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IL-23 Receptor and IL-12 Receptor Expression is Restricted to Distinct Cell Types in the IL-23R-GFP Reporter Mouse

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Mémoire intitulé:

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Abstract

Inflammatory bowel diseases (IBD) are characterised by uncontrolled immune responses in the gut. Genome-wide association studies (GWAS) have identified a protective polymorphism for IBD in the *IL23R* gene. *IL23R* codes for the IL-23r protein, one of the two subunits of IL-23R. IL-23R belongs to the IL-12R family, which contains many heterodimeric receptors. For example, both IL-12R and IL-23R share the IL-12Rβ1 subunit. Nevertheless, IL-12R and IL-23R are associated with different immune processes (Th1 vs. Th17).

This thesis characterizes the cellular patterns of expression of both IL-23R and IL-12R, to further elucidate their roles in inflammation. We established that IL-23R and IL-12R were never co-expressed together, even though they share the IL-12Rβ2 subunit. Analysis of murine splenocytes revealed that IL-23R is expressed by some TCRγδ T-cells, a few B-cells, CD4+ T-cells and several Lti-like cells. IL-12R protein was found in a few B-cells.

The analysis of IL-23R and IL-12R expression in different organs revealed that the lamina propria of the small intestine was the organ containing the largest proportion of IL-23r+ cells. IL-12R+ cells were found in constant numbers throughout the organs.

Finally, *in vitro* cultures showed that IL-23R and IL-12R had crossed reaction to IL-12 and IL-23. Study of IL-23R in IBD should always be accompanied by IL-12R analysis, because both receptors could have complementary roles.

Key words: Inflammatory bowel diseases, IL-23, IL-12, IL-23R, IL-12R, small intestine lamina propria, Lti-like cells, IL-23R-GFP reporter mouse

Résumé

Les maladies inflammatoires de l'intestin (MII) sont caractérisées par des réponses immunitaires incontrôlées dans l'intestin. Des études génétiques ont associé un polymorphisme dans le gène de l'*IL23R* à la résistance aux MII. *IL23R* code pour la protéine de l'IL-23r, une sous-unité du récepteur à l'IL-23 (IL-23R). Ce récepteur appartient à la famille de l'IL-12R, contenant plusieurs récepteurs hétérodimériques. D'ailleurs, IL-12R et IL-23R partagent la sous-unité IL12Rb1. Néanmoins, ces deux récepteurs favorisent des réponses immunitaires distinctes (Th1 vs Th17).

Ce mémoire caractérise les dynamiques d'expression cellulaires de l'IL-23R et l'IL-12R, afin d'élucider leurs rôles dans l'inflammation. Nous avons établi qu'IL-23R et IL-12R ne sont jamais co-exprimés, malgré qu'ils partagent la sous-unité IL-12Rβ1. Parmi les cellules de rates de souris, la protéine IL-23r est trouvée dans certaines cellules T TCRγδ ou T CD4+, quelques cellules B et des cellules Lti-like. La protéine IL-12Rβ2 est exprimée par quelques cellules B.

L'analyse de l'expression de l'IL-23R et l'IL-12R dans différents organes révéla que la plus grande proportion de cellules exprimant l'IL-23R se retrouve dans la lamina propria de l'intestin grêle, alors que les cellules exprimant l'IL-12Rβ2 ont été retrouvées en proportion équivalente dans tous les organes lymphoïdes. Ces observations appuient les études génétiques suggérant un rôle prédominant de l'IL23R dans les intestins.

Finalement, des cultures *in vitro* suggèrent que l'IL-23R ou l'IL-12R avaient des réactions croisées à l'IL-12 ou l'IL-23. L'étude de l'IL-23R dans les MII devrait donc être complémentée par l'étude de l'IL-12R, car les deux récepteurs pourraient avoir des rôles complémentaires.

Mots clés: maladies inflammatoires de l'intestin, IL-23, IL-12, IL-23R, IL-12R, lamina propria de l'intestin grêle, Lti-like cells, souris IL-23R-GFP

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Abbreviations used in this thesis

BM	Bone marrow
CD	Crohn's disease
CIA	Collagen induced arthritis
DC	Dendritic cells
EAE	Experimental autoimmune encephalomyelitis
GFP	Green-fluorescent protein
IBD	Inflammatory bowel diseases
IL-12R	Interleukin-12 receptor
IL-23R	Interleukin-23 receptor
IL-27R	Interleukin-27 receptor
IL-35R	Interleukin-35 receptor
lDC	Lymphoid dendritic cells
LN	Lymph nodes
mDC	Myeloid dendritic cells
MS	Multiple sclerosis
NK	Natural killer cells
pDC	Plasmacytoid dendritic cells
UC	Ulcerative colitis

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Introduction

1. Inflammatory Bowel Diseases

Inflammatory bowel diseases (IBD) are a family of illnesses including Crohn's disease (CD), ulcerative colitis (UC) and indeterminate colitis, among others[1]. This introduction will focus mainly on Crohn's disease and ulcerative colitis, as they are the two most prevalent and most studied in this family of diseases[1]. Even though both diseases share certain hallmark characteristics, there are striking differences between their etiology, genetic predisposition and the clinical presentation of patients.[1]

Crohn's Disease

The prevalence of autoimmune and inflammatory diseases such as Multiple sclerosis (MS) and systemic lupus erythematosus (SLE) is often higher in women than in men, suggesting a role of sex hormones in the pathogenesis of the disease. However, CD is diagnosed in equal number between men and women[2], which could indicate that factors other than sex hormones are determinants in this disease.

The most common symptom of CD is chronic diarrhea, often accompanied by weight loss and abdominal pain. Some CD patients also suffer from symptoms that can manifest outside the GI tract, including pain in joints, bones and muscles[2]. However, those symptoms are not helpful in leading to a direct diagnosis of CD.

Indeed, to diagnose this disease, a variety of tests are required. Ileocolonoscopies, which involves the observation of both the colon and the ileum, as well as biopsies from both are essential to rule out other diseases, including UC[2]. Diagnosis of CD is usually made through the exclusion of known infectious origins and other gastrointestinal diseases, after which CD is confirmed through pathological tests, such as histological analysis of biopsy samples.

[1] Around 10% of people suffering from IBD suffer from indeterminate colitis, a disease with hallmarks of both CD and UC1. Danese, S. and C. Fiocchi, *Ulcerative Colitis.* New England Journal of Medicine, 2011. **365**(18): p. 1713-1725..

Pathology

Inflammation during CD can be found throughout the GI tract, from the mouth to the anus. However, it is not continuous, and areas of unaffected tissue are found interspersed between diseased sections of tissue. The inflammation is transmural, meaning all layers of gut epithelium extending into the lamina propria may be invaded with lymphocytes. Inflammation can also result in granulomas: macrophages and T-cells forming structures where tissues are necrotizing[3].

Treatment

As is the case for many other relapse-remitting disease chronic diseases, treatment of CD consists of induction of remission and maintenance of remission[4]. There are no definitive treatments available right now. Disease remission is first induced using various agents, such as corticosteroids, antibiotics, aminosalicylates and other immunosuppressive drugs. New biological agents, for example anti-TNF-α monoclonal antibodies, are more and more frequently utilized.

However, those immunosuppressive agents cannot be used on a long-term basis for the management of CD, especially in the case of corticosteroids and antibiotics. For instance, continued treatment with corticosteroids is associated with bone demineralization and increased susceptibility to infections. Various studies analyzed the effect of 5-aminosalicylates with anti-TNFα and other drug combinations, but opinions are divergent regarding the best course of treatment and how to maintain remission in CD patients, since no drug combinations are completely devoid of side-effects nor do they effectively prevent relapses[4].

Ulcerative Colitis

Ulcerative colitis(UC) is more common than Crohn's disease[2][1, 5]. Very often, it is also milder than CD and is easier to treat. However, it still severely impedes the quality of life of patients affected by it.

Like CD, UC is a relapsing-remitting disease, where symptomatic and asymptomatic periods alternate, with no specific patterns of timing[5]. The most frequent symptom of UC is bloody diarrhea[6]. Depending on the severity of the disease, it can be accompanied by weight loss, fever, abdominal pain and other symptoms[6].

Because there are no standard hallmarks of the disease, diagnosis relies on a combination of colon biopsy and histological findings, while medical history and stool analysis may be used to rule out known infections causes and other intestinal diseases[6]. Histological findings, such as continuous inflammation contained in the colon and restricted to the first layer of the intestine lining will confirm the UC diagnosis. Currently, one of the main research goals in this field is to identify disease markers that could facilitate UC diagnosis, including genetic markers.

Pathology

Even though CD and UC have many similar characteristics and symptoms, there are also some very important differences amongst the two: UC is restricted to the colon, inflammation is restricted to the mucosal layer and it is usually continuous[1]. Colonoscopy is therefore essential in order to establish a correct diagnosis, as treatment and consequences of UC can be very different from those with CD. For example, as one of the main consequences of UC is colon cancer, and patients who suffer from UC for a decade or more must be followed for possible cancerous lesions in the colon [1].

[2] Reported by Danese et al., incidence of UC is 1.2 to 20.3 cases per 100,000 people, while CD incidence varies from 0.03 to 15.6 cases per 100,000 people.

Treatment

Similar to CD, there are conceptually two steps in the treatment of UC: induction of remission and maintenance of remission.

As most patients are diagnosed during an episode of inflammation, treatment starts by making an attempt to decrease gut inflammation. 5-aminosalicylates, orally and/or rectally, is effective in around 50% of patients in inducing clinical remission[1]. If it does not work, glucocorticoid therapy is given, and as a last resort anti-TNFα monoclonal antibodies are administered. In the second treatment phase, the goal is to maintain the patient in an asymptomatic phase. Various immunosuppressive therapies can be used (but not usually glucocorticoids, for the same reasons mentioned in CD treatment, where the side effects of long-term treatments with glucocorticoids overcome its possible benefits). Many studies have looked at the potential use of infliximab in a long-term treatment option for medium-to-severe disease; these studies showed positive results for many of the patients participating[7]. However, there could be possible risks for adverse events, such as cancer and/or serious infections[7]. The final UC treatment, which leads to complete remission but also brings various serious life-impediments is a colectomy, or the complete removal of the colon[1].

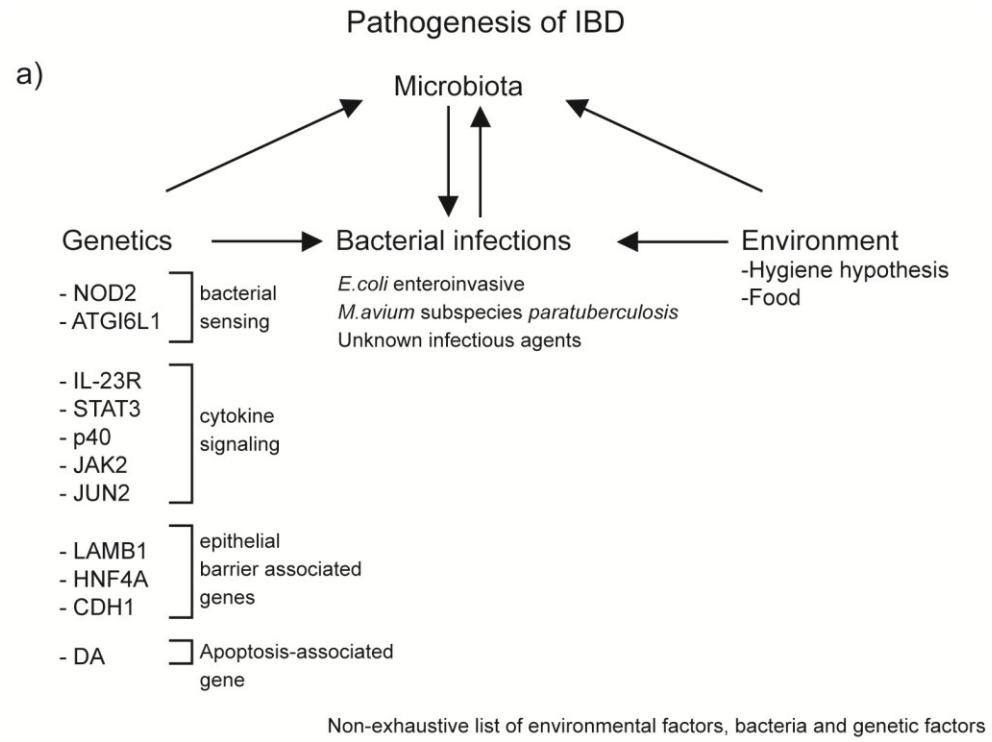
IBD epidemiology

In Western countries, the incidence of inflammatory and autoimmune diseases, such as multiple sclerosis and more relevant to this thesis, CD, has been increasing over the last seventy years. For example, incidence of CD has more than tripled [8] since the 1950s. Danese and Fiocchi characterized IBD as “disorders of modern society”[1]. An interesting study from Molodecki *et al.* reviewed 260 articles from different countries measuring incidence and prevalence of IBD through time and different regions of the world [5]. This analysis concluded that 75% of CD studies and 60% of UC studies showed statistically significant increases of incidence of their respective diseases studied. Perhaps more importantly, no CD studies showed a decrease in the incidence of the disease.

Causes

One of the main problems in the identification of the causes of IBD is that upon their first medical visit, patients already exhibit advanced clinical symptoms of the disease. [9] Early IBD patients are difficult to find. However, it is widely accepted that IBD, in general, are the result of an anormal immune response against the microbiota in the gut, leading to an uncontrolled immune response and inflammation[9]. Nevertheless, there is no consensus on the causative factors of IBD. This is still a "chicken or the egg" dilemma, where some scientists believe that defects in mucosal immune responses in the presence of normal flora in the gut leads to IBD, while others posit that defects in resident gut microflora lead to abnormal immune responses[10].

Evidently, IBD are multifactorial diseases, where there is an interplay between the genetic susceptibility of the host, bacterial infections, inadequate innate and adaptive immune responses and weakness of the mucosal barrier in the gut which may contribute to the pathology of IBD[11]. See Figure 1 for a better understanding of IBD factors.



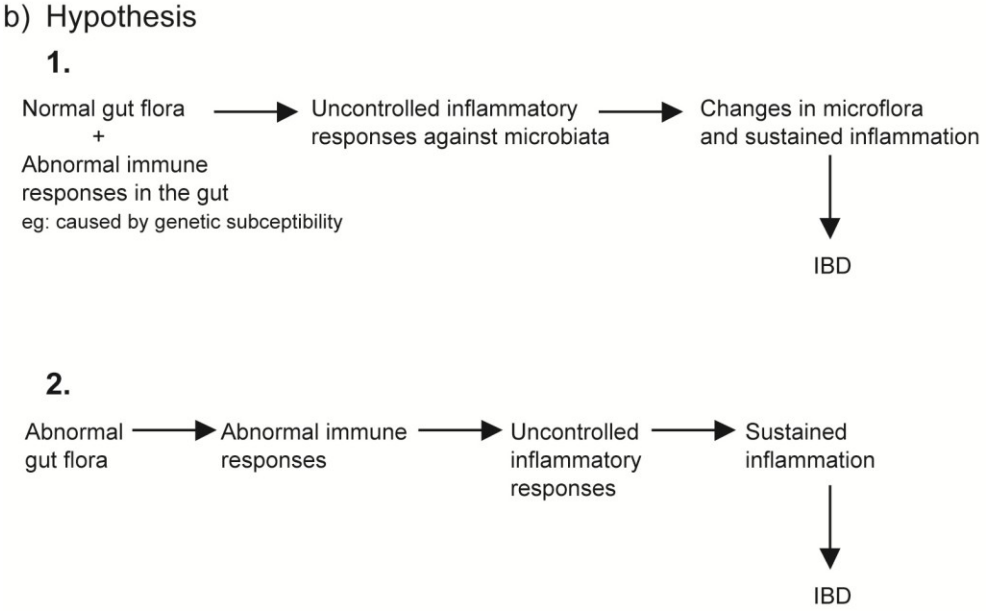


Figure 1: Pathogenesis of IBD

a) There are four factors interacting together that can lead to IBD: genetics, environment, bacterial infections and the microbiota of the individual. Polymorphisms in certain genes can increase susceptibility to some infections, but also influence types of bacteria colonizing the gut. The composition of the microbiota can increase or decrease susceptibility to specific infections, but bacterial infections can modify the gut microbiota. Finally, different environmental factors, including food and cleanliness, can change the frequence of bacterial infections and the composition of the gut microbiota.

b) The order in which the different factors can lead to IBD is not unanimous in the scientific community. In the first hypothesis, the gut microbiota in an individual is normal, but abnormal immune responses, triggered by genetic susceptibility or bacterial infection, disturb the normal immune responses, leading to IBD. The second hypothesis proposes that abnormal gut flora is the first factor leading to abnormal immune responses, which increase in genetically susceptible individuals, leading to IBD.

Hygiene hypothesis

One of the reasons frequently evoked to explain why those autoimmune diseases are more prevalent in industrialized nations is due to the decrease of infectious agents encountered during childhood in those specific environments. Some scientists even suggest that the lower number of infections encountered throughout childhood is the single most important factor[8]. This explanation is also known as the hygiene hypothesis.

For example, the frequency of intestinal infections in children has massively decreased in Western countries when compared to developing countries. These intestinal infections are crucial in shaping the intestinal flora. Parasitic infections can also stimulate Toll-like Receptors (TLR), leading to the production of various pro and anti-inflammatory cytokines, which can help in intestinal repairs and control of bacterial infections, both elements which can be defective in IBD[12].

Bacterial infections

Studies of blood and gut samples from IBD patients sometimes incriminate one or another bacterium, such as adherent enteroinvasive *Escherichia coli* and *Mycobacterium avium* subspecies *paratuberculosis* [3]. For example, biopsy samples from CD patients showed a subspecie of *E. coli* that adhere to intestinal tissue in CD patients than in healthy controls. However, transfer of intestinal bacteria into colitis susceptible monkeys was not sufficient to induce IBD, demonstrating the importance of other factors to induce IBD[13]. Another example of bacteria suspected to play a role in IBD is *M. avium* subspecies *paratuberculosis,* which is known to cause Johne's Disease in cattle, a disease highly similar to CD. Some studies reported identification of *M. avium* subspecies *paratuberculosis* in some CD patients, but this bacterium is not systematically found in patients samples analyzed[13].

Microbiota

Even if we humans like to think that we are "clean", we are in fact colonized by hundreds of billions of bacteria. The gut contains the largest amount of those bacteria,

followed by the skin. Humans have an extremely diversified intestinal flora. One of the first indications that bacteria have a role to play in IBD was demonstrated by the efficacy of antibiotics in inducing remission and decreasing symptoms in IBD patients[3].

Furthermore, many mouse models of IBD are resistant to methods of inducing colitis when they are highly treated with antibiotics or deprived of a normal gut flora[9]. However, in other settings, mice deprived of gut microbiota can be more susceptible to colitis, demonstrating a protective role of gut bacteria against colitis induction. Also, some IBD mouse models, such as the *Helicobacter hepaticus* transfer model, rely on the addition of a specific bacterium to induce gut inflammation[14]. Finally, many genes linked to IBD in humans have roles to play in innate immune responses, and especially in bacterial recognition[3][3].

Genetics

To better understand and identify genes that could be involved in IBD, various genetics studies have been done through the years. More than 30 susceptibility loci have been associated with IBD[15]. A more recent analysis using even more patients and healthy controls increased the number of CD risk loci to 71[16]. CD and UC both share similarities in their pathogenesis yet are different in other ways, as reflected by the genetic polymorphisms linked with the diseases; some are associated with CD only, some are linked with UC only and others are linked with both diseases. [17]. For example, genetic polymorphisms present in genes of the IL-23R pathway, such as STAT3, p40 and IL23R are linked to both UC and CD[1]. However, the first genetic polymorphism linked with increased susceptibility to IBD was NOD2, and is only associated with CD[17]. Polymorphisms in ATG16L1, a gene associated with both autophagy and NOD2 signaling, are also only associated with CD. Polymorphisms in DAP, death associated protein, are often linked with UC[18]. Other genes possibly involved in UC pathogenesis include epithelial-barrier associated genes, such as HNF4A, CDH1 and LAMB1[18]. However, these UC-only genes will not be further

[3] For example, NOD2 and ATG16L1, which will both be discussed later in this section.

discussed in this study, as they are not directly linked to immunology, even if they may be crucial in understanding the full scope of pathogenesis of the disease.

One of the tools used to understand the genetics of IBD is the genome wide association study (GWAS), where genomes from patients are compared to genomes from healthy controls. Using genetic markers, there is an attempt to identify allelic patterns, where rare alleles are more frequently associated with one phenotype[17]. Through GWAS, many genetic loci were identified, revealing candidate genes involved in the pathogenesis of IBD. Only a few of these genes will be discussed herein.

NOD2

The first gene to be associated with CD was NOD2(encoded by *CARD15*)[19]; this gene is only associated with CD and not UC. The NOD2 protein can sense muramyl dipeptide, a subunit of peptidoglycan found in bacteria. So far, three different polymorphisms of NOD2 have been identified, all of which are found in the LRR coding domain which detects the presence of MDP when expressed in monocytes[17]. This leads to a reduced detection of the PAMPs in bacteria and a subsequent decreased activation of the NF-κB pathway. However, NOD2 genetic polymorphism are not enough to induce CD on its own; only 15% of CD patients have one or two polymorphic alleles (SNP identified through GWAS) of NOD2[20], so most CD patients (85%) have a disease independent of NOD2 mutations.

The mechanism by which NOD2 could increase disease risk is poorly understood. One hypothesis is that NOD2 signalling can regulate and decrease TLR2 signaling; without NOD2, TLR2 signaling induces an increased amount of IL-12 and leads to dysregulated immune functions[20]. However, there is increasing evidence that IL-12 is not the most important cytokine involved in the pathogenesis of IBD. In mouse models of colitis, NOD2 deficiency is insufficient to cause colitis by itself; another stimulus is required to induce gut inflammation.

ATG16L1

ATG16L1 is important in autophagy, the biological process by which the cell "recycles" its components. Autophagy is central for antigen preparation, protein degradation and other important processes of immune responses[21]. In a North American-wide study, a polymorphic allele of ATG16L1 was the third most frequent polymorphism associated with CD, after IL-23R and NOD2[21]. Other studies, including one in Germany, replicated these findings[22]. Interestingly enough, ATG16L1 is highly expressed in the epithelium layer of the intestine, as well as in APC and T-cells, and is important for responses against *Mycobacterium* infections, sometimes suspected in the pathogenesis of CD[11].

A link between ATG16L1 and NOD2 signaling has recently been revealed; NOD2 signaling is essential for the formation of the autophagosome following bacterial stimulation[23]. Without autophagosome formation in DCs, antigen presentation is impaired and the immune response against the bacterium is impaired. This could explain how polymorphisms in both ATG16L1 and NOD2 could work together to create susceptibility to IBD.

Polymorphisms in genes involved in bacterial handling and recognition, such as NOD2 and ATG16L1, increase susceptibility to IBD. Those genes are mostly expressed in epithelial cells and DC. Bacterial signaling through NOD2 and ATG16L1 is one of the first steps in the induction of the immune response. Those immune responses, when out of control, can lead to chronic inflammation and IBD. The signaling through NOD2 and ATG16L1 also induces a plethora of inflammatory mediators, but also the induction of the adaptive immune response. An example of an important inflammatory cytokine is IL-23. It is known that DCs are an abundant source of IL-23 and IL-12; could the signaling induced by those cytokines be important in the pathogenesis of IBD? Genetic studies point towards an involvement of IL-23R and its signaling cascade.

IL-23R

The polymorphism identified in IL-23R was the second IBD-associated polymorphism found in humans[24]. After a GWAS study comparing approximately 600 healthy controls with 600 patients having ileal CD, a polymorphism was found in the intracellular domain of the receptor where an arginine at position 381 is modified for a glutamine (R381Q polymorphism). This polymorphism is protective against colitis. In addition to the R381Q polymorphism, nine other polymorphisms were identified in the IL-23R region, including in the intergenic region between IL-23R and IL-12Rβ2 (both genes are next to each other on chromosome 6), but no polymorphism were identified in IL-12Rβ2. Other studies also found involvement of IL-23R with psoriasis and ankylsoing spondylitis[25].

Following the identification of this polymorphism, multiple studies have tried to identify the biological impact of the amino acid substitution. One of them took CD4+CD45RO+ T-cells from patients with either WT or R381Q coding alleles and activated them with αCD3 and αCD28 antibodies[25]. Measurements of INFγ, IL-17 and IL-22 revealed that the cells coming from individuals with the R381Q polymorphism secreted less cytokines than cells coming from individuals with the wild-type allele. R381Q CD8+ T-cells stimulated in the same conditions also produced less cytokines. IL-23 induced STAT3 phosphorylation was also decreased in R381Q cells. The R381Q polymorphism induces a change in the amino acid sequence of the IL-23r[4] subunit. This amino acid substitution is between the transmembrane domain of the receptor and the possible JAK2 recruitment site[26]. This could lead to either impairment of recruitment of the receptor to the cell surface or problems in the downstream signaling cascade. Failure or decrease of JAK2 recruitment could lead to decreased activation of STAT3 (and other factors), as well as decreased cytokine production. This group's findings were also consistent with other studies where IL-23 signaling on R381Q T-cells led to less phosphorylation of STAT3, but also STAT1. Another study demonstrated that the number of circulating Th17 T-cells was not different between the two alleles, but the responses and pathogenicity following IL-23 stimulation was decreased in patients bearing the protective allele[27]. Together, these

[4] IL-23R is the receptor composed of two subunits, IL-23r and IL-12rβ2. Further explanation on the nomenclature will be provided.

findings indicate that IL-23R signaling is essential in the pathogenesis of both UC and CD. Furthermore, less signaling through the receptor following cytokine binding could lead to decreased inflammatory signalling and therefore protects against colitis.

Among other genetic polymorphisms identified in IL-23R which differ between healthy controls and IBD patients, one of them, r10889677, was found to be in the 3' untranslated region (3'UTR) of the receptor. This polymorphism altered the sequence in the UTR of the IL-23r mRNA which led to a different mRNA[28]. People bearing this variant produced more IL-23R protein. One mechanism proposed was that miRNA Let-7 could not bind and regulate the translation of the IL-23R mRNA, leading to increased signaling and immune responses, including feedback regulation. Patients with a difference in the UTR region are more susceptible to colitis, as more signaling through IL-23R could induce more inflammation.

As IL-23R variants seem to have very strong implications with IBD, other genetic studies looked carefully at possible genes involved in the IL-23R signaling cascade and their possible implication in increasing or decreasing susceptibility to IBD. Susceptibility alleles were found in IL12B(p40, which forms IL-23 when paired with p19 or IL-12 when paired with p35), STAT3, IL12RB1, JAK2 and JUN2[29, 30]. Those genes are essential in the activity[5] and signaling cascade of IL-23R[17].

Since the identification of different polymorphisms in IL-23R, many investigators have tried to create new therapies to control IBD by modulating IL-23 and IL-23R responses. One study used ustekinumab, a α-p40 monoclonal antibody against IL-12 and IL-23. This randomized trial did not lead to dramatic effects when compared to placebo. It did have a beneficial effect for some patients, but overall, did not lead to drastic improvements[31], which could indicate the possible need for therapies targeted only to IL-23R and not both IL-12R and IL-23R.

So, even though some genetic variants are found in CD and not UC and vice-versa, IL-23R is common to both diseases. Understanding the roles of IL-23R in one or both

[5] The structure and signaling cascade of IL-23R will be studied in deeper detail in the next section.

diseases is crucial for diagnostics, prevention, and treatment of IBD, which are all currently strongly lacking in the field of IBD.

2. IL-23R and IL-12R biology

IL-12R/IL-23R/IL-35R/IL-27R family of receptors

The IL-12R family, which includes IL-12R, IL-23R, IL-35R and IL-27R, are all heterodimeric receptors : they are made of two different subunits linked together with covalent bonds[32, 33](Figure 2: Receptors of IL-12R family). IL-12Rβ1 and IL-12Rβ2(which form together IL-12R) both have homology to gp130[34], one of the subunits of IL-27R. IL-23r is highly similar to IL-12Rβ1 and IL-12Rβ2: all three subunits contain in their extracellular domain a signal sequence, two cytokine receptor domains, but only IL-23r and IL-12Rβ2 possesses an N-terminal Ig-like domain[35]. However, IL-23r, unlike IL-12Rβ2 and gp130, does not contain three fibronectin-like extracellular domains[35]. In humans and mice, IL-23r and IL-12Rβ2 are 150kbp apart on chromosome 1 or 6, respectively.

It is sometimes hard to differentiate between IL-23 receptor, composed of two different subunits, and the IL-23r subunit itself. The ambiguity between IL-23R and its IL-23r subunit will be resolved in this thesis in the following way: IL-23r with a small r, will denote the subunit, while IL-23R with the capital R, will denote the functional IL-23R protein, made of the IL-23r and IL-12Rβ1 subunits.

IL-27R is composed of gp130 and WSX-1(also known as IL-27Ra[6])[36]. gp130 also possesses an N-terminal Ig-like domain[7]. IL-35R can act through three different receptors: heterodimers made of gp130 and IL-12Rβ2, homodimers made of gp130 or homodimers made of IL-12Rβ2[37]. Each receptor is comprised of subunits highly similar to one another, however, they bind to different cytokines and are expressed in different cell types, leading to very different *in vivo* roles. One must be also cautious when analyzing mice knock-out data; for example, IL-12Rβ2KO mice lack responsiveness to IL-12, but could also show decreased Treg functions through the lack or the decreased number of IL-35R.

[6] http://www.informatics.jax.org/marker/MGI:1355318
[7] Idem

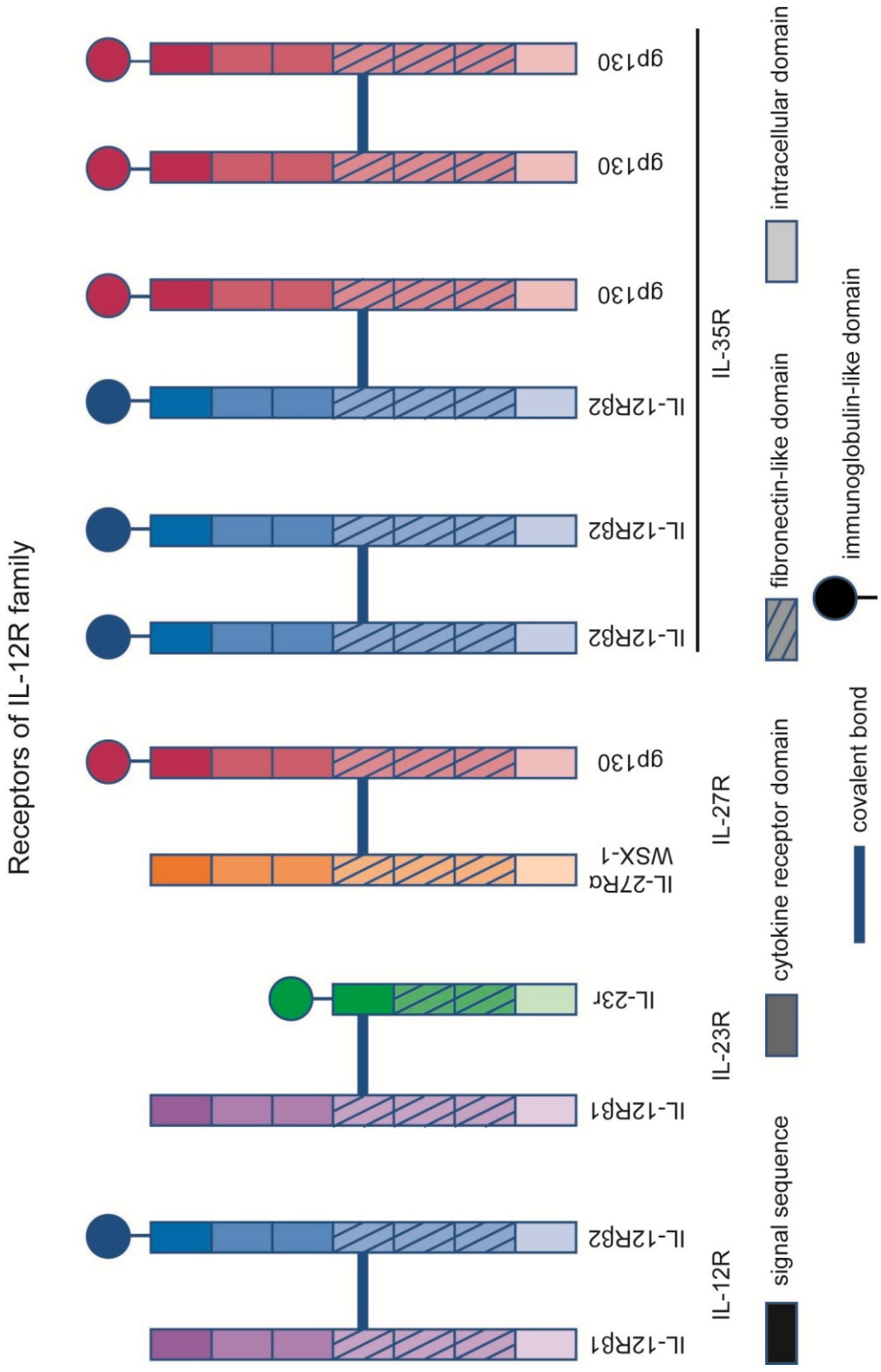


Figure 2: Receptors of IL-12R family

The IL-12R family are all heterodimeric receptors (IL-35R can be composed of IL-12Rβ2 or gp130 homodimers). All the subunits contain a signal sequence, a fibronectin-like domain and an intracellular domain. All receptors contain at least one subunit with an immunoglobulin like domain. The two subunits are linked together through a covalent bond. IL-23r is the only receptor subunit without a cytokine receptor domain, which probably explains why IL-23 is known to mostly bind to the IL-12Rβ1 subunit.

IL-12/IL-23/IL-35/IL-27/p40 homodimer families of cytokines

IL-12 family cytokines are heteromeric complexes, where two different subunits linked with a disulfide bond form a functional cytokine[33]. IL-12 is made up of a p35 chain and a p40 chain. IL-27 is made up of p28 and EBI3, which are homologuous to p35 and p40, respectively[33]. EBI3 can also bind to p35, forming the IL-35 cytokine. IL-23 is composed of p40 and p19, where p19 is also homologuous to p35.

The result is therefore four highly similar cytokines. However, even though they share many similarities they have drastically different roles. IL-35 is essential for the suppressive activity of Tregs in mice. IL-12 induces INFγ, inhibits IL-17 and is characteristic of Th1 response. IL-23 leads to IL-17 cytokine production and Th17 responses and IL-27 can induce Th1, but strongly inhibits the development of Th17[33]. Addition of IL-35 in T-cell proliferation assays will strongly decrease the proliferation of T-cells. This inhibition of proliferation is similar to the effect of the addition of Tregs in the same assay[38]. IL-35 is secreted by Tregs and can act on other T-cells to restrain their proliferation[39].

The last and often overlooked member of this cytokine family is the p40 homodimer. This homodimer can bind to IL-12Rβ1 with both high and low affinity[40]. When injected *in vivo* following LPS injection, p40 homodimer decreases levels of INFγ in the serum[41]. This homodimer can be either an agonist or an antagonist[42] of IL-12 secretion, and thus regulates IL-12 signaling. Because of the strong links and shared subunits between the cytokines, one must be careful when interpreting data from murine cytokine knock-outs[8].

To understand the role of each cytokine, one must understand their source and the stimuli leading to IL-12, IL-23, IL-27 and IL-35 secretion. DCs are a source of IL-12, IL-23 and IL-27 following stimulation through different TLR signaling cascades[33], while IL-35 is mostly secreted by Tregs[39]. The quantities of each cytokine found during an immune reaction depend on the ligands recognized by the DC.

[8] For an overall analysis of mice knock-out experiments, consult section 3 of the introduction.

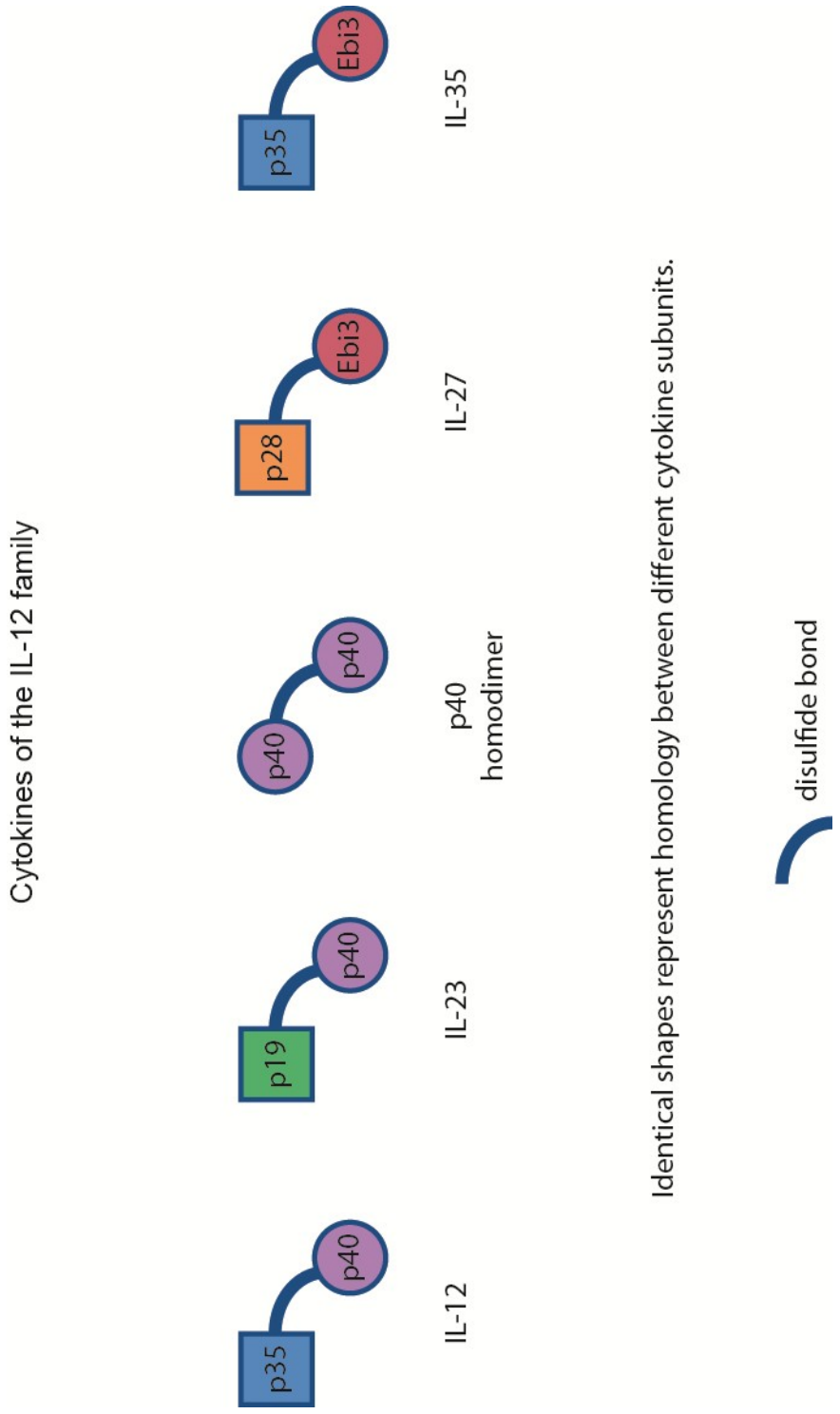


Figure 3 : Cytokine structure

Ebi3 and p40 are homologuous to each other. Both are thought to be constantly produced in various cell types, especially DC and monocytes. Upon signaling through PAMPs or other signals, p35, p19 and p28 are believed to be produced. The different subunits then bind to p40 or Ebi3 through a covalent bond and are secreted as an heterodimer by the various cells. The p40 homodimer secretion and biology is not well understood.

For example, signaling through TLR2 will induce strong secretion of p40 and p19, but no secretion of p35[33]. As is frequently seen in inflammatory responses, NF-κB is known to bind to the p40 promoter[43] and probably regulates the secretion of IL-23 and IL-12. The strongest p40 promoter activity by NF-κB was recorded in the intestine of mice and is therefore of interest when studying the biology and role of IL-23R in the gut. Ebi3 and p40, the α chain of the cytokines, are secreted in high numbers, while the β chain (p19, p35) is the limiting factor of cytokine secretion and depends on the stimulation of both APCs and Tregs[39].

Soon after the identification of p19, mice ubiquitously expressing p19 subunit were created[44]. Those animals suffered from impaired growth, infertility and most importantly, systemic inflammation. In naïve WT mice, p40 mRNA is produced at high levels in many different cells without any inflammation. Levels of p40 mRNA production did not increase in the p19 ubiquitous mice, but high levels of IL-23 were found, as well as systemic inflammation. This indicates that p40 is always secreted, but needs production of p19 to induce its effects. However, it could also mean p19 could have biological effects independent of p40.

IL-23R biology

Signals leading to the upregulation of the receptor

Naïve CD4+ T-cells do not express IL-23R [35]. When naïve CD4+ T-cells were transfected with a hyperactivated STAT3 and cultured with IL-6, TGFβ and αIL-4 antibody, IL-23R mRNA levels increased seven fold compared to cells not transfected, demonstrating that STAT3 is important in the upregulation of IL-23R[45]. One of the downstream signals of IL-23R, IL-17, increased dramatically when compared to controls, showing that mRNA was translated into proteins and affected cell signaling. Under the same conditions, using STAT3 KO naïve T-cells, IL-23R mRNA slightly decreased, but other downstream cytokines such as IL-22 and IL-17 decreased tremendously, demonstrating a relationship between STAT3, IL-17 and IL-22. Another group also showed with the same STAT3 CD4+ T-cell-deficient mouse model that STAT3 was required for the upregulation of IL-23R mRNA following IL-6, TGFβ or IL-21 exposure[46]. Together, these studies indicate an important role of STAT3 in IL-23R upregulation.

APCs can secrete cytokines which induce naïve T-cells to differentiate into Th17; these cytokines include TGFβ, IL-6 and IL-21. Th17 cytokines can also induce IL-23R at the surface of naïve cells. Using RT-qPCR analysis, one group demonstrated that IL-6 (in combination with αCD3/αCD28 stimulation) could lead to up-regulation of IL-23R[46]. IL-21 could also upregulate IL-23R. However, in presence of TGFβ+IL-6 and/or IL-21, IL-23R mRNA did not increase as much. According to the authors of this article, and in contrast to previously published papers, TGFβ actually had an inhibitory effect on the induction of IL-23R mRNA.

Interestingly, even though both IL-23R and NOD2 have been identified as important for the pathogenesis of IBD in genetic studies colitis induction by *C. rodentium* or *S. thyphimurium* have been shown to induce colitis in mice, Nod1 and Nod2 are apparently not important for the upregulation of either IL-23 or IL-23R[47]. The role of ATG16L1 in bacterial colitis is important, but its relationship to IL-23R is

unknown. Very little is known about IL-23R induction in other cell types; other data suggest that those cells may not need to upregulate IL-23R because they constitutively express it, even in a steady state.

Signals at the receptor levels

When IL-23R was first characterized in humans, it was found that IL-23 could bind to both subunits IL-23R and IL-12Rβ1 [35]. In the absence of IL-23, IL-12Rβ1 and IL-23R are not bound together; the binding of the cytokine brings the two subunits in close proximity and leads to the dimerization of the receptor. Following dimerization, signaling can take place through IL-23R and the IL-23 cascade is therefore activated[48].

Signaling cascade

The signaling cascade of IL-23R following IL-23 binding is highly similar to IL-12R, which uses JAK2, TYK2, STAT1, STAT3, STAT4, AND STAT5**[35]**. Signaling through IL-23R mainly induces the phosphorylation of STAT3, but also weakly phosphorylates STAT4 (and STAT1/5)**[45]** in Th17 cells. Tyrosine Kinase 2 ((TYK2) and JAK2 are bound to the IL-12Rβ1 subunit, which will phosphorylate STAT3 upon IL-23 binding. This stimulation induces IL-17 secretion in naïve T-cells; in the absence of Tyk2, IL-17 levels are decreased, indicating an important role for Tyk2 in inducing IL-17 production following IL-23 stimulation**[49]**.

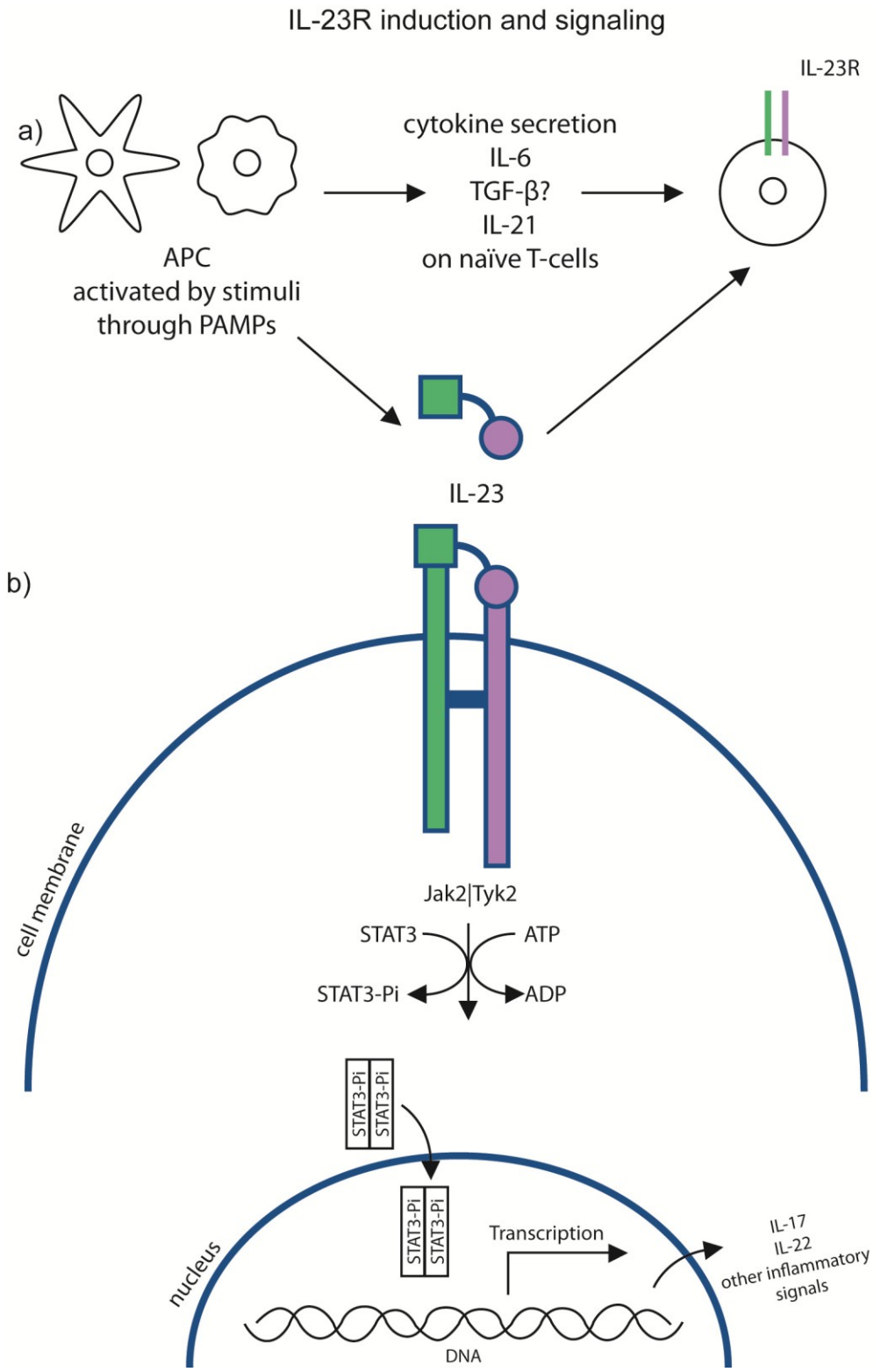


Figure 4: IL-23R induction and signaling cascade

a) APCs are one of the main source of cytokines leading to the upregulation of IL-23R. APC are also a good source of IL-23. The stimuli activating APC will determine the nature of the cytokines.

b) Following IL-23 binding to IL-23R, a covalent bond will be formed between the two subunits of IL-23R and the downstream signaling cascade will be activated. Kinases will phosphorylate STAT3, which will dimerize. Once the dimerized-STAT3 reaches the nucleus, it will activate the transcription of various inflammatory signals, including IL-17 and IL-22.

IL-12R biology

2.1.1 Signals leading to the upregulation of the receptor

IL-18, another very important Th1 cytokine, just like IL-12[50], is known to be important in the upregulation of IL-12Rβ2 on the surface of developing Th1 cells, which assembles with IL-12Rβ1 and produces the functional IL-12R[34, 51]. IL-27, another member of the IL-12 family, is also believed to be an early Th1 cytokine which would lead to the upregulation of IL-12Rβ2. Following this upregulation, IL-12 could then signal through IL-12R and stabilize the Th1 phenotype[32]. Signals that could lead to IL-12R upregulation in cell types other than T-cells are not very well-known.

Signals at the receptor levels

Even though IL-12R is made up of IL-12Rβ1 and IL-12Rβ2 in both mice and humans, the affinity of IL-12 for its receptor is different. In humans, IL-12 binds with low and high affinity to both subunits; in mice, IL-12 seems to bind very strongly with IL-12Rβ1 but only possesses low affinity for IL-12Rβ2[52]. However, in mouse studies, it was showed that without IL-12Rβ2, STAT4 phosphorylation was greatly reduced and INFγ production (a hallmark of IL-12 activity) was inhibited in response to IL-12[52]. So, even though IL-12 only weakly binds to IL-12Rβ2, this subunit is essential for signaling.

Signaling cascade

Upon binding of IL-12 to IL-12R, the Jak2/Tyk2 kinases associated with the receptor phosphorylate the receptor's tyrosine residues [53]. IL-12 binding to IL-12R leads to the activation (dimerization and phosphorylation) of STAT4, but also of STAT1,3,5[34, 45]. The activated STATs can than translocate into the nucleus and activate a plethora of genes, including IFNγ. One of the hallmarks of IL-12 signaling is INFγ secretion; in the absence of Tyk2, INFγ secretion after IL-12 stimulation is greatly reduced[49] (Figure 5).

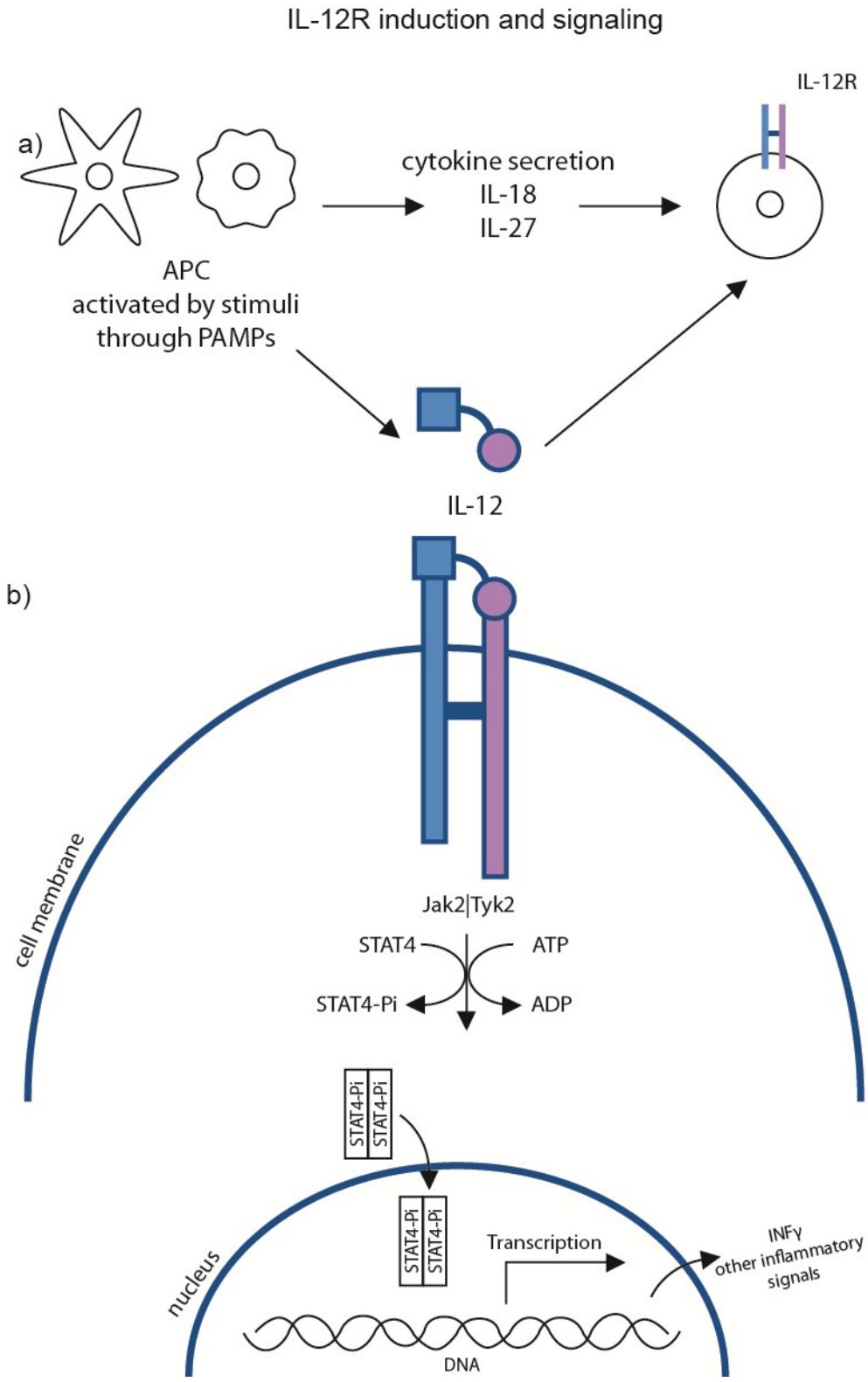


Figure 5: IL-12R induction and signaling cascade

a) APCs are one of the main source of cytokines leading to the upregulation of IL-12R. APC are also a good source of IL-12. The stimuli activating APC will determine the nature of the cytokines.

b) Following IL-12 binding to IL-12R, the downstream signaling cascade will be activated. Kinases will phosphorylate STAT4, which will dimerize. Once the dimerized-STAT4 reaches the nucleus, it will activate the transcription of various inflammatory signals, including INFγ.

IL-23R and IL-12R functions

Th17 T-cells

Th17 T-cells are CD4+ T-cells that secrete IL-17 [54]. Th17 cells may play an important role in defending against extracellular pathogens[54]. Other experiments demonstrated that Th17 cells were highly inflammatory and very important in animal models of inflammatory diseases, including colitis and EAE [54]. IL-23 is required for the maintenance and pathogenicity of Th17[54]. Th17 can also produce IL-22 and other inflammatory cytokines.

Two different types of Th17 have recently been described: the classical Th17, which are differentiated from naïve T-cells using IL-6 and TGF-β, and "alternative" Th17 cells which were induced using IL-6, IL-23 and IL-1β[55]. T-cells can differentiate into Th17 in the absence of IL-23/12 [56], but can also form Th17 pathogenic cells in the absence of TGF-β[57]. Th17 cells differentiated in the presence of IL-23 had a more pathogenic phenotype than the classical ones; this could be one way in which IL-23 is important to induce inflammation, through production of pathogenic Th17 cells. Another mechanism by which IL-23 seems to affect T-cells function is through the inhibition of IL-10; without IL-10, Th17 may lack this feedback inhibition loop which could hinder their immune responses [55]. IL-23 can also induce GM-CSF production from Th17 T-cells; this cytokine has potent effects on APCs, leading to even more IL-23 production and increasing the inflammatory environment [58] and the potency of pathogenic Th17 cells.

γδ T-cells

γδ T-cells are a rare subtype of T-cells, representing less that 5% of total T-cells[59]. However, they are enriched in epithelial tissues, such as the skin and are found in high numbers in the gut. Unlike αβ T-cells, they do not need APC to be activated; their T-cell receptors recognize small bacterial patterns and not specific antigens [59]. γδ T-cells found in epithelial tissues are mature and can either be important in homeostatic processes or in the defence against pathogens.

Using the IL-23R-GFP reporter mouse, it was previously demonstrated that γδT-cells express IL-23R at steady state[60]. IL-23R+ were CCR6+ and INFγ-, while IL-23R- γδT-cells were CCR6+ and INFγ+[61]. γδT-cells stimulated with IL-23 induced IL-17, IL-22 and IL-21; this cytokine production was dependent on IL-23R.

Th1 T-cells.

Th1 T-cells are essential for clearance of intracellular pathogens[62]. They are characterized by their high secretion of INFγ. The most important Th1-inducing cytokines are derived from APCs; they are IL-12 and IL-18[50]. DCs and macrophages, upon activation, will secrete various cytokines; if they are primed to secrete IL-12 and IL-18, it will lead to the up-regulation of IL-12R and differentiation of naive T-cells into Th1 cells.

Up-regulated Th1 T-cell responses have been associated with many diseases, including multiple sclerosis, rheumatoid arthritis, lupus and type I diabetes [62]. On the other hand, down-regulation of Th1 responses leading to a decrease in IL-12 can lead to increased malignancy. It has also been shown that increasing the concentration of IL-12 in tumors can decrease the immunoregulatory environment of the tumor and increase the immune response against the malignancy[63].

a)Th17/Th1 dichotomy

Generally speaking, cytokines leading to the differentiation of a certain Th subtype will also inhibit the differentiation of another subtype. For example, Th17-inducing cytokines will inhibit the formation of Th1[50].

However, even if Th1 cells are associated with INFγ production and Th17 cells with IL-17 production, it was identified that in situations of autoimmunity, for example, Th17 cells actually produced INFγ[51]. In a study by Lee *et al*., OT-II naïve CD4+ T-cells in culture with irradiated splenic cells p40KO, OVAp[9], αINFγ, αIL-

[9] OVA, short for ovalbumin, is the specific peptide recognized by OT-II (CD4+ T-cells).In this experiment, OVAp are peptide charged on MHC molecules class II that can activate OT-II.

4(both antibodies), IL-6 and TGF-β for seven days gave rise to Th17 cells. About half of the Th17 differentiated cells produced IL-17, but few produced INFγ or INFγ and IL-17. Those Th17 differentiated cells were then transferred into new culture conditions. Upon stimulation with IL-12 (and OVAp, irradiated p40KO splenic cells, and αIL-4/αINFγ), more than 60% of the cells started producing INFγ. However, when stimulated with IL-23, 50% of the cells continued the production of only IL-17. This experiment shows that even if Th17 do not express (or express very low levels of) IL-12Rβ2, they can still respond to IL-12 and produce INFγ. In the same study, the authors also showed that TGFβ and not IL-23 was required *in vitro* to maintain IL-17 production by Th17 cells. These experiments cast a shadow on the specific response to IL-12 and IL-23; nevertheless, all of these results were obtained *in vitro* and merits verification in an *in vivo* model. Transcriptome analysis of Th17 cells cultured with IL-12 revealed a Th1-skewed gene expression, which shows a plasticity in Th17 cells depending on the cytokine environment, but most importantly a responsiveness to IL-12 even when expression of IL-12Rβ2 is very low. It could be that this responsiveness to IL-12 *in vitro* is an artefact of the *in vitro* conditions[64]. IL-23 is important in driving inflammatory responses in the gut; this could include the production of INFγ by Th17 following IL-23 stimulation[64].

Other cell types

The importance of IL-23R in certain T-cell subtypes is reasonably well understood, but IL-23R expression is not restricted to T-cells. Experiments measuring IL-23R expression in other cell types are rare. One of the reasons is due the lack of appropriate antibodies to study IL-23R expression. Very often, in order to measure IL-23R presence, cells are stimulated with IL-23 and cytokines such as IL-22 and IL-17 are measured to evaluate IL-23 responsiveness. It is interesting how some articles describe certain cell populations as IL-23 responsive and IL-23R expressing[65], as measured by IL-23R mRNA in certain cells populations, but others respond to IL-23 even in the presence of only very small quantities of IL-23R mRNA. This could indicate that IL-23 may signal through a receptor other than IL-23R; it could also mean that only small quantities of mRNA are required for protein production and signaling.

a) NK and NKT-cells

In a recent paper, DX5+TCRβ+ cells, known as NKT-cells, were found to secrete high amounts IL-17 when stimulated with αCD3 and IL-23. Those cells also expressed IL-23R mRNA before stimulation. NK cells, on the other hand, did not respond to the same stimulation and did not express IL-23R mRNA [66]. Those NKT-cells, when stimulated with either IL-23 or αCD3, still produced IL-17. The authors also isolated NKT-cells from an IL-23RKO mouse and stimulated them with IL-23. IL-23 stimulation IL-23RKO NKTcells did not induce IL-17 production. Further characterization revealed that NK1.1- NKT cells were responsible for producing the most IL-17 following IL-23/αCD3 stimulation. In this article by Rachitskaya *et al*, there is a suggestion that early IL-17 production following splenocyte stimulation with αCD3 and IL-23 is made by NKT-cells and that this mechanism is independent of IL-6, which would be a mechanism different from the one inducing IL-23R in T-cells.

It is generally believed that NK cells can directly respond to IL-12[67]. It is also known that IL-12 stimulation of NK cells in the inflammatory phase of immune responses induces INFγ[34]. IL-12 is usually associated with activation and proliferation of NK cells. IL-12 stimulated NK cells are also strong producers of INFγ.

b) B-cells

No expression of either IL-12R or IL-23R by B-cells in mice has been reported in the literature. Interestingly, it is known that IL-12Rβ2 acts as a tumor suppressor gene in human chronic B cell malignancies[68, 69], which led the authors of that paper to believe that IL-12/IL-12R signaling could be important in the regulation of B cells. Another study from the same group showed that some B cells in human tonsils expressed IL-12Rβ2 mRNA[70]. In the long-term study of IL-12Rβ2KO mice[10][69], it was observed that B220+ B cells were activated and plasma-cell hyperplasia (B-cell malignancies) were high, possibly indicating a role of IL-12Rβ2 in controlling B cell proliferation and malignancy.

[10] Mice were kept in a specific-pathogen free facility for a period of up to 26 months.

c) Dendritic cells, monocytes and macrophages

DCs, monocytes and macrophages are mostly recognized as a source of IL-23 and IL-12, but not as responding to IL-12 and IL-23, which could indicate that they do not express IL-23R and IL-12R[33]. However, there are many reports of INFγ secretion from these cells, including following stimulation with IL-12 alone[71], which would indicate the presence of a receptor to IL-12 on those cells. Others report the requirement of IL-18 and IL-12 through a STAT4 dependent mechanism to induce INFγ secretion[72]. Macrophages are specifically believed to respond to IL-23 and therefore express IL-23R, but data is conflicting about this conclusion[67].

d) Lti-like

Lymphoid tissue inducer cells (Lti) are required during embryonic development to create secondary lymphoid organs, such as lymph nodes [73]. Lti-like cells are cells similar to Lti that continue to be found in both mice and human in adulthood [73]. They can produce IL-22 and IL-17, are dependent on the RORγT transcription factor, can be found in germ-free mice, and can respond to IL-23[73]. They were found to be important in the αCD40 colitis models as a source of INFγ[74]. At the mRNA levels, IL-23R was expressed in high amounts [35]. The definition of Lti-like cells that can respond to IL-23 varies from one research group to another. They usually are Lin-, Thy1 (CD90) ^{high}, Sca-1+, and RORγT[64]. Another name frequently used is innate lymphocytes. Lti-like cells were found to increase in numbers during gut inflammation.

IL-22 production by innate lymphocytes is crucial for gut homeostasis, especially for epithelial cell proliferation, mucus production and overall maintenance of the epithelial barrier in the gut[64].

3. Mouse models

Before the discovery of IL-23 and its receptor, all biological phenotypes of p40 were associated with IL-12. Many experiments had to be re-examined and results were re-analyzed. Very often, roles attributed to IL-12 and IL-12R were actually caused by IL-23 and IL-23R.

Roles and importance of IL-23R and IL-12R in mouse models other than colitis

IL-23R and IL-12R have been studied in animal models of autoimmune diseases, bacterial and fungal infections and malignancies. In this section, a brief overview of the involvement of those receptors in the disease pathogenesis will be given in order to emphasize the importance of studying the biology of IL-12R and IL-23R.

EAE

Experimental autoimmune encephalitis (EAE) is an animal model of MS, where the animals are immunized with MOG (myelin oligodendrocyte glycoprotein), a peptide of myelin which is often the target of autoreactive T-cells in MS[75]. IL-12 was considered the main orchestrator of brain inflammation through generation of Th1-producing INFγ. However, Cua *et al.* showed that EAE was totally inhibited in the p19KO and p40KO animal models but not in the p35KO models[76, 77], showing that IL-23 is a crucial cytokine in the development of EAE, while IL-12 is dispensable[11]. The local IL-23 production in the brain was required for induction of brain inflammation.

γδT cells are IL-23R+ and are known to accumulate in great numbers in the CNS of mice affected by EAE [61]. γδT cells can control and decrease the activity of Foxp3+ Tregs and inhibit the conversion of naïve CD4+ T cells into Tregs [61], which could be one of the ways IL-23R can mediate brain inflammation.

[11] In that study by Cua et al., p35KO actually had a worse clinical state after two weeks than the WT animals, which could indicate a role for IL-35 in regulating inflammatory responses.

Other articles showed that IL-23RKO animals, similar to p19KO, were resistant to EAE induction, demonstrating the crucial role of the receptor in the induction of EAE [60, 78]. IL-12 is known to be important in the generation of Th1 pathogenic T cells; however, induction of EAE in IL-12Rβ2KO mice resulted in a more severe disease than in controls[79]. p19 mRNA was also higher in IL-12Rβ2KO mice, which may indicate a role for IL-12R in the control of IL-23R mediated inflammation. As one review clearly indicated, IL-23 is crucial for the development of encephalitogenic Th17 and IL-17 production, but also through the modulation of an array of inflammatory cytokines, including GM-CSF and IL-22 and through its actions on a variety of cells not limited to autoreactive T-cells[80].

Collagen induced arthritis

Collagen induced arthritis (CIA) is a murine model for rheumatoid arthritis. Genetically susceptible mice are immunized twice subcutaneously at the tip of the tail with collagen and develop a specific immune response against their joints. Using p35KO and p19KO mice, it was demonstrated that mice lacking IL-23 were completely protected from CIA, while IL-12-deficient mice actually had a worse disease than WT mice[81]. Mice lacking both IL-12 and IL-23 (p40KO) were also protected from CIA. The levels of IL-17 and TNF were slightly higher in the p35 knock-out strain compared to the WT animals, which could explain the slightly worse outcome of those animals. In this model, IL-23 seems to be the pathogenic cytokine, probably through the Th17 and IL-17 pathway[82].

IL-23-induced arthritis

The single hydrodynamic injection of IL-23 minicircle DNA resulted in joint-destructive arthritis [83], clearly demonstrating the possible pathogenic effects of increased IL-23.

Lupus nephritis

C57BL/6–*lpr/lpr* are susceptible to lupus erythemathosus and are therefore the animal model of human lupus, a disease characterised by systematic inflammation caused by autoreative T-cells and autoantibodies. When bred with IL-23RKO, the progeny were susceptible to lupus had lower cytokines, lower auto-antibodies levels and no nephritis, showing the importance of IL-23R in the pathogenesis of lupus in mice[84].

Infectious diseases

a) Experimental Cerebral Malaria

The role of IL-12/IL-12R in the development of malaria is still not well understood. In a mouse model of experiment cerebral malaria (ECM), it was shown that IL-12Rβ2KO mice did not suffer from ECM and survived much longer than the controls, but IL-12Rβ2KO mice had an unrestrained parasite proliferation and died of anemia[85]. When p40KO, p35KO, p19KO and IL-12Rβ1 animals were infected, they suffered from strong ECM and died at day 5. When this study was published, the role of IL-12Rβ2 in forming IL-35R was unknown; this could be an indication of the importance of IL-35R in the pathogenesis of ECM.

b) Klebsiella pneumoniae

In a *K. pneumoniae* mouse lung infection model, IL-23 was essential for the production of IL-17 by T cells. Without IL-17, the bacterial burden was much higher. However, using p19KO, p35KO and p40KO mice, they showed that mortality following bacterial infection was the same in p19KO, p35KO and p40KO mice, indicating an important role for IL-12 as well, even if IL-12 was not required for IL-17 production[86].

c) Listeria monocytogenes

Listeria monocytogenes is often used to study immune responses against intracellular bacteria. In a study by Riol-Blanco *et al*., Riol-Blanco et al. examined the importance of IL-23R during the infection of this pathogen[87]. IL-23R was important

in the early stage of the bacterial infection in order to induce the production of IL-17 by γδT-cells and decrease the liver bacterial burden. However, IL-23R was not required for the immune cells to be recruited to the abdomen following the infection. Also, in the absence of IL-23R, the decrease in IL-17 was correlated with an increase in INFγ, showing a switch from Th17 to Th1 response.

Colitis models

There are currently no colitis mouse models that exhibit all characteristics of IBD seen in humans[9]. However, many mouse models demonstrate that the genes identified in genetic studies in humans were as important to the development of the disease in mice, including IL-23R. Mouse models are relevant to human diseases because many immune responses are homologous between the two mammals and very often rely on the same signaling pathways[9].

However, there are many limitations of mouse models when interpreting IBD in humans[9]:

- Murine inflammation is usually found in the colon; most patients suffering from CD have inflammation in the ileum.
- Inflammation in the mouse is chronic, while in humans most suffer from a relapse-remitting disease.

Types of mouse colitis models

Depending on what aspects on intestinal inflammation need to be studied, one must use a specific rodent model. There are different approaches for inducing intestinal inflammation[9]:

1. Chemically-induced colitis (such as DSS, Dextran sulfate sodium and TNBS, 2,4,6-trinitrobenzene sulfonic acid)
2. Mixed techniques (such as αCD40 injections in RagKO mice)
3. Genetic modifications (such as IL-10KO)
4. Transfer of bacteria into genetically susceptible mouse

5. Transfer of cells into a genetically susceptible mouse (such as T-cells transferred into RagKO mice)

a) DSS

DSS is awhite chemical that can easily be dissolved in water[12]. When added to the mice drinking water at concentrations between 1%-5% it induces an acute colitis characterized by colon shortening, neutrophil recruitment, weight loss, and ulcers in the mucosa of the intestine[88]. This model of colitis induction is useful when studying the importance of innate immune responses in the development of colitis in both SCID[88] and RagKO mice[65], which are deprived of T and B-cells and therefore of adaptive immune responses. However, DSS-induced colitis can also be used in immunocompetent mice, such as mice with T and B-cells.

b) TNBS

The TNBS model is another chemically induced model of colitis; however, unlike DSS, it depends on T-cells to induce intestinal damages[88]. Briefly, TNBS is mixed with ethanol and is given through the rectum. This model uses ethanol and TNBS to break the mucosal barrier in the intestine and induce gut inflammation[89].

c) αCD40 antibodies

Injection of an agonistic CD40 monoclonal antibody in a RagKO mouse induces systemic inflammation and rapid weight loss[90]. Lesions and pathology are observed in the liver and the colon, while the spleen increases in size. Thus, this model is not simply a colitis model; however the intestinal pathologies induced by this model are particularly useful in understanding inflammation in an innate immune model.

To understand the pathogenesis of the disease, monoclonal antibodies against different cytokines, including IL-12 and TNFα, were injected *in vivo* to understand the role of each cytokine in the pathogenesisof the disease. Amongst the ones tested, anti-p40, a monoclonal antibody against IL-12 and IL-23, was the most effective. Anti-

[12] MP Biochemicals, http://www.mpbio.com/product_info.php?family_key=02160110

INFγ and-TNF-α inhibited the strong weight loss (wasting disease), but did not have an effect on colitis or cytokine serum levels[90]. The anti-p40 monoclonal antibody treatment indicated a possible role of IL-12 and IL-23 in the inflammation developed after αCD40 injection. In the next section, induction of inflammation with αCD40 in p19KO, p35KO and p40KO mice will be further addressed.

d) IL-10KO

A longitudinal study of IL-10KO mice revealed that many of them spontaneously developed colitis[91]. Inflammation started at three weeks of age, mostly in the colon, but after three months 100% of IL-10KO suffered from intestinal inflammation. Treatment with anti-INFγ decreased the disease severity but did not entirely inhibit it, even if analysis showed a skewed Th1 immune response in the gut of those animals. These findings have been replicated, but demonstrated that IL-23 and not IL-12 was crucial in the development of colitis in IL-10KO animals[92].

e) T-cell transfer

Another interesting model of colitis[13] is the transfer of CD45RBhighCD4+ T cells into a SCID or RagKO mouse. Upon this transfer, the mice suffer from a wasting disease, massive weight loss and strong intestinal inflammation[93].

f) Helicobacter hepaticus

H. hepaticus is an intestinal bacteria used to study to role of the microbiota and infections in the development of colitis. When given orally in 129SvEvRagKO, it induces inflammation of the gut, which is then a T-cell-independent gut inflammation[94]. However, if CD45RBhighCD4+ T cells are transferred into the 129SvEvRagKO mouse and *H. Hepaticus* given orally the gut inflammation is much more severe, thereby showing a T-cell-dependent gut inflammation[94]. In both cases, transfer of Tregs can inhibit the gut inflammation. Transfer of *H. Hepaticus* in IL-10KO mice also induces colitis[95], but infection with *H. Hepaticus* in WT animals

[13] Will be further discussed in section 3.3 of the introduction

does not lead to gut inflammation. These different experiments clearly show that in the *Helicobacter hepaticus* colitis model, Tregs are a crucial element in controlling gut inflammation and/or immune dysregulation.

Importance of IL-23R in different colitis models

Since the identification that IL-23R is implicated in IBD aetiology, many groups have generated different mice that were either lacking some of the important genes in this pathway or cytokine family, including p35KO (absence of IL-12 and IL-35), p40KO (no IL-12 and IL-23), p19KO (no IL-23), as well as IL-12Rβ1KO, IL-12Rβ2KO and IL-23RKO. Then, using different methods to induce colitis, they tested the importance of each of the pathways knocked out in the induction of colitis.

Using DSS induced-colitis, Cox *et al.* tested the importance of IL-23 and IL-23R using p19KO and IL-23RKO mice. When compared to wild-type mice, both KO mice had less weight loss, histological colitis scores were decreased and leukocyte infiltration was lower[65]. All KO mice did not have the same phenotypic response; histological findings for the p19KO were less severe than for the IL-23RKO mice. This highlights, once again, that IL-23 may be acting through another receptor other than IL-23R, and/or IL-23R could be activated through another cytokine other than IL-23[65]. Another group in 2006 had done a similar experiment, also using DSS 2.5% in p19KO mice, but their findings were contradictory to Cox *et al*.. Their results: the p19-KO mice lost more weight during the course of the disease than WT mice [96]. Surprisingly, when using an anti-IL-12/23-p40 antibody in p19KO, the weight loss in DSS-induced colitis was tremendously decreased. The authors hypothesized that IL-23 could actually control IL-12 secretion, and in the absence of IL-23, IL-12 would increase. Alternatively, those results could be explained by the p40 homodimer secretion. Becker *et al.* also used TNBS-induced colitis; p19KO were actually more susceptible to colitis than the controls. Another study of TNBS-induced colitis measured cytokines through the progress of the disease; the early stages of inflammation had high levels of IL-12 and INFγ, but as time progressed the cytokine profile switched to IL-23 and IL-17, which could indicate a role for both cytokines in this colitis model[89].

However, colitis was not totally inhibited in the Cox study, demonstrating that other pathways are likely also important in disease induction. Pro-inflammatory cytokines IL-17 and IL-22 were also decreased. They also tested DSS induced colitis in RagKO mice, deprived of B and T-cells. In this situation, animals lacking IL-23R as well as T and B-cells actually lost more weight than the RagKO control animals. Levels of IL-22 and IL-17 were reduced. However, injection of IL-22 helped to decrease colitis. Depletion of Thy1.2+ cells of RagKO animals worsened the colitis state. Thy1.2+ are definitely one of the most important cell populations expressing IL-23R in RagKO animals, but are probably not the only cells doing so, as evidenced by the residual protection from DSS-induced colitis. IL-22 was previously identified as important in mucosal repairs and this effect is mediated, at least partly, through Thy1+ cells[65].

In another colitis model, upon injection of αCD40 antibodies into RagKO p35KO (IL-12KO) mice systemic inflammation was inhibited, for RagKO p19KO (IL-23KO) mice colitis stopped, and for RagKO p40KO (IL-12 and IL-23KO) mice both types of inflammation were inhibited. This demonstrated a different role for each cytokine in different settings, where IL-23 seems more important for local inflammation in the gut[90] and IL-12 for systemic inflammation. When this study was published, CD4+ cells were the main type thought to respond to IL-23, but there are no T-cells in the αCD40 model. The group went on to identify a population of innate lymphocytes CD90.2+ (Thy1.2+) Sca-1+ population in the gut that responded to IL-23 by secreting IL-17[74]. Depletion of this cell population with a αCD90 monoclonal antibody decreased colitis, demonstrating the importance of this population of innate lymphocytes in the pathogenesis of colitis.

Other colitis models, which consist of *Helicobacter hepaticus* administration and either injection of anti-IL-10R antibody or T-cell transfer into RagKO mice showed that IL-23 was essential for the development of severe intestinal inflammation, while IL-12 was dispensable. However, INFγ production was essential for some aspects of the pathology, and is usually associated with IL-12 production[14]. Th17 cells are known to secrete INFγ, so INFγ coming from Th17 cells could cause severe intestinal inflammation. p40 had an essential role in the gut inflammation, but not p35.

A study using *H. hepaticus* infection in 129SvEvRagKO mice showed that IL-23 and IL-17 cytokines were increased in the intestine, but IL-12p35 was increased in the spleen, here again reinforcing the roles of IL-12 in systemic inflammation and IL-23 in local inflammation[14][97]. Injection of αIL-23p19 greatly decreased inflammation in the colon and caecum and cytokine levels measured in the gut were much lower, but inflammation was not totally inhibited. The αp19 antibody also decreased cellular recruitment to the spleen, this time demonstrating an importance of IL-23 in systemic inflammation. In T-cell independent colitis, IL-23 was an important mediator of inflammation[97]. Again, another study by this group found that the CD90.2+ (Thy1.2+) Sca-1+ population in the gut was responding to IL-23 and secreted both INFγ and IL-17[74]. Those IL-23-responsive innate lymphocytes which seem important in the pathogenesis of animal models of colitis induced by αCD40 and *H. hepaticus* were found in increased numbers in the intestines of IBD patients[98], increasing their pertinence to colitis pathogenesis.

In the IL-10KO colitis models, double KO IL-10KO p35KO mice still suffered from colitis, demonstrating that IL-12 is not required for the development of colitis. On the other hand, IL-10KOp19KO did not develop colitis, indicating an important role for IL-23 in the development of gut inflammation[92].

In a T-cell transfer model, IL-10KO T-cells usually induce colitis in about 10-12 weeks. However, continuous treatment with IL-23 led to development of colitis in four weeks, but IL-23 treatment by itself did not induce colitis. IL-23's effect on T-cells was thus crucial to the development of colitis[92]. To measure the importance of IL-23 on T-cells, another group induced colitis in the T-cell transfer model by transferring IL-23RKOCD4+CD45RBhigh T-cells into RagKO mice[99]. Mice injected with IL-23RKO T-cells only developed a mild colitis when compared to controls. T cells from IL-23RKO mice injected into RagKO mice led to decreased levels of IL-22, IL-17, IL-21, TNF-α and other cytokines in the colon, but the number of CD4+ T-cells was the same and the total number of splenocytes was actually higher in mice injected with IL-23RKO CD4+T-cells than in mice injected with WT CD4+T-cells. INFγ production

[14] As mentioned in the αCD40 colitis model.

was not decreased in IL-23RKO T-cells, so Th1 differentiation was not impaired. They also showed with mixed bone marrow chimeras and transfer of IL-23RKO and WT T-cells that proliferation of T-cells was reduced in IL-23RKO, probably through the induction of Treg and IL-10 secretion. IL-23R signaling could then modulate IL-10 secretion, increasing inflammation.

Another study by the same group also tested the importance of IL-23 over IL-12 secreted by RagKO animals in which WT CD4+CD45RBhigh were transferred. The intestine inflammation score of RagKOp40KO and RagKOp19KO were much lower than RagKOWT animals, but RagKOp35KO was similar to RagKO[97] and intestinal inflammatory cytokines were decreased in p40KO and p19KO animals. However, systemic inflammation (measured by spleen weight, number of cells in the spleen and number of CD4+T-cells) was decreased only in the RagKOp40KO, which could indicate roles for p40 independent of p35 and p19.

IL-23R-GFP reporter mouse

One of the biggest challenges in the study of IL-23R and its biology is the lack of reagents. Monoclonal antibodies against IL-12R and IL-23R are rare and often non-functional. In the studies cited previously, IL-23R expression was usually measured by mRNA quantification, which can be indicative but is not always translated into real protein measurements. It was possible to identify roles for the receptor and the cytokines through knock-out animals, but pin-pointing exactly which cell populations were directly expressing the receptor was much harder.

To overcome this difficulty, an IL-23R-GFP reporter mouse was created by Awasthi *et al*[60]. To generate this mouse, an internal ribosomal entry site (IRES) and enhanced green fluorescent protein (GFP) were inserted directly after exon 8 in the IL-23R gene on chromosome 6 of the mouse genome, removing exon 9 entirely. Exon 9 codes for the transmembrane domain of IL-23R; removal of exon 9 probably inhibits the IL-23R protein from reaching the surface. However, insertion of IRES-GFP assures that GFP is produced instead of IL-23R protein.

When this mouse is heterozygous for the IL-23R-GFP allele, cells that should express IL-23R will produce both GFP and a functional IL-23R, allowing responsiveness to IL-23. If the mouse possesses two GFP alleles, it leads to a functional IL-23R knock-out mouse (IL-23RKO), but cells that should express IL-23R can still be tracked through GFP expression(Figure 6: IL-23r-GFP reporter mouse).

Following the production of the mice, Awasthi *et al.* compared IL-23R-GFP homozygous mice to the IL-23RKO mice and both had no responses to IL-23. They also were equally fertile compared to WT mice and had similar numbers of different immune cell types compared to WT.

They further analysed which cells expressed IL-23R in steady state in the lymph nodes. They showed that TCRγδ T-cells were the main cells expressing the receptor, while some CD4+CD3- cells also expressed it. There was also a small population of CD11c+ and CD11b+ cells that expressed IL-23R. All IL-23R+ cells were CD45+, indicating their hematopoietic provenance. B-cells, NK cells and CD8+T-cells did not express the receptor. Very low numbers of spleen cells in naïve animals expressed IL-23R in this system. Around 3% of CD45+ cells in the lamina propria of the small intestine expressed IL-23R and most(65%) LP γδ T-cells expressed IL-23R, but no DCs expressed the receptor.

They concluded their paper by inducing EAE in IL-23RKO. In the absence of a functional IL-23R, EAE was totally inhibited. Because EAE is caused by myelin autoreactive T-cells, it is possible to transfer EAE simply by transfering T-cells. Transfer of IL-23RKO T-cells did not induce EAE in recipient mice, indicating the importance of IL-23R signaling on T-cells in EAE pathogenesis.

This paper strongly establishes some important facts: IL-23R expression in naive animals is rare, especially in the spleen. However, more cells expressed IL-23R in the gut, which fits nicely with the colitis experiments previously discussed. The EAE experiments clearly indicate a crucial role of IL-23R signaling in the pathogenesis of the disease. The characterization of the mouse is interesting, but as many *Cutting Edge* papers published in the Journal of Immunology point out, a lot of data is not shown and

many interesting cell types and organs are not looked at for IL-23R expression. They also generated a RagKOIL-23R-GFP mouse, but did not show how IL-23R was different in those RagKO animals, which could be interesting to look at rarer cell populations, such as innate lymphocytes.

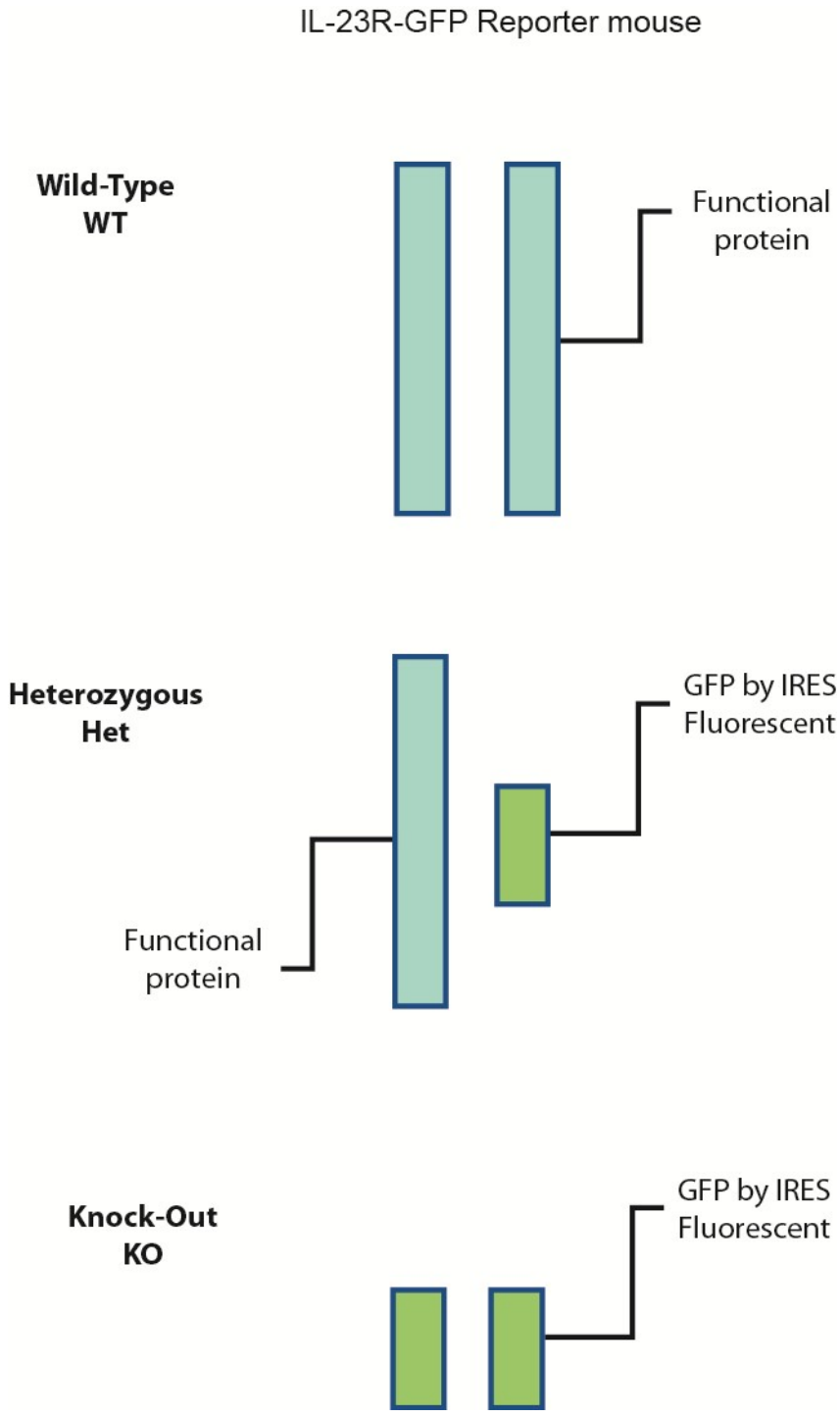


Figure 6: IL-23r-GFP reporter mouse

In wild-type mice, both IL-23R alleles are normal and produce IL-23r protein. However, it is impossible to detect IL-23r+ cells with flow cytometry. In IL-23r-GFP-Het mice, one allele can produce IL-23r, while the other will produce GFP. IL-23R will still be produced and responsiveness to IL-23 through IL-23R is possible. In IL-23r-GFP-KO mice, IL-23r expression through GFP detection can be detected; however, a functional IL-23R will not be produced.

Research question and hypothesis

IBD represents a puzzle in which many of the pieces have been identified. However, figuring out how all those pieces actually fit together is still a tremendous challenge. Understanding IL-23R biology and how it fits in the bigger picture of IBD will definitely help put some pieces of this puzzle together and improve our comprehension of how genetics, environmental triggers and immune responses can lead to inflammatory bowel diseases. It is no small task to tackle.

As shown in the previous sections, IL-23R functions are intrinsically linked to IL-12R and other members of IL-12R. IL-12R could also be involved in IBD pathogenesis as genes important for its biology, such as p40, IL-12Rβ1 and Jak2, have been identified in IBD GWAS. The strong homology between IL-12R and IL-23R and IL-12 and IL-23 leads to many questions: could each cytokine only act through its specific receptor? Which cells express those receptors?

Our laboratory acquired the IL-23R-GFP mouse used in the Awasthi *et al*. article. A thorough characterization of this mouse in order to analyze IL-23R expression in steady state could answer some questions asked in the analysis of the Awasthi paper, such as IL-23R expression in RagKO. For example, colitis in certain RagKO mouse models is IL-23 dependent, so IL-23R expression is definitely not restricted to T-cells or IL-23 can signal through receptors others than IL-23R. Also, tracking IL-23R expression by cells through induction of colitis or cytokine stimulation could be indicative of IL-23R expression and the possible dichotomy between IL-23 responsiveness and IL-23R expression sometimes seen in the literature.

Furthermore, there is a new antibody targeted against the IL-12Rβ2 specific subunit. Analysing the expression and modulation of the two receptors could lead to a better comprehension of the expression dynamics of the two receptors. It was previously shown that both may contribute to different pathogenesis or different components of the same disease model, especially colitis. Mapping IL-23R and IL-12R

pattern expression at steady state could increase our knowledge of the possible interactions between the two receptors.

Characterization of the expression of both receptors could help understand the first cells responding to IL-12 and IL-23 following an insult, such as bacterial or viral infection. Differentiation of naïve T-cells into Th1 and/or Th17 is clearly more complex than first thought, as Th17 cells seem to also secrete INFγ and both be influenced by IL-12 and IL-23, but can also differentiate without either cytokine.

This project will focus on understanding IL-12R and IL-23R expression dynamics in homeostasis state, but also answer some questions raised by the paper by Awasthi *et al*. It will look at expression of both IL-23R and IL-12R in different organs, but also at the responsiveness of each specific cell types to IL-12 and IL-23 and the concordance with the receptors expression.

Objectives

1. Identify the cell populations expressing IL-23R and IL-12R using the IL-23R-GFP reporter mouse and a monoclonal antibody, respectively;
2. Characterize the cell populations expressing IL-23R and IL-12R in different organs, especially the gut; and
3. Understand the specificity of the cytokine responses of different cell types following stimulation with IL-12 or IL-23 in order to understand the specificity of each cytokine.

Methods

1. Lymphocytes extraction from murine organs

Most mice used in the experiments were aged 8 to 13 weeks old at sacrifice. They are sacrificed either by: cervical dislocation alone or with isoflurane anesthesia followed or by cervical dislocation. Following death, organs are harvested.

There is an order by which it is easier to extract organs and blood.

1. Peripheric lymph nodes
2. Thymus
3. Lungs
4. Liver
5. Spleen
6. Mesenteric lymph nodes
7. Intestines
8. Bone marrow

When the lungs and the liver are required, one must perfuse the mouse with fresh cold saline or PBS, which will wash out the organs of their blood. This step will ensure that the cells collected in the organs were resident in the organs and not coming from the periphery. It will also reduce the quantities of red blood cells found in the organs, making it easier for lymphocyte extraction later on. Anesthesia is first induced with isoflurane, then the mouse is cut opened. The hepatic vein is then cut. After opening the thorax, a small syringe is inserted into either the left or the right venticule of the heart. Then, very slowly, cold saline is injected in the heart, which will then circulate all through the organs. The blood and the saline will be ejected through the hepatic vein that was cut open.

1.1 Spleen, lymph nodes, thymus, mesenteric lymph nodes

The spleen, lymph nodes, thymus and mesenteric lymph nodes are organs filled with lymphocytes. Extraction of immune cells from these four organs is simple. Each organ is crushed on a 70μM cell strainer with a syringe piston. Using a 5mL pipette, the cell suspension is filtered many times through the cell strainer. The filtrate is then transferred into a 15mL conical tube. The tube containing the cells is then centrifuged at 1200RPM for 7 minutes.

Following centrifugation, the supernatant is removed and the cell pellet is kept. In organs containing high numbers of red blood cells, such as the spleen, the cells are resuspended in 5mL of a solution containing NH_4Cl which lyses the red blood cells. After addition of the NH_4Cl solution, the cells are again centrifuged during at 1200RPM for 7 minutes.

The thymus, lymph nodes and mesenteric lymph nodes contain very little red blood cells; treatment with NH_4Cl is therefore not required. Following the first centrifugation, supernatant is removed and cells are suspended in FACS buffer, media or PBS, depending on the intended use of the cells.

1.1.1. Collagenase treatment

Specific cell types, especially dendritic cells, require special treatment to be retrieved intact from lymphoid organs and tissues in general. To ensure proper collection, enzymatic treatment of the organs with collagenase is required. Collagenase is an enzyme that can fragment the collagen found in the extracellular matrix and organs in general[15]. Following digestion with collagenase, cells are freed from the organs, intact.

Collagenase is first diluted into a concentration of 1mg/mL. Organs are then placed in a Petri dish. Big organs, such as thymus and spleen, are injected with 1mL of diluted collagenase. Then, 1mL of collagenase is added to the Petri dish itself. With a

[15] Sigma, http://www.sigmaaldrich.com/life-science/biochemicals/biochemical-products.html?TablePage=14573101

razor blade or scissors, the organs are cut into small pieces and incubated at 37°C for 15 minutes. For smaller organs such as lymph nodes, they are simply placed in Petri dish (all of them together), then 1mL of diluted collagenase is added and the mixture is incubated at 37°C for 15minutes. After the digestion, 5mL of complete media (10%FBS, β-mercaptoethanol, HEPES, Streptomycin/Penicillin, RPMI) is added to stop the reaction. The organs are subsequently treated as described in section 1.1.

1.2 Bone marrow

In these experiments, bone marrow coming from the femur and the tibia of the two legs of a mouse were used. The legs are first cut apart from the animal and the skin and muscles are removed. Following bone cleaning, each extremity of the bone is cut with scissors. Using a small needle attached to a syringe, complete media is flushed in the bone. The bone marrow falls into a cell strainer placed in a Petri dish. Each bone is flushed around four times, twice at each extremity[16].

Once each bone is white and therefore completely emptied of marrow, the bone marrow is treated similar to a spleen (section 4.1.1). It is crushed with a syringe piston, filtered through a 70μM cell trainer, centrifuged, treated with NH_4Cl, centrifuged again and suspended in the desired buffer. The bone marrow can also be treated with collagenase (similar to lymph nodes, section 4.1.1.2).

1.3 Lamina propria of the small intestine

The lamina propria of the small intestine is a region situated below the basement membrane of the epithelial layer of the intestine, where there are many different types of lymphocytes. Those cells are different from intraepithelial lymphocytes, which are found between the epithelial cells of the intestinal lining[100].

Hereby is a general view of the protocol; the entire protocol, with the preparation of each solution, can be found in Appendix 1. After the intestines are

[16] Bone marrow cell extraction protocol is adapted from a common lab protocol.

removed from the mouse, they are cut open and washed in cold media (the exact composition of each solution can be found in the protocol in Appendix 1). Once they are thoroughly cleaned of fecal matter, they are incubated at 37^0 C for 20 minutes in a solution composed of DTT, EDTA, and others, which will loosen the epithelium of the intestine and release both the epithelial layer and intraepithelial lymphocytes. Following this incubation, the intestinal fragments are shaken with a mix of EDTA and media and filtered through a kitchen strainer. The liquid obtained contains epithelial cells and intraepithelial lymphocytes. Successive filtrations on 100μM, 70μM and 40μM cell strainers and centrifugation result in obtaining a cell suspension containing intraepithelial lymphocytes and some epithelial cells.

The intestinal pieces that were left in the kitchen strainer are then chopped into very small fragments and incubated at 37^0 C for 36 minutes with liberase, collagenase and DNase. This will allow the collagen in the basement membrane and extracellular matrix of the intestine to be digested and the lymphocytes will be freed. After digestion, the intestines are crushed through a 100μM cell strainer and are the cell suspension obtained is filtered several times through the same 100μM filter. The cell suspension is further filtered on a 70μM cell strainer, then centrifuged and strained again through a 40μM cell strainer and centrifuged one last time. The pellet obtained can then be suspended in the desired buffer. This results in a cell suspension of lamina propria lymphocytes, with some debris and other cells from the extracellular matrix.

1.4 Lungs

The lungs are also covered with an epithelial layer filled with lymphocytes. There is a simple way to extract those cells. The complete protocol for lung lymphocytes extraction can be found in Appendix 2.

Once the mouse is anesthetized with isoflurane, the thoracic cage is opened and cold saline is injected in the heart. As the organs are perfused, blood will exit the lungs and mouse limbs will become pale. After the perfusion, the lungs are removed and placed in complete cold media on ice. The lungs are then cut in small pieces and placed

in a round bottom tube with 2mL of 1mg/mL collagenase and incubated for 30 minutes at 37^0 C. Every 10 minutes, the tubes are vortexed.

After the 30 minutes incubation, 2mL of complete media are added to the tubes. The pieces of tissue are then pipetted up and down and transferred on to a 70µM cell strainer, where the tissues are then crushed with a syringe piston. Using a 5mL pipette, the cell suspension is filtered many times through the cell strainer. The filtrate is then transfer into a 15mL conical tube. The tube containing the cells is then centrifuged during for 7 minutes, at 10^0 C and 1200RPM.

Following centrifugation, the supernatant is removed and the cell pellet is kept. The cells are then resuspended in 5mL of a solution containing NH_4Cl which lyse the red blood cells. After addition of the NH_4Cl solution, the cells are again centrifuged for 7 minutes, at 10^0 C and 1200RPM. The last pellet contains lung lymphocytes.

1.5 Cell counting

Following cell extraction from the different organs, it is important to know how many live cells were obtained. A small portion of each cell suspension is then used. Cells are diluted in Trypan Blue 0.016% and added onto a hemacytometer. The dye will penetrate into the dead cells, making them blue, while live cells will be clear[17]. It is therefore possible to count the number of live cells in the dilution and estimate the total number of cells extracted from the different organs.

1.6 Flow cytometry

One of the main tools employed to characterize cells is flow cytometry. A flow cytometer can detect particles from 1µM to 50µM in size and provide information on their size and granularity. To further characterize cells, it is possible to add antibodies conjugated to fluorochromes. Each antibody can recognize a specific antigen on the surface of the cells (and inside the cell, given the proper treatment). This antibody will bind to the molecule. Then, in the flow cytometer equipped with different lasers, the

[17] Invitrogen Website, http://products.invitrogen.com/ivgn/product/15250061

fluorochrome will be excited by the laser, which will emit a light and will be analyzed by a computer attached to the flow cytometer. Mixing different antibodies with different fluorochromes can then reveal the different cell populations found in different organs. In these experiments, cells coming from IL-23r-GFP-Het or homozygous mice which express IL-23R will emit a positive signal which can be detected by the flow cytometer.

Between 1 and 3 millions cells are added to a V-bottom 96-well plate. The cells are then centrifuged and the pellet is kept. Each antibody is then added to the wells. The proper quantity of antibody to add was previously measured in a titration. If an antibody is needed in a 1:200 dilution, the antibody is diluted to 5:200, then 10µL of the dilution of the antibody for one million cells is added to the well. If three antibodies are required, 10µL of each antibody will be added to the well. Then, the volume will be brought up to 50uL with FACS buffer[18] and the proper dilution for each antibody will be obtained. Very often, an antibody against Fc receptor, 24G2, will be added to the cocktail. Fc receptors bind to most antibody. Blocking the Fc receptor reduces the amounts of unspecific binding by antibodies.

The cells will be incubated for at least 30 minutes at 4^0 C. After the incubation, 100µL of buffer is added to wash the cells, followed by a 3-minutes centrifugation at 1100RPM. This last step is repeated one last time. The cell pellets are then diluted and transferred into FACS tube. If more than five antibodies are required, there will be only one wash after the first antibody incubation and new antibodies will be added, followed by another 30minutes incubation period at 4^0 C and two washes. Cells will then be transferred to a FACS tube.

1.6.1 Antibodies combinations used to identify cells

Most cells used in the different experiments were stained with CD45.2 and IL-12Rβ2 (Research and Development, cat#FAB1959A)[19] monoclonal antibodies. CD45.2 is very important when staining cells coming from organs containing many epithelial

[18] In the example where three different antibodies are used, 20µL of FACS buffer is added to each well and therefore the final volume is 50µL, resulting in a final dilution of 1:200, for example.
[19] Appendix 3 contains a list of all antibodies used.

cells, such as the LP of the small intestine, to make sure the proportions of cells analysed are only hematopoietic cells. The FITC channel was also reserved for IL-23r-GFP+ cells.

Cocktails to identify the different cell types were used, which all contained CD45.2 and IL-12Rβ2 monoclonal antibody. Lti-like cells cocktail contained Sca-1, c-kit and CD90.2. The T-cell cocktail contained CD3, CD4, CD8 and TCRγδ antibodies. There were three B-cells cocktails. CD19 and/or B220, were always present, with a)CD21, CD23, IgM and IgD, b)GL-7 and CD138, or c)CD43. Monocytes and NK cells were often analysed together, with CD11b, CD11c, Gr1 and DX5 (CD49b). Finally the three subtypes of DCs were analysed with CD11c, CD8, CD11b and mPDCA-1. Myeloid DCs (mDC) are CD11b+CD11c+, lymphoid DCs (lDC) are CD8+CD11c+, and plasmacytoid DCs (pDC) are mPDCA-1+CD11c+.

The last staining used was Lin(-) staining. A Lin stain removes T-cells(CD3+ and CD8), B-cells (B220+), NK cells (DX5), monocytes (CD11b and Gr1), CD11c (DC) and Ter19 (developing erythrocytes). Biotin-conjugated antibodies to all the markers are added in the first staining step and streptavidin-conjugated PE (fluorochrome) is added during the second step. All cells positive for Lin are therefore known subtypes, such as T-cells, B-cells, *etc.*. All the cells negative for Lin staining left are therefore called Lin(-), as they are not any of the usual subtypes.

1.6.2 Cell sorting strategies

One of the main limitations of using a regular flow cytometer is that no further experiments can be done on the cells, as they are destroyed following their passage through the flow cytometer. However, there is a specific flow cytometer, called a cell sorter, which allows the recuperation of the cells after their passage through the flow cytometer. Furthermore, cells can be sorted upon their specific markers. Cell sorting is a particularly efficient way to isolate specific cell types for further experiments. The procedure to stain cells for cell sorting is similar to the one described in the previous section, except that everything is done in sterile conditions.

To obtain cells for *in vitro* cell cultures, cells were stained with CD45.2, CD19, IL-12Rβ2, CD3, CD4, TCRγδ, DX5(CD49b), c-kit and CD90.2. This combination of markers allowed for us to obtain all the required cell types in the same experiments. Figure 7 : Sorting Strategy for cell cultures explain the gating strategy to obtain CD4+T-cells, γδT-cells, NK cells, Lti-like cells and B-cells without contamination with the other cell types.

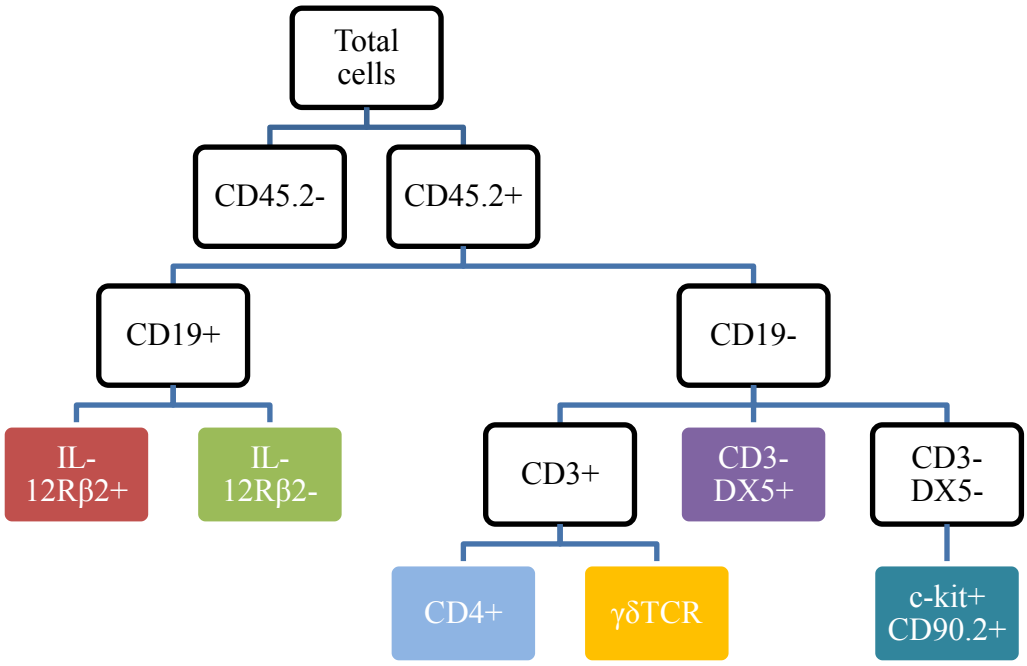


Figure 7 : Sorting Strategy for cell culture

- Color-filled boxes are cells used in further experiments.
  - **CD19+ IL-12Rβ2+** are IL-12Rβ2+ B-cells, while **CD19+IL-12Rβ2-** are IL-12Rβ2-B-cells.
  - **CD19-CD3-DX5+** are NK cells.
  - **CD19-CD3+CD4+** are CD4+ T-cells, while **CD19-CD3+δγTCR** are γδT-cells.
  - **CD19-CD3-DX5-c-kit+CD90.2+** are Lti-like cells.
- Using this strategy, it ensures that NKT-cells, which express CD3 and DX5, are not contaminating DX5+NK-cells. There is also a big population of NKcells and T-cells that express CD90.2. However, they have been removed from the c-kit+CD90.2+ population by taking only CD3-and DX5- cells.

In this strategy, all cells not expressing CD45.2 are thrown away. CD45.2+ cells are further sorted according to CD19 expression. CD19+ cells are B-cells; they are then sorted apart according to IL-12Rβ2 expression. This sorting results in two different cell populations: IL-12Rβ2+ B-cells and IL-12Rβ2- B-cells.

CD19- cells are then separated into three groups: CD3+ cells(containing all T-cells), CD3-DX5+ cells(NK cells) and CD3-DX5- cells (containing monocytes, DCs and Lti-like cells).

CD3+ T-cells are quite heterogenous; they segregated into CD4+ T-cells and TCRγδ T-cells and all the other ones are discarded. NK cells are kept. CD3-DX5- cells are further segregated into c-kit+CD90.2+ cells, which removes all DC and monocytes, while keeping Lti-like cells.

2. *In vitro* culture

Following cell sorting, cells were cultured in different conditions and with different cytokines. Quantities of IL-12 and IL-23 added to each well were always the same. IL-12 and IL-23 were added to wells in quantities of 5ng of cytokines per 100µL, resulting 2.5ng/µL. For IL-2, it is usually recommended to use 1000 units of IL-2 per mL. 10ng of IL-2 were added per 100µL of media, for a final concentration of 1000units/mL. Those different cytokine concentrations were the ones used for all cultures with IL-2, IL-12 or IL-23. All the cells were incubated in the same 37°C incubator at 5% CO_2.

2.1 CD4+T-cells and TCRγδ+T-cells

T-cells were always put in culture on αCD3 and αCD28 coated flat bottom 96-well plate. To coat the plates, αCD3 was diluted to 0.5µg/mL in sterile PBS and αCD28 was diluted to 5µg/mL. 50µL of each dilution was added in each well of the 96 well plate. The plate was then placed overnight at 4^0 C. After the incubation period, each well was carefully washed with complete sterile media, to remove all non-attached antibody.

After washing the wells, sorted T-cells were added to the wells. For CD4+ T-cells, 200000 cells in 200µL of fresh media were put in each well. However, numbers of γδT-cells in spleens of naïve animals were pretty low. Numbers between 60000 and 90000 cells were put per well, with 100µL of fresh media. Then, depending on the conditions, IL-12 or IL-23 was added. After three days in culture, 100µL of media was added with the appropriate cytokine to the CD4+T-cells and 50µL was added to the TCRγδ. On the seventh day, cells were removed from cultures and supernatants were kept for cytokine analysis.

2.2 B-cells (IL-12Rβ2+ and IL-12Rβ2-)

After obtaining the cells, 200000 IL-12Rβ2- B-cells in 200µL of complete media were put in 96 round bottom well plate. Because IL-12Rβ2+ cells are found in

much lower numbers than IL-12Rβ2+ B-cells, around 100000 IL-12Rβ2+ B-cells in one 100μL of complete media were put in each well. The cytokines required were added in each well. After three days in culture, media and cytokine were added to each well, 100μL for β2- B-cells and 50μL for β2+ B-cells. On the seventh day, cells were removed from culture and supernatants were kept for immunoglobulin and cytokines analysis.

2.3 Lti-like cells

Lti-like cells in the spleen are found in very low numbers, so their culture is a challenge. Around 50000 cells in 100μL were added per well of a round-bottom 96-well plate. Adding less than 100μL of media per well was not successful, as the media evaporates in the 37°C incubator. After three days in culture, 50μL of media and the appropriate cytokines were added to the well. Cells were removed from culture on the seventh and supernatants were kept for further analysis.

2.4 NK cells

After obtaining the cells, 200000 NK-cells in 200μL of complete media were added in each well of a 96 round bottom well plate. NK cells do not survive well without IL-2; therefore, most NK cultures contained the previously described dose of IL-2. After three days in cell culture, one well of NK cells was usually split in two different wells. Afterwards, 100μL-200μL of complete media was added to each well with the appropriate cytokines. On the seventh day, cells were removed from culture and supernatants were kept for further analysis. Often, after three days in culture, some NK cells were removed from culture and analyzed for proliferation, while supernatants were kept for cytokine analysis.

2.4.1 Proliferation assay

To understand the effect of the different cytokine treatments on NK cells, a CFSE proliferation assay was done. CFSE is a stain that can enter the membrane of different cells. When CFSE stained cells pass through a cytometer, they emit a signal

that can be detected. As the cells are proliferating, the dye is diluted and the signal detected by the flow cytometer decreased by half after each cell division, which can allow the measurement of the number of cell divisions underwent by the cells[20].

NK cells were suspended so there were 10 millions cells per 1mL of FACS buffer. Then, 1µL of CFSE diluted 1:100 was added per 250000 cells, while vortexing the cells. Afterwards, the cells were incubated 10 minutes at 37°C, then washed two times with cold complete media. The CFSE stained NK cells were put in culture in the same conditions as none-CFSE stained NK cells. After three days in culture, NK cells were washed and analysed with a flow cytometer and supernatants were kept for further analysis[21].

3. Measurements of supernatants

3.1 Ig ELISA

Upon stimulation, B-cells can secrete different kinds of immunoglobulins. Using an immunoglobulin typing test from Invitrogen (Mouse Immunoglobulin Isotyping Kit, catalogue #99200), supernatants from B-cells in culture with different cytokines were analysed to qualify their antibody secretion.

The protocol used in these experiments is the one found in the kit[22]. Briefly, a flat bottom p96 well plate was coated with antibodies diluted in sterile PBS specific for IgG1, IgG2b, IgG2a, IgG3, IgA and IgM. The plate was incubated overnight at 4^0 C. On the next day, the plate was washed and blocked. After blocking, 50µL of cell supernatant is diluted in 550µL of assay diluent. 25µL of each dilution was added to each well. After a one hour incubation at room temperature, the plate was washed several times and an α-mouse-κ coupled to biotin was added. Horseradish peroxidise (HRP) conjugated to streptavidin was then added. After a short incubation and a couple of washes, stabilised chromogen Tetramethylbenzidine (TMB), HRP substrate, was

[20] From eBioscience website, http://www.ebioscience.com/cfse.htm.
[21] Quah, B. J. C., Parish, C. R. The Use of Carboxyfluorescein Diacetate Succinimidyl Ester (CFSE) to Monitor Lymphocyte Proliferation. *J. Vis. Exp.* (44), e2259, DOI: 10.3791/2259 (2010).
[22] From Invitrogen website, http://tools.invitrogen.com/content/sfs/manuals/ms_Ig_isotyping_man.pdf

added. After 10 minutes, the "Stop" reagent solution was added. The plate was then read on an ELISA (Biotek) plate reader at reading absorbance of 450 nm and reference absorbance of 650nm, in a 30 minutes timeframe after adding Stop Solution. Any readings above the background indicates a positive secretion of a specific antibody. Higher optical density (OD) readings indicate higher production of antibodies.

3.2 Cytokine measurements by Flow Cytomix

To identify the possible cytokines secreted by the cells cultured *in vitro*, a Mouse Th1/Th2/Th17/Th22 13plex Kit from eBiosciences was used. The protocol used is the one found in the kit[23]. Here is the rough outline.

Briefly, this kit uses beads covered with antibodies specific to one cytokine. In this experiment, there were 13 beads (of two different sizes) for 13 different cytokines. All beads were added to 50μL of cell supernatants. Cytokines will specifically bind to the antibody-covered beds. Then, 13 different biotin-conjugated antibodies are added to the mixture, to the cytokines that bind to the beads. Streptavidin-PE (fluorochrome) is added to supernatants, which will emit fluorescence when read on a flow cytometer. The stronger the PE intensity, the higher the cytokine concentration. Standard curves of the 13 cytokines will also be read, which will allow for precise measurements of the cytokines secreted by each cell in culture.

[23] Protocol from eBioscience website, http://www.bendermedsystems.org/bm_products/MAN/822FFRTU.pdf

4. Statistics

Unless otherwise stated, p-values were calculated using Mann-Whitney two tailed tests, with a confidence interval of 95%. GraphPad software was used to calculate p-values. Significance is achieved when p-value≤0.05.

< 0.001	Extremely significant	***
0.001 to 0.01	Very significant	**
0.01 to 0.05	Significant	*
>0.05	Not significant	N.S

Table 1 : Table of significance[24]

[24] From GraphPad Prism 5 Help section, "Extremely Significant".

Results

Objective 1: Identify the cell populations expressing IL-23R and IL-12R using the IL-23r-GFP reporter mouse and a monoclonal antibody, respectively

IL-12R and IL-23R expression is often thought to be found only in activated and/or differentiated cells. Finding IL-23R+ and IL-12R+ cells in naïve conditions could indicate which cells first respond upon IL-12 and IL-23 secretion.

Using naïve IL-23r-GFP reporter mice, it is possible to detect the presence cells expressing IL-23R at basal conditions. In Figure 8a, it is possible to see a small population of IL-23R in naïve animals, absent from IL-23r-GFP-Neg animals. All IL-23R+ cells are CD45.2+, demonstrating their hematopoietic origin. On average, around 0.15% of total splenocytes of naïve animals express IL-23R (Figure 17). Further, it was possible to characterize IL-12R expression using a monoclonal antibody against IL-12Rβ2. Here again, a small population of splenocytes (around 0.4%) were identified as IL-12R(Figure 8b). Those cells were also all CD45.2+ cells. On average, 0.25% of splenic cells expressed IL-12Rβ2 (Figure 17).

Finally, because IL-12R and IL-23R share the IL-12Rβ1 subunit, it was expected that both receptors would be expressed on the same cells. However, Figure 8c demonstrates that there is no co-localization between IL-23r and IL-12Rβ2.

The next step was to identify which lymphocyte subsets express IL-23R. In Figure 9, we can see that in comparison to IL-23r- cells, CD3+ T-cells are enriched in the IL-23R+ cells population. About 60% IL-23R+ CD3+ T-cells are TCRγδ T-cells and 40% of total IL-23R+ cells are TCRγδ T-cells. TCRγδ T-cells are actually very rare in total splenic cells, representing around 1% of total splenocytes. Around 5% of IL-23R+ cells are CD4+T-cells. It is important to notice there is a population of IL-

23R+CD4+CD3- cells. Those cells are therefore not T-cells. There also seems to be a population of CD3+CD4-CD8-TCRγδ- T-cells. This population can be deducted from the addition of the three subtypes of T-cells: 62.1% (CD3+ cells)-(47.4% (TCRγδ)+5.77%(CD4+) +0.52% (CD8+) is equal to 8.41%, so around 8% of IL-23r+ cells are CD3+CD4-CD8-TCRγδ- T-cells.

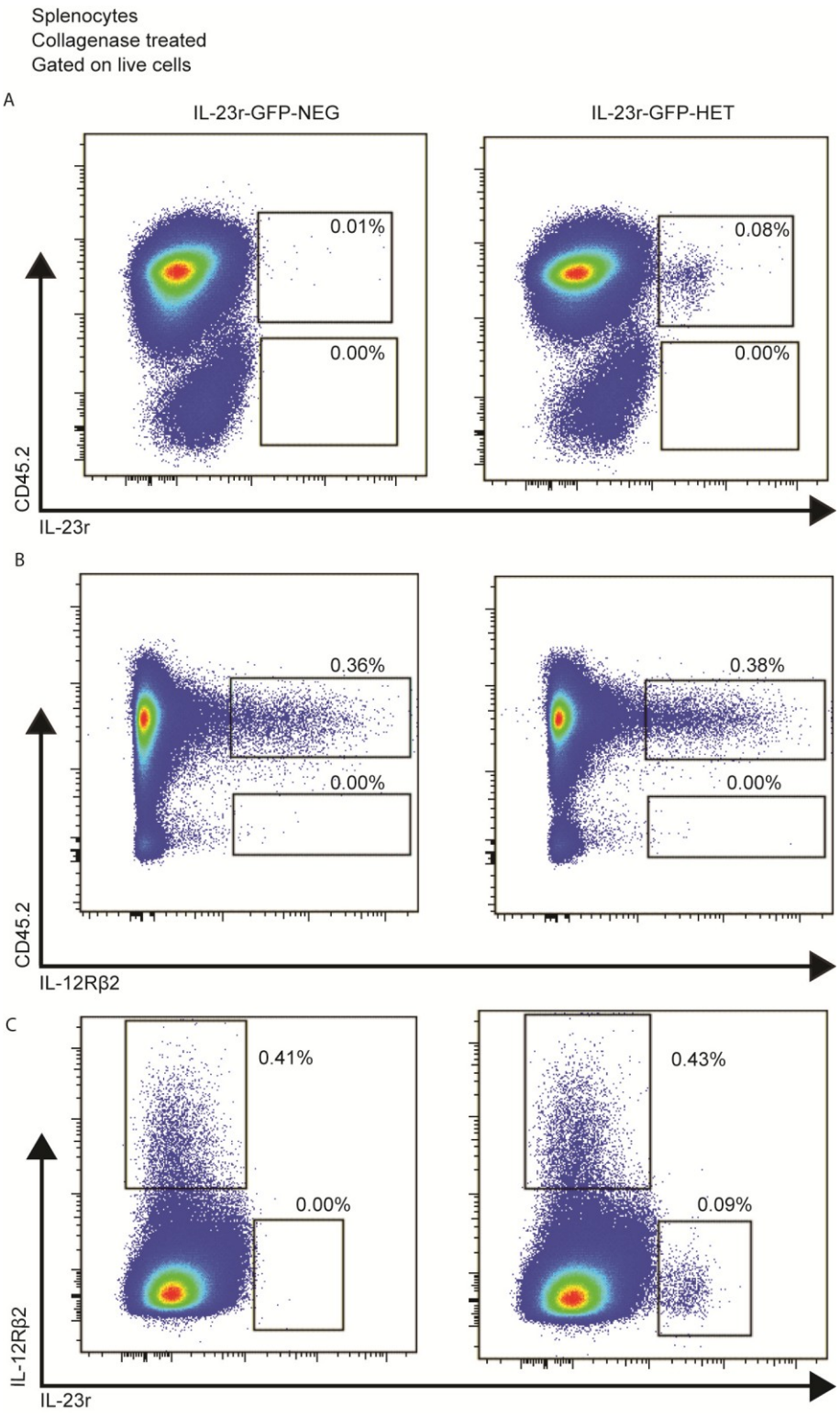


Figure 8: IL-23r and IL-12Rβ2 are of hematopoietic origin, are expressed in naïve mice and are not co-expressed

Total splenocytes staining from naïve IL-23r-GFP-Het or IL-23r-GFP-Neg mice. A) IL-23r expression B) IL-12Rβ2 expression C) IL-12Rβ2 and IL-23r expression.
Representative of 13 experiments.

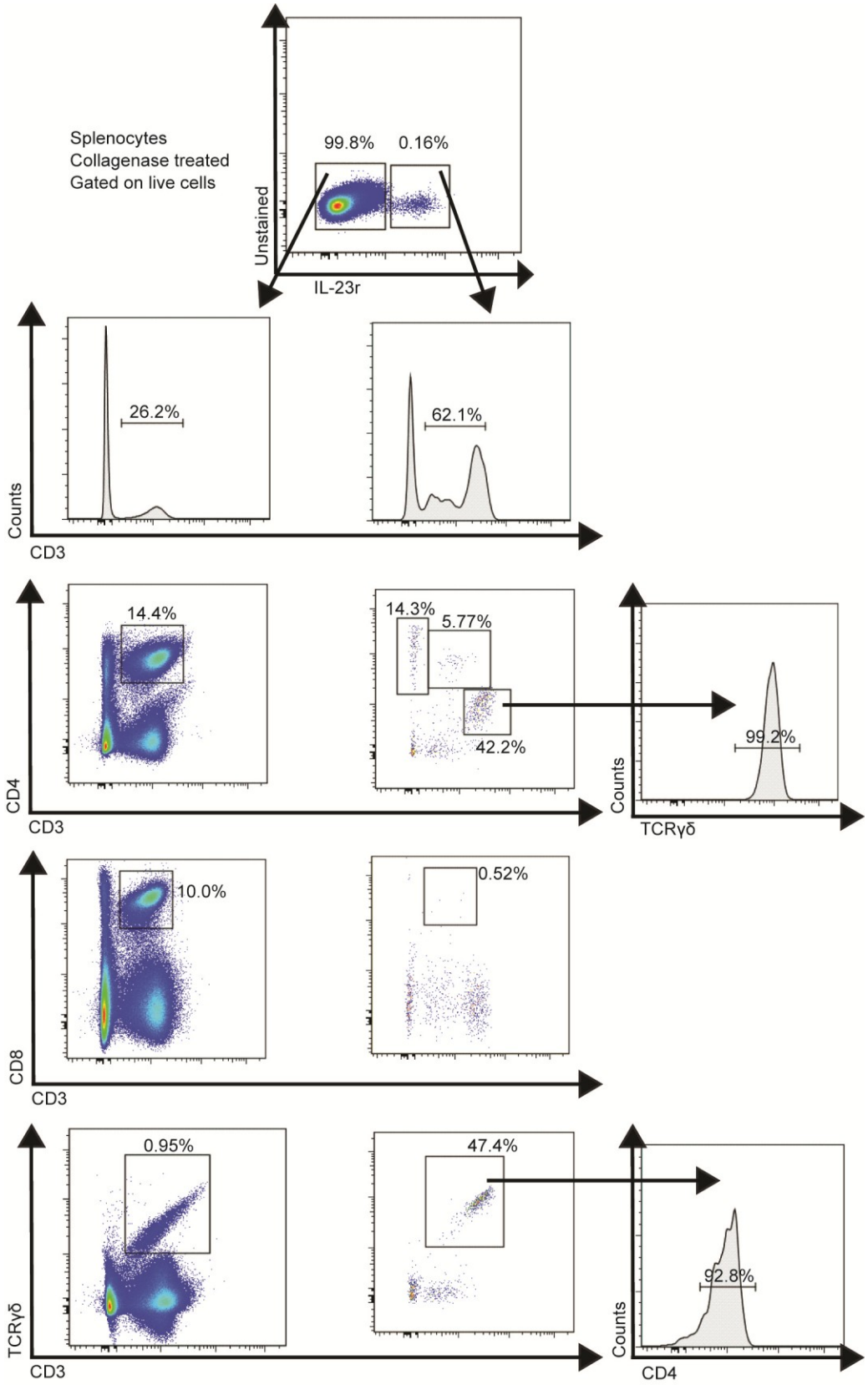


Figure 9: IL-23r is expressed by different T-cell receptor bearing T-cells

Expression of CD3, CD4, CD8 and TCRγδ in either IL-23R- or IL-23R+ splenocytes coming from IL-23R-GFP-Het naïve mice.

Representative of six experiments

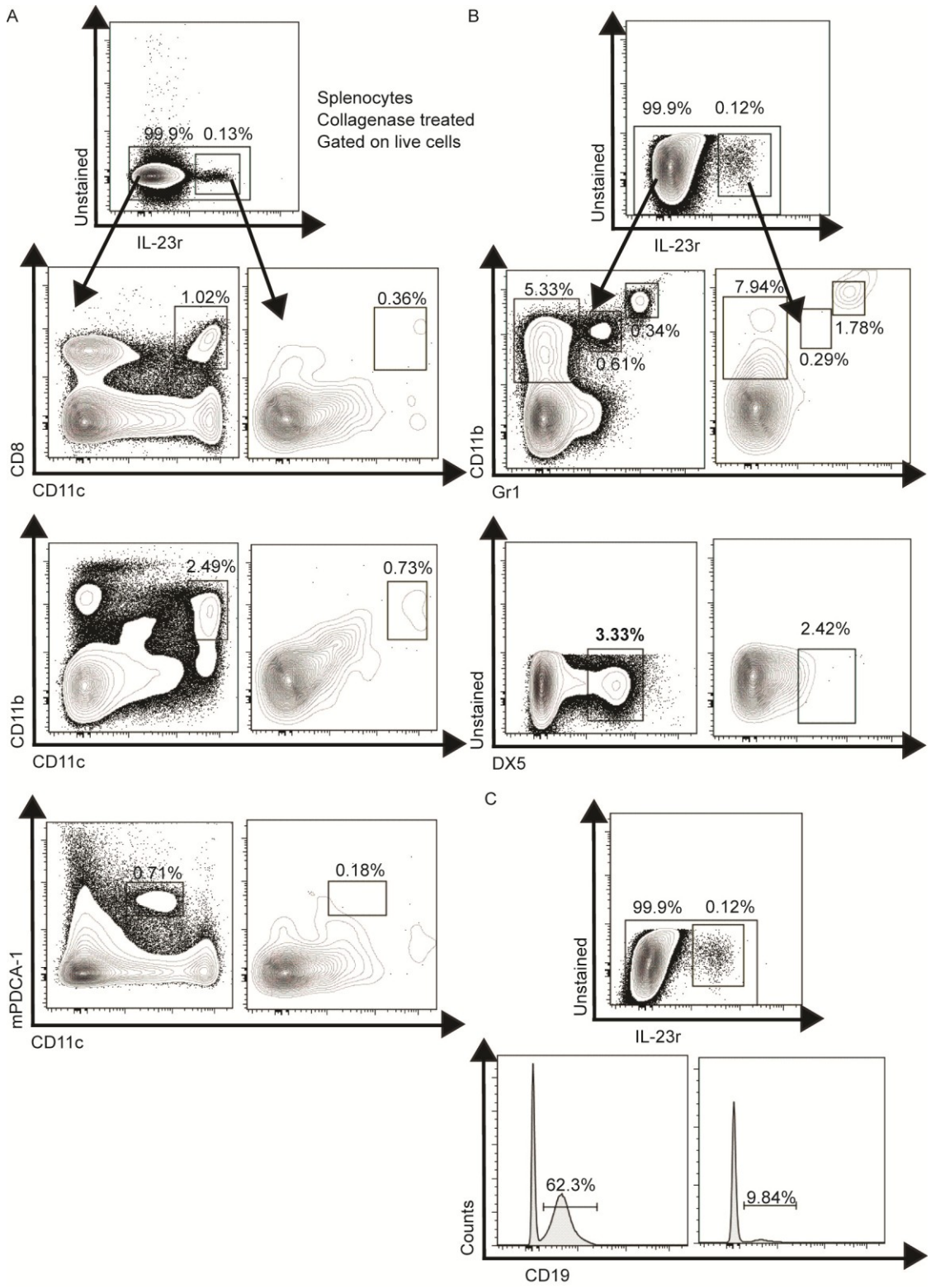


Figure 10 : IL-23R is rarely found in B-cells, DC, NK and monocytes

Gating on total or IL-23R+ cells, it is possible to identify proportions of A)lymphoid DC (lDC, CD8+CD11c+), myeloid DC (mDC, CD11b+CD11c+) or plasmacytoid DC (pDC, mPDCA-1+CD11c+), B)monocytes (Gr1+CD11b+) or NK cells (DX5+CD11b+) or C)B-cells (CD19+) amongst the two populations.
Representative of at least 5 experiments.

Around 60% of IL-23r+ cells were identified as T-cells. It was then necessary to phenotype the remaining 40% of cells. In Figure 10, stainings for B-cells, monocytes, NK cells and DCs were done to identify if those cell populations expressed IL-23R. In Figure 10a, it is possible to see that mDC, pDC and lDC are not the main cell subtypes expressing IL-23r. Added together, less than 1.5% of IL-23R+ were DC and most of them were mDC. When looking at monocytes and NK cells in Figure 10b, IL-23R+ cells were under-represented in NK cells and CD11b+Gr1low, while there seemed to be an enrichment in CD11bGr1high, a certain type of neutrophils. Finally, the last cell subtype analysed was B-cells. Around 10% of IL-23R+ cells were B-cells, which definitely represent an important subtype. However, when the number of B-cells, NK cells, DCs and monocytes are all added together, we find there is around 15% of IL-23R+ cells identified. There is still around 25% of IL23R+ cells left to identify. Compilation of IL-23r+ DC, monocytes, NK and B-cells can be seen in the top left panel of Figure 17b.

Cells of the innate immune system, such as monocytes, DCs, and NK cells are found in increased numbers in RagKO animals. RagKO animals are deprived of T-cells and B-cells. If the low percentages of monocytes, DC and NK cells expressing IL-23R are important in WT, RagKO animals should have increased numbers of those three cell types expressing IL-23R.

IL-23r-GFP-Het animals were bred onto RagKO background, which resulted in RagKO-IL-23r-GFP-Het animals. Using those animals, it was possible to identify a population of IL-23r+ cells in the spleens of naïve RagKO-IL-23r-GFP-Het animals. Figure 11a shows that IL-23r+ cells are found in increased proportion in RagKO IL-23r-GFP-Het animals when compared to RagKO animals. Those cells are once again all CD45.2+ and are therefore of hematopoietic lineage. However, it was surprising to see that IL-12Rβ2+ cells were found in decreased numbers in RagKO animals when compared to WT animals. In Figure 17a, a compilation of IL-23r+ and IL-12Rβ2+ cells shows that there is a significant increase in IL-23r+ cells in RagKO mice (p-value<0.0001), while IL-12Rβ2+ are significantly decreased in RagKO mice (p-value<0.0001). There are more IL-23r+ cells in the spleen of RagKO animals, so the increase cannot be caused by T-cells and B-cells, which are absent from RagKO

animals (Figure 11d). Figure 9c demonstrates again that even though IL-23R+ cells are in higher proportion and IL-12Rβ2+ cells in lower proportion than in WT animals, they are still not found at the surface of the same cells.

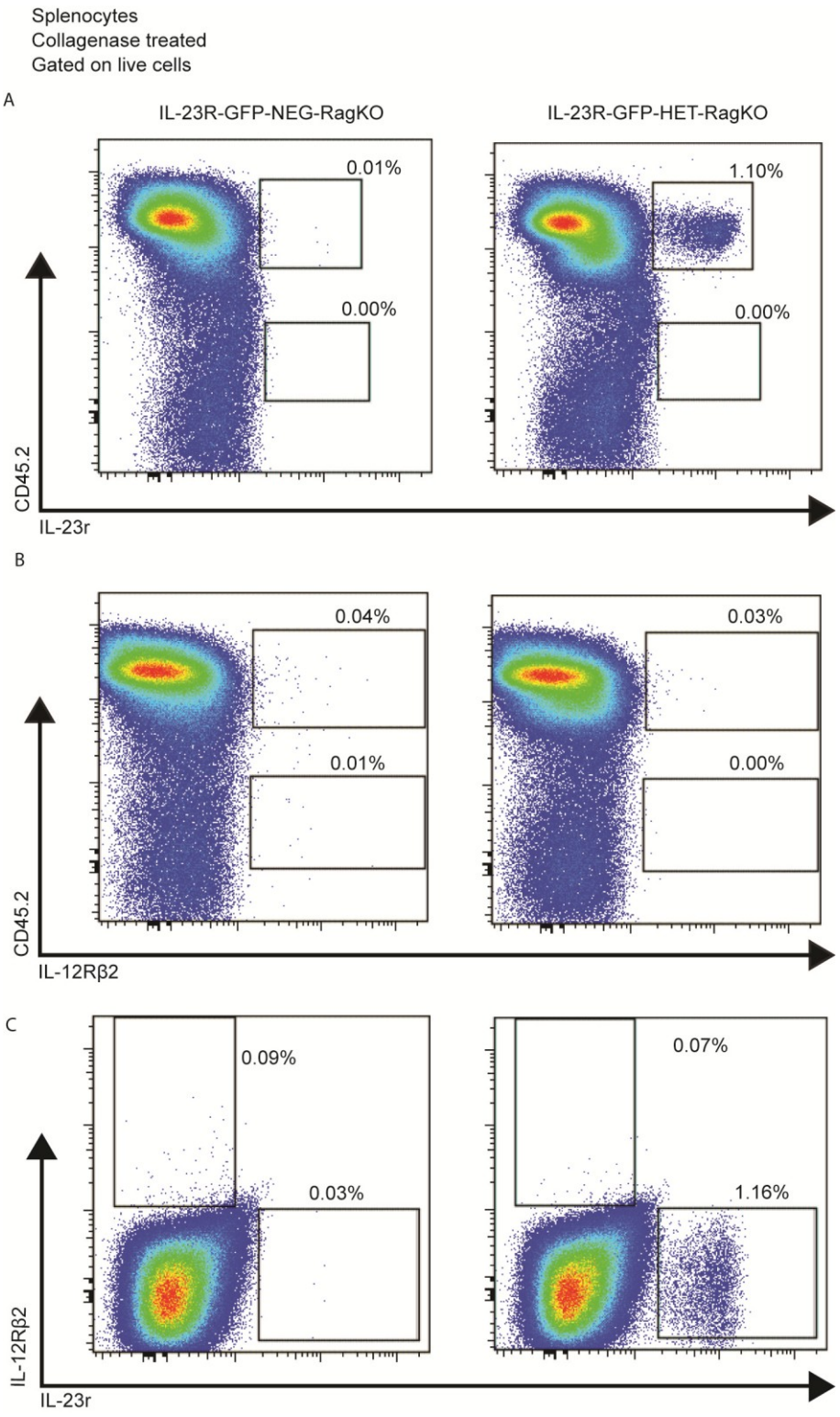


Figure 11: IL-23r is found in increased proportion in RagKO mice despite a lack of T-cells and B-cells, while IL-12Rβ2 is found in very low proportion

Total splenocytes staining of RagKO-IL-23r-GFP-Het or RagKO-IL-23r-GFP-Neg in naïve mice. A) IL-23r expression B) IL-12Rβ2 expression C) IL-12Rβ2 and IL-23r expression. Representative of 10 experiments.

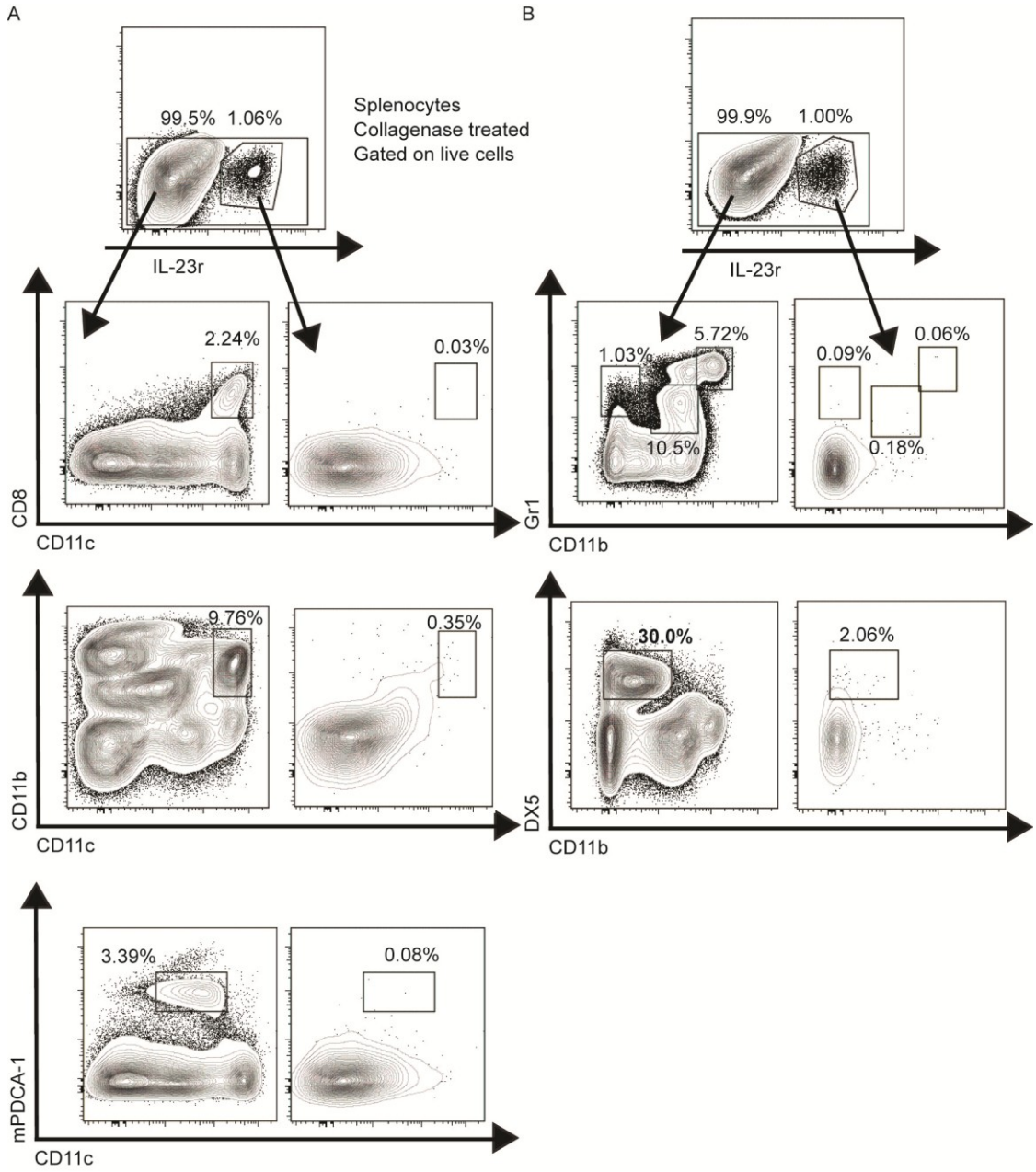


Figure 12: DC, monocytes and NK are not the major cell types expressing IL-23R in RagKO animals

Gating on total or IL-23R+ cells, it is possible to identify proportions of A)lymphoid DC (lDC, CD8+CD11c+), myeloid DC (mDC, CD11b+CD11c+) or plasmacytoid DC (pDC, mPDCA-1+CD11c+), B)monocytes (Gr1+CD11b+) or NK cells (DX5+CD11b+).

Representative of at least 5 experiments.

The next step was then to identify IL-23r+ cells in RagKO-IL-23r-GFP-Het naïve mice. In Figure 12, stainings to identify DCs, monocytes and NK cells were done on total splenocytes from RagKO-IL-23R-Het-GFP in order to identify IL-23R expression in RagKO mice. Those IL-23r+ cells could not be B-cells or T-cells, as they are absent from RagKO mice. In Figure 12a, it is clear that most IL-23R+ are not DCs, where less than 0.50% of IL-23R+ cells are DCs. There are actually less IL-23R+DC in RagKO animals than in WT animals. DCs represent at least 15% of IL-23R- splenocytes in RagKO animals, which is much higher than the proportion of DCs in WT mice. In Figure 12b, it is also obvious that the bulk of IL-23R+ cells in RagKO animals are neither monocytes (0.2%) nor NK cells (2%).

DCs, NK cells and monocytes represent the vast majority of splenocytes in RagKO, but only 3% of IL-23R+ could be accounted for using those three cell types. In both WT and RagKO animals, there was a population of IL-23R+ cells that could not be identified using the different antibody cocktails that identified NK cells, DC and monocytes. To verify which cell subtypes they could be, a Lineage(Lin) stain was made.

In both IL-23r-GFP-Het and RagKO-IL-23r-GFP-Het mice, there was a population of IL-23R+Lin(-). In Figure 13, all Lin(-) cells were c-kit. Those cells were furthermore characterized as four different cell populations, depending on the CD90.2 and Sca-1 expression. In Figure 17, it is possible to see that in IL-23r-GFP-Het, around 25% of IL-23R+ cells are c-kit+. Amongst those IL-23R+c-kit+ cells, more than 80% express CD90.2. In RagKO-IL-23r-GFP-Het mice, virtually all IL-23R+ cells express c-kit. Here again, 80% of IL-23R+c-kit+ cells are CD90.2+. In IL-23r-GFP-Het mice, the 25% of IL-23r+ cells that could not be analysed are therefore c-kit+, where 80% of c-kit+ cells are also CD90.2+. In RagKO-IL-23r-GFP-Het, most IL-23r+ cells are c-kit+ cells and 80% of IL-23R+c-kit+ cells are CD90.2+. Lin(-)CD90.2+IL-23r+ cells can be called Lti-like cells, so this terminology will be used in this thesis from here on[101].

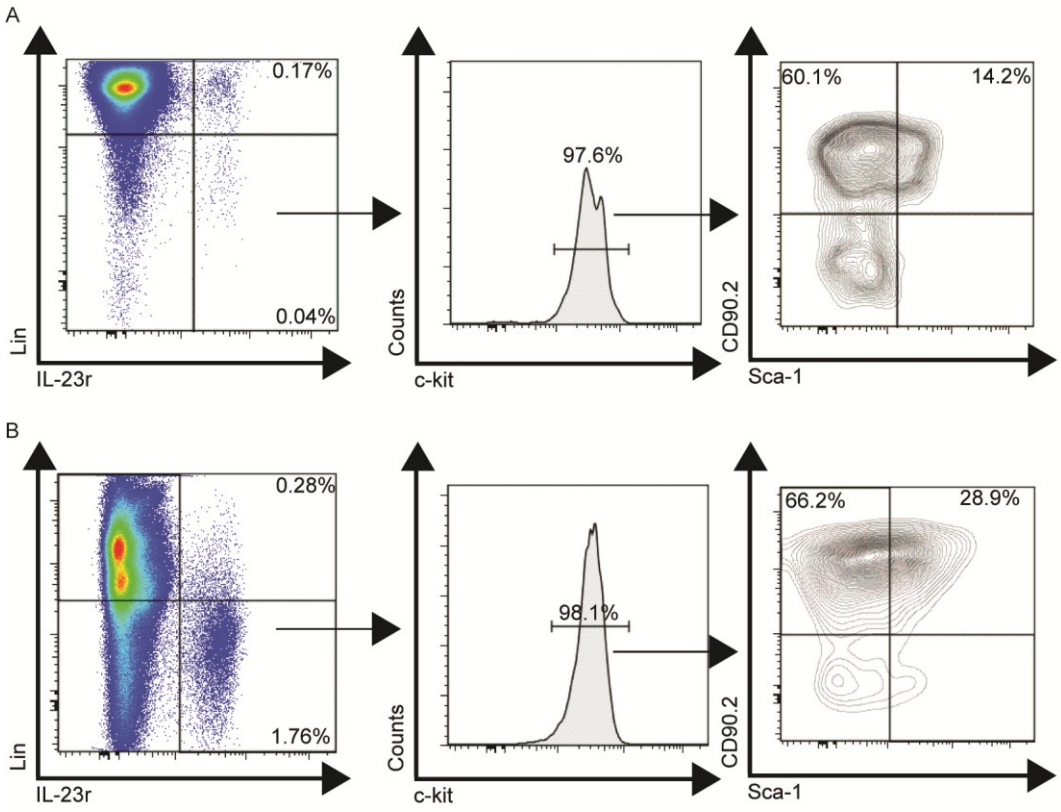


Figure 13: A Lin(-) c-kit+ cell population express IL-23R and can be found both in WT and RagKO mice

IL-23R+ Lin(-) splenocytes from A)WT and B)RagKO naïve mice all express c-kit. Those c-kit+ cells can be further characterized into CD90.2+ and Sca-1+ positive populations. Representative of two experiments.

Upon diverse experiments, it was observed that there were many variations of the proportion of IL-23R+ cells in total splenocytes of naïve cells. It was hypothesized that the collagenase treatment was influencing IL-23r expression. Another experiment was then setup to test this hypothesis. A spleen from RagKO-IL-23r-GFP-Het was cut in half. One half was treated with collagenase and the other was not. With collagenase treatment, the proportion of IL-23R+ went from 0.60% to more than 1.5%, as can be seen in Figure 14a.

Collagenase treatment was not changing the expression of IL-23r+ cells, but treating with collagenase increased the proportion and numbers of IL-23r+ cells recovered from the spleens, increasing IL-23r+ cell yield. IL-23R+ cells from collagenase treated and untreated spleens were characterized. They were still all expressing c-kit and 80% of them expressed CD90.2. In Figure 14b, the total proportion of c-kit+CD90.2+ cells was diminished overall, whether or not they expressed IL-23R. Collagenase is an enzyme that disrupts the extracellular matrix in organs, r

Those c-kit+ CD90.2+ cells are also found in spleens of WT animals. Without the collagenase treatment, they are extremely rare. They also triple in number upon treatment with collagenase, as seen in RagKO animals.

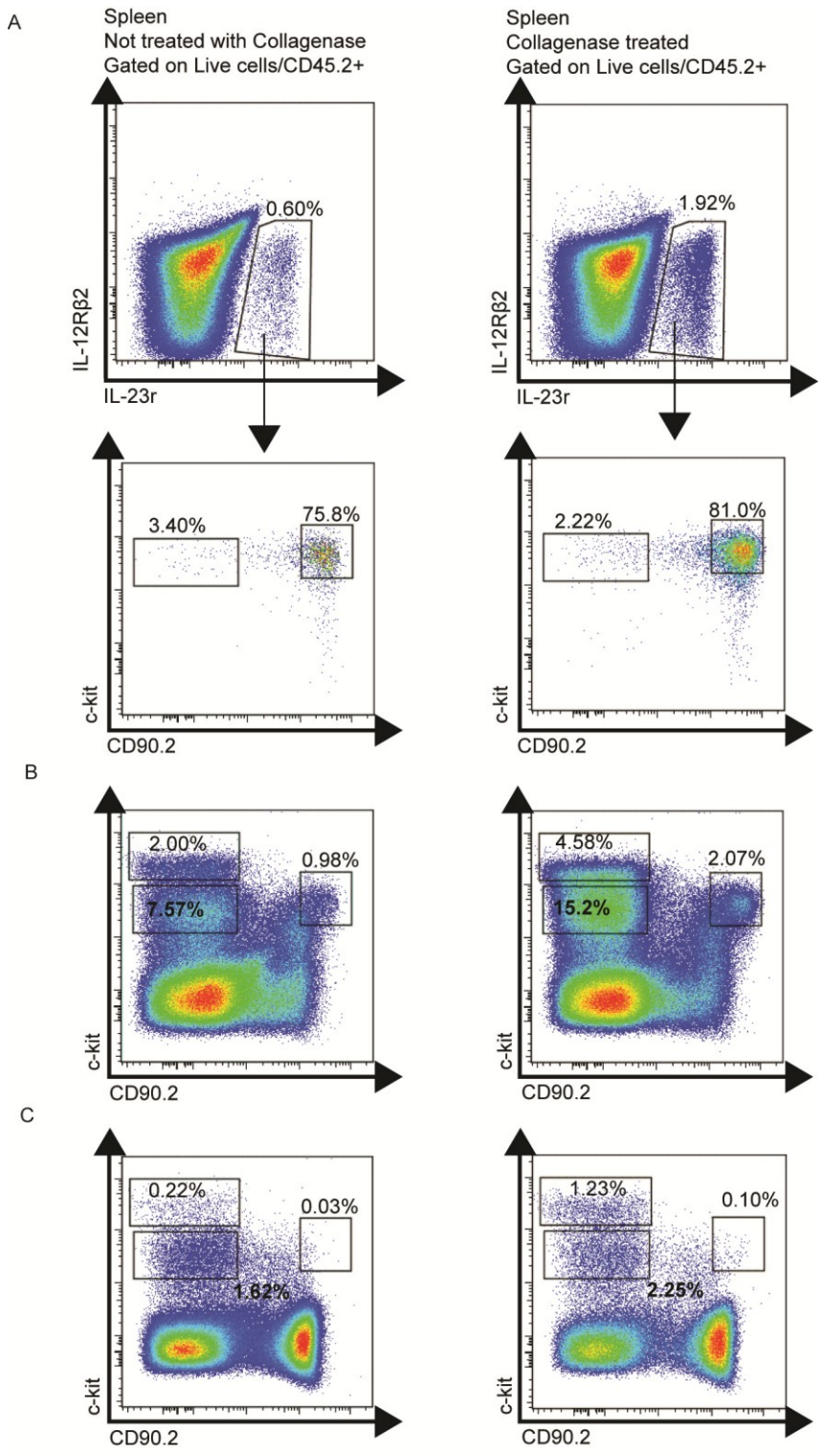


Figure 14 : Lti-like cells are dependent upon collagenase treatment

A) and B): Spleen from IL-23r-GFP-Het RagKO; left panel is not treated with collagenase and right panel is the half spleen treated with collagenase. B) Proportion of c-kit+ CD90.2+ and c-kit+ cells in total spleen, treated or not with collagenase. C) Spleen from WT mice, stained with c-kit+ CD90.2+. Left, collagenase untreated, right collagenase treated. A) and B): Representative of two experiments

After IL-23R+ cells were characterized, it was important to identify the cell populations expressing IL-12Rβ2. It was already noticed that IL-12Rβ2 cells were almost absent in RagKO animals. In Figure 15a, which shows WT animals, the majority of IL-12Rβ2+ cells expressed both CD19 and B220, which make them IL-12Rβ2+ B-cells. Figure 15b reinforces the idea that IL-12Rβ2+ are B-cells, as RagKO animals are deprived of both B-cells and IL-12Rβ2+ cells. This result was consistent. In Figure 17a, right panel, IL-12Rβ2+ cells are less than 0.02% of total cells in RagKO animals and are significantly decreased when compared to WT animals (p-value<0.001).

Because only a few B-cells expressed IL-12Rβ2, they could represent a different subtype of B-cells. Upon characterization of their cell surface markers, it was found that there were no variations in the expression of CD21, CD23, IgD, IgM, GL-7 and CD138 between IL-12Rβ2+ and IL-12Rβ2- B-cells (Figure 15b and 15c). However, one surface marker was enriched in IL-12Rβ2+ B-cells: CD43(Figure 16). CD43 is usually expressed by less than 2% of B-cells and is not expressed by mature B-cells[102]. However, around 40% of IL-12Rβ2+ B-cells expressed CD43. CD43+ B-cells are B1-B-cells, so 40% of IL-12Rβ2+ B-cells could be B1-B-cells.

To conclude this section, IL-23R+ cells are mostly comprised of T-cells, B-cells and c-kit+CD90.2+ cells. In RagKO animals, all IL-23R+ splenocytes are actually Lin(-) cells, expressing c-kit and CD90.2. IL-12Rβ2+ cells are absent from RagKO since it is mostly expressed by B-cells. However, usual B-cell markers do not vary between IL-12Rβ2+ and IL-12Rβ2- B-cells. The only different marker identified so far to distinguish the two populations is CD43. In Figure 17, the compilation of each cell population can be found according to gating strategies in previous figures. Another very important fact is that IL-23r and IL-12Rβ2+ are not expressed on the same cells. Furthermore, they are not only not co-expressed together; they are found in totally different cell subtypes.

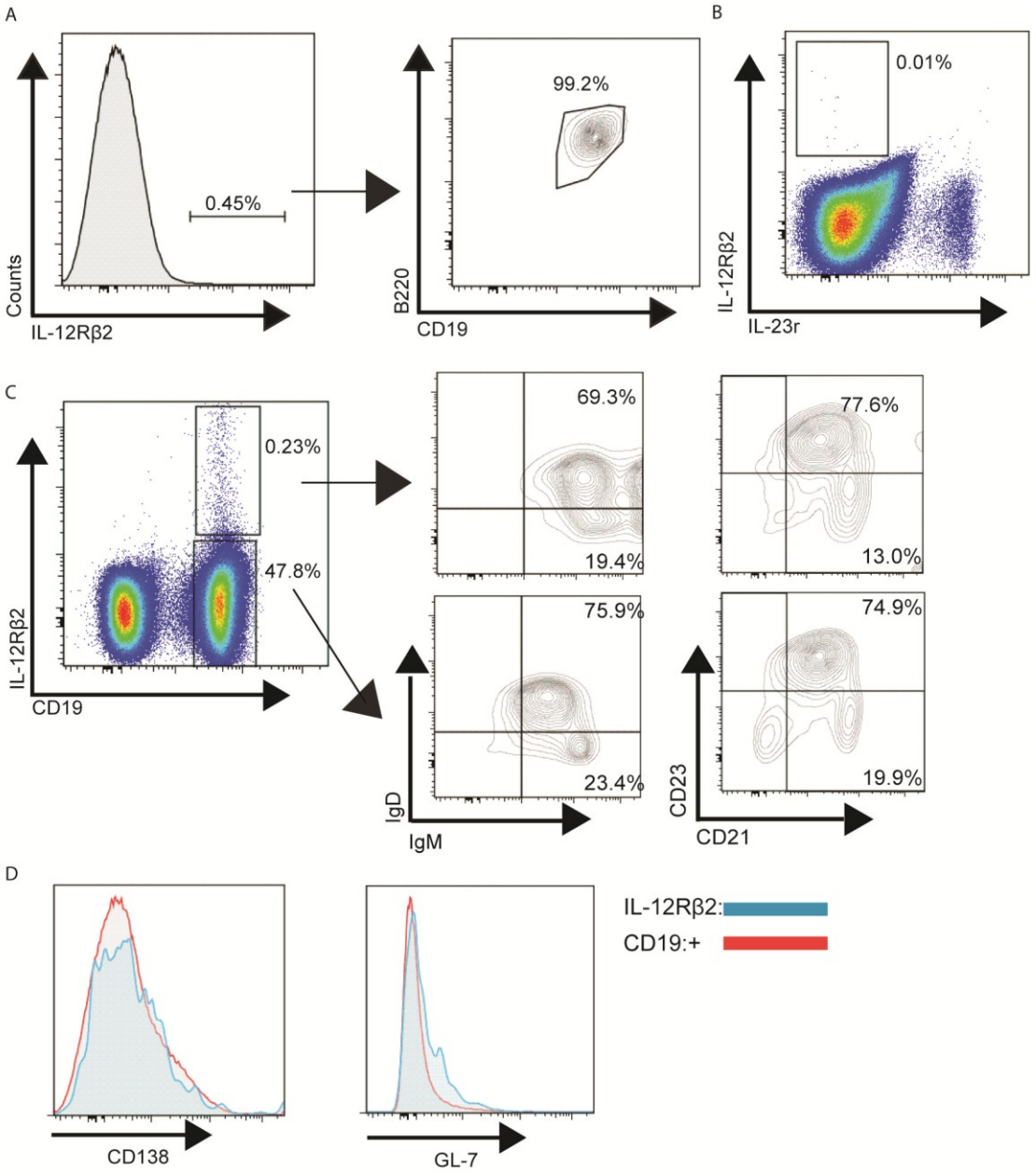


Figure 15: IL-12Rβ2 is expressed by B-cells in naïve mice

Total splenocytes from naïve mice. A) Majority of IL-12Rβ2+ are CD19+B220+ and are B-cells. B) IL-12Rβ2+ cells in RagKO mice. C) and D)Characterization of IL-12Rβ2+ and IL-12Rβ2- B-cells. A and B: representative of 10 experiments. C and D: representative of two experiments.

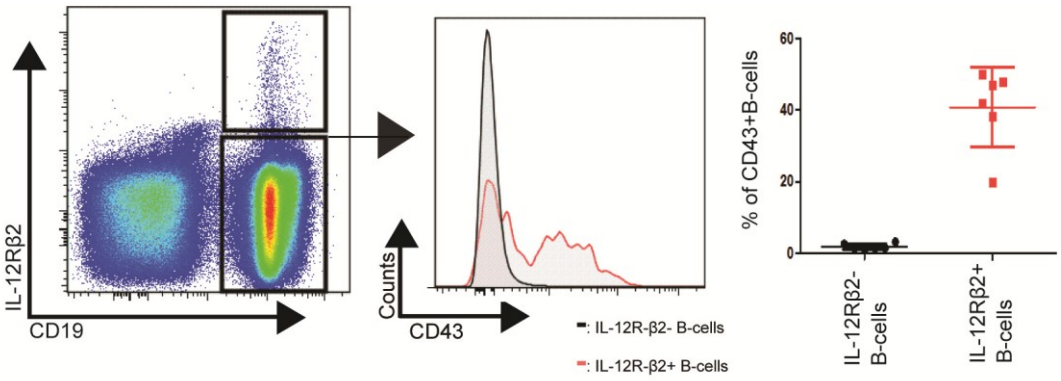


Figure 16: CD43+B-cells are enriched in the IL-12Rβ2+ B-cells population

Total splenocytes from naïve mice. CD43 expression by IL-12Rβ2+ and IL-12Rβ2- B-cells. Representative of 3 experiements; compilation of CD43 expression by IL-12Rβ2+ and IL-12Rβ2- B-cells.

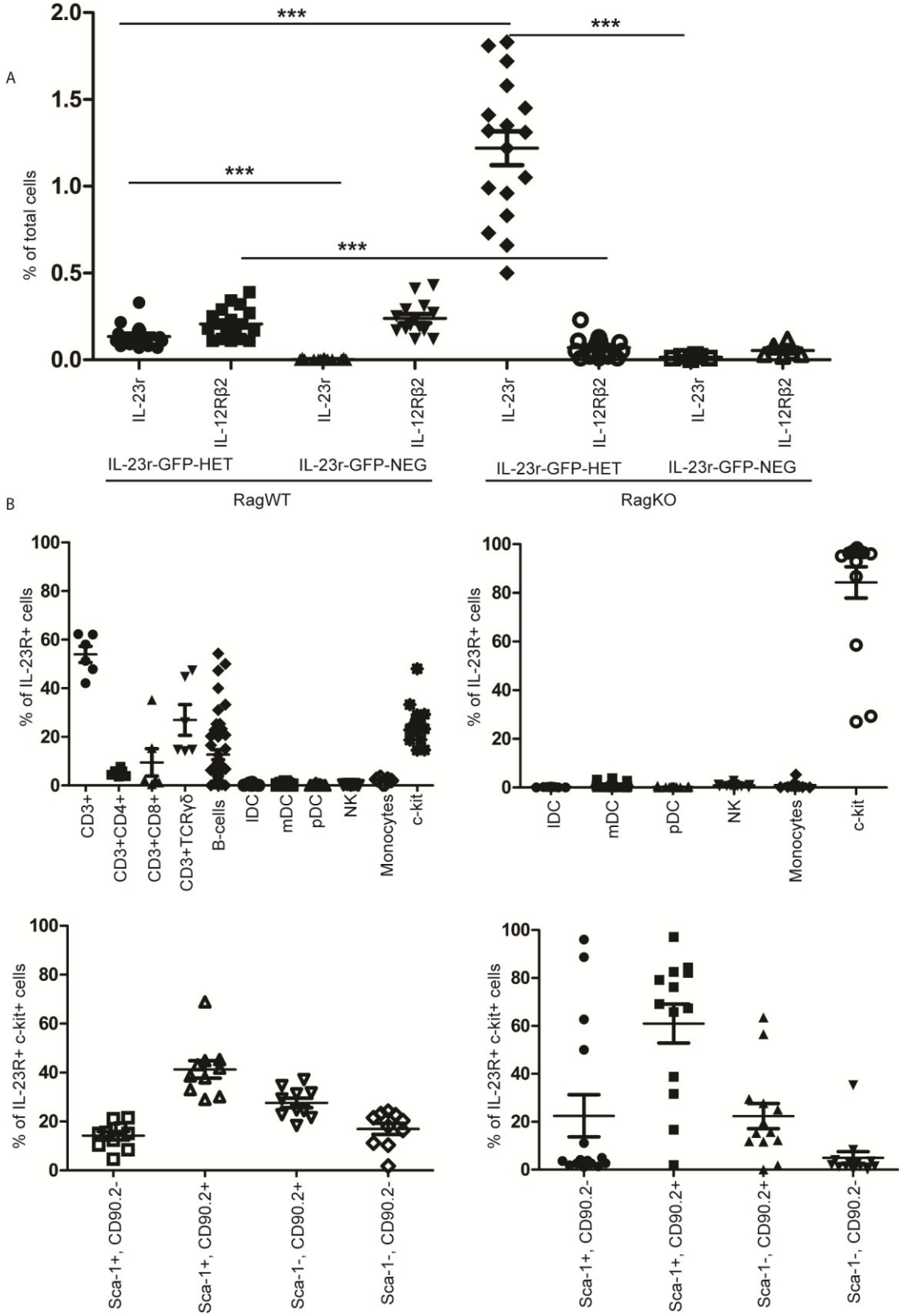


Figure 17: Compilations of IL-23R expressing cells in the spleen

A) Frequency of IL-23r+ and IL-12Rβ2+ cells in total splenocytes in WT(IL-23r-GFP-Het and Neg) and RagKO(IL-23r-GFP-Het and Neg) animals. B) Distribution of IL-23r+ cells in WT and Rag KO IL-23r-GFP-Het animals (top panel). Bottom panel: distribution of c-kit+ cells according to their CD90.2 and Sca-1 expression.

Objective 2: Characterize the cell populations expressing IL-23R and IL-12R in different organs, especially the gut

IL-23R has not only been associated with colitis and gut inflammation, but also with other organ specific inflammation. Therefore, it was important to characterise IL-23R lymphocytes expression throughout the different organs. Also, many other organs contain large (or small) populations of B-cells; examining IL-12R expression through the use of αIL-12Rβ2 monoclonal antibodies in other organs could help to understand if the distribution of IL-12R+ cells in other organs is restricted to B-cells.

The first organ examined was the thymus. T-cells undergoing negative and positive selections undergo various phases of activation and differentiation, which could possibly trigger IL-23R upregulation. In all the steady state IL-23r-GFP-Het animals studied, the proportion of IL-23r+ cells was always very low, below 0.05% of the total cell expression (Figure 18). Thorough characterization of IL-23r+ and IL-12Rβ2+ cells in the thymus was rendered difficult by the very rare expression of the two receptors. Only a third of IL-12Rβ2+ cells in the thymus are CD19+; however there are a very low number of cells that are IL-12Rβ2+ (37, data not shown). It is interesting that cells found in the thymus undergo many different stages and some activation through signaling in their TCR.

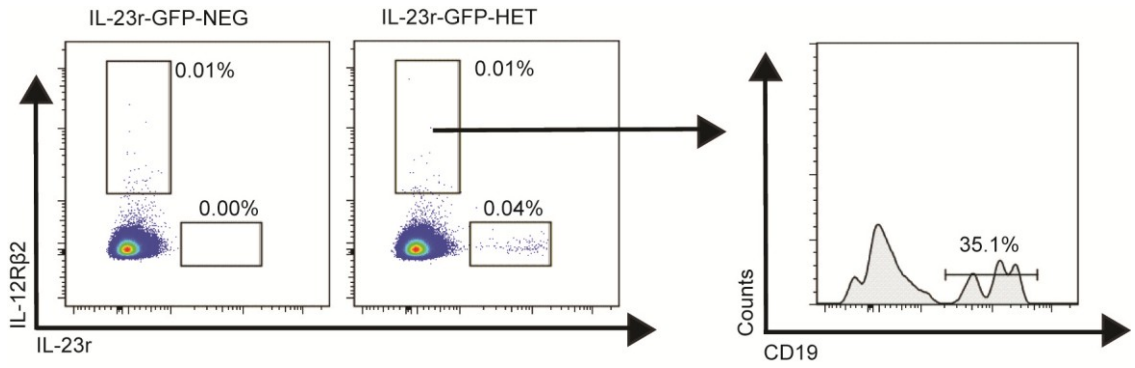


Figure 18: IL-23R and IL-12Rβ2 expressing cells are found in low numbers in the thymus

Thymocytes of IL-23r-GFP-Het naïve mice, stained for IL-12Rβ2. Collagenase treated. Representative of two experiments.

Another organ from which lymphocyte expression of IL-23R and IL-12Rβ2 was examined is the bone barrow. The bone marrow is one of the organs containing the highest amount of progenitor cells; if the IL-23r+ Lti-like cells identified in the other organs are progenitor cells, they should be found in higher numbers in the bone marrow. In Figure 19a, IL-23r+ cells are not found in increased numbers in the bone marrow when compared to the spleen (0.08% in this figure when compared to the 0.15% average in the spleen). IL-23r+ cells were diminished in the BM when compared to the spleen (Figure 29a, p-value<0.05). The BM is also filled with B-cells (Figure 20b shows that 55% of IL-23R- cells in the BM are B-cells), so it is a good organ to analyse IL-12Rβ2 expression. Figure 19a shows that IL-12Rβ2+ is expressed in the same proportion of cells as in the spleen. Three-quarters of IL-12Rβ2+ were B-cells, which is a lower proportion than in the spleen.

BM also contains T-cells. In figure 19b, T-cell analysis reveals that around 75% of IL-23r+ cells in the BM expressed CD3+. Almost no IL-23r+ cells expressed CD3 and CD4 at the same time; around 13% expressed TCRγδ and CD3, and 7% were CD8+ T-cells. However, only 20% of cells expressing CD3 expressed another co-marker of T-cells, such as CD8 and TCRγδ. Those IL-23R+CD3+ cells could therefore be CD3+ cells, such as double negative T-cells and NK T-cells. There is also a small proportion of CD4+CD3- IL-23r+ cells, as seen in Figure 19b.

It was then important to finish characterising IL-23R+ cells. Amongst the three different types of DC (lymphoid, myeloid and plasmacytoid), only pDC are found in high numbers in the BM (Figure 20b). But even amongst the most abundant group of DC in the BM, no DC in the bone marrow actually expresses IL-23R. c-kit+ cells were not overall higher in proportion of the total cells in the bone marrow. A small proportion of IL-23R+ cells expressed c-kit, but none of them expressed Sca-1 and only 14% expressed CD90.2+. CD90.2 expression by IL-23r+ cells in the BM was lower than the expression of CD90.2 by IL-23R+c-kit+CD90.2+ cells in the spleen.

One of the last cell types that could express IL-23r in the BM was B-cells. 55% of the total cells in the BM are B-cells (Figure 20c). Interestingly, the highest proportion of IL-23R+ B-cells was found in the BM, where more than 30% of IL-23r+

were expressing CD19. Overall, the BM did not contain a higher proportion of IL-23R+ cells than other organs. BM IL-23r+ cells did not seem to be of the same nature as IL-23R+ cells found in the spleen (and other organs, which can be seen in the next figures). They did not express CD90.2 at the same levels are IL-23r+ found in other organs, but they also did not express Sca-1 in any proportion.

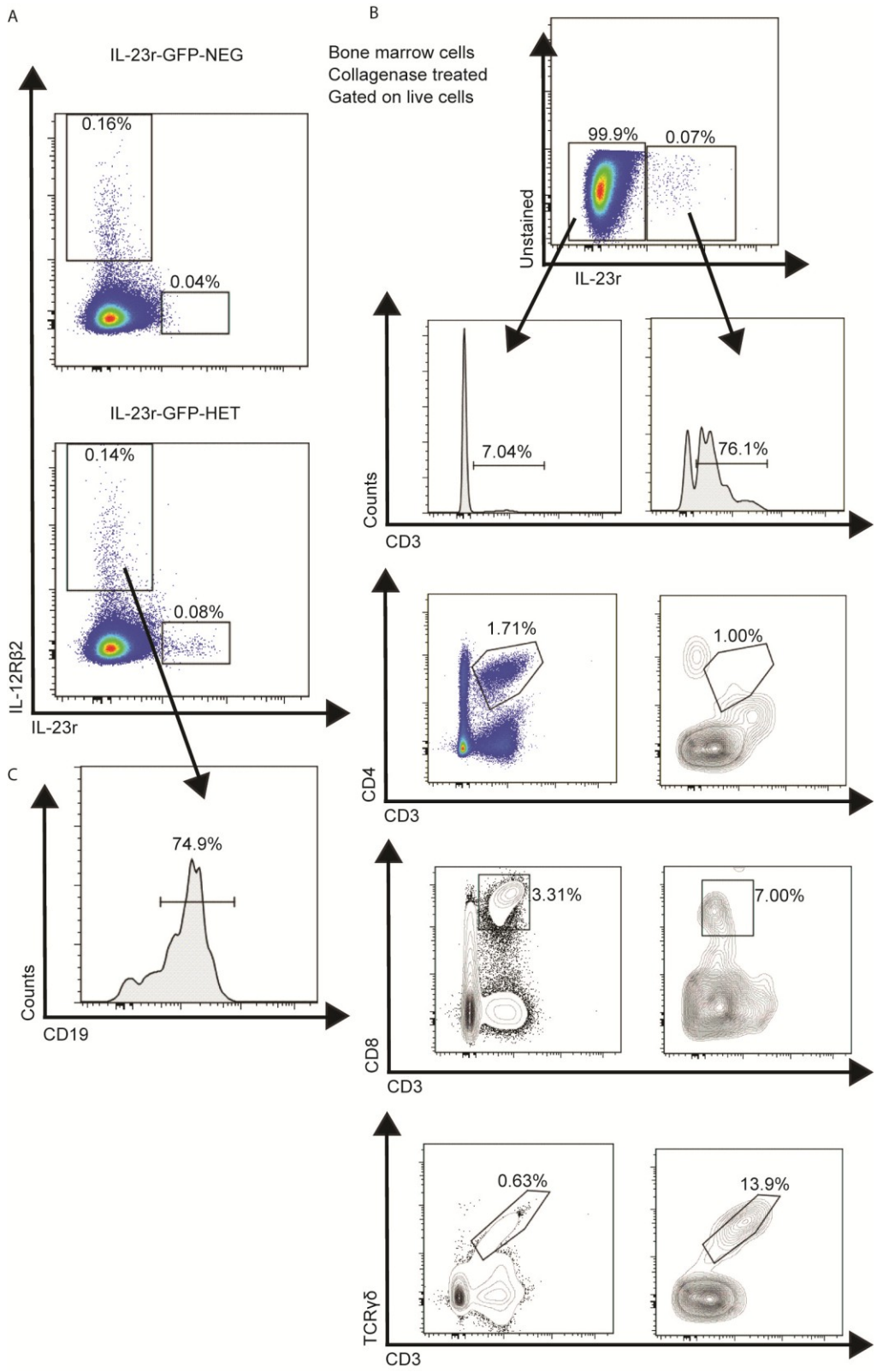


Figure 19: IL-23R and IL-12Rβ2 expressing cells are found in low numbers in the bone marrow and are not co-expressed on the same cells

A) IL-23R+ and IL-12Rβ2+ cells can be found in the bone marrow of naïve IL-23r-GFP-Het animals. B) T-cells staining on IL-23R+ and IL-23R- cells C) CD19 expression on IL-12Rβ2+ cells. Representative of three experiments.

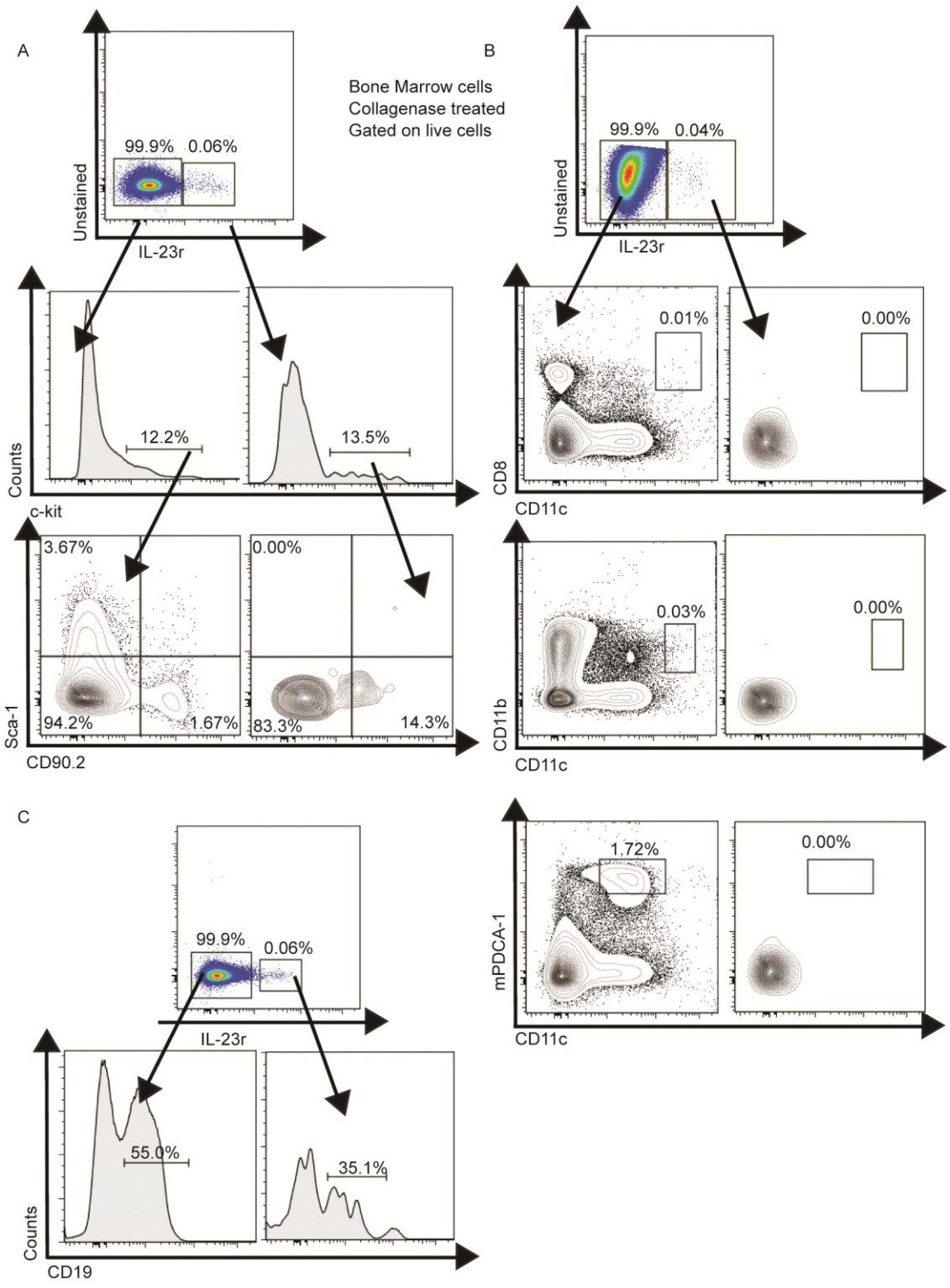


Figure 20: Lti-like are rare in bone marrow and are rare expressors of Il-23R, while a strong proportion of IL-23R+ cells are B-cells

IL-23r-GFP-Het mouse bone marrow cell staining: A) Lti-like staining in the bone marrow B)DC staining C)B-cells staining A) representative of three experiments B) representative of one experiment C) representative of three experiments

Many cells found in the LNs (lymph nodes, secondary lymphoid organs) are activated. Activation of cells could lead to the upregulation of IL-23R in cells, so IL-23r+ cells could be found in higher proportion in LNs than in the spleen. LNs also contains large amounts of T-cells and B-cells which could be activated and express IL-12R. The co-expression between the two receptors could also be observed in the LNs.

Figure 21a demonstrates that a small proportion of the total cells found in the LN of IL-23r-GFP-Het mice are IL-23r+. Around 0.3% of cells in the pooled LNs of a naïve animals expressed IL-23R+, which is slightly higher than the proportion found in the spleen. Figure 29a shows that the average of IL-23r+ cells is significantly higher in LNs than in the spleen. The proportion of IL-12Rβ2+ cells in the LN is similar to that found in the spleen (Figure 21a). Those IL-12Rβ2+ cells are largely B-cells (Figure 21c), similar to what we found in the spleen. Similarly , IL-23r and IL-12Rβ2+ are not co-expressed at the surface of the same cells.

Half of the cells found in the LNs are T-cells; however, 90% of the IL-23r+ cells in the LNs express CD3 and are therefore T-cells, demonstrating a specific enrichment of T-cells in the IL-23r+ population. The TCRγδ population, which is less than 1% of the total LN cells, represents 50% of the IL-23r+ population. One third of IL-23r+ T-cells are also CD4+, while 10% are CD8+, one of the highest proportion seen for IL-23r+ CD8+ T-cells seen so far. Unlike the BM, it does not seem like there is another T-cells population, such as the DN T-cells, that could express IL-23r in the LNs.

T-cells represent around 90% of the IL-23r+ cells in the LNs. Another 10% of cells express c-kit (Figure 22). Of this IL-23r+ c-kit+ population, 80% express CD90.2 only, while the second largest population is CD90.2-Sca-1-. The IL-23r+ Lti-like cells that can be found in the spleen can also be found in the LNs. However, the proportion of Lti-like cells in the LNs (around 10%) is lower than the proportion in the spleen (around 25%).

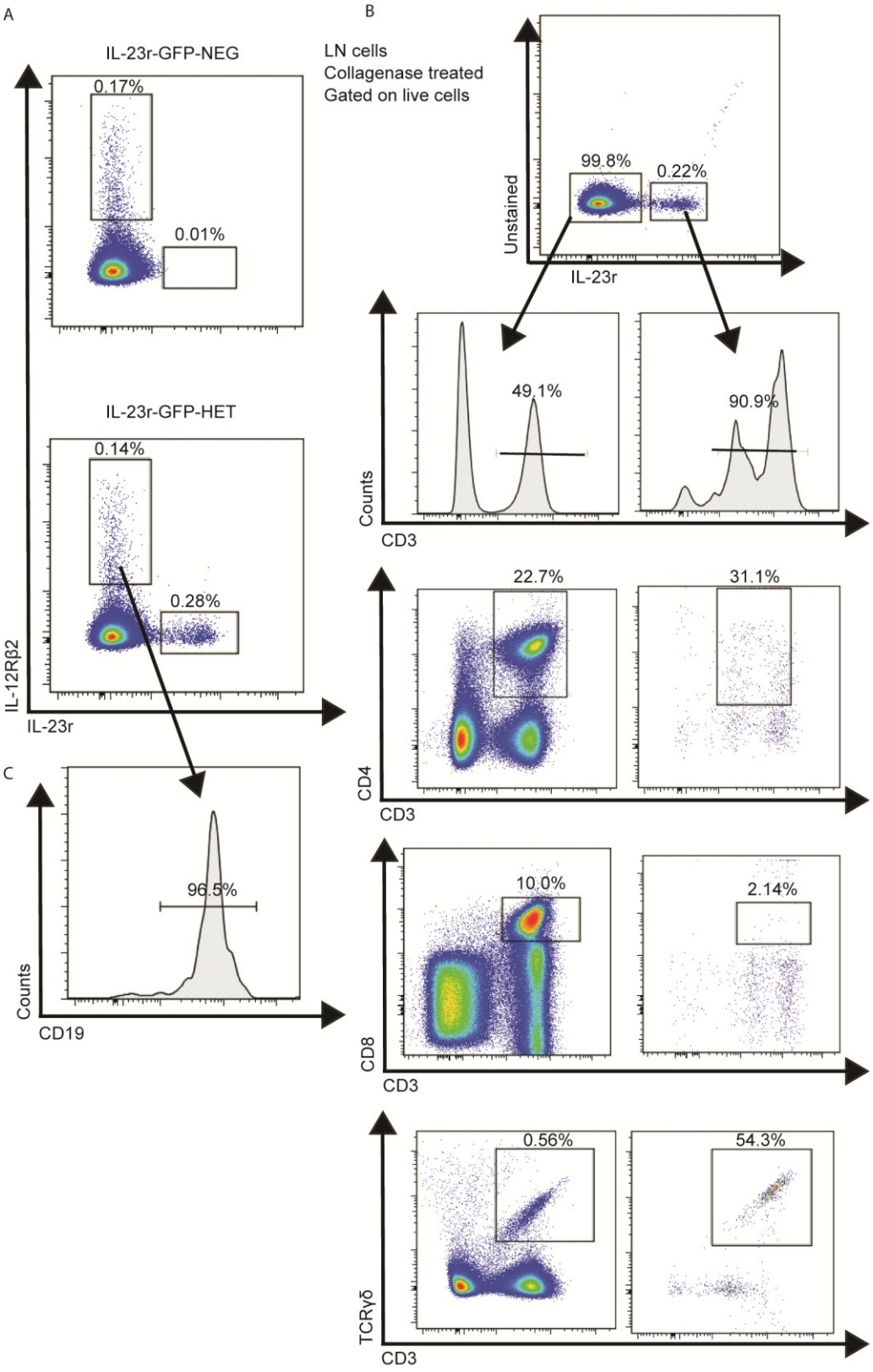


Figure 21: Peripheral lymph nodes contain slightly higher percentages of IL-23R positive cells than the spleen

A) IL-23R and IL-12Rβ2 expression in WT and IL-23r-GFP-Het mice B) T-cells characterisation of IL-23R- and IL-23R+ in IL-23r-GFP-Het C) CD19 staining of IL-12Rβ2+ cells Representative of three experiments

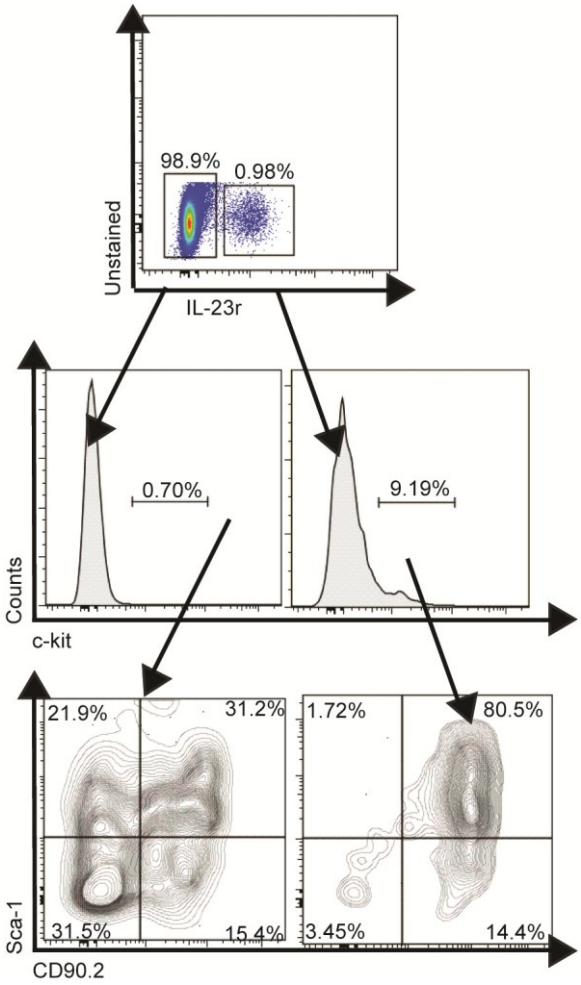


Figure 22: A small proportion of IL-23r+ Lti-cells are found in the LN
CD90.2, c-kit and Sca-1 staining of IL-23r-GFP-Het splenocytes.

Another organ of interest are the lungs. Lungs are an important part of the mucosal immune system because the epithelial lining of the lungs is also filled with lymphocytes. Lung lymphocytes are crucial in coping with the influx of microbes entering the lungs. IL-23R and IL-12R were found on many cells in the lungs because of the possible presence of activated lymphocytes.

In Figure 23a, it is possible to see that IL-12Rβ2+ cells are found here again in a percentage similar to the ones found in other organs. Most of IL-12Rβ2+ cells are B-cells (93%). Another finding is that even in the lungs, IL-12Rβ2 and IL-23r are not co-expressed; no cells are positive for IL-12Rβ2+ and IL-23r at the same time.

Figure 23b shows that 16% of IL-23r+ cells in the lungs express c-kit. 50% of this IL-23r+ c-kit+ cell population expresses CD90.2 and Sca-1, which is similar to what was seen in the spleen (60%) and higher than in the LNs. A small proportion of IL-23r+ cells were B-cells (6%).

Finally, since these animals were WT, the proportion of IL-23r+ T-cells was also examined. In Figure 23c, that the proportion of CD3+ IL-23r+ cells was around 85%. Amongst the CD3+ cells, only 20% of them were expressing other T-cells markers. 13% of IL-23r+ cells were TCRγδ, while 7% of them were CD4+CD3+. There was only a small IL-23r+CD8+CD3+ cell population. In the lungs, we observed a situation similar to what was seen in the bone marrow: a large proportion of CD3+ cells did not express CD8 or CD4, which is consistent with a DN T-cells, NK T-cells, another type of T-cells phenotype, or any combination of those T-cells could be found in the lungs and express IL-23R.

From Figure 23c, it can be seen that there also seems to be a CD4+ CD3- cell population amongst IL-23r+, similar to the population in the spleen and BM.

As previously described, the proportion of the IL-23r+ cells in the spleen of RagKO-IL-23r-GFP-Het naïve animals is increased when compared to IL-23r-GFP-Het animals, probably because of the increased proportion of Lti-like cells in the spleen RagKO. However, when the lungs of RagKO-IL-23r-GFP-Het animals were analysed

for IL-23r expression, the proportion of IL-23r+ cells seemed lower. In Figure 24e, it is possible to see that the percentage of IL-23r+ cells in the lungs decreases from 0.5% to less than 0.2% when moving from WT to RagKO animals (p-value≤0.05).

Only 50% of IL-23r+ cells in the lungs expressed c-kit (Figure 24b), and all c-kit+ cells expressed CD90.2. 25% of IL-23r+ cells were c-kit+CD90.2+Sca-1+. What were the other 50% of IL-23r+ cells? Figure 24d clearly demonstrates that even though monocytes and DCs are found in high numbers in the lungs, no IL-23r+ cells were NKs, DCs or monocytes.

Figure 24c shows that all IL-23r+ cells in the lungs expressed CD90.2. From this IL-23r+CD90.2+, 55% of cells also express c-kit. In other organs, all IL-23r+ that were not B-cells or T-cells express c-kit, which does not seem to be the situation in the lungs. Another important detail to note is that those CD90.2+ cells can be neither T-cells nor B-cells, as we are examining RagKO animals in Figure 24. Interleukin 23 receptor is therefore expressed by cell populations in the lungs that are not found in the spleen. It is also of importance that NK cells are very rare in the lungs.

On the other hand, IL-12Rβ2 seems to be more expressed in RagKO animals, but the findings were inconclusive and those cells could not be identified. The possible identity of those IL-12Rβ2+ cells is DCs.

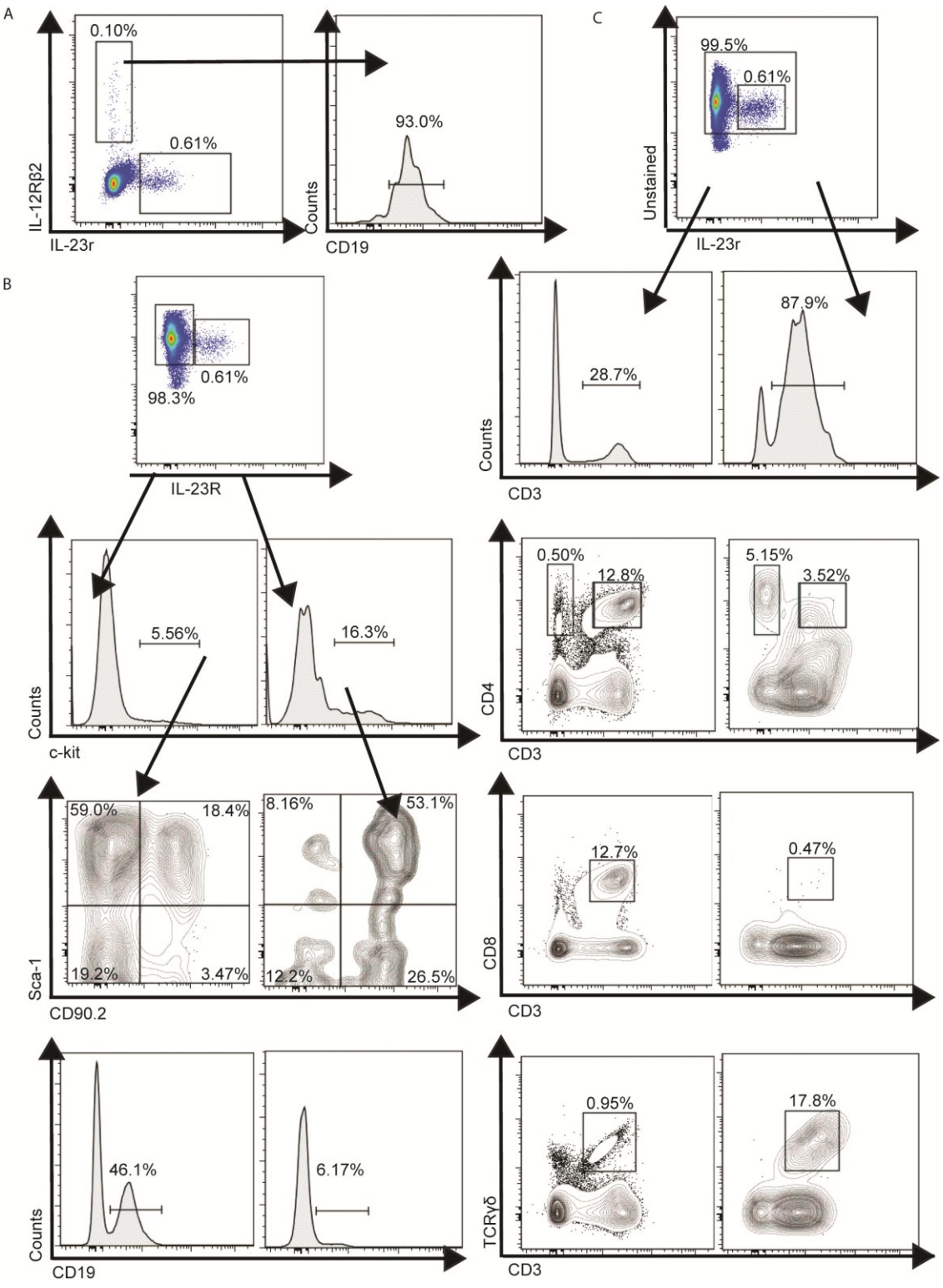


Figure 23: Lungs contain a population of IL-23r positive cells which are maily CD3+ and some Lti-like cells

A) IL-23R and IL-12Rβ2 expression in IL-23r-GFP-Het mouse and CD19 staining of IL-12Rβ2+ cells. IL-23R- and IL-23R+ B)Lti-like staining C) T-cells staining. Representative of one experiment.

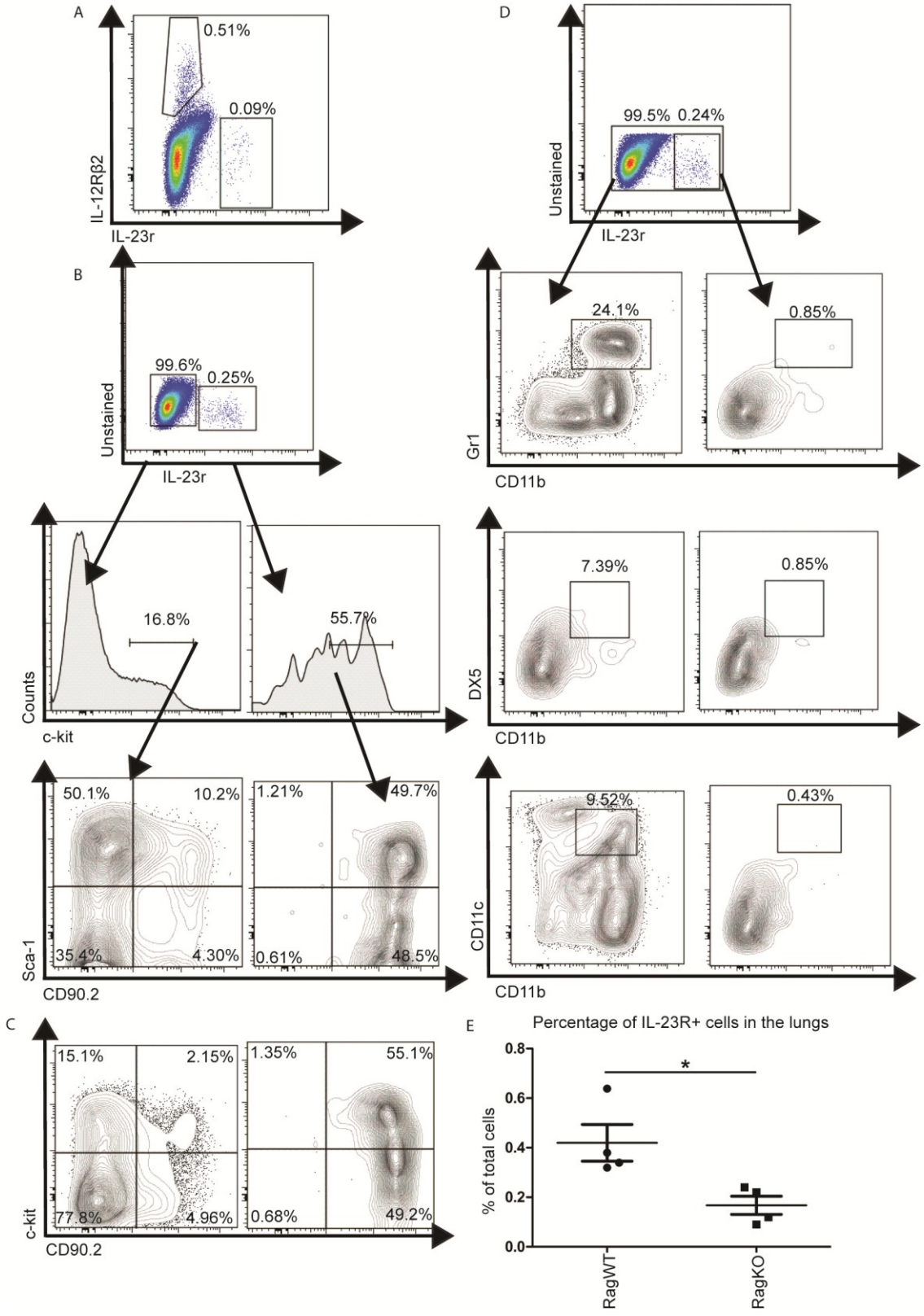


Figure 24: Percentage of IL-23r+ in the lung decreases in RagKO mice

A) IL-23r and IL-12Rβ2 expression in RagKO-IL-23r-GFP-Het mice. B) c-kit expression by IL-23R+ and IL-23R- cells and CD90.2 Sca-1 staining of c-kit+ cells. C) CD90.2 and c-kit staining of IL-23r+ and IL-23r- cells D) DC, monocytes and NK staining of IL-23r+ and IL-23r- E) IL-23r+ total cell expression in lungs of WT and RagKO IL-23r-GFP-Het mice. Representative of four experiments

The last organ examined for the expression of IL-23R and IL-12R was the gut. The lamina propria of the small intestine is a very interesting compartment of the mucosal immune system in the digestive tract and is the site where many inflammatory processes thought to be involved in human colitis occur.

In Figure 25a, IL-12Rβ2+ cell proportion is slightly higher than what was seen in the spleen. Most of those IL-12Rβ2+ were B-cells (84%, Figure 25c), as in other organs.

IL-23r+ cell percentage in Figure 25a was the highest proportion of IL-23r+ cells seen in all of the organs in WT mice. More than 2% of the cells in the lamina propria of the small intestine in IL-23r-GFP-Het mice were IL-23r+. One third (36%) of IL-23r+ in the LP were CD3+ and therefore T-cells. Unlike the spleen, where more than one third of IL-23r+ cells were CD3+TCRγδ+ T-cells, only 7% of IL-23r+ cells in the LP were TCRγδ. An important proportion of IL-23r+ were CD4+CD3+ (18%), but the proportion of CD8+CD3+ T-cells was negligible. There was also a CD4+CD3- cell population and a possible CD3+CD4-CD8- TCRγδ-population.

Characterization of IL-23r+ cells was continued in Figure 26. In Figure 26b, a very small proportion of IL-23r+ cells was CD11c+, but was neither mDC nor lDC. They could represent a small proportion of monocytes[25][26]. Figure 26c also shows that only 4% of B-cells express IL-23r, even though B-cells compose two-thirds of the cells found in the LP. Half of the IL-23r+ cells found in the LP were c-kit+. Upon further characterization, 88% of those cells expressed CD90.2, but only 8% of c-kit+ cells were CD90.2+ Sca-1+, while most IL-23r+ c-kit+ cells in the spleen were both CD90.2+ and Sca-1+ cells.

[25] pDC are not found in the lamina propria of the small intestine and therefore there are no IL-23r+ pDC.
[26] CD8 expression by CD11c+ cells in the lamina propria of the small intestine is different than the expression by mDC in lymphoid organs.

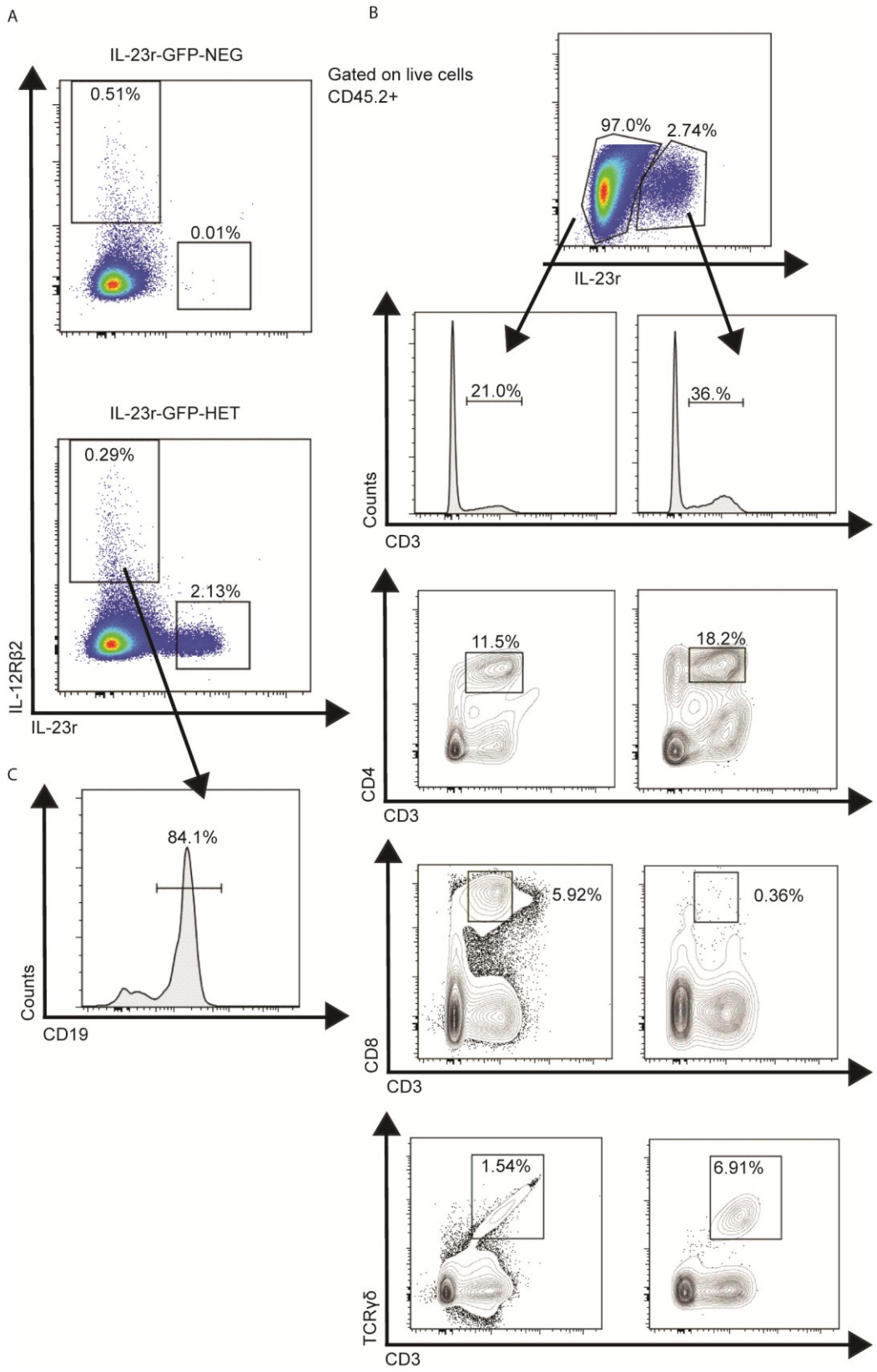


Figure 25: A third of IL-23R+ cells in the lamina propria are T-cells

A) IL-23r and IL-12Rβ2 expression in lamina propria of IL-23r-GFP-Het mice B) T-cells staining of IL-23r+ and IL-23r- cells C)B-cells staining of IL-12Rβ2+ cells. Representative of three experiments.

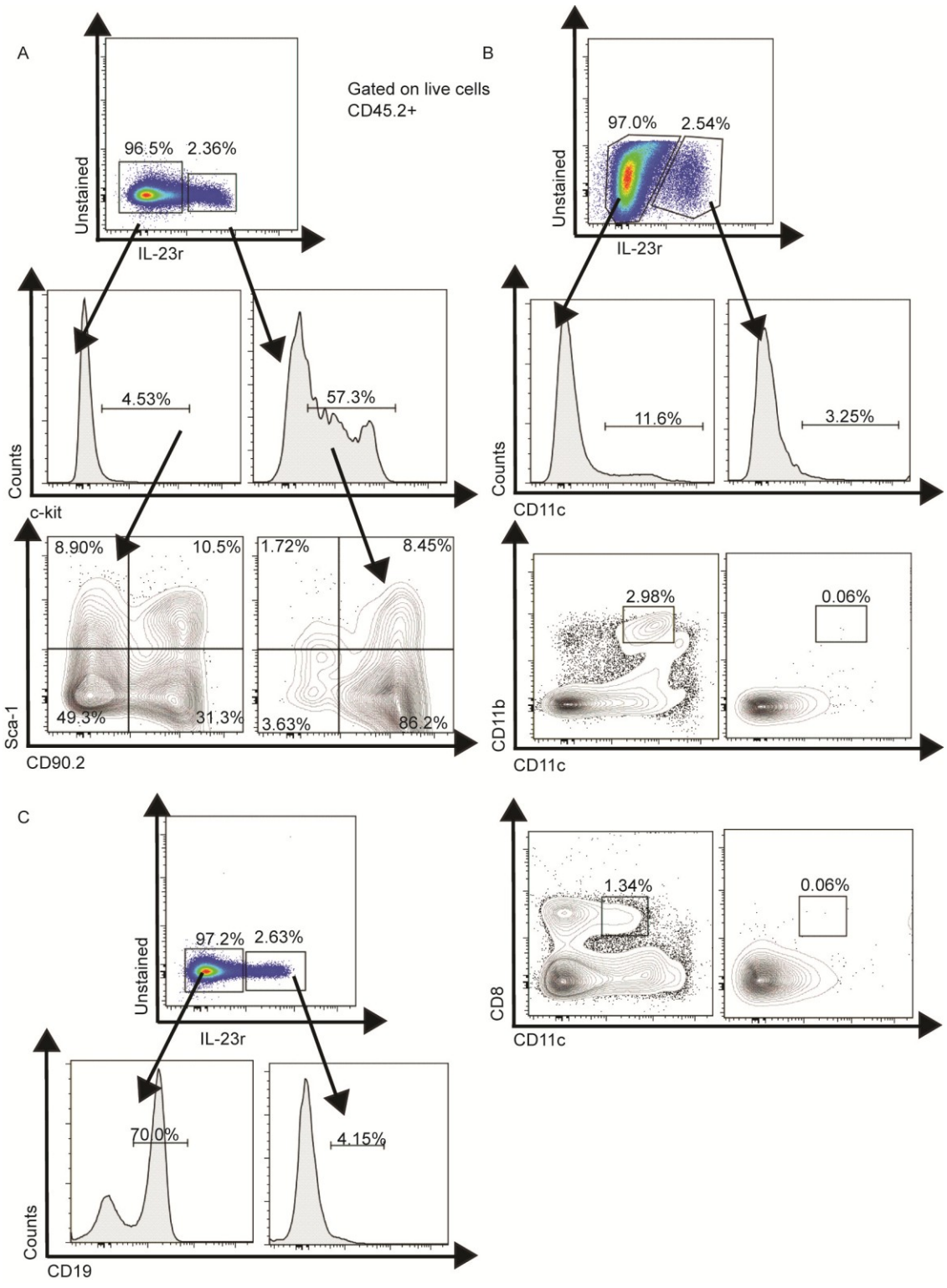


Figure 26: Lamina propria of the small intestine contains high numbers of IL-23R positive cells and those cells are in vast majority Lti-like cells

IL-23r+ and IL-23r- cells staining of A) Lti-like cells, B) DC, C) B-cells. Representative of three experiments

The final step in IL-23R organ characterization was to look at IL-23r expression in the LP of RagKO-IL-23r-GFP-Het mice, because the lamina propria of the intestine would be deprived of T-cells and B-cells and rarer cell populations such as Lti-like cells would be in increased percentages. Figure 27a clearly shows that the proportion of IL-23r+ cells in the LP of RagKO animals represent one third of the total cell counts. In Figure 27a, it is also possible to note that IL-12Rβ2+ cells are increased in the LP of the small intestine. It is, however, very hard to characterise those cells, as they are found in low numbers in an organ from which extracted cells have a low viability. There are suspicions those cells might be dendritic cells (data not shown).

Figure 27b also clearly demonstrates that IL-23r+ cells in the LP are mostly c-kit+ cells. They are CD90.2+ in the same proportion as the c-kit+ cells in the LP of WT animals. In both RagKO and WT IL-23r-GFP-Het mice, 80% of IL-23r+c-kit+ cells are CD90.2 and the rest are CD90.2+Sca-1+ cells.

Figure 28 is a compilation of IL-23r+ cells distribution in the LP of WT and RagKO IL-23r-GFP-Het mice. In Figure 28a, we see that the proportion of IL-23r+ cells increased in the LP of RagKO animals, moving from an average of 3% to around 40% of total cells. In Figure 28b, that c-kit+ cells are the major subtype of cells expressing IL-23r in the LP of both RagKO and WT animals.

In WT animals, IL-23r expression is divided between T-cells, B-cells, and c-kit+ cells. The T-cells were mostly CD4+ and TCRγδ cells, with very little CD8+ cells. The c-kit+ cells can be further separated in two major groups: CD90.2+(80%) and CD90.2+Sca-1+(20%) cells. In RagKO animals, c-kit+ cells are the only cells to express IL-23r at steady state. Similar to WT mice, c-kit+ cells were further characterized as either CD90.2+(80%) or CD90.2+Sca-1+(20%) cells

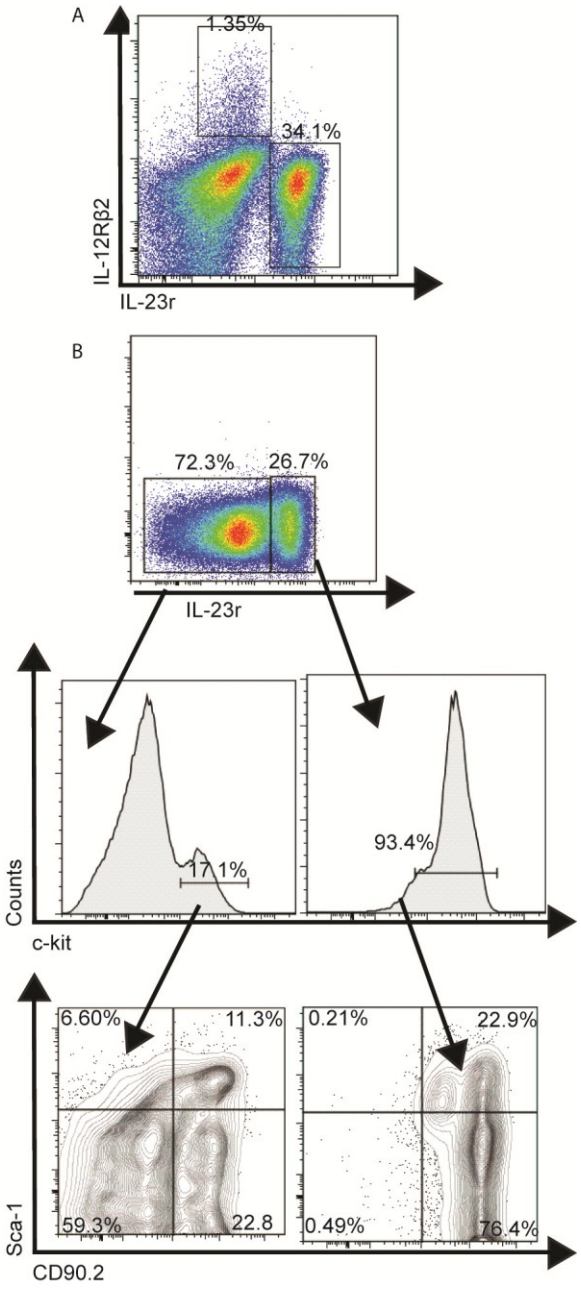


Figure 27: IL-23r+ cells are found in very high numbers in the lamina propria of RagKO mice

Analysis of RagKO-IL-23r-GFP-Het A) IL-12Rβ2 and IL-23r staining of small intestine lamina propria B) Lti-like staining of IL-23r+ cells. Representative of three experiments

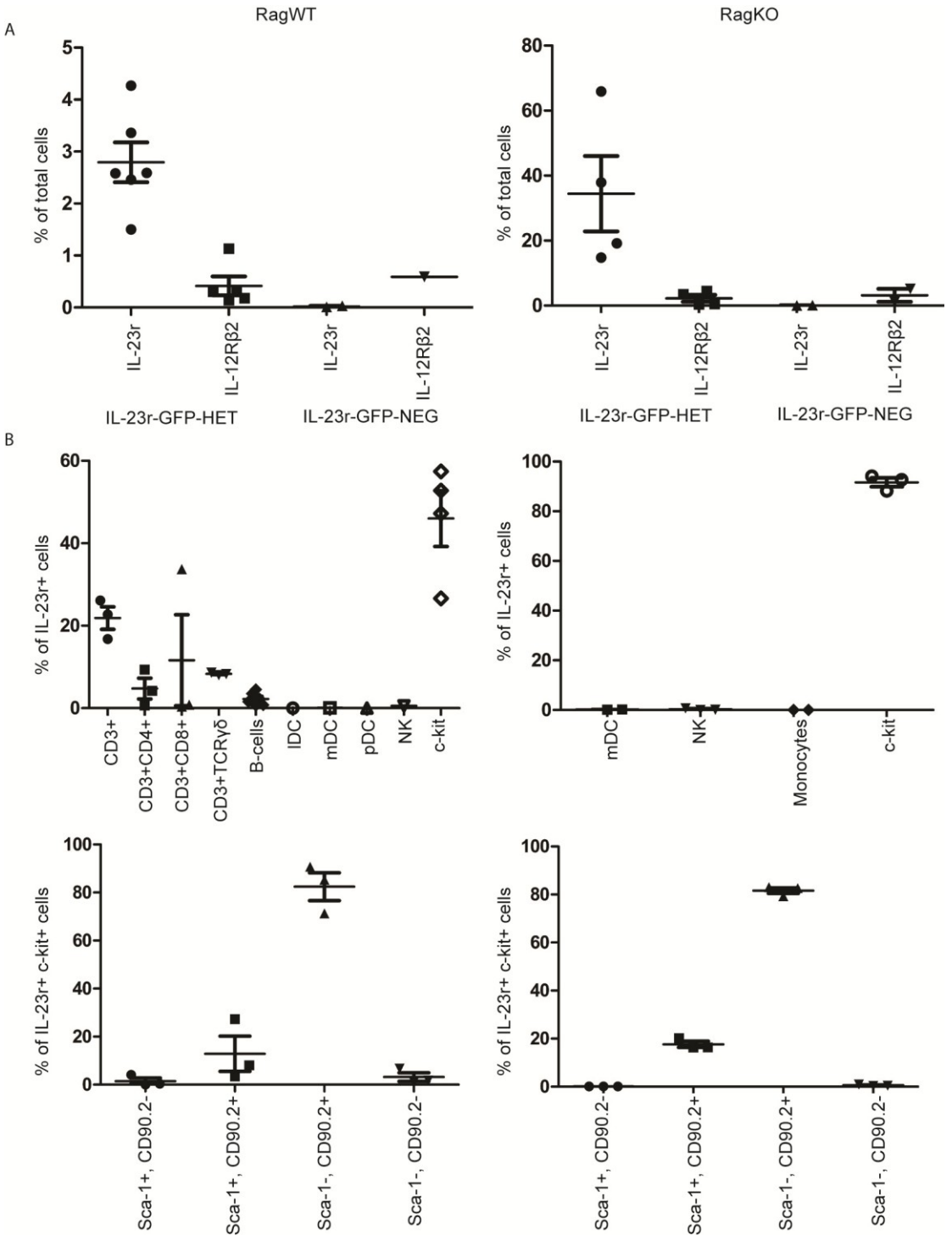


Figure 28 : Distribution of IL-23r+ cells in the small intestine lamina propria

A: IL-23R expression is restricted to IL-23R-GFP heterozygous, while IL-12Rβ2 expression is found in both kind of mice. IL-23R and IL-12Rβ2 is found in both WT and RagKO mice.

B: Gating on IL-23R+ cells in IL-23r-GFP-Het in both WT and RagKO mice, it is possible to identify different cell subtypes expressing the receptor. c-kit+ cells can be further segregated in four groups, depending on their CD90.2 and Sca-1 expression (B, bottom panel).

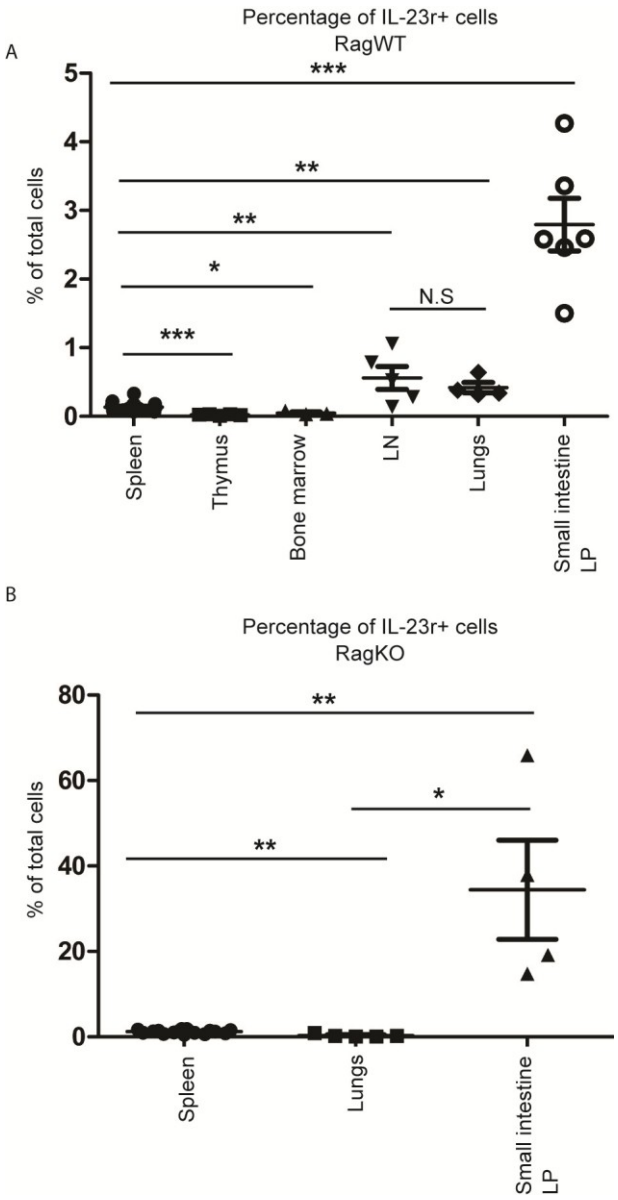


Figure 29 Compilation of IL-23R expression in different organs: the small intestine LP is the organ with the highest proportion of IL-23r+ cells of all the organs analysed.
IL-23R+ cell proportion in different organs coming from A) IL-23r-GFP-Het and B) RagKO-IL-23r-GFP-Het.

To have a better overview of IL-23R expression throughout the different organs, Figure 29 contains the compilation of IL-23r positive cells in all organs. In Figure 29a, the organ containing the most in IL-23r-GFP-Het mice is definitely the LP of the small intestine, followed by the LN and the lungs. The spleen was ranked third for having the most IL-23r+ cells, with the thymus and BM having the lowest proportions of IL-23r+ cells. There was no statistically significant difference in IL-23r expression between the LNs and the lungs.

This increase in IL-23r+ in the LP was even more tangible when looking at RagKO animals. A third of LP cells are IL-23r+ cells, which is more than any other organ examined. The spleen contained more IL-23r+ cells than the lungs in RagKO animals, which was opposite to WT animals. IL-23r+ characterization throughout the different organs reinforced its possible importance in gut immunology.

Objective 3: Understanding the specificity of the cytokine responses of different cell types following stimulation with IL-12 or IL-23

As mentioned previously, IL-23R and IL-12R are expressed on different cell types. Both receptors have different roles, are activated by different triggers, and induce different signaling cascades. However, both receptors have very high similarity in their composition, by sharing one subunit (IL-12Rβ1) and the second subunits are orthologous to one another (IL-23r to IL-12Rβ2). Therefore, an important experiment was to test the specificity of each cell type in response to both IL-12 and IL-23. Because each cell type seemed to express one or the other receptor, it was hypothesized that they should respond to only one of the two cytokines. Cells were sorted and stimulated *in vitro* with IL-12 or IL-23, alone or in combination with other cytokines. Cytokines secretion following cell stimulation was used to measure IL-12 and IL-23 responsiveness.

This last section contains preliminary data generated after identification of the cell types expressing IL-23R or IL-12R. These experiments were designed to answer new questions about the cytokines' capacity to induce signaling through only one receptor. However, most of the experiments were done only once or twice and some important controls might be missing. This is important to mention, as the results may raise new questions and/or indicate new roles for IL-12R and IL-23R which are important for the characterization of the IL-12R family.

1. Lti-like cells

The first cells to be tested were Lti-like cells or DX5-CD3-CD19-c-kit+CD90.2+ cells. Lti-like cells from both the spleen and the LP of the small intestine were extracted, sorted and cultured *in vitro*. In Figure 30, cytokine secretion of Lti-like from the spleen following stimulation with IL-12 or IL-23 is observed. To test the importance of IL-12Rβ2 in IL-23 and IL-12 signaling, Lti-like cells from IL-12Rβ2KO mice were also sorted. Cells from IL-12Rβ2KO mice should not respond to IL-12 if IL-12Rβ2 is required for signaling. However, if IL-23 can interfere with IL-12Rβ2, maybe IL-12Rβ2KO cells will react differently when stimulated with IL-23.

IL-22 and IL-17 are associated with IL-23 stimulation, while INFγ is mostly associated with IL-12 stimulation. IL-22 was effectively secreted by Lti-like cells following IL-23 stimulation, independent of IL-12Rβ2 presence. There was no IL-17 secretion following IL-23 stimulation, which is exactly the opposite of what was expected. IL-17 is often a hallmark cytokine of the IL-23R signaling cascade. IL-12 stimulation induced strong INFγ stimulation, even though Lti-like cells are not supposed to express IL-12R. IL-10 may be secreted by IL-23 stimulation, but this experiment was only done once and the levels were very low. There was no secretion of IL-4, IL-6, IL-21, IL-27 and TNF-α recorded following either IL-23 or IL-12 stimulation (data not shown).

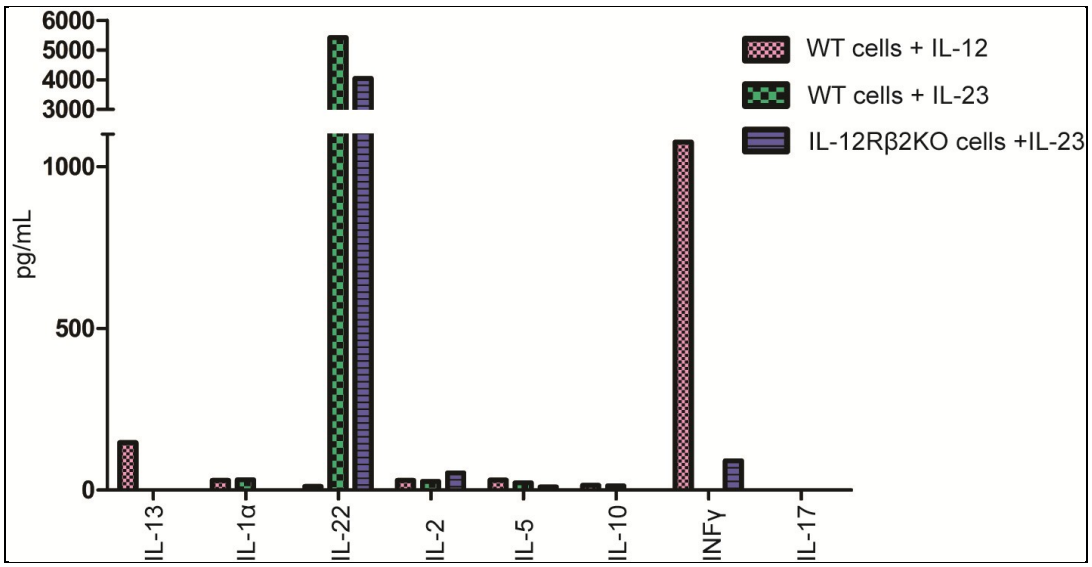


Figure 30: Lti-like cells can produce high amounts of IL-22 following IL-23 *in vitro* stimulation

Cytokine production by CD45.2+CD19-CD3-DX5-c-kit+CD90.2+ after 6 days of in vitro culture. Cells are from the spleen of WT or IL-12Rβ2KO naïve mice. Representative of one experiment.

Lti-like cells from the LP of the small intestine were also stimulated *in vitro* for six days with IL-12, IL-23 or IL-2+IL-23. Figure 31 contains the compilation of the cytokine measurements. Lti-like cells from the LP cytokine secretion profile was slightly more heterogeneous than Lti-like cells from the spleen. IL-22 was secreted in response to both IL-23 and IL-2+IL-23, but slight levels of IL-22 were also detected in response to IL-12. IL-13 was detected only in the presence of IL-2+IL-23. In Lti-like cells from the LP, unlike Lti-like cells from the spleen, IL-17 was detected in response to IL-23 and IL-23+IL-2.

IL-12 stimulation of Lti-like cells from the LP induced INFγ production, but only at very low levels, unlike Lti-like cells from the spleen. However, Lti-like cells from the LP stimulated with IL-2 and IL-23 induced higher amounts of INFγ than IL-12 stimulation by itself. However, IL-23 stimulation by itself did not induce INFγ, so IL-2 could be the real inducer of INFγ.

IL-6 and IL-2 levels were significant only in response to IL-2 and IL-23. However, as IL-2 is added directly into the wells of culture, IL-2 measurements cannot be considered as being secreted from Lti-like cells. IL-2 was included in the graph to show that it was efficiently added only to that well. IL-1α, IL-4 and IL-27 were also measured, but no cytokines were detected and therefore the data is not shown.

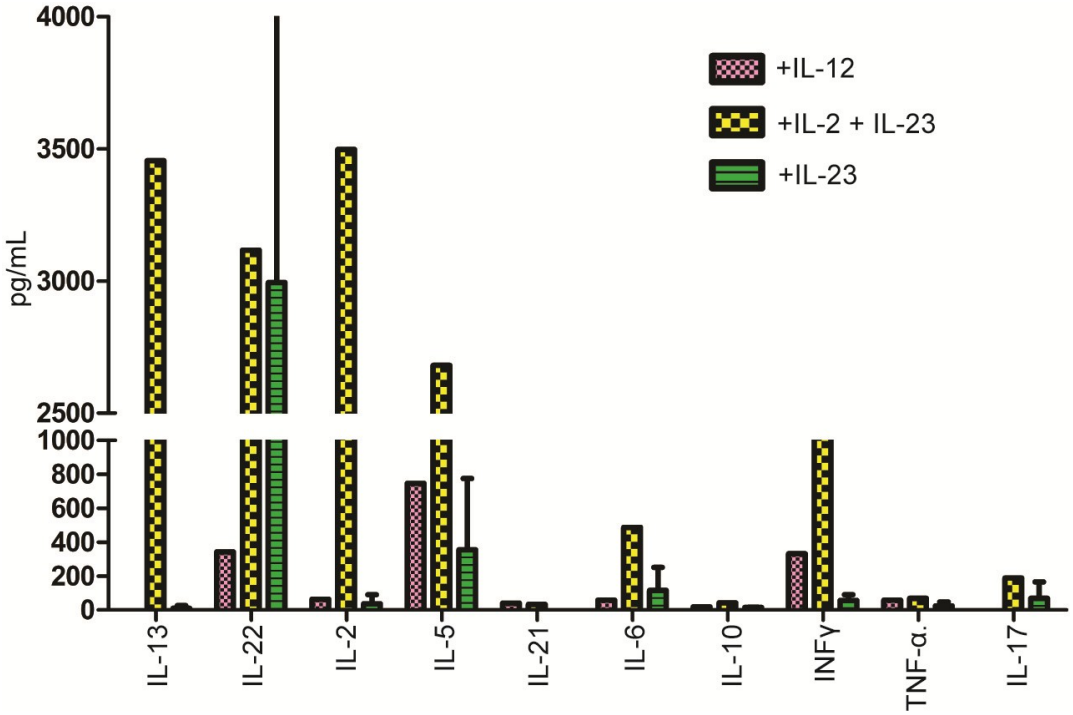


Figure 31: Lti-like cells from the lamina propria respond to IL-23 and IL-2

Cytokine production by CD45.2+CD19-CD3-DX5-c-kit+CD90.2+ after 6 days of in vitro culture. Cells are from the LP of the small intestine of WT naïve mice. Representative of one experiment.

2. T-cells

Th17 and Th1 T-cells are thoroughly characterized. It is very often thought that IL-23 and IL-12 are crucial in the differentiation into those two effective T-cells subtypes. However, other cytokines are required to induce Th17 and Th1. IL-12 and IL-23 are produced in early immune responses, but do they have an effect directly on the cells? Could they both have an effect on T-cells, even though very few T-cells express IL-12R and IL-23R?

In Figure 32a, two different types of CD4+ T-cells were used: WT and IL-12Rβ2KO. Each subtype of CD4+T-cells were either stimulated with IL-12, IL-23, both cytokines, or no cytokines. We first examined the cytokine production following IL-12 stimulation. INFγ secretion following IL-12 stimulation is well-documented in the literature; INFγ production were seen in CD4+T-cells stimulated with IL-12. Addition of IL-23 did not change INFγ secretion. However, IL-12Rβ2KO mice stimulated with IL-12 (or IL-12 and IL-23) produced much lower levels of INFγ, but above the detection limit nonetheless. IL-23 stimulation of WT CD4+T-cells, IL-12Rβ2KO CD4+T-cells, or CD4+T-cells without any cytokine stimulation did not result in any increase of INFγ production. Unless IL-12 and IL-12Rβ2 were present, INFγ production did not increase from background secretion. Even without any Th1 inducing conditions, IL-12 induced an increase in INFγ production through IL-12R.

IL-23 stimulation is thought to induce IL-17 and IL-22 production by various cell types, including CD4+ T-cells. However, we showed that in Figure 32a IL-23, IL-12 or IL-12 in combination with IL-23 induced neither IL-17 nor IL-22 production by CD4+ T-cells. IL-23 alone failed to induce secretion of those cytokines *in vitro*.

IL-13 production was the highest in the presence of IL-12Rβ2 and in the absence of IL-12. Addition of IL-23 did not change IL-13 levels. However, IL-12, independently of IL-12Rβ2 expression, seemed to decrease IL-13 levels.

IL-2 levels were all similar, except in the presence of IL-12 or in the absence of IL-12Rβ2; IL-2 levels were very high in the circumstances. On the other hand, IL-23

added to IL-12Rβ2KO CD4+T-cells seemed to inhibit all IL-2 production. Others cytokines such as IL-1α, TNF-α, IL-6, and IL-10 were all measured but were not absent or in very low quantity in cell supernatants.

Another important subtype of T-cells in IL-23 biology is TCRγδ T-cells. Figure 32b is the analysis of splenic TCRγδ T-cells from either WT or IL-12Rβ2KO naive mice. Each subtype of TCRγδ T-cells were either stimulated with IL-12, IL-23, both cytokines, or no cytokines.

Very surprisingly, IL-12 had an effect on INFγ secretion by TCRγδ T-cells, but this effect was the opposite on CD4+ T-cells. Addition of IL-12 to WT TCRγδ T-cells strongly decreased INFγ production. TCRγδ T-cells stimulated with IL-23, with IL-12 and IL-23, or without IL-12Rβ2 all had similar INFγ production.

IL-22 production in response to IL-23 by TCRγδ T-cells was much lower than IL-22 production by Lti-cells. However, the highest production of IL-22 was by WT TCRγδ T-cells stimulated with IL-23 only. IL-22 secretion was not detectable in IL-12Rβ2KO TCRγδ T-cells stimulated with IL-12, while it was similar in all other culture conditions.

IL-17 secretion was also very low, but was only detectable following stimulation with IL-23, whether or not the TCRγδ T-cells were WT or IL-12Rβ2. TCRγδ T-cells seem more prone to IL-17 secretion at steady state than CD4+ T-cells. IL-2 and IL-5 secretion were similar in all the different cell cultures. There is also a trend where TNF-α production by TCRγδ T-cells depends on IL-12 and IL-12Rβ2 presence, but more experiments will be needed to confirm this. IL-1α, IL-6, IL-21 and IL-27 were also measured but were not different under any of the conditions tested.

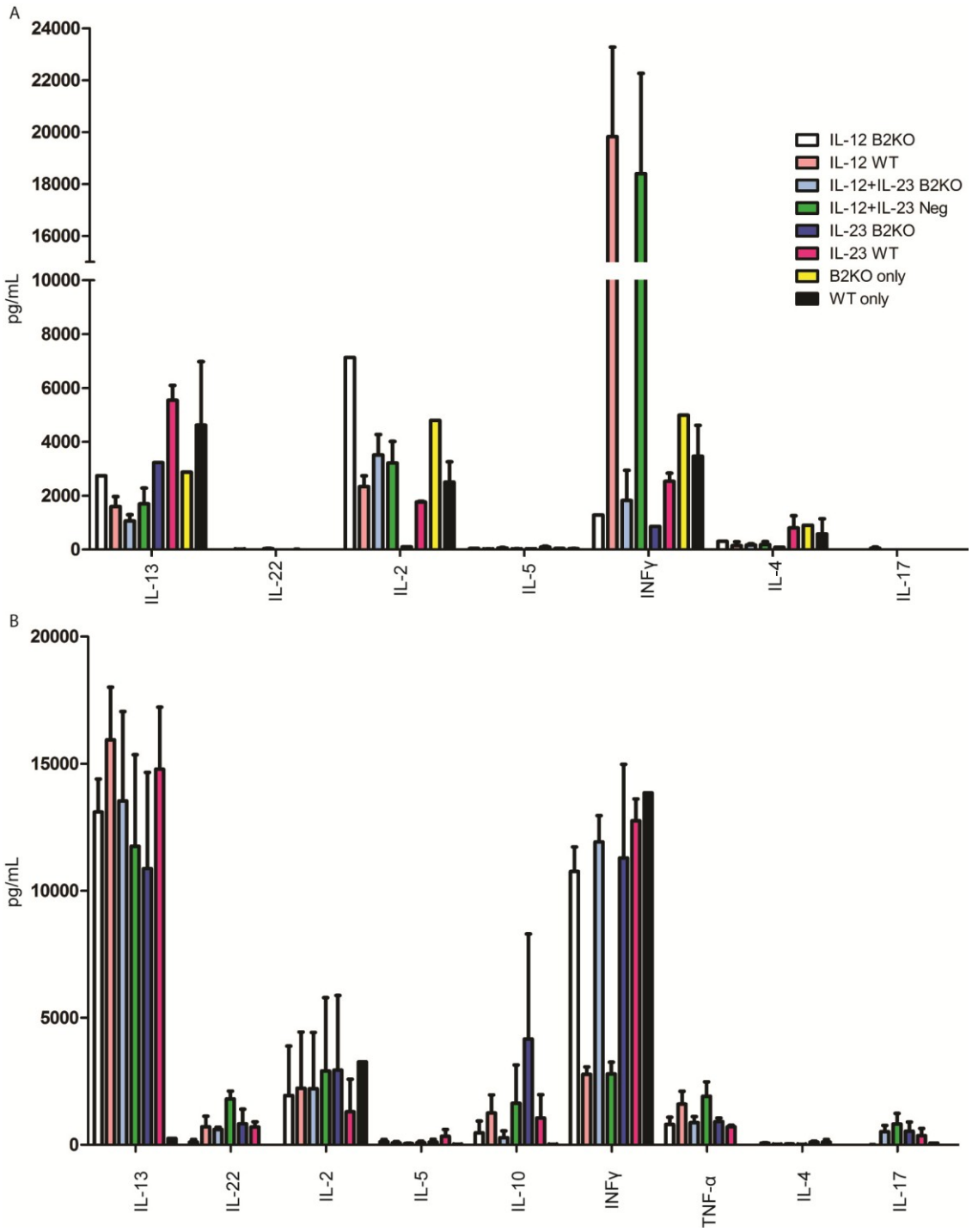


Figure 32: CD4+ and γδ T-cells cytokine secretion following *in vitro* stimulation

Sorted spleen T-cells from naïve WT or IL-12Rβ2KO animals. Six days in vitro culture and supernatants analysis A)CD3+CD4+T-cells and B)CD3+TCRγδ T-cells

3. B-cells

A problem was encountered throughout B-cell sorting. The monoclonal antibody IL-12Rβ2 was binding to B-cells in the IL-12Rβ2KO mouse. This was extremely surprising, but it also lead to many different problems. All of the IL-12Rβ2 characterizations done with the monoclonal antibody could therefore be erroneous.

However, as "IL-12Rβ2+" B-cells were consistently seen, the following experiment was done. Splenic B-cells from both WT and IL-12Rβ2KO mice were sorted, resulting in four different populations: WT IL-12Rβ2+, IL-12Rβ2- B-cells, IL-12Rβ2KO IL-12Rβ2+ and IL-12Rβ2- B-cells. All the populations were stimulated with IL-12, IL-23 or no cytokine. Supernatants were then measured for cytokine production.

In Figure 33, cytokines measurements of *in vitro* stimulation are shown. WT IL-12Rβ2- B-cells stimulated with IL-12 produced large amounts of INFγ and IL-2. However, those same cells stimulated with IL-23 also produce IL-2 and INFγ, even though some B-cells are known to express IL-23r. However, in the absence of IL-12Rβ2, IL-23 and IL-12 did not induce any significant amounts of IL-2 and INFγ. IL-23 stimulation of any cells did not induce IL-17 or IL-22, which is surprising as they are important downstream cytokines of IL-23 signaling.

WT IL-12Rβ2+ stimulated with IL-12 did not produce any of those cytokines. Those cells did not produce any other cytokines, except IL-27. IL-27 is highly similar to IL-12 and is therefore very interesting. IL-27R is composed of gp130, a subunit orthologous to IL-12Rβ2. Other cytokines were measured: IL-10, IL-6, IL-1α, IL-21, IL-4, IL-5 and TNF-α, but they were not shown as they were not detected or not different between the cell types.

Another important characterization of B-cells was identifying immunoglobulins secreted by B-cells in *in vitro* culture. In presence of IL-12 only, only IgM were significantly measured. However, IgM production by WT IL-12Rβ2+ was higher than the IgM production by IL-12Rβ2- following IL-12 stimulation (Figure 34).

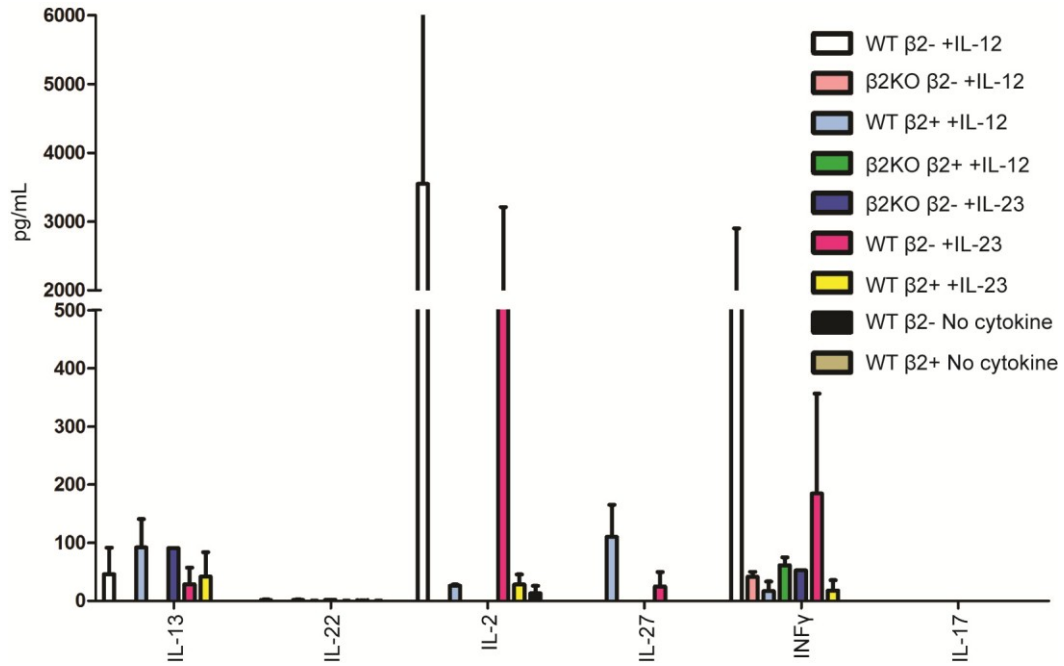


Figure 33: B-cells cytokine secretion following *in vitro* stimulation

Sorted splenic IL-12Rβ2+ or IL-12R-β2- B-cells from naïve WT of IL-12Rβ2KO mice were cultured in vitro with IL-12, IL-23 or no added cytokine. After six days, supernatants were removed analysed for cytokines secretion.

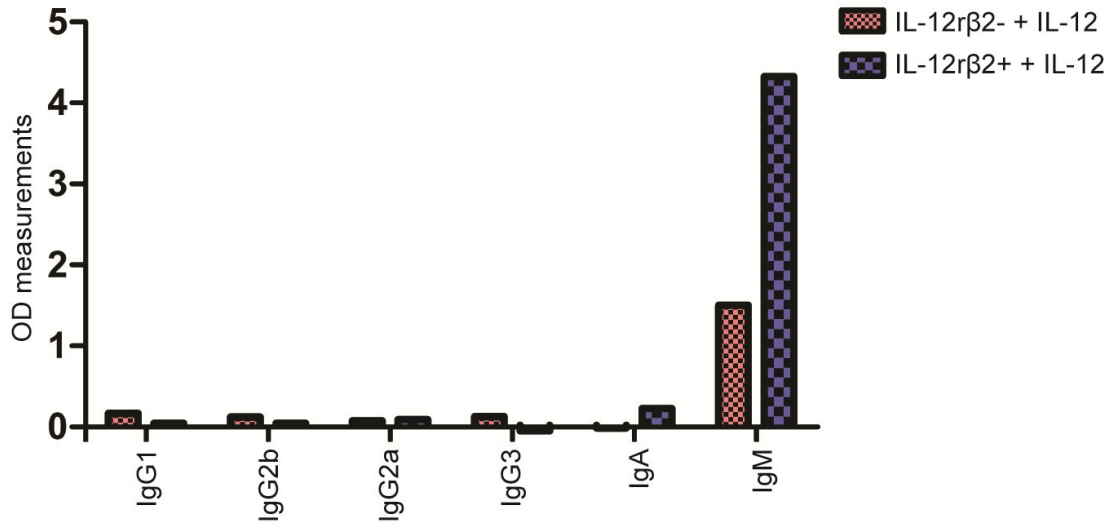


Figure 34: Ig production by IL-12Rβ2+ B-cells

Splenic IL-12Rβ2+ and IL-12Rβ2- B-cells were sorted from naïve WT mice and put 6 days in culture, stimulated with IL-12. Surnatants were harvested and κ-Immunoglobulins were measured. Optical density measurements.

4. NK cells

NK cells and IL-12 have often been linked together, where IL-12 is thought to activate and help NK cells proliferate. IL-12 is also thought to be essential for INFγ secretion from NK cells.

To test the influence of IL-12 and IL-23 on different NK cells, two experiments were set up. In the first one, NK cells from WT or IL-12Rβ2KO mice were put in culture with IL-2, IL-2+IL-23, or IL-2+IL-12. Surprisingly, addition of IL-12 and IL-2 to WT NK cells slowed down cell proliferation, as can be seen in Figure 35a. CFSE was not diluted in the presence of IL-12. However, when IL-12Rβ2KO NK cells were placed in the same conditions, NK cells proliferated, as can be seen in Figure 35b. Actually, not only did IL-12Rβ2KO NK cells proliferate the same as IL-12Rβ2KO NK cells cultured with IL-2 or IL-2+IL-23, but IL-12Rβ2KO NK cells proliferated more than WT NK cells in comparison.

It was also important to measure cytokine production by NK cells. Cytokines were measured after three and six days in culture. Figure 36a and b demonstrate that only INFγ and IL-2 were found in NK cell supernatants. However, as IL-2 is added into the NK wells it is hard to measure IL-2 as either being produced by the cells or not consumed by the NK cells. INFγ was only produced by WT NK cells in the presence of IL-12 and with functional IL-12Rβ2 subunit. IL-12Rβ2KO NK cells cultured with IL-12 did not produce INFγ. IL-2 or IL-2+IL-23 treatment could not induce INFγ production by NK cells. The readings for NK cells after six days in culture are also similar to those after 3 days, only with higher background cytokines secretion and higher amounts of INFγ.

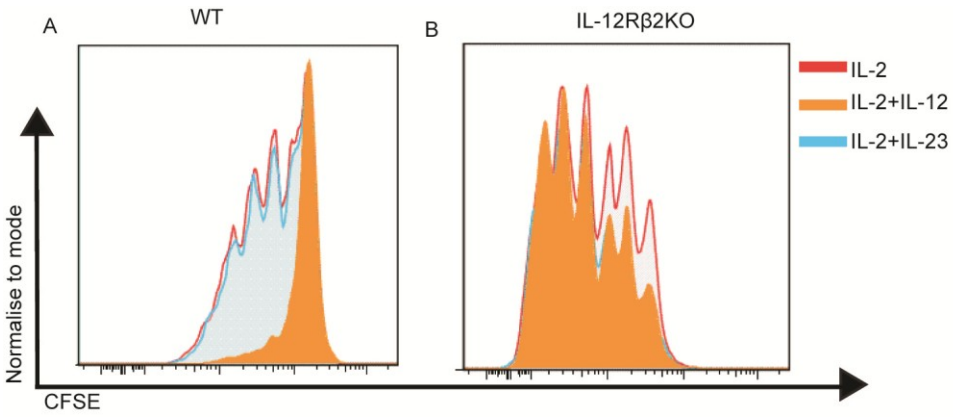


Figure 35: NK proliferation is impaired when stimulated with IL-12

After three in culture *in vitro*, NK cells stimulated with IL-12 and IL-2 proliferate less than NK cells stimulated with IL-2 only or IL-2 and IL-23. However, in NK cells coming from IL-12Rβ2KO mice, dilution in CFSE is increased whether cells are stimulated with IL-2 only or in combination with IL-12 or IL-23. Representative of one experiment.

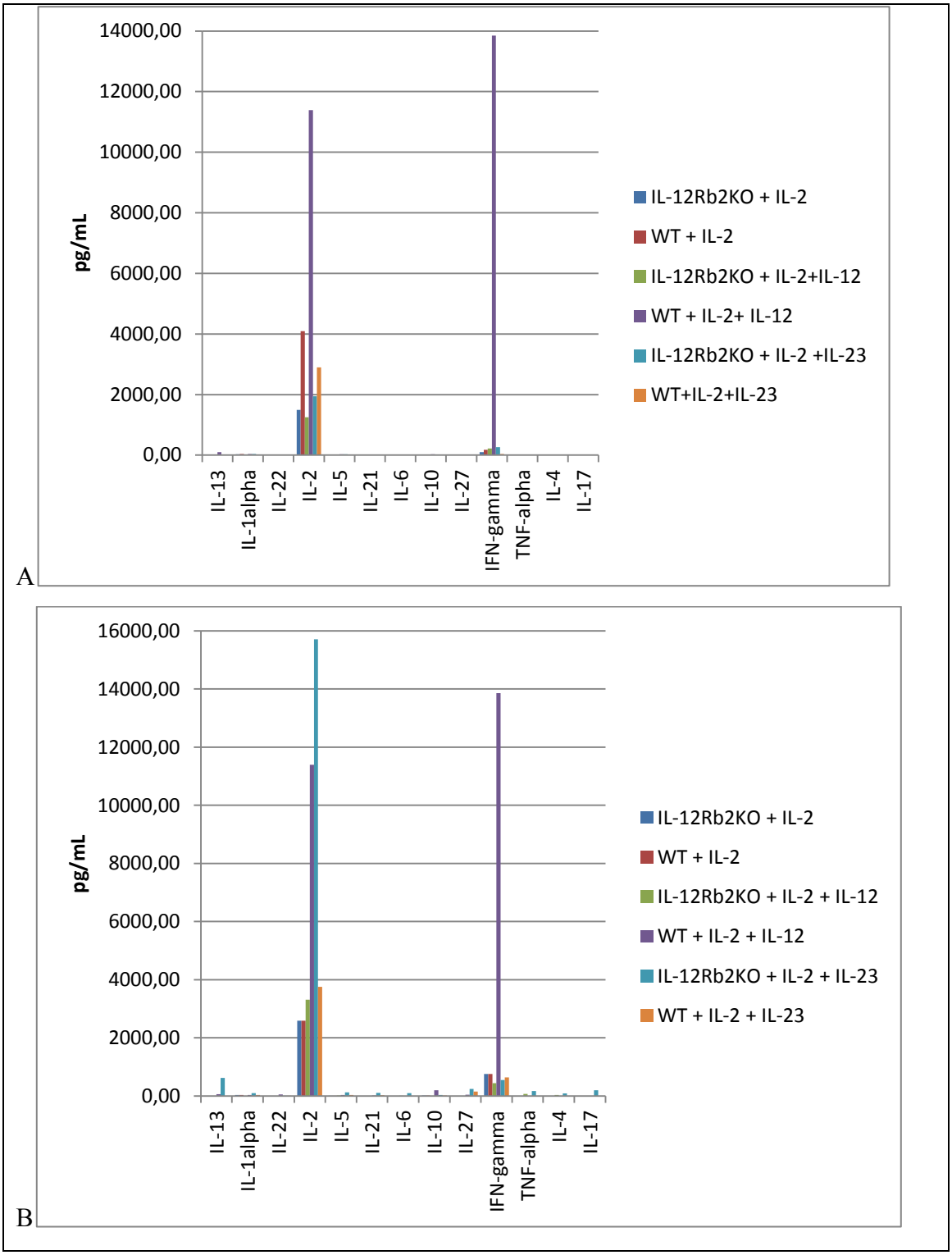


Figure 36: Presence of IL-12 and IL-12Rβ2 is required for INFγ production by NK cells

Splenic NK cells from WT and IL-12Rβ2KO naïve mice after A: 3 days in culture and B: 6 days in culture, stimulated with IL-12 or IL-23 and IL-2 for all conditions. Representative of one experiment.

Discussion

IL-12R expression was hypothesized to be easily analysed with the use of a IL-12Rβ2 monoclonal antibody. Identifying a small proportion of B-cells expressing IL-12Rβ2 was extremely surprising, as the literature review did not impressively reveal an IL-12-responding or an IL-12R-responding B-cell population. B-cells expressing IL-12R could be the first cell population responding to early IL-12 secretion *in vivo*, before IL-12R is upregulated at the surface of other cells, such as T-cells differentiating into Th1. Those IL-12R+ B-cells were rare by comparison, but found throughout the different organs examined, so they could respond in different areas of the body wherever the initial insult inducing IL-12 secretion was. Another indication that IL-12R+ cells found in the spleen were B-cells was the strong decrease of IL-12Rβ2+ cells in RagKO animals. As RagKO animals do not produce any B-cells, it seems logical that IL-12Rβ2+ cells would be dramatically decreased in RagKO animals. After thorough characterization of B-cells with the usual subset markers, such as CD21, CD23, IgM, IgD, GL-6, and CD136, IL-12Rβ2+ B-cells did not seemed neither enriched nor decreased in any subtype. CD43 was the only one to stand. Almost all lymphocytes express CD43 except mature B-cells. However, a special type of B-cell, called B-1 B-cells, are known to express CD43[103]. As reported here, less than 2% of splenic B-cells express CD43, in contrast with IL-12Rβ2+ B-cells where 40% of cells expressed CD43.

It would be interesting to further characterize IL-12Rβ2+ B-cells. B-1 B-cells are found in the spleen, but are found in even higher numbers in the peritoneal cavity[103], so staining peritoneal B-cells with IL-12Rβ2 monoclonal antibody could be a good way to continue the characterization. CD5 is another important marker for B-1 B-cells; staining with CD5 and IL-12Rβ2 antibodies at the same time could be informative. B-1 B-cells are really interesting as they arise during fetal development; they are known to react to different antigens than conventional B-cells and are known to be amongst the first responders to certain bacterial and fungal antigens such as polysaccharides[102]. Those B-1 B-cells can almost be qualified as “innate B-cells”

and they would fit perfectly this model. At basal state they are the first cells that respond to IL-12 secretion by activated APCs and induce primary immune responses. It was logical that IL-12Rβ2+ cells were B-cells and were very rare in the spleen of RagKO animals.

On the other hand, other organs still contain high amounts of IL-12Rβ2+ cells; those cells seemed to be DC, which is surprising. NK cells are usually thought to express IL-12R; this was not observed in any of our experiments. Another reason that could explain the high amounts of IL-12Rβ2+ cells found in other organs is due to monocytes. RagKO organs, such as the lungs, have very high amounts of monocytes. Monocytes can often bind to antibodies in an unspecific way which could artificially increase the apparent number of IL-12Rβ2+ cells. Another important issue was the low proportion of B-cells amongst IL-12Rβ2+ cells of the thymus. Only a third of IL-12Rβ2+ thymocytes were B-cells and the other two-thirds could not be identified. On the other hand, IL-12Rβ2+ cells were very rare, so it was hard to analyze.

Nevertheless, characterization of IL-12Rβ2+ cells seemed conclusive. They were mostly B-cells, with an enrichment of B-1 B-cells which would be the first cells to respond to IL-12 coming from APCs, which seems to be an appropriate role for “innate-like B-cells".

However, finding that the monoclonal IL-12Rβ2 antibody could bind to the same B-cell population in IL-12Rβ2KO mice was quite a drawback. IL-12Rβ2KO animals do not express IL-12Rβ2, so the monoclonal antibody is not supposed to bind to cells from IL-12Rβ2KO animals. This means that the monoclonal antibody was binding to something that was not IL-12Rβ2, but it was consistently identifying the same subset of B-cells in the spleen, as they had the same characteristics throughout the experiments and IL-12Rβ2+ B-cells were absent from RagKO. This is indicative that this IL-12Rβ2 monoclonal may be recognizing something specific, but it is not IL-12Rβ2. Another important experiment would be to sort the IL-12Rβ2+ cells from WT, RagKO, WT-IL-12Rβ2KO and RagKO IL-12Rβ2KO mice and measure IL-12Rβ2 mRNA levels in each of those cells subtypes. By doing so, it would be possible to measure if IL-12Rβ2 actually binds to IL-12Rβ2 or if it is only some unspecific

binding. Furthermore, because of the strong homology between IL-12Rβ2, IL-12Rβ1, IL-23r, gp130 and IL-27rα, all of those subunits should also be measured, to make sure the antibody is not recognising another similar protein.

Those "apparent" IL-12Rβ2+ cells did have some particularly special characteristics, such as an enrichment in CD43+ B-cells. They also produced less INFγ when stimulated with IL-12, which is different from what was expected. IL-12 action through IL-12R is thought to induce INFγ. However, IL-12 stimulation of TCRγδT-cells decreased INFγ production, so this could present a regulatory pathway to controlling INFγ production. Interleukin-12 stimulation of IL-12Rβ2+ B-cells also induced very strong IgM production, which was not seen in the stimulation of IL-12Rβ2- B-cells. They therefore have a certain responsiveness to IL-12.

IL-12Rβ2 was rare in spleens of RagKO animals, but it was more frequent in the lungs and LP of RagKO animals. They could be monocytes or DCs, but it was difficult to characterize them. This may be because of the antibody, which was binding to something other than IL-12Rβ2. More thorough characterization of RagKO-IL-12Rβ2KO animals with the IL-12Rβ2 antibody in the LP and lungs could help elucidate which cells bind to the monoclonal antibody. However, one explanation not examined is the limit of detection of the IL-12Rβ2 antibody, or what is the minimum number of cells positive for IL-12Rβ2 required to get positive results over the background. It would be interesting to compare how many cells are positive for IL-12Rβ2 antibody in the spleen of WT and IL-12Rβ2KO mice. Maybe the positive cells found in the KO mouse represent an artefact.

One hypothetical target of the IL-12Rβ2 monoclonal is gp130. The IL-12Rβ2 subunit is highly similar to gp130, which is composed of IL-27R and IL-35R. The antibody could be recognizing this gp130 subunit, which could help render responsiveness to IL-12. The antibody against gp130 exists, but was not used in characterization of cells. An interesting follow-up experiment would be to co-stain splenocytes from both WT and IL-12Rβ2KO splenocytes with gp130 and IL-12Rβ2 antibody and compare the expression pattern. It would also be interesting to stain

splenocytes from IL-23r-GFP-Het mice and verify if gp130 and IL-23r expression happens at the surface of the same cells.

We therefore could not conclude on IL-12Rβ2 expression by cells from the spleen, lungs, LP of the small intestine, bone marrow, and thymus, because of the specificity of the monoclonal antibody. This does not mean that the B-cell characterisations were useless; those cells identified by the antibody never expressed IL-23r in naïve state, even though some B-cells express IL-23r. The "IL-12Rβ2+" B-cells are therefore a specific subset of B-cells that do not express IL-23r, but they probably do not express IL-12Rβ2 either, because they come from the IL-12Rβ2KO mouse.

The characterization of IL-23r was not done using a monoclonal antibody, so the specificity of the expression was not in doubt. IL-23r expression is found in steady state animals, as seen in IL-23r-GFP-Het naïve mice. Those cells are all CD45.2+, suggesting their hematopoietic origin. However, it is impossible to conclude that IL-23r is only expressed by lymphocytes, as IL-23r expression by epithelial cells, for example, was not tested. Confocal microscopy of tissue sections could be used to measure GFP expression and therefore IL-23r expression in different organs and cell types. Flow cytometry of other cell types with specific markers could also be used.

IL-23r is expressed by hematopoietic cells, but is found in a wide variety of cells in WT animals. Unlike what was expected, IL-23r expression is not restricted to one specific cell. Furthermore, not all cells from one specific subtypes express IL-23r. For example, c-kit+CD90.2+Sca-1+ cells are found in both IL-23R+ and IL-23r- cell populations. In the spleen, 50% of IL-23r+ cells are TCRγδT-cells, but that proportion only represents one-third to half of the total TCRγδ T-cells found at steady state. B-cells represent at least 50% of the cells in the spleen; a handful of B-cells express IL-23r in the spleen. This variety of cells expressing IL-23r in IL-23r-GFP-Het mice raises several questions: why do only a few cells in each subset express this receptor? What function do those specific IL-23r+ cells have? Is the IL-23r expression transient? Is it induced by encountering different self-antigen? Is it expressed at certain stages of

development? In WT animals, CD4+T-cells, Lti-like cells, B-cells and TCRγδ were the main types of cells expressing IL-23r.

In Figure 17, there seems to be quite a few variation of the percentage of IL-23r+ B-cells found in the spleen of naïve IL-23r-Het-GFP animals. This could relate to the use of two different antibodies to identify B-cells: CD19 and B220. CD19 is very specific to B-cells. However, B220 can sometimes be upregulated by other cell types, including DCs, monocytes and T-cells, especially upon activation. The next B-cells analysis should distinguish between CD19+ and B220+ to ensure that the variability is not caused by the different antibody used.

However, two unknown populations of IL-23r+cells arose throughout the characterization: CD3-CD4+ cells and CD3+CD4-CD8-TCRγδ- cells. Some CD3-CD4+ cells have been found before in the gut; they are called innate lymphocytes. They have also been implicated in colitis development, IL-17 and IL-22 secretion, and can respond to IL-23, making a case that they probably express IL-23r[74, 98]. Those cells were characterized base on the expression of Sca-1+CD90.2+ (Thy1.2+), but they were not expressing c-kit. As almost all IL-23r+ cells in the gut and spleen of RagKO mice express c-kit, they are evidently not the same cells. On the other hand, in inflammatory conditions, the CD4+CD90.2+CD3-c-kit- innate lymphocytes could be in increased numbers and respond to IL-23 production. There were IL-23r+CD90.2+c-kit- cells in the lungs of RagKO-IL-23r-GFP-Het mice which could be the same CD4+CD90.2+CD3-c-kit- cells seen in the Buonocore article [74]. Another experiment is required to verify whether or not the c-kit+CD90.2+IL-23r+ cells in the lamina propria of the small intestine are CD4+CD3-. Staining specifically splenocytes obtained from IL-23r-GFP-Het and RagKO-IL-23r-GFP-Het mice with Lin(-) staining, CD90.2, c-kit and CD4 could prove whether or not the IL-23r+Lti-like cells identified in this thesis are the same as the innate lymphocytes identified by Buonocore *et al*. As will be discussed later, those c-kit+CD90.2+ Lti-like cells responded to IL-23 by secreting IL-22, but did not produce any IL-17, which is another indication that they are not the same cells described in the Buonocore article, since those cells were described as secreting IL-17 in response to IL-23.

CD3+CD4-CD8-TCRγδ- T-cells can be of different nature, but two main types are known: double-negative T-cells (DNT-cells) and NKT-cells. Both are big contenders for IL-23r+ expression. In the first characterization of the IL-23R-reporter mouse, a small population of DNT-cells were said to express IL-23r, but the data was not shown. NKT-cells are another important contender; another article reported that those cells could respond to IL-23[66]. NKT-cells express both DX5 and CD3; a staining with CD3, CD4, CD8, TCRγδ and DX5 could show whether or not cells can express IL-23r, CD3, and DX5 at the same time. DNT-cells are more probable, because no IL-23r+ cells expressed high levels of DX5. There seemed to be a small population of IL-23r+ cells expressing low levels of DX5, but further characterization is required to be sure. Those CD3+CD4-CD8-TCRγδ- were enriched in the lungs, BM, and the spleen, but seemed to be in low numbers in the LP of the small intestine.

Lti-like cells were not identified in spleens and therefore were not well described and characterized throughout the literature. The roles they could play in immune responses and especially their importance in the spleen remain obscure. However, they must have a specific role in IL-23r signaling; importance of IL-23 in colitis and other disease models in RagKO animals was well described in the previous section of this thesis. All IL-23r+ splenic cells in RagKO-IL-23r-GFP-Het animals are Lti-like cells. Even though DCs, NK cells, and monocytes are found in high proportion in the spleen and have been highlighted before as expressers of IL-23R, it was impossible to find CD11b+IL-23r+, CD11c+IL-23r+ or DX5+CD11b+IL-23r+ cells in the spleen of RagKO animals at steady state. Furthermore, Lti-like cells were the greatest producers of IL-22 following IL-23 stimulation, where IL-22 is a well-known downstream molecule effected by IL-23 signaling. Various publications have shown the importance of IL-22 in either stopping or increasing colitis development.

It is possible that Lti-like cells were not previously identified in the spleen because most reports analysing splenocytes to not treat the spleens with collagenase. Without collagenase treatment, as shown in Figure 14, Lti-like cells cannot be easily identified in the spleen. As not every laboratory systematically treats spleens with collagenase, Lti-like cells can be easily missed. They are also found in very low

numbers. Other organs, such as the lungs and small intestines are always treated with collagenase to extract lymphocytes, so Lti-like cells can be found and characterised.

IL-23r+ cell proportions varied between organs, where the LP of the small intestine clearly contained the highest proportion of IL-23r+ cells. This is of particular interest, as this project all started by identifying IL-23r as a possible locus for predisposition to colitis and therefore gut inflammation. IL-23r expression nicely correlates with the different polymorphisms identified in IL-23r and predisposition to colitis. However, IL-23r was expressed in the lungs, LNs, BMs, thymus, and spleen. In WT mice, IL-23r+ cells were found in higher numbers in organs where there is direct contact with the exterior (such as the lungs and LP), as well as secondary lymphoid organs.

However, in RagKO animals, numbers of IL-23r+ cells were different from WT animals. For example, LP of the small intestine contains higher numbers of IL-23r+ cells; in the absence of T-cells and B-cells, Lti-like cells are increased in proportion, which leads to more cells that can express IL-23r+. However, in the lungs, the proportion of IL-23r+ cells actually decreased in RagKO animals when compared to WT animals. Most IL-23r+ cells in the lungs were T-cells and Lti-like cells were rare. In the absence of T-cells, the proportion of IL-23r+ was decreased in the lungs. In the spleen, IL-23r+ cells were also increased in proportion, probably for the same reasons as the LP of the small intestine.

One of the main missing details of this thesis is IL-23r expression during inflammation. Some preliminary data from our laboratory showed that LPS systemic inflammation did not lead to an increase of IL-23r expression in the IL-23r-GFP-Het mouse. Other experiments which induced inflammatory conditions, such as bacterial infections or colitis induction, need to be done in the IL-23r-GFP-Het mice to understand IL-23r modulation. It is possible that other cell types, such as CD8+T-cells, monocytes, or DC start expressing IL-23r following different inflammatory signals. It would also be interesting to see CD4+T-cells in Th1 and Th17 inflammatory conditions and look at the up-regulation of IL-23r and IL-23 responsiveness during different inflammatory immune responses.

Because of the defective functioning of the IL-12Rβ2 it is impossible to conclude on the possible co-expression of IL-12R and IL-23R at the surface of the different cells examined. However, those "special B-cells" and whatever specific molecules they expressed never expressed IL-23r and responded to IL-12 in a specific way. They will need to be further characterized in the future. A possible comparison of IL-23r+ and "special B-cells" via micro-array, cell culture and stimulation, and cytokine analysis and Ig phenotyping could reveal how those two types of B-cells are different.

IL-12 and IL-23 stimulation of different types of sorted cells is another way that characterization can be indicative of IL-23R and IL-12R expression by those cells. For example, NK cells do not respond to IL-23 and were not found expressing IL-23r in any organs examined. Those two observations agree very well with one-another.

What was not expected from NK cells was their reaction to IL-12. IL-12 did induce INFγ production by NK cells; however, it also seemed to inhibit their proliferation. Many reviews mention that IL-12 could activate NK cells and cause them to secrete INFγ, but also induce NK cells proliferation. When IL-12Rβ2KO NK cells were cultured with IL-12 their proliferation was not inhibited; it actually increased. However, IL-12Rβ2KO NK cells could not produce INFγ upon IL-12 stimulation; this could very well reflect at IL-12-specific action through IL-12Rβ2.

However, IL-12Rβ2KO NK cells proliferate much more than WT NK cells. It was as if IL-12Rβ2 acted as an inhibition feedback loop to control NK cell proliferation. In its absence, NK cells could proliferate without any control. It is possible that upon stimulation with IL-2, NK cells secrete IL-12 (or another cytokine) which can act through IL-12Rβ2 to decrease NK cells proliferation. When IL-12 is added to the culture wells, this decrease of proliferation is even stronger. IL-23 did not seem to interfere in this process, as IL-23 addition to any conditions did not change cytokine secretion or cell proliferation. In this situation, IL-12 had a very specific effect, INFγ production, through its specific receptor using IL-12Rβ2.

On the other hand, Lti-like cells were able to respond both to IL-23 and IL-12. IL-12 stimulation of splenic Lti-like cells induced strong INFγ production, while IL-23 treatment caused only slight INFγ production. However, IL-12 did not induce IL-22 production by Lti-like cells. IL-22 is a hallmark cytokine of IL-23 signaling and was detected in very high amounts in Lti-like cells culture. Lti-like cells could therefore respond to IL-23, as was expected by the analysis of IL-23r-GFP-Het animals, but could also respond to IL-12 production. The combination of IL-2 and IL-23 in LP Lti-like cells even induced more INFγ than IL-12 alone, so Lti-like cells can respond to IL-2 and IL-23 in combination. IL-2 also induced IL-6 and IL-13, cytokines with totally different roles. IL-6 is important for Th1 T-cells development, while IL-13 is predominantly associated with Th2 responses.

Two different types of T-cells were also stimulated with IL-12 and IL-23: CD4+ T-cells and TCRγδ+ T-cells. CD4+T-cells stimulation with IL-12 induced INFγ production, but only in WT CD4+ T-cells. Stimulation of IL-12Rβ2KO CD4+T-cells with IL-12 did not lead to an increase in INFγ production. IL-23, even if highly similar to IL-12, did not impact IL-12 stimulation of CD4+ T-cells. However, there seems to be another receptor through which IL-12 could work. Addition of IL-12 to IL-12Rβ2KO CD4+ T-cells lead to a significant decrease in IL-13 secretion, which was not seen in the absence of IL-12 or in the presence of IL-12Rβ2. Maybe IL-12 affinity for this second receptor is lower than to the functional IL-12R and can only mediate binding to it in the absence of IL-12R. Another clue that IL-12 could bind to another receptor and mediate regulatory function is seen through the addition of IL-12 onto IL-12Rβ2KO CD4+T-cells, where INFγ production is actually reduced when compared to unstimulated cells.

TCRγδ stimulation was also surprising in regards to IL-12 stimulation. Without IL-12 or IL-12Rβ2, INFγ production by TCRγδ T-cells was very high. However, in the presence of IL-12 or IL-12Rβ2, INFγ was greatly decreased, as if IL-12 was a negative regulator of INFγ production by TCRγδ T-cells. NK cells produce high amounts of INFγ following IL-12 stimulation; inhibition of INFγ secretion by TCRγδ T-cells in presence of IL-12 could be a regulatory signal to control INFγ levels.

TCRγδ T-cells could also produce IL-22 in response to IL-23, one of the hallmark cytokines of IL-23. However, there seemed to be some interaction between IL-23 and IL-12Rβ2 for IL-22 production. IL-22 was at its highest when IL-23 and IL-12Rβ2 were present, but IL-12 was absent. Could IL-23 compete with IL-12 to bind to IL-12Rβ2 and induce IL-22? So in the absence of IL-12, there would be no competition for IL-12Rβ2 finding for IL-23 and more IL-22 could be produced.

Another important result obtained from TCRγδ T-cells stimulation with IL-23 was that IL-17 could actually be measured in the cell supernatants, but it could also be measured in response to IL-12 in the presence of IL-12Rβ2. IL-17 secretion is supposed to be a direct downstream effect of IL-23 stimulation; however, in most culture conditions, IL-23 was not able to induce high amounts of IL-17 secretion. Furthermore, IL-23 stimulation of Lti-like cells and CD4+ T-cells did not induce IL-17 secretion, while TCRγδ T-cells produced small amounts. It is possible that TCRγδ are more primed for IL-17 secretion than others, but other cytokines seemed required to induce strong IL-17 secretion by cells, even in the presence of IL-23. It could be important to reconsider IL-17 as a marker of IL-23/IL-23R signaling, as other triggers seem to be required to induce signaling.

All the cytokine stimulation results are very interesting and could indicate that different cells can respond to both IL-12 and IL-23. There are also some indications that IL-12Rβ2 could be important for both IL-12 and IL-23 signaling. IL-12 could also be a negative regulator of INFγ production on TCRγδ T-cells, which seems surprising. However, most cytokine stimulations were done only once or twice; they all need to be repeated in order to make sure the trends seen can be translated into strong data. Another important experiment would be to repeat the stimulation using a IL-23r-GFP-KO mouse, which would allow testing of IL-23 specificity for IL-23R to induce its signaling, but could also indicate if IL-12 can signal through IL-23R receptor, as the KO mouse is deprived of IL-23R at the surface. Different cells from IL-23r-GFP-Het mice could also be used, as IL-23r expression could be tracked through GFP expression. It would be interesting to see if IL-23r can be induced by IL-23, IL-2, IL-12, but also if cells that express it at steady state still express during *in vitro* culture.

In conclusion, IL-23r can be found in steady state animals, without any inflammatory stimulation. IL-23r+ cells are found in greater numbers in the LP of the small intestine, which is highly indicative of a possible role of IL-23R in immune responses in the gut and therefore in the pathogenesis of IBD. The thorough characterization of IL-23r+ cells confirms various results already seen in the literature, such as Lti-like cells responding to IL-23. With the IL-23r-GFP-Het mouse, it was possible to confirm the IL-23r expression in CD4+T-cells, TCRγδ T-cells, and Lti-like cells. However, it also allowed the identification of new IL-23r+ cells, such as B-cells, but also confirmed that NK cells, monocytes, and DCs do not express the IL-23r receptor.

It was extremely disappointing to realize that the IL-12Rβ2+ monoclonal antibody was not specifically binding to IL-12Rβ2. However, the antibody identified a population of B-cells never expressing IL-23r and those cells could respond to IL-12 by producing IgM and IL-27. This population needs to be further characterized.

Specificity of IL-12 and IL-23 action on their respective receptors has always been quite surprising, as both cytokines and receptors are highly similar. The cytokine stimulations showed that some cell types, such as NK cells, can only be activated by one cytokine, IL-12. However, other cell types could react to both IL-12 and IL-23. More surprisingly, the preliminary results in IL-12Rβ2KO mice could indicate that IL-12R can influence not only IL-12 actions, but also IL-23 signaling. The results obtained from cytokine stimulations question the dogma of IL-12 signaling only through IL-12R and IL-23 signaling through only IL-23R. Further characterization of IL-23R and IL-12R in IL-23rKO and IL-12Rβ2KO mice, by cytokine stimulation and RT-qPCR could be informative regarding the relationships between the two receptors.

IL-23R has been highly associated with IBD; however, modulating its effects in the possible treatment of colitis was not always successful. One possibility explaining why IL-23R therapies had mitigated results could be the possible interactions between IL-23R, IL-12R, IL-12 and IL-23, but also IL-27/IL-27R and IL-35/IL-35R, which altogether could be interacting with any therapies targeted against IL-23/IL-23R. To adequately create targeted therapies against IL-23R, it will be important to continue to understand IL-23R biology, its role in intestinal immune responses and in the pathogenesis of IBD.

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Appendix 1

Lamina propria extraction

Solutions required to prepare 1 small or 1 large instestine and ceacum

a) Extraction solution:
    a. For 500mL of RPMI1640, add
        i. Hepes (5 mL)
        ii. Beta-Mercaptoethanol (500uL)
        iii. 5mL of antibiotics (penicillin/streptomycin)
        iv. Do not add FBS
    b. Put 30mL of cold medium in a 50mL tube
    c. This RPMI will be used for all the other solutions
b) Solution 1: 40mL
    a. RPMI1640 (about 28ml)
    b. 12 ml of FBS
    c. 5 mM EDTA (400uL of 0.5M EDTA)
    d. 0.145mg/mL DTT (40uL of 0.145g/mL)
    e. preheated to 37 ° C
c) Solution 2: 2X15mL
    a. Cold RPMI1640 + penicillin / streptomycin (about 15mL)
    b. 2 mM EDTA (60uL of 0.5M EDTA)
d) Solution 3: approximately 50mL for intraepithelial lymphocytes, 15 ml for LP
    a. Cold RPMI1640 + penicillin / streptomycin (approximately 45.5mL)
    b. 3% FBS (19.5mL of 10% FBS)
e) Solution 4: 15mL
    a. Cold RPMI1640 + penicillin / streptomycin (15mL)
    b. 150uL Liberase 5mg/mL
    c. 0.05% DNase (75uL of DNase 4.33mg/mL)
    d. 2 mL of collagenase in PBS 1mL/mg
    e. Do not add the enzymes to the solution
f) Solution 5: 30mL
    a. RPMI1640 + penicillin / streptomycin cold (about 21ml)

b. 3% FBS (FBS 9ml 10%)
c. 0.05% DNase (150uL DNase 4.33mg/mL

Instruments required

1. 2 small Petri dishes
2. 2 medium/large Petri dishes
3. Kitchen strainers (or any type of strainers)
4. Beaker
5. Incubator that shakes
6. Cell strainers (BD): 100um, 70um and 40um
7. Tweezers (tongs)
8. Scissors (for dissection and organ treatment)
9. Centrifuge

Most solutions are put in 15mL or 50mL conic tubes.

A) Isolation of the mesenteric lymph nodes (MLN), small and large intestine and Peyer's patches.

a. Euthanize mice by cervical dislocation. Open the abdomen and expose the intestines.
b. Isolate the mesenteric lymph nodes, if necessary. By moving the small intestine to the left, the GLM will appear as a chain in the fatty tissues.
c. Cut the small intestine at the junction with the pyloric valve at the bottom of the stomach. With tweezers, separate the fat from the intestine to the cecum.
d. Cut the small intestine at the caecum. If the ceacum and the large intestine are required, removed them as well.
e. Put the intestine on a gauze wet with medium.
f. Open the intestine along the length with scissors. Then, using a clamp, delicately empty the intestine.
g. Put some medium in a petri dish. Put the opened gut in the Petri dish and clean.
h. Put the medium in a second Petri dish and clean again.
i. Put the intestine in the tube containing what is left of milieu.

KEEP ON ICE AT ALL TIMES.

B) Isolation of intraepithelial lymphocytes

a. Incubate the intestines on ice for at least 1 hour, up to 5 hours. Two hours of incubation on ice are highly recommended.
b. Cut the intestines into pieces 1 to 3 cm in length with fine scissors.
c. Add the intestines in Solution 1, preheated to 37C. Incubated for 20 min at 37C, with stirring at 500RPM.
d. Place a beaker on ice and put the kitchen strainer on the top of the beaker. Pour the pieces of intestine in a kitchen strainer to filtrate.Shake the strainer several times against the beaker.
e. Using tongs, transfer the pieces of intestine in a 50mL tube. Pour 15 ml of Solution 2.
f. Shake the tube vigorously for 30 seconds.
g. Filter the contents of the tube with the same kitchen strainer into the same beaker.
h. Repeat step e and f.
i. The pieces of intestine will become pink.
    i. Take the pieces of intestines and move to C for the extraction of lamina propria lymphocytes.
    ii. The beaker contains epithelial cells and intraepithelial lymphocytes.
j. Filter the contents of the beaker with a 100um cell strainer placed on the top of a 50mL tube. Rinse the filter with 5 mL of solution 3.
k. Take the filtered solution and strain again through a 70um strainer on top of a 50mL tube. Rinse the filter with a few mL of solution 3.
    i. It will take two 50mL tubes for all intraepithelial lymphocytes.
l. Centrifuge the 50mL conical tubes at 1500RPM for 10 minutes at 4C. Discard the supernatant and retain the precipitate.
m. Resuspend the precipitates in 25 mL of solution 3 in the same tube(transfer the precipitate of one tube into the other one). Filter through a 40um cell strainer.
n. Centrifuge the conical tubes at 1500RPM for 10 minutes at 4C. Discard the supernatant and retain the precipitate.

o. If the cells go to flow cytometry, resuspend in FACS buffer. Staining with CD45 if highly recommended to discriminate between epithelial cells and hematopoietic cells.

C) Isolation of lymphocytes in the lamina propria

a. Place the intestine pieces in a petri dish. Chop the intestines with scissors.
b. Transfer the pieces of pink intestines into the tube containing the solution 4 (without enzyme). Rinse the dish with the solution without enzyme.
c. Add the Liberase, collagenase and DNase.
d. Incubate at 37C for 36 min at 450RPM.
e. Add DNase in solution 5.
f. After digestion, pour the contents of the tube in a 100um filter placed in a petri dish. Add 10 ml of solution 5.
g. Crushed pieces of intestine with a syringe plunger. Filter the cell suspension through the 100um several times. A second 100um filter may be necessary.
h. Take the crushed and filtered tissues and filter through a 70um tube placed on a 50mL tube. Rinse all filters with solution 5.
i. Centrifuge the tube for 10 min for 1500RPM at 4C.
j. Discard the supernatant. Resuspend the precipitate with 10 mL of solution 3 and filtered with a filter placed on a 40um tube 50mL.
k. Centrifuge the tube for 10 min for 1500RPM at 4C.
l. Discard the supernatant. Resuspend the precipitate with 1-3ml of FACS buffer if the cells are going to cytometry. Staining with CD45 if highly recommended to discriminate between the hematopoietic cells and other cells.

Appendix 2

Lung Lymphocyte Extraction

1. Anesthesize mice with isoflurane (*or other anesthetic*)
2. **Lung perfusion**
    1. Prefill all seringes and empty all air from catheters
    2. Expose the abdominal and thoracic compartments
        a. Cut the diaphragm
        b. Cut the ribs on each side of the sternum
        c. Lift the cut ribcage piece
        d. Expose the heart
    3. Carefully insert a 27G needle (plugged into a catheter) into the left ventricle and fix with a clamp.
    4. With scissors, make a tiny incision into the right ventricle (*to your left)* to enable blood flow-out .
    5. Perfuse (through the left ventricle, *on your right*) with 10 mL of **COLD** PBS. If perfusion is OK, the liver and limbs should become pale.
        a. Inject 5mL of COLD saline in the incision previously made in the right ventricule, which is located to your left (using the needle for flowout for the liver perfusion.
            i. The lungs will turn white
        b. Take out the lungs and put in 3mL of **COLD** RPMI medium +1% FBS
            i. Always keep on ice
            ii. If the perfusion worked correctly, the lungs should NOT float when put in PBS. If they do, they should be perfused directly into the lungs.

<u>Lung</u>

a. Cut each lung in little pieces and transfer in a 5 mL polypropylene round-bottom tube, containing 900 µL of **WARM** PBS (37°C).
b. Add 100uL of collagenase V 10X (10mg/mL, Sigma-Aldrich, Cat : C9263-1G).

c. Incubate for 30 min at 37°C.
    a. Vortex the lungs every 10 minutes
d. Add 2mL of COLD complete RPMI :
    1. 500 mL RPMI 1640
    2. 5mL PenStrep
    3. 5 mL HEPES
    4. 50 mL FBS
    5. 500 µL ß-Mercaptoethanol
e. Pipette ups and downs, then transfer solution onto a 70µM cell strainer (BD Biosciences, Cat : 352350)
f. Using a seringe plunger, crush and homogenize lung pieces
    a. Rinse strainer by pipetting to make sure all cells went through.
g. Spin for 7 minutes at 1200 RPM at 4°C.
h. Discard supernatant and resuspend in 5mL of NH_4Cl for erythrocyte lysis.
i. Spin for 7 minutes at 1200 RPM at 4°C. Discard supernatant and resuspend in 1mL FACS buffer and proceed to 1:10 cell count.
j. Cell staining (adding CD45 would be recommended)

Appendix 3

Flow Cytometry Antibodies Used

Antigen	**Antibody Clone**	**Company**	**Fluorochrome**	**Catalog Number**
CD3	17A2	Biolegend Homemade	Alexa 700 Biotin	100215
CD4	GK1.5	Biolegend	Pe-Cy7	100421
CD8	53-6.7	Biolegend	Pe Biotin	100708 100704
CD11b	M1/70	Biolegend	Pacific Blue Biotin	101223 101204
CD11c	N418	Biolegend	Pe-Cy7 Biotin	117317 117304
CD19	6D5	Biolegend	Percp	115532
CD21	7E9	Biolegend	Percp/Cy5.5	123415
CD23	B3B4	Biolegend	Pe	101607
CD43	1B11	Biolegend	Pe-Cy7	121217
CD45.2	104	Biolegend	APC-Cy7 Pacific Blue	109813 109819
CD45R (B220)	RA3-6B2	Biolegend	Pacific Blue	103230
CD49b (DX5)	DX5	Biolegend	APC Biotin	108910 108904
CD138	281-2	BD	Pe	553714
IgD	11-26c.2a	Biolegend	Pacific Blue	405711
IgM	RMM1	Biolegend	Pe-Cy7	406513
IL-12Rβ2	305719	Research and Development	PE APC	FAB1959P FAB1959A
GL-7 (Ly-77)	GL-7	eBioscience	APC	51-5902-80
Gr1	RB6-8C5	Biolegend	Percp Biotin	108425

mPDCA-1	JF05-1C2.4.1	Mylteniy-Biotec	APC	130-091-963
TCRγδ	GL3	Biolegend	Percp/Cy5.5	118117
Ter119	Ter119	Homemade	Biotin	

Streptavidin: PE, Biolegend, Cat # 405203