of CD patients. These data indicate that, as in mLNs, Slan DCs expressed CD172a and the frequency of CD14^{bright}Slan DCs increased in the colon of CD patients.

The accumulation of Slan DCs seen in the inflamed CD mucosa was associated, like in mLNs, with a spontaneous increase in their pro-inflammatory cytokine production (**Figure 3-4F**). In fact, *ex vivo* examination of LPMC from CD showed a significant increase in the percentage of IL1β^{+}Slan DCs in inflamed relative to non-inflamed tissue. Furthermore, the frequency of TNFα-producing Slan DCs also showed a trend towards an increase. In fact, whenever patients treated with anti-TNFα mAb (red symbols) are excluded from the cohort analysis, the increase in percentage of TNFα^{+}Slan DCs was significant in inflamed CD mucosa. Similar to mLNs, the cytokine producing Slan DCs were CD14^{bright}.

In CD patients, Slan DCs that accumulate in the colonic tissue resemble those found in mLNs of CD patients in terms of their phenotype and cytokine profile.

3.2.3.4 *Increased frequency of cytokine-producing Slan DCs, but not CD14^{bright} monocytes, in in vitro-stimulated PBMC of CD patients.*

Slan DCs produce pro-inflammatory cytokines *ex vivo* in colon and lymph nodes from CD patients. We also investigated the function of circulating Slan DCs in IBD patients versus healthy subjects in response to NOD2 and TLR2 *in vitro* stimulation. To this end, PBMC from CD, UC and control donors were stimulated with muramyl dipeptide (MDP) and Pam3Csk4, and the production of various cytokines was assessed among Slan DCs by intracytoplasmic staining. MDP and Pam3Csk4 are NOD2 and TLR2 stimuli respectively that are known to mimic colonic microbiota (Brain et al., 2013). The overall cytokine production in Slan DCs was higher in response to the combined stimuli than when MDP and Pam3Csk4 were added separately (data not shown). *In vitro* stimulation increased the percentage of IL1β^{+} and TNFα^{+} Slan DCs in CD as well as UC patients (**Figure 3-5A**). As depicted in **Figure 3-5B**, the

frequency of TNFα, IL23p19 and IL12/23p40-producing Slan DCs was significantly higher in CD compared to UC patients and control donors reflecting the increase in the percentage of Slan DCs that produce cytokines seen in stimulated PBMC. The percentage of $IL1\beta^{+}Slan^{+}$ cells tended to augment in CD compared to control and UC patients. Similar to data in mLN and colonic mucosa, the cytokine producing Slan DCs have a higher expression of CD14 (**Figure 3-S3**). Finally, this differential cytokine production between IBD and control donors was not noted for the $CD14^{bright}$ monocyte population (**Figure 3-5C**).

Taken together, the frequency of circulating pro-inflammatory cytokine-producing Slan DCs in response to *in vitro* stimulation with TLR2 and NOD2 agonists is higher in CD relative to UC patients.

3.2.4 DISCUSSION

Our present study revealed an increased frequency of IL1β- and TNFα- producing Slan DCs in the mLNs of CD patients relative to mLNs of non-IBD donors. Furthermore, pro-inflammatory Slan DCs accumulated in inflamed colons of CD, but not UC, patients. The accumulation of Slan DCs in peripheral tissues was associated with a significant reduction in the frequency of circulating Slan DCs in CD patients refractory to immunosuppressive agents or TNFα blockers treatment, relative to untreated CD, UC or healthy subjects. These data suggest that Slan DCs have been recruited to inflamed CD, but not UC, tissues and thus may contribute to the local inflammatory process in CD.

The accumulation of Slan DCs in inflammatory or autoimmune diseases provokes the hypothesis that these cells are implicated in disease immunopathogenesis. Quantification by IHC revealed an increased frequency of Slan DCs in the dermis of SLE patients (Hansel et al., 2013) as well as in inflammatory demyelinating brain lesions and cerebrospinal fluid of patients with multiple sclerosis (MS) (Thomas et al., 2014). As observed in CD patients, Slan DCs accumulated in highly active lesions when compared to less inflamed tissues in SLE and MS patients. However, similar percentage of circulating Slan DCs was observed between untreated SLE and MS patients and healthy subjects, in agreement with our data in untreated IBD patients. These observations suggest that disease severity is better correlated with the frequency of Slan DCs in tissue rather than in blood, with the exception of psoriatic disease in which the number of Slan DCs are increased in both active plaques and blood (Hansel et al., 2011).

Furthermore, impact of therapeutic drugs on the frequency of circulating Slan DCs does not necessarily reflect the clinical outcomes of the patients. Administration of anti-TNFα mAb (Adalimumab) in psoriasis patients improves disease and decreases the numbers of Slan DCs

in dermis as well as in blood with no induction of Slan DC apoptosis (Brunner et al., 2013). By contrast, successful treatment of psoriatic patients with soluble TNFα receptor (Etanercept) reduces the number of Slan DCs in resolved lesions while augmenting their frequency in blood, suggesting an impaired migratory activity of Slan DCs to tissue in responder patients (Gunther et al., 2013). Finally, long-term therapy with IFNβ, but not with natalizumab (anti-α4β1), significantly reduced the frequency of circulating Slan DCs in MS patients with remitting relapsing stable disease (Thomas et al., 2014). Our present study established that the percentage of Slan DCs significantly decreased in the blood, but accumulated in inflamed colonic mucosa and mLNs of CD patients refractory to treatment. The latter further highlights the importance of examining, whenever feasible, tissues rather than PBMC to evaluate the potential contribution of cells to disease process and the impact of drug therapy.

Using flow cytometry, we detected and quantified for the first time Slan DCs in mLN of CD patients. Slan DCs were rarely observed in non-inflamed mLN of non-IBD donors, corroborating their paucity in LN and spleen using IHC (de Baey et al., 2003). Their increased percentage in LN relates to their previously reported functions: priming of naïve T cells and induction of Th17/Th1 response (Hansel et al., 2011), a hallmark of CD pathogenesis (Kaser et al., 2010). Slan DCs are endowed with reverse migratory properties suggesting that they travel from the tissue, where they are in contact with the antigen, to LN to encounter naïve T cell population (Randolph et al., 2002). However, Slan DCs could also make their journey to the mLN arriving directly from the blood. This is strongly suggested in carcinoma patients where Slan DCs were not present within the primary carcinomas, but an increase in their frequency was noted in the metastatic tumor draining LN (Vermi et al., 2014). C5a, CXCL12 and CX3CL1 are involved in the migration of Slan DCs to psoriatic plaques and possibly to metastatic LN

(Hansel et al., 2011). Furthermore, CD16 expression provides Slan DCs with the capacity to handle immune complexes (Dobel et al., 2013) and the latter are shown to promote CCR7-dependent DC migration to LN in mice (Clatworthy et al., 2014). However, the mechanisms that govern the selective accumulation of Slan DCs to inflamed CD mLNs as well as in colon of CD, but not UC, patients warrant further investigation.

Two opposite functions are attributed to Slan DCs. These cells display either protective or pro-inflammatory properties. Pro-inflammatory cytokine production of circulating Slan DCs was established in psoriatic and SLE patients, following stimulation with ligands that bind to toll like receptors 2, 4, 7 and 8 (Gunther et al., 2012; Hansel et al., 2013; Hansel et al., 2011). In the present study, we evaluated the pro-inflammatory properties of Slan DCs in IBD patients. On one hand, Slan DCs that spontaneously secrete IL1β and TNFα after *ex vivo* isolation significantly accumulated in mLNs as well as in inflamed colons of CD patients. On the other hand, the percentage of IL1β, TNFα as well as IL23p19 and IL12/23p40 cytokine-producing Slan DCs in response to *in vitro* stimulation was highest in blood of CD patients, compared to that of UC and healthy subjects. In fact, the augmentation of cytokine production observed in Slan DCs largely results from an increase in the percentage of cytokine-producing cells and not in the cytokine producing capacity of each cell (data not shown). Notably, the clinical response to Etanercept in psoriasis appears to be more related to a reduction in the percentage of cytokine-producing Slan DCs in the dermis than to the decreased ability of these cells to secrete TNFα or IL23p19 (Gunther et al., 2013).

Pro-inflammatory cytokines were mainly produced in Slan DCs that expressed CD172a and had up-regulated CD14 expression in blood, mLN and colon, i.e. the $CD14^{bright}CD172a^{+}$ Slan DCs. Our previous study revealed that pro-inflammatory cytokine production is restricted to the

CD47Var-1^{+} cells in mLNs as well as colonic mucosa of CD patients (Baba et al., 2013). This CD47Var-1^{+} cells in CD colon is a heterogeneous population that comprises the minor Slan DCs and other DCs as well as macrophage subsets, that remain to be further characterized. Although Slan expression was described on CD14^{+}CD163^{+} and CD14^{+}CD163^{dull} APC subsets in normal colonic mucosa of colorectal cancer patients (Ogino et al., 2013), Slan DCs were CD11c^{+}CD163^{-} in inflamed CD colons (data not shown), in agreement with their phenotype reported in skin (Gunther et al., 2012). Notably, exposure of inflamed colonic tissue explants to CD47Var-1 suppresses the release of pro-inflammatory cytokines that include IL1β, TNFα, IL12 and IL23 (Baba et al., 2013). Also, administration of CD47-Fc fusion protein in mice prevents the relapse of experimental colitis, which correlated with impaired DC recruitment to mLNs as well as decreased pro-inflammatory cytokine expression in tissues (Fortin et al., 2009; Van et al., 2006). As such, targeting CD172a using CD47-Fc might alter both the cytokine producing capacity as well as recruitment of Slan DCs into tissues in CD patients.

In conclusion, we demonstrate that pro-inflammatory CD14^{bright}CD172a^{+}Slan DCs accumulate in mLNs and colons of CD. Since our data did not reveal a role for Slan DCs in UC, we propose that Slan DCs may uniquely contribute to CD immunopathogenesis.

3.2.5 REFERENCES

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3.2.6 FIGURES AND TABLES

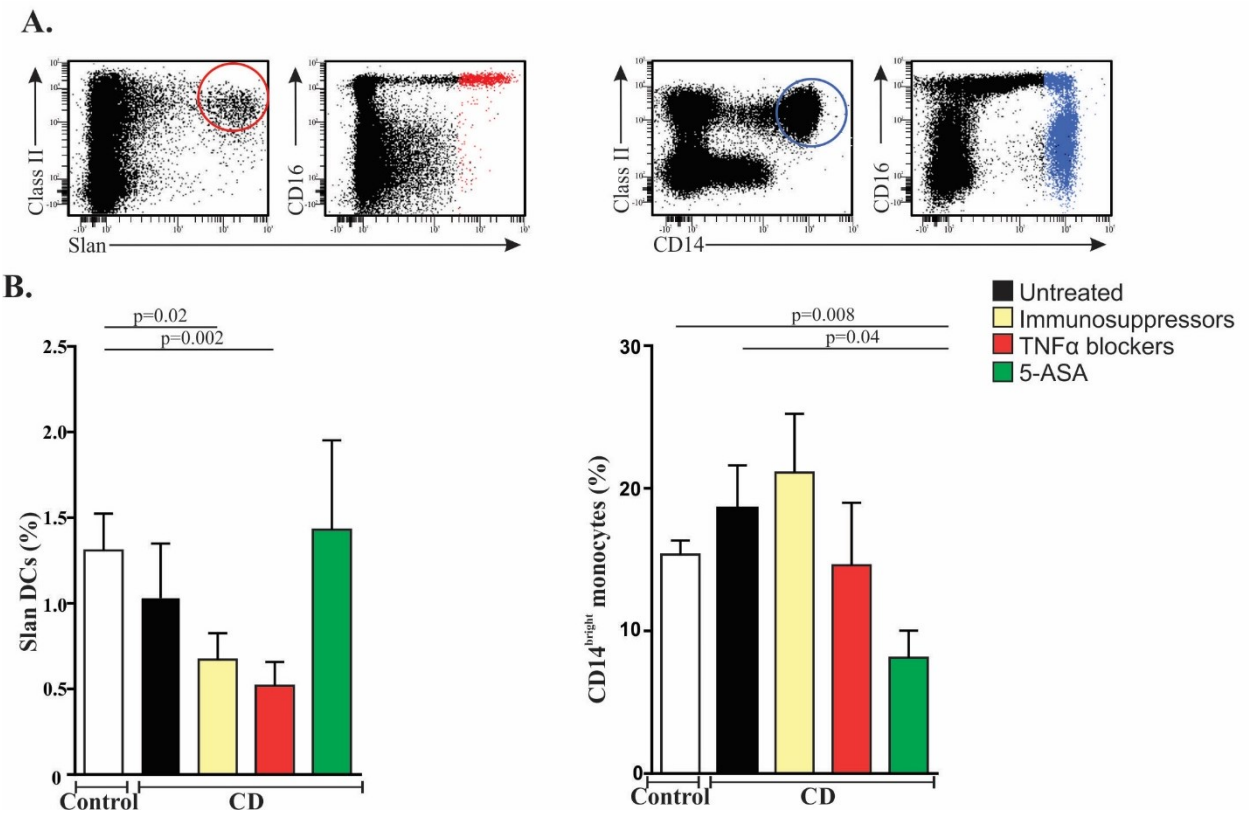

Figure 3-1: Slan DCs decrease in frequency in the blood of CD patients refractory to treatment.

PBMC isolated from CD and healthy subjects (control) were stained for CD14, CD16, MHC class II and Slan. ***(A)*** *Representative flow cytometry plot of CD16 expression in ClassII^{+}Slan^{+} cells (left panels) and ClasII^{+}CD14^{bright} cells (right panels).* ***(B)*** *Percentage of Slan DCs (left panel) and CD14^{bright} monocytes (right panel) in healthy (control) subjects (n=44), untreated CD patients (n=14), as well as refractory CD patients to immunosuppressors (n=9), TNFα blockers (n=8) and 5-ASA (n=5) treatment. Data are represented as mean ± s.e.m. Mann-Whitney test was used to assess significance.*

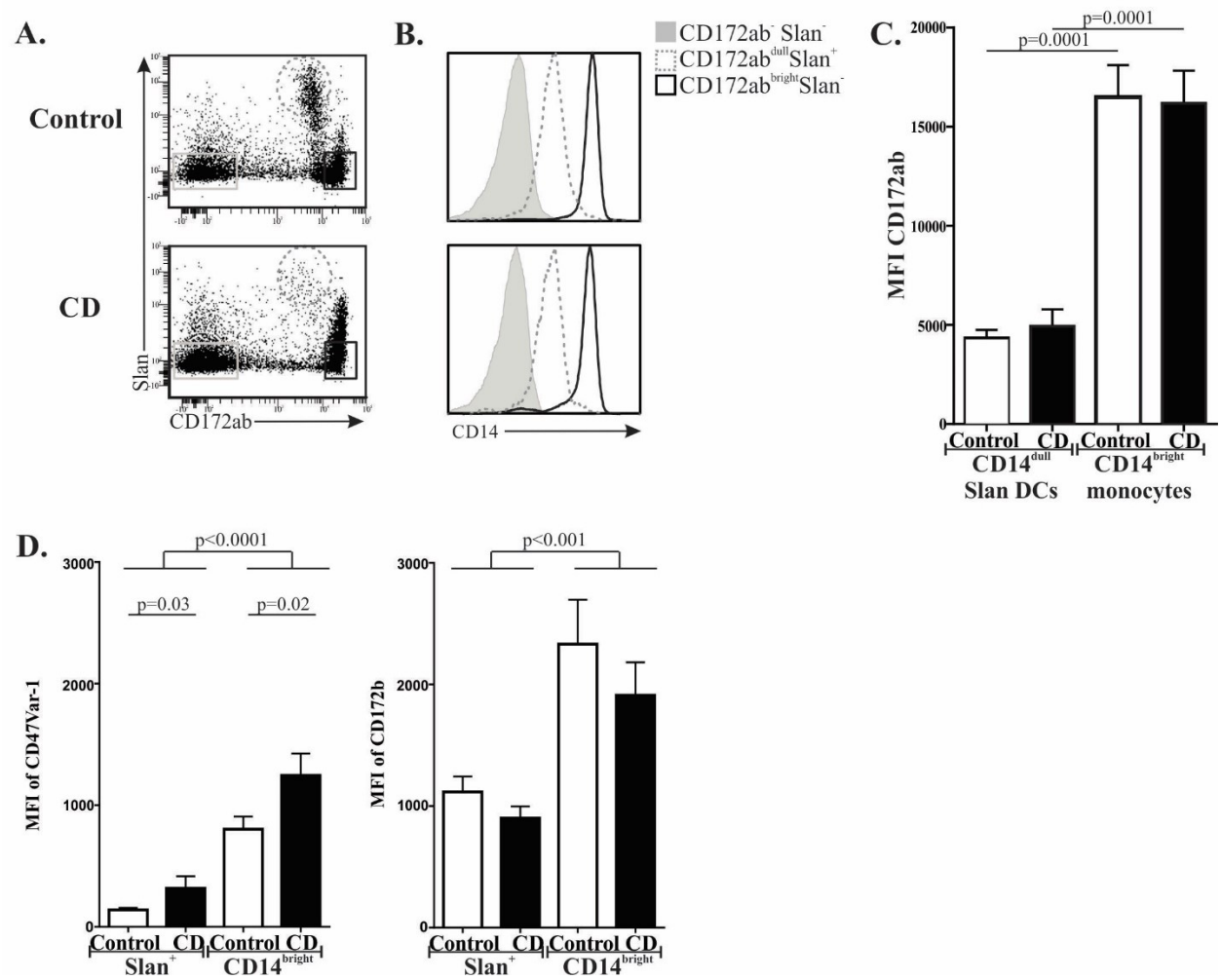

Figure 3-2: ClassII$^+$Slan$^+$ express CD172a in peripheral blood of CD patients.

PBMC isolated from CD patients and healthy subjects (control) were stained for CD172ab, CD172a, CD172b, CD14, MHC class II and Slan. ***(A)*** *Representative flow cytometry plot of CD172ab expression in relation to Slan on ClassII$^+$ gated cells.* ***(B)*** *Representative flow cytometry histogram of CD14 expression on the CD172ab^{dull}ClassII$^+$Slan$^+$ (grey dashed line), CD172ab^{bright}ClassII$^+$Slan$^-$ (black line) and CD172ab$^-$ClassII$^+$Slan$^-$ (filled histograms).* ***(C)*** *Mean MFI of CD172ab (control n=33 and CD n=36).* ***(D)*** *Mean MFI of CD47Var-1 (control n=25; CD n=21) and CD172b (control n=16; CD n=13) on Slan$^+$ and CD14^{bright} cells. Data are represented as mean ± s.e.m. Paired as well as Unpaired Student t test and Mann-Whitney test were used to assess significance.*

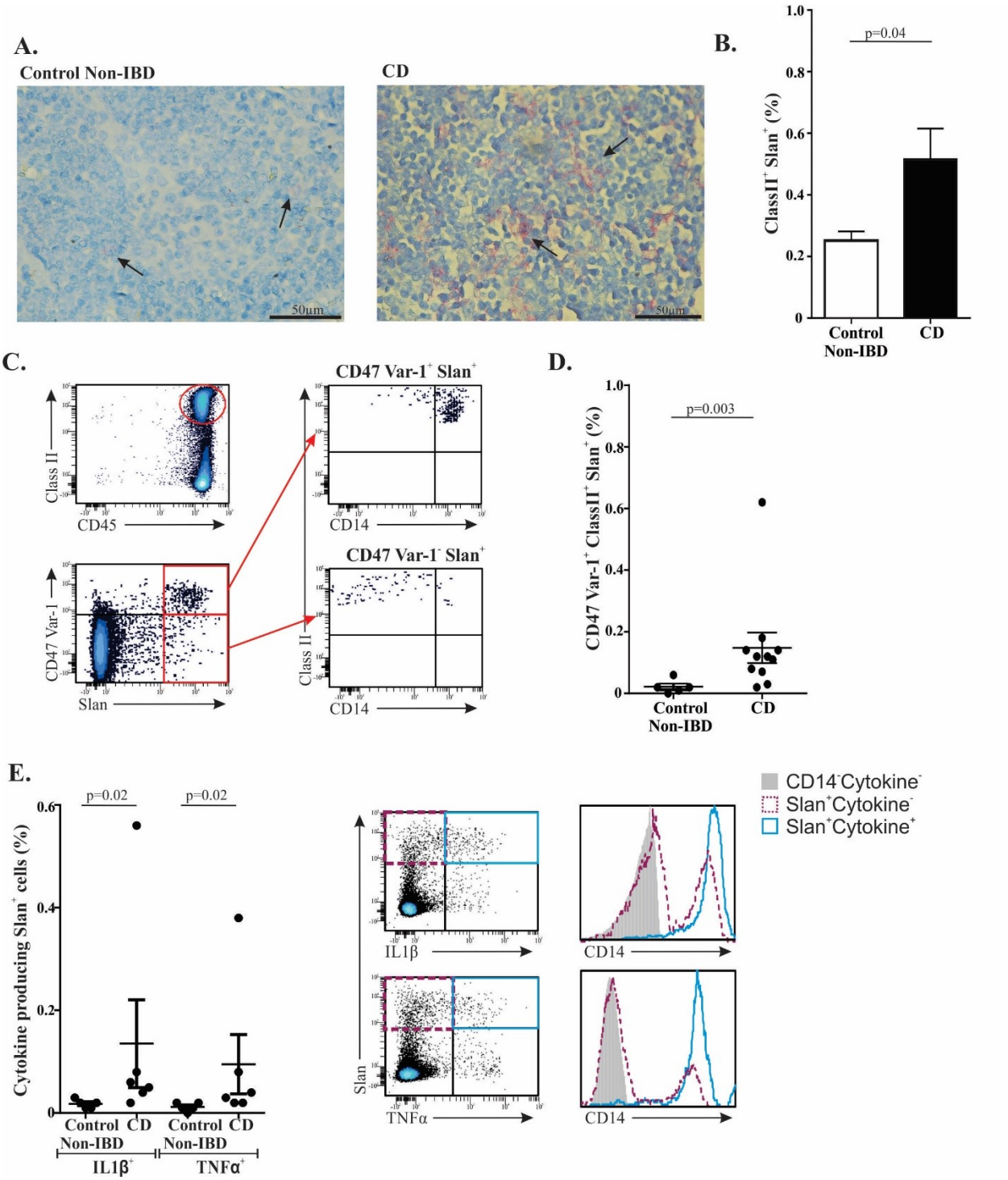

Figure 3-3: CD14^{bright}CD172a^{+} Slan DCs accumulate in the mLNs of CD patients and produce IL1β and TNFα.

(A) *DD2^{+} Slan cells are detected in the mLN of CD (right panel) but not non-IBD (left panel) patients. One representative staining out of 5 is shown.* ***(B-E)*** *Freshly isolated mLN from CD*

patients and non-IBD control donors were stained for CD47Var-1, CD14, CD45, MHC class II and Slan. ***(B)*** *Percentage of ClassII^{+}Slan^{+} among CD45^{+} cells (n=6 non-IBD control patients and n=10 CD patients).* ***(C)*** *Representative gating strategy showing CD47Var-1^{+and-} Slan^{+} population among ClassII^{+}CD45^{+} cells in mLN of CD patient. The expression of CD14 is represented on Slan^{+} populations.* ***(D)*** *Percentage of CD47Var-1^{+}ClassII^{+}Slan DCs (n=5 non-IBD control patients and n=11 CD patients).* ***(E)*** *Freshly isolated mLN were stained intracytoplasmically for IL1β and TNFα. Percentage of IL1β^{+}Slan^{+} and TNFα^{+}Slan^{+} cells in control (n=5) versus CD patients (n=6). Flow cytometry histogram of CD14 staining on the Slan^{+}Cytokine^{-} (purple dashed-line), Slan^{+}Cytokine^{+} (blue line) and CD14^{-}Cyokine^{-} (grey filled) populations. Data are represented as mean ± s.e.m. Mann-Whitney test was used to assess significance.*

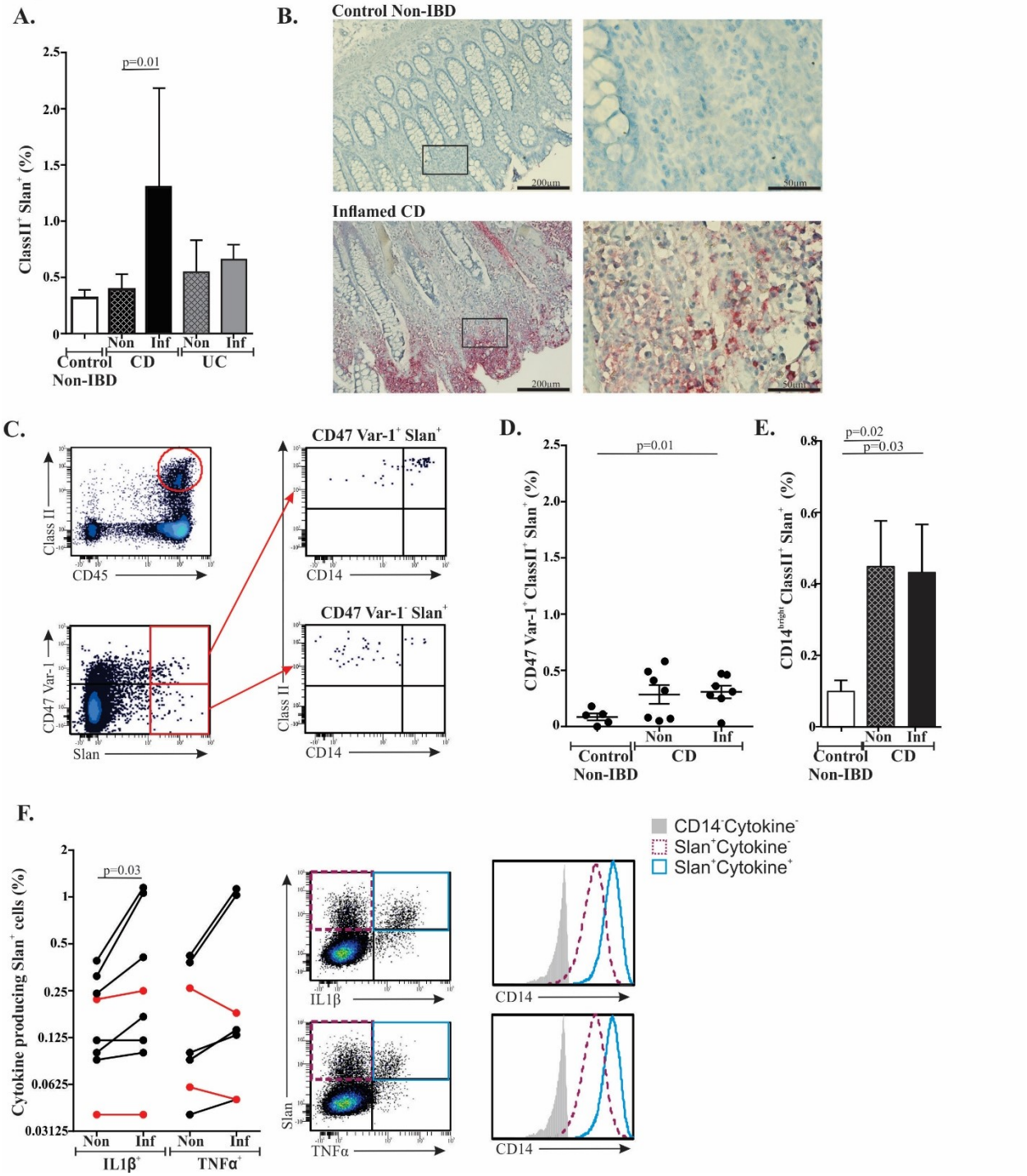

Figure 3-4: CD14^{bright}CD172a^{+} Slan DCs accumulate in the inflamed colonic tissue of CD, but not UC, patients and produce IL1β and TNFα.

LPMC were isolated from the inflamed and non-inflamed colonic mucosa of CD (n=11, which included 4 biopsies) and UC (n=6, which included 5 biopsies) patients and compared to non-IBD (control) patients (n=6). ***(A)*** *Frequency of ClassII^{+}Slan DCs among CD45^{+} cells.* ***(B)***

DD2^{+} Slan cells are concentrated at the apical part of villi in CD colonic sections. One representative staining out of 5 is shown. ***(C)*** *Freshly isolated LPMC from non-inflamed and inflamed CD patients and healthy control donors were stained for CD47Var-1, CD14, CD45, MHC class II and Slan. Representative flow cytometry plot depicting the CD47Var-1^{+and-}Slan^{+} population among ClassII^{+}CD45^{+} cells in inflamed LPMC of CD patients. The expression of CD14 is depicted on Slan^{+} populations.* ***(D)*** *Percentage of CD47Var-1^{+}ClassII^{+}Slan DCs (n=5 non-IBD control patients and n=7 CD patients).* ***(E)*** *Percentage of CD14^{bright}ClassII^{+}Slan DCs (n=5 non-IBD control patients and n=7 CD patients).* ***(F)*** *Freshly isolated LPMC were stained ex vivo for IL1β and TNFα by intracytoplasmic staining. The percentage of cytokine producing Slan^{+} cells in inflamed versus non-inflamed tissue of CD patients (n=8) is depicted. Patients undergoing anti-TNFα treatment at the time of sample collection are represented by red symbols. Flow cytometry histogram of CD14 staining on the Slan^{+}Cytokine^{-} (purple dashed-line), Slan^{+}Cytokine^{+} (blue line) and CD14^{-} Cytokine^{-} (grey filled) populations. Data are represented as mean ± s.e.m. Paired and Unpaired Student t tests, Mann-Whitney and Wilcoxon signed rank tests were used to assess significance.*

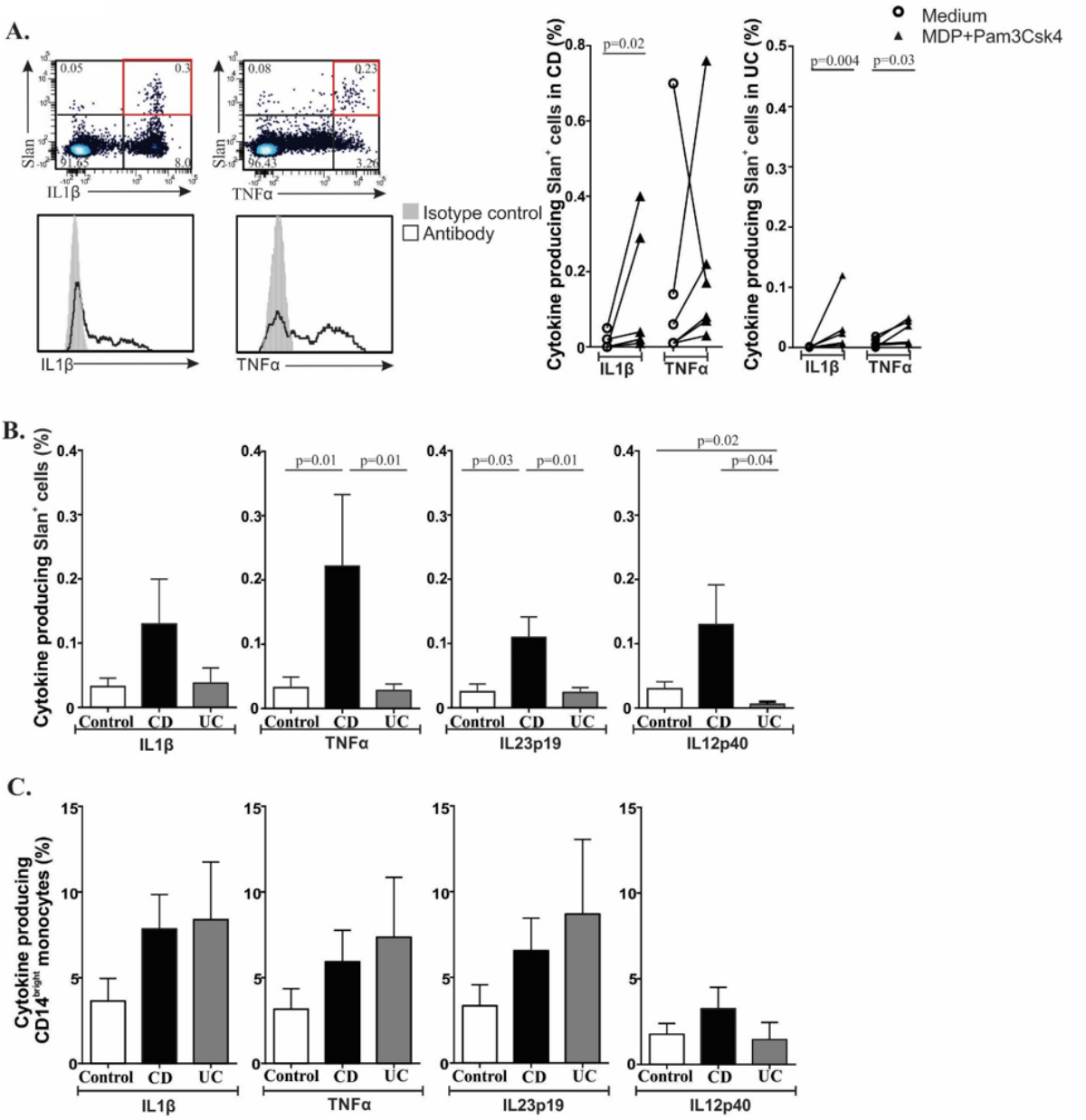

Figure 3-5: Increased frequency of cytokine-producing Slan DCs, but not CD14^{bright} monocytes, in in vitro-stimulated PBMC of CD patients.

PBMC were stimulated for 24 hours with MDP and Pam3Csk4. BrefeldinA was added for the last 3 hours. ***(A)*** *Representative flow cytometry dot plot showing the Slan$^+$IL1β$^+$ and Slan$^+$TNFα$^+$ among PBMC. Flow cytometry histogram of IL1β and TNFα (black line) and their respective isotype controls (grey filled) on the Slan$^+$ population. Percentage of IL1β and TNFα producing Slan DCs in PBMC of CD (n=6) and UC (n=5) patients in MDP+Pam3Csk4 (triangle symbol) versus medium (circle symbol) conditions.* ***(B)*** *Percentage of IL1β$^+$, TNFα$^+$, IL23p19$^+$ and IL12/23p40$^+$ Slan DCs in stimulated PBMC of CD (n=6), UC (n=5) patients and*

healthy subjects (n=4 control). ***(C)*** *Percentage of IL1β^{+}, TNFα^{+}, IL23p19^{+} and IL12/23p40^{+} CD14^{bright} monocytes in stimulated PBMC of CD (n=6), UC (n=5) patients and healthy subjects (n=4 control). Data are represented as mean ± s.e.m. Mann-Whitney and Wilcoxon signed rank tests were used to assess significance.*

Table 3-1 Study participant information

Variable	CD	UC	Non-IBD
n	48	14	20
Females, *n* (%)	29 (60.4)	7 (50)	9 (45)
Age, median (range)	45 (21–76)	39 (17–62)	54 (22–88)
Age at diagnosis			
<16	5	0	
17–40	32	5	
>40	11	9	
Treatment			
None	17	7	
5-ASA alone	6	5	
Thiopurine or methotrexate	14	2	
TNF-α inhibitor	7	1	
Corticosteroid	11	0	
Disease location—CD			
Terminal ileum	0		
Colon	14		
Ileocolonic	34		
Upper GI tract	0		
Disease location—UC			
Proctitis		2	
Left-sided colitis		9	
Pancolitis		3	
Proximal colitis		0	
Disease behavior—CD			
Nonstricturing–Nonpenetrating	30		

Variable	CD	UC	Non-IBD
Stricturing	13		
Penetrating	5		
Perianal disease	4		
Diagnosis–control			
Colonic neoplasia			7
Cold diverticulitis			4
Screening colonoscopy			9

3.2.7 SUPPLEMENTAL INFORMATION

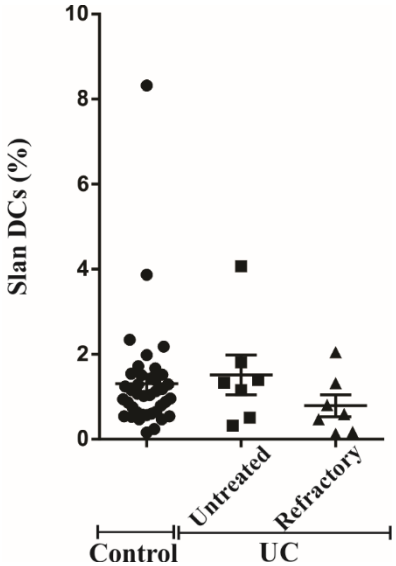

Figure 3-S1: Percentage of circulating Slan DCs.
Percentage of Slan DCs in healthy (control) subjects (n=44), untreated UC patients (n=7), as well as refractory UC patients (n=7). Data are represented as mean ± s.e.m. Mann-Whitney test was used to assess significance.

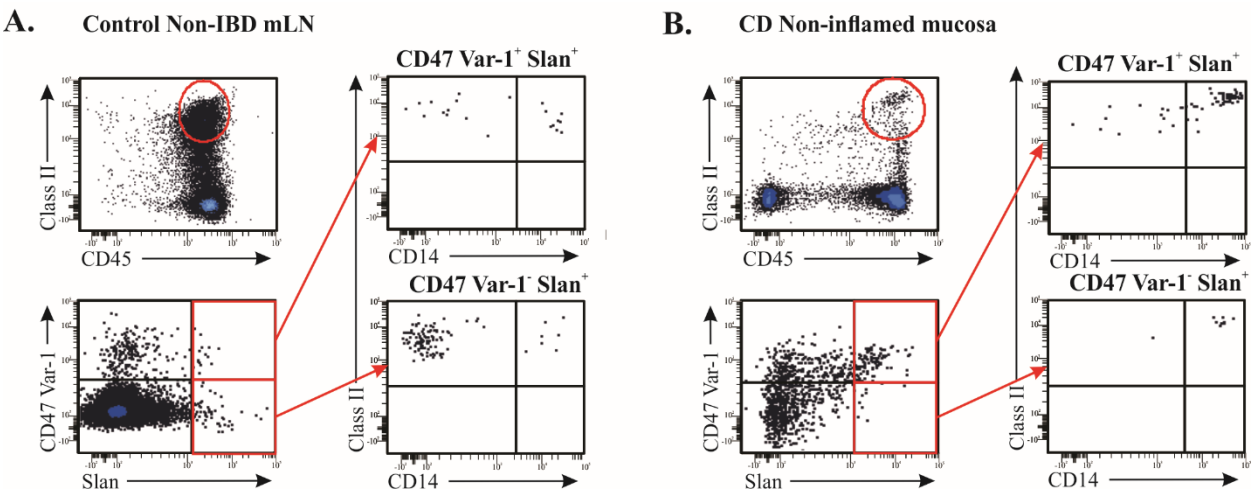

Figure 3-S2: Gating strategy of Slan$^+$ cells in mLN.

Representative gating strategy showing CD47Var-1$^+$and Slan$^+$ population among ClassII$^+$CD45$^+$ cells in mLN of non-IBD patient (A) and non-inflamed mucosa of CD patient (B). The expression of CD14 is represented on Slan$^+$ populations.

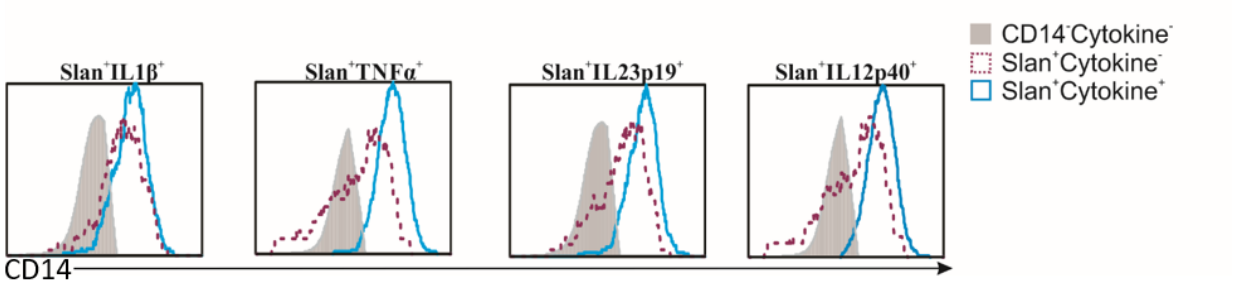

Figure 3-S3: Phenotype of cytokine-producing Slan$^+$ cells.

PBMC were stimulated for 24 hours with MDP and Pam3Csk4. BrefeldinA was added for the last 3 hours. Flow cytometry histogram of CD14 staining on the Slan$^+$Cytokine$^+$ (blue line), Slan$^+$Cytokine$^-$ (purple dashed line) and CD14$^-$Cytokine$^-$ (grey filled) populations.

Chapter 4 :

Differential pathogenic Th17 profile in mesenteric lymph nodes of Crohn's disease and ulcerative colitis patients

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Author's contribution	I participated to the design of the study (50%) and conducted the experimental work (70%), data analysis (80%) and the writing of the manuscript (80%).
Journal	Frontiers in Immunology
Status	Published May 2019
DOI	10.3389/fimmu.2019.01177
Key words	Th17 cells; human; inflammatory bowel disease; mesenteric lymph nodes; plasticity

4.1 Abstract

The drug targets IL23 and IL12 regulate pathogenicity and plasticity of intestinal Th17 cells in Crohn's disease (CD) and ulcerative colitis (UC), the two most common inflammatory bowel diseases (IBD). However, studies examining Th17 dysregulation in mesenteric lymph nodes (mLNs) of these patients are rare. We showed that in mLNs, CD could be distinguished from UC by increased frequencies of $CCR6^{+}CXCR3^{-}ROR\gamma^{+}Tbet^{-}CD4^{+}$ (Th17) memory T cells enriched in $CD62L^{low}$ effector memory T cells (T_{EM}), and their differentially expressed molecular profile. Th17 T_{EM} cells (expressing *IL17A, IL17F, RORC* and *STAT3*) displayed a higher pathogenic/cytotoxic (*IL23R, IL18RAP,* and *GZMB, CD160*, *PRF1*) gene signature in CD relative to UC, while non-pathogenic/regulatory genes (*IL9*, *FOXP3, CTLA4*) were more elevated in UC. In both CD and UC, IL12 but not IL23, augmented IFNγ expression in Th17 T_{EM} and switched their molecular profile towards an ex-Th17 (Th1*)-biased transcriptomic signature (increased *IFNG,* and decreased *TCF7, IL17A*), suggesting that Th17 plasticity occurs in mLNs before their recruitment to inflamed colon. We propose that differences observed between Th17 cell frequencies and their molecular profile in CD and UC might have implications in understanding disease pathogenesis, and thus, therapeutic management of patients with IBD.

4.2 Article

4.2.1 INTRODUCTION

Lymph nodes (LNs) are the key sites to initiate an effector response and generate memory T cells. However, human lymphoid tissue samples available for research use is quite limited. Recent studies examined several organs of deceased healthy donors, which expanded our knowledge on T cell compartmentalization throughout the body under homeostatic conditions (Kumar et al., 2018; Thome et al., 2016b). Naïve versus memory $CD4^+$ T cell balance shifts with age, with memory cells gaining numbers in adulthood in mucosal and lymphoid tissues (Senda et al., 2018). Mesenteric lymph nodes (mLNs) home gut migratory DCs that dictate the type of helper T (Th) responses by driving naive T cell polarization towards Th1, Th2, Th9, Th17, T follicular helper (Tfh) or regulatory T (T_{reg}) cells, each specialized in immunogenic or tolerogenic immune responses (Stadhouders et al., 2018). Migratory DCs further induce the expression of gut homing receptors such as CCR9 or α4β7 on antigen-specific T cells, which enable their return to the intestine (Iwata et al., 2004). MLNs are thus considered a primary site for generation of mucosal Th responses, including Th17 cells that are important contributors to gut homeostasis. Indeed, an essential role was demonstrated for murine mLNs in the generation of pro-inflammatory $IL17A^+CD4^+$ T cells that are found in the small intestine (Kawabe et al., 2016). However, Takebayashi *et al.* found that absence of mLNs did not affect IL17 cytokine production by $CD4^+$ T cells isolated from the colonic lamina propria in murine IBD models (Takebayashi et al., 2011). Furthermore, it is proposed that Th17 cells are generated in the gut and rarely observed in mLNs and Peyer's patches (Atarashi et al., 2008). Studies investigating Th cells in mLNs of patients with inflammatory bowel diseases (IBD) remain scarce (Baba et al., 2013; Chapuy et al., 2014; Sakuraba et al., 2009; Saruta et al., 2007).

Crohn's disease (CD) and ulcerative colitis (UC) are the two most frequent chronic, remitting and relapsing IBD forms (Zhang and Li, 2014). Both diseases share common features but are distinct entities with CD developing in the entire gastrointestinal tract and UC in colon and rectum. The immune mechanisms that govern UC and CD disease process include the recruitment of pathogenic Th17 cells in the gut. Pathogenicity of mucosal Th17 cells is not defined by IL17 secretion per se but rather by their plastic nature, a hallmark of IBD (Stockinger and Omenetti, 2017). Th17 conversion to ex-Th17 (Th1*) is predominantly controlled by two pro-inflammatory cytokines IL12 and IL23 that share a common p40 chain (Oppmann et al., 2000). Yet, the potential contribution of mLNs to the generation of pre-committed pathogenic Th17 cells during intestinal inflammation in CD and UC remains to be investigated. In the present study, we examined the distribution of memory Th17 cells in the mLNs of UC and CD patients, their molecular characteristics, and determined their plasticity in response to IL12 and IL23.

4.2.2 MATERIALS AND METHODS

4.2.2.1 *Human clinical samples*

MLNs were collected from surgical resections. This study included 25 patients with CD and 9 patients with UC (clinical information is shown in **Table 4-S1**). No histological data or bacterial infections suggested a differential diagnosis.

4.2.2.2 *Cell purification and analysis*

MLNs were digested mechanically to obtain cellular suspensions (Baba et al., 2013). Antibodies used for flow cytometry are listed in **Table 4-S2**. Their respective Fluorescence minus one (FMO) or isotype controls are shown in **Figure 4-S1**. FCS Express 6 (DeNovo Software) or *t*-SNE (*t*-Distributed Stochastic Neighbor Embedding) plugin available in FlowJo version 10.5.3 (FlowJo, LLC) (Quinn et al., 2015) were used for data analysis.

4.2.2.3 *Cell sorting and culture*

$CD62L^{low}CD45RO^{+}CD45RA^{-}CD25^{-}CD8^{-}CD4^{+}$ T cell subsets: $CCR6^{+}CXCR3^{-}$, $CCR6^{+}CXCR3^{+}$, and $CCR6^{-}CXCR3^{+}$ were FACS sorted for functional studies according to the gating strategy depicted in **Figure 4-2A**. Transcriptomic studies examined sorted $CCR6^{+}CXCR3^{-}CD62L^{low}CD45RO^{+}CD45RA^{-}CD25^{-}CD8^{-}CD4^{+}$ T cells treated in the absence or presence of IL12. Cell isolation was performed using FACS Aria II cell sorter and data were analyzed using FACS Diva 6 (BD Biosciences).

The three purified $CD4^{+}$ T cell subsets were stimulated with anti-CD3/CD28 beads (Miltenyi Biotec) and cultured with or without IL12 (20 ng/ml, R&D system) or IL23 (10 ng/ml, R&D system) for 6 days. Cultures were performed in RPMI 1640 medium supplemented with 10%

fetal calf serum and 1% penicillin/streptomycin; 20 000 – 50 000 cells per well. For intracytoplasmic staining, PMA-ionomycin was added for 6 hours in cell cultures and Brefeldin A for the last 3 hours, cells were then fixed and stained with CD3 monoclonal antibody followed by intracytoplasmic staining for IL17 and IFNγ.

4.2.2.4 *NanoString*

NanoString was performed at the LDI Molecular Pathology Research Core. RNA was isolated using the NucleoSpin RNA extraction protocol followed by nCounter Low RNA Input Amplification Protocol (nanoString).

Differential gene expression was assessed using the NanoString Human Immunology v2 panel according to the manufacturer's specifications. In brief, amplified RNA was used for Sample Preparation. The samples were then processed with the nCounter Preparation Station to purify the hybridized targets and affix them to the cartridge for imaging using the nCounter Digital Analyzer (CCD camera). Barcodes were counted for each target molecule at High Resolution.

4.2.2.5 *NanoString statistical analysis*

The mRNA expression matrix for 583 genes was normalized using a list of house-keeping genes including *ABCF1, ALAS1, EEF1G, G6PD, GUSB, HTPRT1, HTPRT1, OAZ1, POLR2A, PPIA, PPIA, RPL19, TBP, TUBB*. However, it excluded *GAPDH* for having a high expression SD in our dataset. Subsequent PCA analysis revealed that the house-keeping normalized data was primarily clustered by diseases (UC and CD) which is of biological significance. In order to validate the inclusion of a patient covariable in the association model, we performed

normalization using the R program (R Core Team, 2016): R limma (Ritchie et al., 2015) and EdgeR (McCarthy et al., 2012; Robinson et al., 2010) library that removed the effect of the patient identity on the PCA expression pattern. The resulting PCA analysis graph showed the samples being clustered by conditions (control and IL12) for which we want to analyze the expression.

A differential expression analysis was done with the R limma package with three contrast matrices:

1. ContUC vs ContCD (Differential expression analysis between Control samples from UC and CD)
2. IL12CD vs ContCD (Different expression analysis between IL12 stimulated cell versus control for CD)
3. IL12UC vs ContUC (Different expression analysis between IL12 stimulated cell versus control for UC)

The association model included the contrast sample condition plus a covariate for the patient identity to reflect what was detected on the PCA analysis.

Graphics and visualization of the differential expression analysis metrics where done using the gplots, ggplot2 and beanplot libraries.

4.2.2.6 *Statistical analysis*

Statistical analysis was performed with Prism version 6 (GraphPad Software). Data were checked for normality using Shapiro-Wilk test and then the appropriate test was applied as indicated. For all tests, 1 symbol means $P< 0.05$, 2 symbols mean $P< 0.01$, and 3 symbols mean $P<0.001$. Bar graphs are shown as mean ± SEM.

4.2.2.7 *Study approval*

This study was approved by the Institutional Ethics Research Committee of the Centre Hospitalier de l'Université de Montréal (CER-CHUM). The patients provided written consent to the study protocol.

4.2.3 RESULTS

4.2.3.1 *Predominance of CCR6$^+$CXCR3$^-$CD4$^+$ T cells in mLNs of CD when compared to UC patients*

The human T cell compartment is heterogeneous with variable distribution in different mucosal and lymphoid tissues (Senda et al., 2018) that is further altered upon inflammation. We investigated whether the distribution of CD4$^+$ T cell subsets in inflamed mLNs distinguished CD from UC. The percentage of CD4$^+$ T cells and memory CD45RA$^-$CD4$^+$ T cells was similar in both diseases (**Figure 4-1A**). Memory CD4$^+$ T cells were next stratified using CCR6 and CXCR3 which are Th17 and Th1-associated markers respectively (**Figure 4-1B**) (Annunziato et al., 2007). Accordingly, CCR6$^+$CXCR3$^-$CD4$^+$ T cells expressed RORγ but not Tbet, and conversely, CCR6$^-$CXCR3$^+$CD4$^+$ T cells expressed Tbet but not RORγ (**Figure 4-1C**). CCR6$^+$CXCR3$^+$CD4$^+$ T cells co-expressed RORγ and Tbet. Interestingly, the percentage of memory CCR6$^+$CXCR3$^-$CD4$^+$ T cells was significantly higher in CD relative to UC, and additionally, it predominated over both CCR6$^-$CXCR3$^+$ and CCR6$^+$CXCR3$^+$ CD4$^+$ T cell subsets in CD only (**Figure 4-1D**). However, there were no differences between CD and UC in the frequencies of CCR6$^-$CXCR3$^+$ or CCR6$^+$CXCR3$^+$ CD4$^+$ T cells (**Figure 4-1D**). Memory Th cell subsets were further subdivided into CD62L^{low} effector memory (T_{EM}) and CD62L^{high} central memory (T_{CM}) T cells. As expected, inflamed mLNs comprised more T_{EM} than T_{CM} cells among all Th subsets examined (**Figure 4-1E**). However, only in CD the frequencies of CCR6$^+$CXCR3$^-$ T_{EM} cells were significantly higher than CCR6$^-$CXCR3$^+$ T_{EM} cells.

Noteworthy, mLN CD4$^+$ T cells also comprised minor T subpopulations that were equally distributed in CD and UC, they included T_{reg} (CD25$^+$Foxp3$^+$), and invariant T cells (**Figure 4-**

S2A). The latter comprised MAIT (93-97%) (TCRVα7.2^{+}TCRVα4.24^{-}), γδ T (1.5-3%) (TCRγϱ^{+}TCRVα7.2^{-}) and iNKT (1.5-3%) (TCRVα7.2^{-}TCRVα4.24^{+}) cells. T_{reg} and invariant T cell subpopulations were more represented in the CD4^{+} compared to CD8^{+} compartment (**Figure 4-S2A-C**). Within these invariant subpopulations, only 20% of cells expressed CCR6 (**Figure 4-S2D**). Furthermore, T follicular helper cells (Tfh) were detected as rare ICOS^{+}CXCR5^{+}Ki-67^{+}cells in both diseases (**Figure 4-S2E**).

Taken together, CD mLNs comprised more CCR6^{+}CXCR3^{-}CD4^{+} T cells relative to UC and T_{EM} cells predominated over T_{CM} population.

4.2.3.2 *MLN Th17 T_{EM} cells differentially expressed a pathogenic/cytotoxic molecular profile in CD relative to UC*

Next, we thought to compare the cytokine and molecular profile of CCR6^{+}CXCR3^{-} CD62L^{low}CD4^{+} T cells (Th17 T_{EM}) in mLNs of UC and CD patients. To this end, mLN Th17 T_{EM}, purified as depicted in **Figure 4-2A**, expressed high IL17 and low IFNγ while CCR6^{+}CXCR3^{+} CD62L^{low}CD4^{+} T cells (Th17/Th1 T_{EM}) produced both, and CCR6^{-} CXCR3^{+}CD62L^{low}CD4^{+} T (Th1 T_{EM}) cells secreted IFNγ only (**Figure 4-2B**). However, unlike with unfractionated CD4^{+} T cells (Sakuraba et al., 2009), no significant differences were noted in the frequencies of IL17 or IFNγ -producing cells in purified Th T_{EM} subsets between CD and UC patients. Th17 T_{EM} identity was further confirmed at the molecular level by equally elevated expression of *IL17A, IL17F, RORC, STAT3* and *CCL20* in CD and UC (**Figure 4-2C**) (Stadhouders et al., 2018; Stockinger and Omenetti, 2017; Uniken Venema et al., 2019).

Unexpectedly, mLN Th17 T_{EM} subset in CD was distinguished by a set of differentially expressed genes when compared to UC (**Figure 4-2D** (FDR< 0.005) and **Table 4-S3**). In fact, *IL23R*, *CCL3, IL22, DPP4*, *GZMB* and *IL18RAP*, reported to be associated with a pathogenic Th17 signature in humans and mice (Bengsch et al., 2012; Ramesh et al., 2014; Wang et al., 2015), were over-expressed in Th17 T_{EM} from CD relative to UC (**Figure 4-2E**). *GZMB* and *IL18RAP* along with *PRF1, CSF1, CD160, CXCR6, CD3E, KLRB1* further delineated a pro-inflammatory/cytotoxic Th profile in CD relative to UC (**Figure 4-2D** and **Figure 4-2E** and **Table 4-S3**) (Patil et al., 2018; Uniken Venema et al., 2019). In contrast, Th17 T_{EM} in UC, when compared to CD, had a greater expression of *IL9*, *IL10*, *IL1RN, CTLA4* and *FOXP3*, genes that are considered non-pathogenic or regulatory (Lee et al., 2012; Ramesh et al., 2014; Wang et al., 2015). Interestingly, augmented *IL9* along with low *CD96* and *DPP4* expression (**Figure 4-2D**) observed in UC relative to CD mimics a Th9 pro-inflammatory profile associated with chronic intestinal inflammation in mice (Gerlach et al., 2014; Stanko et al., 2018). Moreover, a Th9-biased profile has also been reported in UC mucosa (Nalleweg et al., 2015).

In conclusion, Th17 T_{EM} cells are associated with a pathogenic/cytotoxic molecular profile in CD and a non-pathogenic/regulatory profile in UC.

4.2.3.3 *IL12 shifts mLN Th17 T_{EM} cells towards ex-Th17 (Th1*) in CD and UC*

IL23 favors Th17 effector function while IL12 down-regulates IL17 and promotes IFNγ expression in circulating and intestinal Th17 cells (Annunziato et al., 2007; Kleinschek et al., 2009; Kobayashi et al., 2008; Ramesh et al., 2014). Furthermore, mucosal pathogenic Th17 cells that contribute to IBD pathogenesis are best defined by their ability to acquire IFNγ and thus, ultimately switch to Th1* (Ramesh et al., 2014). We therefore asked whether Th17 T_{EM}

in mLNs could be shifted towards Th1*. Th17 T_{EM} exposure to IL12 increased the percentage of $IL17^{-}IFN\gamma^{+}$ cells as well as IFNγ production per cell, as measured by the mean fluorescence intensity (MFI), in both CD and UC (**Figure 4-3A**). Frequencies of $IL17^{+}IFN\gamma^{-}$ cells were significantly reduced by IL12 in CD only, further demonstrating a shift of Th17 T_{EM} cells to Th1*. In addition, we noticed that IL12 augmented frequencies of $IL17^{+}IFN\gamma^{+}$ cells in 7 out of 9 CD, and 6 out of 8 UC samples. In contrast, IFNγ and IL17 expression was not significantly modified by IL12 in Th17/Th1 T_{EM}, and, IFNγ expression was marginally increased in Th1 T_{EM} in UC only (**Figure 4-S3A**).

Furthermore, Th17 conversion to Th1* under the influence of IL12 was associated with *IL17A, TCF7* and *IL9* downregulation while pro-inflammatory and cytotoxic gene expression (*IFNG*, *IL21*, *GNLY, DPP4*, *GZMB)* increased in both CD and UC (**Figure 4-3B** and **Figure 4-3C**). *TCF7* downregulation was consistent with the emergence of $IL17^{-}IFN\gamma^{+}$ (Th1*) cells and increase of Th1 genes (Muranski et al., 2011; Oestreich et al., 2011). *IFNG* and *HAVCR2* gene expression, which were augmented, best defined Th1-like T cells in colorectal cancer (Zhang et al., 2018), while *PDCD1*, an immune checkpoint inhibitor, was decreased. The gene encoding *IL17F*, that promotes colitis in mice (Tang et al., 2018), was not inhibited upon IL12 stimulation; reinforcing the concept that IL12 induces the generation of pathogenic Th1* cells in inflamed mLNs. IL12 is likely contributed by mature DCs that accumulate in mLNs of CD (Jaensson et al., 2008); these cells also produce IL23 along with IL12 (Sakuraba et al., 2009). Unlike exposure to IL12, modulation of IL17 and IFNγ expression was unchanged in Th17, Th17/Th1 and Th1 T_{EM} cells in response to IL23 (**Figure 4-S3B**). Failure of IL23 to augment

IL17 or IFNγ in Th17 T_{EM} cells was not attributed to absence of IL23 receptor since *IL23R* was expressed in CD, and at higher levels relative to UC (**Figure 4-2D**).

Taken together, IL12 but not IL23 promotes plasticity of mLN Th17 T_{EM} cells.

4.2.4 DISCUSSION

It is established that mucosal Th cells regulate gut homeostasis and inflammation, but few reports examined mLNs of IBD patients. Overall, the present study revealed that CD and UC could be distinguished by examining the frequencies and molecular profile of Th17 cells in mLNs. MLNs in CD were characterized by a predominant Th17 T_{EM} population displaying a pathogenic/cytotoxic gene signature relative to Th17 T_{EM} cells in UC that expressed a profile biased towards regulatory genes. Under IL12 exposure, mLN Th17 T_{EM} cells from both CD and UC shifted towards a Th1* cytokine and molecular profile, suggesting that Th17 plasticity is taking place in this inductive site before T cell homing to gut tissues.

A previous study indicated that frequencies of IL-17^+ cells are augmented in CD when compared to UC using plastic-coated CD3/CD28 activated unfractionated mLN $CD4^+$ T cells (Sakuraba et al., 2009). We showed here that the proportion of $IL17^+$ cells was similar in both diseases using anti-CD3/CD28 beads activated $CCR6^+CXCR3^-$ effector memory $CD4^+$ T cells while the expression of IL17 per cell (MFI IL17) tended to augment in CD.

Pathogenicity of Th17 cells is best defined by their capacity to elicit disease after *in vivo* adoptive transfer, their selected gene expression profile and their plastic nature (Ahern et al., 2010; Ramesh et al., 2014; Wang et al., 2015). In mice, Th17 cells gradually progress to a pre-Th1 effector phenotype in the LN and, to a Th17/Th1-like effector phenotype in non-lymphoid tissues (Gaublomme et al., 2015), suggesting that Th17 conversion is already initiated in LN. Indeed in CD mLN, Th17 T_{EM} cells displaying a pathogenic, "pre-Th1" inflammatory gene (*IFNG*, *HAVCR2, CD26)* profile (Bengsch et al., 2012) corroborate the progression observed

in mice LN. Furthermore, Th17 T_{EM} cells isolated from the mLN of IBD patients converted to Th1* under the influence of IL12. Th17 program inhibition by IL12, as shown here by decreased *TCF7* expression (Oestreich et al., 2011), also depends on *Eomes* up-regulation that inhibits *RORC2* and *IL17A* expression while maintaining *IFNG* (Mazzoni et al., 2019). Interestingly, *Eomes*-expressing Th1* and Th1 cells have a more stable phenotype and do not up-regulate IL17 under the influence of IL1β, IL6, IL23 and TGFβ Th17-polarizing cytokines (Geginat et al., 2016; Mazzoni et al., 2019), consistent with a lack of modulation of IL17 and IFNγ expression observed in CD and UC Th1 T_{EM} cells. The limitation of our study is that *Eomes* was not part of the nanostring expression matrix.

IL23 expression is required on T cells to trigger colitis, which is associated with IFNγ and IL17 co-expression (Ahern et al., 2010). Furthermore, administration of anti-IL23p19 monoclonal antibody attenuates development of colitis in Abcb1a$^{-/-}$ mice (Ahern et al., 2010; Maxwell et al., 2015). However, Th17 T_{EM} cells from IBD mLNs did not modulate IL17 and IFNγ expression in response to IL23, differing from increased Th17 responses to IL23 observed in human colonic CD4^{+} T cells from CD patients (Chapuy et al., 2019b). Failure of IL23 to augment IL17 or IFNγ in mLN Th17 T_{EM} cells was not attributed to absence of IL23 receptor since *IL23R* was expressed in CD, and at higher levels relative to UC. These data suggest that tissue-dependent IL23 responsiveness requires additional signals provided by the cytokine milieu and/or environment, which might be absent or low in mLNs. In fact, serum amyloid A proteins 1 and 2 (SAA1/2), secreted by epithelial cells, have been shown to promote robust IL17A production in RORγ^{+} T cells in the mucosa (Sano et al., 2015). Moreover, a recent study demonstrates that induction of severe chronic remitting/relapsing UC-like colitis in

immunocompetent mice requires not only IL23 and pathogenic $CD4^+$ T cells in mLNs and colon, but also intestinal dysbiosis (Chen et al., 2018).

Owing to the importance of IL23/Th17 axis in IBD pathogenesis, it is not surprising that these cells are deemed to be therapeutic targets. However, their inherent plasticity adds difficulty in targeting them directly in inflammatory settings. Antibodies that block IL12 might be suitable targets, as IL12 promotes Th17 plasticity towards pathogenic Th1* in mucosa (Annunziato et al., 2007), and as shown here in mLNs of CD and UC. In fact, anti-IL12p40 drugs are part of the therapeutic arsenal of CD, and clinical trials are ongoing in UC patients (Allocca et al., 2018). However, therapeutic efficacy of both anti-IL12p40 and anti-IL23p19 in ameliorating CD argues for a predominant role for IL23 in disease pathogenesis. Hence, anti-IL23p19 is also in clinical trials for UC (Allocca et al., 2018). Collectively, studying the role of immune cells in IBD mLNs warrants further investigation to better understand differences between CD and UC pathogenesis, and thus, open avenues for personalized medicine.

4.2.5 CONTRIBUTION TO THE FIELD

While being extensively studied in the mucosa, few studies examined helper T cell subsets in the mesenteric lymph nodes (mLNs) of Crohn's disease (CD) and ulcerative colitis (UC) patients. Lymph nodes are the key sites to initiate an effector response and generate memory T cells, emphasizing the need to investigate immune cells in these lymphoid tissues.

Briefly, we showed that mLNs of CD and UC can be distinguished by frequencies of $CXCR3^{-}$ $CCR6^{+}$ Th17 memory T cells, enriched in $CD62L^{low}$ effector memory T cells (T_{EM}), and differentially expressed Th17 T_{EM} molecular profile. Drug targets IL23 and IL12 regulate the pathogenicity and plasticity of intestinal Th17 cells in IBD. Our data further revealed that IL12, but not IL23, shifted mLN Th17 T_{EM} towards a pathogenic Th1* cytokine and molecular profile in both CD and UC, suggesting that Th17 plasticity is taking place in this inductive site before T cell homing to gut tissues.

Therefore, investigation of $CD4^{+}$ helper T cell subsets in the IBD mLNs, which are not easily accessible for research use, has clear implications in further understanding disease pathogenesis and thus, open avenues for personalized medicine.

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4.2.7 Figures

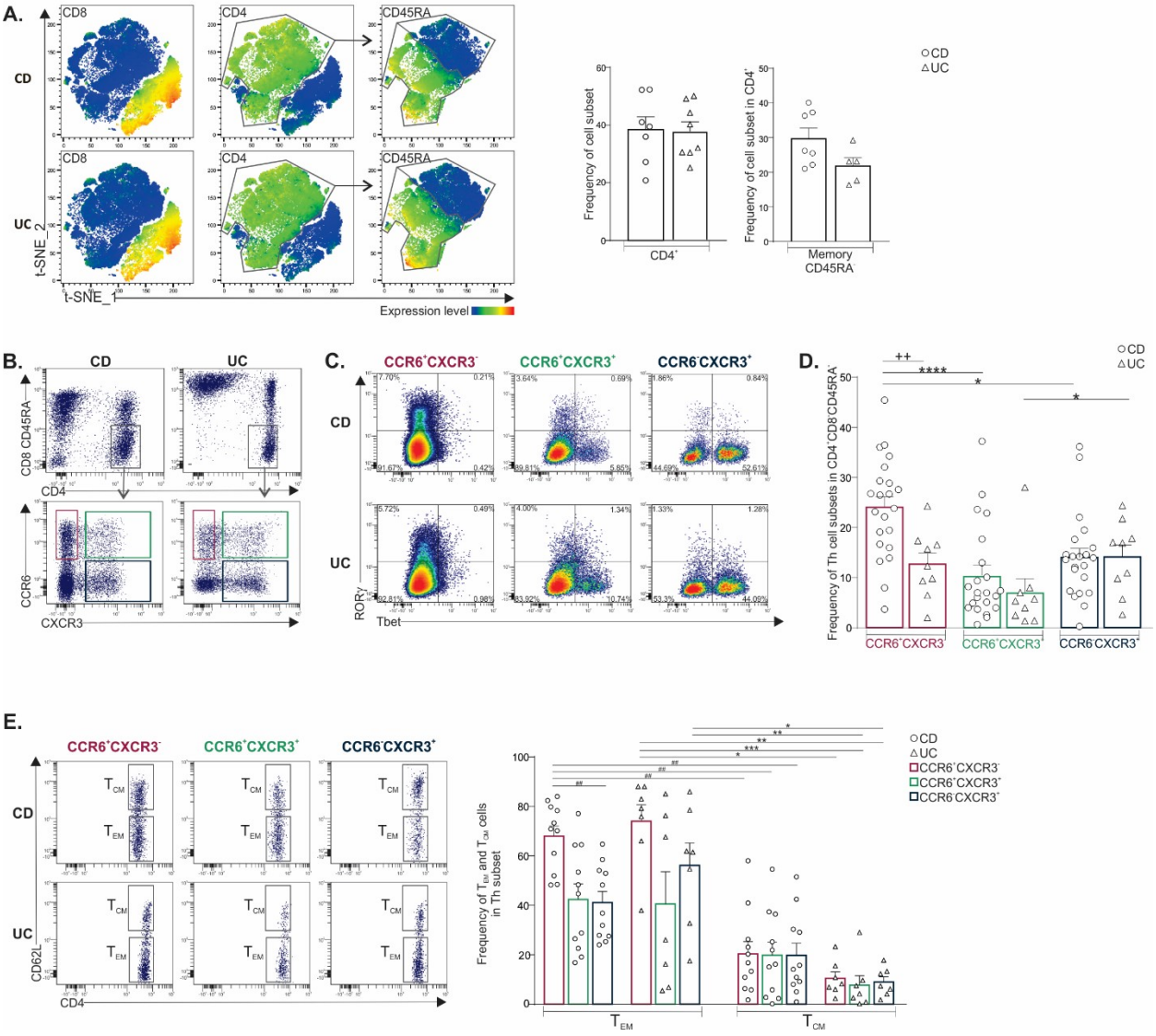

Figure 4-1: Predominance of CCR6$^+$CXCR3$^-$CD4$^+$ T cells in mLNs of CD when compared to UC patients.

(A) CD3$^+$ T cells isolated from mLNs of CD and UC patients were concatenated for t-SNE analysis. Feature plots of the indicated antigens (left panels). Frequencies of CD4$^+$ and memory CD45RA$^-$ T cells (right panels). (B) Representative dot plots of CCR6$^+$CXCR3$^-$, CCR6$^+$CXCR3$^+$ and CCR6$^-$CXCR3$^+$ CD4$^+$ subsets, (C) their expression of RORγ and Tbet, and (D) frequencies of indicated Th subsets. (E) Representative dot plots and frequencies of TEM (CD62L^{low}) and TCM (CD62L^{high}) among Th subsets. Unpaired t-test (+), Friedmann test followed by Dunn's test (*) and repeated measures one-way ANOVA followed by Tukey's test (#). 1 symbol means P< 0.05, 2 symbols mean P< 0.01, and 3 symbols mean P<0.001.

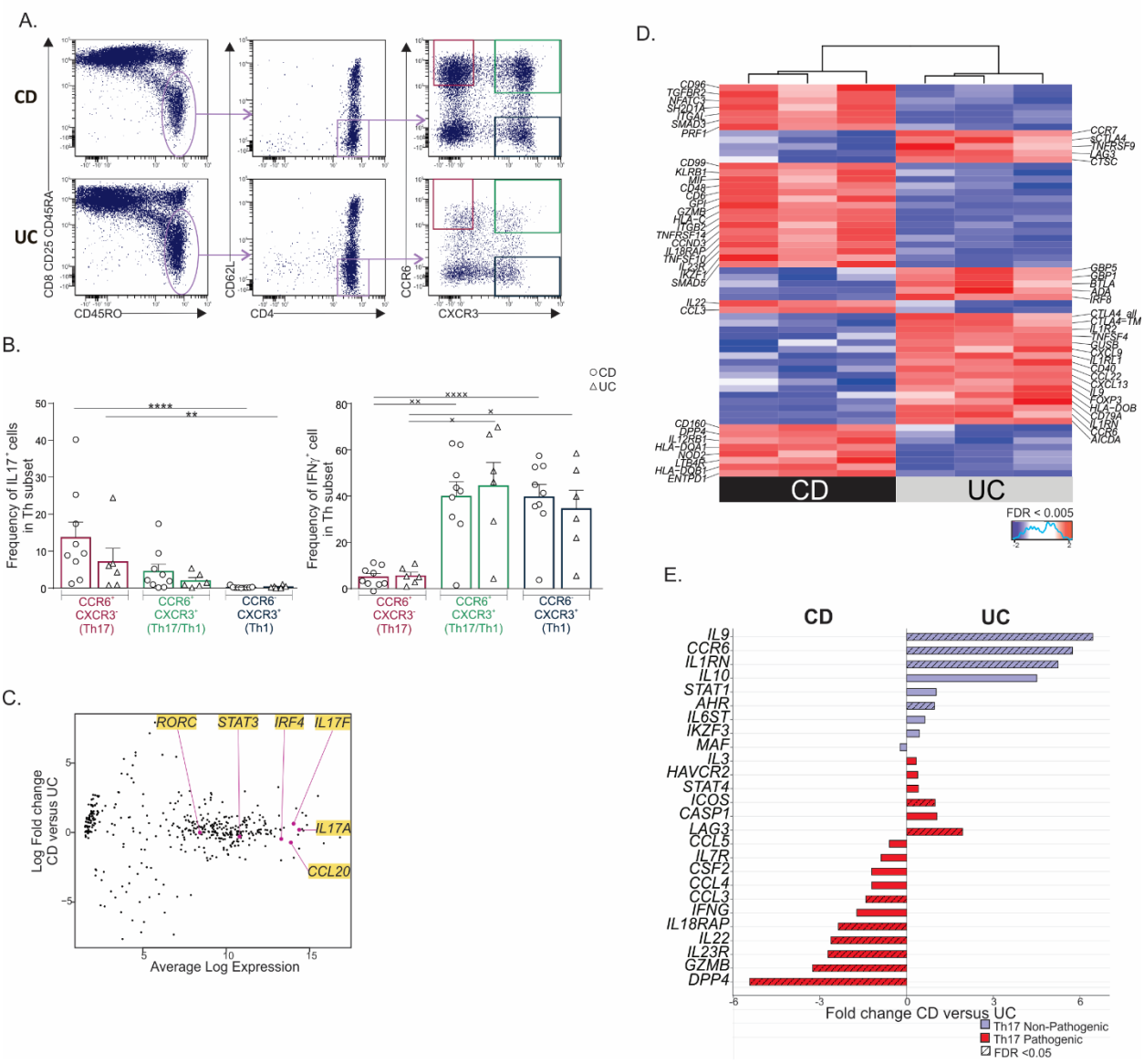

Figure 4-2: MLN Th17 TEM cells of CD differentially express a pathogenic/cytotoxic molecular profile relative to UC.

(A) Gating strategy for sorting CCR6$^{+/-}$CXCR3$^{+/-}$ TEM subsets in mLN. Cells were activated with anti-CD3/anti-CD28-beads for 6 days. On the last day, PMA-ionomycin was added for 6 hours and Brefeldin A for the last 3 hours. (B) Frequencies of IL17 and IFNγ-expressing cells by intracytoplasmic staining in sorted Th TEM subsets. (C-E) Cells were activated with anti-CD3/anti-CD28-beads for 6 days. Molecular profiling of mLN Th17 TEM cells in CD (n=3) and UC (n=3) by Nanostring. (C) Expression of key Th17 genes in CD versus UC. (D) Heatmap of differentially expressed genes in CD relative to UC (FDR<0.005). (E) Fold change of Th17-associated pathogenic and non-pathogenic genes. Friedmann test followed by Dunn's test (*)

and one-way ANOVA followed by Tukey's test (×). 1 symbol means P< 0.05, 2 symbols mean P< 0.01, and 3 symbols mean P<0.001.

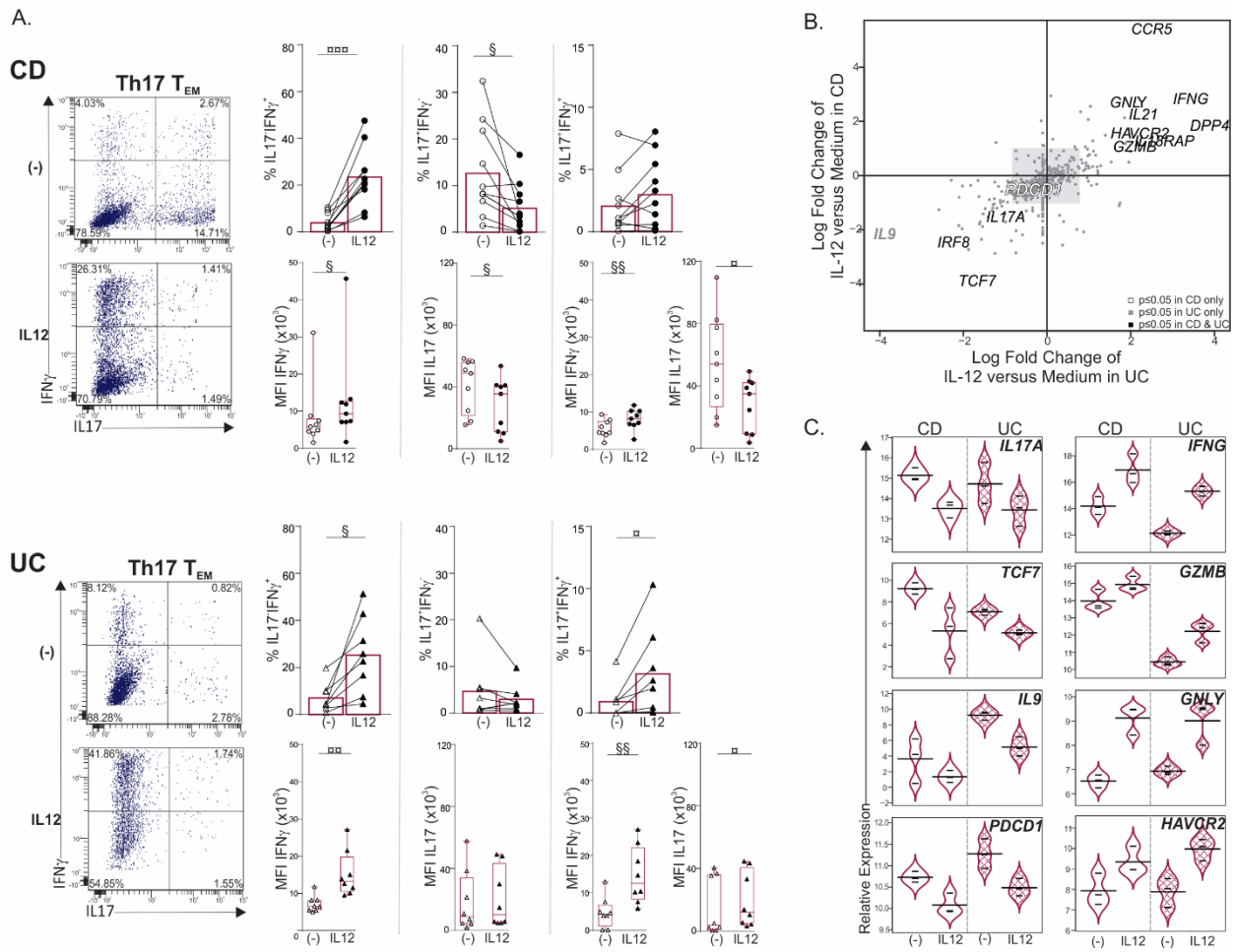

Figure 4-3: IL12 shifts Th17 TEM cells towards Th1* in mLNs of CD and UC.

Sorted mLN Th17 TEM cells were cultured with or without IL12 for 6 days. PMA-ionomycin was added for 6 hours and Brefeldin A for the last 3 hours. (A) Representative dot plots, frequencies and mean fluorescence intensity (MFI) of IL17 and IFNγ. (B-C) Molecular profiling of mLN Th17 TEM cells treated with medium or IL12 from CD (n=3) and UC (n=3) by Nanostring. (B) Quadrant analysis for differentially expressed genes in Th17 TEM, with IL12 treatment relative to medium, in CD versus UC. (C) Violin plots illustrating relative expression of indicated genes. Paired t-test (§) or Wilcoxon signed rank test (¤). 1 symbol means P< 0.05, 2 symbols mean P< 0.01, and 3 symbols mean P<0.001.

4.2.8 SUPPLEMENTAL INFORMATION

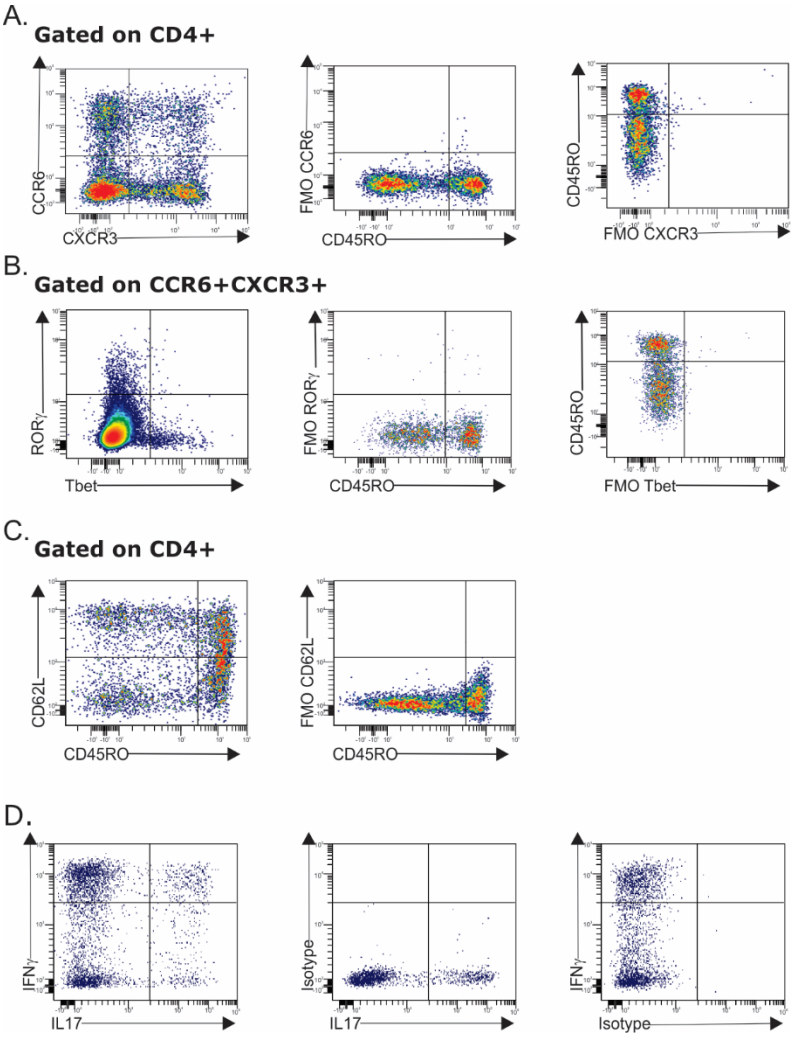

Figure 4-S1: Fluorescence minus one (FMO) and isotype-matched control antibodies.
(A and C) Surface staining on mLN from IBD patients.
(B) Intra-nuclear staining on mLN of IBD patients.
(D) $CCR6^{+}CXCR3^{+}$ TEM cells from CD donor were culture for 6 days with anti-CD3/CD28 beads. On the last day, PMA-ionomycin was added for 6 hours and Brefeldin A for the last 3 hours.

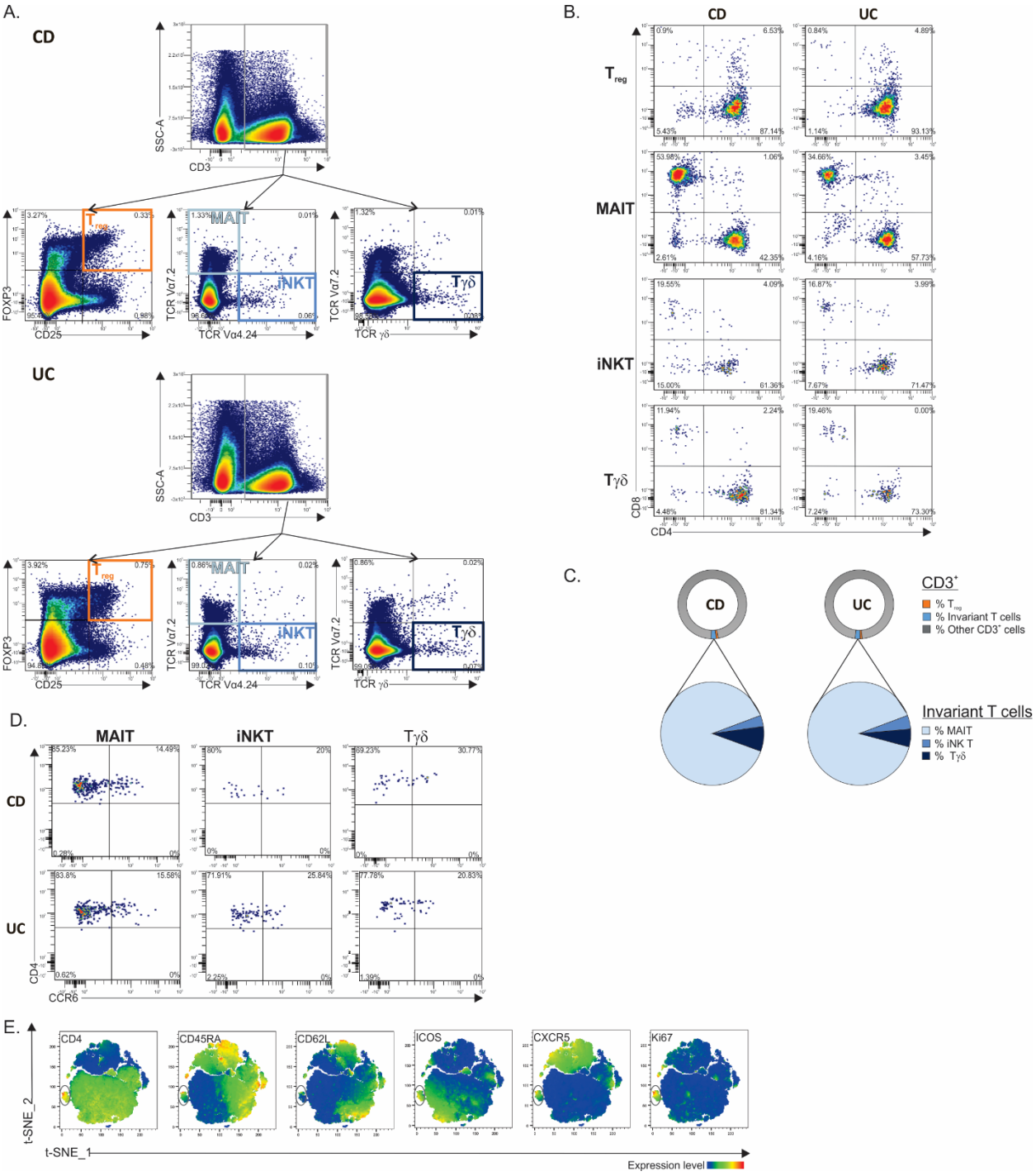

Figure 4-S2: Tfh, T_{reg} and invariant T cell populations in mLNs of IBD patients.
(A) Representative dot plots for identifying T_{reg} ($CD25^+Foxp3^+$), MAIT ($TCR\ V\alpha7.2^+TCR\ V\alpha4.24^-$), iNKT ($TCR\ V\alpha7.2^-TCR\ V\alpha4.24^+$) and γδ T ($TCR\gamma\delta^+TCR\ V\alpha7.2^-$) cells among $CD3^+$ T cells. (B) Representative dot plots showing CD4 and CD8 expression on various T cell subpopulations. (C) Pie chart representing the proportion of T cell subsets among $CD3^+$ T

cells. (D) Representative dot plots showing CCR6 expression on invariant T cell populations. (E) CD3$^+$ T cells were concatenated for t-SNE analysis. Feature plots of the indicated antigens.

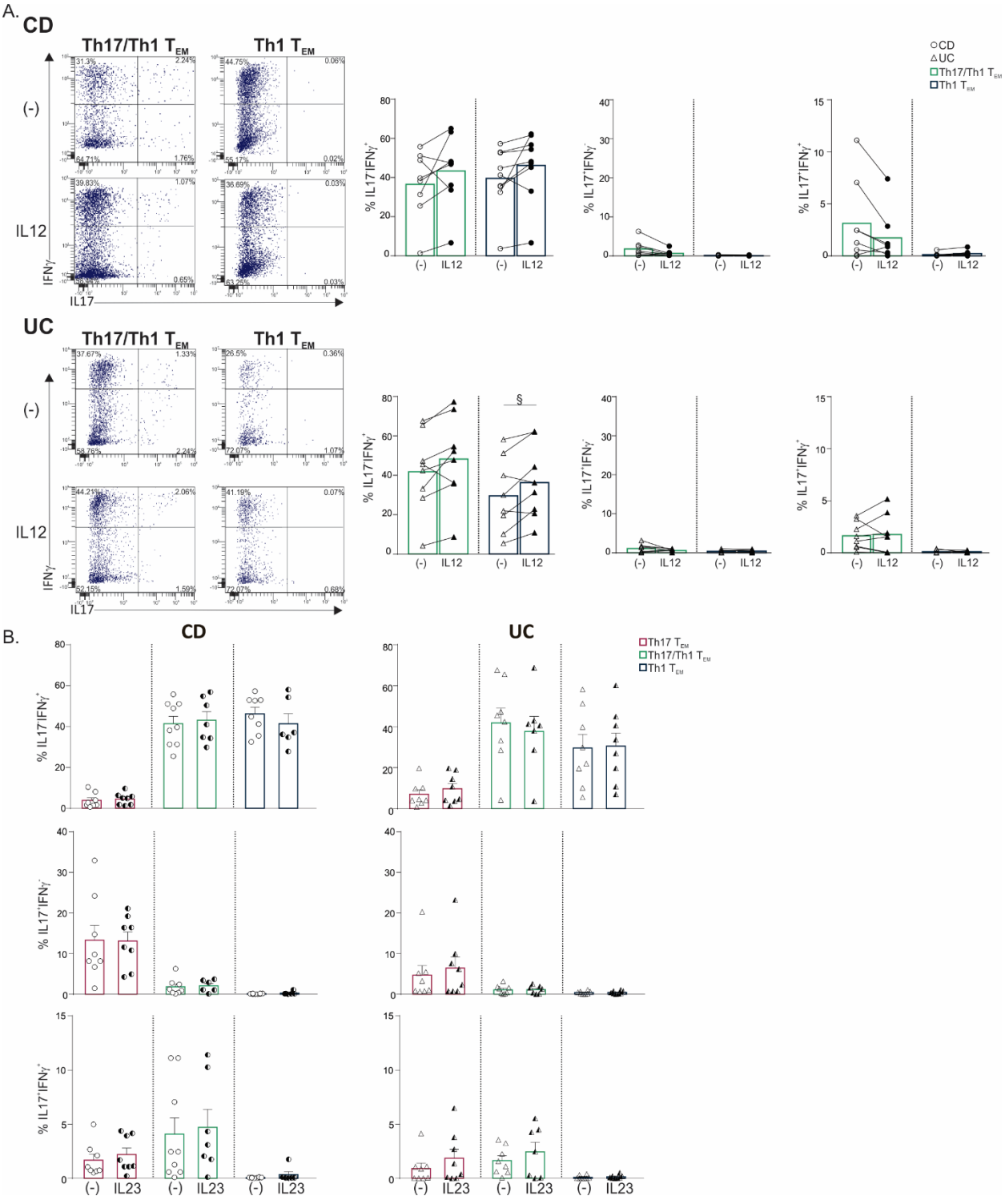

Figure 4-S3: Effect of IL12 and IL23 on Th17, Th17/Th1 and Th1 cells in mLNs of IBD patients.

Th TEM cell subsets in mLNs of CD and UC were sorted according to the gating strategy shown in Figure 4-2A. Th TEM cell subsets cultured with or without (A) IL12 or (B) IL23. Representative dot plots and frequencies of IL17 and IFNg expression. Paired t-test (§).

Table 4-S1: Clinical information.

	CD	UC
N	24	9
Females, n (%)	16 (66.7)	5 (55.5)
Age, median (range)	39 (24-67)	33 (18-80)
Age at diagnosis		
< 16	5	2
17-40	17	4
> 40	2	3
Treatment		
None	4	1
Thiopurine or methotrexate	14	3
TNFα inhibitor	10	2
Anti-IL-12p40	0	0
Anti-α4β7 integrin	1	2
5-ASA	2	1
Corticosteroid	7	7
Disease location - CD		
Terminal ileum	1	
Colon	8	
Ileocolonic	15	
Perianal	7	
Disease behavior - CD		
Non-stricturing - Non-penetrating	0	
Stricturing	15	
Fistula	16	
Abscess	8	
Disease location - UC		
Proctitis		1
Left side colitis		1
Pancolitis		7
Proximal colitis		0

Table 4-S2: List of antibodies.

Antibody	Conjuguate	Clone	Company
CCR6 (CD196)	PE	G034E3	Biolegend
		M-A251	BD
CD25	APC		biosciences
CD25	BV605	BC96	Biolegend
CD3	BV510	UCHT1	Biolegend
		UCHT1	BD
CD3	BUV496		biosciences
CD4	BV510	RPA-T4	Biolegend
CD4	BV785	OKT4	Biolegend
CD45RA	APC	HI100	Biolegend
CD45RA	APC FIRE	HI100	Biolegend
CD45RO	PerCP/Cy5.5	UCHL1	Biolegend
CD62L	PeCy7	DREG-56	Biolegend
CD62L	BV421	DREG-56	Biolegend
CD8	APC	RPA-T8	Biolegend
		SK1	BD
CD8	BUV737		biosciences
	Alexa Fluor	G025H7	
CXCR3	488		Biolegend
		1C6	BD
CXCR3	BUV395		biosciences
FoxP3	APC	PCH101	e-bioscience
ICOS (CD278)	BV421	C398.4A	Biolegend
IFNg	PerCP/Cy5.5	4S.B3	Biolegend
IFNg	BV711	4S.B3	Biolegend
IFNg	BV421	4S.B3	Biolegend
IFNg	AF700	4S.B3	Biolegend
	Alexa Fluor	BL168	
IL17A	647		Biolegend
IL17A	AF700	BL168	Biolegend
IL17A	BV421	BL168	Biolegend
Ki67	BV711	Ki-67	Biolegend
TCR Vα4.24	APC	6B11	Biolegend
TCR Vα7.2	PerCP/Cy5.5	3C10	Biolegend
TCR gd	FITC	B1	Biolegend
RORg	APC	AFKJS-9	e-bioscience
Tbet	FITC	4B10	e-bioscience

Table 4-S3: Genes differentially expressed between activated Th17 cells from CD and UC (FDR value between 0.005 and 0.05).

Higher in CD	Higher in UC
APP	*AHR*
ARHGDIB	*BATF*
BCL2	*CTNNB1*
CD163	*EBI3*
CD3E	*ICOS*
CD40LG	*IFNGR1*
CD5	*IL1A*
CD53	*IL1R1*
CD9	*IL4R*
CSF1	*LIF*
CXCR6	*MX1*
DEFB4A	*PTK2*
ETS1	*SELL*
FCGR3A/B	*SLAMF1*
GFI1	*TIGIT*
HLA−DRB3	
ICOSLG	
IL16	
IL26	
IRAK2	
ITGA5	
ITGAX	
LCP2	
MBP	
MCL1	
PML	
PTPRC_all	
STAT5B	
TNFRSF4	

Chapter 5 :

A two-step human culture system replicates intestinal monocyte maturation cascade: conversion of tissue-like inflammatory monocytes into macrophages

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Author's contribution	I participated to the design of the study (90%) and conducted the experimental work (100%), data analysis (90%) and the writing of the manuscript (90%).
Journal	European Journal of Immunology
Status	Accepted June 2020
DOI	10.1002/eji.202048555
Key words	Macrophages, Crohn's Disease, IL23, TGFβ, IL10

5.1 Abstract

Monocyte maturation program into macrophages (MΦ) is well-defined in murine gut under homeostatic or inflammatory conditions. Obviously, *in vivo* tracking of monocytes in inflamed tissues remains difficult in humans. Furthermore, *in vitro* models fall short in generating the surrogates of transient extravasated tissue inflammatory monocytes. Here, we aimed to unravel environmental cues that replicated the human monocyte "waterfall" process *in vitro* by first, generating tissue-like inflammatory monocytes, which were then shifted towards MΦ. Purified $CD14^{+}CD16^{-}$ monocytes, cultured with granulocyte-macrophage colony-stimulating factor (GM-CSF), IFNγ and IL23, differentiated into $CD14^{+}CD163^{-}$ cells that displayed a monocyte-like morphology. *In vitro* generated inflammatory $CD14^{+}CD163^{-}$ (Infl mo-like) cells promoted IL-1β-dependent memory Th17 and Th17/Th1 responses, like the $CD14^{+}CD163^{-}$ mo-like cells that accumulate in inflamed colon of Crohn's disease patients. Next, *in vitro* generated Infl mo-like cells converted to functional $CD163^{+}$ MΦ following exposure to TGFβ and IL10. Gene set enrichment analysis further revealed a shared molecular signature between converted $CD163^{+}$ MΦ and MΦ detected in various inflamed non-lymphoid and lymphoid diseased tissues. Our findings propose a two-step *in vitro* culture that recapitulates human monocyte maturation cascade in inflamed tissue. Manipulation of this process might open therapeutic avenues for chronic inflammatory disorders.

5.2 Article

5.2.1 INTRODUCTION

Mononuclear phagocyte (MNP) populations, which encompass monocyte-derived cells, macrophages (MΦ) and conventional dendritic cells (DCs), play an important role in the maintenance of tissue homeostasis (Joeris et al., 2017). Intestinal mucosa is home to one of the largest MΦ populations in the body (Lee et al., 1985). Several studies have uncovered the ontogeny of murine intestinal MNPs by *in vivo* tracking of monocyte fate at steady state and during inflammation (Bain et al., 2014; Desalegn and Pabst, 2019). Intestinal MΦ are initially embryo-derived but from the time of weaning originate almost entirely from circulating Ly6C^{high} monocytes (Bain et al., 2014; Bain et al., 2013; Liu et al., 2019; Tamoutounour et al., 2012). At steady state, classical monocytes, which arrive from circulation into tissue, differentiate through a series of short-lived CX3CR1^{int} intermediate cells into mature CX3CR1^{high}CD11b^{+}CD11c^{+}MHCII^{+} MΦ (Bain et al., 2013; Smythies et al., 2010; Tamoutounour et al., 2012). This process begins shortly after monocyte arrival to the gut mucosa (Bain et al., 2013; Schridde et al., 2017). At each stage within the differentiation cascade, cells are imprinted by the gut environment, and thus, become distinct from their dermal or lung counterparts derived from the same precursor (Schridde et al., 2017; Tamoutounour et al., 2012). This monocyte differentiation cascade is interrupted during inflammation, leading to a buildup of intermediate CX3CR1^{int}Ly6C^{+}CD64^{+} pro-inflammatory population (Bain et al., 2013; Platt et al., 2010; Rivollier et al., 2012; Tamoutounour et al., 2012; Zigmond et al., 2012).

In vivo tracking of monocytes in patients with inflammatory bowel diseases (IBD) is obviously difficult. Nonetheless, in 1995, Grimm *et al.* provided direct evidence of monocyte recruitment

to inflamed mucosa in IBD patients using radiolabeled circulating autologous $CD14^+$ monocytes that were re-infused in Crohn's disease (CD) patients and traced to the inflamed gut mucosa as $CD14^+$ MΦ-like cells (Grimm et al., 1995). Recently in the context of tissue transplantation, Bujko *et al.* has shed light on the ontogeny of macrophage populations in human small intestine by identifying four $CD14^+$ MΦ subsets at steady state (Bujko et al., 2018b). Therefore, most studies have been limited to phenotypic characterization of $CD14^+$ cells in gut mucosa of IBD patients using a combination of biomarkers (Baba et al., 2013; Kamada et al., 2008). A $CD14^+$ MΦ-like population that secretes IL1β, IL23, IL6, TNFα and IL10 was identified in inflamed mucosa of IBD patients (Kamada et al., 2008). Recently, two $HLADR^+SIRP\alpha^+CD64^+CD14^+$ subsets, distinct from conventional $CD14^-$ DCs, were molecularly and functionally characterized in inflamed colon of CD patients (Chapuy et al., 2019a). Specifically, $CD14^+CD64^+CD163^-$ (P3) monocyte-like (mo-like), but not $CD14^+CD64^+CD163^+$ (P4) MΦ, subpopulation was shown to accumulate in inflamed colon of CD patients, in proportions that are positively correlated with endoscopic disease severity (Chapuy et al., 2019a). However, the environmental conditions that drive the potential plasticity of recruited monocytes into inflammatory cells which give rise to anti-inflammatory MΦ warrant full elucidation in humans. Hence, attempts to replicate conversion of tissue-like inflammatory monocytes into MΦ *in vitro* are key for studying their respective functions.

Human monocytes differentiate into either DCs or MΦ following exposure to granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL4 (MoDC), or macrophage colony-stimulating factor (M-CSF) respectively (Chow et al., 2017; Mills et al., 2000; Murray et al., 2014; Orecchioni et al., 2019; Sallusto and Lanzavecchia, 1994). Furthermore, classically

activated (M1) MΦ and alternatively activated (M2) MΦ are two well-studied, functionally distinct populations (Italiani and Boraschi, 2014). The M1 MΦ display a pro-inflammatory profile and are generated following *in vitro* monocyte exposure to M-CSF and IFNγ, or lipopolysaccharide (LPS) stimulation *in vivo* (Mills et al., 2000). On the other hand, M2 MΦ generation requires M-CSF plus stimuli such as IL4, IL10 and IL13 (Italiani and Boraschi, 2014; Orecchioni et al., 2019). M1 and M2 MΦ are highly plastic resulting in M1 conversion into M2 and *vice versa* (Das et al., 2015; Stout et al., 2005). However, MΦ complexity and plasticity observed in tissue is oversimplified by the M1/M2 functional paradigm; M1 and M2 represent two extremes with a wheel of MΦ activation states in between (Sica and Mantovani, 2012). Indeed, Xue *et al*. described a wide spectrum of human MΦ activation, with different transcriptional clusters associated with different stimuli, portraying a higher level of MΦ diversity (Xue et al., 2014). Whether M1 and M2 MΦ are part of the monocyte differentiation cascade remains unclear.

In the present report, we first aimed to generate the surrogates of murine extravasated monocytes *in vitro*, which share morphological, phenotypic and functional features with human $CD14^{+}CD64^{+}CD163^{-}$ mo-like cells detected in chronically inflamed colonic tissue but are distinct from MoDC. Next, we elicited the maturation process of *in vitro* generated inflammatory $CD14^{+}CD64^{+}CD163^{-}$ (Infl mo-like) cells towards prototypical tissue $CD14^{+}CD64^{+}CD163^{+}$ MΦ.

5.2.2 MATERIALS AND METHODS

5.2.2.1 *Human clinical samples*

All participants signed an informed consent form that had been approved by the Institutional Ethics Research Committee of the Centre Hospitalier de l'Université de Montréal (CER-CHUM). Blood was obtained from healthy donors or cord blood. CD patient recruitment was based on clinical and histological criteria. Inflamed colonic tissue was obtained from endoscopic biopsies and MLN from surgical resections.

5.2.2.2 *MLN and colonic lamina propria mononuclear cells purification and sorting*

The colonic mucosa was first processed by enzymatic digestion with DNase I (Roche) and Collagenase D (Roche) followed by mechanical digestion with gentle MACS (Miltenyi Biotec) to isolate lamina propria mononuclear cells (LPMC) (Baba et al., 2013). MLN were mechanically digested to obtain cellular suspension (Baba et al., 2013). LPMC and MLN were stained with CD14, CD64, CD163, CD172α, HLADR monoclonal antibodies (mAb) and sorted with FACS Aria II (BD Biosciences) to purify $CD163^{-}CD14^{+}CD64^{+}CD172\alpha^{+}HLADR^{+}$ (P3), $CD163^{+}CD14^{+}CD64^{+}CD172\alpha^{+}HLADR^{+}$ (P4), and $CD163^{-}CD14^{-}CD64^{-}CD172\alpha^{+}HLADR^{+}$ populations.

5.2.2.3 *Peripheral blood mononuclear cell purification and sorting*

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation of heparinized peripheral or cord blood. PBMC were stained with CD14, CD16 and HLADR mAb and sorted with FACS Aria II to purify $CD14^{+}CD16^{-}HLADR^{+}$ classical monocytes. $CD45RO^{-}CD45RA^{+}CD4^{+}CD8^{-}$ naïve T cells (from cord blood) and $CD62L^{-}CD25^{-}$

$CD45RO^+CD45RA^-CD4^+CD8^-$ effector memory T cells (T_{EM}) (from adult peripheral blood) were also sorted for functional studies.

5.2.2.4 *Cell culture and sorting*

$CD14^+CD16^-$ classical monocytes were cultured in RPMI 1640 medium with 10% fetal calf serum (FCS) and 1% Penicillin-Streptomycin for 6 days. They were differentiated in the presence of cytokines: GM-CSF (2.5 ng/mL, R & D systems), IL4 (40 ng/mL, R & D systems), IL23 (10 ng/mL, R & D systems) and IFNγ (50 ng/mL, R & D systems). All cytokines were replenished at days 2 and 4. MoDCs were generated by culturing monocytes with GM-CSF+IL4.

Following 6 days culture with GM-CSF+IFNγ+IL23 (Gγ23), TGFβ (1 ng/mL, R & D systems) and IL10 (10 ng/mL, Peprotech) were added for another 6 days. TGFβ and IL10 were replenished at days 8 and 10.

Cultured cells were sorted at days 6 and 12 for morphological, functional and molecular studies.

5.2.2.5 *Flow cytometry*

In vitro differentiated cells were stained for surface markers, then fixed, permeabilized, and stained for intracytoplasmic cytokines expression using mAb listed in **Table 5-S5**, in adherence with the guidelines for the use of flow cytometry and cell sorting in immunological studies (Cossarizza et al., 2019). Data were analyzed with FCS Express 6 (DeNovo Software).

5.2.2.6 *Co-culture with T cells*

Carboxyfluorescein succinimidyl ester (CFSE) (Bioprobe) labeled naïve $CD4^+$ T cells (10^5 cells) were co-cultured with a 1:2 serial dilution of FACS sorted MoDC or Infl mo-like cells, starting with 30 000 cells in the presence of anti-CD3 (1µg/mL; Biolegend) and *Staphylococcus aureus Cowan1* (SAC; Calbiochem at 1/10000 dilution for 6 days. CFSE labeled naïve $CD4^+$ T cells (10^5 cells) were co-cultured at a 1:10 ratio with FACS sorted $CD163^-$ $CD14^+CD64^+CD172\alpha^+HLA\text{-}DR^+$ (P3), $CD163^+CD14^+CD64^+CD172\alpha^+HLA\text{-}DR^+$ (P4), or $CD163^-CD14^-CD64^-CD172\alpha^+HLA\text{-}DR^+$ cells from the inflamed mucosa of CD patients in the presence of human IgG1 and SAC (1/10000 dilution) for 6 days.

T_{EM} ($35x10^3$ cells) were co-cultured with FACS sorted Infl mo-like, at a 1:7 ratio for 6 days. They were cultured in the presence of soluble anti-CD3 (1µg/mL; Biolegend), human IgG1 (10 µg/mL, Biolegend) and peptidoglycan from Staphylococcus aureus (PGN) (10 µg/mL; Sigma-Aldrich) or muramyl dipeptide (MDP, 1 mg/mL; InvivoGen) and Pam3Csk4 (PAM, 1mg/mL; InvivoGen). Anti-IL1β receptor (10 µg/mL), anti-IL-12p40 (10 µg/mL) or IgG1 (10 µg/mL) was added to some co-cultures. At day 6, cells were re-stimulated with phorbol 12-myristate 13−acetate (PMA, 5ng/ml; Sigma-Aldrich) and ionomycin (500ng/ml; Calbiochem-Behring) for 6 hours and with brefeldin A (1 µg/mL; Calbiochem-Behring) for the last 3 hours. Next, cells were stained with CD3, fixed and permeabilized (Invitrogen) for IL17 and IFNγ intracytoplasmic cytokine staining, using mAb listed in **Table 5-S5**.

For all cultures, RPMI 1640 medium with 10% FCS and 1% Penicillin-Streptomycin was used.

5.2.2.7 *Phagocytosis assay*

FACS sorted Infl mo-like and MΦ-like cells were cultured with or without 1/100 FITC-labeled latex beads (Cayman Chemical Company) in 96 well plates for 1 hour. Samples were quenched with trypan blue for 2 minutes. Cells were analyzed by flow cytometry.

5.2.2.8 *Quantitative reverse transcriptase-PCR*

RNA was extracted using Nucleospin® RNA XS kit (Macherey-Nagel) according to the manufacturer's instruction from sorted classical monocytes, and monocytes differentiated *in vitro* with GM-CSF+IFNγ for 2, 4 or 6 days. TaqMan *IL23R* probe and *GAPDH* (housekeeping gene) were purchased from Thermo Fisher.

5.2.2.9 *Morphology*

For morphological studies, FACS sorted MNPs were cytospun and stained according to Wright Stain procedure. Leica DM4000B microscope, equipped with Leica DFC300FX camera was used to visualize cells.

5.2.2.10 *Whole-Transcript Expression Analysis*

Total RNA was quantified using a NanoDrop Spectrophotometer ND-1000 (NanoDrop Technologies, Inc.) and its integrity was assessed using a 2100 Bioanalyzer (Agilent Technologies). Sense-strand cDNA was synthesized from 9 ng of total RNA, and fragmentation and labeling were performed to produce ssDNA with the GeneChip® WT PicoTerminal Labeling Kit according to manufacturer's instructions (Thermo Fisher Scientific). After fragmentation and labeling, 5 µg DNA target was hybridized on

GeneChip® Clariom™ D human (Thermo Fisher Scientific) and incubated at 45°C in the Genechip® Hybridization oven 640 (Affymetrix) for 17 hours at 60 rpm. GeneChips were then washed in a GeneChips® Fluidics Station 450 (ThermoFisher) using GeneChip Hybridization Wash and Stain kit according to the manufacturer's instructions (ThermoFisher). The microarrays were finally scanned on a GeneChip® scanner 3000 (ThermoFisher).

The data are available at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE149722, GSE149722.

5.2.2.11 *Gene expression data analysis*

5.2.2.11.1 Microarrays:

The preprocessing of the microarray data was done in R (R Core team, 2017) and its derived library for genomic data analysis, Bioconductor (Huber et al., 2015). Bioconductor package *oligo* was used to load the CEL files in R environment and the *rma* function implemented in *oligo* for background subtraction, normalization and log2 transformation. Quality control was performed by inspecting various diagnostic plots of the intensity distribution.

5.2.2.11.2 Differential gene expression:

Differential gene expression analysis was done using Bioconductor package *limma*. A linear model was fit to each gene separately and a moderated t-test, comparing paired populations ($CD163^-$ d6 versus $CD163^+$ d12, $CD163^-$ d6 versus $CD163^-$ d12, and $CD163^-$ d12 versus $CD163^+$ d12) was derived. P-values were adjusted using Benjamini-Hochberg (Benjamini and Hochberg, 1995) method for multiple test correction.

5.2.2.11.3 Gene Set Enrichment Analysis (GSEA):

GSEA was performed to assess whether a known biological pathway or sets of individual genes were significantly enriched among the genes ranked by the moderated t-test following the differential gene expression analysis between $CD163^+$ d12 and $CD163^-$ d6 groups. We tested gene sets from the Molecular signature Database (MsigDB, http://www.broad.mit.edu/gsea/msigdb), Hallmark collection (h.all.v5.0.symbols.gmt), and C5 (C5.all.v6.2.symbols.gmt) collections. We also tested two distinct $CD14^+$ genes clusters (Chapuy et al., 2019a) identified in inflamed CD colon recently using scRNA sequencing, a gene expression signature of MΦ (Mo-Mac) and inflammatory DC (Mo-DC) (Goudot et al., 2017), inflammatory MΦ (Inf MΦ) and inflammatory DCs (Inf DC) gene expression signature (Sander et al., 2017), as well as monocyte/macrophage like cells (Mono/MΦ-like) (Chapuy et al., 2020a). The GSEA was performed using the Bioconductor's package fgsea (Korotkevich et al., 2019). The p-values associated to the pathways were adjusted for multiple test correction with a False Discovery Rate cut-off of 0.05.

5.2.2.11.4 Enrichment Map Analysis:

Gene sets within GSEA are often overlapping and represent the same biological process. The Enrichment Map (Merico et al., 2010) was used to reduce redundancy and to organize the gene-set enrichment analysis results into networks. Highly connected gene-sets are grouped together as clusters of functionally related pathways. Nodes represent gene-sets and edges represent the gene overlap that exists between two gene sets. A and B as measured by the Jaccard Coefficient

defined as: size of (A intersect B) / (size of (A union B)). A Jaccard Thresholds of 0.3 was used in this study and a False Discovery Rate cut-off of 0.05.

Enrichment Map Analysis and networks visualization were performed using Cytoscape (Shannon et al., 2003), a network data analysis and visualization software.

5.2.2.12 *Statistical analysis*

Statistical analysis was performed with GraphPad Prism version 6 (GraphPad Software). Data were checked for normality using Shapiro-Wilk test and then the appropriate test was applied as indicated. For multiple unpaired samples, one-way ANOVA was applied followed by Tukey's test (represented by ¤) or Kruskal-Wallis test followed by Dunn's test (represented by #). For paired two columns comparison, paired t-test (represented by *) or Wilcoxon signed rank test (represented by +) were used. For all tests, 1 symbol means *P-value* < 0.05, 2 symbols mean *P-value* < 0.01, and 3 symbols mean *P-value* < *0.001*. Bar graph data are shown as mean ± SEM.

5.2.3 RESULTS

5.2.3.1 *In vitro generation of CD14$^+$CD64$^+$CD163$^-$ cells with monocyte morphology using GM-CSF, IFNγ and IL23*

Circulating CD14$^+$CD16$^-$ classical monocytes were purified following the gating strategy depicted in **Figure 5-1A** and cultured for 6 days in the presence of GM-CSF+IL4. Classical monocytes differentiated into CD14$^-$CD64$^-$CD163$^-$ cells (**Figure 5-1B**), and thus were not considered surrogates for tissue CD14$^+$CD64$^+$CD163$^-$ mo-like (P3) cells that accumulated in inflamed colon of IBD patients (Chapuy et al., 2020b; Chapuy et al., 2019a). We, therefore, thought to develop culture conditions that mimicked gut inflammatory milieu. Replacing IL4 by IL23 maintained CD14 expression but generated CD14$^+$CD64$^-$CD163^{dim} cells, while substituting IL4 with IFNγ allowed the differentiation of monocytes into CD14$^+$CD64$^+$CD163$^-$ cells (**Figure 5-1B**). However, GM-CSF+IFNγ monocyte-derived cells appeared to express CD14 at lower intensity when compared to GM-CSF+IL23. Furthermore, since we noticed that GM-CSF+IFNγ up-regulated *IL23R* mRNA levels on monocytes after 2 days of culture (**Figure 5-1C**), purified monocytes were cultured with the three cytokines: GM-CSF, IFNγ and IL23 (Gγ23). This cytokine cocktail reproducibly generated a large population of HLADR$^+$CD64$^+$CD163$^-$cells (>80%) (**Figure 5-1D**) that expressed CD14, TREM-1, CD89, CD172α and CD172β (**Figure 5-S1**). When purified, Gγ23 monocyte-derived CD14$^+$CD64$^+$CD163$^-$ cells showed a kidney-shaped nucleus and very few dendrites (**Figure 5-1E**) and, unlike *in vitro* GM-CSF+IL4 monocyte-derived DCs (MoDC), did not induce naïve CD4$^+$ T cell proliferation (**Figure 5-1F**). Similarly, CD14$^+$CD64$^+$CD163$^-$ mo-like (P3) cells isolated from inflamed colon of CD patients, in contrast to CD14$^-$CD64$^-$CD163$^-$ DCs, did not display antigen-presenting function (**Figure 5-S2**).

Thus, classical monocytes cultured with Gγ23 differentiate into $CD14^+CD64^+CD163^-$ cells that are distinct from MoDC but share some phenotypic and functional characteristics with colonic $CD14^+CD64^+CD163^-$ mo-like (P3) cells.

5.2.3.2 *$CD163^-$ mo-like cells promote IL1β- and IL12p40- dependent Th17 and Th17/Th1 responses*

$CD163^-$ mo-like (P3) cells isolated from inflamed colon of IBD patients, but not $CD163^+$ MΦ (P4), augmented intestinal Th17 and Th17/Th1 responses in an IL1β-dependent manner (Chapuy et al., 2020b; Chapuy et al., 2019a). We, therefore, examined how *in vitro* Gγ23 monocyte-derived $CD14^+CD64^+CD163^-$ cells regulated the function of effector memory T cells (T_{EM}) isolated from PBMC. These cells augmented the percentage of $IFN\gamma^+IL17^-$, $IFN\gamma^+IL17^+$ and $IFN\gamma^-IL17^+$ cells in both autologous or allogeneic T_{EM} using PGN or MDP and PAM (**Figure 5-2A**). Furthermore, IL1β and IL12p40 blockade inhibited the generation of $IFN\gamma^+IL17^+$ and $IFN\gamma^-IL17^+$ T_{EM} cells only, sparing $IFN\gamma^+IL17^-$ cells (**Figure 5-2B**). The amount of IL17 and IFNγ expression per cell (mean fluorescence intensity, MFI) were reduced by anti-IL1β and anti-IL12p40, respectively (**Figure 5-2B**).

Collectively, Gγ23 culture condition differentiates classical monocytes into functional $CD163^-$ inflammatory mo-like (Infl mo-like) cells that promote IL1β and IL12p40-dependent memory Th17 and Th17/Th1 responses.

5.2.3.3 *In vitro generated CD163$^-$ Infl mo-like cells shift to CD163$^+$ MΦ in response to TGFβ plus IL10*

Next, we thought to define the *in vitro* culture conditions that replicate the monocyte "waterfall" in human inflamed tissue and result in the generation of MΦ. For that purpose, following the first step with Gγ23, culture medium was supplemented at day 6 with TGFβ, IL10 or TGFβ combined with IL10 for another 6 days (**Figure 5-3A**). CD163 expression could not be upregulated with the addition of increasing amounts of TGFβ, while 10 ng/ml of IL10 was sufficient to induce up to 5% of CD64$^+$CD163$^+$cells (**Figure 5-3B**). Only the anti-inflammatory cocktail (TGFβ+IL10) generated a large population of CD64$^+$CD163$^+$ cells (**Figure 5-3C**), expressing MERTK and CD16 (**Figure 5-3D**). High CD14 expression on CD163$^+$ cells at day 12 relative to CD163$^-$ Infl mo-like cells at day 6 was reminiscent of the phenotype of CD163$^+$ MΦ (P4) versus CD163$^-$ mo-like (P3) cells observed in IBD colon (Bain et al., 2013; Chapuy et al., 2019a). Indeed, *in vitro* generated CD14$^+$CD64$^+$CD163$^+$ cells displayed a typical MΦ morphology at day 12 (**Figure 5-3E**). Furthermore, these CD163$^+$ cells efficiently phagocytosed latex beads when compared to CD163$^-$ Infl mo-like cells at day 6 (**Figure 5-3F**), reinforcing their MΦ nature.

Taken together, monocytes cultured with Gγ23 first differentiate into CD14$^+$CD64$^+$CD163$^-$ Infl mo-like enriched cells (CD163$^-$ d6), which under the combined effect of TGFβ and IL10 progress towards CD14$^+$CD64$^+$CD163$^+$ MΦ (CD163$^+$ d12), thus replicating *in vitro* the tissue monocyte maturation cascade seen *in vivo* in mice.

5.2.3.4 *CD163$^-$ Infl mo-like cells and shifted CD163$^+$ MΦ express distinct molecular profile*

We next determined the molecular profile of highly purified CD163$^-$ d6 and CD163$^+$ d12 cells (**Figure 5-4A**). Notably, monocytes that were first exposed to Gγ23, and then TGFβ+IL10 also generated CD64$^+$CD163$^-$ cells at d12 (CD163$^-$ d12) that displayed low CD14, CD16 and MERTK expression compared to CD163$^+$ d12 (**Figure 5-S3**).

Differential gene expression analysis resulted in 1796 differentially expressed genes (DEGs) between CD163$^-$ d6 and CD163$^+$ d12 (**Table 5-S1A**), 1115 DEGs between CD163$^-$ d6 and CD163$^-$ d12 (**Table 5-S1B**), and 1 DEG between CD163$^-$ d12 and CD163$^+$ d12 at a 5% false discovery rate (FDR) and a fold change absolute value higher than 1.3. We next compared CD163$^-$ d12 and CD163$^+$ d12 populations at 1% nominal (non-FDR adjusted) *p-value* using the same fold change cut off, it resulted in 696 DEGs (**Table 5-S1C**). **Figure 5-4B** shows a gene expression heatmap of a supervised hierarchical clustering analysis combining the three sets of DEGs: CD163$^-$ d6 versus CD163$^+$ d12 (1796), CD163$^-$ d6 versus CD163$^-$ d12 (1115), and CD163$^-$ d12 versus CD163$^+$ d12 (696). The clustering shows that CD163$^-$ d6 population is segregating apart from CD163$^-$ d12 and CD163$^+$ d12 populations, that have a high transcriptional similarity. Similar observations were also made from an unsupervised analysis using the top 500 most varying probes across all samples (**Figure 5-S4A**). Elevated expression of *CD14, CD163, MERTK, MARCO, MS4A4A* and *FCGR3A* was noted in CD163$^+$ d12 relative to CD163$^-$ d6. CD163$^-$ d12 cells expressed a transcriptomic profile closely related to CD163$^+$ d12 with an intermediate expression of *CD163*, *MARCO* and *FCGR3A* (**Figure 5-S4B**).

MAFB versus *IRF4* transcription factors regulate monocyte cell fate to MΦ (Mo-Mac) or inflammatory DC (Mo-DC), defined by Goudot *et al.* using bulk RNAseq, respectively (Goudot

et al., 2017). In fact, CD163^{+} d12 expressed *MAFB* at higher intensity relative to CD163^{-} d6, and vice versa for *IRF4* as well as *BATF3* expression (**Figure 5-4C**). Unexpectedly, *AHR* expression, which reportedly impaired M-CSF treated monocyte differentiation into Mo-Mac (Goudot et al., 2017), was more expressed in CD163^{+} d12 than in CD163^{-} d6 cells. *NCOR2* that controls differentiation into inflammatory DCs (Inf DC) and not inflammatory MΦ (Inf MΦ) defined by Sander *et al.* (Sander et al., 2017), was expressed at similar intensity in all three *in vitro* generated cell populations. Gene set enrichment analysis (GSEA) revealed that CD163^{+} d12, when compared to CD163^{-} d6, had a significant enrichment of Mo-Mac or Inf MΦ gene set, whereas CD163^{-} d6, when compared to CD163^{+} d12, had an overrepresentation of Mo-DC or Inf DC genes (FDR adjusted *p-value*<0.005) (**Figure 5-4D** and **Table 5-S2A** to **Table 5-S2E**). Furthermore, CD163^{+} d12 were enriched, when compared to CD163^{-} d6, in genes differentially expressed in CD14^{+}CD163^{+} Mono/MΦ-like cells isolated from mesenteric lymph nodes (MLN) of IBD patients (Chapuy et al., 2020a) (FDR adjusted *p-value*<0.005) (**Figure 5-4E** and **Table 5-S2A** and **Table 5-S2F**).

Taken together, CD163^{-} d6 shares a gene expression signature with Mo-DC/Inf DC detected in synovial fluid and tumor ascites, while CD163^{+} d12 cells express a common transcriptomic profile with Mo-Mac/Inf MΦ/Mono/MΦ-like cells detected in inflamed non-lymphoid and lymphoid tissue.

5.2.3.5 *Shifted CD163^{+} MΦ display functional repair and resolving gene signatures*

To gain further insight into the potential function of CD163^{+} MΦ, we next performed GSEA using the MsigDB's Hallmark database and identified up- and down- regulated pathways in

CD163$^+$ d12 versus CD163$^-$ d6 cells. TGFβ+IL10 up-regulated functional pathways involved in complement (*C1QC, C1QA, C2, C3*), coagulation (*FN1, SPARC*), and TGFβ signaling (*SMAD6, ID3*) in CD163$^+$ d12 when compared to CD163$^-$ d6 (**Figure 5-5A** and **Table 5-S3**). Genes implicated in inflammatory response (*IL6, IL18*), IL6 signaling (*IL6, PIM1*), IFNγ response genes (*CCL5*) or associated with tryptophan to kynurenine pathway best characterized by *IDO1* (Smillie et al., 2019), were downregulated in CD163$^+$ MΦ (**Figure 5-5A** and **Table 5-S3**). Further analysis of CD163$^+$ MΦ transcriptomic profile corroborated a MΦ function best characterized by up-regulation of Gene-Ontology (GO) pathways for phagocytosis, apoptotic clearance and wound healing processes in CD163$^+$ d12 cells (**Figure 5-5B**, **Table 5-S4** and **Figure 5-S5A**). Moreover, TGFβ+IL10 up-regulated GO categories involved in TGFβ signaling, IL10 response and "response to bacterial compounds" (**Figure 5-5C** and **Figure 5-5D**, **Table 5-S4** and **Figure 5-S5B**).

Overall, these complementary analyses define CD163$^+$ d12 cells as prototypical MΦ detected in inflamed tissue and endowed with functional repair and resolving gene signature.

5.2.3.6 *In vitro converted CD163$^+$ MΦ share a molecular signature with colonic CD14$^+$CD163$^+$ MΦ in CD patients*

We next tested if the gene signature of the two distinct CD14$^+$ clusters, CD163$^-$ mo-like cells (P3; cluster E) and CD163$^+$ MΦ (P4; cluster F) identified in inflamed CD colon using scRNA sequencing (Chapuy et al., 2019a), were also enriched in *in vitro* generated CD163$^-$ d6 and CD163$^+$ d12 cells respectively. As depicted in **Figure 5-6A**, *in vitro* shifted CD163$^+$ d12 cells shared a transcriptional profile with P4-enriched cluster F in CD colon, which was best

represented by *C1Q* family members and MΦ gene signature (*CD163L1, OLFML2B, MERTK, SLCO2B1, STAB1)* (FDR adjusted *p-value*<0.005) (**Figure 5-6A** and **Table 5-S2A** and **Table 5-S2G**). Noteworthy, $CD163^-$ d6 cells had a higher expression of several genes associated with $CD163^-$ mo-like cells P3-enriched cluster E of CD patients, including *IL1RN, PLAUR, CCRL2, APOBEC3A, FCN1* and *SLC2A3,* when compared to $CD163^+$ d12 (**Figure 5-S6**) but the Normalized Enrichment Score (NES) did not reach statistical significance (**Table 5-S2A**).

Finally, in order to demonstrate the plasticity of $CD163^-$ d6 cells into $CD163^+$ MΦ *in vitro*, the $CD163^-$ Infl mo-like cells were first sorted at day 6 by applying the gating strategy depicted in **Figure 5-1E**, and then cultured in the presence of TGFβ and IL10 until day 12. As shown in **Figure 5-6B**, converted $CD163^+$ MΦ phenotypically and morphologically resembled purified colonic as well as MLN $CD14^+CD163^+$ MΦ isolated from CD patients (Chapuy et al., 2020a; Chapuy et al., 2019a).

As depicted in the schematic model (**Figure 5-6C**), Gγ23 monocyte-derived $CD14^+CD163^-$ cells overexpressed *IL23R* and its associated transcription factor *JAK2* as well as pro-inflammatory cytokines including *IL6* and *IL18*. As $CD163^-$ Infl mo-like cells progressed towards $CD163^+$ MΦ in response to an anti-inflammatory milieu, TGFβ and IL10, the cells upregulated *IL10*, *IL10RB* and *TGFβR2* expression, together with a MΦ gene signature associated with apoptotic clearance and resolving function.

5.2.4 DISCUSSION

The *in vitro* environmental factors that control monocyte fate into surrogates of extravasated inflammatory tissue monocytes which progressively mature towards anti-inflammatory MΦ are not well understood in humans. Using functional and molecular studies, we have defined a two-step culture condition that could mimic the journey of monocytes which arrive in inflammatory tissue and progress towards MΦ. We first showed that *in vitro* Gγ23 monocyte-derived $CD14^+CD64^+CD163^-$ cells shared morphology and functional properties but not their molecular signature with $CD163^-$ mo-like (P3; cluster E) cells that accumulate in inflamed IBD tissue (Chapuy et al., 2019a). These *in vitro* Gγ23 generated $CD163^-$ cells are distinct from *in vitro* GM-CSF+IL4 MoDCs. Next, the data revealed that following exposure to TGFβ plus IL10, Gγ23 monocyte-derived $CD14^+CD64^+CD163^-$ cells switched to large $CD14^+CD64^+CD163^+$ cells that displayed morphology and phagocytic function of MΦ. Converted $CD163^+$ MΦ robustly expressed genes associated with MΦ detected in inflamed colon of CD patients, rheumatoid arthritis synovial fluid and tumor ascites (Chapuy et al., 2019a; Goudot et al., 2017; Segura et al., 2013).

GM-CSF, IFNγ and IL23 expression is significantly elevated in inflamed intestinal tissues of CD patients (Noguchi et al., 2001; Sasaki et al., 1992; Schmidt et al., 2005). Gut epithelial and T cells secrete GM-CSF, natural killer T and T helper cells produce IFNγ, and $CD11c^+$ monocytes and MΦ are the main sources of IL23 during inflammation (Kamada et al., 2008; Longman et al., 2014). We showed here that exposure to GM-CSF and IFNγ upregulated *IL23R* mRNA expression on circulating monocytes, suggesting that they respond to IL23. These observations corroborated earlier studies using IL23 receptor green fluorescent reporter mice

showing that myeloid cells express IL23R, and secrete IL23 and IL6 (Awasthi et al., 2009). Moreover, an autocrine/paracrine IL23 amplification of pro-inflammatory cytokine production was recently reported in human intestinal myeloid cells (Sun et al., 2019). *In vitro* Gγ23 generated $CD163^-$ cells, like their potential *in vivo* counterpart in inflamed colon of CD and ulcerative colitis patients, did not induce naïve T cell proliferation but promoted IL1β and IL12p40 -dependent memory Th17 and Th17/Th1 responses, a hallmark of IBD (Chapuy et al., 2020b; Chapuy et al., 2019a).

Murine $Ly6C^{high}$ monocytes differentiate through a sequence of intermediary populations to generate $CX3CR1^{high}$ MΦ in gut (Bain et al., 2014; Bain et al., 2013; Tamoutounour et al., 2012), a maturation program defined as tissue monocyte waterfall. Here, we demonstrated that TGFβ plus IL10 shifted Gγ23 monocyte-derived $CD163^-$ Infl mo-like cells towards $CD163^+$ MΦ without supplementing the culture medium with M-CSF. Noteworthy, Gγ23 monocyte-derived $CD163^-$ Infl mo-like cells expressed more *CSF1* than $CD163^+$ MΦ. Moreover, our unpublished observations indicated that monocytes exposed for 12 days to only Gγ23 remained $CD163^-$ and did not differentiate into MΦ, suggesting that monocyte differentiation into $CD163^+$ MΦ is not a default pathway. A recent study favors the concept of monocyte plasticity by showing that monocytes modulate their nature in response to M-CSF or GM-CSF present in the environment, with cross-talk between these two pathways (Rodriguez et al., 2019). $CD163^+$ MΦ displayed a suppressed inflammatory gene signature and expressed genes associated with regulatory and repair functions, and apoptotic cell clearance. Morphological and molecular analyses further revealed that *in vitro* converted $CD163^+$ MΦ were closely related to colonic or MLN $CD163^+$ MΦ from CD patients (Chapuy et al., 2019a).

We also generated a significant proportion of $CD64^{+}CD163^{-}$ cells after exposure of Infl mo-like cells at day 6 to TGFβ+IL10. Whether these cells at day 12 resemble the intermediate $CD14^{+}CD64^{+}CD163^{dim}$ population which did not accumulate in the inflamed mucosa of IBD patients, remains to be investigated (Chapuy et al., 2019a) , remains to be investigated. Taken collectively, we postulate that in IBD colon, and similar to what has been observed in mice in the context of inflammation, a single and highly plastic monocyte population differentiates into pro-inflammatory monocyte-like cells, and progress from transitioning cells to give rise to low numbers of protective MΦ.

Gγ23 monocyte-derived $CD163^{-}$ Infl mo-like cells and *ex vivo* isolated colonic $CD163^{-}$ mo-like cells (P3) in IBD patients showed a monocyte-like morphology, and thus, were not considered type 1 MΦ (M1). Generation of M1 or M2 MΦ have been extensively investigated *in vitro* (Chavez-Galan et al., 2015; Italiani and Boraschi, 2014; Martinez and Gordon, 2014; Orecchioni et al., 2019). Nonetheless, *in vitro* models of human monocyte differentiation into MΦ fall short in first generating the surrogates of transient extravasated tissue inflammatory monocytes that can be subsequently shifted to MΦ.

In vitro converted $CD163^{+}$ MΦ highly expressed *IL10R* and *TGFBR2.* The respective role of IL10 or TGFβ in the generation of gut anti-inflammatory MΦ and protection from colitis stems from several *in vivo* studies in mice and humans (Girard-Madoux et al., 2016; Krause et al., 2015; Kuhn et al., 1993; Rivollier et al., 2012; Schridde et al., 2017; Shouval et al., 2014; Xiao et al., 2019; Zigmond et al., 2014a). High levels of IL10 released under homeostatic conditions

retain intestinal MΦ in a "post-inflammatory anergy" state (Smythies et al., 2005). The critical role of IL10 in maintaining gut homeostasis is revealed in IL10R deficient patients who develop very early onset IBD (Glocker et al., 2009; Moran et al., 2013), associated with uncontrolled IL1β production by IL10R-deficient MΦ (Shouval et al., 2016). IL10 secreted by MΦ in the large intestine is important in limiting excessive inflammation (Krause et al., 2015). However, MΦ production of IL10 is not fundamental in maintaining gut homeostasis (Shouval et al., 2016), it is rather the failure of MΦ to respond to paracrine IL10 that leads to the development of spontaneous colitis (Shouval et al., 2014). The role of TGFβ signaling in generating anti-inflammatory MΦ during homeostasis is less clear. At steady sate, TGFβR1 signaling controls colonic monocyte accumulation (Schridde et al., 2017). Recent studies demonstrate that TGFβ/TGFβR2 is key for the development of embryonically-derived alveolar lung MΦ but also appears to contribute to the replenishment of the pool of resident MΦ during inflammation (Yu et al., 2017). Here, we provided evidence that TGFβ was essential but not sufficient to convert $CD163^-$ Infl mo-like cells into $CD163^+$ MΦ. Finally, lipid mediators and metabolic factors are additional molecular components that alter MΦ differentiation, and are therefore involved in the resolution of chronic inflammatory response (Na et al., 2019; Xue et al., 2014). In that regard, *in vitro* converted $CD163^+$ MΦ expressed some genes associated with butyrate-treated MΦ (Schulthess et al., 2019).

Collectively, understanding the molecular pathways implicated in the monocyte waterfall and change from pro-inflammatory to anti-inflammatory function in tissue might open therapeutic avenues in patients with chronic inflammatory disorders. Individual augmentation of IL10 or TGFβ by administration of recombinant IL10 or smad7 inhibitor respectively, has not proven

clinically efficacious (Giuffrida et al., 2019). Thus, combined therapeutic approaches warrant further investigations to induce and/or sustain remission in chronic inflammatory diseases.

5.2.5 REFERENCES

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5.2.6 FIGURES

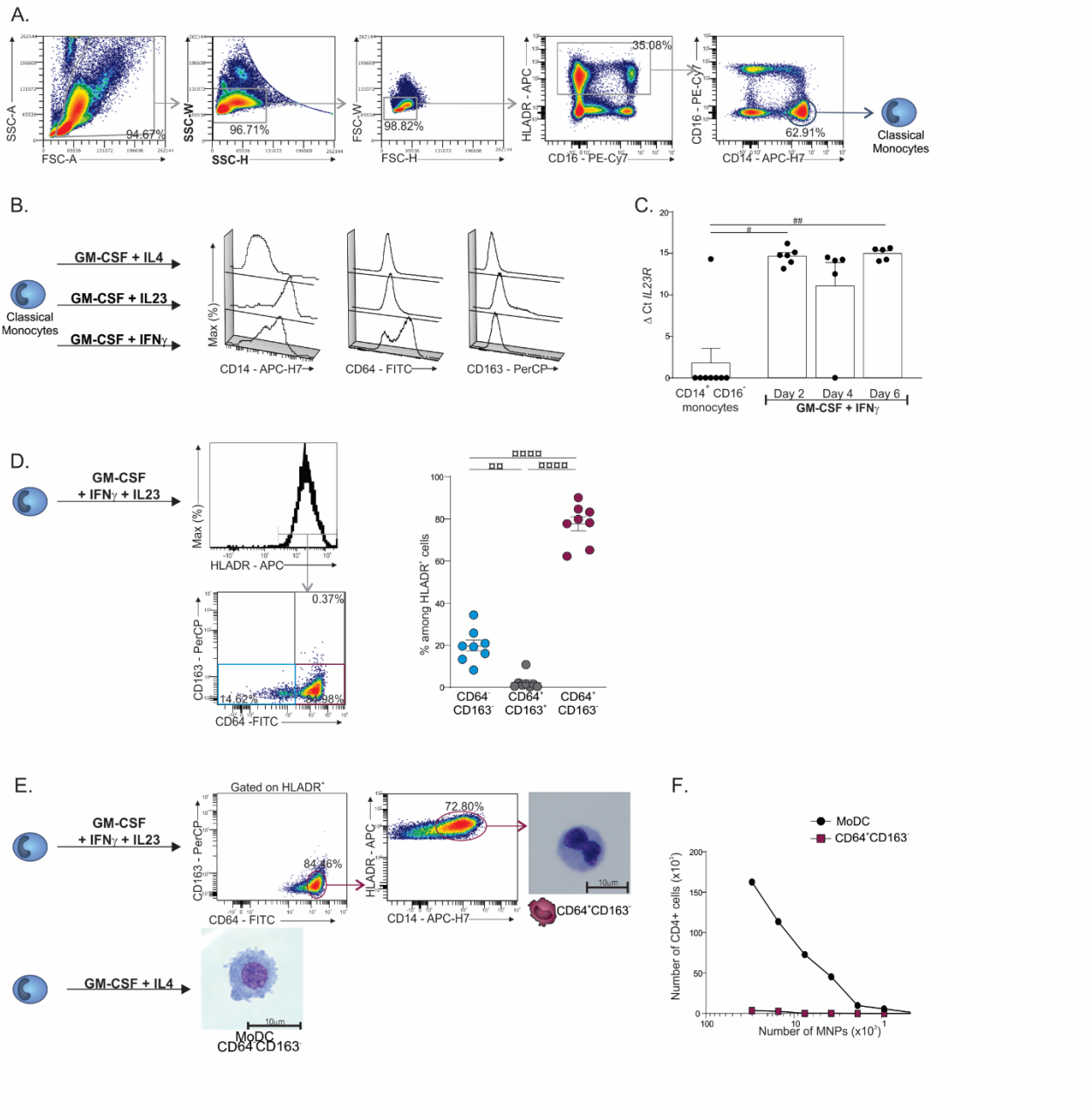

Figure 5-1: In vitro generation of CD14⁺CD64⁺CD163⁻ cells with monocyte-like morphology using GM-CSF, IFNγ and IL23.

(A and B) Human monocytes were sorted (A) and cultured with GM-CSF and either IL4, IL23 or IFNγ for 6 days (B). CD14, CD64 and CD163 Mean fluorescent intensity (MFI) was examined by flow cytometry (one representative experiment out of 4, with one donor per experiment) (C) Relative expression of IL23R as compared to GAPDH by real time-qPCR on

freshly isolated, sorted classical monocytes and monocytes cultured in the presence of GM-CSF+IFNγ for 2, 4 or 6 days (mean ±SEM of 5 experiments, with one donor per experiment). (D-F) Classical monocytes were cultured with GM-CSF+IFNγ+IL23 (Gγ23) for 6 days. The expression of CD64 and CD163 on HLADR^{+} cells delineated 3 subpopulations (D). The major CD64^{+}CD163^{-}CD14^{+} population generated in Gγ23 culture condition was sorted and examined morphologically in comparison to GM-CSF+IL4 (MoDC) (n=3 experiments, with one donor per experiment) (E). Dose response curve with 10^{5} CSFE-labeled CD45RO^{-}CD45RA^{+}CD8^{-}CD4^{+} naïve T cells and 1:2 serial dilution of CD64^{+}CD163^{-}CD14^{+} cells or MoDC, starting with $2x10^{4}$ MNP in the presence of anti-CD3 and SAC (one representative experiment out of 4, with one donor per experiment) (F). Kruskall-Wallis ($^{\#}$) and ANOVA (¤), $^{¤,\#}$ P-value<0.05, $^{¤¤,\#\#}$ P-value<0.01, and $^{¤¤¤¤}$ P-value<0.0001.

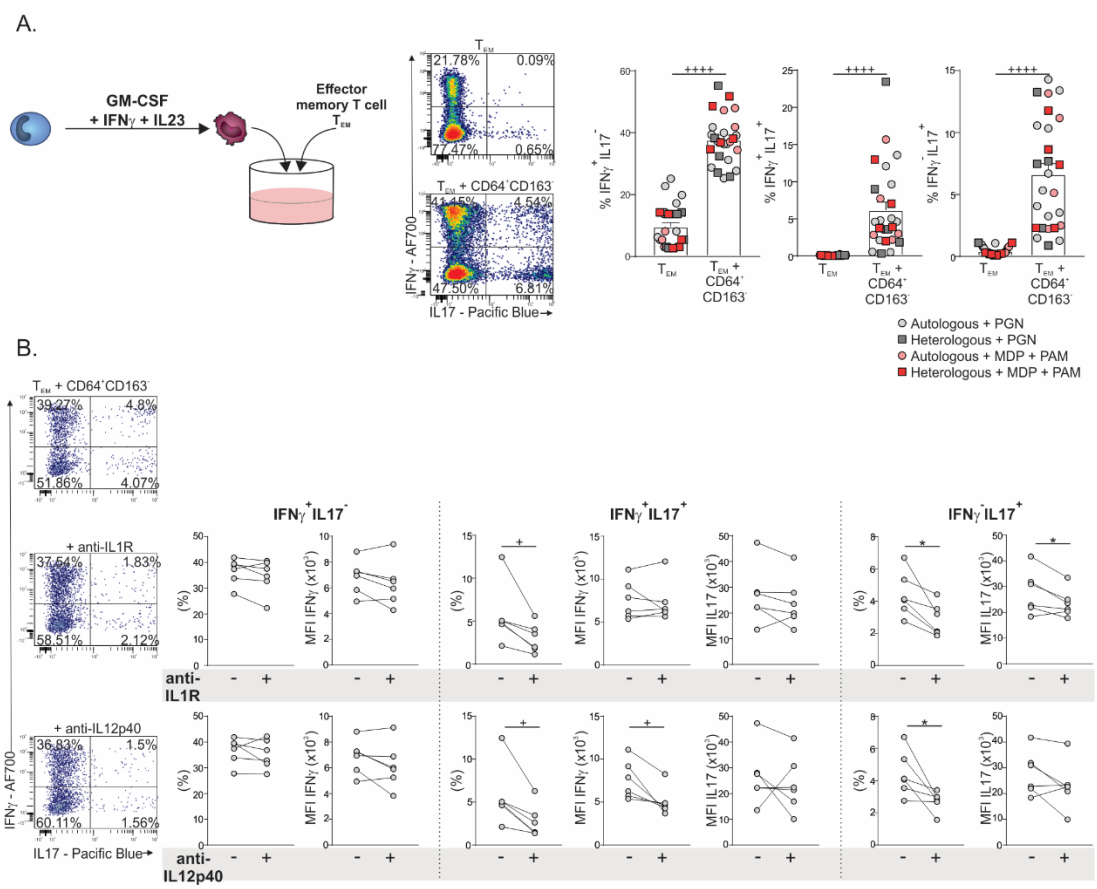

Figure 5-2: In vitro generated CD163$^-$ mo-like cells favor IL1β- and IL12p40- dependent Th17 and Th17/Th1 responses.

(A and B) Following 6 days culture with Gγ23, CD64$^+$CD163$^-$CD14$^+$HLADR$^+$ cells were sorted and co-cultured with CD62L$^-$CD25$^-$CD45RO$^+$CD45RA$^-$CD4$^+$CD8$^-$ effector memory T (T_{EM}) cells at a 1:7 ratio in the presence of PGN, or MDP+PAM for 6 days without (mean ± SEM of 26 experiments, with one donor per experiment) (A) or with anti-IL1R (n=6 experiments, with one donor per experiment) or anti-IL12p40 (n=6 experiments, with one donor per experiment) antibodies (B). On the last day, PMA-ionomycin was added for 6 hours and brefeldin A for the last 3 hours. Frequencies and MFI of IL17 and IFNγ in IFNγ$^+$IL17$^-$, IFNγ$^+$IL17$^+$ and IFNγ$^-$IL17$^+$ T_{EM} cells are represented. (A) Wilcoxon, $^{++++}$ P-value<0.0001 and (B) Wilcoxon (+) and paired t-test (*), P-value <0.05.

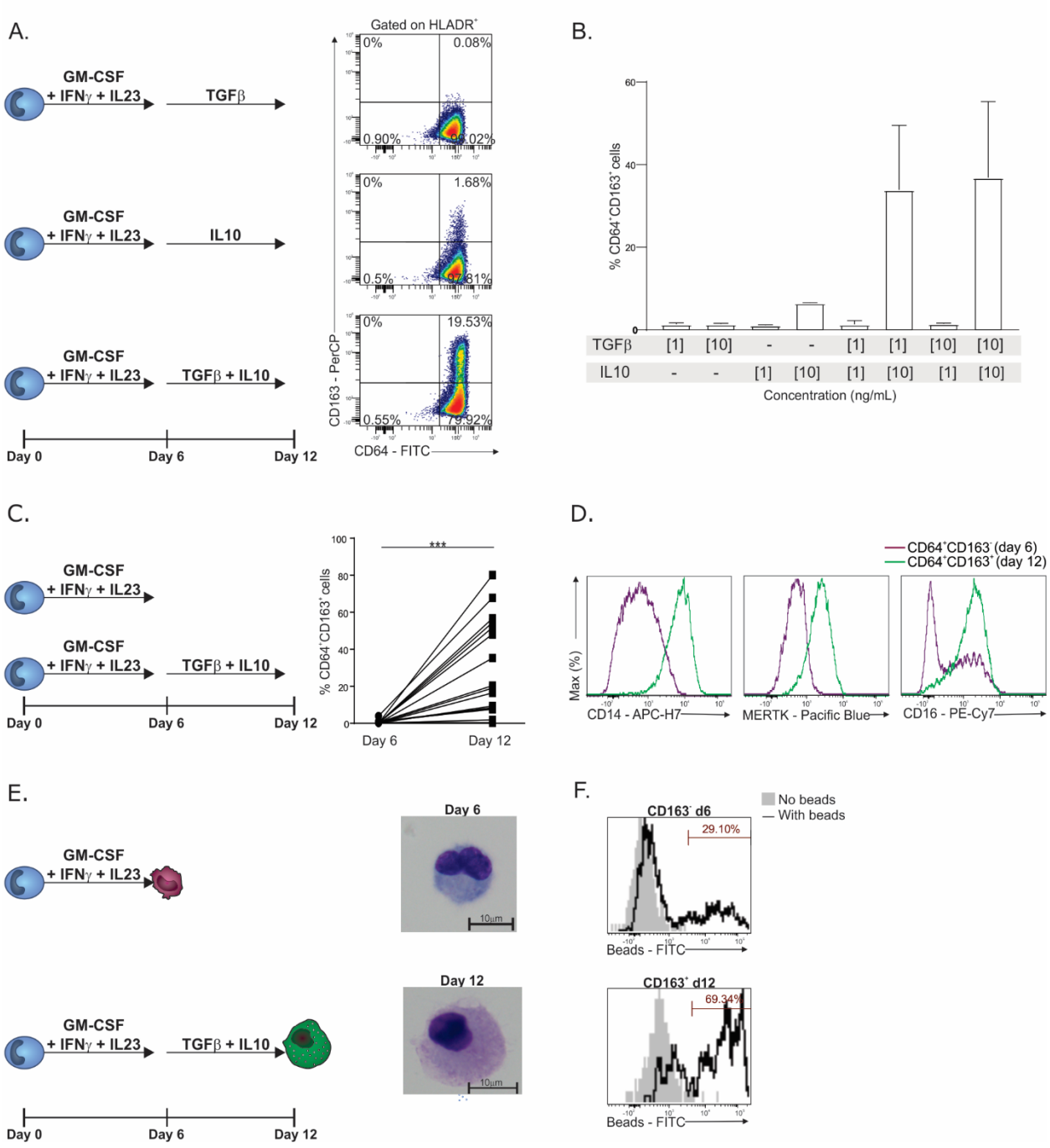

Figure 5-3: In vitro generated CD163⁻ Infl mo-like cells shift to CD163⁺ MΦ in response to TGFβ plus IL10.

(A-D) Following 6 days culture with Gγ23, TGFβ, IL10 or TGFβ+IL10 were added for another 6 days. CD64 and CD163 expression was examined on HLADR⁺ cells at day 12 (one representative experiment out of 4) (A). Varying concentrations of IL10 and TGFβ added at day 6 and % of CD64⁺CD163⁺ cells was examined at day 12 (mean ± SEM of 4 experiments, with one donor per experiment) (B). Frequency of CD64⁺CD163⁺ cells before (day 6) or after

*adding TGFβ+IL10 (day 12) (C). Phenotype of $CD64^{+}CD163^{-}$ cells at day 6, and $CD64^{+}CD163^{+}$ cells at day 12 (one representative out of 4 experiments, with one donor per experiment) (D). (E and F) Sorted $CD14^{+}CD64^{+}CD163^{-}$ (Gγ23, day 6) and $CD64^{+}CD14^{+}CD163^{+}$ (TGFβ+IL10, day 12) cells were examined morphologically (E) and cultured for 1h with latex beads to examine phagocytosis function (F). (E and F) One representative experiment of 5 is shown, with one donor per experiment. (C) paired t-test **** P-value <0.0001.*

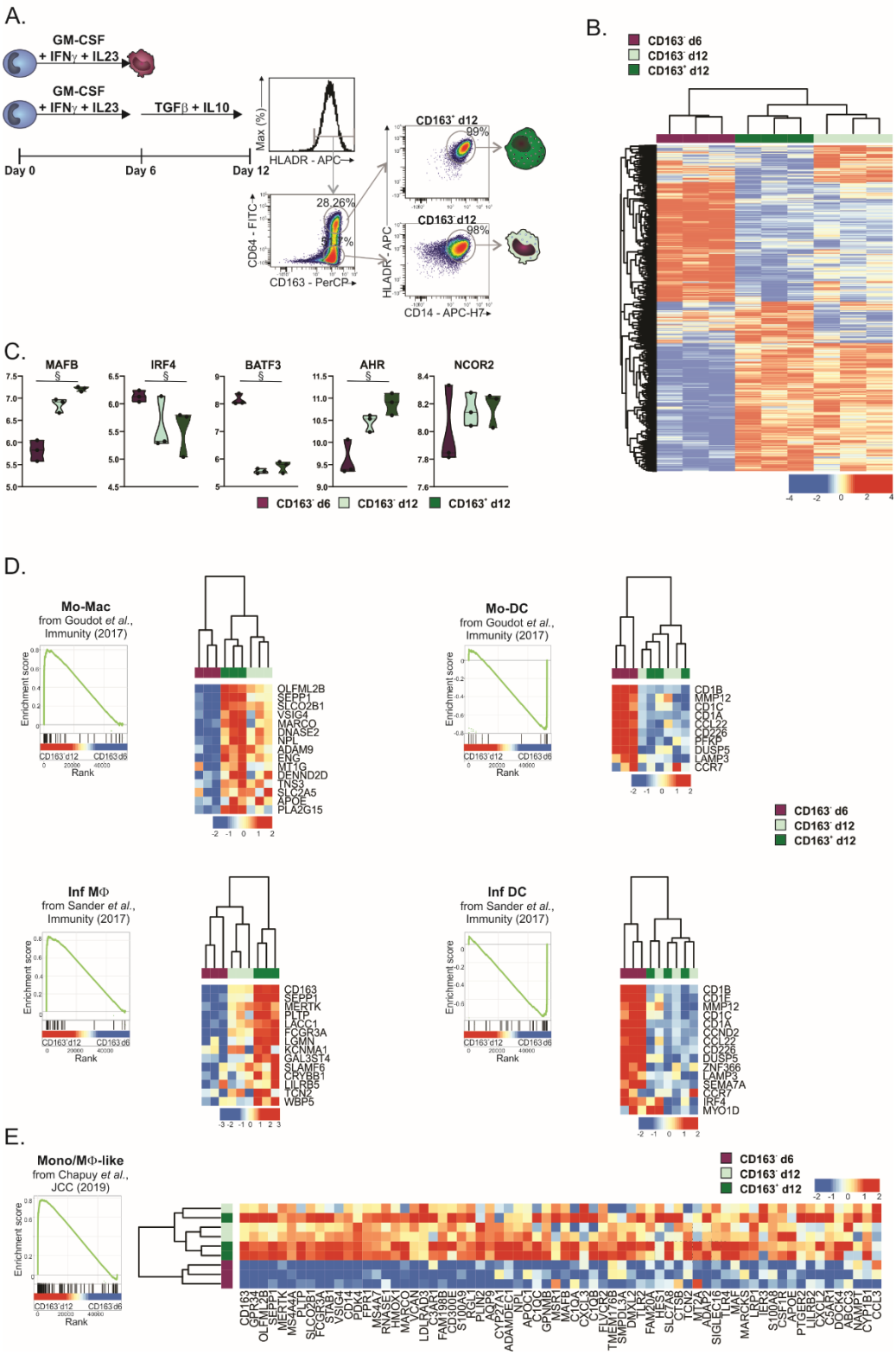

Figure 5-4: CD163⁻ Infl mo-like cells and shifted CD163⁺MΦ express distinct molecular profile.

(A) $CD14^+CD64^+CD163^-$ (Gγ23, day 6), $CD14^+CD64^+CD163^-$ (TGFβ+IL10, day 12) and $CD14^+CD64^+CD163^+$ (TGFβ+IL10, day 12) populations were sorted for microarray analysis (n=3 experiments, with one donor per experiment). (B) Heat map representing the genes significantly different between $CD163^-$ d6 and $CD163^+$ d12, and $CD163^-$ d6 and $CD163^-$ d12 (FDR adjusted p-value <0.05 and fold change >1.3), as well as $CD163^-$ d12 and $CD163^+$ d12 (nominal p-value <0.01 and fold change >1.3). (C) Violin plots illustrating gene expression levels of key genes. P-value <0.05 represented by § (D-E) Gene set enrichment plots for Mo-Mac and Mo-DC (Goudot et al., 2017), Inf MΦ and Inf DC (Sander et al., 2017) (D), and Mono/MΦ-like (Chapuy et al., 2020a)(E) gene signatures (left panels). Strength of enrichment is represented by normalized enrichment score (NES). Heat map of differently expressed genes (right panels). Significance of enrichment is shown in ***Table 5-S2A-F*** *(FDR< 0.05).*

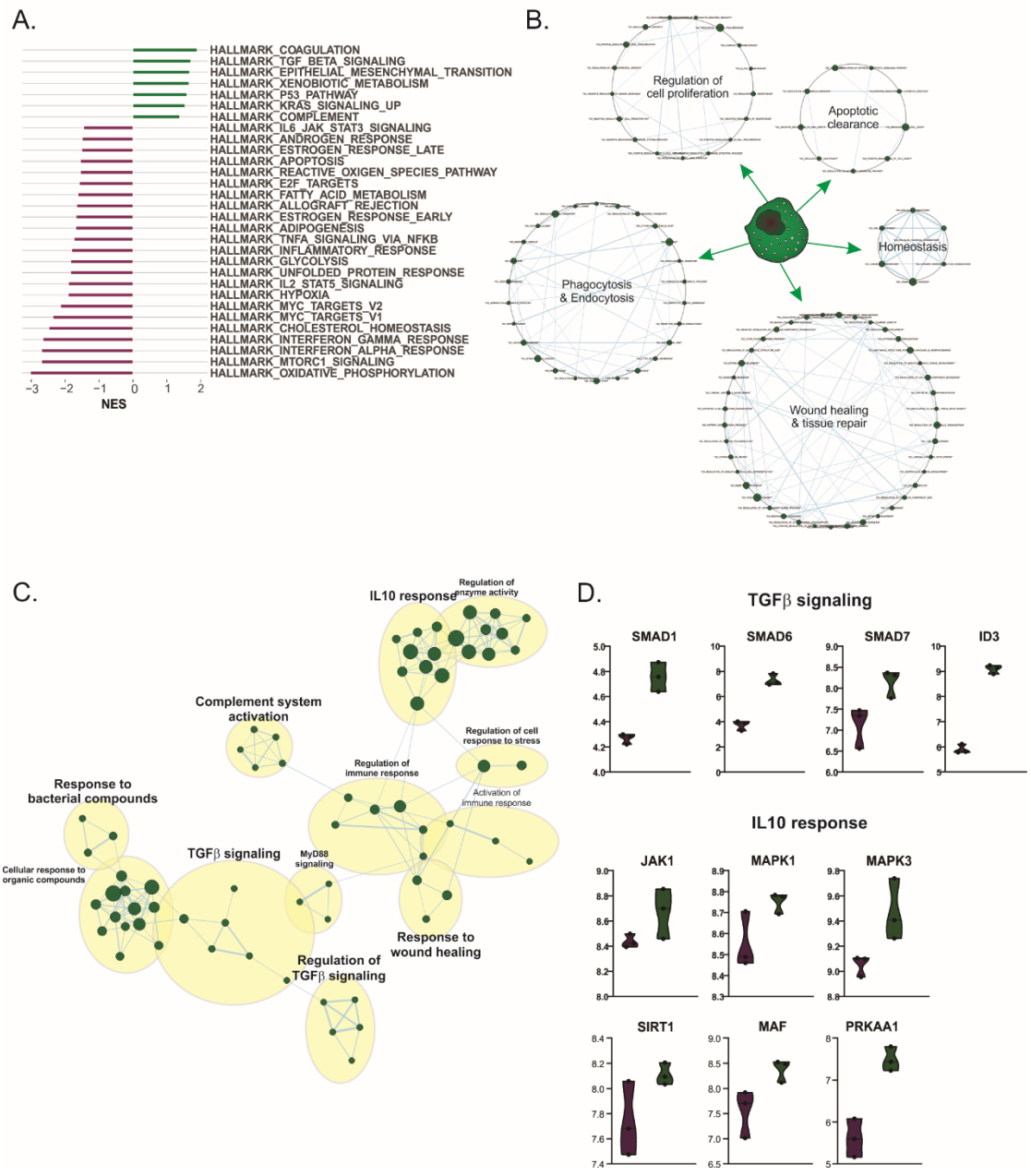

Figure 5-5: Shifted CD163$^+$ MΦ display functional repair and resolving gene signatures.
(A) Enriched MsigDB's Hallmark categories up-(green) or down-(purple) regulated in CD163$^+$d12 versus CD163$^-$d6 cells (FDR <0.05). (B) Selected examples of GO pathways upregulated in CD163$^+$ d12 versus CD163$^-$ d6 cells (FDR <0.05). (C) Network-based visualization of GO pathways overexpressed in CD163$^+$ d12 versus CD163$^-$ d6 cells (FDR <0.05) illustrating the emergence of IL10, TGFβ response and complement activation in CD163$^+$ d12 macrophages. (E) Violin plots illustrating gene expression levels of key genes. 3 experiments were performed, with one donor per experiment. P-value <0.05 for all genes presented.

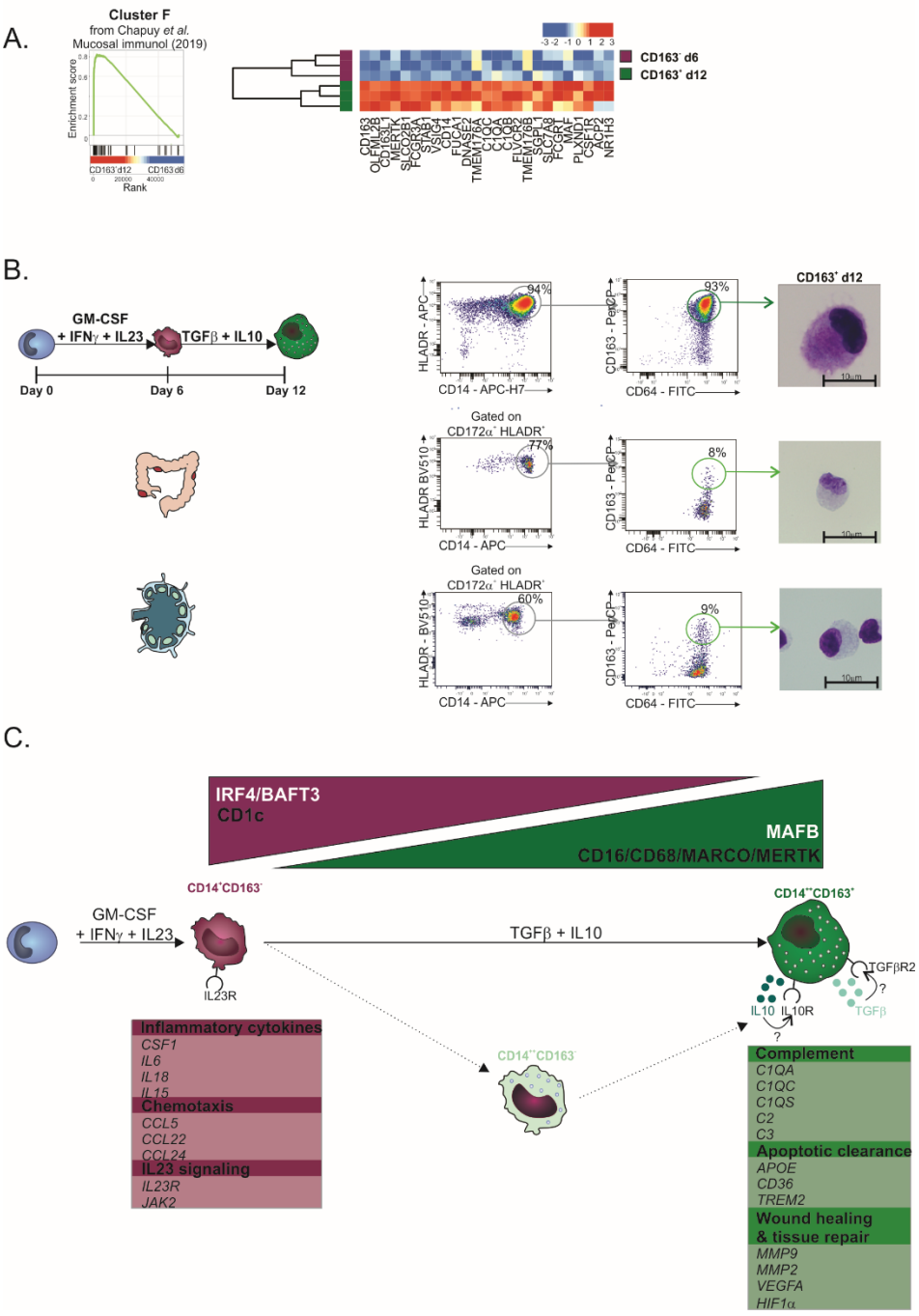

Figure 5-6: In vitro converted CD163$^+$ MΦ molecularly resemble colonic CD14$^+$CD163$^+$ MΦ in CD patients.

(A) Gene set enrichment plot for macrophages (cluster F enriched in colonic CD163$^+$ (P4) cells) (Chapuy et al., 2019a) (left panel). Strength of enrichment is represented by NES. Heat map of differently expressed genes (right panel) (n=3 experiments, with one donor per

experiment). Significance of enrichment is shown in ***Table 5-S2A*** *and G (FDR <0.05). (B) Following 6 days culture with Gγ23, CD163$^-$ d6 cells were sorted and cultured with TGFβ+IL10 for another 6 days generating CD163$^+$ d12 cells (upper panels; n=6 experiments, with one donor per experiment). Representative dot plot of CD163$^+$ MΦ isolated from inflamed colon (P4) (middle panels; n=10 experiments, with one donor per experiment) or mesenteric lymph nodes (lower panels; n=10 experiments, with one donor per experiment) of CD patients. Morphology of in vitro converted and ex vivo tissue CD163$^+$ MΦ sorted populations (one representative out of 3). (C) Schematic model of Gγ23 monocyte-derived CD163$^-$CD14$^+$ cells and their progression, under the influence of TGFβ+IL10, towards CD163$^+$ MΦ expressing MΦ markers.*

5.2.7 SUPPLEMENTARY INFORMATION

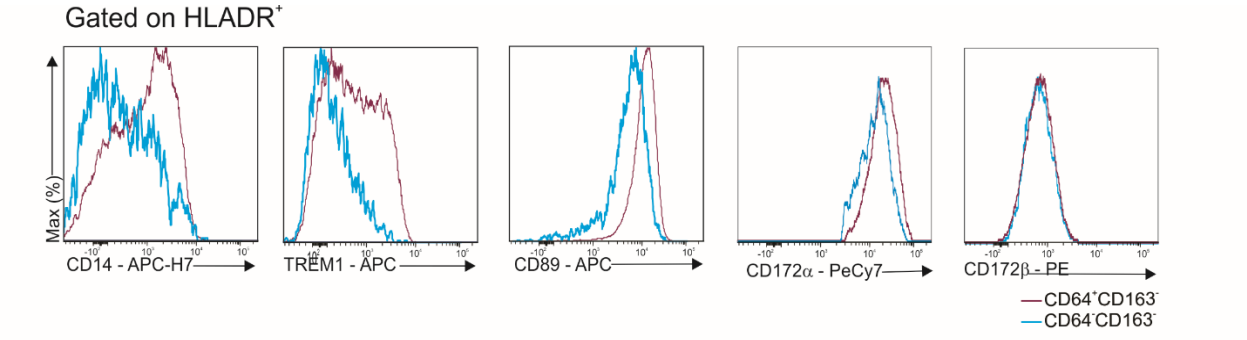

Figure 5-S1:. Phenotype of CD64$^-$CD163$^-$ and CD64$^+$CD163$^-$ day 6 populations.

Sorted human monocytes were cultured with GM-CSF+IFNγ+IL23 for 6 days. Mean fluorescent intensity (MFI) of CD14 (one representative experiment out of 30), and TREM1, CD89, CD172α and CD172β (one representative experiment out of 4) on CD64$^+$CD163$^-$ d6 and CD64$^-$CD163$^-$ d6 populations are represented.

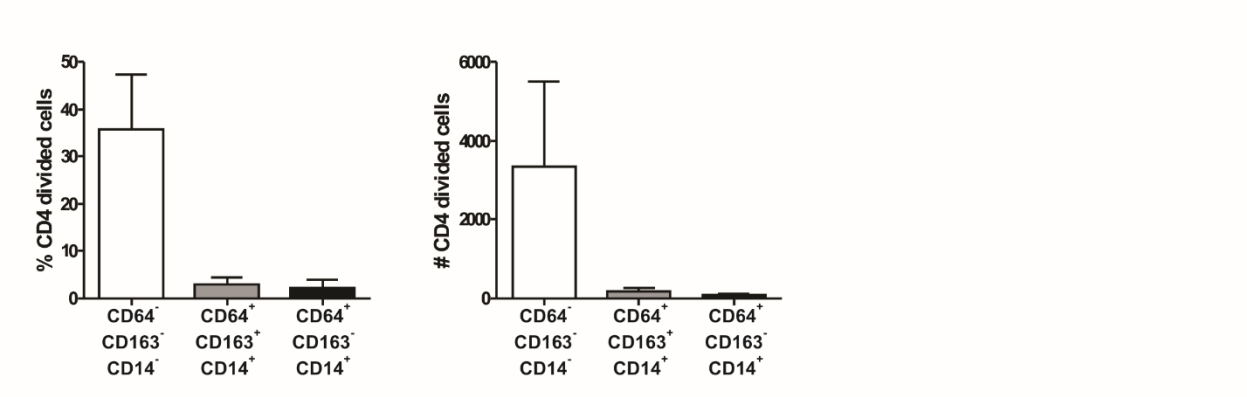

Figure 5-S2: Antigen presentation capacity of ex vivo MNP from inflamed mucosa of CD patients.

Naïve T cells ($CD45RO^-CD45RA^+CD8^-CD4^+$, 10^5) were sorted from blood of healthy donors and co-cultured at a ratio of 1:10 with either $CD64^-CD163^-CD14^-$ or $CD64^+CD163^+CD14^+$ or $CD64^+CD163^-CD14^+$ cells, isolated from inflamed mucosa of CD patients, in the presence of SAC for 6 days.

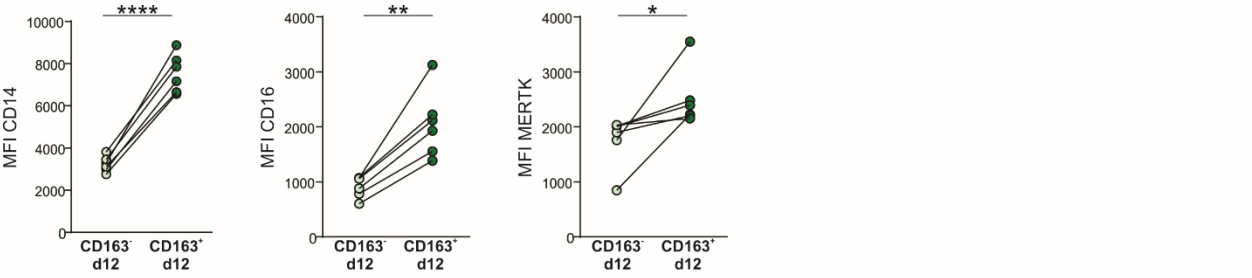

Figure 5-S3: Phenotype of CD163⁻ d12 and CD163⁺ d12 populations.

*Following 6 days culture with Gγ23, TGFβ and IL10 were added for another 6 days. Mean fluorescent intensity (MFI) of CD14, CD16 and MERTK on indicated populations. paired t-test *P-value< 0.05, **P-value0.01, **** P-value<0.0001.*

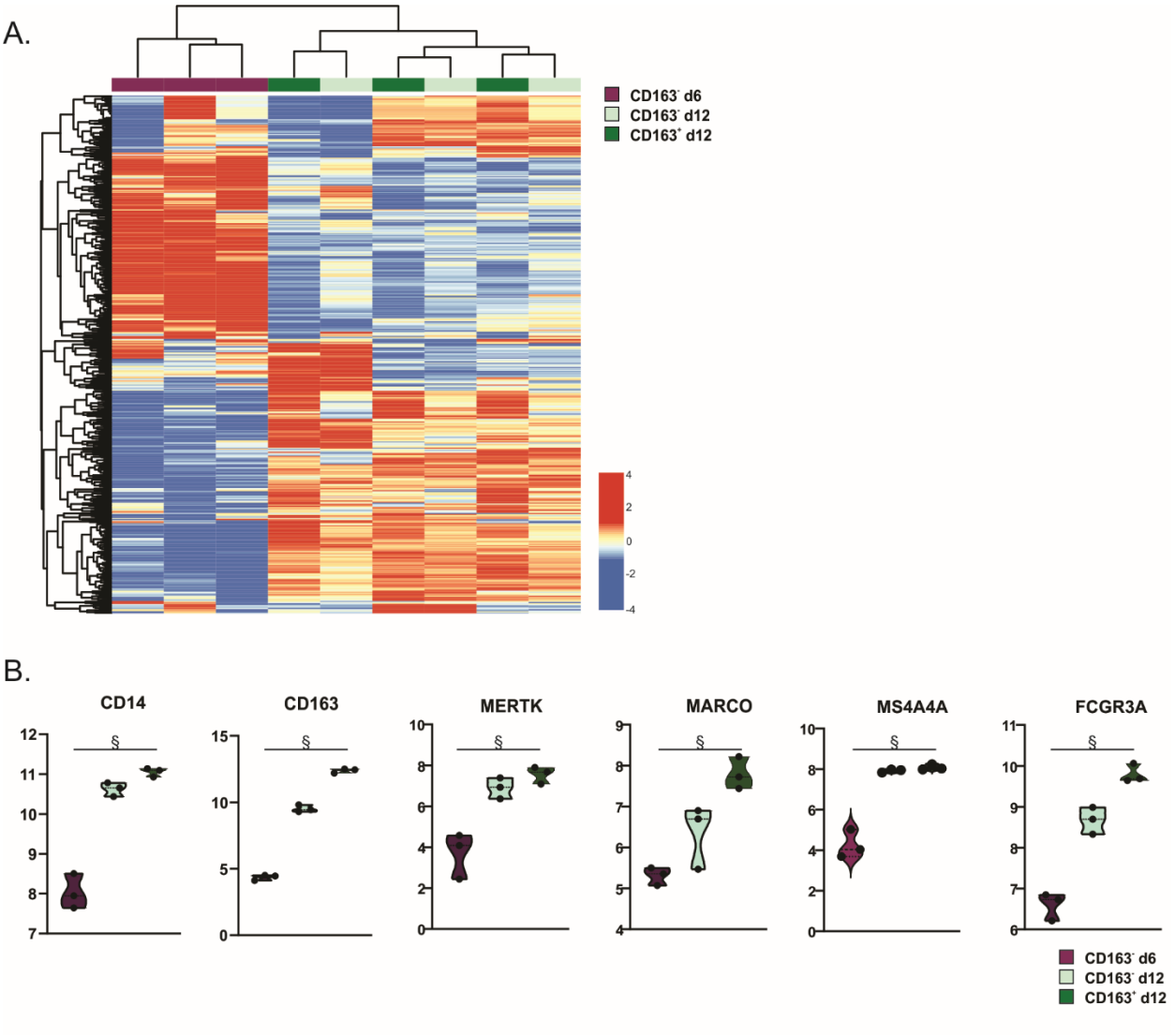

Figure 5-S4: Differentially expressed genes across CD163$^-$ d6, CD163$^-$ d12 and CD163$^+$ d12 populations.

(A) Heat map of the top 500 genes differentially expressed across CD163$^-$ d6, CD163$^+$ d12 and CD163$^-$ d12 cells. (B) Violin plots illustrating gene expression levels of key genes. P-value <0.05 represented by §.

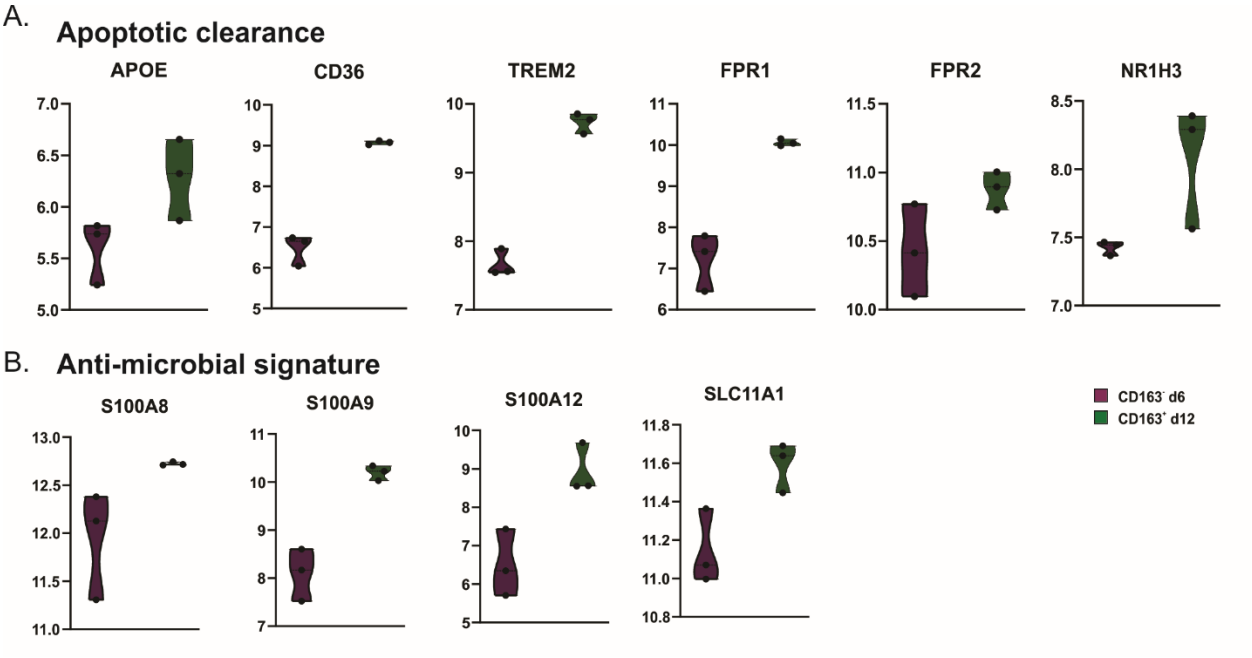

Figure 5-S5: CD163^{+}d12 cells have a higher expression of apoptotic clearance and anti-microbial gene signatures.

(A-B) Violin plots illustrating gene expression levels of key genes. P-value < 0.05 for all genes presented.

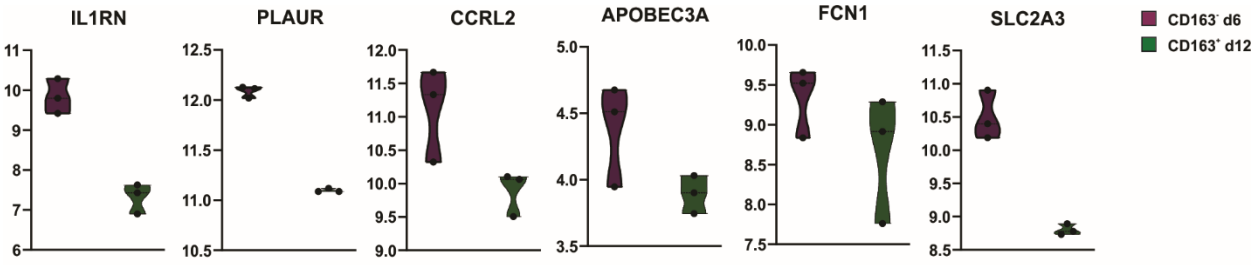

Figure 5-S6: CD163- d6 cells have a higher expression of genes associated with cluster E. *Violin plots illustrating gene expression levels of key genes. P-value < 0.05 for all genes presented.*

Table 5-S1: Database of differentially expressed genes (DEG) between in vitro generated cells.

Available at https://onlinelibrary.wiley.com/doi/full/10.1002/eji.202048555

Table 5-S2: GSEA of selected pathways in CD163^{+} d12 versus CD163^{-} d6 populations.

Available at https://onlinelibrary.wiley.com/doi/full/10.1002/eji.202048555

Table 5-S3: GSEA on MsigDB's Hallmark pathways in CD163^{+} d12 versus CD163^{-} d6 populations.

Available at https://onlinelibrary.wiley.com/doi/full/10.1002/eji.202048555

Table 5-S4: GSEA of GO pathways in CD163^{+} d12 versus CD163^{-} d6 populations.

Available at https://onlinelibrary.wiley.com/doi/full/10.1002/eji.202048555

Table 5-S5: Antibodies for flow cytometry

Antibody	Conjugate	Clone	Company
Anti-HLADR	APC	L243	BioLegend
Anti-HLADR	AF700	L243	BioLegend
Anti-HLADR	BV510	L243	BioLegend
Anti-CD3	BV510	UCHT1	BioLegend
Anti-CD3	BV496	UCHT1	BD Biosciences
Anti-CD4	BV510	RPA-T4	BioLegend
Anti-CD4	BV785	OKT4	Biolegend
Anti-CD8a	APC	RPA-T8	BioLegend
Anti-CD8a	BV787	SK1	BD Biosciences
Anti-CD14	APC	M5E2	BioLegend
Anti-CD14	APC/H7	MφP9	BD Biosciences
Anti-CD14	Pacific blue	HCD14	BioLegend
Anti-CD14	BUV737	M5E2	BD Biosciences
Anti-CD16	PE/Cy7	B73.1	BioLegend
Anti-CD16	PE	B73.1	BioLegend
Anti-CD25	APC	M-A251	BD Biosciences
Anti-CD25	BV510	M-A521	BD Biosciences
Anti-CD45	APC-H7	2D1	BD Biosciences

Anti-CD45RA	Alexa fluor 488	HI100	BioLegend
Anti-CD45RO	PerCP/Cy5.5	UCHL1	BioLegend
Anti-CD62L	PeCy7	DREG-56	Biolegend
Anti-CD64	FITC	10.1	BioLegend
Anti-CD89	APC	A59	BioLegend
Anti-CD163	PerCP/Cy5.5	GHI/61	BioLegend
Anti-CD172α	PE-Cy7	SE5A5	BioLegend
Anti-CD172β	PE	B4B6	BioLegend
Anti-CD183 (CXCR3)	AlexaFluor 488	G025H7	BioLegend
Anti-CD196 (CCR6)	PE	G034E3	BioLegend
Anti-IFNγ	PerCP/Cy5.5	4S.B3	BioLegend
Anti-IFNγ	AF700	4S.B3	BioLegend
Anti-IFNγ	BV711	4S.B3	BioLegend
Anti-IL-1β	PE	8516	R&D
Anti-MERTK	BV421	590H11G1E3	BioLegend
Anti-TREM1	APC	193015	R&D systems
Anti-IL-17A	Pacific Blue	BL168	BioLegend
Anti-IL-17A	Alexa 647	BL168	BioLegend
Anti-IL-17A	PE-Cy7	BL168	BioLegend

Chapter 6 :

Discussion

6.1 Investigating inflammatory intestinal MNP subsets and generating their *in vitro* surrogates

MNPs play a critical role in the pathogenesis of IBD as they interact, via the cytokines they produce, with innate and adaptive immune cells. Genetic studies provided evidence for the important role of MNP-derived cytokines in CD and UC. In fact, single nucleotide polymorphisms in cytokines and their signaling molecules, such as IL12R, IL23R, STAT3, STAT4 and JAK2, have been identified in GWAS (Cleynen et al., 2016; Jostins et al., 2012). In addition, antibodies blocking IL12, IL23 and TNF decreased intestinal inflammation in numerous mouse models of colitis (Neurath et al., 1995; Uhlig et al., 2006; Yen et al., 2006).

Accordingly, therapies in the treatment of IBD, as well as other inflammatory disorders such as rheumatoid arthritis and psoriasis, aimed at impairing the MNP-mediated pro-inflammatory immune response. Anti-TNF therapy is the first choice of biologics used in CD and UC patients unresponsive to first line therapy (Colombel et al., 2010; Panaccione et al., 2014). Furthermore, antibodies targeting IL12 and/or IL23 are approved for CD treatment and have positive clinical results in UC patients (Feagan et al., 2016; Sandborn et al., 2012). Therefore, studies contributing to this thesis work, examined MNP subsets in inflamed colonic mucosa and MLN of IBD patients (**Chapter 2** and **Annex 1 and 2**). It proved to be quite challenging due to inherent patient variability, number of cells recovered from the tissue and difficulties in accessing samples, especially MLN as frequencies of surgical resection decreased with the development of biologics.

6.1.1 THE $CD14^{+}CD64^{+}CD163^{-}$ MONOCYTE-LIKE (P3) POPULATION

We examined the $CD172\alpha^{+}HLA\text{-}DR^{+}$ population, expressing CD11c, previously noted to accumulate in CD colonic mucosa and MLN (Baba et al., 2013), and confirmed their presence in UC patients (**Chapter 2** and **Annex 1 and 2**). One of the subpopulations, the $CD14^{+}CD64^{+}CD163^{-}$ (referred to as P3), with monocyte-like morphology and molecular signature accumulated in inflamed versus paired non-inflamed colonic tissue of IBD patients. Noteworthy, it was also observed in the MLN of CD and UC (**Annex 2**). Morphological, phenotypical and molecular analyses of P3 population were reminiscent of the intermediates in the intestinal monocyte "waterfall" of extravasated classical monocytes that accumulate during inflammation (Bain et al., 2013). The human counterpart of the CX3CR1 intermediate populations have recently been described in healthy ileum as $CD14^{hi}CCR2^{+}CD11c^{hi}$ (Bujko et al., 2018a). This suggests a classical monocyte origin of the $CD163^{-}CD206^{-}CD209^{-}MERTK^{-}$ $TREM1^{dim}HLA\text{-}DR^{dim}CCR2^{+}CD11b^{bright}$ (**Chapter 2**) monocyte-like pro-inflammatory P3 population.

The P3 population was the predominant IL1β and IL23 producing $CD14^{+}$ subset in inflamed colon (**Chapter 2** and **Annex 1**), and we believe that it plays a pathogenic role in IBD. P3 cells expressed TREM1 known to amplify inflammation in colitis models (Brynjolfsson et al., 2016; Kokten et al., 2018; Schenk et al., 2007). More importantly, the percentage of P3 cells positively correlated with the simple endoscopic score for CD (SES-CD) severity index. Finally, the accumulation of P3 cells observed in inflamed CD mucosa of untreated and refractory patients was not noted in patients in remission. Thus, characterizing and understanding the function of

monocyte-like CD14^{+}CD64^{hi}CD163^{-} P3 cells might open novel avenues for therapeutic intervention in IBD.

6.1.1.1 *P3 monocyte-like population are not Slan^{+} cells*

In the study that contributed to this Ph.D. work, we demonstrated that IL1β and TNF producing CD14^{hi}SIRPα^{+} Slan^{+} cells accumulated in MLN and inflamed colon of CD but not UC patients (**Chapter 3**). The phenotypic similarities raise the question of whether the accumulated colonic P3 population observed is due to an increase in Slan^{+} cells, especially now that Slan^{+} cells are considered a monocyte subset.

6.1.1.1.1 Slan^{+} cells: monocytes or DCs? Solved.

Up until recently, Slan^{+} cells were considered members of the DC compartment based on their antigen presenting capacities and cytokine secretion. Despite that most reports asserted that Slan^{+} cells are DCs, their overlap with the CD16^{+} monocyte populations was demonstrated early on (de Baey et al., 2001; Siedlar et al., 2000). Furthermore, Cros *et al.* showed that human Slan^{+} cells were closely related to the murine Ly6C^{lo}CX3CR1^{hi} monocyte population and considered them part of the non-classical monocytes (Cros et al., 2010). Indeed, transcriptional analysis clustered Slan^{+} cells with non-classical monocytes, and away from cDC1 and cDC2 subsets (Hofer et al., 2015; van Leeuwen-Kerkhoff et al., 2017).

Furthermore, functional data reinforce Slan^{+} cells clustering away from cDC populations. First and foremost, DCs are defined by their naïve T cell priming capacities (Jakubzick et al., 2017). Slan^{+} cells had inferior antigen presenting capacities when compared with cDC populations

consistent with their monocytic identity (van Leeuwen-Kerkhoff et al., 2017). Furthermore, and similar to our observations (**Chapter 3**), their HLA-DR expression paralleled that of monocytes and was lower than that of DCs (van Leeuwen-Kerkhoff et al., 2017). Moreover, CD80 and CD86 co-stimulatory molecules expression, upon stimulation, was lower on Slan$^+$ cells when compared to cDC1 or cDC2. Finally, in allogenic mixed lymphocyte reaction (MLR), inferior CD4$^+$ and CD8$^+$ T cell proliferation was noted with Slan$^+$ and classical monocytes as opposed to cDC2 (Cros et al., 2010; van Leeuwen-Kerkhoff et al., 2017). Thus, these data support segregation of Slan$^+$ cells away from cDC subsets and relatedness to non-classical monocytes.

6.1.1.1.2 Update on function of Slan$^+$ cells

Identification of Slan$^+$ cells in the tissue of inflammatory or autoimmune diseases such as psoriasis, multiple sclerosis, systemic lupus erythematosus and CD (Ahmad et al., 2019) rendered them the subject of many studies aimed at understanding their function for therapeutic purposes. A deep understanding of the function of this population in tissue has been challenging in part due to the absence of a murine counterpart. However, some advances have been made in elucidating the role of Slan$^+$ cells' in inflammatory settings. Since Slan$^+$ cells were not further examined in Crohn's disease, I will speculate on their potential significance in CD tissue based on the reported functional role of Slan$^+$ cells, and what we had previously described.

Slan$^+$ cells are potent producers of pro-inflammatory cytokines and are capable of driving naïve T cell polarization into Th effectors cells. Firstly, Slan$^+$ cells secrete high levels of IL12 upon TLR stimulation (Hansel et al., 2011; Schakel et al., 2006). However, contrary to what was previously established, they are not the main producers in the blood, which are cDC2 (Nizzoli

et al., 2013; van Leeuwen-Kerkhoff et al., 2017). Upon MDP and Pam3CSk4 stimulation, circulating Slan$^+$ cells from CD patients had a higher expression of IL12p40 and IL23p19 compared to healthy donors (**Chapter 3**), and thus might play a role in the induction of Th17/Th1 and Th1 responses. Secondly, circulating Slan$^+$ cells, as well as classical monocytes, are the main producers of IL1β (van Leeuwen-Kerkhoff et al., 2017). This production was further increased in Slan$^+$ cells from blood, colonic tissue and MLN of CD patients (**Chapter 3**). IL1β was shown to promote Th17/Th1 response in CD4$^+$ T cells from IBD patients (**Chapter 2** and **Annex 1**). Therefore, Slan$^+$ cells could drive Th17/Th1 response in CD patients, as was observed in psoriasis (Hansel et al., 2011). Finally, Slan$^+$ cells were also found to secrete pro-inflammatory IL6, IL8, IL23 and TNF (Baran et al., 2018; Hansel et al., 2011; van Leeuwen-Kerkhoff et al., 2017). The presence of IL1β and IL6 induce IL17 production by T_{regs}, a population found in high proportions in IBD patients (Basu et al., 2015; Ueno et al., 2018; Ueno et al., 2013). Collectively, the cytokine secretion profile of Slan$^+$ cells suggests that they play a key role in the induction of pro-inflammatory Th17/Th1 subsets, a hallmark of IBD pathogenesis.

Slan$^+$ cells play a role in amplifying the innate inflammatory response. They expressed high levels of CD16 (**Chapter 3**), which equips them to bind IgG immune complexes, phagocytose and mediate antibody-mediated cellular toxicity (Schmitz et al., 2002). Additionally, Slan$^+$ cells are involved in the complement-associated response, a common monocytic function. Indeed, they have high expression of complement receptors, secretion of C3a and C5a, and enrichment in complement-associated factors at the transcriptional level (van Leeuwen-Kerkhoff et al., 2017). The complement system is involved in the detection, opsonization and elimination of

bacteria, and thus, Slan$^+$ cells play an important role in maintenance of intestinal homeostasis (Sina et al., 2018). The localization of Slan$^+$ cells in the apical part of the villi of inflamed colonic mucosa of CD patients (**Chapter 3**) strategically position them to intercept pathogen translocation across the epithelium. One could hypothesize that in intestinal inflammation, Slan$^+$ cells bind bacterial product through their many TLRs (Hansel et al., 2013), and activate the complement system, leading to recruitment of innate immune cells, and thus, further amplifying the innate immune response at the intestinal barrier.

Since classical monocytes are the contributors to the intestinal CX3CR1 intermediates observed in mice and their human counterpart (Bain et al., 2013), non-classical CD16$^+$Slan$^+$ monocyte subset likely does not contribute to the CD16$^-$ P3 population (**Chapter 2** and **Annex 1**). More importantly, Slan$^+$ cells from inflamed CD mucosa are CD64$^-$CD163$^-$, and thus not part of CD64$^+$CD163$^-$ P3 cells (**Annex 3-Figure 1**). Furthermore, contrary to P3 cells, *ex vivo* Slan$^+$ cells from inflamed colonic mucosa of CD patients not undergoing anti-TNF therapy had a higher frequency of TNF$^+$ when compared to paired non-inflamed tissue (**Chapter 3** and **Annex 1**). Collectively, these data support that P3 cells are not the Slan$^+$ cells described in CD mucosa.

6.1.1.2 *P3 monocyte-like population are not in vitro macrophage 1 (M1)*

Similar to P3 population, M1 macrophages differentiate under inflammatory conditions (CSF1 + IFNγ), and express abundant quantities of pro-inflammatory cytokines such as IL1, IL6 and IL23 (Murray et al., 2014). However, M1 cells possess typical macrophage morphology with large size and abundant vacuoles (**Annex 3-Figure 2**).

6.1.1.3 *P3 monocyte-like population are not monocyte-derived DCs*

Classical monocytes arriving to the inflamed intestinal mucosa can also differentiate into DCs referred to as "inflammatory DCs" (Tang-Huau and Segura, 2019). Phenotypically, they are $CD11c^{+}HLA\text{-}DR^{+}$ and express cDC markers (such as CD1c and FcεRI). However, they are also $CD206^{+}CD14^{+}$, markers associated with macrophages, but they lack CD16 and CD163 (Segura et al., 2013). Furthermore, they express the monocyte tissue-recruiting marker CCR2 (Boring et al., 1997), although in the murine intestine a $CCR2^{+}$ DC population was shown to derive from pre-DC (Scott et al., 2015). Moreover, MoDCs from ascites share transcription factors expressed by cDC (such as ZBTB46, BATF3 and IRF4) and macrophages (such as EGR1 and EGR2) but not MAFB which is only found on macrophages (Segura et al., 2013). Transcriptional studies segregated them from cDC populations; they were found to closely resemble classical monocytes (Goudot et al., 2017; Segura et al., 2013), strongly suggesting their monocyte origin. Finally, the human inflammatory MoDCs possess the morphology (**Annex 3-Figure 2**) and function of *bona fide* DCs with their dendrites and ability to stimulate naïve T cells (Segura et al., 2013). Therefore, the P3 population cannot be identified as inflammatory DCs, and does not resemble the GM-CSF+IL4 *in vitro* differentiated MoDCs (**Chapter 5**).

Collectively, P3 cells identified in the colonic mucosa of IBD patients are monocyte-like and distinct from $Slan^{+}$ and inflammatory MoDCs.

6.1.2 P3 MONOCYTE-LIKE CELLS INTERACT WITH CD4$^+$ T CELLS AND DRIVE INFLAMMATION

P3 monocyte-like cells isolated from the inflamed intestinal mucosa of IBD patients secrete cytokines that drive memory CD4$^+$ T cell response.

6.1.2.1 *P3-secreted IL1β augment Th17/Th1 response*

IL1β cytokine that is secreted by P3 cells augmented mucosal effector Th17/Th1 cell responses in the colonic mucosa, but not MLN, of CD and UC patients (**Chapter 2, Annex 1** and **Annex 3-Figure 3**). In addition, it promoted a differential pathogenic Th17 phenotype in CD and UC that was defined by IFNγ, GM-CSF, TNF and IL6 secretion in the former, whereas IFNγ and IL8 secretion was observed in UC. IL8 secretion by Th17 and Th17/Th1 cells might play a role in attracting neutrophils to UC inflamed mucosa and along with GM-CSF induce epithelial barrier destruction (Coccia et al., 2012; Dabritz et al., 2015; Kryczek et al., 2016).

These data suggest a role for IL1β in IBD induction. In fact, IL1β favors the survival of colonic Th17 cells in a T cell-dependent model of colitis (Coccia et al., 2012), upregulates IL23R expression on pathogenic T cells (Kleinschek et al., 2009), and activates local neutrophils (Dinarello, 2011). Moreover, IL1RN genetic variants have been associated with increased susceptibility to CD and UC (Carter et al., 2004; Stankovic et al., 2015). Furthermore, IL1β levels correlate with disease activity in CD patients (Casini-Raggi et al., 1995) and increases prior to disease relapse (Schreiber et al., 1999). Finally, IL1R antagonists, such as anakinra, have been used in very early onset IBD patients (Shouval et al., 2016), and are in clinical trials for severe manifestations of UC (Thomas et al., 2019).

6.1.2.2 *IL12 promote Th17 pathogenicity*

IL12 promotes colonic Th17 cells pathogenic signature by inducing the production of pro-inflammatory cytokines such as IFNγ, GM-CSF, TNF and IL6 (**Chapter 2** and **Annex 1**). The pathogenic role of GM-CSF during inflammation includes triggering hematopoiesis, and thus, accumulation of myeloid cells in inflamed organs (Griseri et al., 2012). Furthermore, GM-CSF enhances IL23 secretion by APCs *in vitro (El-Behi et al., 2011)* which might further contribute to Th17 cell pathogenicity. It is worth noting the dual role of GM-CSF, as it has been shown to ameliorate colitis via its effect on monocytes that led to bacterial clearance and epithelial healing (Dabritz et al., 2015). IL6 enhances the survival of pathogenic IL23R-expressing T cells and drives the development of colitis-associated colonic carcinoma (Punkenburg et al., 2016). Finally, IL12 was also responsible for IL8 induction in colonic $CD4^+$ T cells of UC, but not CD, patients (**Chapter 2**).

6.1.2.3 *IL23 promotes a pathogenic Th17 profile*

Following the discovery of IL23 (Oppmann et al., 2000) and the *IL23R* variant that protects from IBD development (Duerr et al., 2006), the positive effect of IL12p40 antagonists were studied to better understand the contribution of IL23 in triggering intestinal inflammation. Firstly, this cytokine is needed for the maintenance, but not development, of Th17 cells during the inflammatory response (Ahern et al., 2010; Langrish et al., 2005; McGeachy et al., 2009). Secondly, IL23 drives GM-CSF and IFNγ secretion by Th17 cells (Griseri et al., 2012). Indeed IL23 increased the frequency of $IL17^+IFN\gamma^+$ cells in $CD4^+$ T cells isolated from CD mucosa (**Annex 1**). Thus, mice with transgenic expression of IL23p19 have severe inflammation in the small intestine and colon (Wiekowski et al., 2001). Furthermore, loss or blockade of IL23

reduces intestinal inflammation in mice (Elson et al., 2007; Kullberg et al., 2006; Yen et al., 2006).

IL23-mediated intestinal tissue injury can be seen in the absence of IL17 and T cells (Izcue et al., 2008; Uhlig et al., 2006). In fact, IL23 also modulates the pathogenicity of non-Th17 cells, such as natural killer T cells, γδ T cells and ILC3 (Gaffen et al., 2014; Mizuno et al., 2014; Pearson et al., 2016; Qiu et al., 2012). IL23 promotes inflammation by inhibiting intestinal T_{reg} response (Izcue et al., 2008). Finally, we also presented a role for IL23, along with GM-CSF and IFNγ, in the *in vitro* generation of inflammatory monocyte-like cells (**Chapter 5**).

6.1.3 *IN VITRO* MODEL FOR THE GENERATION OF TISSUE INFLAMMATORY MONOCYTE-LIKE CELLS

The P3 population isolated from the mucosa of CD and UC patients is monocyte-derived and pathogenic, as they promote pathogenic Th17 profile, so they could represent a target in IBD therapy. Therefore, a better understanding of their differentiation and function is required. This elucidation is however limited by the difficulty in patient recruitment and low cell number acquired. Therefore, we hunted for surrogates of these cells, especially since previously described culture conditions do not generate monocyte-like cells. Other than the classic MoDC, M1 and M2 macrophages, the majority of *in vitro* work relies on differential activation of M-CSF macrophages (M0) (Gharib et al., 2019; Goudot et al., 2017; Schulthess et al., 2019; Xue et al., 2014).

In our *in vitro* model, we isolated the homogenous $CD14^{+}CD16^{-}$ classical monocytes (Villani et al., 2017) from peripheral blood of healthy donors and exposed them to the effect of GM-CSF, IFNγ and IL23 (**Chapter 5**). The abundance of these cytokines in inflamed tissue of CD patients (Noguchi et al., 2001; Sasaki et al., 1992; Schmidt et al., 2005) reinforced their employment in mimicking the IBD inflammatory milieu. Although an effect of IL23 on monocytes is unusual, we showed that exposure to GM-CSF and IFNγ upregulated *IL23R* mRNA expression on circulating monocytes, suggesting that they can respond to IL23 (**Chapter 5**). Furthermore, IL23R expression has been noted on myeloid cells in a study using IL23R GFP reporter mice (Awasthi et al., 2009). Finally, recent work on human intestinal myeloid cells demonstrated an autocrine/paracrine IL23 amplification of pro-inflammatory cytokine production (Sun et al., 2019).

The $CD163^{-}$ cells generated in our *in vitro* model resembled the P3 monocyte-like cells, and therefore, referred to as Infl mo-like cells (**Chapter 5**). First, Infl mo-like cells displayed a monocyte-like morphology and resembled the P3 population found in IBD mucosa. Furthermore, like their potential *in vivo* counterpart, they promoted an IL1β-dependent Th17 and Th17/Th1 responses, a hallmark of IBD (**Chapter 2** and **Annex 1**). They also expressed *TREM1,* similar to murine tissue $Ly6C^{high}$ monocytes and P3 population (**Annex 1**) (Kokten et al., 2018; Schenk et al., 2007). However, in contrast with the later, Infl mo-like cells had a high expression of CD209 when compared to macrophages. This discrepancy might be inherent to *in vitro* culture that does not obviously represent all the players in the tissue. For instance, a higher concentration of IL4 in the culture medium could upregulate CD209 expression (Teles et al., 2010). The higher concentration might be due to lack of IL4R expressing cells in culture

during the 6 days-culture, resulting in no consumption of IL4. Noteworthy, the use of circulating monocytes from IBD patients did not generate different frequencies of Infl mo-like cells following GM-CSF+IFNγ+IL23 treatment (**Annex 3-Figure 4**), although an upregulated response to IFNγ has been noted in CD monocytes (Nakanishi et al., 2018).

Collectively, we have identified culture conditions that generate monocyte-like cells resembling pathogenic P3 cells isolated from inflamed mucosa of IBD patients.

6.2 The second $CD14^+CD64^+$ population in IBD mucosa: the $CD163^+$ macrophages (P4)

A second $CD14^+CD64^+$ population was identified in the inflamed mucosa of CD and UC patients, the $CD163^+$ (referred to as P4) with macrophage morphology and signature, and therefore, distinct from P3 cells (**Chapter 2** and **Annex 1**). This population displayed features associated with an anti-inflammatory, pro-resolving function in the mucosa. First, they did not accumulate in inflamed UC and CD colon, or correlate with SES-CD. Second, they expressed high levels of IL10 in inflamed UC mucosa (**Chapter 2**). IL10 plays a critical role in maintaining gut homeostasis since IL10R deficient patients develop very early onset IBD (Glocker et al., 2009; Moran et al., 2013) due to uncontrolled IL1β production by IL10R-deficient macrophages (Shouval et al., 2016), and IL10 knockout mice lack regulation of IL23 production (Krause et al., 2015). Third, in contrast to the monocyte-like P3, P4 cells expressed higher levels of TNF (**Annex 1**) which has a dual role in intestinal inflammation. Although APC-secreted TNF mediates inflammation by promoting T cell survival, low levels contribute to the maintenance of epithelial barrier integrity (Billmeier et al., 2016). Fourth, they do not drive intestinal Th17, Th17/Th1 responses, or IL8 secretion (**Chapter 2** and **Annex 1**). Finally, scRNAseq demonstrated that P4 cells expressed genes associated with mature and/or regulatory macrophages such as *CD209, MRC1, CD163, CD163L1* and *STAB1* (Gonzalez-Dominguez et al., 2015). Thereby, it might be an attractive therapeutic avenue to increase the proportion of this population in IBD mucosa to favor repair, and consequently remission.

6.2.1 P4 MACROPHAGES ARE DISTINCT FROM INTESTINAL RESIDENT MACROPHAGES

Although P4 macrophages possess anti-inflammatory properties, they are distinct from macrophages generated during homeostasis. Unlike resident macrophages they had increased *ex vivo* cytokine secretion of IL23 in inflamed tissue (**Annex 1**). They also expressed genes such as *IDH1, FOLR2, DNASE2, SLCOB21, DAB2* and *VSIG4* reported to be associated with tissue inflammatory macrophages (Goudot et al., 2017). Finally, they do not resemble macrophages isolated from healthy human small intestine at the phenotypic or molecular level (Bujko et al., 2018b). More specifically, $CD14^{hi}CD64^{hi}$ P4 cells are distinct from the two mature macrophage populations: the $CD14^{lo}$ Mf3 (as defined by Bujko et al., 2018) and the $CD14^{hi}CD64^{lo}$ Mf4 (as defined by in Bujko et al., 2018). The latter is mainly localized in the submucosa and muscularis propria; therefore, it is unlikely that we isolated it given our tissue processing protocol which excludes these layers (**Chapter 2**).

Noteworthy, P4 population also included a subset of $TIM4^{+}CD4^{+}$ cells (**Chapter 2**) which were recently reported in mice as embryonically-derived, self-maintaining, resident macrophages (De Schepper et al., 2018; Shaw and Houston, 2018). Generally, embryonically-derived, tissue-resident macrophages play an important role in resolution of inflammation, as they continuously produce anti-inflammatory cytokines, lipid mediators, and engulf dying cells regardless of inflammation (Okabe and Medzhitov, 2016; Uderhardt et al., 2012). It would be interesting to further examine the P4 population to better understand its function and contribution to the anti-inflammatory process.

6.2.2 P4 MACROPHAGES ARE NOT *IN VITRO* MACROPHAGE 2 (M2)

Similar to P4 macrophages, M2 are $CD64^{+}CD163^{+}CD209^{+}$, and express *FOLR2* gene (**Annex 1**) (Puig-Kroger et al., 2009). However, M2 macrophages possess an anti-inflammatory cytokine profile with a high secretion of IL10 and TGFβ, paralleled with low production of IL12 and IL23 (Italiani and Boraschi, 2014). Furthermore, P4 macrophages do not express the IRF5 gene, specific to M2 macrophages (Krausgruber et al., 2011). Finally, arginase 1 production is a key feature of M2 macrophages (Pauleau et al., 2004) that plays a role in parasite infections (Anthony et al., 2006). Arginase 1 mRNA expression was not highlighted in colonic P4 population (**Annex 1**). These data suggest that P4 macrophages do not resemble *in vitro* M2 macrophages.

6.2.3 P4 MACROPHAGES DIFFERENTIATE FROM MONOCYTE-LIKE P3 CELLS

The morphological, phenotypical and molecular analyses of P3 and P4 populations remind the intestinal $CD14^{+}$ MNPs and their murine counterparts identified by Bain *et al.* during homeostasis and inflammation (Bain et al., 2013). Consequently, we hypothesized that P3 and P4 subsets represent two distinct functional phenotypes of a single population displaying a high degree of plasticity, with pro-inflammatory P3 converting towards anti-inflammatory P4 macrophage-like cells, transitioning through a $CD14^{+}CD64^{+}CD163^{dim}$ population (referred to as Px). Noteworthy, this intermediate population does not accumulate during inflammation (**Annex 1**). This conversion would be slowed down during inflammation leading to the buildup of pro-inflammatory P3 cells in inflamed tissue. Indeed, pseudo-time reconstruction in scRNAseq analysis showed that P3 cells progressively acquired CD163, CD14 and MERTK while down-regulating TREM1, CD11b and CCR2 (data not shown). Furthermore, an un-

supervised Monocle analysis, utilizing minimum spanning trees (Ji and Ji, 2016), portrays P3 transitioning to CD163^{int} Px cells, and finally to P4 subset (data not shown). The environmental cues driving monocyte-like P3 cells that potentially give rise to anti-inflammatory P4 macrophages are still not entirely understood and warrant full elucidation in humans in order to increase the number of pro-resolving macrophages.

6.2.4 HYPOTHESIS FOR THE RESOLUTION OF INFLAMMATION

Plasticity, a key characteristic of macrophages demonstrated *in vitro* (Italiani and Boraschi, 2014) could be leveraged for the generation of pro-resolving macrophages. Whether and how it occurs *in vivo* remains questionable, especially since macrophage populations in the tissue are observed in snapshots. Several hypotheses attempt to explain this issue. Although M1 and M2 macrophage concept is outdated, most of what is known regarding macrophage plasticity stems from these *in vitro* models.

The first hypothesis suggests that the different monocyte or macrophage populations assume distinct functional phenotypes. In other words, Ly6C^{-} non-classical monocytes and tissue resident macrophages become M2, while Ly6C^{+} classical monocytes and monocyte-derived macrophages become M1. However, it is not completely supported as non-classical monocytes could give rise to M2 macrophages and M1 cells could trans-differentiate into M2 macrophages (Arnold et al., 2007; Crane et al., 2014; Murray et al., 2014). Notably, we generated Infl mo-like cells using classical and non-classical monocytes from healthy donors and found similar cell distribution (**Annex 3-Figure 5**).

The second hypothesis argues for macrophage trans-differentiation, i.e. switching between the polarized macrophages. *In vitro* studies back up this view as inflammatory M1 macrophages mature into M2 repair macrophages upon exposure to specific factors, for example IL4 and IL13 cytokines, efferocytosis of apoptotic cells, phospholipase C, the miRNA let-7c, among others (Das et al., 2015; Italiani et al., 2014). It was also demonstrated that upon exposure to IFNγ or TLR ligands, M2 macrophages switch to M1 (Mylonas et al., 2009; Stout et al., 2005).

The third hypothesis discusses monocyte recruitment in consecutive waves throughout the inflammatory reaction. Thus, depending on the inflammatory stage in which they are recruited, monocytes are exposed to different microenvironments that guide their differentiation. In the early stage of inflammation, they polarize into M1 macrophages, whereas in the later phase into M2 macrophages (Arnold et al., 2007; Nahrendorf et al., 2007). This is supported by the niche model during inflammation, creating empty and accessible niches for monocyte recruitment, due to macrophage loss and increased leakiness (Guilliams and Scott, 2017). Moreover, monocytes exhibit higher plasticity when compared to resident macrophage populations, as revealed by fate mapping experiments, irrespective of their origin (Bowman et al., 2016; Lewis et al., 2014; Yamasaki et al., 2014; Zigmond et al., 2014b).

Hypotheses two and three are not exclusive and probably have a role at different stages of inflammation. For instance, efferocytosis induces the switch of inflammatory macrophages to a pro-resolving "M2" phenotype that will contribute to the establishment of a "less-inflammatory milieu." Hence, newly recruited monocytes, trying to fill the empty macrophage

niches, will develop into anti-inflammatory/resident macrophages further contributing to resolution of inflammation (***Figure 6-1***).

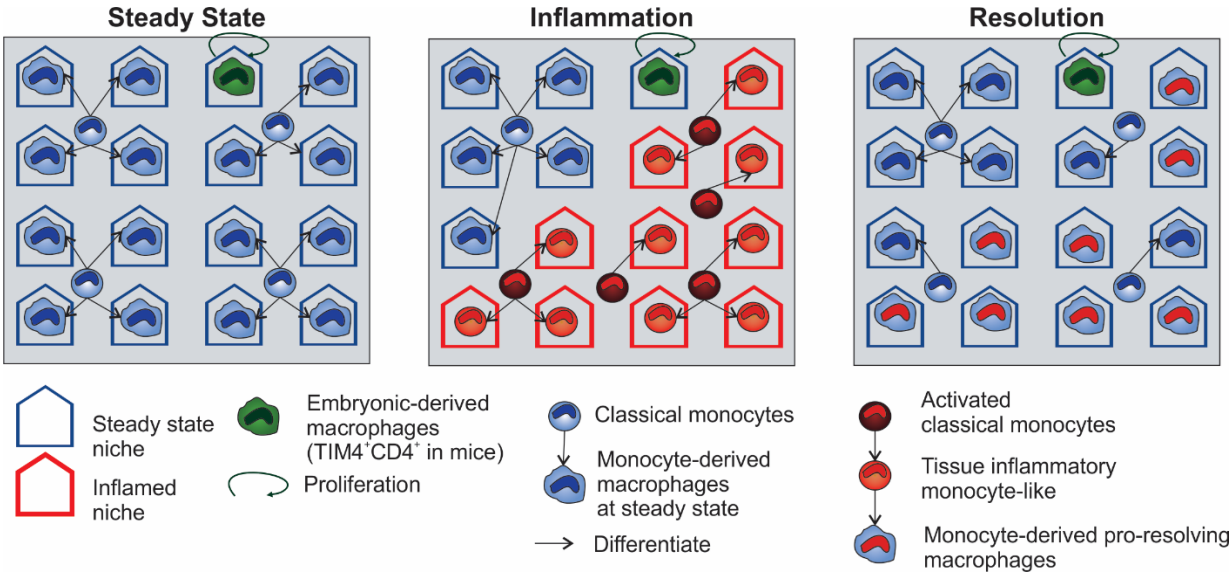

Figure 6-1: Hypothesis 2 and 3 explaining intestinal inflammation and its resolution. *During homeostasis, macrophages in the intestine are mainly derived from classical monocytes, except for the newly identified embryonically-derived $TIM4^{+}CD4^{+}$ macrophages in mice that maintain their homeostatic function regardless of inflammation. In inflammatory context, macrophage death leads to empty niches that are filled by recruited activated monocytes. In the inflammatory milieu, monocytes do not complete their maturation cascade, leading to an increase in the number of pro-inflammatory monocyte-like cells in the tissue. During the resolution phase, inflammatory monocyte-like cells shift to an anti-inflammatory, pro-resolving macrophage phenotype. Furthermore, the empty niches are repopulated by newly recruited classical monocytes that arrive into a less/non-inflammatory milieu and differentiate into non-inflammatory macrophages, resembling those at steady-state. (Influenced by (Guilliams and Scott, 2017)).*

6.2.5 *IN VITRO* MODEL FOR THE GENERATION OF TISSUE MACROPHAGES FROM IBD MUCOSA:

We presented a two-step culture model in this thesis that pushed the *in vitro* differentiated Infl mo-like cells to anti-inflammatory macrophages. For that purpose, after monocyte

differentiation for 6 days by exposure to GM-CSF + IFNγ + IL23, the combination of TGFβ and IL10 was added for another 6 days.

CSF1 (or M-CSF) is known to play a vital role in intestinal macrophages differentiation, survival and proliferation. The number of intestinal macrophages is markedly decreased in CSF1-deficient or mice receiving anti-CSF1 receptor treatment (Arnold et al., 2016; Dai et al., 2004; MacDonald et al., 2010; Ryan et al., 2001). Indeed, most *in vitro* models employed it to generate macrophages from monocytes before activating them or inducing their plasticity (Gharib et al., 2019; Goudot et al., 2017; Schulthess et al., 2019; Xue et al., 2014). However, CSF1 was not employed in our model for the generation of $CD163^+$ macrophages (**Chapter 5**). Nonetheless, $CD163^-$ Infl mo-like cells expressed *CSF1* at higher intensity when compared to $CD163^+$ macrophages which expressed more *CSF1R*, suggests a potential involvement of CSF1. It would be interesting to block CSF1 or its receptor to better understand its role in our model.

As an alternative to CSF1, we used the combination of TGFβ and IL10, as each cytokine was not sufficient to induce a significant increase in $CD163^+$ population (**Chapter 5**). Both cytokines have been implicated in macrophage differentiation. TGFβ upregulates the expression of *Cx3cr1*, *Il10,* and genes encoding αvβ5 integrins, thus imprinting the genetic signature of colonic macrophages in mice (Schridde et al., 2017). Moreover, TGFβ inhibit pro-inflammatory cytokine secretion, such as IL1β, IL6, IL8 and TNF, aiding in macrophage hypo-responsiveness to TLR stimulation (Maheshwari et al., 2011; Smythies et al., 2010). On the other hand, IL10 and its receptor have a more prominent role in governing macrophage regulatory phenotype. For instance, IL10R deficient macrophages exhibited higher expression of iNOS, IL12 and IL23

that drive spontaneous colitis (Zigmond et al., 2014a). Moreover, TLR hyper-responsiveness is observed when IL10-IL10R signaling is disturbed in mice and early onset IBD patients (Shouval et al., 2016; Ueda et al., 2010; Zigmond et al., 2014a). Thus, the concomitant addition of the cytokines should ensure the molecular and functional development of anti-inflammatory macrophages.

Indeed, *in vitro* converted $CD163^+$ cells possess key features of *bona fide* macrophages, including morphology, phagocytic function (**Chapter 5**), and molecular profile defined by the expression of *MARCO*, *MERTK*, *CD163* and *MAFB* (Goudot et al., 2017). The macrophage identity and the highly significant enrichment in P4 genes support the resemblance of converted $CD163^+$ cells with P4 macrophages from the intestine.

As *in vitro* converted $CD163^+$ macrophages expressed *IL10* and *TGFβ*, we thought of testing whether they induce their own differentiation. In fact, we co-cultured CFSE-labeled converted macrophages with purified Infl mo-like cells and failed to induce conversion, probably due to the low quantity of cytokines secreted. Therefore, we attempted to use culture supernatant of LPS-stimulated converted macrophages, but it did not induce CD163 expression on Infl mo-like cells (data not shown). In fact, Gharib *et al.* recently reported that repolarization of M1 macrophages with IL10 induced higher expression of pro-inflammatory cytokines and improved efferocytosis upon LPS challenge, as compared to IL4 (Gharib et al., 2019). Another possibility is the role of macrophage-derived IL10 in maintenance of homeostasis that is secondary to T_{reg}-secreted IL10 (Bain et al., 2013; Zigmond et al., 2014a).

A limitation of the study entitled “A two-step human culture system replicates intestinal monocyte maturation cascade: conversion of tissue-like inflammatory monocytes into macrophages” is the use of gene set enrichment analysis that depends on pre-determined gene sets prone to selection bias and limits the extent of the analysis (**Chapter 5**). Nevertheless, this approach has been efficiently employed and its feasibility tested (Gharib et al., 2019; Xue et al., 2014). Another weakness is the small sample size used for the molecular study that could benefit from further validation.

6.3 Therapeutic target: cell plasticity and/or cell recruitment?

MNPs and more particularly P3 cells, play a critical role in pathogenesis of IBD. In fact, Baillie *et al.* perceived that several IBD susceptibility loci identified in GWAS coded for promoters involved in regulation of monocyte-macrophage differentiation (Baillie et al., 2017). Defects in the later have been linked with reduced bacterial clearance (Smith et al., 2009), high cytokine secretion (Bain et al., 2013; Bernardo et al., 2018; Kamada et al., 2008; Smythies et al., 2005), and thus, defects in resolution of intestinal inflammation leading to IBD development (Na et al., 2019). Targeting MNPs, their recruitment, differentiation, plasticity as well as their effect on T cell plasticity via their cytokine secretion could be a good therapeutic option in the treatment of IBD.

6.3.1 IS PLASTICITY A ONE-WAY STREET? AIM FOR THE GOOD!

Plasticity is a key characteristic of macrophages and Th17 cells that could be exploited to increase the number of protective cells, such as pro-resolving macrophages and non-pathogenic Th17 subsets in the treatment of IBD.

6.3.1.1 *Macrophage plasticity: P3 to P4?*

The shift of inflammatory monocyte-like P3 phenotype to P4 macrophages might lead to resolution of the inflammatory response.

6.3.1.1.1 Role of macrophages in the resolution of inflammation and tissue repair

The universal components of resolution of inflammation, as discussed by Schett and Neurath (Schett and Neurath, 2018) are: (1) blocking influx of neutrophils to the tissue, (2) removing apoptotic neutrophils, and (3) switching macrophage function to limit inflammation. Thus,

highlighting the role of macrophages, capable of removing bacteria and apoptotic cells, in the tightly coordinated resolution phase of inflammation (Fullerton and Gilroy, 2016; Na et al., 2019). This process is initiated with the clearance of apoptotic neutrophils by macrophages, a crucial step to avoid necrosis and further aggravation of inflammation. Macrophages follow the "find me" signals (such as sphingosine 1-phosphate and CX3CL1) released by apoptotic neutrophils and engulf the cell displaying the "eat me" signal (such as annexin-1 and phosphatidylserine). Upon efferocytosis, monocyte-derived macrophages switch their metabolic mechanism to adapt an anti-inflammatory phenotype (Gordon and Pluddemann, 2018; Schett and Neurath, 2018). Defects in efferocytosis, the initiating step of the resolution program, contribute to IBD pathogenesis. Indeed, intestinal macrophages bearing apoptotic epithelial cells overexpressed genes associated with increased susceptibility to IBD (Cummings et al., 2016).

The microbiota is another factor that promotes anergic macrophage function via the secretion SCFA and AhR ligands. For instance, administration of the SCFA butyrate restored homeostasis in the intestine by modulating macrophage metabolism and promoting anti-inflammatory alternative activation of macrophages (Scott et al., 2018). The microbiota could also induce TGFβ release by intestinal epithelium and T_{reg} cells, contributing to anergic macrophages (Atarashi et al., 2008; Ishikawa et al., 2008; Smythies et al., 2005). Actually, oral inoculation of *Clostridium* strains increased TGFβ levels in colon supernatant, which was associated with inhibited colitis induction by DSS treatment (Atarashi et al., 2011).

The pro-resolving function of macrophages is ensured by the production of lipoxin A4 (LXA_4) that enhances their phagocytic function as it inhibits synthesis of ROS and pro-inflammatory cytokines. Furthermore, LXA_4 limits neutrophil recruitment, while promoting monocyte chemotaxis (Na et al., 2019). Noteworthy, patients with severe UC have defective lipoxin synthesis (Mangino et al., 2006). Also, pro-resolving macrophages play a role in epithelial barrier repair by supporting epithelial cell proliferation. In fact, $TREM2^+$ macrophages are necessary for proliferation of epithelial cells and mucosal healing in murine colonic punch-biopsy-induced injury (Seno et al., 2009). The $CD163^+$ converted macrophages significantly up-regulate their expression of TREM2 (**Chapter 5**), suggesting that they may play a role in promoting epithelial repair.

However, increasing tissue repair is a double edge sword, as it could lead to tissue fibrosis, a symptom seen in highly treated IBD patients. In fact, infiltration of macrophages and high TGFβ concentration was observed in fibrotic colonic sections from CD patients (Scharl et al., 2015). Macrophages are the primary cellular source of IL36 that play an important function in intestinal wound healing by increasing proliferation of epithelial cells, and activating myofibroblasts (Scheibe et al., 2017). The macrophage-myofibroblast communication is essential to the healing of intestinal tissue but could also be pathogenic when aberrant. For instance, high production of connective tissue growth factors by macrophages led to constant activation of fibroblasts, and thus, fibrosis in a mice model of radiation-induced fibrosis (Yeh et al., 2016).

6.3.1.1.2 Increasing pro-resolving macrophages in IBD

Overall, increasing pro-resolving macrophages and tissue repair appears to play an important role in IBD therapy. Indirect evidence from currently available therapies in IBD support increasing the frequency of pro-resolving monocyte-derived macrophages. First, anti-TNF therapy (known as infliximab) induces regulatory macrophages. Anti-TNF responders possessed higher frequency of $CD68^{+}CD206^{+}$ regulatory macrophages (Vos et al., 2012). Of note, colonic P4 macrophages expressed CD206, a key feature of M2 macrophages, suggesting an anti-inflammatory role for these cells (**Annex 1**). Moreover, anti-TNF therapy ligates Fcγ receptor, thus inducing alternative macrophage polarization (Vos et al., 2011). The JAK inhibitor, tofacitinib, shapes macrophage polarization towards a pro-resolving phenotype, as well as increases anti-inflammatory function while decreasing IFNγ secretion (De Vries et al., 2019a). Corticosteroids have a wide range of effects on macrophages including promoting alternatively activated macrophage differentiation and increasing efferocytosis (Ehrchen et al., 2007; Giles et al., 2001). Finally, a suggested treatment for IBD involving macrophage plasticity is induction of helminth infection (Summers et al., 2005a; Summers et al., 2005b). The latter induces a type 2 immune response that contributes to pro-resolving macrophage differentiation with tissue repair function (Wynn and Vannella, 2016), and have been shown to improve colitis in mice (Smith et al., 2007). Consequently, increasing pro-resolving macrophages represents an attractive therapeutic target in IBD. This approach will promote the resolution process, rather than dampening the effector/pro-inflammatory pathways currently employed in treatment of IBD (Schett and Neurath, 2018).

Our *in vitro* model supports the concomitant increase of TGFβ and IL10 in the mucosa of IBD in order to promote anti-inflammatory macrophage maturation (**Chapter 5**). Although TGFβ1 is increased in intestinal mucosa of IBD patients (Babyatsky et al., 1996), the levels are insufficient to reduce inflammation. More specifically, TGFβ is unable to ensure inhibitory effect on target population leading to increased SMAD7 that interacts with TGFβR1, and thus, prevents optimal signaling upon TGFβ1 binding (Abraham et al., 2017; Boirivant et al., 2006). Mongersen is an oligonucleotide that block SMAD7 protein, thus increasing cell responsivity to TGFβ (Ardizzone et al., 2016). In a recent phase 3 clinical trial, Mongersen showed no beneficial results in treatment of active CD resulting in the termination of the trial (Giuffrida et al., 2019).

We have previously discussed the role of IL10 in IBD, and therefore, it seemed natural to supplement IL10 in CD and UC treatment (Marlow et al., 2013). Tenovil, the IL10 supplement, was tested in CD treatment and induced varying results in patients. Marlow et al. attempted to explain the inefficacy of Tenovil in CD and suggested that IL10 alone is unable to inhibit all the pro-inflammatory mediators in CD. This theory is supported in our model where IL10 addition induced a low percentage of $CD163^+$ macrophages when compared to the combined effect of TGFβ and IL10 (**Chapter 5**). Thereby, we propose the combined administration of Mongersen, and an IL10 supplement with higher mucosal bioavailability such as a bioengineered *Lactococcus lactis* that secretes IL10 (Braat et al., 2006; Steidler et al., 2000). Finally, IL10 supplementation can be maintained after treatment of inflammation to prevent relapse as suggested by a colitis model in rats (Barbara et al., 2000).

While further studies are necessary to fully comprehend the mechanisms of intestinal inflammation resolution, pro-resolving macrophages are central to re-establish homeostasis in the intestinal environment. Furthermore, research point to a flaw in monocyte transition to mature anti-inflammatory macrophages in patients with IBD. Consequently, therapeutic strategies that aim to re-educate pro-resolving macrophages are appealing.

6.3.1.2 *T cell plasticity*

We present evidence that P3 cells, together with pathogenic Th17 cells, drive intestinal inflammation. Pathogenicity of Th17 cells is best defined by their capacity to elicit disease and their plastic nature (Ahern et al., 2010; Ramesh et al., 2014; Wang et al., 2015). Several variants in the Th17 pathway have been linked to higher risk for IBD, such as *IL12B*, *IL23R*, *TYK2*, *JAK2*, *STAT3*, *CCL20*, *CCR6* and *RORC* (Barrett et al., 2008; Jostins et al., 2012).

The extensive infiltration of Th17 cells observed in IBD mucosa evoked the idea of neutralizing the main cytokine they produce, IL17A, as it promotes inflammation via the recruitment and activation of neutrophils, DCs and macrophages, and concommitant production of inflammatory cytokines (Abraham et al., 2017). Contrary to its impressive therapeutic efficacy in treatment of psoriasis, IL17A neutralization - by either blocking IL17A (secukinumab) or IL17RA (brodalumab) (Argollo et al., 2017; Targan et al., 2016)- was inefficient and sometimes even deleterious in IBD patients; thus, a phase 2 clinical trial was halted (Colombel et al., 2013; Hueber et al., 2012; Mozaffari et al., 2015). In fact, murine models of colitis reported mixed results with IL17 targeting (Maxwell et al., 2015; O'Connor et al., 2009; Ogawa et al., 2004). The increased intestinal inflammation observed was due to impaired regulation of the epithelial

tight junction protein occludin, decreased anti-microbial proteins as well as blockade of the protective IL17A secreted by lamina propria γδT cells in an IL23 independent manner (Lee et al., 2015; Maxwell et al., 2015). Additionally, fungal infections were reported in CD patients following IL17 neutralization (Hueber et al., 2012) which is consistent with the role of Th17 in protection against fungi and intracellular bacteria at mucosal surfaces (Abraham et al., 2017). Taken together, the harmful effects of blocking IL17 prevail over its benefits in treatment of intestinal inflammation.

Considering the protective role of Th17 cells, it is important to maintain this population. However, Th17 cells present with a range of phenotypes and display plasticity under the influence of the environment leading to the pathogenic IL17 and/or IFNγ secreting Th17 cells, referred to as Th17/Th1 and Th1* (Globig et al., 2014; Ramesh et al., 2014). Indeed, in the inflamed colonic mucosa of CD patients, we have shown a predominance of Th17/Th1 and Th1 cells over Th17 cells, suggesting the plasticity of Th17 cell lineage (**Annex 1**). Therefore, IL12 and IL23, secreted mainly by monocytes, macrophages and DCs, represent suitable targets as they act as a "switch" promoting Th17 plasticity towards the pathogenic Th17 phenotype (**Figure 6-2**). (Hirota et al., 2013; Ramesh et al., 2014).

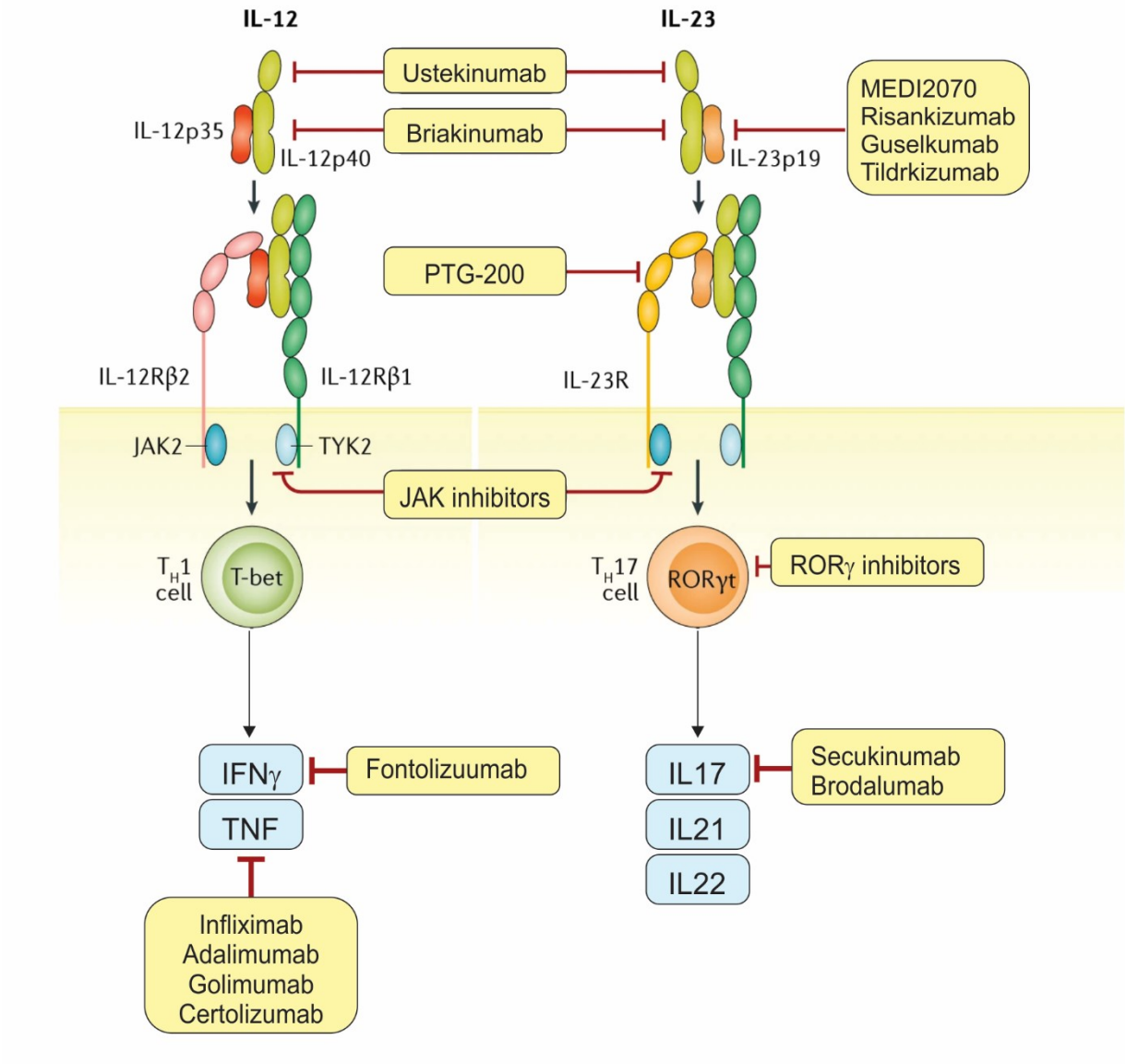

Figure 6-2: Targeting the IL12, IL23 and Th17 cell pathway.

IL23 plays a crucial role in the maintenance of Th17 cells that contribute to intestinal inflammation. The herterodimeric IL23 (p19 and p40) cytokine binds to its receptor (made up of IL23R and IL12Rβ1) and activates the JAK2-STAT3 signaling pathway. The latter controls the transcription of IL17, IL21 and IL22. In contrast, IL12 is more involved in Th17 plasticity. It is made up of p35 and p40 subunits, binds to its receptor (composed of IL12Rβ1 and IL12Rβ2), and utilizes JAK1, JAK2 and STAT4. IL12 contributes to Th1 and Th1 generation. Thus, biologics that target the p40 subunit disrupt Th1, Th1* and Th17 cells, whereas targeting p19 subunit blocks IL23-dependent regulation of Th17 cells.*
(Adapted from (Moschen et al., 2019); authorization code 4892431277928).

IL12 is known to promote plasticity of Th17 cells into Th1* by down-regulating IL17 and promoting IFNγ expression in circulating and intestinal Th17 cells (Annunziato et al., 2007; Kleinschek et al., 2009; Kobayashi et al., 2008; Ramesh et al., 2014) (**Chapter 2** and **Annex 1**).

We have extended the IL12 effect to include Th17 TEM cells isolated from the colon-draining MLN of IBD patients (**Chapter 4**). In mice, Th17 cells gradually progress to a pre-Th1 effector phenotype in the LN, and Th17/Th1-like effector phenotype in non-lymphoid tissues (Gaublomme et al., 2015), suggesting that Th17 conversion is already initiated in LN. Indeed, in CD MLN, Th17 T_{EM} cells displaying a pathogenic, "pre-Th1" inflammatory gene (*IFNG*, *HAVCR2, CD26)* profile (Bengsch et al., 2012) corroborate the progression observed in mice LN. Furthermore, the identification of low percentages of $CCR6^{+}CXCR3^{+}$ Th17/Th1 and $CCR6^{-}CXCR3^{+}$ IFNγ-secreting $CD4^{+}$ T cells in MLN of IBD patients (**Chapter 4** and **Annex 1**) supports the concept of plasticity of Th17 in LNs. Furthermore, Th17 TEM cells isolated from MLN of IBD patients converted to Th1* under the influence of IL12 (**Chapter 4**). This was independent of the differential molecular profile of Th17 TEM cells from CD or UC patients. Th17 program inhibition by IL12, as shown by decreased *TCF7* expression (Oestreich et al., 2011), depends on *Eomes* up-regulation that inhibits *RORC2* and *IL17A* expression while maintaining *IFNG* (Mazzoni et al., 2019).

Due to the role IL12 plays in promoting colitis, IL12p40 neutralizing antibody was effective in decreasing colitis in various mouse models. For example, pro-inflammatory cytokines and intestinal inflammation were suppressed in a IL12p40 treated TNBS model (Neurath et al.,

1995). Supported by extensive research, IL12p40 antagonists were tested in CD patients (Tait Wojno et al., 2019). Briakinumab and ustekinumab (targeting IL12p40 subunit) induced positive response when compared to placebo (Feagan et al., 2016; Fuss et al., 2006; Mannon et al., 2004; Sandborn et al., 2012). IL12p40 neutralization also suppressed other pro-inflammatory cytokines in mucosa of CD patients (Fuss et al., 2006; Mannon et al., 2004). Ustekinumab is FDA-approved for the treatment of CD patients, and in clinical trial for its employment in UC treatment (Verstockt et al., 2017).

Since IL23 also mediates pathogenic Th17 cell phenotype, it raises a fundamental question regarding treatment of IBD: should we target IL23 alone, or both IL12 and IL23 by neutralizing IL12p40? Both cytokines possess complementary functions in the generation of IFNγ and IL17 secreting T cells that play a role in protection against fungi and mycobacteria (Martinez-Barricarte et al., 2018; Zielinski et al., 2012). When Imamura *et al.* compared the suppression of mucosal inflammation in a T cell transfer model of colitis, using neutralizing mAb against IL23R, receptor specific for IL23, versus anti-IL12p40, they found comparable improvement in colitis and decrease in IL17 (Imamura et al., 2018). However, only IL23R mAb managed to decrease IFNγ expression. Likewise, *H. hepaticus*-triggered T cell dependent model of colitis in p35, p19 or p40 deficient mice demonstrates a role for IL23, rather than IL12, in induction of maximal grade of intestinal inflammation by driving IFNγ and IL17 secretion (Kullberg et al., 2006). These results suggest that IL23 and its receptor play a more important role than IL12 in colitis development (Uhlig and Powrie, 2018).

Data from our laboratory support targeting both IL12 and IL23 (**Chapters 2 and 4**, and **Annex 1**) (**Figure 6-3**), even though murine studies suggest a more prominent role for IL23 in colitis induction. First, IL12 induced pathogenic Th17 cells in mucosa and MLN of CD and UC patients. Second, IL23 was able to increase $IL17^{+}IFN\gamma^{+}$ in $CD4^{+}$ T cells from CD mucosa, without increasing GM-CSF, IL6 or TNF. Third, Th17 TEM cells from IBD MLNs did not modulate IL17 nor IFNγ expression in response to IL23. The lack of response to IL23 was not attributed to the absence of IL23R expression on Th17 cells in MLN, suggesting that tissue-dependent IL23 responsiveness requires additional signals provided by the cytokine milieu and/or environment, which might be absent or low in MLN. These results are consistent with clinical studies with risankinumab (targeting IL23p19) that downregulates genes linked with the IL23/IL17 axis and Th1 pathway in the mucosa of CD patients (Visvanathan et al., 2018). Although risankinumab induced endoscopic remission, it is not as effective as ustekinumab in treatment of CD patients (Feagan et al., 2017), suggesting a more advantageous outcome by targeting both IL12 and IL23. Anti-IL23p19 is in clinical trials for UC patients (Allocca et al., 2018); however the lack of response to IL23 we observed in the mucosa and MLN of UC patients advocates for anti-IL12p40 antibody. The apparent contradiction with mice studies strongly highlights the importance of studying immune response in humans, and more particularly in tissues.

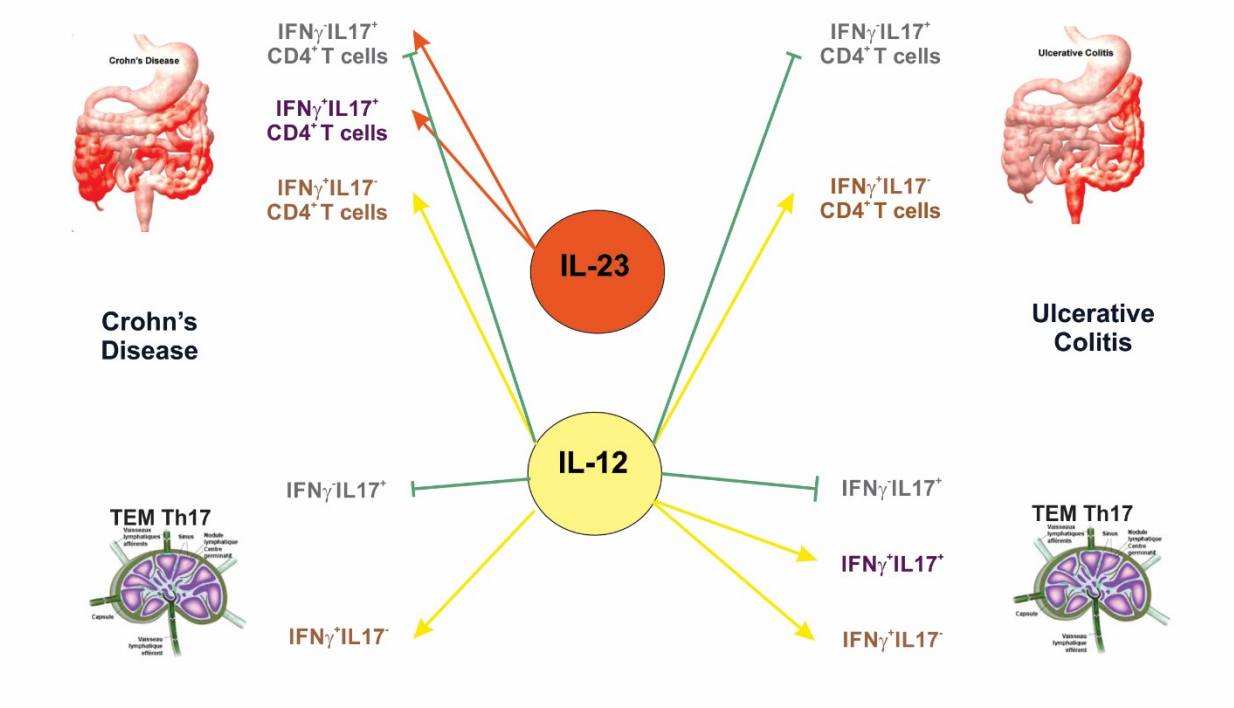

Figure 6-3: A summary of IL12 and IL23 induction of IFNγ and IL17 secretion by CD4⁺ T cells in mucosal and lymphatic tissue of IBD patients.
We have shown that while IL12 increased IFNγ and decreased IL17 expression in all tissues examined; IL23 only promoted Th17 and Th17/Th1 responses in CD mucosal tissue.

Finally, blocking IL23p19 is an attractive therapeutic approach in CD patients resistant to anti-TNF treatment. CD patients refractory to anti-TNF therapy presented with an upregulation of IL23p19 and IL23R expression (Sands et al., 2017; Schmitt et al., 2019), implying a role of IL23 in the resistance to TNF blockers. Indeed, a very recent study by Schmitt *et al*. found a buildup of IL17A and IFNγ expressing $TNFR2^{+}IL23R^{+}$ T cells in the mucosa of CD patients with endoscopic resistance to anti-TNF therapy (Schmitt et al., 2019). IL23 blocked anti-TNF-induced apoptosis of these Th17/Th1 cells leading to their accumulation. Treatment of these patients with MEDI2070, an IL23p19 blocker, proved efficacious as it showed significant clinical improvement compared to the placebo group.

Collectively, Th17 plasticity could be a valuable target in the treatment of IBD.

6.3.2 TARGETING CELL RECRUITMENT

Another therapeutic avenue in IBD pathogenesis involves interfering with immune cell trafficking between the blood vessel, lymphoid and mucosal tissue, especially T cells. Indeed, numerous clinical trials targeting integrins, selectins, immunoglobulin superfamily and chemokine receptors have been conducted for IBD (Gerhardt and Ley, 2015; Neurath, 2019; Zundler et al., 2019a) (**Figure 6-4**).

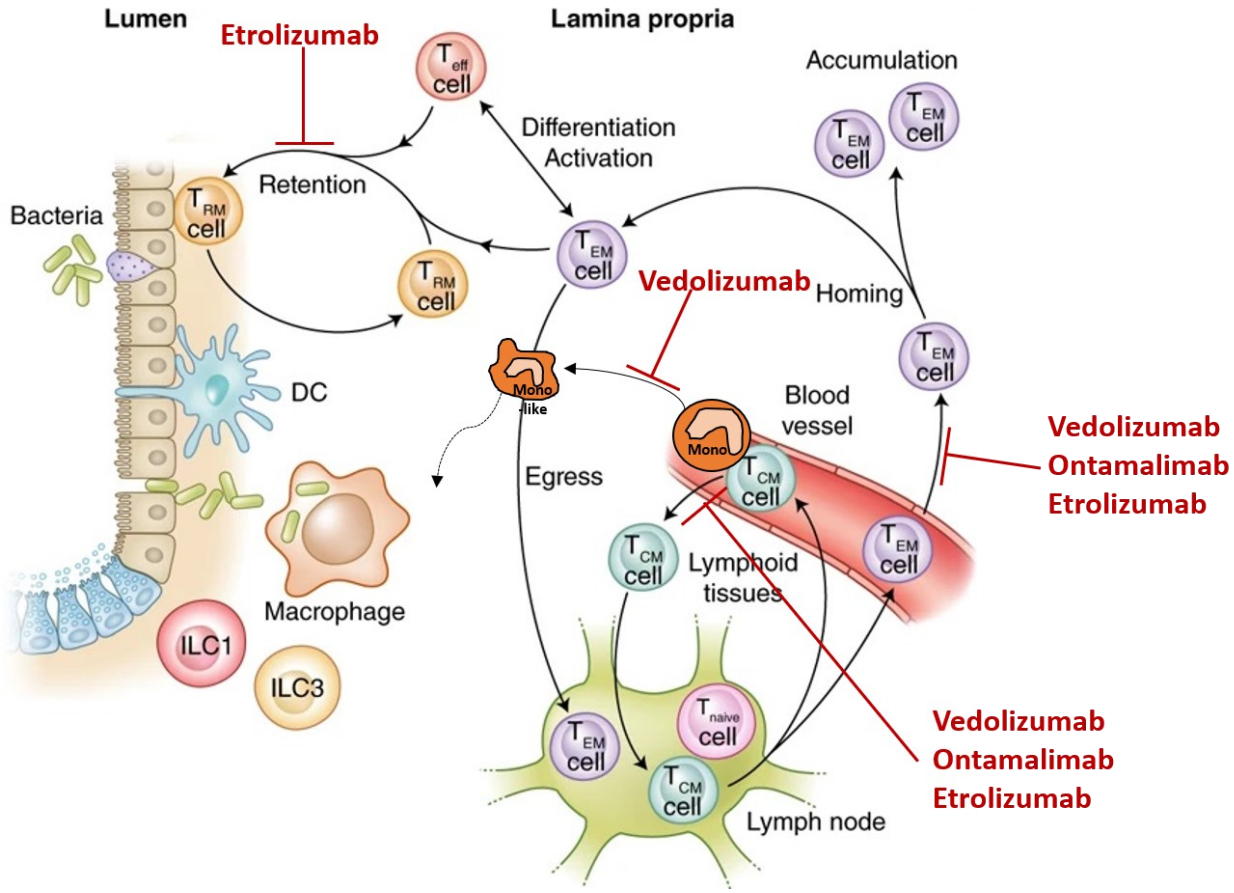

Figure 6-4: Targeting T cell and monocyte trafficking in IBD.
Several therapeutic agents have been developed to target T cell and monocyte recruitment to the inflamed intestine in IBD.

(Adapted from (Neurath, 2019); authorization code: 4660151258991).

The integrin α4β7 is key in mediating gut homing via its adhesion to MAdCAM-1 addressin on endothelial cells. Blocking the α4 subunit suppressed inflammation in a T cell transfer model of colitis in mice (Sugiura et al., 2013), implying a therapeutic benefit in targeting lymphocyte homing. Indeed, natalizumab, an anti-α4 monoclonal antibody, was efficient in CD (Ghosh et al., 2003; Sandborn et al., 2005). The anti-α4 antibody targeted the α4β1 integrin as well, which binds to the widely expressed VCAM-1 and lead to undesirable side effects and eventual discontinuation (Van Assche et al., 2005). Therefore, vedolizumab targeting the α4β7 heterodimer was developed and FDA-approved for the treatment of CD and UC in 2014 (Feagan et al., 2013; Sandborn et al., 2013). It specifically blocks *in vivo* lymphocyte homing to the gut - as MAdCAM-1 expression is mainly gut-specific – resulting in decrease of naïve and TEM accumulation and expansion in the gut (Wyant et al., 2015; Zundler et al., 2019a). Additionally, vedolizumab interferes with the α4β7-dependent gut homing of monocytes (Schleier et al., 2019), ILCs (Kim et al., 2015b), and may also hinder with the recruitment of α4β7-expressing plasmablasts (Bowman et al., 2002; Schippers et al., 2012). Also, a monoclonal antibody against the α4β7 ligand MAdCAM-1 (ontamalimab) is currently under study for the treatment of IBD (Sandborn et al., 2018; Vermeire et al., 2017). Other molecules involved in cell trafficking to the gut have been tested in the context of IBD such as blockers of CCR9 and ICAM-1,but did not give a desirable outcome (Zundler et al., 2019a). Taken together, the α4β7-MAdCAM-1 axis is pertinent in IBD therapy.

Another β7 inhibitor is etrolizumab that blocks α4β7 as well as E-cadherin ligand αEβ7 (CD103). The latter is expressed on pro-inflammatory Th17, Th17/Th1 and Th9 cells in the intestine (Zundler et al., 2017) but CD103 or α4β7 expression was not observed on colonic $IL8^+$ T cells in UC, suggesting a differential mode of recruitment (**Chapter 2**). In addition to targeting Th9 cells, CD103 blockade interferes with lymphocyte retention close to the epithelium, consequently targeting $CD103^+CD69^+$ intestinal TRM shown to play a role in chronic intestinal inflammation (Zundler et al., 2019b; Zundler et al., 2017). Thereby, targeting cell trafficking and tissue retention represents a therapeutic avenue in IBD.

Interfering with monocyte recruitment to the intestinal tissue may present as an attractive option since monocytes and their derivatives play a crucial role in the development of intestinal inflammation. Furthermore, inhibition of monocyte recruitment by targeting CCR2, CCL2 or CCR5 protected mice from DSS-induced colitis (Bain et al., 2013; Tacke et al., 2007; Zigmond et al., 2012). However, these cells also give rise to pro-resolving macrophages that heal the tissue and promote an anti-inflammatory response. Therefore, targeting the recruitment of monocytes to the inflamed tissue could be double-edged and dependent on the stage of inflammation or resolution process.

6.4 Conclusion and perspectives

The research that contributed to this thesis aimed at better understanding the immune-mediated pathogenesis of CD and UC by examining MNP subpopulations and $CD4^+$ Th cells in the colonic mucosa and MLN of IBD patients, as well as developing a two-step culture model that mimiced the intestinal monocyte differentiation cascade.

We identified a monocyte-like $CD14^+$ population (P3) that accumulated in the inflamed mucosa of IBD patients in correlation with disease severity. This augmentation was not associated with the infiltration of pro-inflammatory $CD14^+CD16^+$ $Slan^+$ cells observed in CD mucosa. Monocyte-derived P3 cells drive inflammation in part by promoting a pathogenic T cell response, distinguished in CD and UC by IL8 production (**Chapter 2**). This observed difference could be related to the differential molecular profile seen in Th17 TEM cells from MLN (**Chapter 4**) that travel to the mucosa and get shaped by the P3 cells.

A second $CD14^+$ population was observed in inflamed tissue of IBD patients, the anti-inflammatory P4 macrophages. The latter derive from inflammatory monocyte-like cells under the combined influence of TGFβ and IL10, as demonstrated by our *in vitro* model.

A better understanding of the immunopathology of IBD would help identify novel therapeutic targets and shed light for the development of personalized treatments. Although we have shed some light on MNPs and Th17 cells in the context of intestinal inflammation, there are still a lot of unsolved questions.

$CD4^+$ T cell populations have been the main targets in IBD treatments. We have identified a few differences in the $CD4^+$ T cell response in CD and UC patients beyond the now outdated classical Th1 and Th2 paradigm. Differences were noted in the colonic mucosa and MLN in terms of Th distribution, cytokine secretion and molecular signature. Single cell RNA sequencing could prove very valuable in better segregating, characterizing and identifying the various T cell populations in the mucosa and lymphoid tissues from healthy versus CD versus UC patients. The function of the "better defined" T cell subpopulations would require verification and plasticity in response to cytokines and APC populations would be assessed. Single cell RNA sequencing analysis should also examine the various MNP subsets in CD versus UC, in both mucosal and lymphoid tissues. These studies might segregate the treatments employed for CD and UC.

Another therapeutic avenue in IBD could be targeting monocyte plasticity "aiming for the good" macrophages. The *in vitro* model developed could be a great tool to test some molecules that will push the Infl mo-like cells into the non-pathogenic $CD163^+$ macrophages, such as Mongersen, IL10 supplements, JAK inhibitors and monoclonal antibodies available on the market. Although the model recapitulates some of the key phenotypic, morphological, functional and molecular features observed in the P3 and P4 populations isolated from inflamed IBD colonic mucosa, it still lacks the multi-part composition in the tissue environment. Colonic organoids starting from healthy, CD or UC biopsies could stimulate one extra layer of the complexity found *in vivo*. Furthermore, this improved model could account for the contribution of the microbiota in shaping monocyte development. The *in vitro* model could also perhaps aid in the development of personalized patient treatments. For instance, the model could be

developed starting from peripheral blood monocytes, colonic biopsies and microbiota samples from the same IBD donor; different molecules could thus be tested for the best patient response.

Monocytes, macrophages, Th17, Th17/Th1 and Th1* cells are only a few players in the complex network of innate and adaptive immune cells contributing to IBD pathogenesis, and being targeted in IBD therapy. Despite the plethora of treatments, many IBD patients are still suffering.

Treatments should be developed with the realization that human beings are not syngeneic mice raised in a controlled environment. They are complex resulting in expected "unexpected outcomes" that should be tolerated and conceptually dealt with ahead. This understanding will require the integration of data using computational and systems approach (Davis et al., 2017) that will examine data from colitis models, healthy humans and IBD patients taking into account the patient's genetic background and course of treatment. Eventually, this information will advise on the best employment of a particular therapeutic treatment in the management of CD and UC.

Chapter 7 :

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Chapter 8 :

ANNEX 1 to 3

Annex 1.

Two distinct colonic CD14^{+} subsets characterized by single-cell RNA profiling in Crohn's disease

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Author's contribution	I participated to the experimental work (50%), some data analysis (40%) and drafting of the manuscript (40%).
Journal	Mucosal Immunology
Status	Published May 2019
DOI	10.1038/s41385-019-0242-5
Key words	Crohn's disease, mononuclear phagocytes, macrophages, Th17 cells, Th1 and single cell RNA sequencing.

Two distinct colonic CD14$^+$ subsets characterized by single-cell RNA profiling in Crohn's disease

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Inflammatory bowel diseases are associated with dysregulated immune responses in the intestinal tissue. Four molecularly identified macrophage subsets control immune homeostasis in healthy gut. However, the specific roles and transcriptomic profiles of the phenotypically heterogeneous CD14$^+$ macrophage-like population in inflamed gut remain to be investigated in Crohn's disease (CD). Here we identified two phenotypically, morphologically and functionally distinct colonic HLADR$^+$SIRPα$^+$CD14$^+$ subpopulations that were further characterized using single-cell RNA-sequencing (scRNAseq) in CD. Frequencies of CD64^{hi}CD163$^{-/dim}$ cells selectively augmented in inflamed colon and correlated with endoscopic score of disease severity. IL-1β and IL-23-producing CD64^{hi}CD163$^{-/dim}$ cells predominated over TNF-α-producing CD64^{hi}CD163^{hi} cells in lesions. Purified "inflammatory monocyte-like" CD163$^-$, but not "macrophage-like" CD163^{hi} cells, through IL-1β, promoted Th17/Th1 but not Th1 responses in tissue memory CD4$^+$T cells. Unsupervised scRNAseq analysis that captures the entire HLADR$^+$SIRPα$^+$ population revealed six clusters, two of which were enriched in either CD163$^-$ or CD163^{hi} cells, and best defined by *TREM1/FCAR/FCN1/IL1RN* or *CD209/MERTK/MRCl/CD163L1* genes, respectively. Selected newly identified discriminating markers were used beyond CD163 to isolate cells that shared pro-Th17/Th1 function with CD163$^-$ cells. In conclusion, a molecularly distinct pro-inflammatory CD14$^+$ subpopulation accumulates in inflamed colon, drives intestinal inflammatory T-cell responses, and thus, might contribute to CD disease severity.

Mucosal Immunology _####################_; https://doi.org/10.1038/s41385-018-0126-0

INTRODUCTION

The interplay between genetic, environmental, and immunological factors contributes to the pathogenesis of Crohn's disease (CD), a chronic inflammatory bowel disease (IBD) that results from an inappropriate immunologic response to the commensal microflora.[1,2] Several experimental models of colitis were developed in syngeneic mice to unravel CD pathogenesis.[3] However, none of them entirely recapitulates the complex clinical and histopathological features that are observed in CD patients, emphasizing the need to examine the immune response in human gut disease tissues.[4] Intestinal immune infiltrate is composed of T helper (Th) cell subsets,[5] granulocytes,[6,7] mononuclear phagocyte (MNP) populations[8], as well as rare innate lymphoid cells (ILCs).[9,10] Mucosal MNPs, which includes macrophages (Mϕ), monocyte-derived cells and dendritic cells (DCs), expressing or not CD14 and SIRPα,[11–13] contribute to gut homeostasis. Therefore, investigating the function and molecular profile of the MNP population, which is phenotypically heterogeneous in inflamed gut mucosa, has clear implication in better understanding IBD pathophysiology.[14]

A large population of human intestinal resident Mϕ actively maintains healthy steady-state conditions. Previous studies reported that these Mϕ lack CD14 and CD64 expression, are anergic in response to Toll-like receptor (TLR) stimulation, and retain phagocytic functions.[15] However, it was recently proposed that recruitment and maturation of human monocytes into four Mϕ subsets expressing various levels of CD14 and retaining endocytic function occurred in jejunum at homeostasis.[13] A similar maturation process was previously demonstrated in murine gut lamina propria where Mϕ arise from Ly6C^{hi} monocytes, which are continuously recruited to the mucosa. These cells differentiate towards TLR-hyporesponsive Mϕ that secrete IL-10 and express CD64 at steady state.[16]

In addition to intestinal resident Mϕ, conventional DC (cDCs) subsets that drive the polarization of naive CD4 T cells[12,17] are key players in the maintenance of regulatory T-cell-mediated intestinal homeostatic conditions.[18] Briefly, two cDC subsets stratified by SIRPα and CD103 expression levels in humans and mice have been reported in gut homeostatic state.[12,19–21] More specifically, human SIRPα$^-$ cDC1s that express CD103 represent a minor population in the gut,[20,22] which is related to circulating CLEC9A$^+$CD141$^+$ cDC1.[12] The CD103$^+$SIRPα$^+$ cDC2 are the predominant cDC population in the ileum[22] and are related to circulating CD1c$^+$ cDC2.[23] In contrast, CD103$^-$SIRPα$^+$ cDC2s predominate in the colon but are the less characterized cDC population.[24,25] In fact, these CD103$^-$SIRPα$^+$ cells, which reportedly clustered with blood monocytes, might include monocyte-derived cells.[12] Noteworthy, markers commonly associated with circulating monocytes and cDC2 appeared to be more broadly expressed than previously

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Received: 3 October 2018 Revised: 5 December 2018 Accepted: 11 December 2018
Published online: 22 January 2019

appreciated. For example, CD14$^+$ monocytes were shown to co-express CD1c,[26] a cDC2 marker, while CD1c$^+$ DC subsets were reported to express several monocyte markers, including CD14.[27]

The characterization of the MNP population is far more challenging in human inflamed gut tissue, owing to the heterogeneity of IBD patients with differences in genetic background, disease onset, duration, severity, treatment, and geographic location.[28] In mice, it was shown that the maturation process of Ly6C^{hi} monocytes is interrupted during intestinal inflammation favoring the accumulation of an intermediate Ly6C$^+$CD64$^+$CX$_3$CR1^{int} monocyte-like cell subset that shows a pro-inflammatory profile.[16] Noteworthy, infusion of autologous circulating radiolabeled CD14$^+$ monocytes into CD patients can be retraced as CD14$^+$ Mϕ-like cells in their inflamed gut mucosa.[29] However, analyses deciphering the composition of human CD14$^+$ MNP subpopulations in inflamed intestine remain sparse. Kamada et al. reported the presence of a unique CD14$^+$ Mϕ-like cell population, which produce large amounts of IL-23, IL-1β, IL-6, TNF-α, and IL-10 in the inflamed ileal or colonic mucosa of IBD patients.[30] Our previous report showed that HLADR$^+$SIRPα$^+$ cells, which predominantly include CD14$^+$ cells, but also comprise cDC2, selectively accumulate in inflamed colon of CD patients, and that IL-1β and TNF-α secretion was restricted to HLADR$^+$SIRPα$^+$ cells.[11] Yet, the potential heterogeneity of mucosal CD14$^+$ MNPs under chronic inflammatory conditions is highlighted by the dual function of these cells. The CX$_3$CR1$^+$CD14$^+$ MNPs favor IL-22 production by ILC3 in an IL-23-dependent manner in human mild-inflamed CD mucosa,[31] suggesting their protective function. Furthermore, several groups of investigators have stratified CD14$^+$ MNPs according to their phenotype. CD14$^+$HLADR^{high}CD163^{low} Mϕ are detected in equal proportions in non-inflamed and inflamed CD or UC mucosa[32,33], while CD14$^+$HLADR^{dim} are increased in inflamed ileum and colon of CD or UC patients.[16,34,35] Whether CD14$^+$ HLADR$^{high/dim}$ MNPs represent molecularly distinct subpopulations endowed with functional diversity in inflamed CD tissues remain unanswered.

The efficacy of Vedolizumab, a monoclonal antibody (mAb) that targets the gut homing integrin α4β7, argues for a T-cell-dependent mechanism in CD patients.[36] Studies have provided evidence that HLADR$^+$CD14$^+$MNPs induce human allogeneic circulating naive T-cell polarization into Th17 or Th17/Th1.[32,37] Whether and how these cells drive intestinal inflammatory T-cell responses and more particularly promote Th17/Th1 responses in mucosal effector memory CD4$^+$ T cells, has not been investigated in inflamed gut mucosa. Double IFN-γ/IL-17-secreting cells are considered pathogenic in mice with colitis[38] and in patients with CD.[5,39,40] The IFN-γ$^+$IL-17$^+$ cells might arise from Th17 cells, which display a high degree of plasticity.[41] Indeed, Th17 cells, which exert a protective role in the intestine,[42,43] can acquire a pathogenic signature and function depending on the tissue cytokine environment.[42] In humans, intestinal Th17 clones can be skewed to produce IFN-γ, shifting to IFN-γ$^+$IL-17$^+$ (Th17/Th1) and IFN-γ$^+$IL-17$^-$(non-classic Th1 or Th1*) cells in the presence of IL-12[39] or IL-23.[44]

Here, we assessed the morphology, function and molecular diversity of HLADR$^+$SIRPα$^+$ MNPs that were stratified by CD14, CD64, and CD163 protein expression, in colonic tissues from a large cohort of CD patients. The CD14$^+$CD64^{hi}CD163$^{-/dim}$ and more precisely the CD163$^-$cells selectively accumulated in inflamed CD colon, expressed low levels of TNF-α, and promoted colonic memory Th17 and Th17/Th1 but not Th1 responses in an IL-1β-dependent manner. CD163$^-$ cell accumulation in inflamed mucosa was independent of age, gender, disease location, disease behavior, and remarkably, their frequency correlated with endoscopic disease severity. Isolation of CD14$^+$ subpopulations from two extremes of CD163 expression spectrum, i.e., CD163$^-$ (P3) and CD163^{hi} (P4), led to the identification of two morphologically and molecularly distinct CD14$^+$subsets, while further identifying new discriminating markers that allowed validation of the presence of two distinct functional CD14$^+$ subsets.

RESULTS

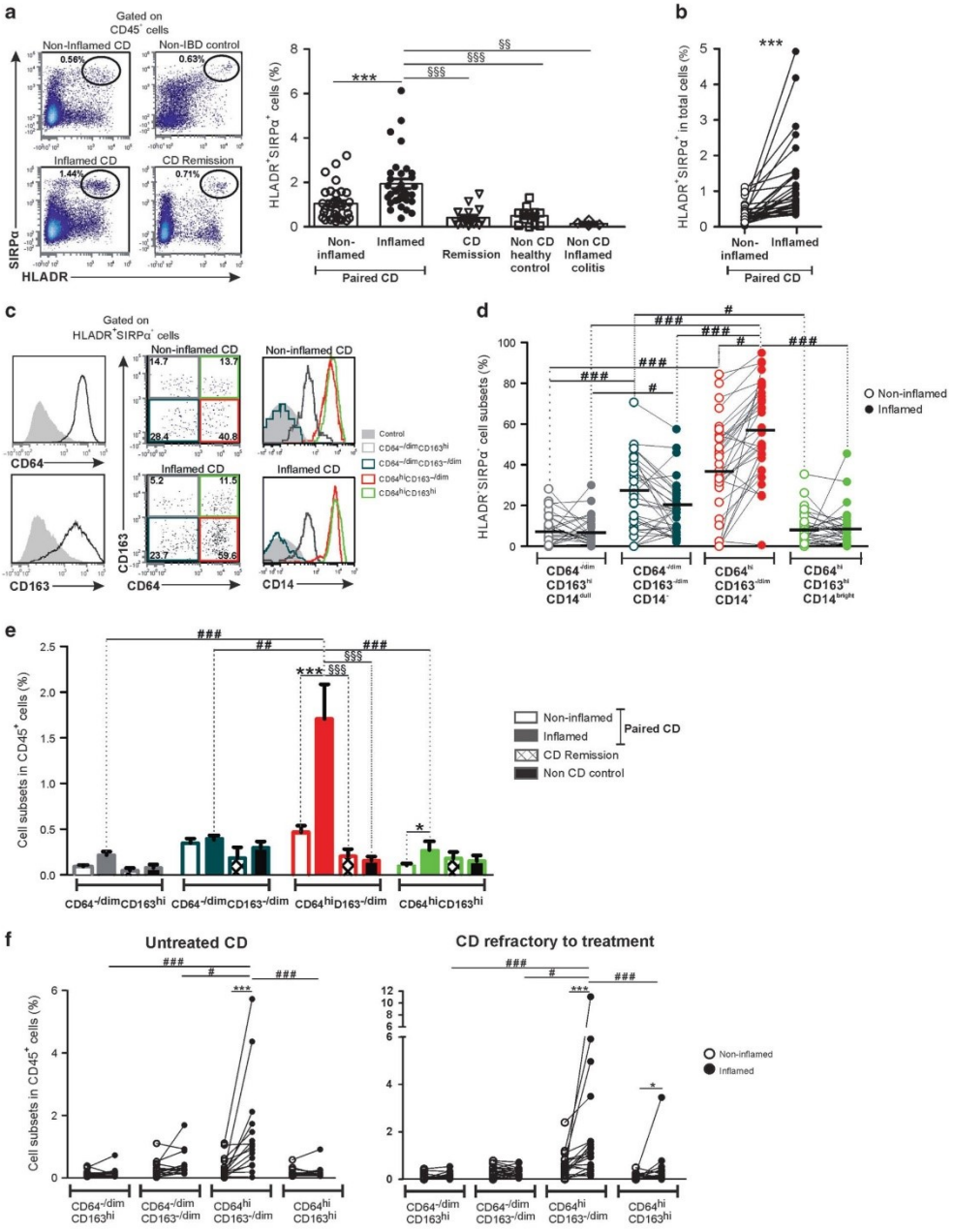
HLADR$^+$SIRPα$^+$ MNPs comprise two CD14$^+$CD64^{hi} subpopulations: accumulation of CD64^{hi}CD163$^{-/dim}$, but not CD64^{hi}CD163^{hi} cells, in inflamed colon of CD patients

After excluding dead cells, doublets and neutrophils and gating on hematopoietic CD45$^+$ cells (Figure S1), the average frequency of HLADR$^+$SIRPα$^+$ MNPs was significantly greater in inflamed tissues relative to paired non-lesional sites in the colon of patients with CD ($n = 36$) ($P < 0.0001$; Fig. 1a). Considering the increased cellular infiltrate in inflamed CD tissues (mean 54.1% (± SD 21.1) versus 34.6% (± SD 16.0) CD45$^+$ cells in inflamed versus non-inflamed colon), the augmentation of HLADR$^+$SIRPα$^+$ MNPs in total cells further reflected their accumulation in lesions ($P < 0.0001$; Fig. 1b). We therefore postulated that HLADR$^+$SIRPα$^+$ MNPs could be associated with disease pathogenesis. The frequency of this population was significantly reduced in healthy colonic mucosa of CD patients in endoscopic remission ($P < 0.0001$; Fig. 1a) to similar levels as those observed in non-inflamed mucosa of CD as well as in healthy non-CD donors ($P < 0.0001$). Moreover, HLADR$^+$SIRPα$^+$ cells were detected at a lower frequency in inflamed colon of patients with infectious or drug-induced colitis ($P < 0.002$; Fig. 1a) relative to inflamed CD colon.

To assess potential heterogeneity within the HLADR$^+$SIRPα$^+$ MNPs infiltrating inflamed colon, this population was subdivided according to CD64 and CD163 expression levels (Fig. 1c). CD163, a scavenger receptor expressed on human gut Mϕ and CD64 (the Fc-gamma receptor 1), has been reported to be an inflammatory DC or Mϕ marker in chronic inflammatory disorders in humans.[45,46] Four subpopulations were identified: two CD64$^{-/dim}$ (left quadrants) as well as two CD64^{hi} cells (right quadrants) expressing variable intensity of CD163 (i.e., CD163$^{-/dim}$ and CD163^{hi}) (Fig. 1c). Further immunophenotyping highlighted that the two CD64^{hi} populations expressed high levels of CD14 (Fig. 1c); mean fluorescence intensity of SIRPα, its isoform SIRPβ, CX3CR1, or CD103 (Figure S2A) could not discriminate these two CD14$^+$ subpopulations. In contrast, HLADR expression was significantly higher in CD64^{hi}CD163^{hi} when compared to CD64^{hi}CD163$^{-/dim}$ cells but reduced in inflamed versus non-inflamed colon in all three subsets examined (Figure S2B). Furthermore, CD64$^{-/dim}$CD163$^{-/dim}$ cells were CD14$^-$ and overrepresented among the HLADR$^+$SIRPα$^+$ MNPs that expressed CD1c (Figure S2C).

Next, we assessed whether any of the 4 HLADR$^+$SIRPα$^+$ MNPs subpopulations accumulated in inflamed CD colonic lesions. As depicted in Fig. 1d, only the frequency of one CD14$^+$subset, i.e., the CD64^{hi}CD163$^{-/dim}$ cells, significantly increased in inflamed versus paired non-inflamed mucosa of CD patients ($P < 0.05$). When accounting for the increased percentage of the entire HLADR$^+$SIRPα$^+$ MNP population in inflamed infiltrate versus non-inflamed colon (Fig. 1a), the accumulation of CD64^{hi}CD163$^{-/dim}$ cells was further highlighted compared to CD in remission and non-CD control donors as well as to paired non-inflamed CD tissue ($P < 0.0001$) (Fig. 1e). The cohort of active CD patients was next subdivided based on treatment history at the time of sample collection (Fig. 1f). The frequencies of CD64^{hi}CD163$^{-/dim}$ cells increased in inflamed when compared to paired non-inflamed colon and remained the predominant HLADR$^+$SIRPα$^+$ cell subset in both treated and untreated groups. Notably, a slight accumulation of CD64^{hi}CD163^{high} cells was noticed in inflamed relative to non-inflamed samples in patients undergoing treatment.

Overall, these data provide evidence that HLADR$^+$SIRPα$^+$ MNPs accumulate in inflamed colon of patients with CD. These MNPs are heterogeneous and comprise two CD14$^+$CD64^{hi} (CD163$^{-/dim}$ and CD163^{hi}), one CD14^{dull} (CD64$^{-/dim}$CD163^{hi}), and one CD14$^-$(CD64$^{-/dim}$CD163$^{-/dim}$) subpopulations. However, only the CD64^{hi}CD163$^{-/dim}$ cells augment in inflamed CD colon.



Frequency of CD64^{hi}CD163$^{-/dim}$ cells correlates with disease severity in CD patients and is not modified by treatment history

To assess the potential biological relevance of CD64^{hi}CD163$^{-/dim}$ cell accumulation in inflamed CD colon, we measured disease severity using the validated Simple Endoscopic Score for CD (i.e., SES-CD) (Table S1)[47] in an independent cohort of CD patients. The data showed that the percentage of CD64^{hi}CD163$^{-/dim}$ but not CD64^{hi}CD163^{hi} cells in HLADR^{+}SIRPα^{+} MNPs positively correlated with SES-CD ($P < 0.0001$; Fig. 2a). Furthermore, frequencies of CD64^{hi}CD163$^{-/dim}$ and CD64^{hi}CD163^{hi} cells were not modified by treatment history that included biologics combined or not with immunosuppressive drugs, in none of the two independent

Fig. 1 Frequencies of CD14$^+$CD64^{hi}CD163$^{-/dim}$ cells increase in inflamed colon of CD patients. **a** Percentage of colonic HLADR$^+$SIRPα$^+$ cells after gating on CD45$^+$ LPMC in active CD patients ($n = 36$) (paired non-inflamed and inflamed samples, Wilcoxon signed rank test); CD in remission ($n = 15$) and non-CD (healthy, $n = 12$; colitis, $n = 4$) patients (Mann–Whitney test). **b** Percentage of HLADR$^+$SIRPα$^+$ cells in total cells in active CD patients ($n = 36$, Wilcoxon signed rank test). **c** HLADR$^+$SIRPα$^+$ MNPs stained for CD64 and CD163 expression, representative histograms with isotype control mAbs (left panels). HLADR$^+$SIRPα$^+$ MNPs subdivided according to intensity of CD64 and CD163 expression into 4 MNPs subpopulations; representative dot plots (middle panels) and CD14 expression (right panels). **d** Frequencies of the 4 MNPs (CD64$^{-/dim}$CD163^{hi}, CD64$^{-/dim}$CD163$^{-/dim}$, CD64^{hi}CD163$^{-/dim}$, CD64^{hi}CD163^{hi} cells) in HLADR$^+$SIRPα$^+$ cells in paired non-inflamed and inflamed CD patients ($n = 36$, Friedman test). **e** Percentage of the 4 MNPs in CD45$^+$ cells in paired non-inflamed and inflamed CD patients ($n = 36$), CD in remission ($n = 15$), and non-CD control ($n = 12$) patients (Wilcoxon signed rank, Mann–Whitney and Friedman tests). **f** Frequencies of the 4 MNPs in CD45$^+$ LPMC in paired non-inflamed and inflamed tissues in untreated CD patients ($n = 15$, left panel) and those refractory to treatment ($n = 21$, right panel) (Wilcoxon signed rank and Friedman tests). For (**a**, **e**, **f**), $P < 0.01$ threshold for significance in Wilcoxon signed rank test and Mann–Whitney test to account for test multiplicity

cohorts examined separately (Fig. 2b). We therefore combined the two cohorts ($n = 97$) and showed that age, gender, age at diagnosis, disease location (L2 and L3) and disease behavior (B1, B3, and B3) did not influence the percentage of HLADR$^+$SIRPα$^+$ MNPs or CD64^{hi}CD163$^{-/dim}$ and CD64^{hi}CD163^{hi} cells in HLADR$^+$SIRPα$^+$ MNPs (Figure S3A, B, C, D and E). Remarkably, the CD64^{hi}CD163$^{-/dim}$ cells remained the predominant HLADR$^+$SIRPα$^+$ cell subset in CD colon, regardless the parameters used for stratification of CD patients, with the exception of colonic tissue from CD patients with penetrating B3 disease, in which the average frequency of CD64^{hi}CD163$^{-/dim}$ was slightly reduced.

Collectively, these observations indicate that one particular CD14$^+$ subpopulation, the CD64^{hi}CD163$^{-/dim}$ cells, accumulates in inflamed CD colon in proportions that correlate with disease severity regardless of treatment history, demographics, and disease classification.

CD64^{hi}CD163$^{-/dim}$ cells are the major contributors to IL-23 and IL-1β secretion but not TNF-α in inflamed CD colon

Building on the previous report of TNF-α and IL-1β production being restricted to SIRPα$^+$ cells in inflamed CD colon,[11] we examined which of CD14$^+$CD64^{hi} cell subset expressed pro-inflammatory cytokines ex vivo (Fig. 3). Frequencies of TNF-α-producing-cells were higher in CD64^{hi}CD163^{hi} cells between non-inflamed and inflamed colonic tissues ($P < 0.05$), undetectable or low in CD64^{hi}CD163$^{-/dim}$ and CD64$^{-/dim}$CD163$^{-/dim}$ cells, and observed in only 2/15 patients in the CD64$^{-/dim}$CD163^{hi} cells (Fig. 3a and Figure S4A). In contrast, the percentages of IL-23-producing-cells were significantly more abundant in CD64^{hi}CD163$^{-/dim}$ and CD64^{hi}CD163^{hi} cells in inflamed mucosal samples ($P < 0.001$ in 13/15 patients and $P < 0.05$ in 9/15 patients, respectively) while their frequencies were lower in the two CD64$^{-/dim}$ subpopulations. Despite the fact that both CD14$^+$CD64^{hi} subpopulations produced IL-1β, only IL-1β–producing CD64^{hi}CD163$^{-/dim}$ cells were significantly enriched in inflamed versus non-inflamed mucosal samples (CD64^{hi}CD163$^{-/dim}$, $P < 0.02$; CD64^{hi}CD163^{hi}, $P = 0.4$), and detected at variable but low frequencies in CD64^{dim} cells. IL-6 levels were highly variable in CD14$^+$CD64^{hi} subsets across the different tissue states tested, and higher in CD64^{hi} compared to CD64$^{-/dim}$ cells. Furthermore, when considering the increased frequencies of HLADR$^+$SIRPα$^+$ MNPs in inflamed colon, TNF-α-producing CD64^{hi}CD163^{hi} cells were more frequent than TNF-α-producing CD64^{hi}CD163$^{-/dim}$ cells ($P < 0.004$). Conversely, IL-23$^-$ ($P < 0.0005$) and IL-1β- ($P < 0.03$) producing CD64^{hi}CD163$^{-/dim}$ cells were higher than CD64^{hi}CD163^{hi} cells in CD45$^+$ cells (Fig. 3b). Treatment including biologics did not influence the frequencies of cytokine-producing-cells in either of the CD14$^+$CD64^{hi} subsets (Figure S4B). We further examined the expression of anti-inflammatory cytokine IL-10 in the two CD14$^+$CD64^{hi} subsets (Figure S4C). The percentage of IL-10-producing-cells, as well as IL-10 expression per cell were higher in CD64^{hi}CD163^{hi} relative to CD64^{hi}CD163$^{-/dim}$ cells in inflamed colon ($P < 0.002$). Finally, single IL-23 or IL-1β-producing-cells predominated in CD64^{hi}CD163$^{-/dim}$ when compared to CD64^{hi}CD163^{hi} cells in 3/4 patients examined (Fig. 3c and Figure S4D).

Overall, these results highlight that among the 4 HLADR$^+$SIRPα$^+$ MNP subsets, the CD64^{hi}CD163$^{-/dim}$ cells accumulate in greater proportions in inflamed CD mucosa and are thus the major contributor to IL-23 and IL-1β. CD64^{hi}CD163^{hi} cells represent the major TNF-α or IL-10-producing-cells in inflamed colon.

Colonic CD64^{hi}CD163$^-$ (P3) cells skew autologous CD4$^+$ T cells towards Th17/Th1 responses in CD patients

We next asked whether and how the colonic CD14$^+$ subsets influenced the Th17/Th1 profile of autologous CD4$^+$ T cells in CD patients. To this end, we simultaneously purified from inflamed CD colon, CD4$^+$ T cells that were depleted of CD25$^+$ regulatory T cells, as well as CD14$^+$CD64^{hi} subpopulations at the two extremes of CD163 expression spectrum (i.e., CD163$^-$ and CD163^{hi}), thus excluding the CD163^{dim} cells (Fig. 4a). Noteworthy, within the CD163$^{-/dim}$ subpopulation, the CD163$^-$ cells remained the predominant subpopulation that significantly infiltrated the inflamed colon while the frequency of CD163^{dim} cells did not increase relative to paired non-inflamed sites (Figure S5A). Furthermore, the frequency of CD163$^-$, but not CD163^{dim} cells correlated with the endoscopic disease severity ($P < 0.0002$, $r = 0.46$, Spearman test). The two highly purified CD14$^+$CD64^{hi} subsets were hereafter named P3 (CD163$^-$) and P4 (CD163^{hi}) cells with P3 cells displaying a kidney shaped nucleus and a reduced cell size when compared to P4 cells, that exhibited typical Mϕ morphology with vacuoles (Figure S5B). The intermediate CD14$^{-/dim}$CD64^{dim} (P1) and the triple negative CD14$^-$CD64$^-$CD163$^-$ cells (P2) were morphologically heterogeneous populations and therefore not further examined in functional studies.

Data in Fig. 4b indicated that in the context of co-culture with P3 cells was a significantly greater proportion of IFN-γ$^+$IL-17$^+$ and IFN-γ$^-$IL-17$^+$ cells in colonic CD4$^+$ T cells observed ($P < 0.002$), with no effect on IFN-γ$^+$IL-17$^-$ T cells. The low yield of P4 cells, which represented on average less than 0.2% of HLADR$^+$SIRPα$^+$ MNPs in CD45$^+$ cells (Fig. 1e), precluded performing more than three co-cultures experiments. However, the P4-Mϕ-like cells did not appear to augment memory intestinal Th17, Th17/Th1, or Th1 responses.

Noteworthy, colonic CD4$^+$ T cells contained higher frequencies of cells displaying phenotypes associated with Th1 (CCR6$^-$CXCR3$^+$) and Th17/Th1 (CCR6$^+$CXCR3$^+$) in inflamed mucosa when compared to their mesenteric lymph node (MLN) counterparts (Fig. 4c). In contrast, Th17 (CCR6$^+$CXCR3$^-$) cells represented more than 25% of CD4$^+$ T cells in inflamed MLNs and less than 10% in inflamed CD colon ($P < 0.001$; Fig. 4c). In this context, we further asked whether CCR6$^+$CXCR3$^-$CD62L^{low}CD4$^+$ (Th17 T_{EM}) MLN cells that recently migrated to the colon could be readily shaped by P3 cells to acquire a Th17/Th1 cytokine profile. To this end, Th17 T_{EM} were purified from either MLN or colon and co-cultured with colonic P3 cells. Th17/Th1, Th17 but not Th1, responses were significantly augmented by P3 cells in CCR6$^+$CXCR3$^-$ T_{EM} cells (Fig. 4d). Note that the minor colonic CCR6$^+$CXCR3$^-$ T_{EM} subset comprised increased frequencies of single IFN-γ-producing-cells (~50%), relative to their MLN (~2%) counterpart. Finally, P3 cells increased the frequency of IL-6$^+$TNF-α$^-$ ($P < 0.03$) and IL-6$^+$TNF-α$^+$ ($P < 0.03$) cells in CCR6$^+$CXCR3$^-$ T_{EM} cells (Figure S6).

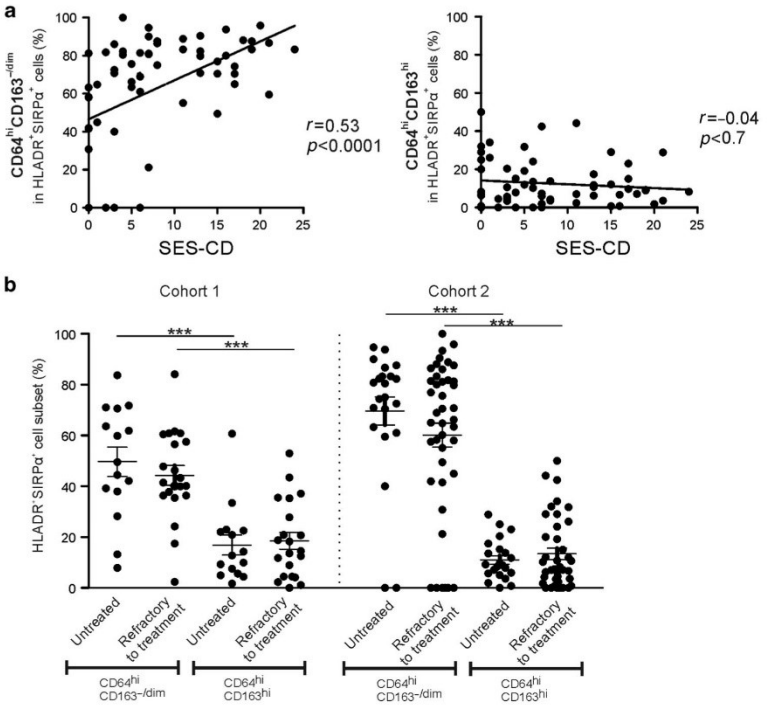


Fig. 2 Frequencies of $CD14^{+}CD64^{hi}CD163^{-/dim}$ cells correlate with disease severity. **a** Correlation between the percentage of $CD64^{hi}CD163^{-/dim}$ (left panel) and $CD64^{hi}CD163^{high}$ cells (right panel) and the endoscopic score of disease severity (inflamed colon of active CD patients, SES-CD > 3, $n = 46$; healed colon of CD in remission SES-CD ≤ 2, $n = 15$) (Spearman's rank correlation test). **b** Frequencies of $CD64^{hi}CD163^{-/dim}$ and $CD64^{hi}CD163^{hi}$ cells in the two treated or untreated independent cohorts examined (cohort 1, $n = 36$ and cohort 2, $n = 61$). Wilcoxon signed rank test and Mann–Whitney test were used. $P < 0.01$ threshold for significance to account for test multiplicity

Collectively, P3 cells shift Th17 towards a Th17/Th1 profile while contribute to further augment the local Th17 response.

$CD64^{hi}CD163^{-}$ (P3) cells promote colonic Th17 and Th17/Th1 responses in an IL-1β-dependent manner in CD patients

We next investigated some of the mechanisms by which P3 cells, along with the IL-1β and IL-23 they secrete, can govern mucosal Th17 and Th17/Th1 profile. Neutralizing IL-1β function in P3/$CD4^{+}$ T-cell co-cultures inhibited Th17 and Th17/Th1 responses elicited by P3 cells ($P < 0.03$) (Fig. 5a). Accordingly, addition of recombinant IL-1β to purified colonic $CD4^{+}$ T cells significantly increased the frequency of IFN-γ^{-}IL-17^{+} ($P < 0.002$), IFN-γ^{+}IL-17^{+} ($P < 0.002$) but not IFN-γ^{+}IL-17^{-} cells (Fig. 5b), along with enhancing IL-17, IFN-γ, GM-CSF, TNF-α, and IL-6 secretion in the culture supernatant (Fig. 5c). The presence of IL-23 augmented the percentage of IFN-γ^{-}IL-17^{+} ($P < 0.04$) and IFN-γ^{+}IL-17^{+} ($P < 0.03$) in mucosal $CD4^{+}$ T cells. In contrast, IL-12 addition decreased IFN-γ^{-}IL-17^{+} ($P < 0.007$) while increasing IFN-γ^{+}IL-17^{-} cells ($P < 0.04$) (Fig. 5d) along with IFN-γ, GM-CSF, and IL-6 production (Fig. 5e). Noteworthy, the opposite IL-17 expression in colonic $CD4^{+}$ T cells stimulated by either recombinant IL-23 or IL-12 (Fig. 5d, e), which both share the IL-12p40 chain, reflects the lack of significant inhibition of Th17 responses when adding neutralizing anti-IL-12p40 mAb to $CD4^{+}$ T/P3 cells co-cultures (Fig. 5f).

Taken collectively, IL-1β secreted by the P3 cells, as well as IL-23, contribute towards amplifying a potential pathogenic Th17 signature in CD mucosa.

Unsupervised single-cell RNA-sequencing (scRNAseq) independently segregates clusters enriched in P3 or P4 cells

To further assess whether P3 and P4 cells indeed represent molecularly distinct subpopulations, we performed unsupervised scRNAseq analyses according to the gating strategy depicted in Fig. 6a. Since this experimental approach aimed to comprehensively sample all the MNPs defined by the $HLADR^{+}SIRP\alpha^{+}$ gate, single cells isolated from inflamed colonic mucosa of three CD patients were thus sorted from five overlapping gates consisting of P3, $CD64^{hi}CD163^{dim}$ (Px), P4 as well as P1 and P2, which collectively capture the full spectrum of $HLADR^{+}SIRP\alpha^{+}$MNPs, and then profiled using a modified version of Smart-Seq2 protocol.[27] Unbiased clustering analyses of all $HLADR^{+}SIRP\alpha^{+}$ single cells revealed six general clusters (Fig. 6b). The single-cell identity making up each cluster was reported in Table S2. Clusters E and F consisted of a majority of P3 and P4 cells, respectively, a significant enrichment over a random assortment of cells into clusters ($P < 0.01$ for both comparisons; chi-square test) (Fig. 6b). Thus, the remaining cells comprised in clusters E and F originated from sorting gates Px and P1 that were designed to capture likely transitioning population (Fig. 6a). More precisely, cluster E comprised 50% P3, 32% Px and 11% P1 while cluster F comprised 50% P4, 17% Px and 13% P1. These two clusters were enriched in CD14-expressing cells with cluster F expressing higher CD14 levels than cluster E (Figure S7A and Table S3), corroborating the data observed at the protein level in the two $CD64^{hi}$ subsets ($P < 0.05$; Figure S7B). Collectively, these observations support the concept that CD163 effectively stratified two $CD14^{+}CD64^{hi}$ subpopulations.

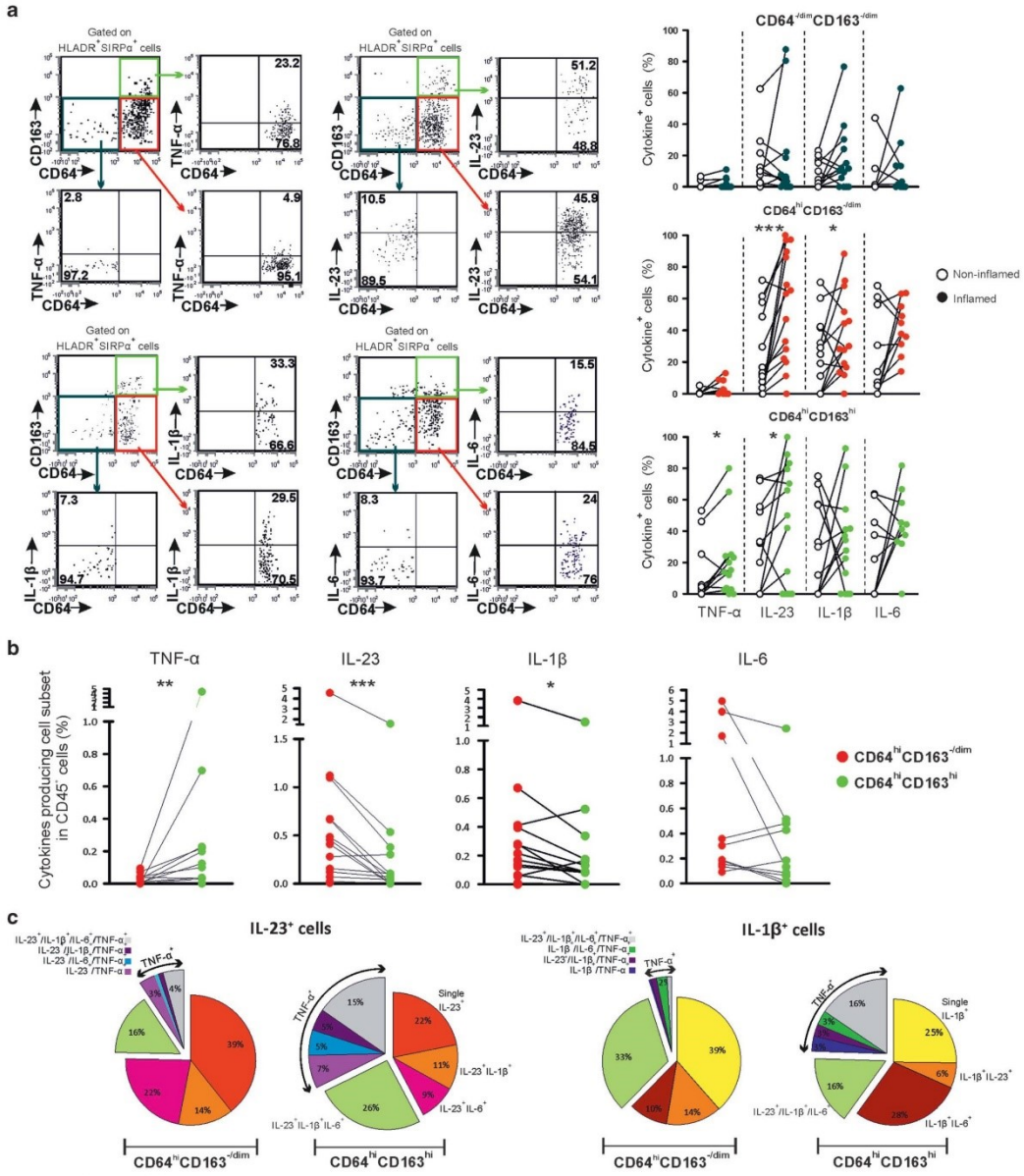


Fig. 3 CD64^{hi}CD163$^{-/dim}$ cells are the major contributors to IL-23 and IL-1β secretion but not TNF-α in inflamed CD colon. **a** Frequencies of TNF-α ($n = 13$), IL-23 ($n = 15$), IL-1β ($n = 14$), and IL-6 ($n = 11$)-producing-cells among CD64$^{-/dim}$CD163$^{-/dim}$, CD64^{hi}CD163$^{-/dim}$, and CD64^{hi}CD163^{hi} cells in paired non-inflamed and inflamed CD colon. Dot plots are one representative experiment in inflamed colon. **b** Frequencies of cytokine-producing CD64^{hi}CD163$^{-/dim}$ and CD64^{hi}CD163^{hi} subsets in CD45^{+} cells in inflamed colon: TNF-α ($n = 13$), IL-23 ($n = 15$), IL-1β ($n = 14$), and IL-6 ($n = 11$). **c** Co-expression of IL-1β, IL-6, IL-23p19, and TNF-α in the two CD14^{+}CD64^{hi} subsets in inflamed CD mucosa ($n = 4$): proportion of single, double, triple, and quadruple producing-cells among IL-23^{+} (left panels) and IL-1β^{+} cells (right panels). **a**, **b** Wilcoxon signed rank test

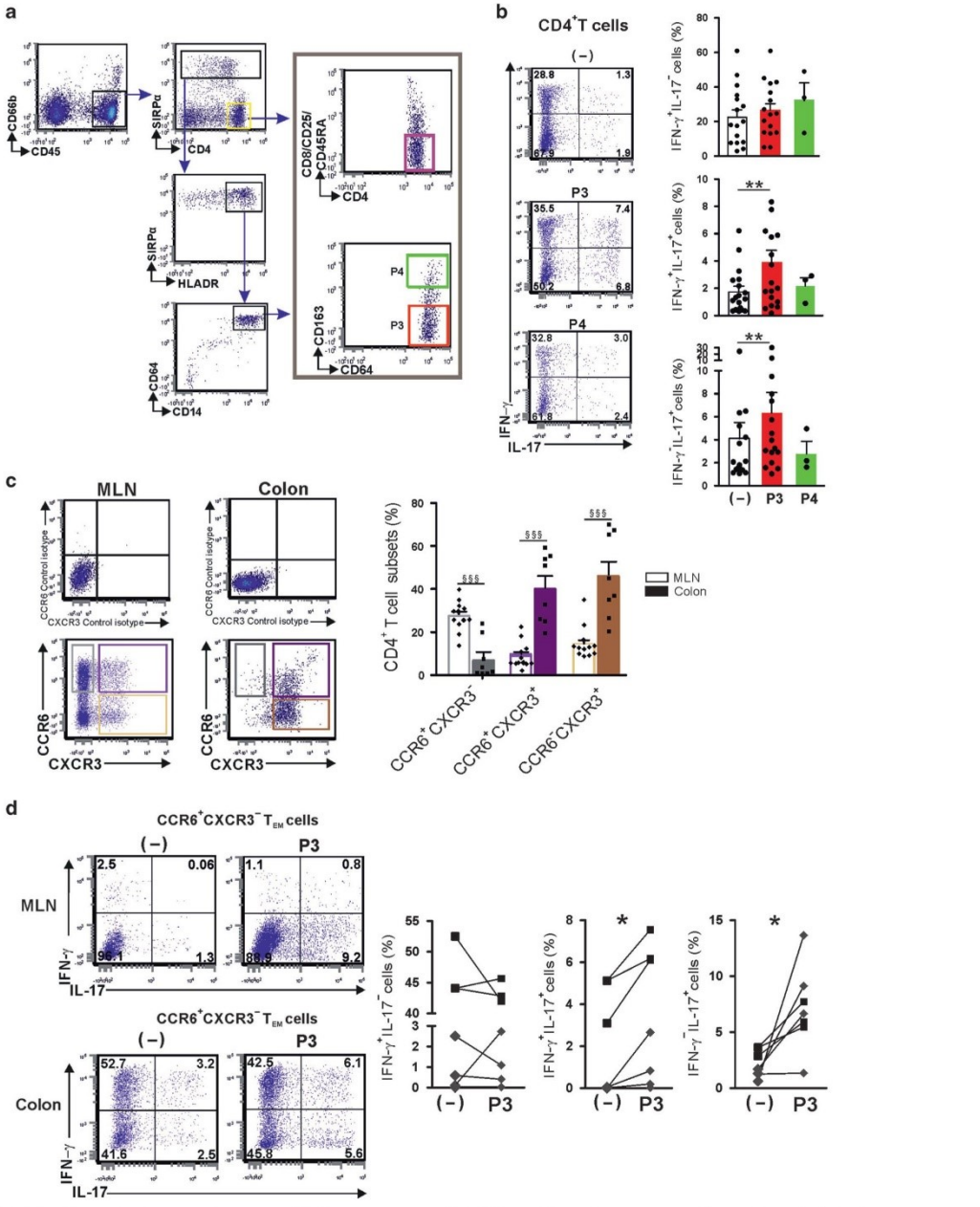


Fig. 4 CD64^{hi}CD163^{-} (P3) cells shape autologous CD4^{+} T cells towards a Th17/Th1 profile. **a** Gating strategy for sorting CD4^{+} T cells (purple gate), P3 (red gate) and P4 (green gate) cells in inflamed CD colon. **b** P3 and P4 cells were co-cultured with autologous colonic CD4^{+} T cells for 6 days. IL-17/IFN-γ expression was measured using intracytoplasmic staining after gating on CD3^{+} T cells (P3, $n = 16$; P4, $n = 3$) (Wilcoxon signed rank test). **c** Distribution of CCR6^{+}CXCR3^{-} (Th17), CCR6^{+}CXCR3^{+} (Th17/Th1), CCR6^{-}CXCR3^{+} (Th1) cells in MLN ($n = 8$) and colon ($n = 12$) of CD patients (Mann–Whitney test). **d** CCR6^{+}CXCR3^{-}CD62^{low}CD4^{+} cells isolated from MLN (diamond, $n = 4$) or inflamed CD colon (square, $n = 3$) were co-cultured with autologous P3 cells for 6 days. IL-17 and IFN-γ expression assessed using intracytoplasmic staining after gating on CD3^{+} T cells (Wilcoxon signed rank test)

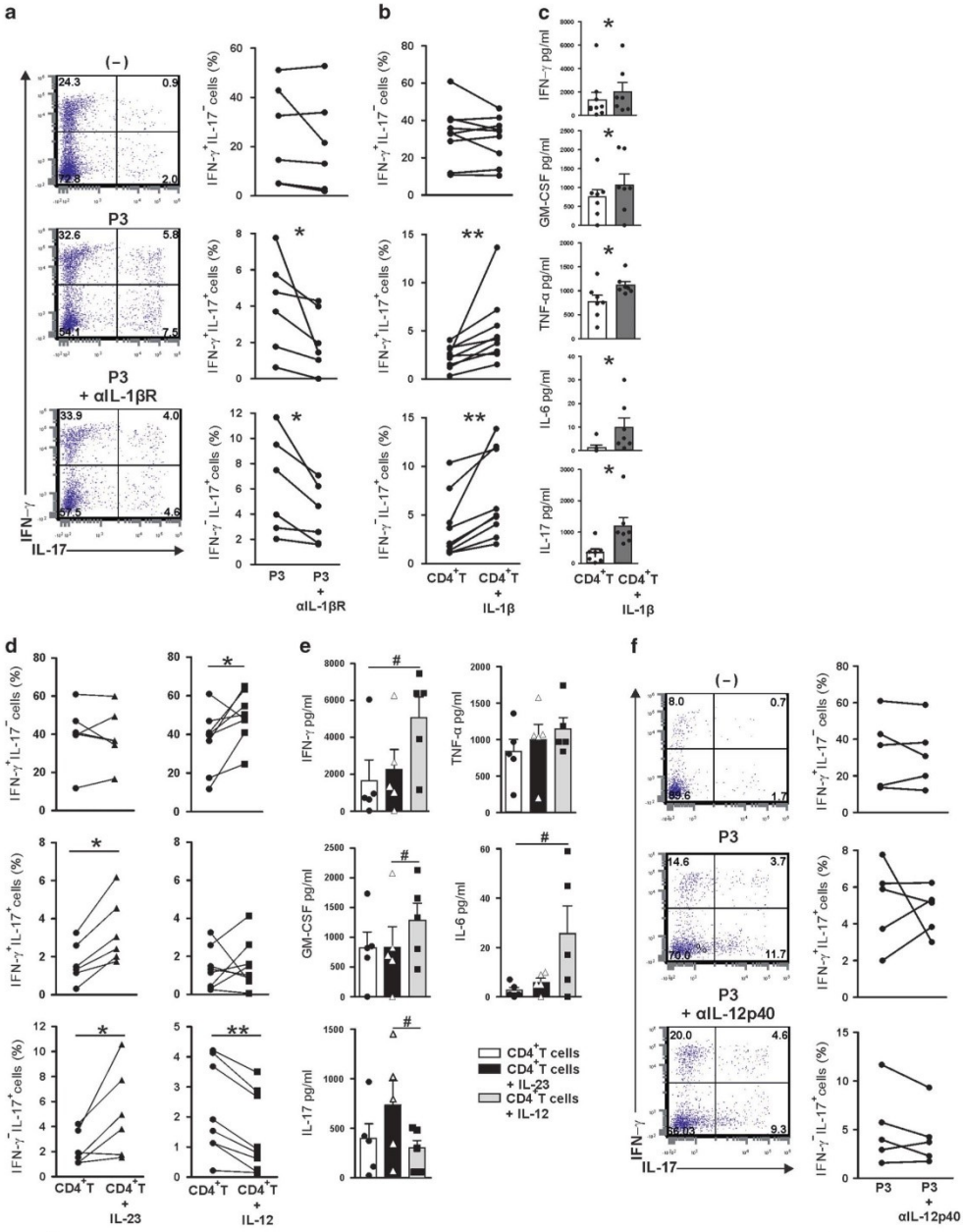


Fig. 5 CD64^{hi}CD163^{-} (P3) cells augment colonic Th17 and Th17/Th1 responses in an IL-1β-dependent manner. CD4^{+} T cells, P3 and P4 cells were purified from inflamed CD colons as in Fig. 4A. CD4^{+} T cells were stimulated with anti-CD3 and anti-CD28 beads and: **a** co-cultured with P3 cells in the presence or absence of αIL-1βR; **b**, **c** cultured with or without IL-1β; **d**, **e** cultured with or without IL-23 or IL-12; **f** co-cultured with P3 cells in the presence or absence of αIL-12p40. **a** ($n = 6$), **b** ($n = 10$), **d** ($n = 7$), and **f** ($n = 5$): intracytoplasmic IL-17/IFN-γ expression after gating on CD3^{+} T cells. **c** ($n = 7$) and **e** ($n = 5$): multiplex dosage in the culture supernatant. **a**–**d**, **f** Wilcoxon signed rank test; **e** Friedman test

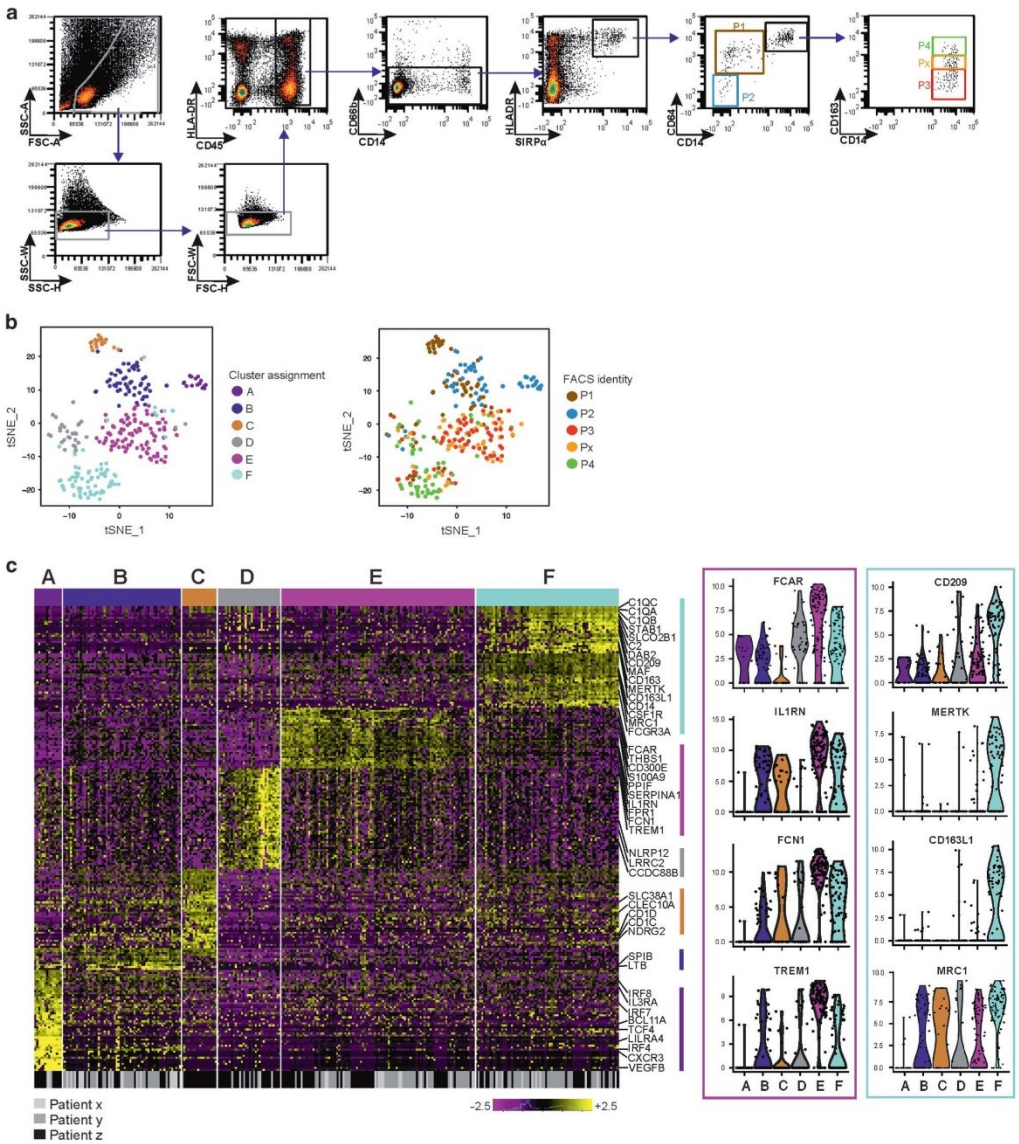


Fig. 6 scRNAseq analysis of HLADR^{+}SIRPα^{+} MNPs in inflamed CD colon. **a** Gating strategy to capture the entire HLADR^{+}SIRPα^{+} cell population for scRNAseq analysis. Single cells were sorted across overlapping gates labeled as P1 (brown), P2 (blue), P3 (red), Px (orange), and P4 (green) from 3 patients. **b** *t*-SNE visualization of isolated single HLADR^{+}SIRPα^{+} cells from all five gates ($n = 294$ cells that passed QCs), colored by cluster assignment (left) and FACS identity (right). **c** Heatmap reports scaled expression (log TPM + 1 (Transcripts Per Million) values) of discriminative gene set (AUC cutoff ≥ 0.75; average log fold change expression difference ≥ 1.2) for each cluster defined. Heatmap color scheme is based on z-score distribution from −2.5 (purple) to 2.5 (yellow). Color legend at the top of the heatmap indicates the unbiased cluster assignment as defined in panel (**b**) and color legend at the bottom of the heatmap indicates the CD patient identity ($n = 3$; labeled "x", "y", and "z"). Violin plots illustrate gene expression level distribution of discriminating surface marker genes (*y*-axis, (log2(TPM + 1))) across each of the six identified clusters (*x*-axis)

As shown in Fig. 6c and Table S3, cluster A encompassed a subpopulation of the P2 gate bearing pDC markers (e.g., *BCL11A*, *TCF4*, *LILRA4*, *VEGFB*), while genes in cluster B (e.g., *SPIB*, *LTB*) and C (e.g., *CD1C*, *SLC38A1)* characterized conventional DCs, and contained most of the remaining P2 along with P1 cells (Fig. 7). While cluster D also expressed myeloid cell markers (*NLRP12*, *LRRC2*, *CCDC88B*), these cells were best defined by their general downregulation of several genes associated with the ubiquitin-

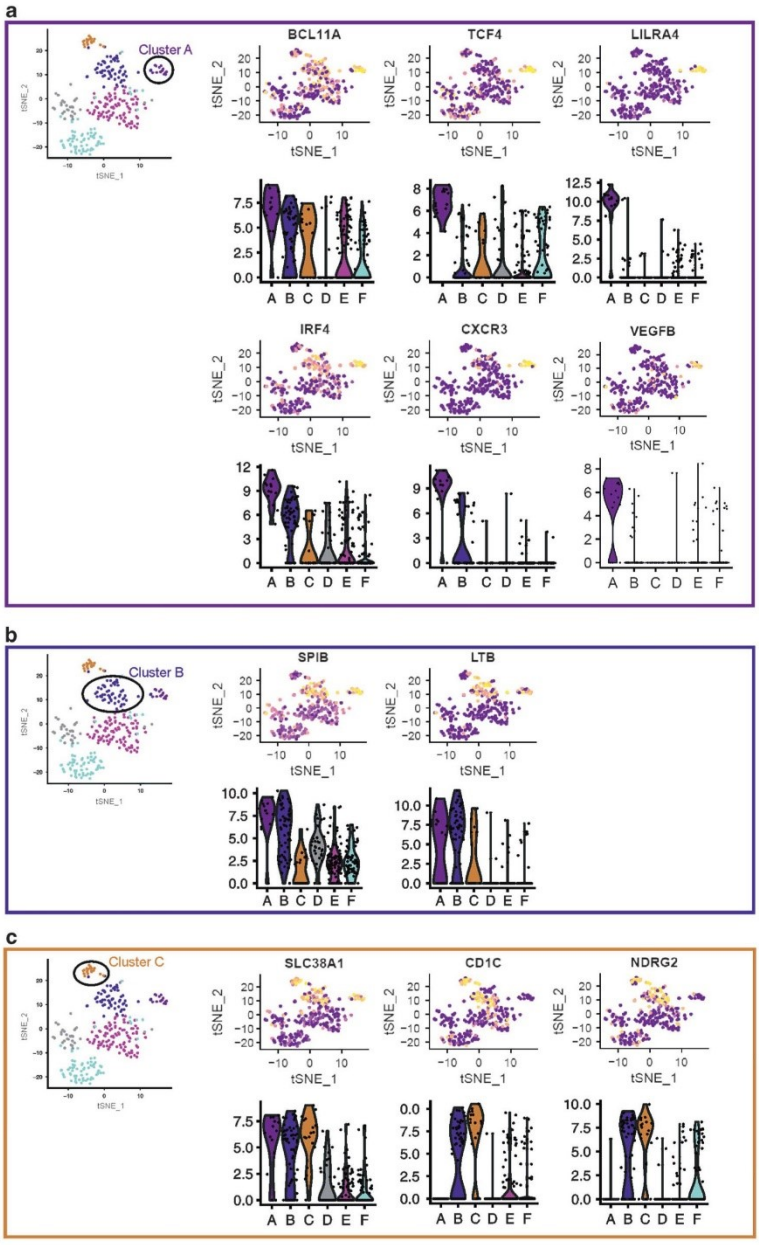


Fig. 7 Gene expression of discriminating genes of cluster (**a**, **b**, **c**). Feature plots of key discriminating genes expressed in particular single-cell cluster, consisting of the *t*-SNE plot from Figure 6b on which is overlaid the gene expression level of selected markers for cluster (**a**, **b**, **c**). Color scheme is according to log2(TPM + 1) expression value, with color gradient from purple (low) to yellow (high) expression. Violin plots illustrate expression level distribution of discriminating surface marker genes (*y*-axis, (log2 (TPM + 1))) across each of the six identified clusters (*x*-axis). Genes overexpressed in **a** cluster A, **b** cluster B, and **c** cluster C are depicted

proteasome system (UPS) (e.g., *PSMA6*, *PSMB1*, *UBE2L6*) (Figure S7C). Clustering analysis was consistent across the three CD patients profiled and allowed the identification of additional discriminating markers beyond CD163 that could be used to isolate cells for the functional analyses. Examples of markers for cluster E (enriched for P3 cells) included *TREM1*, *FCAR*, *FCN1*, *IL1RN*, and *FPR1*, while those for cluster F (enriched for P4 cells) included *CD163L1*, *MERTK*, *CD209*, *MRC1* and a set of complement genes (e.g., *C2*, *C1QA*, *C1QB*, *C1QC*) (Fig. 8).

Together, single cell RNA-sequencing analyses empowered a finer cellular classification of the HLADR^{+}SIRPα^{+} MNPs, showing the existence of six clusters in CD inflamed colon and potentially identified new discriminative markers for P3 and P4 cells.

TREM^{+}CD209^{-}MERTK^{-}(P3/b) cells promote colonic Th17 and Th17/Th1 responses in CD patients

Our subsequent analyses focused on the newly identified surface markers that were differentially expressed in cells included in clusters E and F. Single cells expressing *TREM1* and *FCAR* predominated in cluster E, while *MRC1*, *MERTK*, and *CD209*-expressing cells were observed in cluster F (Fig. 9a). In fact, TREM1 and CD89 (*FCAR*), as well as CD206 (*MRC1*), CD209, and MERTK

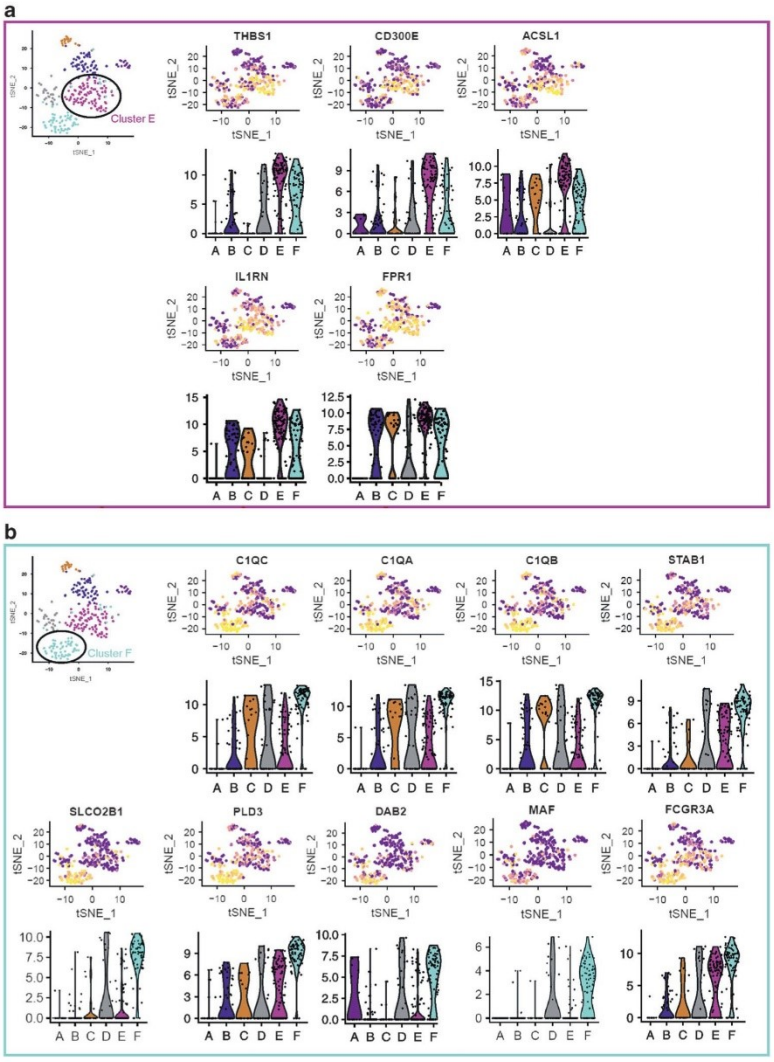


Fig. 8 Gene expression of selected discriminating genes of cluster E and F. Feature plots of key discriminating genes expressed in particular single-cell cluster, consisting of the t-SNE plot from Figure 6b on which is overlaid the gene expression level of selected markers for cluster E and F. Color scheme is according to log2(TPM + 1) expression value, with color gradient from purple (low) to yellow (high) expression. Violin plots illustrate expression level distribution of discriminating surface marker genes (*y*-axis, (log2(TPM + 1))) across each of the six identified clusters (*x*-axis). Genes overexpressed in (**a**) cluster E and (**b**) cluster F are depicted in feature plots and associated violin plots

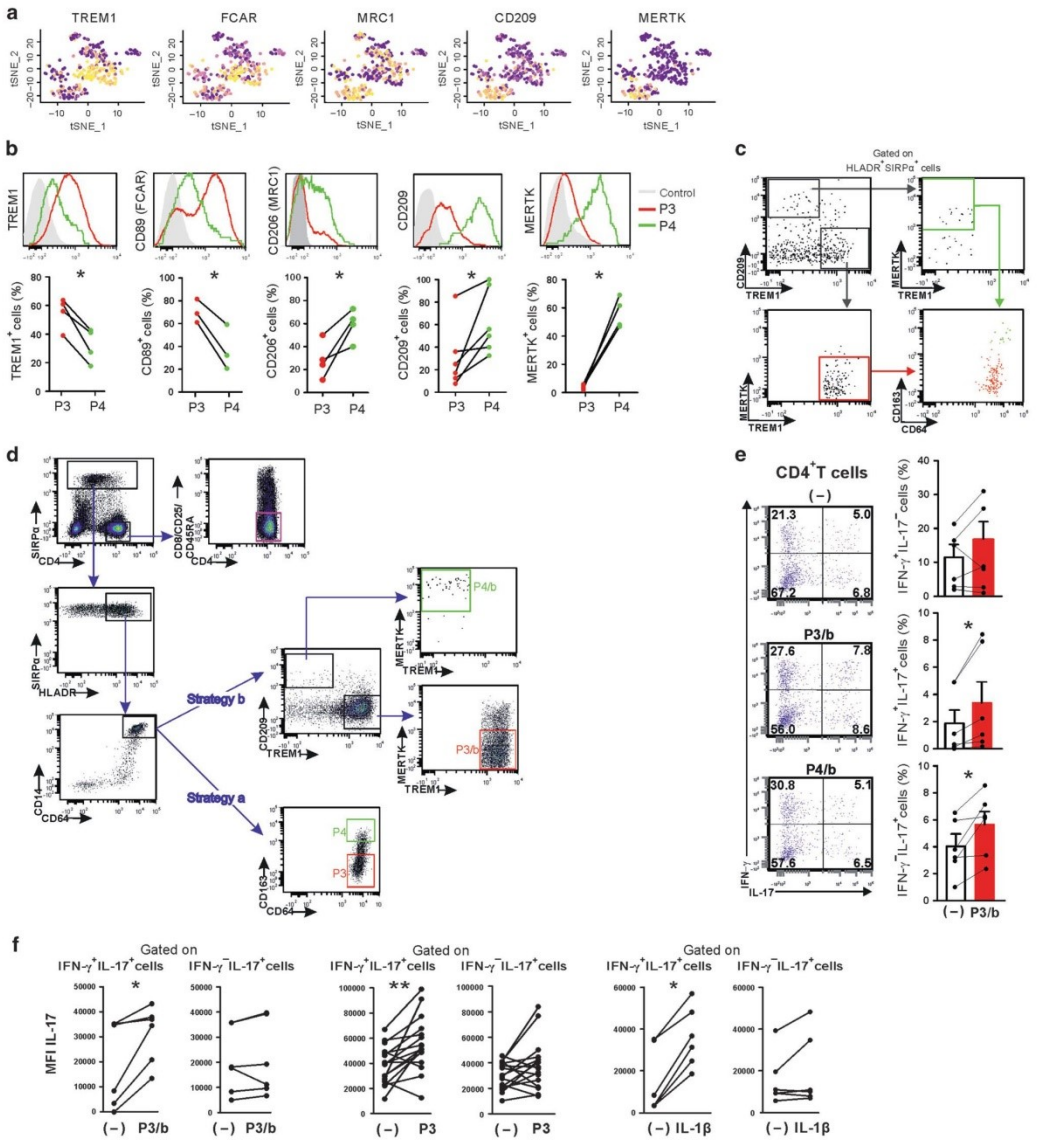


Fig. 9 Functional validation of P3 and P4 cells using the newly identified phenotype by scRNAseq. **a** Feature plots of key discriminating genes consisting of the *t*-SNE plot from Figure 6B on which is overlaid the gene expression level of selected markers genes (TREM1, FCAR, MCR1, CD209, and MERTK) for cluster E and F. Color scheme is according to log2(TPM + 1) expression value, with color gradient from purple (low) to yellow (high) expression. **b** TREM1 ($n = 4$) and CD89 ($n = 3$), CD206 ($n = 4$), CD209 ($n = 6$), and MERTK ($n = 4$) protein expression in the two $CD14^{+}CD64^{hi}CD163^{-}$, subsets after gating on $HLADR^{+}SIRP\alpha^{+}$ cells. **c** $TREM1^{+}CD209^{-}$MERTK- cells display P3 ($CD64^{hi}CD163^{-}$) phenotype and $TREM1^{-}CD209^{+}MERTK^{+}$ cells show P4 ($CD64^{hi}CD163^{hi}$) markers. Shown is one representative experiment among four. **d** Gating strategy for isolation of $CD4^{+}$ T cells and MNPs: strategy b ($TREM1^{+}CD209^{-}MERTK^{-}$, P3/b and $TREM1^{-}CD209^{+}MERTK^{+}$, P4/b) in comparison to strategy a, as depicted in Fig. 4. **e** Representative dot plots of $CD4^{+}$ T cells co-cultured with autologous colonic P3/b ($n = 6$) and P4/b ($n = 2$) cells for 6 days (left panels); IL-17/IFN-γ expression measured via intracytoplasmic staining after gating on $CD3^{+}$ T cells (right panels). **f** Mean fluorescence intensity of IL-17 in colonic $CD4^{+}$ T cells co-cultured for 6 days with autologous P3/b ($n = 6$), P3 ($n = 16$) or in the presence of IL-1β ($n = 6$). **b**, **e**, **f**, Wilcoxon signed test

protein expression was validated as P3 and P4 cells markers, respectively (Fig. 9b), with TREM1$^+$CD209$^-$MERTK$^-$ (P3/b) cells expressing P3 (CD64^{hi}CD163$^-$) phenotype and TREM$^-$CD209$^+$MERTK$^+$ (P4/b) cells expressing P4 (CD64^{hi}CD163^{hi}) phenotype (Fig. 9c). Colonic P3/b and P4/b cells were next purified following the gating strategy b when compared to strategy a used to isolate P3 cells (Fig. 9d). P3/b, but not P4/b cells, augmented Th17 and Th17/Th1 but not Th1 responses ($P < 0.03$ and $P < 0.04$, respectively) in autologous CD4$^+$ T cells (Fig. 9e), corroborating the function of P3 cells (Fig. 4). The low frequency of P4/b, like P4, cells, limited the study of their function to only two out of six experiments. Interestingly, P3/b and P3 cells along with IL-1β significantly upregulated the mean fluorescence intensity of IL-17 (IL-17 MFI) ($P < 0.03$, $P < 0.004$, $P < 0.03$, respectively) in colonic IFN-γ$^+$IL-17$^+$ but not IFN-γ$^-$IL-17$^+$ CD4$^+$ T cells, highlighting an increase in IL-17 expression in Th17/Th1 cells only (Fig. 9f). Collectively, scRNAseq analyses identified new discriminative markers, confirming the existence of one HLADR$^+$SIRPα$^+$ subpopulation, namely the inflammatory monocyte-like CD14$^+$CD64^{hi}CD163$^-$-TREM1$^+$CD209$^-$MERTK$^-$cells that promoted Th17 and Th17/Th1 responses in inflamed colon of CD patients.

DISCUSSION

Several attempts to characterize the phenotype and function of HLADR$^+$CD14$^+$ MNPs infiltrating inflamed CD mucosa highlight their potential heterogeneity.[11,30–32,34,35,48] In the present study, we identified two functionally distinct HLADR$^+$SIRPα$^+$CD14$^+$CD64^{hi} MNPs that differentially accumulate in inflamed CD colon and further characterized their transcriptomic profile using scRNAseq. Firstly, the frequencies of CD163$^{-/dim}$ subpopulation, which was the major contributor of IL-1β and IL-23 but not TNF-α, and more particularly the CD163$^-$cells significantly augmented in inflamed CD colon when compared to CD163^{hi} cells that did not accumulate and represented the predominant TNF-α-producing cell subset. The CD14$^+$CD163$^-$ subpopulation appeared to be part of the inflammatory landscape of CD since these cells did not infiltrate the paired non-inflamed CD colon or the colonic tissue of patients with infectious or drug-induced colitis. Notably, similar frequencies of HLADR$^+$SIRPα$^+$CD14$^-$ cells were detected in inflamed and non-inflamed colonic CD mucosa, unlike previous report of increased numbers of SIRPα$^+$CD11b$^+$CD14$^-$ bona fide cDC2s in the mucosa of patients with hyperemic epithelium.[12] Secondly, highly purified CD163$^-$(P3) cells that morphologically resemble "monocyte-like cells", but not CD163^{hi} (P4) cells resembling MΦ, promoted memory Th17 into Th17/Th1 responses in an IL-1β-dependent manner. Thirdly, scRNAseq revealed that P3 and P4 cells displayed a molecularly distinct signature. Finally, since our present findings further indicated that the percentage of CD163$^-$ cells significantly correlated with SES-CD in an independent cohort of patients, we propose that mucosal P3 cells are associated with pathogenicity in CD.

The P3 cells appeared functionally distinct from the previously described CD14$^+$CD163^{low} cells.[32] As such, healthy, non-inflamed and inflamed CD colon comprise equal proportion of HLADR^{bright}CD14$^+$CD163^{low} and CD163^{high} cells while HLADR^{dim}CD14$^+$CD163^{low} cells were detected but not quantified in inflamed mucosa relative to healthy mucosa.[32] In apparent contradiction with that study and our present findings, immunohistochemical analysis indicates that CD163-expressing cells accumulate in areas of active inflammation in IBD colon.[49] Furthermore, Ogino et al. provide evidence that only the HLADR^{bright}CD163^{low} or CD163^{hi} but not the HLADR^{dim}CD163^{low} cells polarize allogeneic naive peripheral blood CD4$^+$ T cells and induce their differentiation into IL-17- or IFNγ- producing T cells in a IL-1β, IL-6, IL-23, and TGFβ-dependent manner,[32] corroborating earlier observations using unfractionated CD14$^+$ MNPs.[37] The role of IL-1β, IL-6, and TGFβ in human Th17 cell polarization is well established, whereas IL-23 only promotes Th17 cell expansion.[50] Noteworthy, the frequencies of naive CD4$^+$ T cells are extremely low in intestinal mucosa[51] and these two studies did not investigate the propensities of CD14$^+$ MNPs to regulate colonic memory Th17 and Th17/Th1 responses, and thus to drive intestinal inflammation.

Here, we report that highly purified P3 cells, which are the predominant IL-1β and IL-23-producing CD14$^+$ subset in the inflamed colon, augmented mucosal effector Th17/Th1 cell responses with pathogenic potential. This effect was mediated by IL-1β without excluding the implication of IL-23. Several lines of evidence argue for a key role of IL-1β and IL-23 in driving CD. First, IL-1β levels correlate with disease activity in CD patients[52] and increases prior to disease relapse.[53] Second, IL-1β upregulates IL-23 receptor on pathogenic T cells,[44] and vice versa, IL-23 augments IL-1β receptor on T cells.[54] Third, IL-23 promotes gut inflammation in experimental colitis.[43] Finally, IL-1β favors the accumulation and survival of colonic IL-17-secreting cells in a T-cell-dependent model of colitis.[54] This pro-survival function of IL-1β on intestinal effector T cells might represent one of the mechanisms that governed the increased frequency of IL-17$^+$IFN-γ$^-$ T cells observed in our study. Nonetheless, P3 cells as well as IL-1β also augmented IL-17 expression per cell. In fact, drugs aiming to impair the effects of IL-1β, such as IL-1 receptor antagonist (Anakinra), have been used in very early onset IBD patients, including chronic granulomatous disease and patients with IL-10 receptor mutations.[55] Furthermore, some authors[56] proposed that blocking IL-1β could be beneficial in patients carrying *ATG16L1* or *NOD2* mutations, two genes associated with increased risk of CD. However, IL-1β blockade could trigger or worsen IBD.[57] Yet, ongoing clinical trials indicate that IL-23 blockade appears to be beneficial in patients with CD.[58]

TNF-α, which is abundantly produced by local effector T cells, still represents the major target in the therapeutic management of patients with CD.[1] We show here that frequencies of TNF-α-producing CD64^{hi}CD163^{hi} cells were significantly higher when compared to CD64^{hi}CD163$^{-/dim}$ cells that expressed very low levels of TNF-α. Although ~30% of CD patients were refractory to TNF-α antagonists in our cohort, treatment with biologics only or in combination with immunosuppressive drugs did not deplete either CD64^{hi}CD163$^{-/dim}$ cells, CD64^{hi}CD163^{hi} cells or TNF-α, IL-1β, and IL-23-producing-cells. Nonetheless, TNF-α, like IL-1β and IL-23, may exert a dual role in the intestinal mucosa. While TNF-α has pro-inflammatory properties, notably via interactions between membranous TNF-α on the surface of CD14$^+$MNPs and TNFR2 expressed on T cells that results in T-cell survival, low levels of TNF-α may directly contribute to the maintenance of the epithelial cell barrier integrity.[59] IL-1β as well as IL-23 participate in maintaining the integrity of gut epithelial barrier at least through the role they play on IL-22 secretion by ILC3.[31] However, the protective function of ILC3 and IL-22 is also controversial since ILC3 drive experimental colitis through IL-1β and IL-23- producing gut resident macrophages and IL-22.[60–62] Collectively, targeting the IL-1β pathway could be considered in the therapeutic arsenal for the management of CD, though caution should be exercise as the protective role of IL-1β in the intestine could limit the safety of using IL-1β pathway antagonists.

We further present evidence that P3 but not P4 cells might be implicated in the shift of Th17 cells towards Th17/Th1 profile. Although it would be very valuable to increase the number of experiments that compare P3 and P4 as well as P3/b and P4b functionalities, the frequencies of these rare MΦ-like cells (P4 and P4/b), and thus their yield, are very low in inflamed mucosa, precluding their purification in sufficient numbers from fresh biopsies to perform co-cultures experiments. Furthermore, surgical samples from CD patients heavily treated (multiple therapeutic failure) results in low cell recovery. Nonetheless, P4 cells that morphologically and molecularly resemble MΦ, did not

accumulate or correlate with SES-CD, and were superior to inflammatory monocyte-like P3 cells in their propensity to express TNF-α and IL-10 in inflamed CD mucosa. Thus, P4 cells most likely do not drive intestinal memory Th17 responses corroborating Mϕ function in tumor ascites.[63]

IL-17 is secreted by intestinal Th17 cells as well as γδT cells, both of which participate in mucosal homeostasis.[64] In that regard, the administration of anti-IL-17 mAb exacerbates colitis in mice[43] and humans.[65] However, IL-17/IFN-γ-secreting $CD4^+$ T cells have been described as the pathogenic Th cell subset in the gut of IBD patients.[5,40] Th17/Th1 and Th1 cells appear to predominate over Th17 cells in inflamed colon of CD patients, suggesting the plasticity of Th17 cell lineage. P3 cells and IL-1β not only increased the frequency of IL-17/IFN-γ-secreting cells in colonic $CD4^+$ T cells, but also promoted their pathogenic signature as shown by increased GM-CSF, TNF-α, and IL-6 secretion in memory $CD4^+$ T cells. In that regard, IL-6 is known to enhance pathogenic T cell survival and promote the development of colonic carcinoma,[66] which is one of the long-term devastating complications associated with IBD.

Remarkably, unsupervised scRNAseq analysis that capture the entire $HLADR^+SIRPα^+$ MNP population succeeded in providing finer cellular classification, with unbiased defined single cells clusters being significantly enriched for cells that were isolated according to the pre-defined gating strategy that used presence or absence of CD64, CD14, and CD163 expression. This analysis indicates that P3 and P4 cells, the two extreme subpopulations in CD163 expression spectrum within $CD14^+CD64^{hi}$ cells that omit the $CD163^{dim}$ (Px), are both transcriptionally and functionally distinct. Interesting biology can be inferred from the discriminating genes for each cell cluster identified by scRNAseq. For example, markers for cluster E (significantly enriched for P3 cells) include *TREM1*, which is a member of the "Triggering Receptors Expressed on Myeloid cells" family, and *FCAR* reported to be highly expressed by unfractionated $CD14^+$ MNPs and recently suggested to contribute to IBD pathogenesis.[67–69] *FPR1*, also expressed by cluster E cells, was shown as a player involved in colorectal cancer tumor cell invasion, being also expressed in colorectal epitheliums and tumor infiltrating MNPs.[70] A recent integrative genomic approach identifies macrophage gene expression as a common signature, which includes *FPR1* among key regulators in IBD.[71] Cluster F (significantly enriched for P4 cells) classifiers included candidates previously reported to be associated with mature and/or regulatory tissue Mϕ (e.g., *CD209*/DC-SIGN, *MRCI*/CD206, CD163, *CD163L1*, and *STAB1*);[72,73] members of the complement system (e.g. *C2*, *C1QA*, *C1QB*, and *C1QC*) that may play a role in cellular cross-talk and polarization of MNPs; *MERTK*, and *Nr1h3*/LXRα, which is expressed on Mϕ and involved with clearance of apoptotic cells[45,74], as well as *IDH1, FOLR2, DNASE2, SLCOB21, DAB2*, and *VSIG4*, expressed by tissue inflammatory Mϕ and in vitro generated (M-CSF) Mϕ.[75] Furthermore, a more thorough analysis of scRNAseq data of the three patients allowed identification of four additional clusters beside clusters E and F in $HLADR^+SIRPα^+$ MNPs. Cluster A, B, C were characterized by pDCs (cluster A) and conventional DCs (cluster B and C). Gene expression downregulation of several members of the UPS distinctively observed in cluster D cells may highlight the presence of non-reactive anergic macrophage phenotype, which has previously been reported in IBD.[76] Noteworthy, cross-presenting DCs (cDC1) were not identified as a separate cluster since these cells are $HLADR^+SIRPα^-$ MNPs and obviously excluded from the captured population that was analyzed.

Importantly, the gating strategy used to initially isolate the P3 and P4 populations for functional studies excluded P1 intermediate as well as Px transitioning cells, which did not accumulate in the inflamed CD colon. P3 and P4 cell subsets are closely related and, analogous to monocyte-derived cells in mice and humans,[13,16] could represent two distinct functional phenotypes of a single population displaying a high degree of plasticity, with pro-inflammatory P3 converting towards Px transitioning cells, and then finally towards more anti-inflammatory P4 Mϕ-like cells. In the context of IBD, inflammation might slow down this maturation process, resulting in the accumulation of pro-inflammatory P3 cells in lesional sites. This hypothesis is currently being investigated, and out of the scope of the present study. Therefore, isolation of cells from each extreme of the spectrum defined by CD163 antigen intensity contributed to studying more "pure" sorted population and ultimately highlighted distinct roles of P3 and P4 cells. Remarkably, this molecular analysis identified novel classifiers beyond CD163 that enabled prospective isolation of $TREM\text{-}1^+MERTK^-CD209^-$(P3/b), displaying similar functional characteristics to P3 cells.

Based on their phenotype and morphology, the P3 cells ($TREM1^+CD89^+CD163^-CD209^-$ $CD206^-$ $MERTK^-$) resemble Mϕ 1 ($CD14^+CD64^+CD163^{dim}CD206^-CD209^{dim}MERTK^{dim}$), while P4 cells are comparable to Mϕ 4 ($CD14^+CD64^+CD163^{high}$ $CD206^+CD209^{high}MERTK^{high}$) subset, which are detected in healthy jejunum of patients with pancreatic cancer,[13] or to $HLADR^{high}CD14^+CD163^{high}CD209^{high}$ cells in non-inflamed ileum of CD patients.[16] However, neither Mϕ 1 nor Mϕ4 in healthy human mucosa display the transcriptomic signature of P3 or P4 cells found here in inflamed CD colon.[13] Interestingly, the human monocyte-like P3 and Mϕ-like P4 cells characterized in the present study shared common genes with murine colonic P1 (*TREM1*) and P4 (*MERTK, C1QA, C1QB,* and *C1QC*) $CD11b^+CD64^+$ cells, respectively.[77]

Taken together, the functional snapshot analysis of $HLADR^+SIRPα^+$ subpopulations in the colonic mucosa over a large cohort of CD patients yielded consistent results, regardless of the genetic background, age, gender, and disease states of our cohort, which included patients who were refractory to conventional anti-TNF-α or anti-IL-12p40 therapy. This highlights the broader biological relevance of our observations and the potential contribution of one particular mucosal $HLADR^+SIRPα^+CD14^+CD64^{hi}$ subpopulation (i.e., the $TREM1^+CD209^-MERTK^-CD163^-$(P3) cells), also identified by its molecular signature, to gut inflammation. Furthermore, we reproducibly showed the effect of P3 cells in promoting intestinal pathogenic Th17 cell profile. Based on our findings, we propose that impairing the accumulation and/or function of pathogenic $CD14^+CD64^{hi}CD163^-$ cells might attenuate ongoing colonic inflammation that have escaped TNF-α and/or IL-23 control, and thus might open avenues for novel therapeutic approaches in CD.

MATERIALS AND METHODS

Human clinical samples

All participants ($n = 186$) signed informed consent forms that have been approved by the Institutional Ethics Research Committee of the Centre Hospitalier de l'Université de Montréal and the Broad Institute of MIT and Harvard. Non-inflamed and inflamed colonic tissues (from the same patient) and MLNs were obtained from endoscopic biopsies or surgical resections. Five biopsies from non-inflamed and five biopsies from inflamed regions of the same patient were collected for staining, except for figure 1e ($n = 2$ biopsies); 10 biopsies were collected from inflamed region for co-culture experiments and scRNA-sequencing. Clinical information of all patients included in Figs. 1 to 9 and supplementary figures are shown in Table S4.

Cell isolation and flow cytometry

The colonic mucosa was first processed by enzymatic digestion with DNase I (Roche) and Collagenase D (Roche) followed by mechanical digestion with gentle MACS (Miltenyi Biotec) to isolate lamina propria mononuclear cells (LPMC). MLNs were digested mechanically to obtain cellular suspensions.[11]

Ex vivo isolated LPMC cell suspension were immediately stained for surface markers, then fixed, permeabilized, and stained for intracytoplasmic cytokines expression using mAb listed in Table S5. FACS Aria II cell sorter was used for sorting MNPs and T-cell populations in inflamed CD colon or MLNs. For single cell RNA-sequencing (scRNAseq) analysis, single cells were sorted into 96-well full-skirted Eppendorf plates chilled to 4 °C, pre-prepared with lysis buffer consisting of 10 µl of TCL buffer (Qiagen) supplemented with 1% β-mercaptoethanol. Single-cell lysates were sealed, vortexed, spun down at 300× *g* at 4 °C for 1 min, immediately placed on dry ice, and transferred for storage at −80 °C.

MNPs and CD4$^+$ T cells co-cultures

CD4$^+$ T cells (20 to 30×10^3) or CCR6$^+$CXCR3$^-$CD62L^{low}CD4$^+$ effector memory T cells (T_{EM}) (50×10^3) isolated from inflamed CD colon or MLNs were stimulated with anti-CD3 and anti-CD28 coated beads (Miltenyi Biotec) or soluble anti-CD3 (1 µg/ml; Biolegend), respectively, with no expansion in IL-2, and cultured: (a) with or without IL-1β (10 ng/ml; Peprotech), IL-12 (10 ng/ml; Peprotech), or IL-23 (10 ng/ml; R&D systems) for six days; (b) with or without autologous MNP subsets isolated from inflamed colonic mucosa at a 10:1 ratio for six days, in the presence of PGN (10 µg/ml; Sigma-Aldrich) and IgG1 (10 µg/ml; Biolegend). Anti-IL-1β receptor (10 µg/ml) or anti-IL-12p40 (10 µg/ml) mAbs was added to some co-cultures. For all cultures: (a) RPMI 1640 medium with 10% fetal calf serum (FCS), 1% Penicillin/Streptomycin was used; (b) for intracytoplasmic staining, cells were re-stimulated after six days, with phorbol 12-myristate 13$^-$acetate (PMA) (5 ng/ml; Sigma-Aldrich) and ionomycin (500 ng/ml; Calbiochem-Behring) for 6 h in the presence of brefeldin A (1 µg/ml; Calbiochem-Behring) for the last 3 h, then stained with CD3, fixed then permabilized for intracytoplasmic cytokine staining (IL-17, IFN-γ, IL-6, and TNF-α) using mAb listed in Table S5.

Multiplex ELISA

IL-17, IFN-γ, IL-6, TNF-α, GM-CSF quantities were measured by multiplex assay (Eve Technologies, Calgary, AB, Canada) in the co-culture supernatants.

Single-cell RNA-sequencing—raw data samples and processing

The 414 sequenced cells consisted of 115 P1, 79 P2, 77 P4, 78 P3, and 65 Px samples (as depicted in Fig. 6a) from three patients, with 56 cells from patient *x*, 128 cells from patient *y*, and 230 cells from patient *z* (Fig. 6c). The raw sequencing reads were demultiplexed with standard bcl2fastq2 Illumina software (version 2.17.1.14) and aligned to the UCSC hg19 transcriptome with bowtie (version 0.12.7).[78] RSEM (version 1.2.1) was used to quantify gene expression level in transcripts per million (TPM)[79] and these expression data were log-transformed ($\log_2$(TPM + 1)) before further analysis.

Single-cell expression data analyses were performed with the Seurat R package (version 1.4.0.5) following the strategy described in Villani et al, 2017.[27] The data were visualized in two dimensions by running t-distributed stochastic neighbor embedding (*t*-SNE) on the cell loadings for the first nine principle components. Heatmaps were used to visualize scaled (*z*-score) gene expression level ($\log_2$(TPM + 1)) of identified markers.

Statistical analysis

Except for scRNAseq data, statistical analysis was performed with GraphPad Prism version 6 (GraphPad Software, La Jolla, CA, USA). Wilcoxon signed rank test (represented by *) and Mann–Whitney test (represented by §) were used. Threshold for significance was adjusted when indicated to account for test multiplicity. Friedman test and Kruskal–Wallis test were employed followed by Dunn's test (represented by #). Repeated measure Anova was employed followed by Bonferroni test (represented by ¤). For all tests, 1 symbol means $P < 0.05$; 2 symbols mean $P < 0.01$; 3 symbols mean $P < 0.001$. Bar graph data are shown as mean ± s.e.m. unless otherwise stated. Spearman test was used to assess correlation.

ACKNOWLEDGEMENTS

We thank H. Mehta for critical reading and comments. We thank the subjects for donating the samples used in our study; the physicians who provided the samples: E.-J. Bernard, M. Boivin, S. Bouchard, J. Coté-Daigneault, L. Daoust, E. Deslandres, J. Dorais, B. Faulques, R. Lahaie, R. Leduc, M. Lemoyne, B. Panzini, P. Poitras, S. Sidani, and D. Von Renteln from gastroenterology unit; E. Debroux, R. Lougnarath, R. Ratelle, C. Richard, F. Swchenter, and R. Wassef from digestive tract surgery unit; B. Nguyen, G. Soucy from pathology department, at CHUM, Montreal; and C. Deslandres from gastroenterology unit at CHU Sainte-Justine, Montreal. We also thank the hematology department and the nurse C. Bergeron for their help. Canadian Institutes of Health Research (MOP#130533 (M.S.) and Banting Postdoctoral Fellowship (A.-C.V.), Fonds de Recherche en Santé du Québec (L.C., M.Bs.), National Human Genome Research Institute Centers of Excellence in Genomics Science grant P50 HG006193 (N.H.), and the National Human Genome Research grant T32 HG002295 (S.S.; PI: Park, Peter J).

AUTHORS CONTRIBUTIONS

Conceptualization: L.C., M.Bs., M.R., S.S., A.-C.V., N.H., and M.S. Methodology and investigation: L.C., M.Bs., M.R., S.S., and A.-C.V. Resources: M.B., K.O., A.W., A.T., E.W., and L.C. Writing: L.C., M.Bs., A.-C.V., and M.S. Supervision: N.H., A.-C.V., and M.S. Funding acquisition: N.H. and M.S.

ADDITIONAL INFORMATION

The online version of this article (https://doi.org/10.1038/s41385-018-0126-0) contains supplementary material, which is available to authorized users.

Competing interests: The authors declare no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

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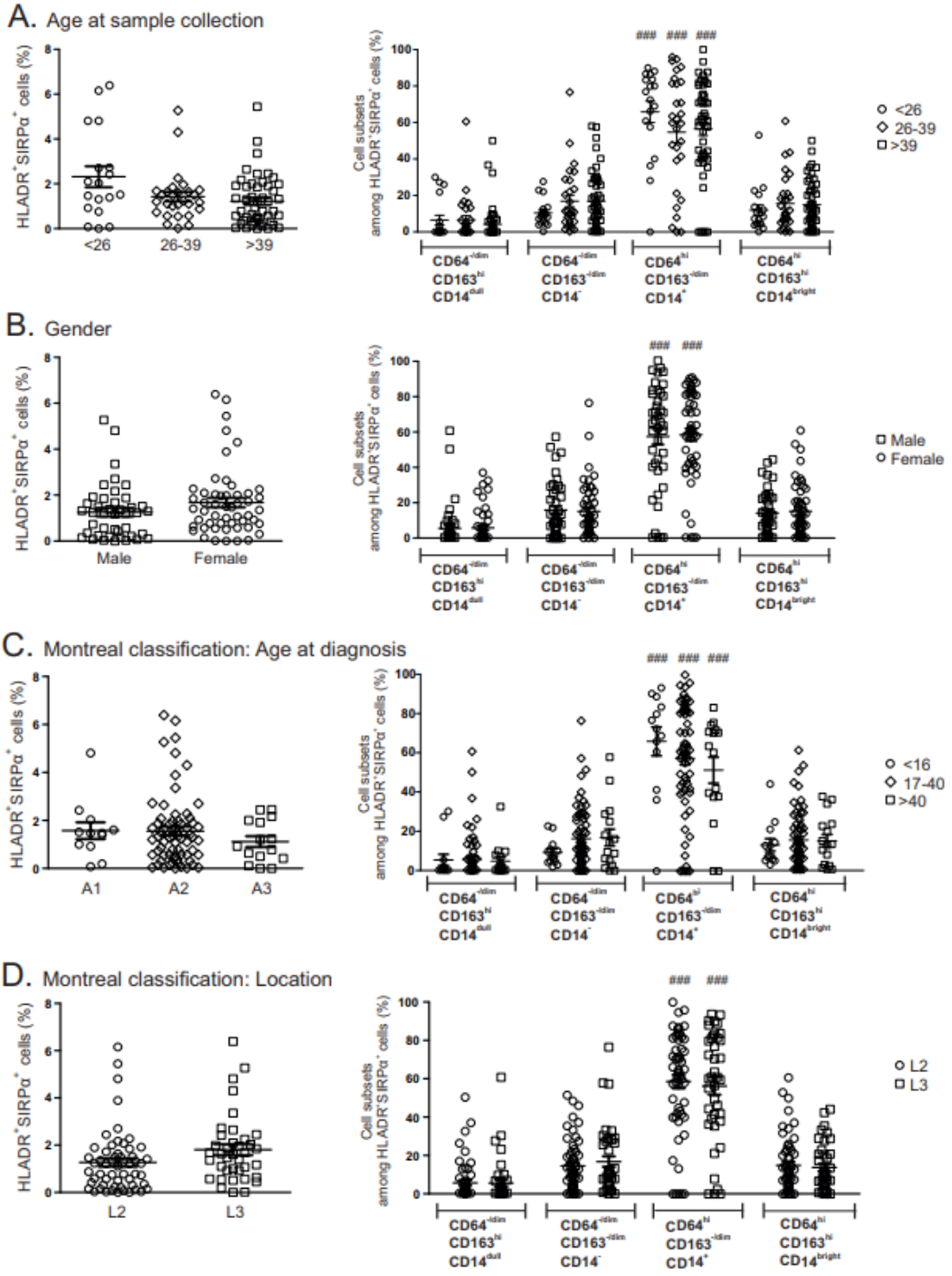
Figure S3


A. Age at sample collection
HLADR+SIRPα+ cells (%)
<26
26-39
>39
Cell subsets among HLADR+SIRPα+ cells (%)
CD64-/dim CD163hi CD14dull
CD64-/dim CD163-/dim CD14-
CD64hi CD163-/dim CD14+
CD64hi CD163hi CD14bright
###
B. Gender
Male
Female
C. Montreal classification: Age at diagnosis
A1
A2
A3
<16
17-40
>40
D. Montreal classification: Location
L2
L3

E. Montreal classification: Behavior

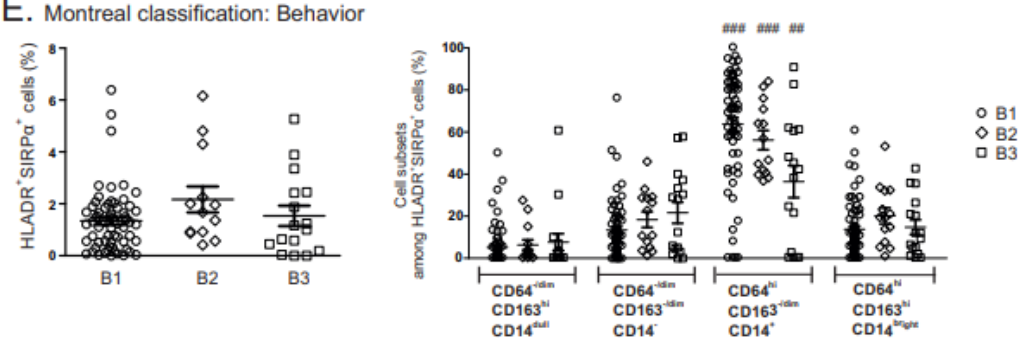


Table S3. List ofdiscriminating genesidentified through scRNAseq analysis

Gene	myAUC	avg_logFC	pct.1	pct.2	Cluster
TREM1	0,900	2,502	0,939	0,362	E
FCN1	0,890	2,785	0,949	0,515	E
PLAUR	0,883	2,247	0,990	0,832	E
RAB20	0,861	1,634	0,980	0,587	E
OLR1	0,858	2,535	0,939	0,48	E
SERPINA1	0,857	1,468	0,990	0,714	E
SLC11A1	0,854	2,181	0,908	0,464	E
TIMP1	0,838	1,976	0,980	0,821	E
GPCPD1	0,836	2,082	0,980	0,684	E
CCRL2	0,826	1,883	0,908	0,51	E
THBS1	0,825	2,327	0,908	0,495	E
IL1RN	0,821	3,037	0,898	0,536	E
C5AR1	0,819	1,618	0,949	0,551	E
LCP2	0,814	1,540	0,969	0,776	E
CD300E	0,809	2,195	0,867	0,464	E
C15ORF48	0,803	1,845	0,949	0,582	E
PPIF	0,802	1,804	0,949	0,765	E
ACSL1	0,791	2,677	0,827	0,541	E
G0S2	0,789	1,339	0,959	0,648	E
APOBEC3A	0,786	2,948	0,837	0,556	E
GK	0,785	1,699	0,969	0,689	E
KYNU	0,784	1,219	0,980	0,684	E
FPR1	0,784	1,213	0,949	0,571	E
IVNS1ABP	0,772	1,964	0,918	0,755	E
EREG	0,771	2,369	0,867	0,5	E
S100A9	0,763	1,645	0,908	0,587	E
FCAR	0,760	2,636	0,857	0,694	E
SLC2A3	0,757	1,552	0,980	0,806	E
UPP1	0,754	1,414	0,888	0,577	E
SAMSN1	0,751	1,553	0,908	0,673	E
PLD3	0,920	2,785	0,958	0,495	F
C1QC	0,918	2,529	0,944	0,446	F
SLCO2B1	0,916	2,174	0,944	0,266	F
TMEM176B	0,905	1,645	1,000	0,631	F
C1QA	0,901	2,009	0,944	0,491	F
CSF1R	0,897	1,596	0,986	0,775	F
C1QB	0,896	2,281	0,917	0,473	F
CTSD	0,895	2,459	1,000	0,779	F
CTSA	0,885	1,673	0,986	0,694	F
LIPA	0,881	2,535	0,944	0,613	F
STAB1	0,868	1,973	0,931	0,45	F
TMEM176A	0,862	1,372	0,986	0,563	F
FUCA1	0,859	4,223	0,819	0,243	F
FCGRT	0,849	1,385	1,000	0,784	F

C2	0,848	2,395	0,847	0,378	F
ACP5	0,846	2,461	0,972	0,626	F
SCPEP1	0,843	1,449	0,958	0,545	F
CMKLR1	0,842	1,868	0,792	0,14	F
CD209	0,841	1,906	0,903	0,577	F
PRDX1	0,839	1,538	0,972	0,775	F
SLC7A8	0,838	2,700	0,722	0,09	F
CD14	0,830	1,230	0,958	0,631	F
GM2A	0,827	1,756	0,931	0,64	F
IDH1	0,826	1,709	0,861	0,315	F
PLA2G7	0,826	1,626	0,944	0,486	F
AKR1B1	0,821	1,809	0,861	0,342	F
SGPL1	0,820	1,758	0,931	0,644	F
FCGR3A	0,815	2,249	0,889	0,5	F
LGALS3BP	0,814	1,762	0,861	0,369	F
SLC40A1	0,812	1,400	0,778	0,243	F
VSIG4	0,810	2,545	0,764	0,207	F
OLFML2B	0,809	2,188	0,694	0,117	F
NRP1	0,808	1,965	0,833	0,342	F
NR1H3	0,806	3,357	0,681	0,122	F
MRC1	0,803	1,236	0,958	0,509	F
DAB2	0,802	1,332	0,806	0,284	F
LPAR6	0,800	1,630	0,861	0,32	F
DNASE2	0,799	1,874	0,806	0,401	F
ACP2	0,794	1,803	0,736	0,198	F
MAF	0,789	1,297	0,708	0,149	F
MERTK	0,787	2,545	0,653	0,104	F
CD163	0,787	1,679	0,861	0,423	F
CREG1	0,786	1,491	0,875	0,495	F
PLXND1	0,782	1,295	0,778	0,252	F
CORO1B	0,781	1,246	0,917	0,514	F
FOLR2	0,776	1,893	0,681	0,144	F
CD163L1	0,773	2,771	0,611	0,108	F
VAT1	0,768	1,970	0,667	0,162	F
CAPG	0,768	1,432	0,903	0,572	F
FLVCR2	0,766	1,379	0,722	0,189	F
CST7	0,862	2,183	0,917	0,355	B
NAPSB	0,850	1,392	1,000	0,556	B
LTB	0,826	3,021	0,750	0,137	B
ADAM19	0,806	1,381	0,933	0,496	B
TUBA1A	0,797	1,413	0,950	0,765	B
SPIB	0,796	2,033	0,983	0,829	B
PKIB	0,788	3,194	0,650	0,111	B
RALA	0,788	1,260	0,950	0,65	B
FAM110A	0,780	2,043	0,817	0,41	B
PPA1	0,778	1,599	0,950	0,782	B
SPINT2	0,766	1,586	0,933	0,692	B

LOC100131551	0,870	3,452	0,938	0,744	D
DICER1-AS1	0,858	2,301	0,906	0,706	D
PDE6A	0,858	2,197	0,969	0,813	D
GREB1	0,856	2,038	1,000	0,821	D
CDHR4	0,846	3,201	0,938	0,74	D
OSBPL10	0,846	2,110	0,938	0,71	D
AK055666	0,846	1,914	0,938	0,626	D
AKAP5	0,846	1,613	0,906	0,721	D
PNMA2	0,844	2,215	0,938	0,687	D
ERCC4	0,843	1,288	1,000	0,802	D
PTK6	0,841	2,921	0,906	0,607	D
AX746683	0,836	2,175	0,938	0,771	D
AX746734	0,835	3,079	0,906	0,74	D
CCDC120	0,834	2,565	0,938	0,725	D
KDELC2	0,834	2,185	0,938	0,664	D
PRR19	0,829	1,822	0,969	0,786	D
AX746567	0,828	3,245	0,938	0,706	D
ZNF713	0,820	2,381	0,844	0,599	D
AK056524	0,818	2,566	0,875	0,725	D
FER	0,818	2,386	0,906	0,634	D
ZNF329	0,815	2,545	0,875	0,664	D
AK092965	0,815	1,897	0,906	0,588	D
MED18	0,815	1,763	0,906	0,74	D
TMEM213	0,812	2,128	0,844	0,679	D
ZNF526	0,812	1,650	0,906	0,687	D
KCNJ11	0,811	2,438	0,844	0,611	D
EMX2OS	0,809	2,520	0,875	0,649	D
PPP1R13L	0,808	1,694	0,938	0,752	D
XRCC2	0,807	3,043	0,906	0,748	D
ZNF273	0,807	1,263	0,906	0,725	D
PIWIL2	0,806	2,937	0,844	0,618	D
CCDC150	0,806	1,981	0,938	0,782	D
BC045769	0,805	2,412	0,906	0,721	D
SYT17	0,803	2,417	0,844	0,66	D
LRRC2	0,803	2,159	0,812	0,527	D
GLIPR1L2	0,802	2,391	0,938	0,786	D
AX746995	0,802	1,920	0,844	0,668	D
LOC440300	0,802	1,786	0,906	0,66	D
DNASE1	0,800	1,739	0,875	0,641	D
CCDC88B	0,799	1,352	0,906	0,584	D
WFDC6	0,797	2,627	0,875	0,714	D
BX647715	0,797	1,705	0,875	0,691	D
PPIEL	0,797	1,445	0,969	0,809	D
DMC1	0,796	2,556	0,844	0,607	D
NLRP12	0,794	1,943	0,875	0,714	D
MAP1LC3C	0,793	1,737	0,938	0,737	D
KANK2	0,792	2,666	0,844	0,649	D

CCDC113	0,792	1,763	0,844	0,641	D
LOC727896	0,791	1,492	0,875	0,676	D
AK124832	0,790	3,245	0,812	0,653	D
AX746699	0,790	2,614	0,875	0,706	D
WDR62	0,790	2,394	0,812	0,58	D
AX747172	0,789	2,689	0,875	0,653	D
TNFAIP8L3	0,785	2,473	0,812	0,588	D
PGM2L1	0,784	2,718	0,844	0,618	D
LINC00485	0,784	2,686	0,844	0,553	D
DNAJC27-AS1	0,783	1,948	0,844	0,599	D
GNRHRII	0,782	2,848	0,938	0,786	D
SEC14L4	0,781	2,128	0,875	0,691	D
RAD23B	0,780	1,717	0,844	0,676	D
LOC100289341	0,778	1,954	0,844	0,618	D
ULK2	0,778	1,834	0,875	0,691	D
SLC14A2	0,777	2,053	0,844	0,599	D
LPO	0,776	2,289	0,812	0,611	D
CCL16	0,771	1,975	0,844	0,664	D
HP09025	0,771	1,889	0,844	0,679	D
AX746787	0,769	2,687	0,844	0,687	D
PSMG4	0,769	2,134	0,875	0,706	D
CENPN	0,765	2,835	0,906	0,725	D
ZNF677	0,764	3,407	0,844	0,691	D
SKP2	0,762	1,329	0,812	0,496	D
ANKRD16	0,761	2,124	0,844	0,687	D
LOC100128288	0,761	1,794	0,812	0,615	D
FLJ90757	0,756	1,908	0,812	0,656	D
KBTBD12	0,756	1,854	0,812	0,641	D
ZNF527	0,753	1,780	0,812	0,63	D
GCLM	0,753	1,237	0,812	0,588	D
CCDC122	0,752	1,692	0,844	0,66	D
PCBD2	0,751	1,499	0,875	0,702	D
CPVL	0,939	1,977	1,000	0,71	C
SAMHD1	0,916	1,499	1,000	0,783	C
PTEN	0,909	1,857	1,000	0,764	C
ZNF652	0,888	1,523	1,000	0,692	C
P2RY13	0,878	2,167	0,944	0,424	C
HLA-DQB2	0,869	3,646	0,889	0,38	C
TOMM7	0,868	1,365	1,000	0,667	C
LGALS2	0,862	1,933	0,944	0,453	C
NMI	0,851	1,204	1,000	0,525	C
NDUFA12	0,847	1,360	1,000	0,511	C
FAM26F	0,847	1,261	0,944	0,746	C
CD1C	0,843	2,284	0,833	0,312	C
SLC38A1	0,841	1,916	0,944	0,518	C
RUFY3	0,841	1,356	0,944	0,449	C
AF007147	0,834	1,729	0,833	0,196	C

RGS18	0,826	1,652	0,833	0,279	C
OAS3	0,824	1,528	0,944	0,663	C
MNDA	0,821	1,789	0,889	0,634	C
C1ORF162	0,820	1,404	0,889	0,707	C
CAMK2D	0,817	1,801	0,833	0,38	C
C1QBP	0,814	1,319	0,889	0,627	C
PID1	0,812	1,487	0,889	0,431	C
NDRG2	0,807	1,772	0,833	0,308	C
DDB2	0,802	1,363	0,778	0,17	C
ANKRD22	0,797	1,721	0,833	0,424	C
RARRES3	0,797	1,721	0,833	0,464	C
AK293020	0,797	1,524	0,889	0,551	C
NDUFA1	0,797	1,221	1,000	0,667	C
SFT2D2	0,796	1,549	0,833	0,37	C
CLEC10A	0,792	1,268	0,889	0,612	C
SPDYE5	0,791	1,302	0,889	0,467	C
ANKRD36BP1	0,786	1,240	0,944	0,587	C
NAA20	0,779	1,709	0,833	0,424	C
SNRPE	0,774	1,444	0,833	0,359	C
CD1D	0,773	1,538	0,833	0,482	C
UBE2K	0,765	1,242	0,833	0,402	C
IFITM1	0,764	1,567	0,889	0,482	C
SPTLC2	0,758	1,527	0,889	0,656	C
TP53	0,755	1,204	0,833	0,569	C
TSPAN13	0,996	4,590	1,000	0,043	A
PTPRS	0,982	4,155	1,000	0,468	A
ALOX5AP	0,973	3,129	1,000	0,343	A
TCF4	0,972	3,133	1,000	0,371	A
CXCR3	0,960	5,276	0,929	0,096	A
CYBASC3	0,959	2,586	1,000	0,536	A
PLAC8	0,958	2,204	1,000	0,821	A
CARD11	0,957	4,834	0,929	0,05	A
MAP1A	0,956	4,711	0,929	0,036	A
IRF4	0,956	3,605	1,000	0,454	A
LILRA4	0,954	5,113	0,929	0,182	A
GZMB	0,954	4,535	0,929	0,15	A
SERPINF1	0,948	2,701	1,000	0,232	A
SEPT1	0,945	2,643	0,929	0,089	A
IL3RA	0,945	2,060	1,000	0,571	A
SLC20A1	0,940	3,678	1,000	0,732	A
ITM2C	0,933	3,358	0,929	0,257	A
CLIC3	0,923	5,891	0,857	0,05	A
IRF8	0,923	2,654	1,000	0,775	A
EGLN3	0,920	5,928	0,857	0,05	A
INPP4A	0,919	2,069	1,000	0,225	A
SEL1L3	0,918	3,345	0,929	0,211	A
C12ORF75	0,917	3,394	0,857	0,043	A

AK128525	0,916	-3,105	1,000	0,439	A
MGLL	0,909	2,823	0,929	0,207	A
IRF7	0,908	2,928	0,929	0,675	A
DCK	0,907	2,388	0,929	0,364	A
APP	0,906	2,851	1,000	0,486	A
PIM2	0,906	2,065	1,000	0,661	A
SRSF7	0,895	2,536	1,000	0,793	A
LILRB4	0,895	1,551	1,000	0,729	A
DSTN	0,894	2,983	0,929	0,457	A
SLC7A5	0,894	2,045	1,000	0,536	A
C18ORF1	0,893	3,263	0,929	0,486	A
NAPSB	0,892	1,536	1,000	0,629	A
P2RX1	0,891	2,841	0,929	0,3	A
SMPD3	0,887	5,332	0,786	0,039	A
CDH1	0,876	2,774	0,857	0,386	A
PLD4	0,873	2,952	0,857	0,386	A
LOC100190986	0,872	3,221	1,000	0,714	A
SLC44A2	0,872	2,488	0,929	0,575	A
AK093551	0,869	2,349	0,857	0,3	A
BCL11A	0,868	2,429	0,929	0,461	A
SELL	0,867	2,255	0,857	0,404	A
VEGFB	0,866	2,412	0,786	0,118	A
LOC641298	0,865	2,645	1,000	0,768	A
USP11	0,861	1,850	0,857	0,343	A
SIVA1	0,860	1,342	1,000	0,611	A
DNAJC4	0,858	1,442	1,000	0,493	A
ARHGEF7	0,857	2,215	0,929	0,396	A
PLP2	0,856	1,760	1,000	0,461	A
KRT5	0,855	9,023	0,714	0,011	A
CCDC50	0,855	2,694	0,857	0,511	A
LUC7L	0,853	1,409	1,000	0,579	A
CLEC4C	0,848	5,778	0,714	0,054	A
SEC61B	0,848	1,927	1,000	0,764	A
SLC5A3	0,844	2,497	0,857	0,261	A
LOC100271836	0,844	2,404	0,929	0,625	A
SLC15A4	0,842	3,081	0,786	0,207	A
ANKRD36BP1	0,841	1,289	1,000	0,589	A
TOB1	0,839	2,031	0,929	0,643	A
NUB1	0,839	1,410	1,000	0,639	A
GOSR2	0,839	1,407	0,929	0,343	A
SOX4	0,838	3,591	0,786	0,246	A
POLB	0,836	3,235	0,786	0,25	A
MCOLN2	0,836	2,154	0,857	0,343	A
CHAF1A	0,833	1,827	0,857	0,282	A
MKNK2	0,832	1,984	0,929	0,468	A
SEMA7A	0,831	2,553	0,786	0,171	A
EIF2AK4	0,828	2,421	0,857	0,439	A

ACTN4	0,827	2,433	0,857	0,425	A
TRAF4	0,826	3,760	0,714	0,132	A
DERL3	0,826	2,961	0,714	0,118	A
FCHSD2	0,826	2,112	0,857	0,364	A
ZDHHC17	0,824	2,497	0,786	0,189	A
NUP88	0,823	1,774	0,786	0,243	A
AHCY	0,821	1,706	0,857	0,446	A
OFD1	0,820	2,007	0,786	0,268	A
SEPHS1	0,819	3,903	0,714	0,164	A
TP53I11	0,819	2,934	0,714	0,125	A
NOTCH4	0,819	2,308	0,929	0,632	A
HVCN1	0,819	2,153	0,857	0,368	A
OPTN	0,819	1,474	0,786	0,279	A
TSPYL2	0,816	1,539	0,857	0,368	A
BCL2L11	0,814	1,424	0,857	0,5	A
FAM160A1	0,811	4,497	0,643	0,032	A
HSPH1	0,811	2,519	0,929	0,707	A
MZB1	0,811	1,460	0,714	0,093	A
HIGD1A	0,811	1,452	0,929	0,625	A
RNF126	0,810	2,046	0,714	0,111	A
UGCG	0,809	4,190	0,714	0,146	A
ANKRD11	0,808	1,671	0,857	0,461	A
GPBP1L1	0,808	1,407	0,857	0,45	A
KIF5B	0,808	1,350	0,929	0,725	A
PPP1R2	0,807	1,258	0,929	0,779	A
CORO1C	0,806	2,119	0,929	0,654	A
GNA15	0,806	1,850	0,929	0,693	A
JOSD1	0,805	1,731	0,929	0,75	A
PDE7A	0,804	1,816	0,929	0,611	A
MAP3K8	0,804	1,553	1,000	0,661	A
TERF1	0,804	1,296	0,929	0,55	A
SEMA3C	0,801	4,032	0,643	0,054	A
SLC38A1	0,801	1,475	0,857	0,529	A
SPCS1	0,798	1,313	0,929	0,686	A
TOR3A	0,796	2,169	0,857	0,486	A
P2RY6	0,796	1,376	0,857	0,454	A
SLC25A29	0,795	3,436	0,643	0,086	A
NPIPL3	0,795	2,673	1,000	0,843	A
C4ORF26	0,795	1,204	0,857	0,511	A
C1ORF186	0,794	4,217	0,643	0,096	A
ST6GALNAC4	0,794	2,091	0,714	0,171	A
KLHDC4	0,793	1,594	0,786	0,232	A
SELS	0,793	1,539	0,929	0,65	A
LIME1	0,790	2,780	0,643	0,075	A
PTPRCAP	0,790	2,375	0,643	0,089	A
AMIGO3	0,785	1,765	0,714	0,179	A
NOP58	0,785	1,544	0,929	0,639	A

RFTN1	0,785	1,541	0,857	0,489	A
PACSIN1	0,784	5,284	0,571	0,007	A
WDFY4	0,784	1,411	0,929	0,429	A
SLC32A1	0,783	4,900	0,571	0,014	A
PLXNA4	0,782	4,505	0,571	0,011	A
MYBL2	0,781	6,173	0,571	0,018	A
OGT	0,781	1,563	0,857	0,55	A
EPHB1	0,780	4,798	0,571	0,021	A
GALNT3	0,776	1,907	0,643	0,136	A
LOC100507173	0,776	1,338	1,000	0,8	A
TPM2	0,773	3,845	0,571	0,039	A
BTG2	0,773	2,156	0,929	0,779	A
SH3BP4	0,769	4,065	0,571	0,054	A
TTC3	0,769	1,463	0,929	0,743	A
CPNE1	0,769	1,275	0,857	0,593	A
POLR2A	0,765	1,358	0,929	0,707	A
TXNDC3	0,764	2,783	0,571	0,046	A
GALNT4	0,763	1,363	0,929	0,636	A
TCP1	0,760	1,705	0,929	0,754	A
PRMT10	0,758	1,649	0,929	0,614	A
RAB33B	0,752	1,891	0,857	0,596	A
IRF2BP2	0,752	1,569	0,857	0,657	A
UBE2D2	0,751	1,699	0,857	0,489	A

Annex 2.

Transcriptomic analysis and High dimensional phenotypic mapping of mononuclear phagocytes in mesenteric lymph nodes reveal differences between ulcerative colitis and Crohn's disease

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Author's contribution	I participated to the experimental work (50%), data analysis of transcriptome data (40%) and drafting of the manuscript (30%).

Journal	Journal of Crohn and Colitis
Status	Published September 2019.
DOI	10.1093/ecco-jcc/jjz156
Key words	Mesenteric lymph nodes, humans, macrophage, dendritic cells, inflammatory bowel disease

Journal of Crohn's and Colitis, 2019, 1–13
doi:10.1093/ecco-jcc/jjz156
Advance Access publication September 21, 2019
Original Article

Original Article

Transcriptomic Analysis and High-dimensional Phenotypic Mapping of Mononuclear Phagocytes in Mesenteric Lymph Nodes Reveal Differences Between Ulcerative Colitis and Crohn's Disease

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Abstract

Background and Aims: Crohn's disease [CD] and ulcerative colitis [UC] are distinct forms of inflammatory bowel disease. Heterogeneity of HLA-DR$^+$SIRPα^+ mononuclear phagocytes [MNPs], including macrophages [MΦ], monocyte-derived [Mono] cells, and dendritic cells [DCs], was reported in gut tissue but not yet investigated in mesenteric lymph nodes [MLNs] of IBD patients. We here compared the phenotype, function, and molecular profile of HLA-DR$^+$SIRPα^+ MNPs in CD and UC MLNs.

Methods: Cell distribution, morphology, immune function, and transcriptomic [bulk RNAseq] and high-dimensional protein expression profiles [CyTOF] of HLA-DR$^+$SIRPα^+ MNPs were examined in MLNs of UC [$n = 14$], CD [$n = 35$], and non-IBD [$n = 12$] patients.

Results: Elevated frequencies of CD14$^+$CD64$^+$CD163$^+$ [Mono/MΦ-like] MNPs displaying monocyte/MΦ morphology and phagocytic function were a distinct feature of UC MLNs. In CD, the proportion of CD14$^-$CD64$^-$CD163$^-$ [DC-like] cells was augmented relative to Mono/MΦ-like cells; DC-like cells drove naïve T cell proliferation, Th1 polarisation, and Th17 T_{CM} plasticity. Gene expression profile corroborated the nature of DC-like cells, best represented by *BTLA*, *SERPINF*, *IGJ* and, of Mono/

MΦ-like cells, defined by *CD163, MARCO, MAFB, CD300E, S100A9* expression. CyTOF analysis showed that CD123$^+$ plasmacytoid cells predominated over conventional DCs in DC-like cells. Four CD163$^+$ clusters were revealed in Mono/MΦ-like cells, two of which were enriched in MARCO$^-$ CD68^{dim}HLA-DR^{dim} monocyte-like cells and MARCO^{hi}CD68^{hi}HLA-DR^{hi} Mϕ, whose proportion increased in UC relative to CD.

Conclusions: Defining the landscape of MNPs in MLNs provided evidence for expansion of CD163$^+$ Mono/MΦ-like cells in UC only, highlighting a distinction between UC and CD, and thus the potential contribution of monocyte-like cells in driving colitis.

Key Words: Mesenteric lymph nodes; dendritic cells; monocytes; macrophages; RNAseq; mass cytometry; Crohn's disease; ulcerative colitis

1. Introduction

Crohn's disease [CD] and ulcerative colitis [UC] are the two most predominant forms of inflammatory bowel disease [IBD]. Several factors contribute to IBD development including genetic predisposition, environmental triggers, gut dysbiosis, and immune dysfunction.[1] Priming of naïve T cells, and education of memory T cells to home to gut tissue, occur in mesenteric lymph nodes [MLNs] through interactions with mononuclear phagocytes [MNPs].[2,3] Furthermore, MLNs are essential for induction of oral tolerance and prevention of microbial dissemination under homeostatic conditions.[2,4]

In mice, MLNs that drain the small intestine and colon are anatomically and functionally segregated.[5,6] Tolerogenic and pro-inflammatory T cell responses are generated in proximal and distal gut-draining MLNs, respectively.[7] Three CD14$^-$ dendritic cell [DC] populations have been reported in the small intestine and draining MLNs[3,8–11]: BATF3/IRF8-dependent CD103$^+$CD11b$^-$ SIRPα$^-$CX3CR1$^-$CLec9a$^+$CD141$^+$XCR1$^+$ [CD103$^+$cDC1], IRF4-dependent CD103$^+$CD11b$^+$SIRPα$^+$CX3CR1^{low} [CD103$^+$cDC2], and CD103$^-$CD11b$^+$CCR2$^+$ [CD103$^-$cDC2] cells. DC populations in the gut upregulate the expression of CCR7 and HLA-DR, and migrate to MLNs where they join resident HLA-DR^{low} cDC1 and cDC2 populations.[9,12] Functionally, migratory CD103$^+$ cDC1 and CD103$^+$ cDC2 can present orally derived antigens, induce the polarisation of naïve T cells into regulatory FoxP3$^+$ T cells, and imprint CCR9 and α4β7 homing molecules on T cells in a retinoid acid-dependent fashion.[2,13] In contrast, CD103$^-$ cDC2 secrete IL-12 and IL-23 in the absence of overt stimulation, and induce strong inflammatory Th1 and Th17 responses from naïve T cells.[3,14] CD103$^+$ cDC1 and CD103$^-$ cDC2 are the major DC populations found in colonic MLNs, consistent with the very low frequency of CD103$^+$ cDC2 in the colon.

The distribution of DC populations in human gut appears to be analogous to that reported in mice.[15] However, functional properties of DCs in MLNs are extrapolated from data obtained using DCs isolated from intestine. Granot *et al.* reported the presence of resident and migratory cDC1 and cDC2 in MLNs of deceased organ donors.[16] The tissue origin of migratory DCs is inferred by their elevated CCR7 expression acquired in gut. Nonetheless, there are limited studies examining DCs in MLNs of IBD patients. Immunohistochemistry identified cDC1 [BDCA3$^+$] and cDC2 [BDCA1$^+$] in MLNs of CD, UC, and non-IBD patients.[17] By conventional flow cytometric analysis, it was shown that equivalent frequencies of CD103$^+$DCs are observed in MLNs draining the small intestine of CD and non-IBD patients.[18] Furthermore in CD MLNs, CD11c$^+$ DCs, which are stratified by their level of HLA-DR expression, produce high amounts of IL-23 and low amounts of IL-10 upon stimulation, and induce strong Th1/Th17 immune responses, a key feature of CD.[19]

Several macrophages [MΦ] subsets that occupy distinct niches cohabit with DCs in LNs. Ontogenically, murine MΦ are thought to be derived from adult circulating monocytes that replenish the LN pool, since their embryonic origin is not established in LNs.[20] CX3CR1$^+$F4/80$^-$ subcapsular sinus MΦ [SSM] are known to easily capture and transfer lymph-borne substances to B cells, whereas CX3CR1$^-$ F4/80$^+$ medullary sinus MΦ [MSM] are highly phagocytic. In addition to phagocytosis of apoptotic plasma cells, CX3CR1$^-$ F4/80$^+$ medullary cord MΦ [MCM] provide trophic support to these cells.[21] Recently, another subset of MΦ has been reported in the skin-draining LN T cell zone [TZM]. These CX3CR1$^+$ F4/80$^-$ MΦ, which are long-lived in utero and replaced by circulating monocytes in adult mice, are specialised in efferocytosis of numerous apoptotic cells present in the T cell zone.[22] Finally, the germinal centre in reactive LN hosts highly phagocytic tangible body MΦ [TBM] that eliminate apoptotic cells. Like in the mice, human SSM and MSM are both CD68$^+$CD169$^+$, whereas paracortex MΦ express CD68 but not CD169.[23] Similar to DCs, MΦ and monocyte-derived cells [MCs] are poorly characterised in MLNs of IBD patients.

We previously identified a heterogeneous HLA-DR$^+$SIRPα$^+$ population that includes CD14$^+$ and CD14$^-$ cells in colonic MLNs of CD patients.[24] Furthermore, adoptively transferred SIRPα$^+$ MNPs, isolated from colonic MLNs of chemically-induced colitic wild-type mice, elicit colitis in otherwise protected CD47$^{-/-}$ mice,[25] highlighting the relevance of investigating MNPs in MLNs of human IBD. In this report, we uncovered the entire phenotypic landscape of CD14$^-$ and CD14$^+$ HLA-DR$^+$SIRPα$^+$ MNPs in MLNs of CD and UC patients, using mass cytometry in parallel with molecular and functional studies. These data revealed differences and similarities between CD and UC patients, highlighting potentially distinct functionalities of MNPs in MLNs of these two subtypes of IBD.

2. Materials and Methods

2.1. Human clinical samples

All participants signed an informed consent form that had been approved by the Institutional Ethics Research Committee of the Centre Hospitalier de l'Université de Montréal. MLNs were acquired from surgical resections. Patient recruitment was based on clinical and histological criteria. This study includes 14 patients with UC, 35 patients with CD, and 12 non-IBD patients. Clinical information is shown in Table S1a and b, available as

Supplementary data at *ECCO-JCC* online. Cord blood, used for isolation of naïve CD4⁺ T cells, was obtained from healthy subjects.

2.2. Cell purification and data analysis

MLNs were digested mechanically to obtain cellular suspensions.[24] Monoclonal antibodies used for flow cytometry [10 parameters] and CyTOF [37 parameters] are listed in Tables S2 and S3, respectively, available as Supplementary data at *ECCO-JCC* online. Data were analysed with either FCS Express 6 [DeNovo Software] or FlowJo version 10.5.3 [FlowJo, LLC, BD].

2.3. Cell sorting

HLA-DR⁺SIRPα⁺CD14⁺CD64⁺CD163⁺ [monocytes, monocytes-derived and macrophages [Mono/MΦ]-like cells], and HLA-DR⁺SIRPα⁺CD14⁻CD64⁻CD163⁻ [dendritic cells [DC]-like cells] MNPs were FACS-sorted [fluorescence-activated cell sorting] for morphological, functional, and molecular studies.

2.4. Morphology

For morphological studies, FACS-sorted MNPs were cytospun and stained according to the Wright Stain procedure. A Leica DM4000B microscope, equipped with Leica DFC300FX camera, was used to visualise cells.

2.5. Phagocytosis assay

Sorted DC-like and Mono/MΦ-like cells were cultured with or without 1/100 fluorescein isothiocyanate [FITC]-labelled latex beads [Cayman Chemical Company, Ann Arbor, USA] in 96-well plates for 4 h. Samples were quenched with trypan blue for 2 min. Cells were analysed by flow cytometry.

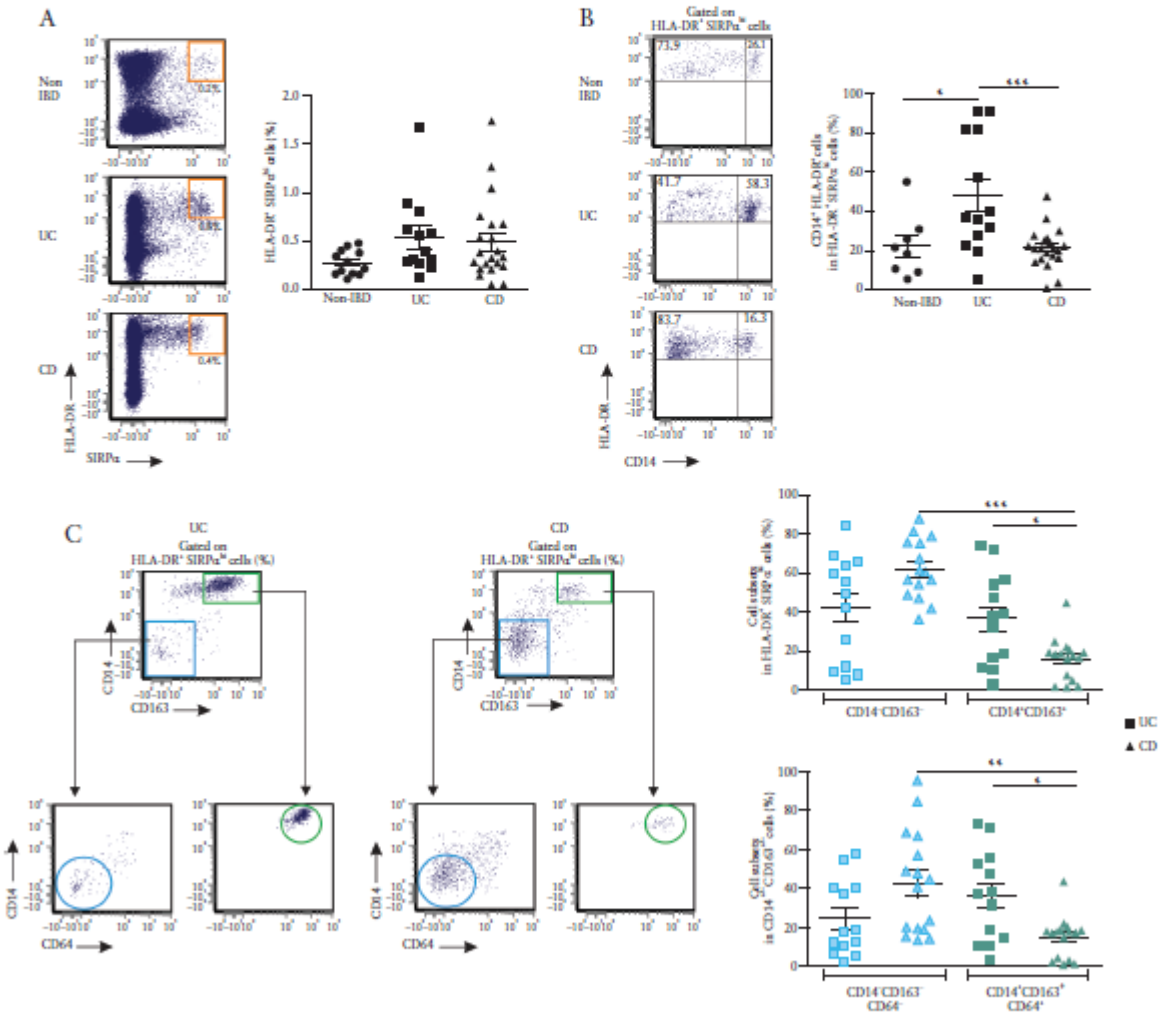

Figure 1. Increased frequencies of CD14⁺CD163⁺ in MLNs of UC relative to CD patients. Representative dot plots and frequencies in MLNs of as follows. A. HLA-DR⁺SIRPα^hi MNPs of control [$n = 12$], UC [$n = 13$], and CD [$n = 21$], Kruskal_Wallis test. B. CD14⁺HLA-DR⁺ in HLA-DR⁺SIRPα^hi MNPs of control [$n = 8$], UC [$n = 13$], and CD [$n = 21$], [$p < 0.0007$]. C. CD14⁻CD163⁻ versus CD14⁺CD163⁺ cells in HLA-DR⁺SIRPα^hi MNPs [top right panel], [$p < 0.0001$], or CD14⁺CD64⁺CD163⁻ versus CD14⁺CD64⁺CD163⁺ in CD14^{+/-}CD163^{+/-} cells [bottom right panel] of UC [$n = 13$] and CD [$n = 15$], [$p < 0.002$]. For B to D, one-way ANOVA followed by Bonferroni's test [$^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$]. [See also Figure S1, available as Supplementary data at *ECCO-JCC* online]. MLN, mesenteric lymph node; UC, ulcerative colitis; CD, Crohn's disease; MNP, mononuclear phagocytes; ANOVA, analysis of variance.

2.6. Cell cultures

Mono/MΦ-like and DC-like MNPs were co-cultured with allogeneic CFSE-labelled naïve CD4$^+$ T cells at different MNs/T cell ratios, and cell proliferation was assessed by CFSE dilution. For Th cell polarisation, allogeneic naïve CD4$^+$ T cells were co-cultured with MNP subsets at a 25:1 ratio for 6 days. For T_{CM} plasticity, Th17 T_{CM} cells were stimulated with anti-CD3/CD28 coated beads [Miltenyi Biotec], and co-cultured with autologous MNP subsets purified from CD MLNs at a 10:1 ratio for 6 days in the presence of PGN [10 µg/ml]. For all cultures: a] RPMI 1640 medium supplemented with 10% fetal calf serum and 1% penicillin-streptomycin was used; b] for intracytoplasmic staining, cells stimulated *ex vivo* or after culture with PMA-ionomycin for 6 h, with brefeldin A added for the last 3 h, were then fixed and stained with CD3 monoclonal antibody followed by intracytoplasmic staining for IL-17 and IFN-γ. Cytokines in culture supernatants were measured by multiplex assay [Eve Technologies, Calgary, AB, Canada].

2.7. RNA sequencing

RNA samples were isolated using the Qiagen RNeasy Mini kit, following the manufacturer's recommendations. RNA quality check was done using Agilent RNA Nano 6000 chip [Agilent Technologies]. Whole transcriptome amplification [WTA] was performed using a modified SMART-Seq2 protocol, as described previously,[25] with Maxima Reverse Transcriptase [Life Technologies] used in place of Superscript II. Whole transcriptome amplification [WTA] products were cleaned with Agencourt XP DNA beads and 70% ethanol [Beckman Coulter, Brea, CA], and Illumina sequencing libraries were prepared using Nextera XT [Illumina, San Diego, CA], as previously described.[26] The libraries of each samples were pooled together and cleaned with two rounds of 0.8x DNA SPRIs [Beckman Coulter]. Library quality was assessed with a high-sensitivity DNA chip [Agilent] and quantified with a high-sensitivity dsDNA Quant Kit [Life Technologies]. Samples were sequenced on an Illumina NextSeq 500 instrument using the 75 cycles NextSeq 500/550 High Output Kit v2 [37 base pairs paired-end reads]. Refer to Supplementary methods for further details, available as Supplementary data at *ECCO-JCC* online

A differential expression analysis was performed using RNA-sequencing data counts for 23 686 genes. The count dataset was produced from two experimental batches, so the batch removal procedure from the limma R package had to be used. Samples having less than 6000 genes expressed, as well as an outlier revealed by PCA [principal component analysis], were removed. Genes with no observable expression count for more than half of the samples were also removed from the dataset. A TMM [trimmed mean of M values] normalisation [EdgeR package] was then applied to the remaining 12 231 genes in all samples.

A differential expression analysis was performed on all samples with limma. A contrast matrix was applied to assess for the differential expression of FACS-sorted DC-like versus Mono/MΦ-like populations from MLNs of five UC and seven CD patients, respectively. Gene set enrichment analysis [GSEA] was obtained using the regression data from limma using the Camera algorithm [Camera: a competitive gene set test accounting for inter-gene correlation] to scan the publicly available MySigDB database. R package Gplots was used to generate heatmaps of significant pathways.

2.8. Statistical analysis

Statistical analysis was performed with GraphPad Prism version 6 [GraphPad Software, La Jolla, CA, USA]. Data were checked for normality using ShapiroWilk test, and the appropriate test was next applied as indicated. For multiple unpaired samples, aoneway ANOVA [analysis of variance] test was applied followed by a Bonferroni test [normal distribution], and a KruskalWallis test followed by Dunn's test [no normal distribution]. A Friedman test followed by Dunn's test was applied for multiple paired samples [no normal distribution]. For two columns comparison, a two-tailed Wilcoxon signed rank test [paired] and a MannWhitney test [unpaired] were used. A chi square test was used as indicated. For all tests, one symbol means $p < 0.05$, two symbols mean $p < 0.01$, and three symbols mean $p < 0.001$. Bar graph data are shown as mean ± SEM [standard error of the mean]or SD [standard deviation]. For molecular analysis, false discovery rate [FDR] p-values adjustment was used as a method to account for multiple testing on both differential expression [DE] and gene set enrichment analysis [GSEA] experiments, as it is implemented into the limma analysis software functions. R package Gplots was used to generate heatmaps of significant pathways.

3. Results

3.1. Increased frequencies of CD14$^+$CD64$^+$CD163$^+$ in MLNs of UC relative to CD patients

The frequencies of HLA-DR$^+$SIRPα$^+$ MNPs were examined in colonic MLNs of UC [$n = 13$] relative to CD [$n = 21$] and diverticulosis [non-IBD; $n = 12$] patients. The HLA-DR$^+$SIRPα$^+$ population was first subdivided into HLA-DR$^+$SIRPα^{hi} and HLA-DR$^+$SIRPα^{low} cells. The frequencies of HLA-DR$^+$SIRPα^{hi} cells tended to increase in colonic MLNs of UC and CD patients when compared with non-IBD donors [Figure 1A]. Notably, the difference between IBD and non-IBD patients was significant only when the UC or CD groups were individually compared with the non-IBD control group [unpaired t test: $p < 0.05$ and $p < 0.04$, respectively], corroborating and extending our previously published observations.[24] In contrast, frequencies of unfractionated HLA-DR$^+$SIRPα$^{low/hi}$ as well as HLA-DR$^+$SIRPα^{low} cells were equivalent among the three groups of patients [Figure S1A, available as Supplementary data at *ECCO-JCC* online]. Since the HLA-DR$^+$SIRPα$^+$ population includes CD14$^-$ and CD14$^+$ cells in MLNs of CD,[16,24] we examined the percentage of CD14$^+$ among HLA-DR$^+$SIRPα$^+$ cells in the three groups of patients. Unexpectedly, only in UC patients was the frequency of CD14$^+$ MNPs significantly increased when considering either the HLA-DR$^+$SIRPα^{hi} or the HLA-DR$^+$SIRPα$^{low/hi}$ populations, but not HLA-DR$^+$SIRPα^{low} cells [Figure 1B and Figure S1B], suggesting that the increase of CD14$^+$ MNPs in the HLA-DR$^+$SIRPα^{hi} subset discriminates between UC and CD.

In light of these observations, HLA-DR$^+$SIRPα^{hi} MNPs were further subdivided based on expression of CD14 and CD163, which marks MΦ in tissues or LNs.[27] The proportion of CD14$^+$CD163$^+$ cells was significantly augmented in UC when compared with CD patients [Figure 1C, upper panels]. In CD only, the percentage of CD14$^-$CD163$^-$ cells was elevated relative to CD14$^+$CD163$^+$ cells. Noteworthy, the percentages of CD14$^-$CD163$^+$ and CD14$^+$CD163$^-$ subsets, which both represented ~10% of HLA-DR$^+$SIRPα^{hi} MNPs, were equivalent in UC and CD patients [Figure S1C]. Since CD64 expression reportedly delineated MΦ in mice[28] and inflammatory monocyte-derived cells in humans, CD14$^+$CD163$^+$ and CD14$^-$CD163$^-$ MNPs were further stratified using CD64. As depicted in Figure 1C [bottom panels], all CD14$^+$CD163$^+$ co-expressed CD64 at high intensity, whereas CD14$^-$CD163$^-$ MNPs predominantly comprised CD64$^-$ cells. The frequencies of CD14$^+$CD163$^+$CD64$^+$cells

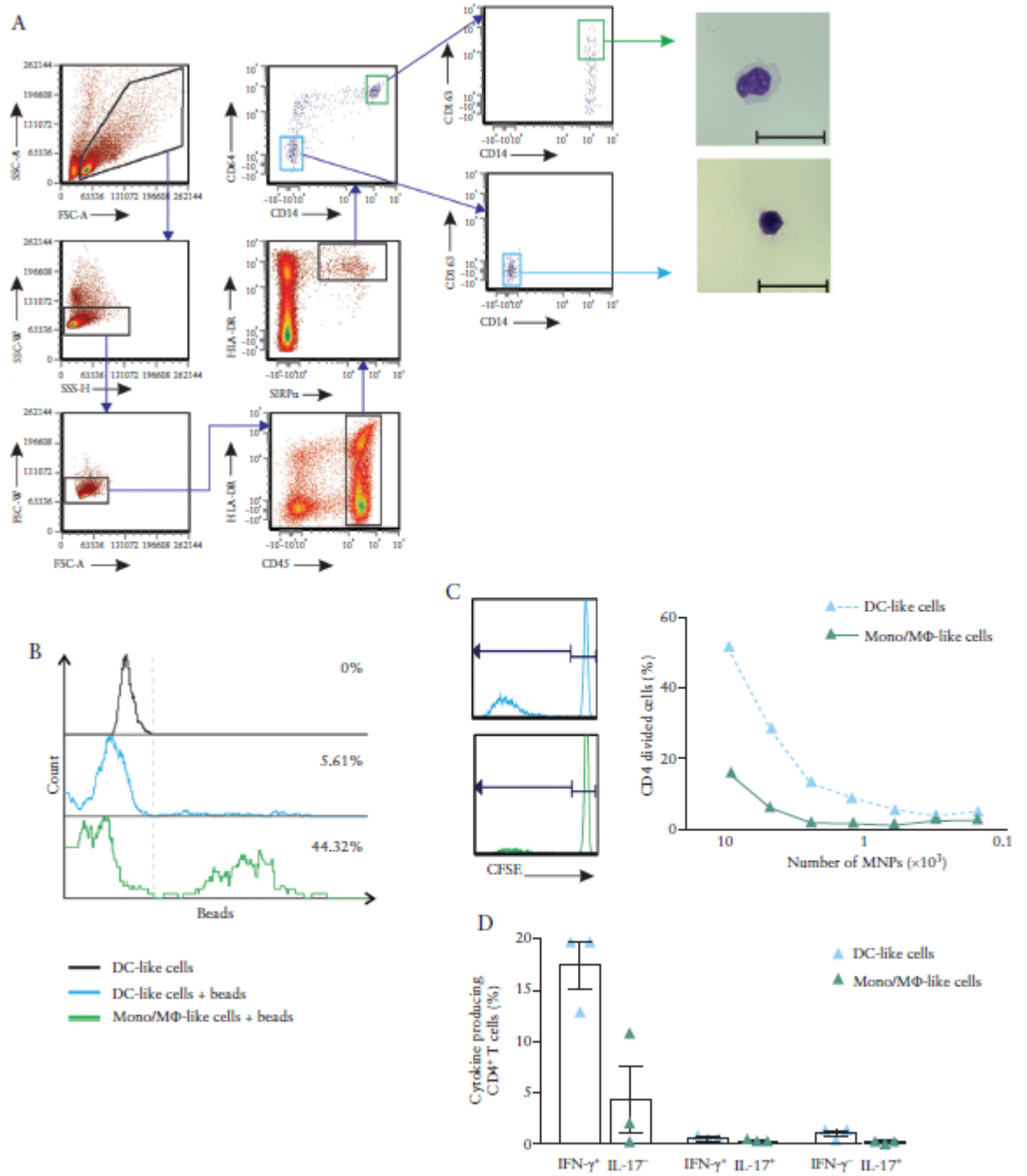

Figure 2. CD14⁺CD64⁺CD163⁺ and CD14⁻CD64⁻CD163⁻ MNPs displayed morphology and function of macrophages and dendritic cells, respectively. A. Gating strategy for isolation of CD14⁺CD64⁺CD163⁺ and CD14⁻CD64⁻CD163⁻ MNPs from IBD MLNs, cell size and morphology [scale bar = 20 μm]. B. Phagocytosis of rabbit IgG–FITC coupled beads by Mono/MΦ-like versus DC-like MNPs from UC MLNs [*n* = 2]. C. For proliferation assay [CFSE dilution], percentage division of fixed number of naïve CFSE-labelled CD4⁺T cells when co-cultured with increasing numbers of allogeneic activated MNP subsets for 5 days. D. Purified naïve CD4⁺T cells were co-cultured with allogeneic activated MNP subsets at 25:1 ratio for 6 days, followed by 4 h with PMA-ionomycin and brefeldin A. Percentage of IFN-γ and IL-17 producing CD4⁺T cells. [See also Figure S2, available as Supplementary data at *ECCO-JCC* online]. MLN, mesenteric lymph node; UC, ulcerative colitis; MNP, mononuclear phagocyte; IBD, inflammatory bowel disease.

were selectively augmented in UC when compared with CD, wherease CD14$^-$CD163$^-$ CD64$^-$cells predominated in CD. In conclusion, increased frequencies of CD14$^+$CD163$^+$ cells that co-expressed CD64 distinguished UC from CD in MLNs.

3.2. CD14$^+$CD64$^+$CD163$^+$ and CD14$^-$CD64$^-$CD163$^-$ MNPs displayed morphology and function of macrophages and of dendritic cells, respectively

In order to investigate the nature and function of CD14$^+$CD64$^+$CD163$^+$ and CD14$^-$CD64$^-$CD163$^-$ MNPs, we purified these cells according to the gating strategy depicted in Figure 2A. A kidney-shaped nucleus or a large cytoplasm with vacuoles, consistent with monocyte and MΦ morphology, respectively, characterised CD14$^+$CD64$^+$CD163$^+$ [Mono/MΦ-like] cells, whereas dendrites were seen on CD14$^-$CD64$^-$CD163$^-$ [DC-like] cells [Figure 2A].

Since the Mono/MΦ-like cells were found in high proportions in UC MLNs, some of their functionalities were assessed in UC patients only. The Mono/MΦ-like cells were phagocytic when compared with DC-like cells [Figure 2B]. Conversely DC-like, and not Mono/MΦ-like, MNPs isolated from CD induced allogeneic naïve CD4$^+$ T cell proliferation, reflecting their antigen-presenting function [Figure 2C]. Furthermore, these cells polarised naïve CD4$^+$ T cells into IFN-γ$^+$IL-17$^-$ [Th1], but not IL-17$^+$IFN-γ$^-$ [Th17] or IL-17$^+$IFN-γ$^+$ [Th1/Th17], cells [Figure 2D]. We next investigated the cytokine expression profile of Mono/MΦ-like and DC-like subsets in both diseases. Although IL-1β, IL-6, IL-10, IL-12p40, IL-23p19, and TNF-α expression was low in DC-like as compared with Mono/MΦ-like MNPs using *ex vivo* cell staining [Figure S2, available as Supplementary data at *ECCO-JCC* online, left panels], TLR2 stimulation significantly augmented cytokine secretion in both cell types [Figure S2, right panels].

Collectively, these data suggested that phagocytic CD14$^+$CD64$^+$CD163$^+$ MNPs, that preferentially accumulated in MLNs of UC when compared with CD, comprised some MΦ, whereas CD14$^-$CD163$^-$CD64$^-$ cells that predominated in MLNs of CD were enriched in *bona fide* DCs.

3.3. DC-like MNPs regulated plasticity of autologous Th17 T_{CM} in MLNs

Double IL-17/IFN-γ-secreting cells [Th17/Th1] display pathogenicity in mice with colitis,[29] and similar cells are detected in gut of patients with CD.[30,31] We therefore asked whether DC-like cells, which did not induce naïve T cell polarisation into Th17/Th1, regulate plasticity of autologous recirculating Th17 central memory T cells [T_{CM}] towards the Th17/Th1 profile [Figure 3]. T_{CM} have been reported to be less differentiated and more prone to conversion than Th17 effector memory T cell [T_{EM}] responses in MLNs.[32] To this end, CD4$^+$CD45RO$^+$CD62L^{high} T_{CM} were subdivided and purified from the MLNs of CD patients, according to surface markers associated with the Th17 [CCR6$^+$CXCR3$^-$], Th1 [CCR6$^-$CXCR3$^+$] and Th17/Th1 [CCR6$^+$CXCR3$^+$] profile [Figure S3A and B, available as Supplementary data at *ECCO-JCC* online]. Indeed, *ex vivo*

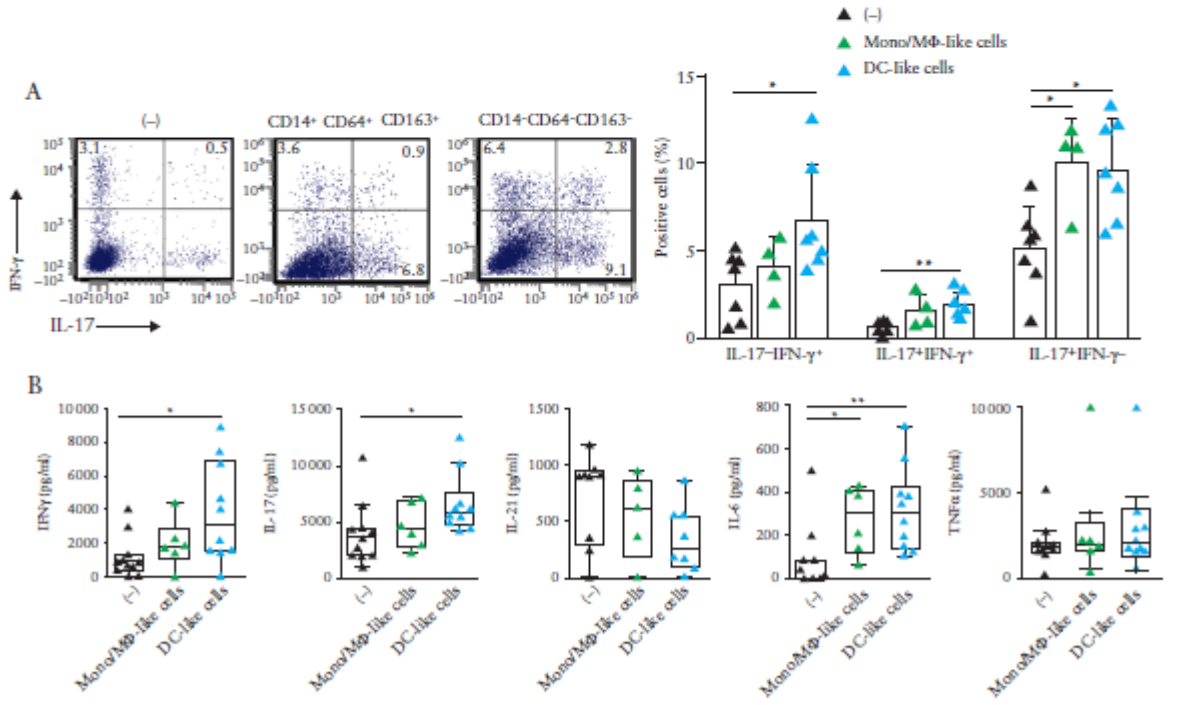

Figure 3. DC-like MNPs regulated plasticity of autologous Th17 T_{CM} in MLNs. Purified Th17 T_{CM} were co-cultured with anti-CD3/CD28 coated beads, in the absence or presence of HLA-DR$^+$SIRPα$^+$ MNP subsets isolated from CD MLNs, for 6 days, followed by PMA-ionomycin for 6 h, and brefeldin A was added for the last 3 h. **A.** Representative dot plots and percentages of IFN-γ and IL-17-producing cells [DC-like MNPs [$n = 7$] and Mono/MΦ-like MNPs [$n = 4$]], Kruskal/Wallis test [IL-17$^-$IFN-γ$^+$ $p < 0.05$, IL-17$^+$IFN-γ$^+$ $p < 0.009$, IL-17$^+$IFN-γ$^-$ $p < 0.006$], followed by Dunn's test [$^*p < 0.05$, $^{**}p < 0.01$]. B. Cytokines in culture supernatants measured by multiplex assay (DC-like MNPs [$n = 8$–10], and Mono/MΦ-like MNPs [$n = 5$–6]). Kruskal/Wallis test [IFN-γ $p < 0.02$, IL-17 $p < 0.03$, IL-21 $p = 0.15$, IL-6 $p < 0.005$, TNF-α $p < 0.6$], followed by Dunn's test [$^*p < 0.05$, $^{**}p < 0.01$]. [See also Figure S3, available as Supplementary data at *ECCO-JCC* online. MLN, mesenteric lymph node; CD, Crohn's disease; MNP, mononuclear phagocyte.

stimulated T_{CM} cells induced a cytokine profile [IL-17 and IFN-γ expression] that concurred with their phenotypic signature, which is in agreement with previous studies in blood of healthy donors.[33] Furthermore, Th17 T_{CM} were converted into IFN-γ-secreting T cells in the presence of IL-12, whereas addition of IL-23 together with IL-1β increased IL-17 expression [Figure S3C and D], corroborating their plasticity. As depicted in Figure 3A, DC-like but not Mono/MΦ-like MNPs cells in co-culture with Th17 T_{CM} favoured the emergence of IL-17$^-$IFN-γ$^+$[ex-Th17 or Th1*] and IL-17$^+$IFN-γ$^+$ [Th17/Th1] T cells, though both cell types increased frequencies of IL-17$^+$IFN-γ$^-$ T cells [Th17]. Cytokine measurement in culture supernatant corroborated the intracytoplasmic staining for IFN-γ and IL-17 [Figure 3B]. IL-6 but not TNF-α was augmented by MNPs, whereas IL-21 tended to decrease [Figure 3B].

Taken together, we propose that DC-like cells contributed to Th17 T_{CM} plasticity towards a Th17/Th1 and Th1* profile in MLNs, and both MNP subsets amplified Th17 responses.

3.4. Molecular signature of DC-like and Mono/MΦ-like cells in MLNs of CD and UC patients

We next performed genome-wide transcription profiling on FACS-sorted Mono/MΦ-like and DC-like populations from MLNs of five UC and seven CD patients [Figure 4]. Principal component analysis [PCA] of gene expression segregated these two subpopulations independently of disease type [Figure 4A]. Comparing gene expression profiles of the two HLA-DR$^+$SIRPα$^+$ MNP subsets indicated that CD163$^+$Mono/MΦ-like cells expressed, as expected, significantly higher levels of *CD14*, *FCGR1A*, and *CD163* than CD163$^-$ DC-like cells in both UC and CD [Figure 4B], thus validating our gating strategies for cell subset isolation. We next identified the gene signature that distinguished Mono/MΦ-like and DC-like populations for all UC or CD patients individually [Tables S4 and S5, available as Supplementary data at *ECCO-JCC* online, and Figure 4C, left [UC] and right [CD] panels, FDR < 0.05, DC-like versus Mono/MΦ-like cells]. Top genes significantly differentially upregulated in DC-like cells relative to Mono/MΦ-like cells included *PTPRS*, *CDH1/e-cadherin*, *IRF4*, *SERPINF*, *IGJ*, *TRAF4*, *GZMB*, *RUNX2*, *BCL11A* [FDR < 0.1], whereas examples of significantly downregulated genes included *MAFB*, *MARCO*, *STAB1*, *SEPP1*, *C2*, *CSF1R*, *CQ1B*, *CD300E*, *S100A8/A9*, *APOE*, *IL1B*, *THBS1*, *VCAN*, *C5AR1*, *CSTL1* [FDR < 0.003] for both diseases and across all patients examined [Figure 4D, volcano plots, heatmap, and Tables S6 and S7, available as Supplementary data at *ECCO-JCC* online, [0.05 < FDR < 0.1].

To gain further insight into the identity of Mono/MΦ-like and DC-like cells in UC and CD, we next performed cross-species analysis by comparing gene expression of these two populations with data obtained from murine LN MΦ and DCs.[22] MΦ genes [*CD14, CD68/, MERTK*] were significantly enriched in the Mono/MΦ-like population, whereas DC genes [*BTLA*, *FLT3*, *CCR7*] were enriched in DC-like MNPs [Figure 4E]. Furthermore, violin plots showed elevated *BTLA*, *FLT3*, *CCR7*, and *IRF4* expression in DC-like populations [Figure S4A], and *SIGLEC1/CD169*, *MARCO*, *CD209*, *MERTK*, *MSR1*, *MRC1*, *ITGAM1*, *CD300E*, *TREM-1*, *TLR4*, *IL1B*, and *IL10* in Mono/MΦ-like cells [Figure S4B, available as Supplementary data at *ECCO-JCC* online]. Analysis of functional pathways (as indexed in the Gene Ontology [GO] database) further indicated that Mono/MΦ-like cells were endowed with MΦ capacities, corroborating their phagocytic activity. Indeed, Mono/MΦ-like cells expressed genes involved in phagocytosis and its regulation [GO:0006909; GO:0050766] [Figure S5A, available as Supplementary data at *ECCO-JCC* online]. Further examination of the gene signature of CD14$^{+/-}$CD64$^{+/-}$CD163$^{+/-}$ MNPs showed that the Mono/MΦ-like subset overexpressed genes implicated in leukocyte chemotaxis [GO:0002690] [Figure S5B].

The gene expression pattern of CD14$^+$CD64$^+$CD163$^+$ cells accumulating in UC concurred with the functional and morphological observations that these cells comprised at least typical MΦ. A characteristic DC-enriched signature also highlighted the heterogeneity of CD14$^-$CD64$^-$CD163$^-$SIRPα$^+$ cells that included conventional cDC2 and plasmacytoid DCs [pDCs], but did not capture SIRPα$^-$ cDC1. Overall, Mono/MΦ-like and DC-like cells expressed a quite similar molecular signature in UC and CD MLNs.

3.5. High-dimensional single cell protein expression analysis of HLA-DR$^+$SIRPα$^+$ MNPs revealed the heterogeneity of CD14$^-$CD64$^-$ populations in MLNs of UC and CD

Based on differentially expressed gene signatures in DC-like and Mono/ MΦ-like cells, we designed a monocytes/MΦ/DC panel to further explore the heterogeneity of HLA-DR$^+$SIRPα$^+$ MNP populations at the protein level, using CyTOF [Figure 5]. After manual gating for CD45$^+$ haematopoietic cells, *t*-distributed stochastic neighbour-embedded [*t*-SNE] analysis of concatenated UC and CD samples identified clusters that included the two main cell types, CD3$^+$ T cells [60.4% ± 18.6] and CD19$^+$ B cells [35.6.4% ± 17.69], CD66b^{hi}SIRPα$^+$ neutrophils [0.022% ± 0.024], and rare cells including FcεRI$^+$CD123$^+$SIRPα$^+$HLA-DR$^-$CD11c$^-$ basophils [0.007% ± 0.007] and SIRPα$^-$HLA-DR$^+$BLTA$^+$CLec9A$^+$ cDC1 [0.007% ± 0.004]. HLA-DR$^+$SIRPα$^+$ MNPs [lineage$^-$: CD3$^-$, CD19$^-$, and CD66b$^-$] represented 0.46% ± 0.2 of CD45$^+$ cells. The concatenated HLA-DR$^+$SIRPα$^+$ MNP population was further subjected to *t*-SNE analysis that resulted in three main clusters [Figure 5A]; expression feature *t*-SNE plots of CD14, CD64, CD11b expression identified a monocyte-derived cell/macrophage [Mono-Mac] cluster, which was distinct from the pDC-like cluster best defined by CD123 with some cells co-expressing CD303, and cDC cluster characterised by CD1c, FcεRI, and BTLA expression. HLA-DR$^+$SIRPα$^+$ MNPs were enriched in the Mono-Mac cluster, and the proportion of pDC-like cluster predominated over cDCs [Figure 5B].

We next investigated the heterogeneity of the CD14$^-$ subpopulation in MLNs from both diseases. To this end, HLA-DR$^+$SIRPα$^+$ MNPs were next stratified by CD14 and CD64 expression [Figure 5C]. FlowSOM [self-organising map] analysis applied to the concatenated UC and CD CD14$^-$CD64$^-$ population identified four major clusters. These included one major CD11c$^-$ CD123$^+$ pDC-like cluster [Cluster D; orange in the figure], and three CD11c$^+$ cDC2 subsets that comprised a predominant CD33$^+$CD1c^{hi} resident DC cluster [Cluster A; blue], a minor HLA-DR^{hi} CCR7$^+$ migratory DC cluster [Cluster B; red], and an additional cluster that did not express CD1c but expressed CD11b [Cluster C; green] [Figure 5D]. In order to compare the two diseases, the FlowSOM analysis map of the combined CD14$^-$CD64$^-$ population was applied on the CD14$^-$CD64$^-$ subset of UC or CD [Figure 5E]. The four clusters were then visualised on the *t*-SNE plot of concatenated HLA-DR$^+$SIRPα$^+$ MNPs, and as a minimal spanning tree [MST] [Figure 5E]. The MST depicts the relative frequencies of all clusters, since circle size is proportional to the number of cells in each node with background colour to indicate the cluster. Triangle size in the star chart depicts the mean intensity of each marker for all cells assigned

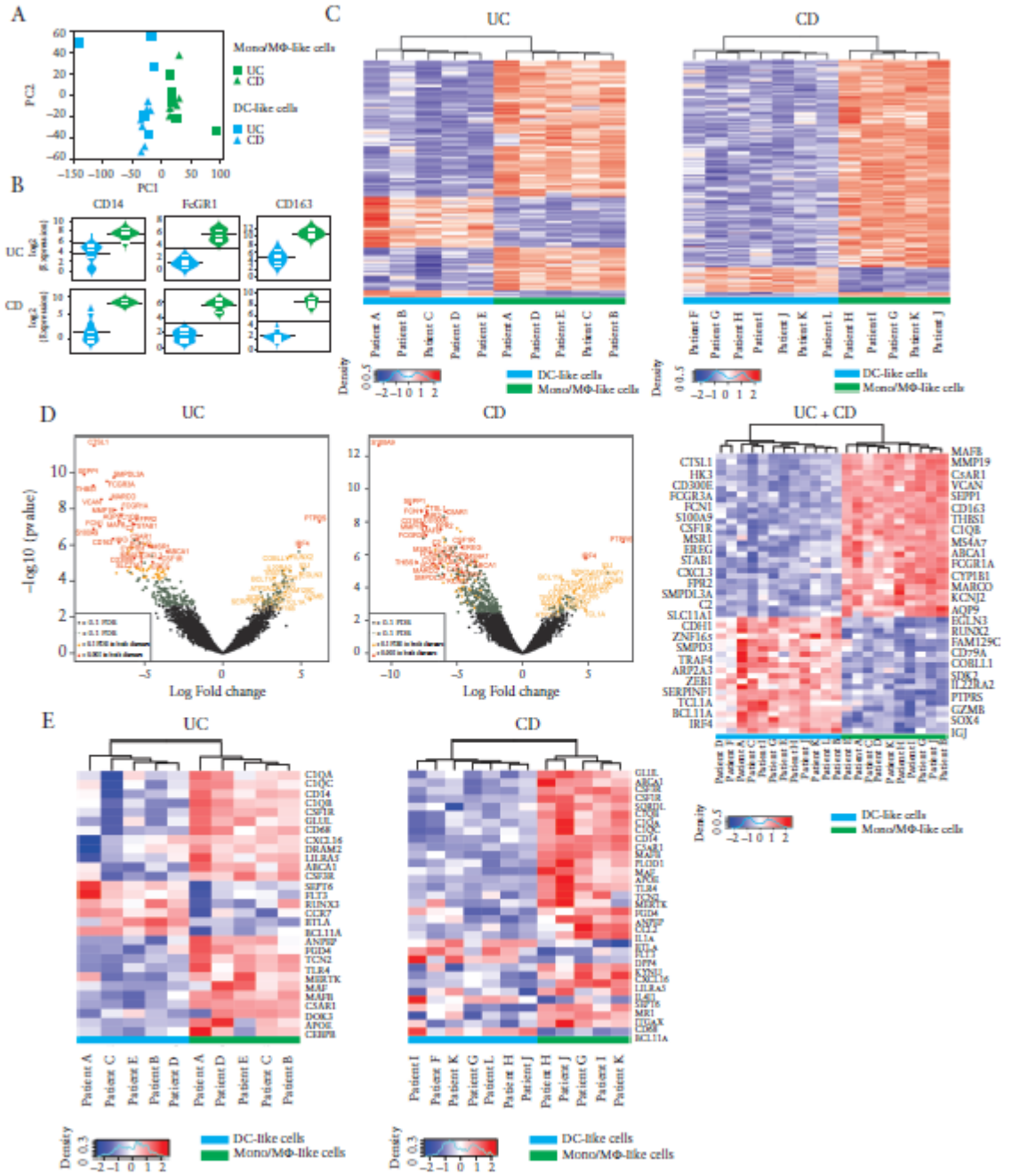

Figure 4. Molecular signature of Mono/MΦ-like and DC-like cells in MLNs of UC and CD patients. **A.** Principal component analysis [PCA] of FACS-sorted Mono/MΦ-like and DC-like populations from five UC and seven CD MLNs, respectively. B. Violin plots illustrate gene expression level distribution of CD14, CD64 [FcγR1], and CD163 surface marker genes (y-axis, $\log_2$ [Expression]) for DC-like [blue] and Mono/MΦ-like [green] MNPs [x-axis]. C. Heatmaps of discriminative genes between DC-like and Mono/MΦ-like MNPs from UC and CD for all patients [FDR < 0.05]. D. Volcano plots [UC and CD, left panels] and heatmap [right panel]. Top genes differentially co-regulated in DC-like relative to Mono/MΦ-like MNPs [downregulated genes, FDR < 0.003, and upregulated genes, FDR < 0.1] in both diseases and across all patients examined. E. Functional enrichment in pathways as indexed in murine LNs[22] [downregulated genes in DC-like versus Mono/MΦ-like + MNPs with $p < 0.05$]. [See also Figures S4 and S5 and Tables S1–S4, available as Supplementary data at *ECCO-JCC* online]. MLN, mesenteric lymph node; UC, ulcerative colitis; CD, Crohn's disease; MNP, mononuclear phagocyte; FDR, false discovery rate.

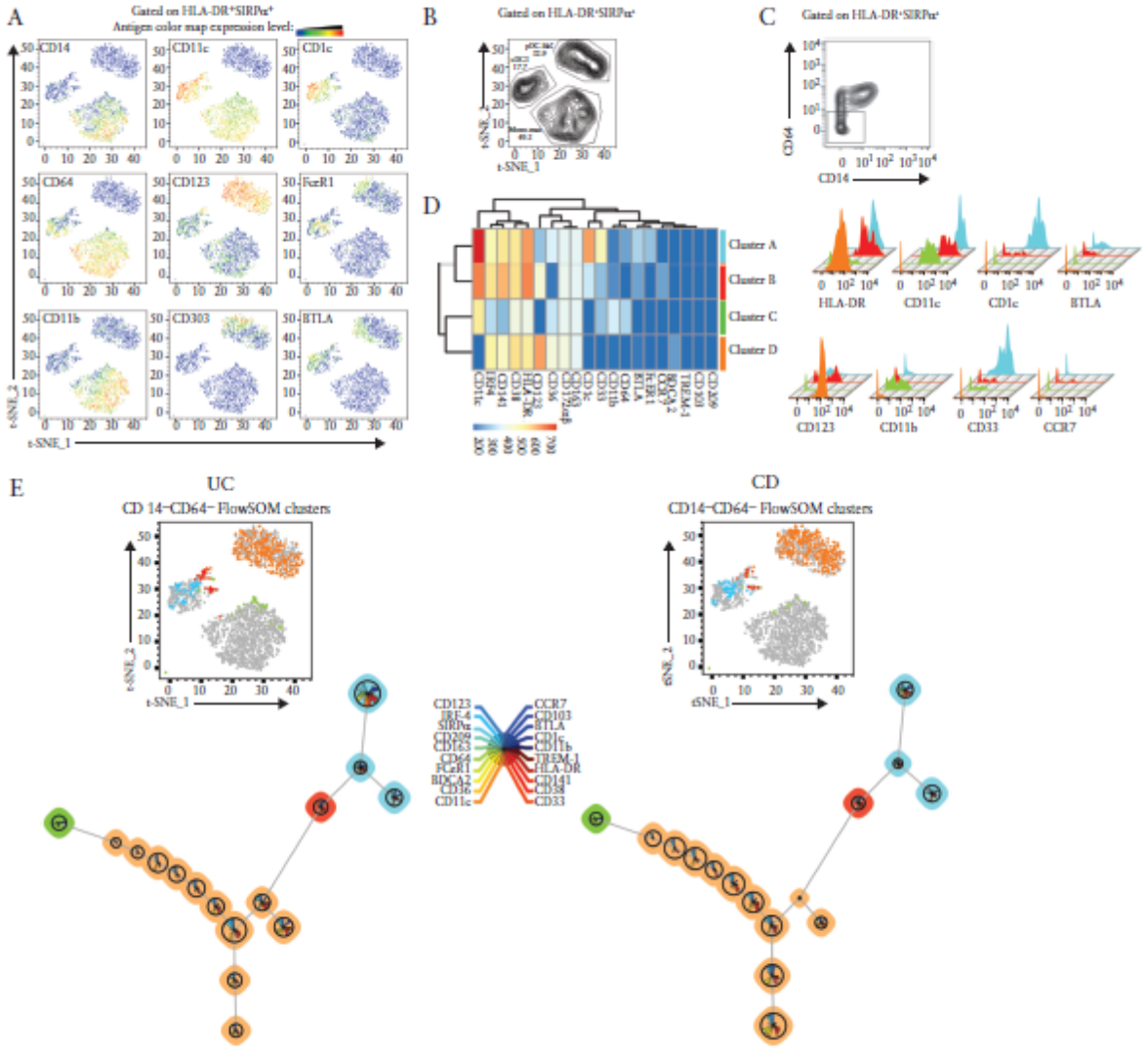

Figure 5. High-dimensional single cell protein expression analysis of CD14$^-$CD64$^-$ HLA-DR$^+$SIRPα$^+$ MNPs in MLNs of UC and CD. Cells isolated from MLNs were stained for CyTOF analysis. A. *t*-SNE analysis of concatenated HLA-DR$^+$SIRPα$^+$ population from eight patients [four UC and four CD]. Expression feature plots of the indicated surface markers. B. Frequencies of pDC-like, cDC, and Mono-Mac clusters in HLA-DR$^+$SIRPα$^+$ *t*-SNE plot. C. CD14$^-$CD64$^-$ subset gated on concatenated HLA-DR$^+$SIRPα$^+$ population. D. Heatmap and histogram overlays of indicated markers of clusters generated by FlowSOM analysis of concatenated CD14$^-$CD64$^-$ subset. E. FlowSOM analysis map of the combined CD14$^-$CD64$^-$ subset was applied on the concatenated CD14$^-$CD64$^-$ subset of only UC or CD, to compare diseases. UC and CD FlowSOM clusters visualided on HLA-DR$^+$SIRPα$^+$ *t*-SNE plots, and as minimal spanning trees. [See also Table S5, available as Supplementary data at *ECCO-JCC* online]. MLN, mesenteric lymph node; UC, ulcerative colitis; CD, Crohn's disease; MNP, mononuclear phagocytes.

to the node. This unsupervised analysis showed that CD14$^-$CD64$^-$ clusters in UC and CD displayed similar phenotypes but differential cell distribution [Table S8, available as Supplementary data at *ECCO-JCC* online: χ^2, p = 0.0001]. The pDC-like cluster D, which appeared to be more abundant in CD than in UC [80.7% versus 66.8%] included CD123$^+$ cells co-expressing or not BDCA2/CD303, whereas CD1c$^+$ cells were best defined by BTLA, FcεRI, CD33, CD11c^{high}, and CD1c^{high} expression.

Collectively, high-dimensional mass cytometry analysis has shown that the CD14$^-$CD64$^-$ cells from UC and CD MLNs were differentially distributed into four DC clusters, with pDC-like cells being the predominant population over cDC2 in both diseases.

3.6. High-dimensional single cell protein expression analysis of HLA-DR$^+$SIRPα$^+$ MNPs revealed the heterogeneity of CD14$^+$CD64$^+$ populations in MLNs of UC and CD

In order to identify which CD14$^+$ subsets contributed to the observed increased proportion of these cells in UC relative to CD [Figure 1B], we next compared the heterogeneity of CD14$^+$CD64$^+$ population in MLNs from both diseases [Figure 6A] using similar analysis strategies as for the CD14$^-$CD64$^-$ population. FlowSOM analysis on concatenated UC and CD CD14$^+$CD64$^+$ MNPs separated this population into seven clusters, four CD163$^+$ and three CD163$^-$clusters [Figure 6B]. FlowSOM clusters were visualised on a *t*-SNE plot of concatenated CD14$^+$CD64$^+$

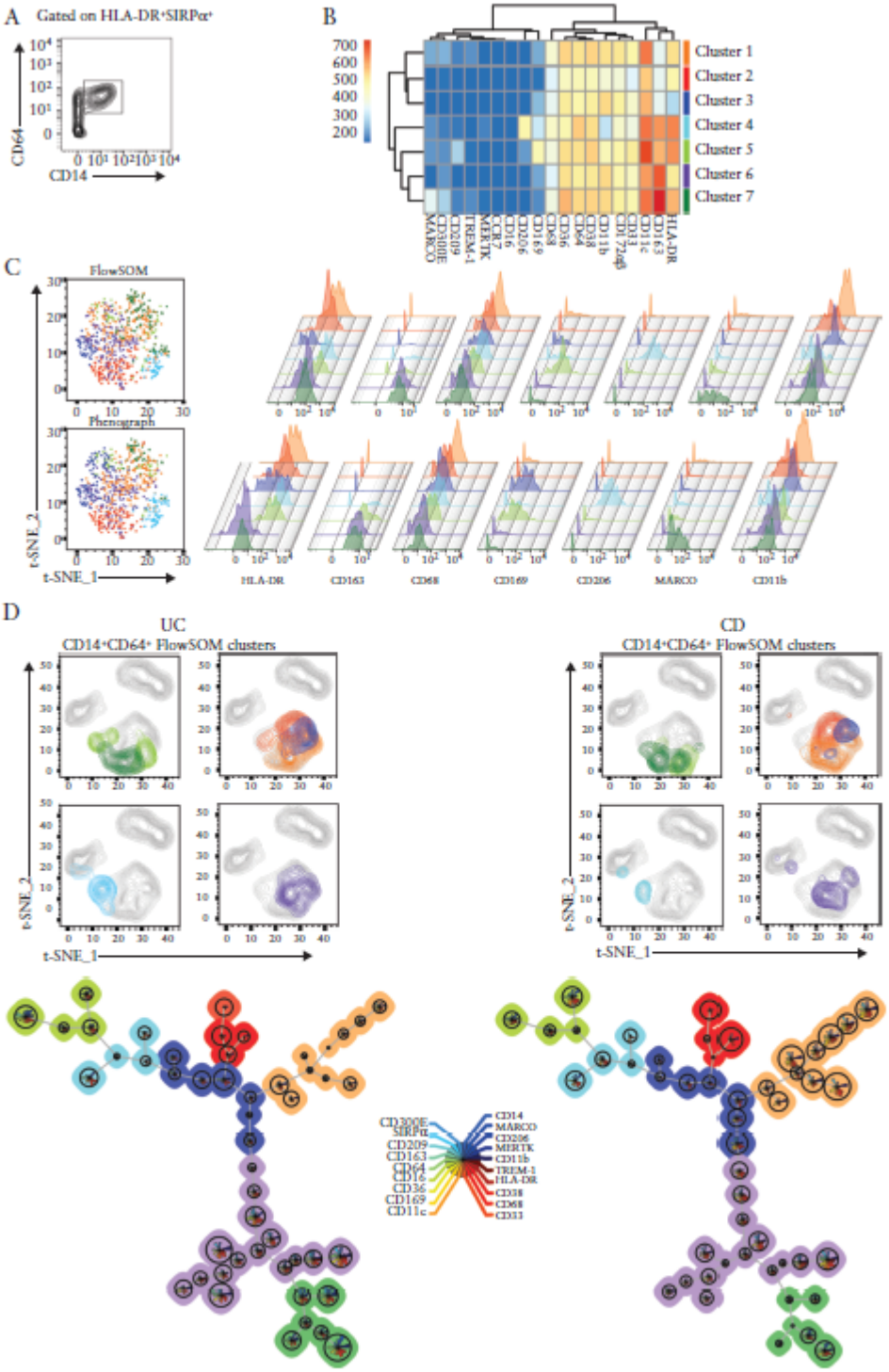

Figure 6. High-dimensional single cell protein expression analysis of CD14+CD64+ HLA-DR+SIRPα+ MNPs in MLNs of UC and CD. MLNs from four UC and four CD patients were stained for CyTOF analysis. A. CD14+CD64+ subset gated on concatenated HLA-DR+SIRPα+ population. B and C. Concatenated CD14+CD64+ subset was subjected to *t*-SNE, FlowSOM, and Phenograph analysis. Heatmap of FlowSOM clusters (B). FlowSOM and Phenograph clusters overlaid on *t*-SNE plots, and histogram overlays of the indicated markers from both algorithms are shown beside the corresponding plot (C). D. For the concatenated CD14+CD64+ subset of only UC or CD, FlowSOM analysis map of the combined CD14+CD64+ subset was applied to compare diseases. UC and CD FlowSOM clusters visualised on HLA-DR+SIRPα+ *t*-SNE plots, and as minimal spanning trees. MLN, mesenteric lymph node; UC, ulcerative colitis; CD, Crohn's disease; MNP, mononuclear phagocytes.

population [Figure 6C, top panels]. Applying Phenograph, another clustering algorithm also identified seven clusters with similar phenotypes [Figure 6C, bottom panels]. CD163$^+$ clusters, 4 [light blue in the figure], 5 [light green], and 7 [dark green] were best defined by cells co-expressing CD68 and HLA-DR at a high intensity. Specifically, cluster 5 was characterised as CD206^{dim}CD209$^+$MARCO^{dim}CD169$^+$CD163$^+$ cells, whereas cluster 7 was enriched in CD206$^-$CD209$^-$MARCO^{hi}CD169$^-$CD163^{hi} cells. Interestingly, cluster 4 included CD206^{hi} cells, and mainly differed from cluster 5 by low CD11b expression. The fourth CD163$^+$ subset, cluster 6 [purple], was enriched in CD206$^-$CD209$^-$MARCO$^-$CD169$^+$CD11b$^+$CD68^{dim}HLA-DR^{dim} cells. The three CD163$^-$ clusters expressed CD68 and HLA-DR at variable intensities: CD68$^+$HLA-DR$^+$ [cluster 1; orange], CD68^{dim}HLA-DR^{dim} [cluster 2; red], and CD68^{dim}HLA-DR^{low} cluster 3; dark blue]. All CD163$^-$ cells co-expressed CD11b, whereas cluster 3 [dark blue] was enriched in CD169$^+$cells.

We next asked which of the four CD163$^+$ cell-enriched clusters contributed to the increased frequency of CD14$^+$ subpopulation observed in UC relative to CD MLNs. Therefore, FlowSOM analysis map of the combined CD14$^+$CD64$^+$ population was applied on the CD14$^+$CD64$^+$ population of UC or CD. Clusters were visualised on *t*-SNE plots of concatenated HLA-DR$^+$SIRPα$^+$MNPs, and as MSTs [Figure 6D]. Cells in the seven identified clusters were differentially distributed in both diseases [Table S5: χ^2, $p < 0.0001$]. CD11b$^+$CD68^{dim}HLA-DR^{dim} cells in cluster 6 [purple in the figure], which was enriched in UC MLNs, represented the major CD163$^+$ population [38.3% versus 24.3%]. Overall, cells belonging to this cluster displayed a similar phenotype [TREM-1$^+$] in both diseases. The relative frequency of MARCO^{hi}CD169$^-$CD68$^+$HLA-DR^{hi}CD163^{hi} cells in cluster 7 [dark green], considered to be *bona fide* MΦ since they also co-expressed MERTK, appeared to be augmented in UC; whereas MARCO^{dim}CD169$^+$CD68$^+$HLA-DR^{hi}CD163$^+$ MΦ [cluster 5; light green] were found in equal proportions in UC and CD. Accordingly, among the three CD163$^-$ clusters that displayed a similar phenotype in both types of MLNs, only the proportion of MARCO^{dim}CD169$^+$TREM-1$^+$HLA-DR$^+$ [cluster 1; orange] increased in CD relative to UC.

Taken together, the heterogeneity of the CD14$^+$CD64$^+$ population revealed that UC MLNs were best characterised by an increased proportion of CD11b$^+$TREM-1$^+$CD68^{dim}HLA-DR^{dim}CD163$^+$ monocyte-like cells and of *bona fide* CD169$^-$MERTK$^+$MARCO^{hi} CD163$^+$ MΦ, which altogether might contribute to the elevated frequencies of Mono/MΦ like cells detected in UC relative to CD.

4. Discussion

Lymph nodes are uniquely positioned at the interface between lymph and blood. Induction of immune response in MLNs results from interactions between resident or migratory MNPs, which carry antigen from peripheral intestinal tissues, and naïve or central memory T cells.[24] The data revealed that a high frequency of CD14$^+$HLA-DR$^+$SIRPα$^+$ MNPs that expressed CD163 was a distinct feature of MLNs in UC. Although Mono/MΦ-like cells appeared to display a common Mono/MΦ gene signature in UC and CD, high-dimensional single cell phenotypic analysis revealed that UC MLNs, when compared with CD MLNs, were enriched in CD11b$^+$CD68^{dim}HLA-DR^{dim}CD163$^+$ cells, a phenotype that resembles tissue monocytes, and in CD169$^-$MERTK$^+$MARCO^{hi} CD163$^+$cells that best characterise *bona fide* MΦ.

The DC-like population comprised conventional DCs [cDCs] and pDC-like clusters. In agreement with a previous study,[35] pDCs predominated over cDCs in MLNs. Circulating CD123$^+$CD303$^+$CD11c$^-$ pDCs represent a minor DC population, best characterised by *TCF4*, *IGJ*, *GZMB*, *SERPINF1* molecular signature.[26,36] However, the selected CyTOF panel did not include AXL and SIGLE6 makers recently reported to best define the minor AXL$^+$SIGLEC6$^+$ [AS] DC population, described as partially captured within the blood CD123$^+$ pDC population.[26] Thus, it remains possible that some of the CD123$^+$ cells in MLNs [defined as CD11c$^-$] also comprise AS DCs. CCR7$^-$FcεR$^+$CD33$^+$CD1c$^+$ resident cDC2 that resemble circulating CD1cA/cDC2 are best defined by *FcerR*, *CD33*, and *CD1c* expression using scRNAseq analysis.[26] The minor CD14$^-$CD64$^-$CD11b$^-$CD36$^-$CD141$^+$CD1c$^+$CCR7$^+$HLA-DR^{high} migratory cDC2s were also observed in UC and CD MLNs. In that regard, CD14$^-$CD64$^-$CD11b$^-$DEC205$^+$CD11c$^+$CD1c$^+$HLA-DR$^+$cells that are detected in lung draining LNs [LLNs] express high levels of CCR7 and HLA-DR, and reside within the T cell zone in close proximity to the B cell zone.[37] Granot *et al*. examined a large cohort of deceased organ donors, and reported that LLNs contain the highest frequencies of mature and migratory SIRPα$^+$CD1c$^+$ cells when compared with other LNs, including MLNs, and their numbers remain stable over life.[16] In the present study, we further identified CD14$^-$CD64^{dim}CD11b$^+$CD36$^+$CD11c$^+$ cells that were CD1c$^-$ in both diseases. These cells share some characteristics with circulating CD11c^{dim}CD36$^+$CD1c^{dim}CD1cB/DC3, which are identified as a separate cell subset in blood.[26]

DC-like cells primed naïve T cells and induced Th1 polarisation in CD, a function likely attributed to cDC2 [resident or migratory] that are included in this cell population, since pDCs are devoid of the ability to present antigen.[26] Naïve T cell priming and differentiation occurs in MLNs and likely not in gut tissue, because these cells are rare in intestinal mucosa.[38] Th17 cell lineage plasticity has been demonstrated *in vivo* in mice.[39] In humans, Th17 clones generated from mucosal T cells that are isolated from gut of CD patients produce IFN-γ when cultured in presence of IL-12.[30] Here, we demonstrated that the DC-like cell-enriched population also shifted Th17 T_{CM} into Th17/Th1 and Th1*. Thus, these results provide evidence that cDCs are implicated in the priming of naïve T cells and the plasticity of Th17 T_{CM} towards Th17/Th1 in the MLNs.

Unexpectedly, the two common IBDs could be distinguished by cell distribution and phenotype of CD14$^+$HLA-DR$^+$SIRPα$^+$MNPs subsets in MLNs. UC MLNs comprised the highest frequency of Mono/MΦ-like cells relative to CD. This population includes cells endowed with phagocytic activity, and resembled CD163$^+$ MΦ detected in axillary skin-draining LNs that are capable of apoptotic cell uptake but do not induce proliferation of naïve T cells under homeostatic conditions.[27,40] CD14$^+$ cells could be subdivided into CD68$^+$HLA-DR^{hi} MΦ that expressed either CD163, MARCO, or CD169. CD169 expression defines subcapsular [SSM] and medullary [MSM] sinus MΦ as opposed to paracortex MΦ that express CD68 but not CD169 in humans and mice.[23,41] Furthermore, MSM are best defined as CD209$^+$CD206$^+$MARCO$^+$CD169$^+$ cells in murine LNs. In the present study, CD68$^+$HLA-DR^{hi}CD163^{hi} MΦ were subdivided into CD209$^+$CD206$^+$MARCO^{+dim}CD169$^+$ [cluster 5] and CD209$^-$CD206$^-$MERTK$^+$MARCO^{hi}CD169$^-$ MΦ [cluster 7]. Therefore, cluster 7 might include MARCO^{hi}CD169$^-$ T cell zone MΦ [TZM], which appeared to be increased in UC relative to CD and, cluster 5 CD169^{hi}CD163^{hi} sinusoidal MΦ. Murine resident TZM MΦ were reported to proliferate in skin-draining LNs under inflammatory conditions.[22] Noteworthy, depletion of CD169$^+$ MΦ in MLNs ameliorates dextran sodium sulphate [DSS]-induced UC-like colitis,[42] suggesting a pro-inflammatory role for these MΦ. An additional feature of MLNs resides in their capacity to restrict pathogen

dissemination.[4] This filtering function of LNs is attributed to CD169$^+$ SSM that poorly support cytomegalovirus replication, and thus prevent spread of infection.[42] Finally, CD169$^+$ MΦ in MLNs contribute to anti-tumour immunity, with high densities being associated with better prognosis in colorectal carcinoma.[44] Therefore, functional studies of CD169$^-$ and CD169$^+$ MΦ in UC and CD MLNs warrant further investigation.

CYTOF analysis provided further evidence that the CD14$^+$CD64$^+$ population in IBD MLNs included, as in murine MLNs,[45] a large population of CD68^{dim}HLA-DR^{dim} cells. Notably, UC MLNs comprised increased frequency of CD11b$^+$CD68^{dim}HLA-DR^{dim}CD163$^+$ cells when compared with CD, mostly contributing to the enrichment in Mono/MΦ-like cells. These CD163$^+$ monocyte-like cells in MLNs might represent either recently recruited circulating monocytes, that have not yet downregulated CD163, or migratory mucosal inflammatory CD163$^-$ monocyte-like cells that acquired CD163.[46] Adoptively transferred Ly6C^{hi} monocytes are retraced with similar kinetics in mucosa and MLNs in colitis, suggesting that monocytes enter MLNs directly from blood rather than migrating from the mucosa.[28] These massively recruited MLN monocytes differentiated into MΦ that contribute to colitis independently of migratory DCs. However, CD11b$^+$CX3CR1^{int} effector monocytes could also migrate from colon to MLNs during DSS-induced colitis,[47] and dysbiosis favours the migration of murine mucosal CD103$^-$CD14$^+$ cells to MLNs in inflammatory conditions.[48] In that regard, MLNs are also the site of bacterial translocation, and reduced microbial diversity is observed in IBD. Microbiota profiling of MLNs reveals differences between CD and UC patients, with increased relative abundance of *Firmicutes* and *Proteobacteria* in UC and CD, respectively.[49] A differential gut dysbiosis in IBD patients is also reflected by an altered bacterial composition and lower diversity in CD relative to UC mucosa.[50]

To the best of our knowledge, no studies have yet examined the high-dimensional protein and transcriptomic profile of HLA-DR$^+$SIRPα$^+$ MNPs in human IBD MLNs. Here, we compared the molecular profile of Mono/MΦ-like cells and DC-like cells, using bulk RNAseq. Our study revealed that the Mono/MΦ-like population over-expressed, relative to DC-like cells, genes that were previously reported in either MΦ [*MAFB*, *CSFR1*, *C1QA*, *C1QB*, *C1QC*, *MRC1*, *MAF*, *STAB1*, *SLCO2B1*, *FOLR2*, *FCGR3A*, *C2*, *VSIG4*] or inflammatory monocyte-derived cells [*CD300E*, *SERPINA1*, *FCN1*, *FPR1*, *S100A9*, *SLC11A1*, *THBS1*, *IL1RN*, *PLAUR*, *CCRL2*, *OLR1*, ,*EREG*, *ACSL1*] in inflamed colon of CD patients.[51] Noteworthy, Mono/MΦ-like cells did not display the gene signature of circulating monocytes [*SELL*, *CLEC4D*, *CD48* but no *MERTK* and *CD209* expression], whereas they shared molecular features with *in vitro* MCSF-monocyte-derived human MΦ [*SEPP1*, *MERTK*, *CD169*, *CD163* expression].[52] In humans, tissue monocytes are best defined by high *S100A9*, *FCAR*, *SERPINB2*, *TNFAIP3* and low *C1QA/B*, *VSIG4*, *MARCO* gene expression relative to MΦ in heart,[53] and by *CD14*, *S100A12*, *S100A8/9*, *TREM* expression in gut under homeostatic conditions.[10] Since CD11b$^+$CD209$^-$HLA-DR^{dim}CD163$^-$ inflammatory monocyte-like cells expressing TREM and *FCAR*/CD89, at protein and molecular levels, accumulate in inflamed relative to non-inflamed colon, these cells may be included in the pool of recruited effector CD163$^-$ monocyte-like cells [cluster 1] which predominated in inflamed CD MLNs.

Taken together, the distribution and detailed phenotypic characterisation of MNPs in human colon-draining MLNs revealed differences between UC and CD. UC MLNs comprised increased frequencies of CD14$^+$CD64$^+$CD163$^+$ cells that displayed a monocyte/ MΦ gene signature and predominantly included CD11b$^+$CD68^{dim}HLA-DR^{dim} monocyte-like cells. MLNs from IBD patients are difficult to access for research investigations. Future studies, that combine imaging and molecular analysis at the single-cell level, are warranted to further discriminate HLA-DR$^+$SIRPα$^+$ MNPs in UC and CD, and define the mechanisms that regulate preferential expansion of the CD163$^+$ monocyte/ MΦ population in UC, thereby providing insights about immunopathogenesis of these two distinct forms of IBD.

Funding

Canadian Institutes of Health Research [MOP#130533 [MS] and Banting Postdoctoral Fellowship [A-CV], Fonds de Recherche en Santé du Québec [LC, MB], National Human Genome Research Institute Centers of Excellence in Genomics Science grant P50 HG006193 [NH], and the National Human Genome Research grant T32 HG002295 [PI: Peter J. Park].

Conflict of Interest

The authors declare no competing interests.

Acknowledgements

We thank the subjects for donating the samples used in our study; the physicians who provided the samples: E. Debroux, R. Lougnarath, and R. Ratelle, from the digestive tract surgery unit at CHUM, Montreal; we also thank the haematology department staff and nurse C. Bergeron for their help.

Author Contributions

Concept: LC, MB, MR, FH, A-CV, NH, and MS. Methodology and investigation: LC, MB, MR FH, VM, JF, A-CV, and MS. Resources: FS, RW, CR, BN, GS, and CD. Writing: LC, MB, HM, and MS. Supervision: NH, A-CV, and MS. Funding acquisition: NH and MS.

Supplementary Data

Supplementary data are available at *ECCO-JCC* online
Data Accessibility GSE138364 at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE138364

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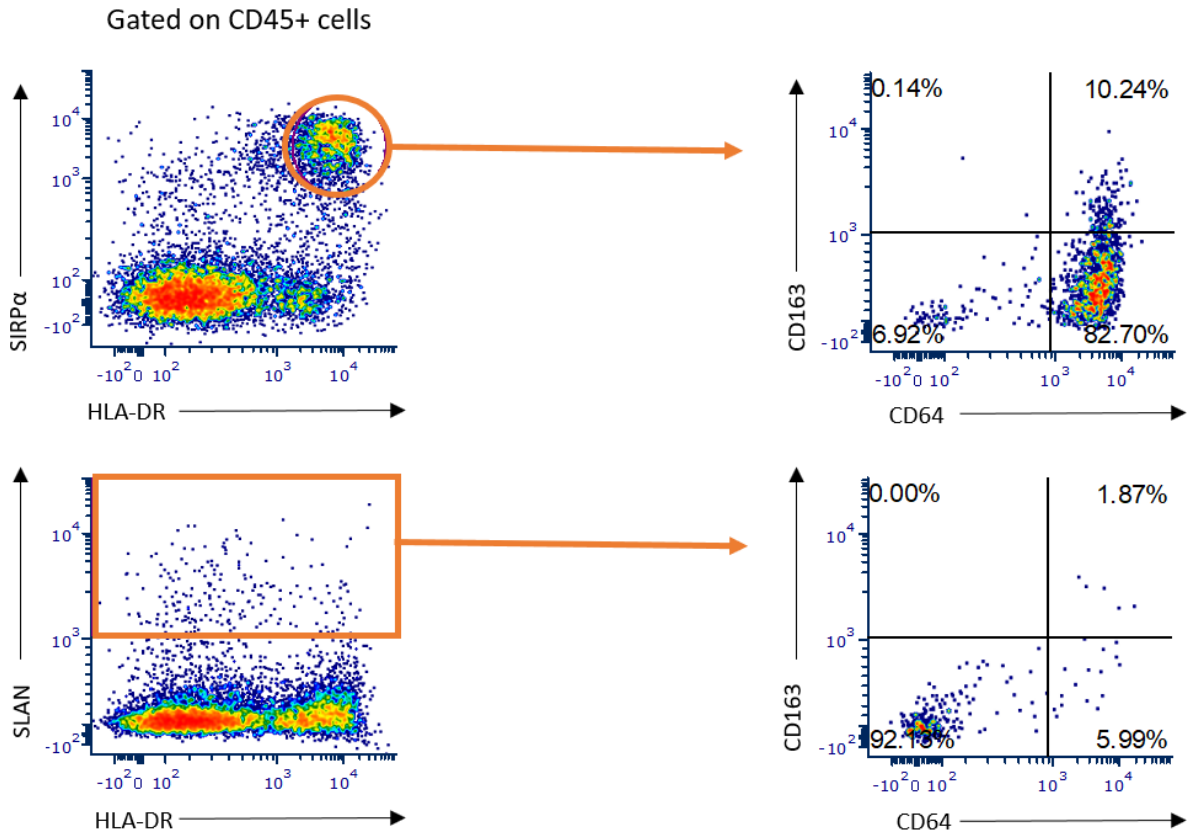
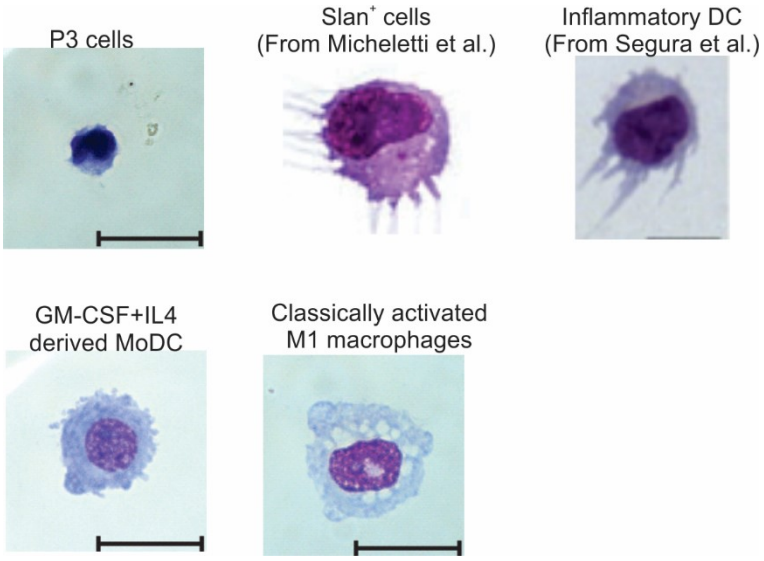
Annex 3.

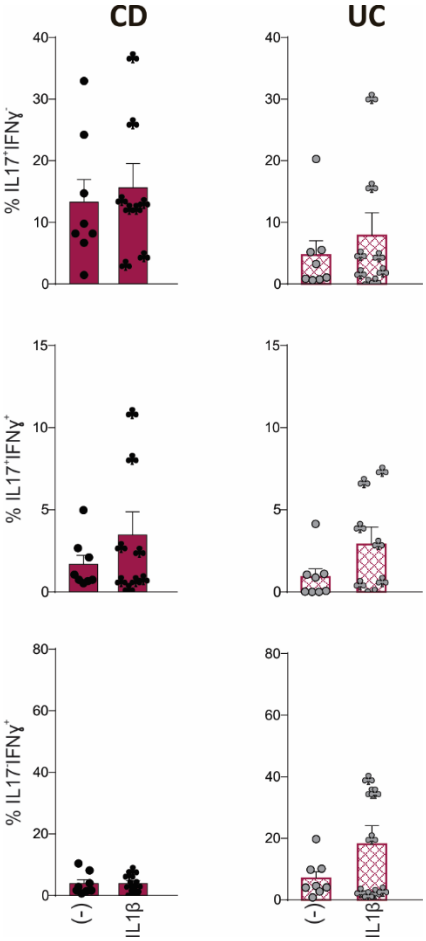
Thesis Supplementary Figures



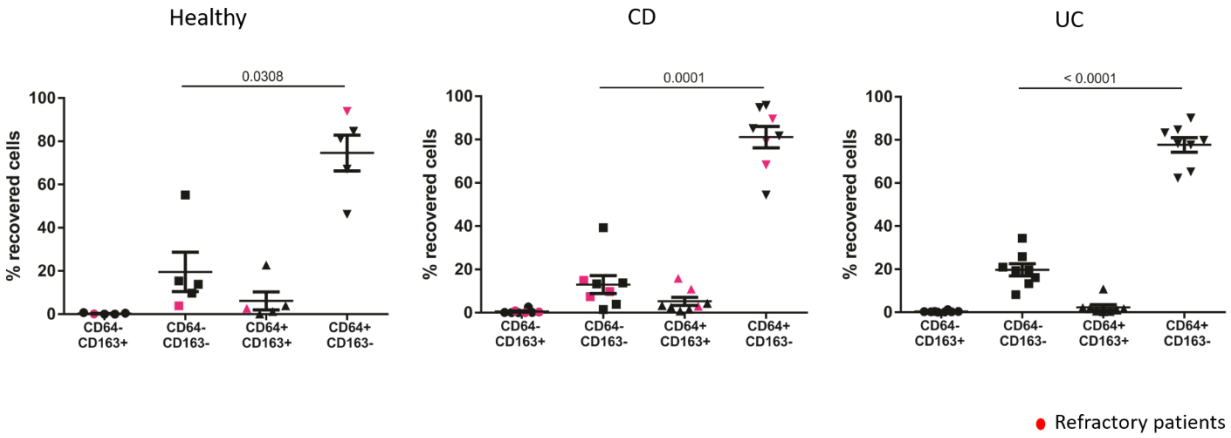
Annex 3-Figure 1: Slan^{+} cells in mucosa of Crohn's disease patients are CD64^{-}CD163^{-}.



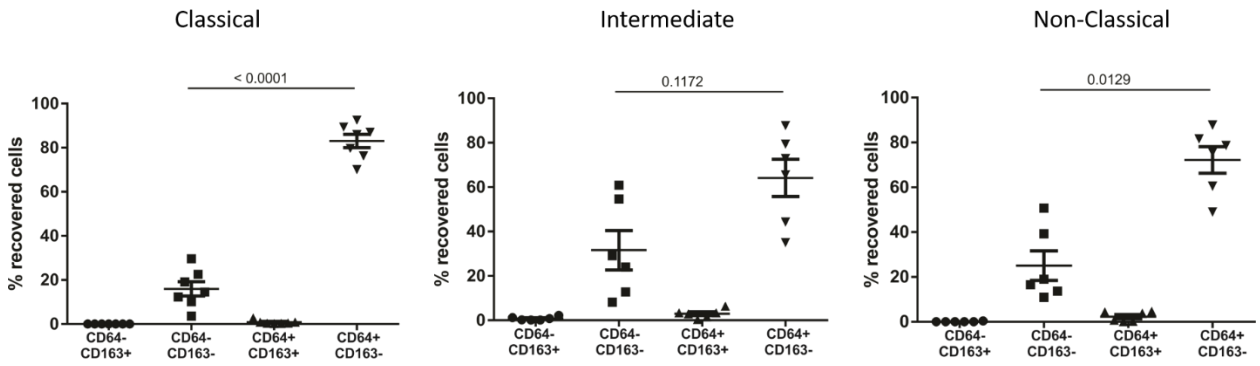
Annex 3-Figure 2*: Morphology of: P3 cells from inflamed CD mucosa. Slan+ T cells from tonsils (Micheletti et al., 2016), the inflammatory DC from synovial fluid (Segura et al., 2013), authorization code 4661460596861) and in vitro derived MoDC and M1 macrophages.*



Annex 3-Figure 3: *IL17 and IFNγ secretion by Th17 TEM cells from MLN of CD and UC patients following a 6 days culture with 10ng/mL of IL1β and anti-CD3/CD28 beads. PMA/Iono was added for the last 6 hours and Brefeldin A for the last 3 hours.*



Annex 3-Figure 4: *Frequency of $CD64^{+/-}CD163^{+/-}$ cells in a 6 days culture with GM-CSF+IFNγ+IL23 starting with $CD14^{+}CD16^{-}$ monocytes from blood of healty donors, CD and UC patients.*



Annex 3-Figure 5*: Frequency of $CD64^{+/-}CD163^{+/-}$ cells in a 6 days culture with GM-CSF+IFNγ+IL23 starting with $CD14^{+}CD16^{-}$ classical, $CD14^{+}CD16^{+}$intermediate and $CD14^{lo}CD16^{+}$non-classical monocytes from blood of healthy donors.*