Université de Montréal

ROLE OF CD4+ T CELLS IN THE REGULATION OF THE IMMUNE RESPONSE AGAINST ENCAPSULATED GROUP B *STREPTOCOCCUS*

par

DAMIAN CLARKE

Département de pathologie et microbiologie

Faculté de médecine vétérinaire

Mémoire présenté à la Faculté de médecine vétérinaire

en vue de l'obtention du grade de

maître ès sciences (M. Sc.)

en sciences vétérinaires

option microbiologie

Août 2012

RÉSUMÉ

Le *Streptocoque* de groupe B (GBS) est un important agent d'infection invasive pouvant mener à la mort et demeure la cause principale de septicémie néonatale à ce jour. Neuf sérotypes ont été officiellement décrits basés sur la composition de la capsule polysaccharidique (CPS). Parmi ces sérotypes, le type III est considéré le plus virulent et fréquemment associé aux maladies invasives graves, telle que la méningite. Malgré que plusieurs recherches aient été effectuées au niveau des interactions entre GBS type III et les cellules du système immunitaire innées, aucune information n'est disponible sur la régulation de la réponse immunitaire adaptative dirigée contre ce dernier. Notamment, le rôle de cellules T CD4+ dans l'immuno-pathogenèse de l'infection causée par GBS n'a jamais été étudié. Dans cet étude, trois différents modèles murins d'infection ont été développé pour évaluer l'activation et la modulation des cellules T CD4+ répondantes au GBS de type III : *ex vivo, in vivo,* et *in vitro*. Les résultats d'infections *ex vivo* démontrent que les splénocytes totaux répondent à l'infection en produisant des cytokines de type-1 pro-inflammatoires. Une forte production d'IL-10 accompagne cette cascade inflammatoire, probablement dans l'effort de l'hôte de maintenir l'homéostasie. Les résultats démontrent aussi que les cellules T sont activement recrutées par les cellules répondantes du système inné en produisant des facteurs chimiotactiques, tels que CXCL9, CXCL10, et CCL3. Plus spécifiquement, les résultats obtenus à partir des cellules isolées T CD4+ provenant des infections *ex vivo* ou *in vivo* démontrent que ces cellules participent à la production d'IFN-γ et de TNF-α ainsi que d'IL-2, suggérant un profil d'activation Th1. Les cellules isolées T CD4+ n'étaient pas des contributeurs majeurs d'IL-10. Ceci indique que cette cytokine immuno-régulatrice est principalement produite par les cellules de l'immunité innée de la rate de souris infectées. Le profil Th1 des cellules T CD4+ a été confirmé en utilisant un modèle *in vitro.* Nos résultats démontrent aussi que la CPS de GBS a une role immuno-modulateur dans le développement de la réponse Th1.

En résumé, cette étude adresse pour la première fois, la contribution des cellules T CD4+ dans la production d'IFN-γ lors d'une infection à GBS et donc, dans le développement d'une réponse de type Th1. Ces résultats renforcent d'avantage le rôle central de cette cytokine pour un control efficace des infections causées par ce pathogène.

Mots-clefs : Groupe B *Streptococcus*, cellules T CD4+, réponse Th1*,* capsule polysaccharidique, cytokines, IFN-γ, CD69, souris, *ex vivo*, *in vivo*, *in vitro*.

ABSTRACT

Group B *Streptococcus* (GBS) is an important agent of life-threatening invasive infections and remains the leading cause of neonatal sepsis to this day. Nine serotypes have been officially described based on capsular polysaccharide (CPS) composition. Among them, capsular type III is considered one of the most virulent and frequently associated with severe invasive diseases, such as meningitis. Although extensive research has been done on the interactions between GBS type III and various cells of the innate immune system, no information is available on the regulation of the adaptive immune response against this pathogen. In particular, the role of CD4+ T cells in the immuno-pathogenesis of the infection caused by GBS has never been assessed. In this study, three different models of murine infection were developed to evaluate activation and modulation of responding CD4+ T cells against GBS type III: *ex vivo, in vivo,* and *in vitro*. *Ex vivo* analysis of total splenocytes showed that GBS induces the release of type-1 pro-inflammatory cytokines. A strong IL-10 production follows this inflammatory cascade, indicating the host effort to maintain homeostasis. Results also indicate that T cells were actively recruited by responding innate immune cells via the release of chemotactic factors such as CXCL9, CXCL10, and CCL3. More specifically, results obtained from isolated CD4+ T cells from *ex vivo* or *in vivo* infections showed that they actively participate in the production of IFN-γ and TNF-α, as well as IL-2, suggesting a Th1 profile of activation. On the other hand, isolated CD4+ T cells were not main sources of IL-10. This observation suggests that this immuno-regulatory cytokine is produced mainly by cells of the spleen innate immune system of infected animals. The CD4+ Th1 cell profile was confirmed using an *in vitro* model of infection. Our results also suggest that the GBS CPS plays an immuno-modulatory role in the development of a Th1 response.

In summary, this study addresses for this first time the contribution of CD4+ T cells in IFN-γ production during GBS infection, and thus, in the development of a Th1 response. Our data further highlight the central role of this cytokine for effective control of GBS infections.

Keywords: Group B *Streptococcus*, CD4+ T cells, Th1 response, capsular polysaccharide, cytokines, IFN-γ, CD69, mice, *ex vivo*, *in vivo*, *in vitro.*

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LIST OF ACRONYMS AND ABBREVIATIONS

AAP	American Academy of Paediatrics
ACOG	American College of Obstetricians and Gynaecologist
ACP	Alpha C Protein
Ag	Antigen
AP	Activator Protein
APC	Antigen Presenting Cell
BBB	Blood Brain Barrier
BCR	B Cell Receptor
β-H/C	Beta Hemolysin/cytolysin
BM	Bone Marrow
BMEC	Brain Microvascular Endothelial Cells
BP	Backbone Protein
BSA	Bovine Serum Albumin
CAMP	Christie, Atkin, Murch-Peterson
CARD	Caspase activation recruitment domain
CD	Cluster of Differentiation
CDC	Center for Disease Control
CD33rSiglecs	CD33-related Siglecs
CFU	Colony Forming Units
CLIP	Class II-associated Invariant-chain Protein
CLP	Common Lymphoid Precursor
CLR	C-type Lectin Receptor
CMP	Common Myeloid Precursor
CNS	Central Nervous System
COX	Cyclooxygenase
CPS	Capsular Polysaccharide
CR	Complement Receptor
CSR	Class Switch Recombination
CTLA	Cytotoxic T-Lymphocyte Antigen

DAG	Diacylglycerol
D-Ala	D-Alanine
DC	Dendritic Cell
DNA	Deoxyribonucleic Acid
ECM	Extracellular Matrix
ELISA	Enzyme linked immunoabsorbent assay
EOD	Early-onset Disease
ERK	Extracellular signal-regulated Kinase
FLT	Fms-related Tyrosine Kinase
Gal*p*	Galactose
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GAS	Group A *Streptococcus*
GBS	Group B *Streptococcus*
GITR	Glucocorticoid-induced tumour necrosis factor receptor related protein
Glc*p*	Glucose
Glc*p*NAc	N-acetylglucosamine
GM-CSF	Granulocyte-Macrophage Colony Stimulating Factor
GPI	Glycosylphosphatidylinositol
GTP	Guanosine Triphosphate
IAI	Intra-Amniotic Infection
ICAM	Intracellular Adhesion Molecule
ICOS	Inducible Co-stimulatory Protein
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inductible Nitric Oxide Synthase
IP$_3$	Inositol-1,4,5-triphosphate
IP-10	Interferon-inducible Protein-10
IRF	Interferon Regulatory Factor
ITAM	Immunoreceptor Tyrosine-based Activating Motif
ITIM	Immunoreceptor Tyrosine-based Inhibitory Motif

JAK	Janus Kinase
JNK	c-Jun N-terminal Kinase
LC	Langerhans Cell
Lmb	Laminin-binding protein
LOD	Late-onset Disease
LPS	Lipopolysaccharide
LTA	Lipoteichoic Acid
MAPK	Mitogen-Activated Protein Kinase
MCP-1	Monocyte Chemotatic Protein-1
MHC	Major Histocompatibility Complex
MIIC	MHC class II-rich Compartments
MIP-1α	Macrophage Inflammatory Protein-1α
MyD88	Myeloid Differentiation Primary Response Protein-88
Neu5Ac	N-acetylneuraminic acid/Sialic acid
NFAT	Nuclear Factor of Activated T cells
NF-κB	Nuclear Factor kappa-light-chain-enhancer of activated B cells
NK	Natural Killer cell
NKT	Natural Killer T cell
NLR	NOD-like Receptor
NLRP3	NLR family pyrin domain containing protein-3
NO	Nitric Oxide
NOD	Nucleotide-binding Oligomerization Domain
PAMP	Pathogen-Associated Molecular Pattern
PBP	Penicillin-Binding Protein
PBS	Phosphate Buffer Saline
PCR	Polymerized Chain Reactions
pDC	Plasmacytoid Dendritic Cell
PG	Peptidoglycan
PGE2	Prostaglandin E2
PIP$_2$	Phosphatidylinositol-bis-phosphate
PI3K	Phosphoinositide-3 Kinase
PRR	Pattern Recognition Receptor

PRU	Polysaccharide Repeat Units
RANTES	Regulated on Activation Normal T cell Expressed and Secreted chemokines
Rha*p*	Rhamnose
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
Siglecs	Ig Super-family lectins
SLC	Secondary Lymphoid Chemokine
STAT	Signal Transducer and Activator of Transcription
STSS	*Streptococcal* Toxic Shock Syndrome
TCR	T Cell Receptor
TD	T cell Dependant
T_{FH}	Follicular Helper T cells
TGF-β	Transforming Growth Factor-β
Th1/2/17	T helper cell type 1/2/17
TI	T cell Independant
TLR	Toll-like Receptor
TNF	Tumor Necrosis Factor
TRAF	TNF Receptor Associated Factor
Treg	Regulatory T cell
TRIF	TIR-domain-contaning adapter-inducing Interferon-β
TSLP	Thymic Stromal Lymphopoietin
UTI	Urinary Track Infection
VLA	Very Late Antigen
WT	Wild Type

ACKNOWLEDGMENTS

I – INTRODUCTION

Group B *Streptococcus* (GBS) or *Streptococcus agalactiae* is a Gram-positive β-hemolytic encapsulated bacterium and is the main cause of life-threatening invasive infections in pregnant women and newborns in North America and Western Europe (133). Recently, however, GBS is becoming increasingly associated with invasive disease in non-pregnant adults, in particular the elderly and individuals with underlying chronic illnesses (83). Clinical manifestations of GBS infection are mainly associated with the development of pneumonia, septicemia, and meningitis. Although several strategies have been implemented to reduce the prevalence of GBS infections in certain parts of the world, GBS remains a serious cause for concern in health care (195).

As mentioned, GBS is a encapsulated bacterium that possesses a think capsular polysaccharide (CPS) shell. This CPS is widely considered as the most important virulence factor and is the basal unit used for serotyping. Officially, nine GBS serotypes have been described (Ia, Ib, II-VIII) as well as a recently proposed serotype IX, all of which are capable of causing human infection (83, 236). From these ten serotypes, serotype III is not only one of the most virulent but also the most commonly isolated type from cases of meningitis (133). The structure of most GBS CPS share similarities across different serotypes such as they are formed by different arrangements of the monosaccharides glucose, galactose and *N*-acetylglucosamine assembled into repeated sequences with a molecule of sialic acid (Neu5Ac) at the terminal position. Interestingly, virtually all mammalian epithelial cells also widely display sialic acid on their surface and serve in the recognition of self by various cells of the immune system (54). It has therefore been suggested that sialic acid at the terminal position on the CPS might be involved in immune evasion as a result of molecular mimicry of host epitopes and/or by decreasing access of host pattern recognition receptors to cell wall components hidden beneath the CPS (73). In this regard, much worked has thus focused on the interactions of GBS and cells of the innate immune system such as monocyte/macrophages, neutrophils and dendritic cells (DCs), as well as the effects of CPS on these interactions. However, the relative ability of encapsulated GBS vs non-encapsulated strains to induce pro-inflammatory events by the aforementioned cells is conflicting (42, 73, 142, 157, 266).

DCs are of particular importance in immunity as they are recognized as the most powerful antigen-presenting cells (APCs) that initiate immune responses against pathogens and are considered an essential link between innate and adaptive immunity. In addition, they are an

important player in T cell activation, the central theme in this project. Before priming T cells, DCs capture and process antigens, and under an infected state, will undergo a maturation process. As APCs are the first responders at the infection site, their activation profiles can largely dictate the ensuing adaptive immune response. Studies on DCs and GBS have revealed that encapsulated GBS uses different endocytic routes to gain entry to DCs and, seems to circumvent bactericidal DC functions by a yet unidentified mechanism (143, 160). Consequently, GBS phagocytosis was shown to be significantly required for optimal DC production of IL-12p70, IL-6, IL-10, IL-1β, TNF-α, CCL2, CXCL1 and CXCL10. In contrast, GBS induces increased expression of MHC-II and the co-stimulatory molecules CD40 and CD86 in a phagocytosis-independent manner. The GBS CPS seems to differentially affect these responses.

These observations are of particular importance when considering T cell activation as it has been demonstrated that the route of bacterial uptake may influence the repertoire of epitopes presented to T cells and, consequently, the ensuing T cell-mediated response (261). However, in any case, after activation DCs will provide T cells with three important signals: T cell receptor stimulation, co-stimulation and cytokine stimulation. These signals are in turn pivotal for T cell activation. Based on the signals received from the APC, CD4+ T cells differentiate into several distinct phenotypes, such as Th1, Th2, Th17 and regulatory T cells (Treg). Each phenotype is described as having particular functions and is associated with the release of signature cytokines. For example, Th1 cells are described as pro-inflammatory cells and release IFN-γ whereas Th2 cells participate in the humoral response by releasing IL-4. Th17 cells are associated with pro-inflammatory response via the production of IL-17. Lastly Treg cells have been described as important mediators of immune regulation after the clearance of infection by producing, for exemple, high amounts of IL-10.

In spite of the large number of studies on GBS interactions with phagocytes and the few recently reports on DC modulation by this pathogen, there is currently no information available on the status of T cell activation during GBS infection. Several *in vivo* studies with animal models have demonstrated a crucial role of IFN-γ in the immuno-pathogenesis of GBS disease. For instance, IFN-γ has been related to enhanced resistance to GBS infection by controlling GBS dissemination in the host (62). However, IFN-γ production can also be detrimental if not appropriately controlled by IL-10 production (61). Nevertheless, the cellular source of this IFN-γ response remains unknown.

Based on these observations, we hypothesized that GBS infection induces CD4+ T cell differentiation into a Th1 phenotype with concomitant release of large amounts of IFN-γ. Based on the aforementioned effects of GBS CPS modulation of DC functions, it is further hypothesized that this major virulence factor influences the T cell response as well.

The general objective of this thesis was to evaluate CD4+ T cell activation in response to encapsulated GBS infection. To this aim, three different approaches were developed to study CD4+ T cell behaviour: *ex vivo, in vivo* and *in vitro* models of infection. Consequently, three specific objectives were proposed:

1. To provide contextual understanding of GBS-induced T cell activation by analyzing *ex-vivo* total splenocyte responses.

2. To evaluate in vivo the cytokine profile of CD4+ T cells during GBS infection.

3. To develop an *in vitro* DC-T cell co-culture model to evaluate the effects of GBS CPS on CD4+ T cell activation and differentiation.

Within this document we show for this first time a specific engagement of CD4+ T cells against GBS via the production of a Th1 inflammatory response, in particular IFN-γ. As CD4+ T cells are known to be crucial mediators for the development of adaptive immunity and memory immunity, understanding the type of immune response engaged by these cells in the context of GBS infection may provide novel insights for the development of vaccines or improved therapeutic strategies.

II – SCIENTIFIC LITTERATURE REVIEW

1. Group B *Streptococcus*

1.1 History and epidemiology

Group B *Streptococcus* (GBS), was first identified by Lancefield in 1933 in a publication which described the serological difference between various hemolytic streptococci strains (138). GBS was known to be an important pathogen that caused bovine mastitis at the time, but was only described as a human pathogen five years later by Lancefield and Hare (139).

Shortly after being identified as a human pathogen, clinical studies on GBS were performed. Due to the lack of information on incidence and prevalence of GBS infections in humans at the time, it remained mostly studied in dairy cattle than in humans for its veterinary and economic importance (6, 94). Work on GBS infections in human thus slowly continued until a series of studies released in the 1970's reported an important emergence of neonatal death related to this particular pathogen (6, 19, 92). A mortality rate of 55% was calculated using medical reports between 1962 and 1976 in neonates infected with GBS rendering it the leading cause of serious bacterial infections in newborns (6).

These shocking statistics resulted in extensive work on GBS infection prevention due to its newly recognized relevance in human disease. In the 1980's, a publication released in the *Journal of Infectious Diseases* by Boyer *et al*. calculated that GBS was found in up to 30% of women's vaginal and anorectal flora (32). This group of patients, considered as at-risk women, was thus selected for clinical trials of antibiotic therapy that consisted of administering intrapartum antibiotics, such as penicillin and ampicillin. These treatments proved to be very efficient in preventing GBS related disease during the newborn first week (78). This finding was, however, somewhat alarming as the neonatal mortality rate was still quite elevated at 10-15% but could be preventable (271). This resulted in the collaboration of health care providers, researchers, parent advocacy groups, the public health community and professional organizations to establish specific GBS prevention protocols.

It wasn't until 1991, however, that such protocols related to screening, diagnosis and prevention of neonatal GBS were presented and recommended to the medical community (128). These initial guidelines developed by Katz *et al*. were proven to be extremely effective, and

consisted primarily of antepartum GBS screening to all pregnant women who attended clinics at the University of North Carolina Hospitals and intravenous antibiotic treatment during labour. In the first two years of their implementation, this group delivered 1681 women identified as positive GBS carriers and claimed to observe no infants infected with this pathogen and no adverse reactions or complications among women who were treated with antibiotics (128). Interestingly, these results were not published until 1994 during which other organisations were developing in parallel their own neonatal GBS protocols and were thus never officially adopted by the medical community. Furthermore, it was later shown by these same organisations that the 100% success rate published by Katz *et al*. could not be correct (22).

Thus, in 1996, the American College of Obstetricians and Gynaecologists (ACOG), the Center for Disease Control (CDC), and 1 year later the Amercian Academy of Pediatrics (AAP), presented their own recommended protocols for intrapartum prophylactic antibiotic administration to GBS positive pregnant women for prevention of vertical transmission to the foetus (67). Candidates for this therapy were selected based on risk factors for GBS or by positive screen test for GBS (22, 35). Shortly after the adaption of the 1996 guidelines, the CDC released a report indicating that the mortality rate of neonates due to GBS disease had dropped to 5% although it continued to be the leading cause of neonatal sepsis (45).

This prompted the CDC to revise the adopted guidelines and protocols in 2002 in the hope to further reduce neonatal GBS sepsis. Both the 1996 and 2002 guidelines share many similarities due to the proven efficiency of the 1996 protocols in reducing neonatal GBS sepsis. However, the biggest changes between the two protocols were made to the screening and treatment process. For example, practitioners were now recommended to screen all pregnant women between 35-37 weeks of gestation, and provided with an updated treatment for women allergic to penicillin (46). The implementation of these newly revised protocols was followed by an important decrease in the incidence of neonatal GBS disease which was estimated at 0.32 cases per 1000 births in 2003 in the USA (47).

The 90's were also a time period where certain researchers shifted their interest to GBS related disease in non-pregnant adults due to surveillance studies reporting an increase of invasive GBS disease in this population by 2 to 4 times (2.4 to 4.4 cases per 100 000 adults) compared to the two previous decades (228). It was estimated that among persons aged 15 through 64 years, disease incidence increased from 3.4 per 100 000 population in 1999 to 5.0 per 100 000 in 2005 (X^2_1 for trend, 57; P < 0.001), a relative increase of 48% (95% CI, 32%-65%). Among adults of 65 years or older, incidence increased from 21.5 per 100 000 to 26.0 per 100 000 (X^2_1 for trend, 15; P < 0.001), a relative increase of 20% (95% CI, 8%-35%) (195).   Currently, most infections are unrelated to pregnancy, and the majority occur in adults >65 years of age. This demography now accounts for more than 2/3 of cases of invasive GBS disease and more the 50% of all death attributed to it (83). This trend can be partly explained by a growing number of patients with predisposing factors, in particular, chronic medical conditions such as diabetes mellitus and compromised immunity. The following figure resumes the incidence and death rates of invasive GBS in 2005 of different age and ethnic groups (195).
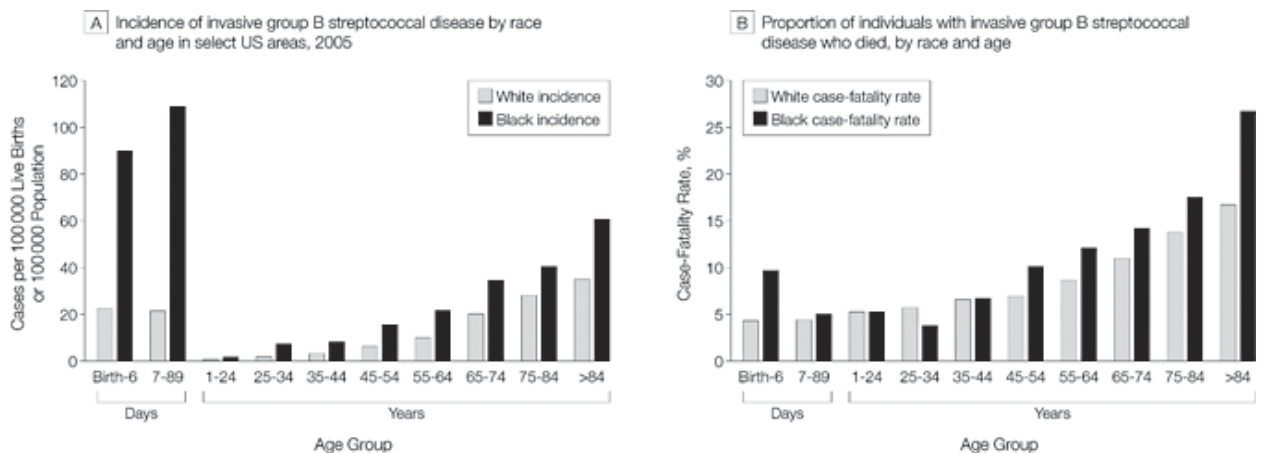
Figure 1: Incidence of invasive Group B streptococcal disease and proportion of individuals who died, by race and age in select US areas, 2005. Adapted from Phares *et al*., JAMA. 2008, 299(17): 2056-2065 [195]

1.2 Pathogen characteristics and serotyping

1.2.1 Characteristics of GBS

GBS is a Gram-positive encapsulated diplococcus bacterium whose basic ultrastructure is similar to other Gram-positive cocci such as streptococci, staphylococci and *Listeria*. The term hemolytic *Streptococcus*, a term often used to describe GBS and other members of this family, was coined from its ability to completely destroy red blood cells when grown on sheep blood agar. Colonies of such bacteria have a characteristic appearance being surrounded by zones of transparent or light yellow colour due to hemolysis of red blood cells.

As previously mentioned, GBS is found in the genital and gastrointestinal track of approximately 30% of women and 20% of men without causing disease (163). Adaptation to coexistence implies that selection pressure on either site of the GBS–host interface has promoted mechanisms through which host immunity can discriminate between colonization and invasion (266). This would also imply that GBS can also rapidly adapt to different environmental changes such as pH, osmolarity, temperature, and chemical concentrations when transitioning from asymptomatic colonization to invasive infection. This has been demonstrated in recent studies by comparative transcriptome analysis of GBS in different growth conditions such as Todd Hewitt-yeast extract medium and human amniotic fluid, highlighting the importance of specific genes which mediate metabolism of amino acids, carbohydrates and nucleotides. The majority of the observed changes in transcripts affects genes involved in basic bacterial metabolism and is connected to growth environment composition and nutritional requirements of the bacterium (234).

In addition to the Lancefield B antigen (Ag), GBS can be further categorized into smaller serologically distinct sub-groups by exploiting structural and chemical differences of the capsular polysaccharide (CPS). This constitutes the base of the serotyping system for this particular pathogen.

1.2.2 Capsular serotyping

Nine different serotypes of GBS have been identified and completely characterized: Ia, Ib, and II-VIII. Although antigenically distinct from one another, striking similarities exist between the

nine classes of CPSs. The constituent monosaccharides included in the various capsular polysaccharide repeat units (PRUs) are conserved, including the presence of *N*-acetylneuraminic acid (Neu5Ac or sialic acid) residues at terminal position on side chains in all serotypes (54). Indeed, glucose (Glc*p*), galactose (Gal*p*), and Neu5Ac are found in all nine characterized capsule serotypes. Except in types VI and VIII, *N*-acetylglucosamine (Glc*p*NAc) is also present. Rhamnose (Rha*p*) occurs only in the type VIII repeating unit (Figure 2). Thus, the difference in chemical (monosaccharide) composition of the PRUs and their polimerization into PRUs are what serologically differentiate GBS into nine classes. Figure 2 better illustrates these variations between PRUs and their corresponding GBS serotype.
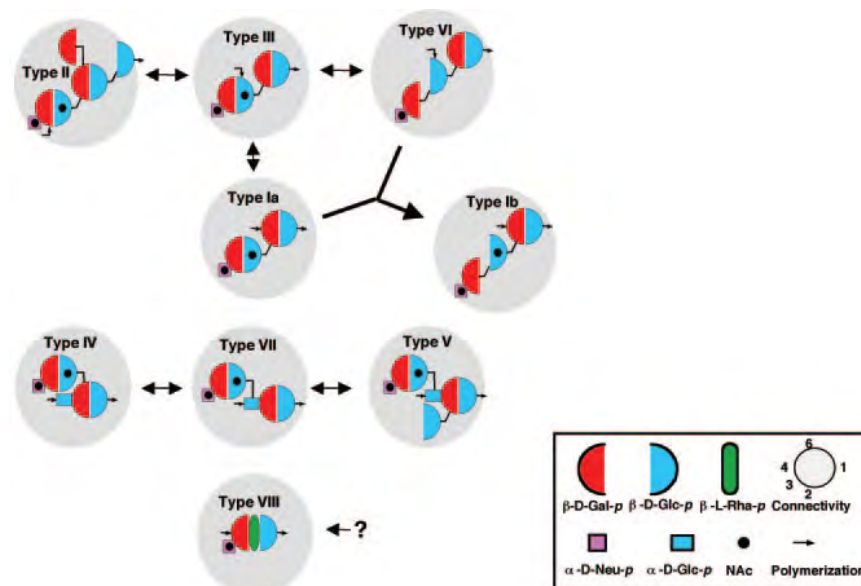
Figure 2: Polysaccharide repeat unit (PRU) structures of Group B *Streptococcus*. Arrows represent potential relationships between PRUs. In the figure, each individual monosaccharide is represented by simple shapes, the acetylation of sugars is represented by a black dot within the shapes, and glycosidic linkages between individual monosaccharides is represented by a circular assignment system. Adapted from Cieslewicz *et al.,* Infect. Immun. 2005, 73(5): 3096-3103 [54]

The terminal side chain residue Neu5Ac (sialic acid) α2,3 linked to Gal found in all nine PRUs is of particular interest because their configuration is identical to the predominant sialic acid

found on human cells (149). It is therefore perhaps not surprising that many pathogenic bacteria have evolved to incorporate sialic acid in their CPS as this could confer important phenotypes regarding their ability to resist the host immune response via molecular mimicry and also their ability to interact specifically with different host cells (229). It has also been observed that GBS can modify its capsular sialic acid by the enzyme *O*-acetyltransferase which adds an *O*-acetyl to the seventh carbon position of sialic acid (149). Despite the potential of sialic acid *O*-acetylation to affect pathologic processes (immunogenicity, polysaccharide epitopes, among others) little is known of its role at the host-pathogen interface (149).

In order to further explain variations between PRUs of the different serotypes, we will focus on the genetic element that codes them: the *cps* locus (Figure 3). The general organisation of this locus is very similar across the different GBS serotypes. The 5' region contains genes involved in CPS expression and chain length regulation, the 3' region contains gene responsible for sialic acid synthesis, and the central region contains the PRU oligosaccharide structural and polymerization genes (50).
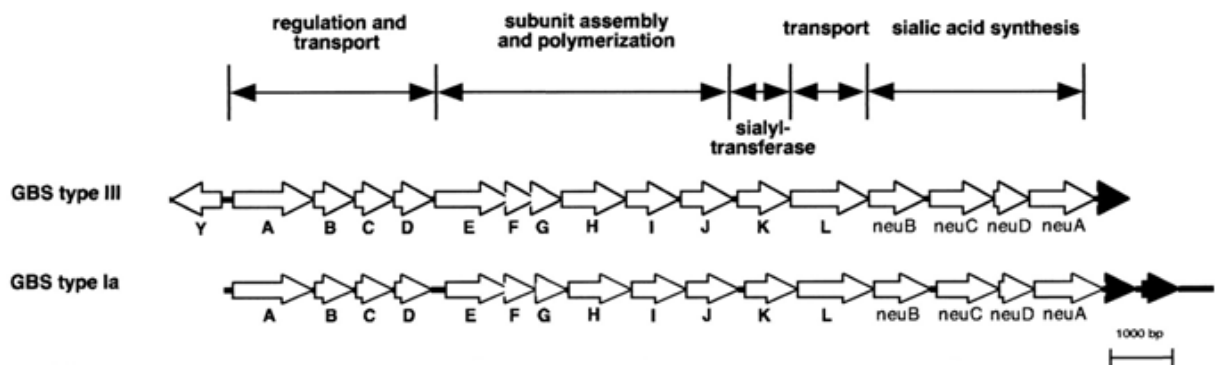
Figure 3: Schematic representation of the organization of the CPS synthesis loci of GBS type III and GBS type Ia. Adapted from Chaffin *et al.,* J. Bacteriol. 2000, 182(16): 4466-4477 [50]

In the figure above, we provide a comparative example of the *cps* loci found in GBS type III and type Ia. At first glance, the genetic organizational similarities between loci of different strains are remarkably similar. Genes from *cpsA* to *cpsD* region of the *cps* locus are involved in expression regulation and transportation of the CPS subunits. The role of transport to the surface of the

bacteria is attributed to *cpsL*. Genes contained within the *neuB* to *neuA* region are involved in sialic acid synthesis and post-synthesis modifications, with a particular importance of *neuA* and *neuD* genes in *O*-acetylation of sialic acid (148, 149). The *cpsK* gene, which was described to possess *N*-acetyltransferase activity, catalyses the linkage of sialic acid to Gal via α2,3 link as a terminal side chain visible in each serotype of GBS (51). These genes, of critical importance in GBS, are highly conserved among the different serotypes and homologues can even be identified in different families of bacteria. For example, 18 species of bacteria were found to possess homologues to *neuD*, due to its central role in sialic acid biosynthesis (149). This same study also found with complementation analysis that *Escherichia coli* K1 *neuD* also functions as a sialic acid *O*-acetyltransferase in GBS, highlighting the homology between *cps* genes in different species (149).

The most variable part of the *cps* locus is contained within the *cpsE* to *cpsJ* region of the operon. Figure 4 below illustrates this variance. Genes found within this region of the locus possess a glycosyltransferase enzymatic activity meaning that they are responsible for catalyzing the specific sugar linkages between monosaccharides of the PRUs, thus defining CPS composition and structure (50). They also vary considerably in both numbers and sequence. In many encapsulated bacteria, genes conserved across diverse capsular serotypes flank genes encoding enzymes unique to a specific capsular serotype. This particularity of the *cps* locus is likely due to genetic horizontal transfers and recombination events. This hypothesis is supported by analysis of the pneumococcal CPS which account for over 90 serotypes and may reflect a higher frequency of exogenous DNA acquisition through natural competence in pneumococci (54).
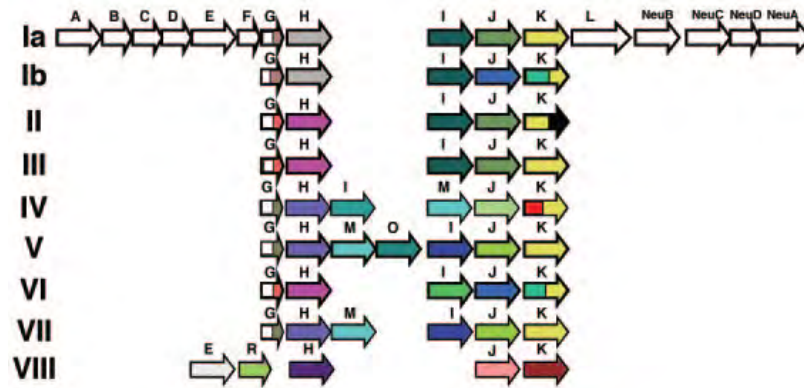
Figure 4: Sequence analysis of all nine GBS capsular serotypes. CpsA to -E and CpsL as well as NeuB, -D, -A, and -C are conserved in all nine serotypes and are shown only in the type Ia capsule cluster. The color inside each arrow indicates the degree of similarity of the amino acid sequence to those encoded by other open reading frames. A gap was introduced between CpsH and CpsI to permit an alignment of corresponding open reading frames. Adapted from Cieslewicz _et al.,_Infect. Immun. 2005, 73(5): 3096-3103 [54]

The grand majority of GBS isolates can be classified according to these serotypes characteristics, although 4 to 7% remain non-typeable. Several hypothesis might explain this observation such as mutations within the _cps_ locus, reversible nonencapsular phase variation or simply can be due to an uncharacterized CPS. In this regard, a recent study proposed a new, provisional "GBS serotype IX," which may have evolved as a result of mutation and/or recombination between serotype Ib and serotype V and/or IV (236). Non-typeable GBS isolates are actually an increasing problem nowadays and extensive studies at the genetic and molecular level have been, and continue to be performed in an attempt to counteract this. As a matter of fact, molecular serotyping of GBS based on detection of serotype specific genes of the capsular region have been rapidly developed, and in certain cases, have now replaced traditional antiserum-based typing methods. PCR and pulsed-field gel electrophoresis have shown that certain non-typeable GBS isolates possess strong similarities to defined serotypes (206).

1.2.2.1 Serotype distribution

GBS serotype distribution tends to vary slightly among the different risk groups although tendencies can be observed. In non-pregnant adults and the elderly infected with GBS, type Ia, III, and V are isolated more than 70% of the time, with an interesting emergence of type V as one of the most commonly CPS types recovered (approximately 25% of cases) from this population (83, 228). In infants, serotype distribution changes slightly with types Ia, II and III isolated in 85% of cases (230). If we divide these infections according to the early-onset disease (EOD) and late-onset disease (LOD) definitions (see section 1.3.2), type Ia is isolated in more than 30% of cases and type III in more than 50% of cases respectively (230). Perhaps most striking is that the serotypes that most affect infant are the same ones most commonly isolated from the genitourinary of colonized women proposing an interesting correlation between the two (17). In all cases, however, serotype III is the most common cause of meningitis (184).

Worldwide, serotype distribution of invasive and colonization strains of GBS vary slightly from country to country and is continuously evolving and changing demonstrating not only a regional but also temporal variation. For example, in the USA and European countries types Ia, Ib, II, III, and V are reported to cause over 85% of all GBS related disease, compared to Japan where over 40% of isolates are types VI and VIII (228). As well, a Danish study revealed an increasing importance of serotype VIII in causing invasive disease in this country (228). Due to its recent discovery, no data is yet available on the distribution of serotype IX (236).

1.3 Disease and clinical manifestations

GBS can cause a plethora of medical conditions in infected individuals such as bacteremia, septicemia, pneumonia and meningitis, among others clinical manifestations. Symptoms and disease manifestation by GBS greatly depends on the individual age, health status, sex and race. These criteria will be used to define risk groups who will make up the sub-sections of this passage in order to facilitate description of GBS disease.

1.3.1 Pregnant women

The severity of GBS disease greatly varies in pregnant women going from mild urinary tract infections (UTI) to life threatening bacteremia. The isolation of GBS from urine, often with low bacterial count, complicates up to 7% of pregnancies, of which 70% are asymptomatic (178). Bacteremia on the other hand, is described as the isolation of pathogens from usually sterile sites such as the blood stream or cerebrospinal fluid, and occurs in 31 to 35% of maternal GBS infections (178). Subsequently, 5 to 25% of these bacteremia cases progress to sepsis while septic shock is rarely observed. Although puerperal sepsis remains a major cause of maternal mortality, the incidence of death related to GBS sepsis is estimated at 0-3% in obstetric patients in contrast to 10 to 81% in non-pregnant adults. Other illnesses, albeit less common, have also been associated to GBS infection such as vulvovaginitis, intra-amniotic infection, mastitis, meningitis, endometritis, osteomyelitis, endocarditis, and post operative wound infections (178, 224, 230).

1.3.2 Neonates and Infants

As mentioned above, GBS disease in neonates and infants is divided into two separate and distinct categories: early-onset disease (EOD) and late-onset disease (LOD). As their names clearly indicate, the difference between the two categories is defined by the age of the child at time of infection and is accompanied by different sets of symptoms and syndromes.

1.3.2.1 Early-onset disease

EOD accounts for 75% of neonatal GBS cases and is defined as to happen within the first 7 days of age, although most cases are evident in the first 24 h of life (184). In these clinical cases, GBS is transferred vertically from the mother to the infant in two major ways. The first transmission mechanism is called Intra-Amniotic Infection, or IAI, and is characterized by the presence of pathogen in placenta and other membranes after an ascending spread from the vagina. The incidence of IAI based on clinical diagnosis is approximately 1 - 2% of all term deliveries with GBS found in 15.4% of the total number making it one of the most frequently isolated species in infected newborns delivered of mothers with IAI (178). The risk for IAI increases with the duration of the membrane rupture although GBS has been isolated from intact membranes as well (178). The second transmission mechanism occurs at birth by aspiration of infected vaginal fluids during the passage of the infant through the birth canal. Disease resulting

from such transmission most often presents itself as a strong pneumonia and respiratory failure which has the potential to quickly progress to bacteremia and septic shock if not diagnosed and treated properly (157).

1.3.2.2 Late-onset disease

Although the definition of LOD is much broader than EOD, it generally occurs in infants between the ages of 3 weeks and 7 months, and is associated with a very different pathogenesis. As opposed to EOD, transmission of the pathogen from host to host is by horizontal mechanisms and is much more gradual in its progression. This can in part be explained by the fact that the entry route into host is not as direct and thus bacterial loads and exposure times are smaller requiring GBS more time to establish itself within the host. Resulting manifestations by GBS is this setting is less associated with lung disease and usually leans towards bacteremia and meningitis (approximately 50% of cases) (195). Susceptibility of these infants to meningitis is higher due to an underdeveloped blood brain barrier (BBB) which GBS can readily penetrate (83). Thirty to 50% of GBS meningitis survivors will develop permanent neurological damages such as hearing deficits, mental retardation, cortical blindness and seizures (230). Many other less frequent neurological complications have also been described such as dementia, hydrocephalus, cerebral infarction, cerebral venous thrombosis, and brain abscess (178).

1.3.3 Adults and elderly people with compromised immunity

After the implementation of the CDC guidelines to successfully reduce neonatal GBS related sepsis, adults and elderly people with compromised immunity, especially those with underlying medical conditions became the number one risk associated with the highest morbidity and mortality rates related to GBS infection. Manifestation of GBS disease in this group expresses itself in a large variety of ways (Table 1).

Table 1: Clinical diagnosis for non-pregnant adults with invasive Group B streptococcal infection (From Edwards *et al*., Clin. Infect. Dis. 2005, 41(6): 839-847)[83]

Underlying condition	Younger adults ($n = 82$)	Older adults ($n = 83$)
Skin or soft-tissue infection	++++	++++
Urinary tract infection	+	++++
Pneumonia	++	+++
Bacteremia with no identified focus	+++	+++
Arthritis	+++	++
Osteomyelitis	++	+
Meningitis	+	+
Endocarditis	+	+
Peritonitis	+	+
Vascular catheter–associated infection	+	+
Postoperative infection	+	+

NOTE. Data are from studies by Tyrrell et al. [17], in which underlying conditions were analyzed on the basis of age (<65 years vs. ≥65 years), and Trivalle et al. [23], which compared persons aged ≤70 years with persons aged >70 years. ++++, Most commonly observed (>20% of subjects); +++, commonly observed (>10% to 20% of subjects); ++, occasionally observed (5%–10% of subjects); +, rarely observed (<5% of subjects).

Without regard to age, skin and soft-tissue infections are the most common expressions of invasive GBS disease in adults (83). Following closely behind is bacteremia with no identified focus affecting younger and older adults about equally. In up to 1/3 of cases, in particular skin and soft tissue infections, GBS is involved in a polymicrobial infection, most often with *Staphylococcus aureus* and other members of the *Staphylococcus* family, highlighting the impact of compromised immunity on protection against opportunistic pathogens (83). Most frequent mild skin diseases observed are cellulitis, infected wounds and ulcers, while more severe manifestations such as myositis, necrotizing fasciitis and toxic shock syndromes are also described (83, 228). Although present in younger adults, pneumonia and UTI caused by GBS are much more important problems in the older populations (83).

1.3.3.1 Skin and soft tissue infections

Skin and soft tissue diseases, in many cases, can be considered stemming from opportunistic pathogen which commonly colonizes its surface. Since skin is one of the most important anatomical barriers against pathogens ubiquitously found in our environment, any alteration of its

integrity promotes infection and invasion of the host. It should therefore not be very surprising that necrotic tissue, abscesses, ulcers and wounds are skin lesions often associated with GBS skin and soft tissue infections (252). From these infections, cellulitis is the most frequent clinical manifestation affecting about 25% of patients. Underlying medical conditions such as lymphedema, vascular insufficiency, chronic dermatitis, or radiation-induced cutaneous injury are frequently present as predisposing factors to cellulitis (83). In addition, infected decubitus ulcers and, in patients with diabetes mellitus, foot ulcers are other skin and soft tissue infections associated to GBS, commonly found in elderly or debilitated persons (83). Vascular device–associated infections can occur as a consequence of intravascular or arterial catheters, and these infections often are polymicrobial highlighting the role of skin as a physical barrier to opportunistic pathogens (83).

1.3.3.2 Bacteremia

Invasive GBS disease presents itself as a bacteremia with no evident source in about 15% of non-pregnant adults (252). Malignancy, diabetes mellitus, and liver cirrhosis are the most frequent underlying medical conditions leading the bacteremia (114). It has been suggested that the association of GBS bacteremia and chronic liver disease might be due to a defective reticulo-endothelial clearance of the pathogen by the host (83). Unrecognized vascular catheter–related infection could be a source of bacteremia in some patients, because coinfection with staphylococci is frequent, and both agents are often associated with health care. Some elderly adults with recurrent GBS infection presented primary bacteremia with the first episode and a focal manifestation of infection, such as cellulitis or pneumonia, with the second episode (83).

1.3.3.3 Urinary track infections (UTIs)

UTIs, including pyelonephritis and prostatitis, are common in older men, especially nursing home residents. In a clinical study it was reported that GBS bacteremic UTI was the most frequent diagnosis among adults >70 years of age, accounting for 13 (39.4%) of 33 cases (252). Most of these elderly men had conditions predisposing to infection, including indwelling urinary catheters, a neurogenic bladder or urologic abnormalities (obstructive uropathy due to prostatic hypertrophy). Abnormalities of urinary flow, kidney stones, chronic renal failure, and diabetes

mellitus were identified as predisposing factors in a prospective study of non-pregnant adults in Spain (83).

1.3.3.4 Meningitis

GBS tends to be able to penetrate the BBB in young infants but is not observed to do so frequently in adults. As a matter of fact, in a population based surveillance study on non-pregnant adults, only 2% of GBS invasive cases led to meningitis (83). A parallel study reported that out of 64 patients with GBS meningitis, 1/4 of the group was aged > 65 year (71). GBS meningitis occurred spontaneously in 94% of patients, and in one-half, a distant focus of infection was found (71). Age seems to be of particular importance in death related to GBS meningitis as Domingo *et al*. cited an overall case-fatality rate of 34%, but among adults >65 years of age, the case-fatality rate increased to 56% (71).

1.3.3.5 Toxic shock syndrome

Streptococcal toxic shock syndrome (STSS) is defined as an acute and febrile illness that begins with mild febrile prodromi but rapidly progresses to shock and multiorgan failure (228). Although STSS is classically associated to Group A *Streptococcus* (GAS), there is an increasing recognition that other streptococci, i.e. Groups B, C and G, can be the causative pathogen. Group B STSS cases are often associated with severe skin infections such as necrotizing fasciitis, although this presentation is not common (83).   However, there is a lower tendency for GBS to manifest disease via STSS in adults below 50 years of age, irrespective of the presence of underlying disease (228).

1.4 Steps of pathogenesis and associated virulence factors

1.4.1 Key virulence factors

GBS expresses a diverse array of surface associated and secreted virulence factors that mediate specific host cell interactions and interfere with the normal development of the immune response, several of which are summarized in Figure 5.
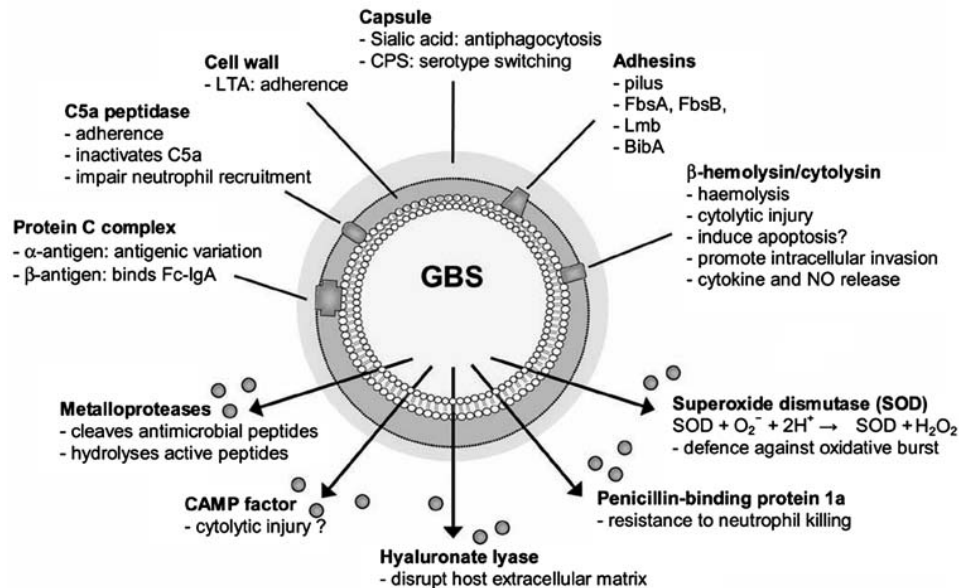
Figure 5: Schematic overview of Group B *Streptococcus* (GBS) virulence factors. This illustration only depicts some of GBS virulence factors as new discoveries continue to be made. Adapted from Sendi *et al*., Infection. 2005, 36(2): 100-111.[228]

The **capsular polysaccharide (CPS)** is considered to be a critical and major virulence factor. At the clinical level, the vast majority of GBS isolates from patients who have progressed to invasive disease are encapsulated. Other studies have shown that non-encapsulated isogenic mutants of GBS display significantly decreased virulence (269). As mentioned in section 1.2.2 the CPS forms the outermost layer of the bacterial surface and is typically composed of repeating subunits of the monosaccharides Glc, Gal, GlcNAc and terminal sialic acid, polymerized in serotype-specific configurations (i.e. Ia, Ib, II-IX) (54). In fact, the GBS oligosaccharide motifs are reportedly very similar to the sialyl-Lewis and related epitopes on human glycoproteins. Thus, GBS CPS is suggested to be involved in immune evasion through molecular mimicry (54). The striking conservation of α-D-Neu5Ac-(2$\rightarrow$3)-β-D-Gal among all GBS capsular types suggests that this structural element is central to the immune evasion function of all GBS CPS (54).

Being an encapsulated pathogen, the CPS was originally thought to play a major role in resistance to phagocytosis, and thus considered as an "anti-phagocytic factor". However, several studies using macrophage/monocyte cell lines or primary macrophages or neutrophils of either human or mouse origin demonstrated that encapsulated GBS is efficiently internalized by these cells, but this remarkable pathogen possesses strong intracellular survival capacity (12, 42, 56, 111, 175, 198, 199, 227, 256). Furthermore, under non-opsonic conditions, non-encapsulated mutants were equally phagocytosed and survived at similar levels within macrophages than wild-type GBS type III, one of the most virulent serotypes (12, 56, 198, 227). These observations challenged the old dogma considering the CPS as a physical barrier impairing bacterial phagocytosis.

On the other hand, the presence of terminal sialic acid on the GBS type III CPS was suggested to inhibit the activation of the alternative complement (81, 164, 196, 241, 263) thus providing further resistance to the intracellular bactericidal functions of neutrophils and macrophages by impairing surface deposition of opsonically active complement C3 on the bacterial surface. One study demonstrated that the resistance of GBS type III to complement-mediated intracellular killing by phagocytes is proportional to the CPS sialic acid content, because its removal by neuraminidase treatment increase surface deposition of opsonically active C3 fragments (C3b and iC3b) (241). As such, complement opsonization has been shown to be critical for efficient intracellular killing of GBS by monocytes/macrophages and neutrophils (5, 80, 82, 157, 189, 237, 267).

In addition, GBS CPS type III was shown to interact with different sialic acid-recognizing Ig superfamily lectins (Siglecs), a family of regulatory receptors expressed on the surface of leukocytes (148). It has been shown that different levels of O-acetylation of sialic acid were present in all GBS capsular types (148, 149). In the case of GBS type III, sialic acid O-acetylation modifies (either reduces or promotes) binding to different Siglecs (148, 263). It has been suggested that production of sialic acid-capped bacterial CPS in order to engage Siglecs represents an example of a previously unrecognized bacterial mechanism of leukocyte manipulation. A phenomenon that could potentially favor pathogen or host, depending on several variables (42, 59, 148, 264). In this regard, a study suggested involvement of sialic acid specific receptors on murine macrophages and lectinophagocytosis in non-opsonic interaction and survival of GBS

within these cells (175). Similarly, it was recently reported that GBS sialic acid binds to Siglec-9 and impairs the bactericidal functions of human neutrophils (42).

In summary, the extent of encapsulated GBS phagocytosis, intracellular survival and the role of the sialylated CPS in these processes remain a matter of controversy. In fact, our knowledge on the specific contribution of sialic acid to the aforementioned interactions of GBS type III with immune cells is hampered by the fact that deletion of *neuA* (CMP-*N*-acetylneuraminic acid synthetase) and *cpsK* (α2,3-sialyltransferase) genes (51, 104, 268, 269), and more recently reported *neuB* (sialic acid synthetase) gene (140), results in considerable loss of CPS expression at the bacterial surface (less than 20% production of CPS compared to the wild-type strain). In view of these data, caution should be used in evaluating the effect of sialic acid mutagenesis on GBS pathogenesis and interactions with host cells, as can be confounded with an overall decrease in CPS production. Nevertheless, recently performed elegant mutagenesis studies (41, 42, 148, 263) allowed the elucidation of the importance of sialic acid O-acetylation levels in GBS capacity to manipulate neutrophil functions and virulence (42, 263, 264).

In spite of these literature contradictions and technical restrictions, GBS sialylated-CPS can be considered, without any doubt, one the most important virulence factors. In fact, the CPS controls the initial non-specific interactions between the pathogen and the innate immunity that are pivotal for the immune system to get an early upper hand on controlling the infection.

As previously mentioned, although the CPS is considered to be a key virulence factor, GBS cell wall components and surface and secreted proteins also play a significant role in the pathogenesis of GBS disease. The next few paragraphs will aim to present and describe currently identified virulence factors important for GBS disease development.

Teichoic acids and **lipoteichoic acids (LTAs)** are two components of Gram-positive bacteria cell wall. Teichoic acids are found covalently bound to peptidoglycans (PG) and LTAs are composed of a polyphosphoglycerol substituted with a **D-Alanine (D-Ala)** ester or a glycosyl residue. The activity of four gene products (DltA to DltD) found in the *dlt* operon assure the role of incorporating D-Ala in LTA. The inactivation of genes within this operon in various Gram-positive bacilli and cocci results in the complete absence of D-Ala ester from LTAs. The main phenotypic

alterations caused by D-Ala ester deprivation are increased susceptibility to the cationic antimicrobial polypeptide colistin, as well the ability to form clumps in standing culture (199). The observation of clump formation is an indicator that alteration of genes within this operon affects bacterial adherence properties, which are very important in pathogen establishment. A study using a GBS DltA- isogenic mutant reported increased susceptibility to host defence peptides such as human defensins and animal-derived cationic peptides. Accordingly, the mutant strain was more susceptible to killing by mouse macrophages and human neutrophils than the wild-type strain. In addition, the virulence of the DltA- mutant was severely impaired in mouse and neonatal rat models (199).

Pore-forming toxins are commonly found in many pathogenic bacteria and promote entry and invasion of host cells. Pathogens defective in expression of these toxins tend to possess a severely attenuated pathogenic power (13, 98). GBS encodes at least two pore-forming toxins, known as β-hemolysin/cytolysin (β-H/C) and Christie Atkins Munch Peterson (CAMP) factor.

β-hemolysin/cytolysin (β-H/C), encoded by *cylE* gene, is a surface-associated, pluripotent toxin that is important for many facets of GBS pathogenesis at both the pathogen and host levels. β-H/C, a potent, oxygen stable, non-immunogenic GBS toxin, comparable to streptolysin S of GAS, promotes GBS invasion of host cell barriers such as the epithelial and endothelial cells of the lung and the BBB (188, 204). Hemolysin-deficient GBS mutants are attenuated for virulence in various animal models of GBS infection, including sepsis, pneumonia, meningitis and arthritis although biochemical and mechanistic insights into the pore-forming abilities of β-H/C and how β-H/C induces inflammatory responses in the host are still largely unknown (188). Interestingly, β-H/C biosynthesis is associated with the production of an orange pigment that promotes pathogen resistance to reactive oxygen species (ROS). Resistance to host-encoded reactive oxygen and nitrogen species is an important component of immune evasion by bacterial pathogens (204).

There is a growing body of evidence that GBS regulates the expression of β-H/C in response to the external environment via a two component system. This system, comprising the DNA-binding response regulator CovR, and its cognate sensor histidine kinase CovS, represses the transcription of *cyl* genes including *cylE* (β-H/ C) and activates the expression of another pore-forming toxin called **CAMP factor** (188).

In fact, CovR activates the expression of **CAMP factor** in GBS, and is an example of a novel paradigm, where members of different signaling families interact to fine-tune gene expression, which was also recently observed in *Streptococcus pneumoniae* (255). In vivo studies have demonstrated the importance of CAMP factor in GBS virulence. Mice inoculated with a sublethal dose of GBS co-administered with partially purified CAMP was sufficient to induce septicemia and death (125, 235). The exact mechanisms underlying CAMP factor functions are still under investigation but it has been shown to oligomerize and form pores on susceptible target membranes such as seen with red blood cells (Rajagopal). Surprisingly, Hensler *et al.* recently observed that a CAMP factor-deficient GBS strain is not attenuated for systemic virulence (112). These data suggest that CAMP factor may be nonessential for GBS pathogenesis or other virulence factors compensate for CAMP deficiency.

The **alpha C protein (ACP)** is a protective surface-associated antigen commonly expressed on the surface of GBS serotypes Ia, Ib and II, but is uncommon in serotype III GBS strains (204). ACP promotes GBS internalization into host cells through its interaction with glycosaminoglycan and/or by binding to $\alpha_1\beta_1$-integrins expressed on the surface of human cervical epithelial cells (204). Mutations that disrupt this interaction between host cell and pathogen render GBS deficient in cervical epithelial cell invasion (204). The protein itself if composed of identical tandem repeats, each comprising 82 amino acids, flanked by N- and C-terminal domains. Clinical isolates of GBS show variable numbers of repeats with a normal distribution and a median of 9 to 10 repeats (100). One interesting study performed with neonatal mice showed that GBS mutants expressing one-repeat ACP were 100-fold more pathogenic than their respective wild-type strain. The authors hypothesized that deletions within the repeat region of the ACP enhance the pathogenicity of GBS in mice by (i) loss of a protective (conformational) epitope(s) and (ii) loss of antibody binding to the ACP due to a decrease in relative antigen size (100). It is possible that a larger repeat content is advantageous in colonization or in some undetermined niches. Most importantly is the recent occurrence of deletions of repeats in the ACP during transmission of GBS from human mother to the neonate, providing further insights in the mechanisms of virulence of GBS and a potential target for immunotherapies.

The GBS **C5a peptidase (ScpB)** is a serine protease that facilitates host immune evasion by the pathogen and contains five distinct domains, including an N-terminal protease domain and

three fibronectin type III binding domains (Fn1–Fn3) at the C-terminus (157). This feature is thought to promote both cellular adherence and complement proteolysis by stabilizing ScpB to allow for C5a binding followed by cleaving just seven amino acids from its C-terminus, so that it can still bind to bacteria, but cannot act as an opsonin (113). As such, ScpB is a bifunctional protein which enzymatically cleaves C5a and thus reduces chemotaxis of polymorphonuclear leukocytes but also mediates adherence to fibronectin. Since binding of fibronectin has been implicated in attachment and invasion of eukaryotic cells by streptococci, these results may imply a second important role for this surface protein in the pathogenesis of GBS infections (21). Finally, C5a peptidase is expressed by most GBS types and is highly conserved and homogenous at the genetic level making it a great potential target for vaccine development. The role of ScpB in virulence has been demonstrated in a model of reconstitution of C5-deficient mice with human C5a since ScpB does not cleave murine C5a (28, 29)

GBS possesses another cell surface-associated serine protease related to ScpB named **CspA** that can cleave fibrinogen, another important extracellular matrix (ECM) protein (204). GBS strains defective in CspA expression exhibit increased susceptibility to neutrophils and decreased virulence in a neonatal sepsis model of infection. This is probably because CspA action produces adherent fibrin-like cleavage products that coat the bacterial surface and interfere with complement-mediated opsonophagocytic clearance (108). Recent studies demonstrated that CspA also readily cleaved chemokines such as CXCL3, CXCL7, and CXCL6 and abolished their ability to attract and activate neutrophils, indicating an important role of CspA in modulation the function of these immune cells (36).

Laminin-binding protein (Lmb) is a lipoprotein expressed by most, if not all, human GBS strains and plays an important role in adherence to human laminin and to human brain microvascular endothelial cells (BMECs) (204, 246). Lmb shows homology to members of the Lra1 family of proteins, which have been implicated in adhesion and metal transport in Gram-positive bacteria (246). When first discovered, the name Lmb was introduced for the GBS protein, although a virtually identical protein called Lsp or Lbp exists in GAS. In both species, the gene is located downstream of the C5a-peptidase gene *scpB* and nucleotides sequence identity between the two species is >98% although adjacent sequences in the two genomes show no homology,

indicating that the region has been horizontally transferred (204). Unfortunately, no information is available on its regulatory mechanisms.

FbsA and **FbsB**, have been identified as important in mediating fibrinogen binding. FbsA is a surface-associated protein encoded by a few GBS strains/serotypes (e.g., serotype Ia and III) whereas FbsB is found in all GBS strains sequenced thus far (204). Interestingly, both share many similarities and differences. For example, at the amino acid level, the proteins FbsA and FbsB are unrelated, but both have surface-exposed localization in the cell wall of the bacterium (245). FbsB was shown to bind to human fibrinogen by its N-terminal 388 amino acids, whereas the FbsA protein interacts with fibrinogen via repetitive units, each 16 amino acids in length (245). Finally, a study reported that GBS mutants lacking FbsA demonstrate decreased binding to human fibrinogen and are more sensitive to phagocytes compared with their respective wild-type strains (223). On the other hand, GBS FbsB mutants are not attenuated for fibrinogen binding but invasion into lung epithelial cells is severely impaired suggesting a particular importance of FbsA for adhesion and FbsB for invasion of host cells (103).

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is another cell-surface associated protein that has dual functionalities including binding and activation of host plasminogen. GBS can bind lysine residues of host plasminogen via GAPDH, activate the bound proenzyme to plasmin, and thus gaining the ability to degrade host ECMs such as fibronectin (157). Pretreatment of GBS with plasminogen and exogenous tissue plasminogen-activator enhances virulence in a mouse model of infection, possibly because of a plasmin-mediated increase in bacterial invasiveness of host tissues (155). In addition, a GBS strain overexpressing GAPDH showed increased virulence as compared with the wild-type strain in C57BL/6 mice. This virulence was markedly reduced in IL-10-deficient and anti-rGAPDH antiserum-treated mice. These results suggest that IL-10 production, which was detected at higher concentrations in the serum of rGAPDH-treated mice, is important in determining the successfulness of the host colonization by GBS and they highlight the direct role of GAPDH in this process (154). Finally, evidence for a novel function of the secreted GAPDH as an inducer of apoptosis of murine macrophages has been recently reported (192). Taken together, these data demonstrate that GBS GAPDH is a virulence-associated immuno-modulatory protein.

As aforementioned in part, GBS possesses an array of extracellular peptidases, proteases, nucleases, and collagenases presumably contributing to the local destruction of tissues and possibly facilitating the spread of GBS. Of these enzymes, **hyaluronate lyase** encoded by the gene *hlyB* is another important secreted protease for GBS pathogenesis capable of cleaving hyaluron (113). Hyalurons are major components of human tissue and ECM and even higher concentrations are found in the placenta, amniotic fluid and in the lung (204). Although the exact mechanism by which hyaluronate lyase exerts its functions is still largely unknown, this protein is thought to facilitate the spread of GBS since high concentrations of hyaluronate lyase were reported in neonates with bloodstream infections compared with neonates that were asymptomatically colonized with GBS (174). The enzyme, however, is not absolutely required for invasion, as strains with *hylB* inactivated by a transposon insertion can still cause disease (272).

Pilus-like structures in GBS were recently discovered during the screening of multiple genomes for surface-exposed protein antigens as possible vaccine targets (204). **Pili**, also called fimbria, are non-flagellar polymeric organelles that mediate many functions such as GBS resistance to antimicrobial peptides and also facilitate adherence and attachment of the pathogen to host cells (135). The genes encoding the GBS pilus machinery are clustered in 3 related genomic islands named pilus island-1, -2a, and -2b, and are located in 2 separate loci flanked by direct repeats and conserved genes. All 3 islands share the presence of 3 genes encoding pilus components: one backbone protein (BP) essential for pilus assembly and 2 accessory proteins (AP1 and AP2). They also all contain 2 genes encoding the sortase enzymes which are responsible for pilus polymerization by catalyzing the covalent linking of the pilus proteins (135). All GBS pilin proteins have an LPXTG-like motif and the 2 pilus consensus motifs YPK-K and E-box first described in *Corynebacterium diphtheriae* pili which are recognized by the sortase enzymes for pilus assembly. Only pilus genes in pilus island-2b do not contain a YPK-K pilin motif (135). In addition to the role of pili in bacterial adhesion, a recent study indicated a role for GBS pilus in macrophage phagocytosis (158). The results showed that the efficiency at which phagocytosis and intracellular survival of GBS occurs in macrophages is dependent on phylogenetic lineage, and this is, in part, related to the presence of **Spb1**, the pilus backbone protein (53).

Superoxide dismutases are molecules often produced by pathogens that are capable of converting superoxide anions to molecular oxygen and hydrogen peroxide that can in turn be

metabolized (204). These enzymes represent an important mechanism for evasion of oxidative stress, a powerful antibacterial clearance mechanism. In particular in GBS, a Mn-cofactored superoxide dismutase coined **SodA** has been reported to play an important role at this level. Mutant strains deficient in SodA were found to be significantly more sensitive to killing by macrophages and showed impaired survival in the bloodstream and brain of mice, but normal survival in the liver and spleen (198). Taken together, these observations suggest that SodA confers survival benefits to GBS in specific host niches (204).

Bacterial penicillin-binding proteins (PBPs) are typically involved in peptidogylcan biosynthesis and are the site of action for the β-lactam family of antibiotics such as penicillin (204). PBP-1a, a surface anchored penicillin-binding protein encoded by *ponA*, was found to be critical in GBS virulence. A study showed that PBP-1a can promote resistance of GBS to innate immunity by protecting the organism from phagocytic killing by cationic antimicrobial peptides of the cathelicidin and defensin families (105, 124).

From this section we can realize that GBS uses panoply of virulence factors, many of which have redundant functions, in order to either colonize or induce disease in the host.

1.4.2 Steps of pathogenesis

The establishment and progression of infection caused by GBS is quite complex. As seen in many other pathogens, the development of disease within a host caused by GBS represents successful colonization of mucosal epithelium, penetrations of epithelial barriers, resistance to immune clearance, followed by systemic invasion, and in the case of meningitis, translocation through the BBB. The constant progressions being made in molecular analysis techniques, genetics, proteomics and refinement of *in vitro* and *in vivo* models, have greatly improved our understanding of this pathogenic process.

In order to adapt to the various environments GBS encounters for disease progression in a host, a plethora of genes and virulence factors need to be regulated at specific times and in specific niches. As mentioned earlier, many of these genetic elements are controlled via two component regulatory systems, with one component responsible for sensing and then communicating the external stimuli, while the second reacts to these signals by activating or

repressing specific genes conferring the bacteria required functions for optimal survival and growth (58). In addition, this system has been recognized as a bacterial strategy to overcome environmental stress factors such as pH, temperature, osmolarity, but also for other important pathogenic processes like adherence to host cells and expression of virulence factors (228).

In the next few pages we will thus be looking at the pathogenesis of GBS type III since this serotype is well identified as one of the most virulent. However, it is important to keep in mind that differences in patho-mechanisms employed among GBS serotypes do indeed exist.

1.4.2.1 Adherence to and colonization of host epithelial surfaces

It is generally well accepted that the first step for disease development by a particular pathogen begins with its entry into the host followed by successful adherence and colonization of host cells. GBS can adhere to a wide variety of human cells including vaginal epithelium, placental membranes and respiratory tract epithelium (73). Of these, maximal adherence occurs at the acidic pH of vaginal mucosa, through the low avidity interactions of cell wall-associated LTA and via higher-affinity interactions mediated by hydrophobic GBS surface proteins (157). Many of these interactions involve attachment of the bacterium to ECM molecules such as fibronectin, fibrinogen and laminin, which can in turn bind host-cell-surface receptors such as integrins (225). At the bacterial level, identified genes coding for adhesins include **ScpB**, , **ACP**, **Lmb**, and **Spb1/pili** (73, 113, 157), which were described in section 1.4.1.

The capacity of GBS to optimally bind vaginal epithelium allows it to occupy a niche that places infants directly at risk of vertical transmission (Doran 2004), with subsequent colonization of pulmonary epithelium and possible disease development (184). Neonates usually become infected during labor, where the risk of aspirating contaminated amniotic fluids is quite elevated, giving GBS access to this host tissue. The pathogen can then travel to the lower respiratory track and damage epithelial cells resulting in pneumonia and respiratory distress within the first few hours following birth (184).

1.4.2.2 Penetration and invasion of host cells

Once GBS has established itself in the host, the next step is to invade target eukaryotic cells such as fibroblasts, endothelial and epithelial cells (228). To this aim, GBS expresses and secretes toxins and surface virulence factors called invasins that promote bacterial uptake, entry and survival within the host by selectively manipulating the ECM and/or cellular signaling pathways (228). Intracellular invasion of both alveolar epithelial and pulmonary endothelial cells by GBS was first noted in newborn macaques following intra-amniotic challenge, and later confirmed in human tissue culture lines (157). Infection of placental cells can promote ascending *in utero* infection, whereas invasion of pulmonary epithelium and endothelium promote systemic dissemination. Ultimately, entry into host cells provides GBS with an intracellular niche for survival and avoidance of recognition by immune cells, although this can also result in compromised integrity of host tissue and activation of the inflammatory response which has been suggested to contribute to disease pathology (157).

Many of GBS invasion factors accomplish their functions by exploiting the ECM. For example, GBS **hyaluronate lyase** is capable of degrading polymeric hyaluronan, an important ECM component that is abundant in placental tissues. This results in bacterial access to the fetus within the amniotic cavity and may induce placental membrane rupture or trigger premature delivery (73). Other invasins that have been identified to promote invasion by changing or interacting with the ECM are **FbsB**, **Lmb** and **ScpB** (Hebert), as described above. The **ACP** protein specifically interacts with host cell glycosaminoglycan on the epithelial cell surface to promote bacterial internalization (157). **ScpB**, **ACP and Spb1** have also been demonstrated to be important for invasion as their eliminations result in a significantly reduced invasion of epithelial cells (157).

Signaling pathways engaged by GBS for its internalization have been studied in some details. Of which, GBS has been identified to increase activation of the Rho family of cytosolic GTPases important in the signaling cascade for actin polymerization and cytoskeletal changes (77). More specifically, levels of activated RhoA, Rac1 and Cdc42 were shown to increase in epithelial cells infected with GBS and invasion was avoided with the use of Rho family inhibitors or dominant-negative expression of these proteins (39). The phosphoinositide-3 kinase (PI3K)/Akt pathway was also identified to be important in GBS invasion of epithelial cells. PI3K is a lipid kinase that catalyzes the recruitment, phosphorylation and activation of the intracellular effector Akt, which

in turn triggers downstream signaling that modulate cytoskeletal activities. GBS infection is accompanied with Akt phosphorylation in epithelial cell and chemical inhibition of PI3K or Akt and genetic inactivation of PI3K results in reduced GBS invasion (38).

In addition to engaging cellular signaling pathways for host cell invasion and dissemination, direct cellular damage caused by GBS to host cells greatly contributes to the spread of infection. As we have seen previously in section 1.4.1, **β-H/C** is a powerful pore-forming toxin that lyses lung epithelial and endothelial cells and compromises their barrier function. In fact, subcytolytic doses are enough to promote lung epithelial cell invasion and triggers release of interleukin-8 (IL-8), a neutrophil chemoattractant (74). In addition, mutants lacking **β-H/C** expression are less able to penetrate pulmonary barriers and produce systemic infection than wild-type strains in a rabbit model of GBS pneumonia. The cytolytic, pro-invasive and pro-inflammatory effects of the GBS **β-H/C** are all neutralized by dipalmotyl phosphatidylcholine, the major phospholipid constituent of lung surfactant. This finding may help to explain the increased risk of premature, surfactant-deficient neonates to develop severe lung injury and invasive disease upon GBS infection (73).

1.4.2.3 Resistance to innate immunity and clearance

After being internalized by host cells, GBS is then capable of accessing deeper tissues and/or the blood stream furthering the establishment of infection. Newly accessed tissues thus expose the pathogen to different effector immune cells and ultimately lead to a broader immunological response to attempt to clear infection. GBS uses various mechanisms to revert normal immune development such as antigenic variation, stealth mechanisms, enzymatic degradation of immune components, molecular mimicry, and inhibition of immunological effectors. The capacity of GBS to produce invasive infections further reflects many virulence factors that allow the bacteria to resist opsonophagocytosis or neutralize the bactericidal activities of neutrophils and macrophages.

Effective clearance of GBS is largely mediated by macrophages and neutrophils and depends greatly upon opsonisation by specific antibodies and/or serum complement. Complement is a system of enzymatic reactions used by the innate immune system to recognize microbes and coat their surfaces with host proteins, making them more easily detected and engulfed by phagocytic cells bearing complement receptors, while simultaneously amplifying other aspects of the inflammatory response (157). It is precisely at this stage of infection that the CPS plays its most

important role in GBS virulence. As discussed in previous sections, capsular sialic acid can bind with high affinity factor H, a host counter-regulator of complement which catalyzes C3b into its inactive form iC3b, ultimately blocking the alternative complement pathway (165). Furthermore, sialic acid-dependent reduction in C3 deposition is correlated with diminished production of C5a, an important complement-derived chemoattractant, which works synergistically with **ScpB**-mediated proteolytic inactivation of C5a to reduce host neutrophil mobilization and activation (241). Studies have showed that isogenic GBS mutants lacking capsule bind far greater amounts of C3b, are more susceptible to killing by human neutrophils and exhibit 100-fold greater LD50 values than wild-type strain in the neonatal rat model (164, 267). Finally, another study has also identified a role of complement for the normal progression of the humoral immune response as marginal zone B cells and dendritic cells (DCs) from $C3^{-/-}$ mice could not internalize GBS CPS and consequently produced low levels of anti-CPS specific IgM and IgG antibodies (200).

Although GBS CPS is largely responsible for modulating bacterial resistance to phagocytic clearance, in part by inhibiting the complement pathway, other proteins also contribute to this process such as the aforementioned ScpB and **BibA,** a new cell-surface GBS immunogenic bacterial adhesin recently determined to mediate inhibition of other complement components. In addition, it was shown that high levels of **β protein** expression are associated with increased virulence of GBS clinical isolates (182). β protein is known to interact with two components of the human immune system, the Fc portion of serum IgA and factor H (11), and recent finding that β protein binds to Siglec-5 identifies a third such interaction. β-protein binding to Siglec-5 functions to impair human leukocyte phagocytosis, oxidative burst, and extracellular trap production, promoting bacterial survival (42). Through non-immune binding of IgA (197), engagement of factor H, and, now, attachment to Siglec5, which is proposed to play an important role in regulating phagocyte function and the link to cellular immunity (59), **β protein** appears to be a multifunctional virulence factor capable of interfering with several aspects of host defense to bacterial infection. Similarly, it has recently been shown that another GBS factor, **C protein,** binds human IgA antibody nonspecifically on bacterial surface and **CspA** targets host fibrinogen, producing adherent fibrin-like cleavage products that also coat the bacterial surface and interfere with complement-mediated opsonophagocytic clearance (73). Perhaps most interesting is that we have already mentioned many of these proteins in previous steps of bacterial pathogenesis demonstrating their flexibility and multifunctionality as GBS virulence determinants.

Although GBS can impair and inhibit various complement components, GBS is nonetheless efficiently internalized by macrophages and can survive for more than 24 hours as discussed above. However, long term survival of streptococci is dependent on the absence of antibodies since pre-treatment of GBS with fresh or heat inactivated serum containing anti-GBS antibodies resulted in a decrease of 90% of intracellular survival (256). However, since others have shown that encapsulated and non-encapsulated GBS are equally susceptible to macrophage uptake, the role of CPS in resisting phagocytosis per se versus other aspects of immune cell killing remains unclear (227).

The entry of GBS contained within a phagosome is rapidly accompanied with the release of toxic ROS produced by oxidative burst of phagocytic cells. Although GBS does not produce catalases, like in certain other streptococcal species to counter ROS, GBS is capable of surviving inside phagolysosomes (243). **SodA** was proposed to participate in this protective function as evidenced by the fact that *sodA* mutants are highly susceptible to macrophage killing and survives poorly *in vivo* (198). As mentioned in section 1.4.1, GBS also produces an orange carotenoid pigment, a property unique among hemolytic streptococci, and it results of β-H/C cytotoxin synthesis. The free-radical scavenging properties of this carotenoid neutralize hydrogen peroxide, superoxide, hypochlorite and singlet oxygen, and thereby provide a shield against several elements of phagocyte ROS killing (204).

Antimicrobial peptides that exhibit broad-spectrum activities, such as cathelicidins and defensins, are produced by many immune and epithelial cell types. The small, cationic nature of most antimicrobial peptides supports the assumption that their mechanism of killing involves their electrostatic attraction to negatively charged microbial cell surfaces, followed by their assembly to create membrane pores or otherwise disrupt membrane integrity (95). GBS increases its intrinsic resistance to antimicrobial peptides by incorporation of positively charged **D-Ala** residues into the cell **LTA**, thereby reducing surface electronegativity and affinity for the cationic peptides (198). A study performed using *dltA* mutants showed that they exhibited decreased negative surface charge which in turn impeded cationic host defence peptides from reaching their cell membrane target of action (157). Surface anchored penicillin-binding protein, **PBP1a**, also enhances GBS resistance to various antimicrobial peptides, thereby reducing GBS susceptibility to killing by alveolar macrophages and neutrophils, and promoting bacterial survival in a neonatal rat model of

aerosolised lung infection (105). GBS mutants with deletion of PBP1a are less virulent after both lung and systemic challenge, which is correlated to an increased susceptibility to cationic anti-microbial peptides (defensins, cathelicidins) produced by host epithelial cells and phagocytes (105). Similarly, expression of the pilus major protein subunit, **PilB**, renders GBS more resistant to killing by cathelicidin, and is associated with enhanced phagocyte resistance and systemic virulence (158).

As it is often said, sometimes the best defense is a good offence, and it appears that in certain circumstances GBS can kill host phagocytes before being killed itself. Apoptosis is a carefully regulated signal cascade involving a group of cysteine proteases known as caspases and several pro- and anti-apoptotic regulators belonging to the Bcl-2 family. In contrast to some cell-death ligands, macrophage apoptosis triggered by GBS requires caspase-3 activation and utilizes unique changes in regulation and localization of Bcl-2 family members (254). On the other hand, GBS can also proceed to activate programmed cell death in macrophages via calpains, independently from caspases, by cleaving and activating Bcl-2 family members resulting in death signaling (88). The fact that GBS can activate apoptosis via two different pathways thus increases its chances of escaping host defense systems before being eliminated itself. Interestingly, the role of β-H/C in inducing apoptosis is still unclear. Although this cytolysin was shown to enhance GBS survival in mouse or human blood, and growth of GBS in high glucose concentrations (which minimises β-H/C synthesis) also reduced macrophages apoptosis (254), *in vitro* infection of macrophages with either wild-type (WT) GBS or β-H/C mutants resulted in similar levels of viability (158). This result suggests that GBS can induce macrophage apoptosis via β-H/C-independent mechanisms.

1.4.2.4 The inflammatory response and sepsis syndrome

When failures in epithelial barrier function and immunological clearance allow GBS to establish bacteremia, development of septicemia often ensues. Studies performed on sheep revealed a biphasic host inflammatory response characterized by the development of an acute phase (<1 h) followed by a more severe late phase (2-4 h) (213). The acute phase is manifested by increased pulmonary artery pressure, decreased arterial oxygenation, and is associated with a rise in serum levels of thromboxanes, a potent vasoconstrictor and hypertensive agent. Conversely,

the late phase is characterized by a progressive pattern of systemic hypotension, decreased cardiac output and metabolic acidosis develop together with hematological abnormalities, organ system dysfunction and an increase in inflammatory markers, such as thromboxanes, prostacyclins, TNF-α, IL-1 and IL-6 (157).

As such, the pro-inflammatory cytokine response to GBS can act as a double edge sword by augmenting bacterial clearance, but also by triggering deleterious changes in host physiology associated with sepsis syndrome. A study using a rat model of infection showed that excessive TNF-α release in response to GBS bacteremia correlated with mortality rate, and *in vivo* TNF-α neutralization was protective against early death (147). The cytokine IL-1 seams to occupy a proximal position in the deleterious cytokine cascade of septic shock as treatment with a receptor antagonist improves cardiac output and mean arterial pressure and increases survival in piglets (258). On the other hand, the cytokine IL-12, which is elevated 12 to 72 h after challenge in animal models, has an important role in regulating the systemic response to GBS infection. Pretreatment with a monoclonal antibody against IL-12 results in greater mortality and intensity of bacteremia, whereas therapeutic administration of IL-12 reverses this phenotype (159). The positive feeding-loop of the pro-inflammatory response is usually followed by a delayed activation of an anti-inflammatory response. IL-10 was thus reported to be synthesized by activated monocytes infected with GBS and was associated with a protective function in septic neonatal mice and piglets (64, 266). It is therefore very tempting to think that an optimal immunological response to GBS requires a fine balance between pro- and anti-inflammatory cascades.

Since the release of the previously mentioned cytokines are stimulated by soluble GBS cell-wall antigens, studies have sought to identify the specific GBS components that trigger the host cytokine cascade and revealed that GBS **PG** is more effective than **LTA** as a stimulator of cytokine release from monocytes (257). Knockout studies in mice indicate that cell wall PG-induced activation of p38 and NF-κB depends upon the cytoplasmic Toll-like receptor (TLR) adaptor protein MyD88 (myeloid differentiation primary response gene 88), but does not proceed via the pattern recognition receptors TLR2 or TLR4 (161). In fact, GBS activation of TLR2 was shown to depend on surface expression of lipoproteins, which also play a significant role in the development of GBS sepsis. Structural differences in the linkage, anchoring and backbone of GBS **LTA** compared with those present in other Gram-positive bacteria may account for its diminished immune activation

properties (157). The course of GBS sepsis in mice with a targeted deletion of TLR2 or MyD88 closely mimics that of wild-type mice that were subjected to an anti-TNF-α injection before the GBS challenge. Hence, TLR2, MyD88, and TNF-α are essential elements in the host immunity to GBS (266). In addition to TLRs, GBS induction of NF-κB signaling and TNF-α release from human monocytes *in vitro* requires CD14 and the receptors for complement C3 and C4. The importance of complement components in amplifying GBS-induction of TNF-α was corroborated when reduced levels of the cytokine were observed in the blood of C3- or C3 receptor-α deficient mice stimulated with GBS (74).

Due to the apparent importance of NF-κB in the development of GBS-induced sepsis, further studies sought to identify the signaling cascades involved in its activation. Inhibitor studies have revealed that the mitogen-activated protein kinase (MAPK)/c-Jun N-terminal kinase (JNK) signaling pathway is essential for the NF-κB-dependent inflammatory response of phagocytes to GBS. It was also suggested that this particular pathway could be of special interest for potential therapeutic treatments since inhibition of JNK did not affect phagocytosis or oxidative killing functions in phagocytes (130).

In addition to cell wall components, the pro-inflammatory effects of the GBS β-H/C were shown to contribute to sepsis pathophysiology. Recently it was discovered that β-H/C and cell wall components act synergistically to induce macrophage production of inducible nitric oxide synthase (iNOS) and generation of nitric oxide (NO) (208), a potent factor in the sepsis cascade. In a mouse model of bacteremia, β-H/C expression is associated with higher mortality, increased bacterial loads, greater degrees of joint injury and intra-articular and systemic release of IL-1 and IL-6 (101). GBS infection was also shown to stimulate cyclooxygenase-2 (COX-2) and prostaglandin E$_2$ (PGE$_2$) expression in lung tissue in vitro and in vivo, probably through MAPK pathway signaling. GBS-induced COX2/PGE2 inflammatory response was reduced by treatment with an iNOS inhibitor and restored by addition of a NO donor, indicating that it is at least partially regulated by the NO pathway (185).

Besides the above mentioned bacterial immunomodulatory components, GBS expresses two distinct polysaccharide species, a group-specific polysaccharide and the CPS. A distinct, albeit relatively weak effect of CPS has been observed, with CPS from GBS serotype VIII exceeding that

from GBS serotype III (266). However, because mutants lacking CPS exhibit normal inflammatory potency to activating phagocytes *in vitro*, this carbohydrate can be excluded as an essential inflammatory factor for GBS. Group-specific polysaccharide has been reported to be a relatively potent inflammatory stimulus, and the adhesion molecule CD11b/CD18 has been implicated in its recognition (266). The generation of highly pure polysaccharide preparations for *in vitro* studies is hampered by its covalent phosphodiester linkage to the PG assembly. Hence, it remains to be clarified if polysaccharides themselves comprise inflammatory potency or whether this is accounted for by co-purified molecules.

1.4.2.5 Blood brain barrier (BBB) penetration and meningitis

Because GBS is the leading cause of neonatal meningitis, much research has been done in this regard. To produce meningitis, GBS must penetrate a specialized structural and functional barrier called the BBB that maintains homeostasis of the central nervous system (CNS). The BBB is mostly composed of BMECs that guard the brain from circulating toxins and pathogens by maintaining tight intercellular junctions and prohibiting pinocytosis. The physiopathology of GBS-induced meningitis can be broken down into two categories depending on the age of onset. In EOD, autopsy studies demonstrate little or no evidence of leptomeningeal inflammation, despite the presence of abundant bacteria, vascular thrombosis and parenchymal haemorrhage. By contrast, infants with LOD usually have diffuse purulent arachnoiditis with prominent involvement of the base of the brain (157). These histopathological differences reflect underdevelopment of the host immunological response in the immediate neonatal period, with a higher proportion of deaths resulting from overwhelming septicemia (178).

The initiation of the CNS inflammatory response is triggered by the BBB endothelium, which activates a specific pattern of gene transcription for neutrophil recruitment, including production of chemokines (IL-8/CXCL8), endothelial receptors and neutrophil activators such as granulocyte-macrophage colony-stimulating factor (GM-CSF) (73). The principal provocative factor for the BBB inflammatory gene response is the GBS β-H/C, and has been shown to contribute to the development of meningitis (Doran, 2003)(72). At high bacterial densities, human BMEC invasion by GBS is accompanied by evidence of β-H/C-induced cellular injury and knockout mutants show decreased BBB penetration and decreased lethality *in vivo* (72). In a parallel study, a library of GBS transposon mutants revealed the importance of FbsA, Lmb and the pilus subunit protein PilB for

the adhesion to and invasion of BMECs *in vitro*. These findings were later confirmed *in vivo* (157).

Although TNF-α appears to play an important role in control infection in the early stages of sepsis, it seams to have a negative effect in the context of meningitis. This was highlighted in a study that revealed that TNF-α production contributed to apoptosis of hippocampal neurons and increase permeability of the BBB in a piglet model of infection (27). GBS-induced microglial apoptosis is triggered via the cysteine protease caspase-8, and is hypothesized to represent a self-dampening mechanism that prevents over stimulation of CNS inflammation. Simultaneous intracisternal administration of dexamethasone with GBS challenge markedly reduces the magnitude of subarachnoid inflammation, vasculopathy, and neuronal injury (73). The following diagram is an excellent resume of several steps of GBS pathogenesis and important virulence factors that contribute to the progression of disease.
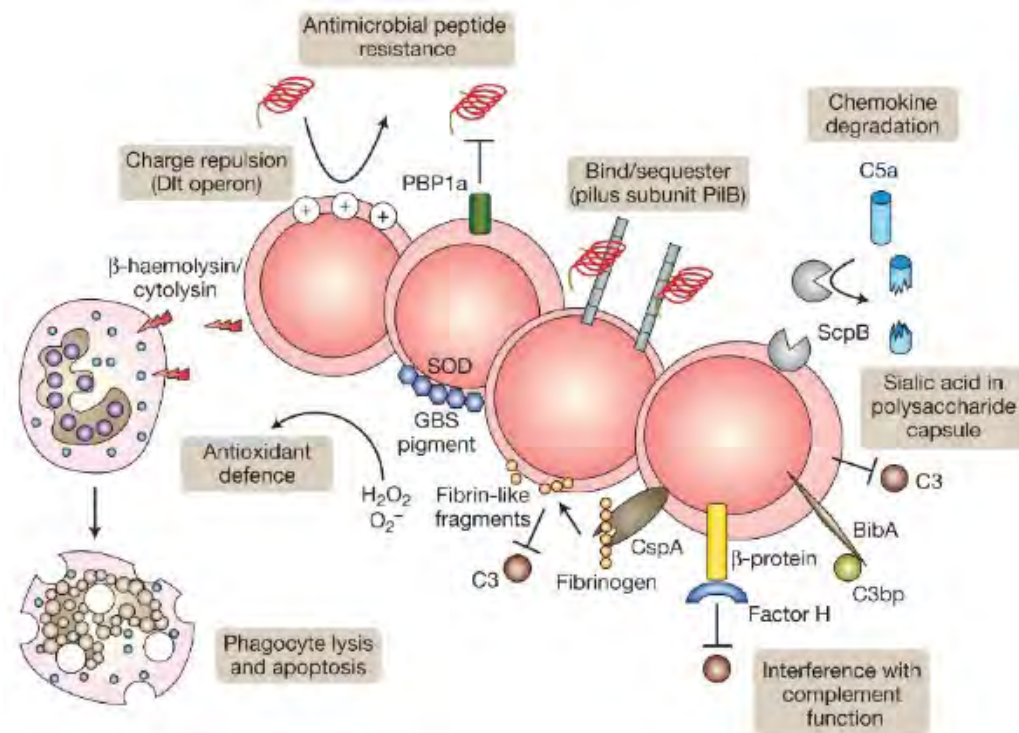
Figure 6: Mechanisms of Group B *Streptococcus* (GBS) immune resistance. GBS express several surface-expressed or secreted factors to evade host immune defences and promote survival. The Dlt operon is responsible for increasing incorporation of D-alanine residues in cell-wall teichoic acids, thereby reducing electronegativity and affinity for cationic antimicrobial peptides. PBP1a and the pilB subunit of GBS pili

also contribute to antimicrobial peptide resistance. ScpB, the sialic acid capsule, BibA, β protein and CspA all inhibit host clearance of GBS by interfering with complement components C5a, C3 and C3bp. SOD as well as the scavenger properties of the orange carotenoid pigment shield GBS from killing by phagocyte-generated reactive oxygen species. Alternatively, β-haemolysin/cytolysin can boost GBS survival by cytolytic or proapoptotic injury to host phagocytes. Abbreviations: BibA, GBS immunogenic bacterial adhesin; CspA, cell-surface protease A; PBP1a, penicillin-binding protein 1a; ScpB, C5a peptidase; SOD, superoxide dismutase. Adapted from Maisey *et al.*, Expert Rev Mol Med. 2008, 10.[157]

1.5 Treatments

1.5.1 Antibiotic therapy

Although the number of pathogens resistant to antibiotics is on the rise, GBS remains susceptible to penicillin. Penicillin G is used as the antibiotic of choice for treating GBS infections due to its effective transplacental passage capacities, its narrow spectrum and its low cost (230). The occurrence of penicillin tolerance in clinical GBS isolates varies among different studies, ranging from 5% to 15%. This *in vitro* phenomenon is observed when bacteria are inhibited by low concentrations of penicillin but are killed at much higher ones (25). GBS strains are also susceptible to ampicillin, ceftriaxone, cefotaxime, meropenem, levofloxacin and vancomycin, which can be used as alternative treatments for patients with penicillin allergies (228).

On the other hand, GBS is resistant to erythromycin and clindamycin is well documented although varies geographically and seams to be on the rise. A Canadian study revealed that 6.7% and 4.4% of clinical isolates from infected adults were resistant to erythromycin and clindamycin respectively in 1996 (252) whereas an American study found a resistance of 32% and 15% respectively from 1999 to 2005 (195). Strains demonstrating resistance to macrolides should be tested for Macrolide-Lincosamide-Streptogramin type B resistance, in particular, when considering clindamycin treatment (228). Finally, aminoglycosides alone have little or no effect on GBS, although synergistic killing with penicillin has been shown in vitro (15).

During labour of an identified GBS positive carrier, penicillin should be administered at least four hours before delivery to achieve adequate placental and amniotic fluid concentration. When the time between the start of antibiotics and delivery is less than one hour, the rate of GBS transmission is 46% compared with 1.2% if the interval is more than four hours (8). For those with

allergies to β-lactams (penicillin), alternative treatments exist and depend on the available information of GBS infection status. For example, for those with penicillin allergies at low risk of anaphylaxis, it is recommended to use Cefazolin for antibiotic treatment. When risk of anaphylaxis is higher, and an antibiotic resistance test indicates that the current GBS strain is susceptible to erythromycin and clindamycin, recommendations are to proceed with their uses. In the final case, when the strain antibiotic resistance profile is unknown, professionals recommend the use of vancomycin (8).

Empiric, universal antibiotic administration to neonates, regardless of maternal colonization status, is not recommended, because it is associated with a 40% increase in overall neonatal mortality despite reducing the GBS attack rate by 68%. The only exception to the rule being in the case of postpartum meningitis where treatment starts empirically with a third-generation cephalosporin (178). Selective neonatal prophylaxis is recommended when maternal GBS carriage has been documented but less than four hours of antibiotic prophylaxis have been given before delivery, chorioamnionitis is suspected, or signs of neonatal sepsis are present (22).

1.5.2 Vaccines

Although efforts for the development of a GBS specific vaccine have been largely reported in the literature, there is still no commercial vaccine available to fight against this pathogen. Conventional approaches have been greatly augmented by novel DNA, genomic and protein technologies. Glycoconjugate vaccines using purified GBS CPSs from serotype Ia, Ib and II–VIII coupled to an immunogenic protein carrier have been synthesized and found to be immunogenic in preclinical trails in mice, rabbits and baboons (157). Immunized humans develop serotype-specific anti-CPS antibodies that function well to promote GBS killing during *in vitro* opsonophagocytic assays. However, the major challenge in developing a potent anti-GBS vaccine will be to develop one that will provide protection against the broad-spectrum antigenic coverage of all GBS serotypes associated with disease in any particular demographic group or geographic area. Much more research and collaborative work will be needed to accomplish this ultimate goal.

Promising avenues do exist in this regard however. Currently investigations are being done to identify potential candidates of surface expressed protein antigens distributed broadly (or

ideally universally) among strains of different GBS serotypes.   Of those who have already been described, **ScpB**, the C5a peptidase, is a universally expressed surface GBS protein capable of eliciting protective IgG antibodies, and may be deliverable in recombinant form within biodegradable polymers (219). Similarly, surface proteins **LrrB** and **Sip** are highly conserved across GBS strains of diverse serotypes and each induces protective immunity in mice (33, 226). The component proteins of newly discovered GBS pili may also represent candidates for a universal vaccine antigen and have been explored as classical antigens and recombinantly expressed in *Lactococcus lactis* as a live-attenuated vaccine (37).

In the field of vaccine development, genome based and reverse vaccinology have greatly accelerated our capacities to analyze and identify potential antigenic proteins in a wide variety of pathogens. A great example is *Neisseria meningitidis* group B, where in less than two years reverse vaccinology achieved what the conventional vaccinology approaches attempted to achieve for decades: identification of surface-exposed proteins able to induce protection and cross-reactivity among distantly related strains and serotypes, suitable to be used in a universal vaccine against this microorganism (176). The success obtained with *N. meningitidis* group B prompted the application of the reverse vaccinology to other pathogens, and was thus applied to GBS. Surprisingly, results were quite unexpected. Only about 80% of the approximately 2100 genes found in any genome were shared by all genomes. About 20% of the genes found in the genomes were lacking in at least one strain. The total number of genes found in the eight strains was almost 50% greater than the size of the average genome. A mathematical analysis of these data indicated that further analysis of more genomes could potentially reveal between 15 and 30 new genes per new sequenced GBS genome. How far this model will hold up is yet unclear, but it is clear that the number of total genes in the global population of GBS may be very high and will need to be taken into account when developing a universal GBS vaccine (170, 244).

2. The T cell-dependant immune response

In order to protect an individual effectively against the plethora of diseases we are constantly exposed to on a daily basis, the immune system must fulfill four main tasks. The first task is characterized by **immunological recognition** of the pathogen where, in order to counter a potential dangerous agent, it must first be recognized as not being a part of the host's normal composition. Following recognition, the immune system must then contain and, in a perfect scenario, eliminate the pathogen by exploiting certain **immune effector functions** such as activation of complement components, secretion of specific antibodies and activation of destructive capabilities of lymphocytes and other white blood cells. As the body is so well known to accomplish, activation of any particular function must be **regulated** in order to maintain homeostasis and failure to do so can potentially result in allergies and autoimmune diseases development. The last task of the host immune system is characterized by the generation of **immunological memory** resulting in a much more rapid and efficient immune response upon a subsequent encounter with the same pathogen. In the field of immunology, generating such memory in a long-term manner represents one of the biggest challenges of vaccinology.

The immune response can be divided into two major categories: **innate immunity** and **adaptive immunity**. Both types of immunity are composed of distinct cellular and molecular components and of different anatomical structures. Innate immunity is the first line of defense and is always available for immediate combat against a wide range of pathogens but does not lead to lasting immunity and is not specific for any individual pathogen. The most important anatomical structure of innate immunity is the skin and mucosal epitheliums as they serve as physical barriers against environmental pathogen and any weakness in these structures can offer a portal of entry into a susceptible host. Phagocytes are the second most important component of the innate immune response because they are the first line of active defense once a pathogen has gained access to the host and also represent the bridge between innate and adaptive immunity. Indeed phagocytes can activate T cells of the adaptive immune response by presenting antigenic epitopes on their surface in association with major histocompatibility complex (MHC) molecules for their recognition and provide additional signalling for the development of an optimal response. Resulting T cells are then capable of interacting with other cells of the adaptive immune response leading to the development of humoral and cellular immunity with acquired memory. For the

sake of this research project, we will thus focus on the adaptive immune response in the context of GBS type III infection.

2.1 The bridge between innate and adaptive immunity: Antigen Presenting Cells (APC)

As mentioned earlier, phagocytes represent the bridge between innate and adaptive immunity. They are considered as such because of their capacity to internalize, process and present pathogen-derived antigens to T cells via MHC molecules. In general, T cells cannot recognize, and therefore react to, 'free' antigen. T cells can only 'see' antigen that has been processed and presented by cells via a MHC molecule. Most cells in the body can present antigen to CD8+ T cells via MHC class I molecules and, thus, act as "APCs"; however, the term is often limited to those specialized cells that can prime T cells (i.e., activate a T cell that has not been exposed to antigen, termed a naive T cell). These cells, in general, express MHC class II as well as MHC class I molecules, and can stimulate CD4+ ("helper") cells as well as CD8+ ("cytotoxic") T cells, respectively. To help distinguish between the two types of APCs, those that constitutively express MHC class II molecules are often called **professional APCs** and include macrophages, DCs and B cells. A non-professional APC does not constitutively express MHC class II proteins required for interaction with naive T cells; these are expressed only upon stimulation of the non-professional APC by certain cytokines such as interferon (IFN)-γ. Non-professional APCs include fibroblasts, thymic and thyroid epithelial cells, glial cells, among others. For the purpose of this literature review, we will limit ourselves to professional APCs.

2.1.1 Dendritic cells: A model APC

Steinman and Cohn were the first to describe and characterized DCs in 1973 when they examined adherent mouse splenic cells and noticed a subpopulation with a unique feature of possessing "dendritic" cellular extensions. Shortly after, the authors named these newly identified cells as "dendritic cells" (220). Since then, much work has focused on this particular immune cell line. DCs are bone marrow (BM)-derived cells that are found in all lymphoid and most non-lymphoid tissues and have been shown to be the most potent APCs. DCs constitutively express MHC class I and II molecules and have a superior capacity in modulating T cell immunity compared to macrophages or B cells (150). They are also involved in the modulation of Natural Killer (NK)

cell and B cell responses and offer an interesting alternative for vaccination, or immunotherapy against cancer, autoimmunity and allergy (9).

Although at first DCs were thought to be a homogenous cell type distributed throughout the body, it quickly became clear that they are a heterogeneous cell line that can be classified into subpopulations. Functional diversity of DC populations is related to their differentiation state as well as their specific location, and is a consequence of differential interactions with antigens and effector cells of the immune system (9). Both mouse and human DC subpopulations have been described with the common ground of being efficient T cell activators (220). Murine DCs have been extensively used by researchers investigating their role in immunity in various infection settings. Even though it is clear that physiological differences exist between murine and human DCs, their utilization in the context of human health cannot be denied as they provide an appropriate model of infection in most cases. The next few pages will describe DC origins and development followed by an overview of DC maturation and activation with a particular focus on murine cells.

2.1.1.1 Murine dendritic cells

2.1.1.1.1 Origins and development

Initial reports on DC origins indicated that they were derived from myeloid precursors due to their functional, phenotypic and morphological similarities with macrophages (9). This observation was further supported by a study of Inaba *et al.* showing that macrophages and DCs could be derived from a common myeloid MHC class II-negative precursor in the presence of GM-CSF (118). However, it was later discovered using *in vivo* models that DC development was actually much more complex then originally believed. A study of DC reconstitution in BM irradiated chimeras revealed that thymic DCs could also be derived from CD4^{low} early thymic precursors and thus led to the concept that some DCs could be of lymphoid origin as well (10). Further studies thus tried to identify if DCs further differentiated into distinct subpopulations reflecting their myeloid or lymphoid origins. For example, when the CD8α protein was identified to be expressed by this cell line, a protein originally thought to be reserved to the T cell subset, researchers thought that CD8$^+$ DCs originated from lymphoid precursors (270). However, this was

later disproven by Weissman *et al.* who showed that common lymphoid precursors (CLPs) and common myeloid precursors (CMPs) could differentiate into both CD8$^-$ and CD8$^+$ DCs (249).

With this in mind, the following thus raises an important question on the relative contributions of CLPs or CMPs in DC generation. In a study by Manz *et al.* it was proposed that both CLPs and CMPs contribute equally to the generation of thymic DCs but that peripheral DCs are mainly derived from CMPs (249). Interestingly, a recent article proposed that DC differentiation activity of BM precursors is correlated with the expression of fms-related tyrosine kinase 3 (FLT3) receptor. In addition, they reported that CLPs (which are mostly FLT3$^+$) are more efficient in the generation of DCs, especially plasmacytoid DCs (pDCs), than FLT3$^+$ CMPs (63). Mice that lack FLT3-ligand have defective development of DCs, but they also have defects in haematopoiesis involving a reduced number of haematopoietic progenitor cells and defective myeloid and B-cell differentiation. These data showed the essential role of FLT3 in the development of DCs (168). Although the experiments on the generation of DCs *in vitro* and *in vivo* allow the study of different precursor populations and their differentiation potential using cytokine combinations, so far, they have provided only limited information on the origin and differentiation of DCs in the mouse. Figure 7 below displays a proposed theoretical model of the developmental origin of mouse DCs.
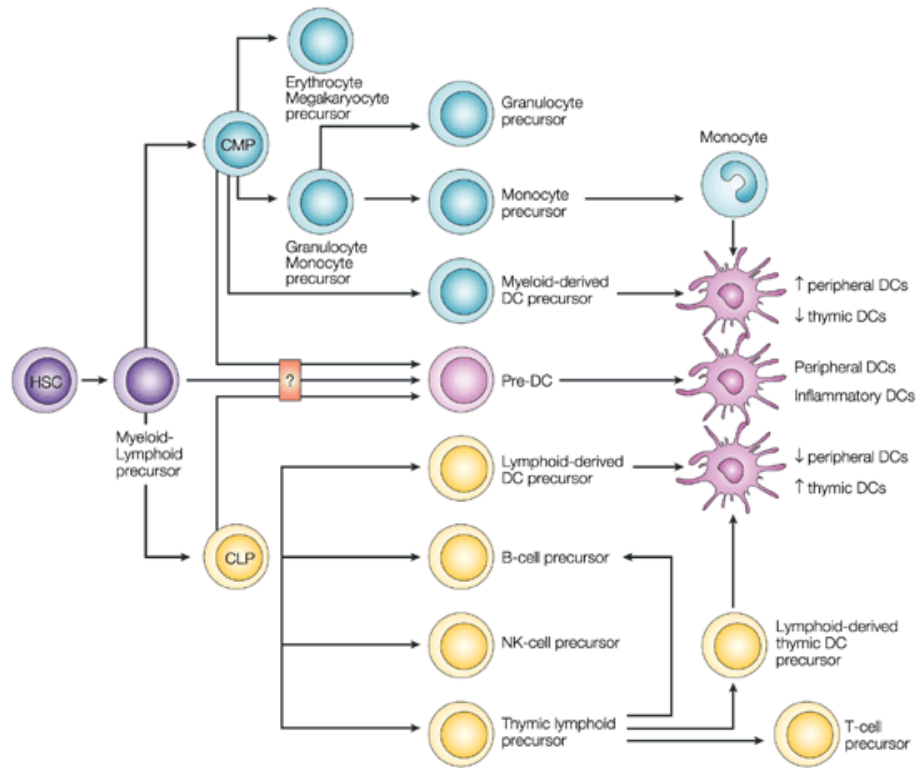
Figure 7: Theoretical model of the developmental origins of mouse dendritic cells (DCs). The differentiation of DCs — including CD8α-, CD8α+, plasmacytoid B220+ DCs and Langerhans cells — has been proposed to proceed directly through myeloid- and lymphoid-derived DC precursors, and through circulating common DC precursors (pre-DCs). On the basis of the relative DC differentiation potential and the absolute number of common lymphoid progenitors (CLPs) and common myeloid progenitors (CMPs), it has been established that thymic DC differentiation results from an equal contribution of both precursors, whereas peripheral DCs are derived mainly from CMPs. Myeloid-derived DC precursors have been shown to be derived from the fraction of CMPs that express fms-related tyrosine kinase 3 (FLT3+). Lymphoid-derived DC precursors could derive from CLPs that are located in the bone marrow or from thymic lymphoid precursors, which give rise to T cells, thymic DCs and B cells. Pre-DCs and monocytes have been proposed to be involved in the generation of DCs after recruitment to reactive sites. The origin of pre-DCs from myeloid and/or lymphoid progenitors, and their relative contribution to the generation of DCs in relation to CMPs and CLPs under steady-state conditions have also to be determined. Adapted from Ardavin, Nat Rev Immunol. 2003, 3(7): 582-591[9]

2.1.1.1.2 Subpopulations

In general, DCs can be grouped into two families, where one family is comprised of DCs that are resident cells in secondary lymphoid organs and the other is made up of DCs in peripheral tissues that migrate to lymph nodes under inflammatory conditions. Conventional DCs are identified as CD11c^{high}MHC-II$^+$ cells that can be further divided into subsets based on expression of myeloid and lymphoid lineage surface molecules. The mouse spleen contains CD8α$^+$CD11b$^-$CD4$^-$, CD8α$^-$CD11b$^+$CD4$^+$, CD8α$^-$CD11b$^+$CD4$^-$ subsets, and the latter two subsets are often analyzed together as a CD8α$^-$ subset, which comprises 75% of mouse splenic DCs (9, 233). These subsets are also found in peripheral lymph nodes, Peyer's patches, and mesenteric lymph nodes as well as in the liver, with the exception that DCs expressing CD4 seem to be rare or absent in tissues other than the spleen. Currently, the functional relevance of the differential expression of CD4 remains a matter for debate.

The controversy stems from two different studies, where in the first, authors stipulate that CD4$^-$CD8α$^-$ and CD4$^+$CD8α$^-$ originate from two developmentally and functionally independent DC subsets, compared to another study which suggests that CD4$^-$CD8α$^-$ might constitutes an activated or more differentiated form of CD4$^+$CD8α$^-$ DCs (127). Which ever the case may be, it has been demonstrated that CD8α$^+$ DCs are mainly localized in the T-cell areas of lymph organs, whereas CD8α$^-$ DCs are predominantly present in the surrounding areas, mainly in zones of antigen uptake, and appear to localize to T-cell areas under inflammatory conditions (150). Peyer's patches, mesenteric lymph nodes and the liver also have a subset of CD8α$^-$CD11b$^-$ DCs (120, 123). Migratory DCs found in non-lymphoid organs include Langerhans cells (LCs) and dermal DCs in the skin as well as interstitial DCs, which is a collective name for migratory DCs in various organs (220). These migratory DCs are also detected in the lymph nodes but not in spleen, which lacks afferent lymphatics (242).

Finally, the expression of the CD45 isoform B220 defines the mouse functional counterpart of human **plasmacytoid DCs** (pDCs). B220$^+$ pDCs are found in all lymphoid organs of the mouse and are characterized by their potential to produce large amounts of type I IFN in response to virus infections (14). It has also been proposed that B220$^+$ pDCs are involved in the maintenance of T cell tolerance by inducing the differentiation of regulatory T cells (Treg), which can block the

activation of naive T cells by an IL-10-dependent mechanism (26). Although B220$^+$ pDCs express various levels of CD8α depending on their localization, this particular dendritic cell line maintains the common nomenclature of pDC due to their type I IFN secreting capacities, which overshadow presence of CD8α since the role of this particular protein in modulating pDC functions is still under investigation (278). Lastly, it is important to consider that in addition to the DC subpopulations described earlier, other DC subsets have been described in specific organs of the mouse, including lungs, heart and kidneys, although available phenotypic and functional data regarding these DCs have not yet allowed them to be ascribed to any particular DC subpopulation (9). Table 2 provides a brief resume of what has just been discussed.

Table 2: Organ distribution of murine DC sub-populations (From Lipscomb and Masten, Physiological Reviews. 2002, 82(1): 97-130)[150].

Dendritic-cell subpopulation	Thymus	Spleen	Lymph node	Peyer's patch	Skin	Liver
CD8$^-$ DCs	*	+	+	+	−	+
CD8$^+$ DCs	+	+	+	+	−	+
CD8^{int} DCs	−	−	+	−	−	−
Langerhans cells	−	−	−	−	+	−
Dermal DCs	−	−	−	−	+	−
B220$^+$DCs	+	+	+	+	−	N.D.

*CD8$^-$ DCs can be detected in the thymus, although they constitute a minute proportion in thymic DCs[73]. +, present; −, absent; CD8^{int}, intermediate level of expression of CD8; DC, dendritic cell; N.D., not determined.

DCs do not only differ phenotypically but functionally as well. For example, CD8α$^+$ and CD8α$^-$ DCs differ in the expression of C-type lectin receptors (CLRs) and express different sets of TLRs, which mediate microbe recognition and DC maturation as part of the innate immune system (see section 2.1.2.1)(242). The DC subsets also differ in their capacity to process and present some but not all types of antigens. For instance, CD8α$^+$ DCs are specialized for cross-presentation of antigen to CD8$^+$ T cells, while CD8α$^-$ DCs may be better at presenting antigens on MHC-II for CD4$^+$ T cell activation (221, 233). Furthermore, CD8α$^-$ DCs seem to have a higher endocytic and phagocytic capacity than CD8α$^+$ (10). These subset-specific features of DCs may optimize their role in triggering adaptive immunity to certain types of pathogens although other signals from the pathogen and environment also influence the ensuing immune response (220).

One might have noticed that we have not discussed about other surface markers such CD80, CD86 and CD40; however, it is important to remember that these proteins are markers of maturity and/or activation and are not used to define specific DC subsets (150). They will be discussed in greater details in section 2.1.3.

2.1.1.2 Human dendritic cells

Human DC sub-populations have been categorized into distinct subtypes based on studies of skin DCs, DCs generated in vitro from CD34+ hematopoietic progenitors, and blood DC precursors (150). Once again the origin of lymphoid or myeloid precursors for DC development and corresponding subsets is a matter of debate, although it seems to be somewhat better defined than in the mouse. Interestingly, human DCs do not express CD8α such as seen in murine DCs, differentially express CD4, CD11b and also show functional heterogeneity (220). Figure 8 below schematically describes what we will be discussing in the next few paragraphs.
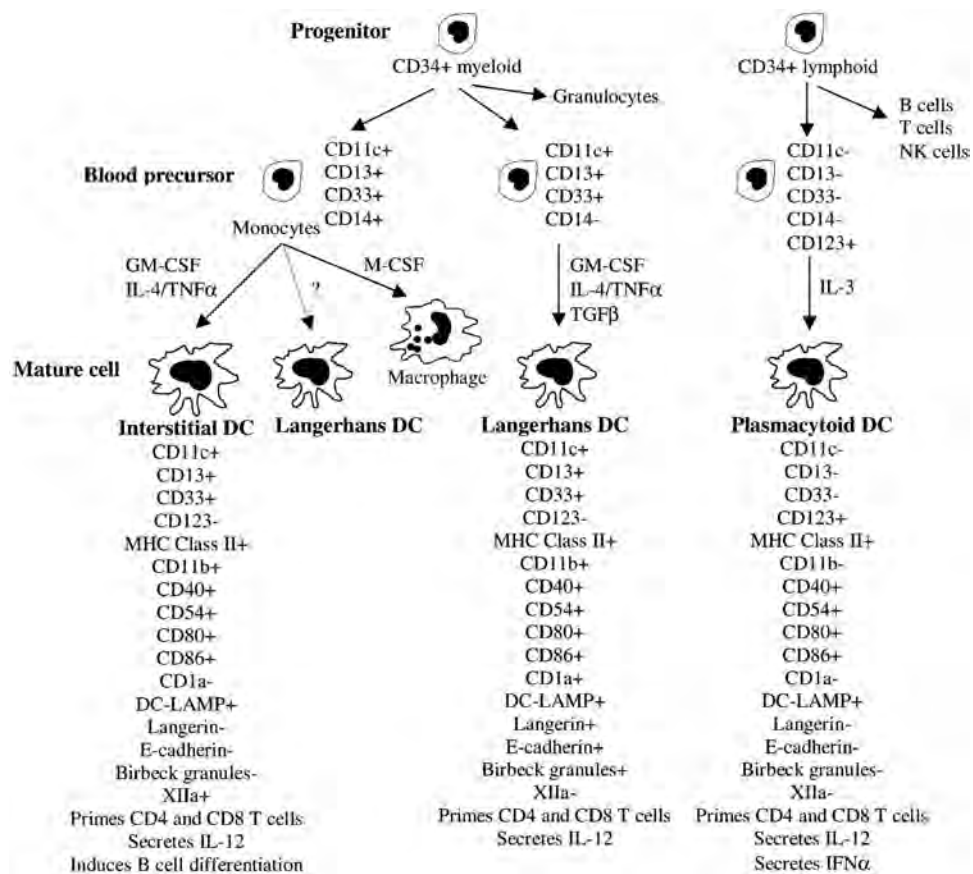
Figure 8: Human DC precursors and sub-populations. Adapted from Lipscomb and Masten, Physiological Reviews. 2002, 82(1): 97-130[150]**.**

As seen in Figure 8, the three human DC subtypes have been labelled as interstitial DCs, LCs, and pDCs. Human skin contains two of the three DC subtypes in immature form: LCs and interstitial DCs. Both subtypes emerge in cultures from CD34+ BM and CD11c+ blood precursors in the presence of GM-CSF and either IL-4 or TNF-α (44). Moreover, CD11c+ precursor cells also express myeloid markers CD13 and CD33 hinting us towards the probable origin of LCs and interstitial DCs. However, LCs also require transforming growth factor beta (TGF-β) and can arise from a CD11c+CD14+ monocyte or a CD11c+CD14- blood precursor, whereas interstitial DCs uniquely arise from a CD11c+CD14+ precursor that can also differentiate into macrophages under certain specific conditions (150). LCs and interstitial DCs subtypes share several markers, although LCs uniquely express CD1a, and interstitial DCs uniquely express the coagulation factor XIIIa. LCs and interstitial DCs also share the capacity to activate both CD4+ and CD8+ naive T cells and secrete IL-12. One striking difference between LCs and interstitial DCs is the ability of interstitial DCs, but not LCs, to induce the differentiation of naive B cells into Ig-secreting plasma cells (76). The pDC equivalent to the ones found in the mouse are also found in humans with certain differences. It is suggested that this particular DC line is generated from lymphoid and myeloid precursors and are found in high concentrations in T cell zones of lymphoid organs and the thymus. As seen in the mouse, these DCs possess the unique ability to secrete large amounts of type I IFN upon viral stimulation (150). Unlike LCs and interstitial DCs, pDCs require IL-3 for their differentiation and are derived from a CD11c- blood precursor that has low expression of GM-CSF receptor (84). On the other hand, pDCs also share the capacity of activating naive CD4+ and CD8+ T cells and secrete IL-12 upon CD40L stimulation (150).

Although it is clear that differences exist between murine and human DCs, it is evident that murine DCs provide an appropriate model for human cells in most cases. Like human DCs, murine DCs: *1*) originate from CD34+ BM stem cells, *2*) are found in blood and tissues, *3*) are able to take up and degrade antigen to antigenic peptides, *4*) express MHC class II molecules complexed with antigenic peptide, *5*) express co-stimulatory molecules, *6*) mature and migrate in response to danger signals, and *7*) are responsive to the microenvironment with a controlled release of chemokines and cytokines (150). Nevertheless, data obtained from murine DCs should always be interpreted with caution, especially in the cases where clear discrepancies exist between murine DC and their human equivalent.

2.1.2 Dendritic cell maturation and activation

Typically, under steady-state conditions, DC progenitors in the BM give rise to fresh immature DC precursors that are free to circulate before homing to a particular tissue, mucosal surface or peripheral organ where they will continuously sample the environment for foreign antigens before slowly returning to lymph nodes (16). DCs under these conditions have high phagocytic capacities and express low levels of surface MHC and co-stimulatory molecules. In the absence of danger signals such as inflammation or pathogens, DCs are important in inducing peripheral tolerance to self-antigens by several mechanisms usually involving T cells (242). However, upon recognition of a particular danger signal, DCs need to undergo an important transformation called maturation in order to further drive the immune system by activating T cells and other accessory cells of the immune response. Thus, DC maturation is associated with many physiological changes and events that will be discussed below.

2.1.2.1 Pathogenic antigen recognition receptors

The first step in DC maturation is sensing that steady state conditions have been replaced by inflammation. That is, an alarm has sounded indicating that an infection is ongoing and the infected host needs to change steady state DCs into immunogenic DCs. DCs have thus evolved to acquire a wide variety of pattern recognition receptors (PRRs) and endocytosis mechanisms for the internalization of pathogens. The principal functions of PRRS include opsonization, activation of complement and coagulation cascades, phagocytosis, activation of pro-inflammatory signaling pathways and induction of apoptosis (121).

Among well known PRRs, **TLRs** contribute significantly to orchestrating an efficient immune response against pathogens. The murine TLR family comprises 11 identified members that are widely distributed among APCs, including DCs. TLR10 is not functional in mice, and the importance of TLR8 is not clear because no natural ligand has been identified (242). Albeit not comparable to T and B-cell receptors, TLRs bring considerable specificity and diversity to the innate immune system (242). Some TLRs can associate as heterodimers or even with non-TLR membrane clusters to further diversify their recognition potential (250). Several pathogen-associated molecular patterns (PAMPs) can simultaneously be recognized by TLRs, such as lipopolysaccharide (LPS) which is recognized by TLR4, as well as flagellin and CpG motifs in DNA, which are recognized by TLR5 and

TLR9, respectively (3, 250). TLR2 recognizes the largest number of ligands. The list includes peptidoglycan, bacterial lipoproteins, a phenol soluble factor from *Staphylococcus epidermidis*, LPS from *Prophyromonas gingivitis* and *Leptospira interrogans* (which differs in structure from the LPS of Gram-negative bacteria), and zymosan, a component of yeast cell walls (121). TLR2 does not recognize these PAMPs independently, but functions by forming heterodimers with either TLR1 or TLR6 (193). A likely consequence of this cooperation is an increased repertoire of ligand specificities. At the molecular signalling level, the protein MyD88 is the most important adapter molecule required for TLR signalling transduction (190). However, a MyD88-independent pathway, mediated by TIR-domain-containing adapter-inducing interferon-β (TRIF), makes an important contribution to TLR signalling. The TRIF pathway, which is activated by TLR3 and TLR4, results in the production of type I IFN, highlighting their role in activating accessory immune cells early in infection (190). Engagement of TLRs leads to activation of transcription factors that promote transcription of inflammatory cytokines and up-regulation of co-stimulatory molecules, the significance of which will be seen in section 2.1.3.

The role and importance of TLRs in the context of GBS has been studied and discussed in part in previous sections. The most compelling evidence for the importance of TLRs for development of the host response to GBS is provided by studies in mice with targeted deletions of TLR2 or MyD88. In normal mice, a targeted local immune response destroys a low subcutaneous GBS inoculum without measurable systemic inflammatory activity in the blood. The same GBS dose induces 20–30% lethality in mice with deletions of either TLR2 or MyD88 (161). Results also correlated with decreased amount of TNF-α production in TLR2 and MyD88 deficient mice. Following these initial findings, additional work has reinforced the importance of this particular TLR for mediation of phagocytic functions and GBS clearance (75, 141, 203, 266). However, it was recently suggested that type I IFN production could potentially be more a result of interaction of GBS DNA with a putative unidentified intracellular DNA sensor or receptor that will be discussed in the next paragraph (52). Mancuso *et al.* have demonstrated a role of TLR7 for the recognition of phagosomal GBS by conventional DCs, but not macrophages. This pathway resulted in high production of the type I IFN-α and IFN-β. This response requires not only TLR7 but also the adaptor MyD88 and the transcription factor IRF1, all of which localized together with bacterial products in degradative vacuoles bearing lysosomal markers. TLR9, in addition to TLR7, may participate in the activation of the Interferon regulatory factor-1 (IRF1)-dependent phagosomal pathway, as

suggested by the moderately but significantly lower type I IFN- production found in response to phagosomal GBS in the absence of TLR9. Therefore, TLR7 and TLR9 may act together in GBS detection, although the effects of TLR7 deficiency outweighed those of TLR9 deficiency in terms of type I IFN- expression. Thus, this DC-specific recognition pathway links lysosomal recognition of GBS RNA with a robust, host-protective IFN response (160). Finally, in contrast to the extensive research in the context of GBS-macrophage interactions, the role of others TLRs in DC recognition of GBS has been poorly addressed. Only one study reported that GBS induces DC production of TNF-α and IL-1β by a MyD88-dependent mechanism that, nevertheless, does not require any of the better-characterized TLRs, such as TLR2, TLR4, TLR7 and TLR9 (57).

Besides TLRs, the second major family of PRRs is the family of nucleotide-binding oligomerization domain (NOD)-like receptors (**NLRs**). Unlike membrane-bound TLRs, which sense PAMPs on the cell surface or in endosomes, NLRs recognize microbial molecules in the host cytosol. After microbial recognition, TLRs and NLRs induce the activation of distinctive host signaling pathways, which lead to innate and adaptive immune responses. NLRs possess a characteristic domain architecture, consisting of an N-terminal pyrin or caspase activation and recruitment domain (CARD), a central NOD, and C-terminal leucine-rich repeats. The CARD or pyrin domains mediate homophilic protein–protein interactions with other CARD or pyrin-containing proteins (129). At least two well-characterized NLRs, that is, nucleotide-binding oligomerization domain-containing 1 and 2 (NOD1 and NOD2), sense molecules produced during the synthesis and/or degradation of bacterial PG, leading to the activation of transcription factor NF-κB and MAPKs (129). Another group of NLRs participates in the formation of a large multiprotein complex called the inflammasome, whose assembly leads to the activation of caspase-1. Such activation involves cleavage of procaspase-1 into the active protease, which, in turn, is responsible for the maturation of the pro-inflammatory cytokines IL-1β and IL-18 (129).

In the context of GBS type III infection, it has been recently reported that IL-1β secretion in GBS-stimulated mouse conventional DCs is critically dependent on the NLR family pyrin domain-containing 3 (NLRP3) inflammasome and on the production of β-H/C by GBS. Moreover, the NLRP3 inflammasome had a crucial role in *in vivo* anti-GBS defense (57). On the other hand, the role of NOD receptors in the recognition of GBS-derived PG is largely unknown.

In addition to TLRs and NLRs, a number of encapsulated bacteria interact with phagocytic cells via surface lectins. In particular, immature DCs express a large variety of **CLRs**, which are often down-regulated upon maturation. CLRs can be divided into two categories based on an amino-acid motif involved in sugar recognition and coordination of Ca^{2+} ion: mannose-type lectins and galactose-type lectins. Mannose type lectins such as the mannose receptor, DC-SIGN, Dectin-1 or Langerins possess a basic specificity for mannose and/or fructose-terminated glycan structures (265). In contrast, galactose-type lectins recognize Gal and GalNAc-terminated glycan structures (265). Furthermore, CLRs form oligomers within the cell membrane to strengthen and limit binding to a specific structure with a certain carbohydrate density and spacing, while maintaining basic carbohydrate specificities that can interact with a large variety of ligands (218). This process has also been shown to facilitate efficient loading of antigen and T cell induction and can greatly influence the ensuing immune response such as by favouring the outcome of a particular T cell phenotype. Due to the large variety of CLRs repertoire, signalling events that mediate their activation are quite extensive and largely surpass the complexity of this review. However, glycan binding to CLRs expressed by DCs and initiation of an inflammatory signalling cascade is nicely demonstrated by work on the β-glucan receptor Dectin-1 (34). Binding of fungal β-glucans to Dectin-1 can directly or in synergy with TLR2 trigger secretion of cytokines such as IL-10, TNF-α and IL-12 (96). All pathways depend on the functional immunoreceptor tyrosine-based activating (ITAM)-like motif in the Dectin-1 cytoplasmic domain and the recruitment of the tyrosine kinase Syk (212). The collaborative response of Dectin-1 can also occur with TLR4, 5, 7 and 9 (265). In spite of the encapsulated nature of GBS, no data is available on GBS interactions with known CLRs.

As mentioned above, **complement receptors** play a very important role in early non-specific pathogen recognition. **Complement receptor 3 (CR3)** is part of the CD11b/CD18 family of integrins and mediates both opsin-mediated and non-opsonic phagocytosis. Two binding sites have been identified on CR3; one site mediates iC3b recognition bound to the surface of foreign cells for phagocytosis and a second lectin-like site with the capacity to bind certain polysaccharides. Engagement of the CR3 lectin site results in priming of CR3-expressing phagocytes for cytotoxicity of iC3b-opsonized targets in the absence of specific antibody. Thus, the engagement of this site is required for the full activation of CR3. This occurs by a two-step process whereby iC3b-opsonized particles bind to CR3 via the first domain and a polysaccharide, either

exogenous or intrinsic to the opsonized particle, engages the lectin site (5). The CR3 lectin site sugar specificity is much broader than originally thought, allowing for a interactions with a number of monosaccharides and certain mannose and Glc containing polysaccharides (110, 248). Coming back to GBS, one study has demonstrated that GBS type III CPS can function as a ligand to promote neutrophil CR3 functions. This was evident as neutrophils were capable of intracellular killing upon ligation of CPS III and iC3b to their respective sites on CR3, representing a powerfull strategy for host cells to avert invasion prior to the development of a specific immune response (5). It is important to remember, however, that GBS possesses multiple virulence factors that can subvert these initial crucial non-specific immune responses as seen in section 1.4.1. The role of complement receptors and/or complement in GBS interactions with DCs has been poorly addressed compared to the amount of publications with other phagocytic cells (5, 7, 80, 82, 158, 164, 165, 189, 237, 267, 268). It has been shown that C3- and CR2-deficient mice exhibited decreased uptake of type III CPS by follicular DCs within the germinal centers with consequent reduced adaptive immune response to type III CPS (200). In a second study, serum-mediated opsonization increased GBS internalization by conventional DCs but did not significantly alter intracellular survival rates of either the encapsulated or the non-encapsulated strain. Furthermore, experiments with heat-inactivated serum suggested that complement does not play an important role in GBS interactions with DCs (142). Once again, these findings suggest important differences in GBS type III interplay with professional phagocytes and conventional DCs.

The final family of PRRS is the **Siglecs**. Siglecs come from a family of type 1 transmembrane proteins that possess variable numbers of Ig domains. Interaction of Siglecs with their corresponding ligand includes the formation of a salt bride between a key arginine residue on the receptor and the carboxylated group of sialic acid (59). Siglecs can be broadly divided into two groups: firstly, CD33-related siglecs (CD33rSiglecs), which are highly related and rapidly evolving; consequently, they differ significantly in composition between mammalian species. Scondly, a group comprising sialoadhesin, myelin-associated glycoprotein and CD22, which are more distantly related, yet have well-conserved orthologues in all mammalian species (59). As we have previously discussed, sialic acid is found on all serotypes of GBS CPSs and has been shown to interact with CD33rSiglecs (41). Functionally, CD33rSiglecs possess immunoreceptor tyrosine-based inhibitory motifs (ITIMs) that are involved in inhibitory signalling that dampens or counteracts activating signals sent by other immunoreceptors, such as those containing ITAMs

(59). Therefore, it is tempting to speculate that GBS and other bacteria have evolved a shared virulence mechanism of decorating their surfaces with a human-like monosaccharide, such as Neu5Ac, which acts as a ligand for both factor H and hCD33rSiglecs. In this fashion, the sialic acid-expressing bacterial pathogens could simultaneously interfere with complement and cellular components of innate immunity (87). As already discussed in section 1.4.1, GBS sialic acid was demonstrated to interact with Siglecs expressed on neutrophils and monocytes (41, 42, 264); however, no single study has addressed the role of Siglecs in GBS interactions with DCs.

2.1.2.2 Endocytosis

APCs, and more importantly DCs, possess a wide variety of endocytic mechanisms to internalize a particular pathogen following its recognition. Internalization mechanisms fall into two major categories: phagocytosis and endocytosis (55).

Specifically speaking, the process of phagocytosis is restricted to a limited class of immunological cells such as DCs, macrophages/monocytes and neutrophils, and usually functions to eliminate pathogens and dead-cell debris. Phagocytosis involves guanosine triphophatases (GTPases) of the Rho family and other signalling molecules that are activated by specific cell-surface receptors and stimulate actin ploymerization/depolymerisation events (30, 55). For example, when Fc receptors on macrophages recognize antibodies bound to surface antigens on bacteria, a signalling cascade involving activation of the Rho family members Cdc42 and Rac triggers actin assembly and the formation of cell-surface extensions that zipper up around the antibody-coated pathogen to engulf it. These signals also activate the cell's inflammatory responses so that once the bacteria are taken up into membrane-bound phagosomes, they are destroyed by the infusion of a barrage of bactericidal weapons, free oxygen radicals and acid hydrolases (1).

In contrast to phagocytosis, endocytosis is a process common to all cell types, except for red blood cells who are deprived of such mechanisms. Classically, endocytosis was considered to mediate the internalization of small molecules although it is becoming increasingly evident that such mechanisms are much more flexible and can be used for the internalization of much bigger particles such as virus and bacteria (70). Endocytosis is divided into at least 4/5 classes on the basis of the molecular machineries that drive the process: (1) clathrin-mediated endocytosis, (2)

caveolae-dependent endocytosis (3/4) clathrin- and caveolae-independent endocytosis (which can either be lipid raft-dependent or independent), and macropinocytosis (30).

Like phagocytosis, the signalling cascades that induce macropinocytosis involve Rho family GTPases, which trigger the actin-driven formation of membrane protrusions. However, unlike phagocytosis, these protrusions do not 'zipper up' along a ligand-coated particle, but instead collapse onto and fuse with the plasma membrane to generate large endocytic vesicles, called macropinosomes, that sample large volumes of the extracellular milieu (55). It has been shown that activation of DCs triggers extensive and prolonged macropinocytic activity (172). The severing of macropinosomes from the plasma membrane is independent of the activity of the fission-inducing protein, dynamin (30).

Clathrin-mediated endocytosis and caveolae-dependent endocytosis are characterized by the formation of their respective protein coats at the membrane invagination site and assemble into a curved rigid scaffold tightly associated with the plasma membrane (30, 31, 55). Both mediate the formation of endocytic precursors, i.e. clathrin-coated or caveolin-coated pits that remain connected to the extracellular environment via constricted necks until dynamin effectuates the scission creating an independently coated vesicle (70, 109). However, an important difference between both mechanisms is that clathrin-mediated endocytosis employs surface receptors that are exposed on the external face of the plasma membrane and bind their receptive molecules in order to activate internalization such the transferrin receptor (70). Comparatively, caveolae-dependent endocytosis is characterized as cholesterol-rich microdomains on the plasma membrane because caveolin binds cholesterol. Caveolae have been experimentally disrupted by depletion of plasma-membrane cholesterol, by overexpression of dominant-negative caveolin mutants, and most recently, through genetic knockout of the caveolin genes, highlighting the importance of caveolin in caveolae-dependent endocytosis (55).

Lastly, caveolin- and clathrin-independent endocytosis occurs without a protein coat and is solely driven by the lipid composition of the membrane patches, although mechanisms by which this occurs are still poorly understood (30). This process can also be cholesterol-dependent or independent and do not require any scaffolding proteins (187). Lipid rafts are dynamic membrane microdomains enriched in cholesterol, glycolipids, glycosylphosphatidylinositol (GPI)-anchored

proteins, where receptors cluster upon activation and initiate their respective signalling cascades (205). Two different subtypes of raft-mediated endocytosis have been identified: one that requires dynamin for the fission of the endocytic carrier and one that is dynamin-independent (30). A number of pathogens including viruses, bacteria and parasites have been reported to require lipid rafts for their internalization as disruption of this pathway inhibits their uptake by phagocytes (162). It has been suggested that bacterial entry via this pathway offers two advantages: avoidance of intracellular degradative pathways (which would obviously result in bacterial destruction), and triggering of the cell signaling that leads to membrane ruffling and rearrangement of cytoskeleton, which are both required for entry of bacteria (162). Figure 9 depicts these various endocytic mechanisms.
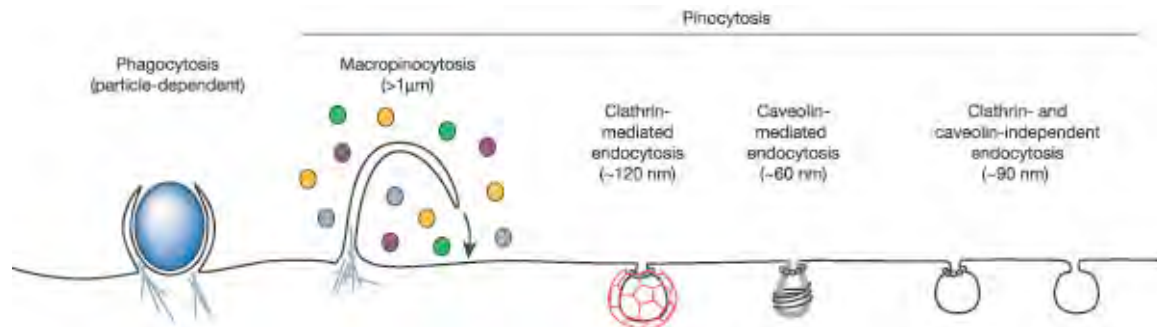
Figure 9: Schematic representation of different mechanisms of endocytosis and phagocytosis. Adapted from Conner *et al*., Nature. 2003, 422(6927): 37-44. [55]

2.1.2.2.1 Endocytosis and GBS

Currently, only three articles are available which have addressed the endocytic mechanisms underlying GBS type III internalization by DCs. In the first article, by using mouse bone marrow-derived dendritic cells (bmDCs) and primary spleen DCs the authors demonstrated that both types of DCs efficiently internalize encapsulated GBS, but the latter possesses strong intracellular survival capacity. GBS devoid of CPS was internalized and killed at higher and faster rates than encapsulated GBS early after infection (143). These data suggest that CPS facilitates, at least in part, GBS survival within DCs. In previous studies, it was shown that under non-opsonic conditions, non-encapsulated mutants were equally phagocytosed and survived at similar levels within

macrophages than wild-type GBS (56, 227). Thus, the contribution of the CPS to the modulation of professional phagocyte functions vs. DC functions might be different. Overall, the capacity of DCs to internalize encapsulated GBS seems similar or lower to that reported for other phagocytes. It is generally accepted that different types of leukocytes differentially perform their specialized tasks. As such, it has been shown that neutrophils and monocytes exhibit a much higher capacity to kill ingested bacteria than DCs (183, 186). In a recent study, Mancuso *et al*. showed that encapsulated GBS survived longer in bmDCs compared to mouse macrophages (160). Finally, co-localization studies and the use of endocytosis inhibitors and caveolin-/- mice, demonstrated that GBS uses multiple endocytosis mechanisms to enter mouse DCs. CPS selectively drives GBS internalization via caveolae-independent but lipid raft-dependent pathways. Non-encapsulated bacteria failed to engage lipid rafts. GBS internalization by DCs also occurs via clathrin-mediated endocytosis in a process independent of bacterial CPS (143). Following internalization, GBS was observed to reside in both acidified and non-acidified compartments (143). It is thus tempting to speculate that perhaps the route of internalization used by GBS could affect avoidance of intracellular degrading mechanisms and the level by which bacteria must actively resist lysosomal fusion, partially explaining why GBS is observed in two different types of vacuoles. The aforementioned study by Mancuso *et al.* beautifully complements the work of Lemire *et al.* In this study authors observed that once GBS was internalized, it could reside in two distinct types of bmDC intracellular compartments: phagosomes that contained single GBS cells that stained positive for bacterial DNA and sequentially acquired early and late endosomal markers but were negative for the lysosomal marker cathepsin D (which they called DNA$^+$ phagosomes); and cathepsin D-positive, DNA-negative phagolysosomes filled with amorphous GBS material (DNA$^-$ phagolysosomes), indicating intracellular bacterial killing (160). As mentioned above, the presence of DNA$^-$ phagolysosomes in bmDCs was required for the induction of a robust type I IFN production, and acted mainly via TLR7 (160). On the other hand, GBS in DNA$^+$ phagosomes did not co-localize with TLR7, and resulting bmDCs were highly defective in mounting an appropriate type I IFN response (160). Although authors did not study internalization pathways used for GBS uptake, once again, it is likely that the route of internalization drives GBS into two distinct intracellular compartments, delays the maturation process of vacuoles into phagolysosomes, consequently leading to impaired DC activation of the ensuing immune response.

2.1.2.3 Peptide loading onto MHC class II for presentation

Antigen presentation can occur via two different types of MHC molecules: MHC class I and MHC class II. MHC class I presentation can be performed by all mammalian cells and mediates peptide recognition by CD8+ T cells (121). Here we will focus our attention on MHC class II presentation for recognition by CD4+ helper T cells (Figure 10). Unlike MHC class I, MHC class II presentation is mainly restricted to professional APCs (121). In this setting, exogenous antigens are internalized by APCs by means of one of the many pathways we have described in previous sections, and gain access to early and late acidic endosomal compartments in which proteases initiate antigen degradation (150). However, before this happens, immature APCs constantly accumulate nascent MHC class II molecules in lysosome-related compartments identified as MHC class II-rich compartments (MIIC). MHC class II α- and β-peptide chains are synthesized in the endoplasmic reticulum, where they associate with the invariant chain (43). The invariant chain protects the peptide-binding groove of the MHC class II heterodimer from being prematurely filled with self-proteins. The MHC class II/invariant chain complex is then transported from the endoplasmic reticulum to the Golgi where vesicles deliver the newly synthesized complexes to the MIIC. There, the invariant chain is partially cleaved by cathepsin, leaving a small fragment called CLIP (class II-associated invariant-chain peptide) in the peptide-binding groove of the MHC class II molecule (231). This is followed by the action of HLA-DM in humans, or H-2M in mice, which removes MHC class II-associated CLIP (150). The action of cathepsin is regulated by cystatin, which inhibits cathepsin. Cystatin action is down-regulated during DC maturation process (215). Upon antigen internalization, resulting antigenic polypeptides contained within the phagolysosome fuse with MIICs where the peptides will associate with preformed MHC class II molecules within the MIIC. Finally, MHC class II molecules with the new antigenic peptide in its binding groove traverse the cytoplasm in exocytic vacuoles for display on the cell surface. In immature DCs, MHC class II molecules are rapidly internalized and have a short half-life. Maturation and inflammatory signals lead to a burst of synthesis of MHC class II molecules that translocate to the cell surface where they remain stable for the remainder of the APC life and are available for recognition by CD4+ T cells (48). It has recently been demonstrated that members of the B7 family of co-stimulatory molecules (which will be seen further) are embedded in the vesicular lipid along with MHC class II molecules and are delivered to the cell surface in association with the MHC class II/peptide complexes (251).
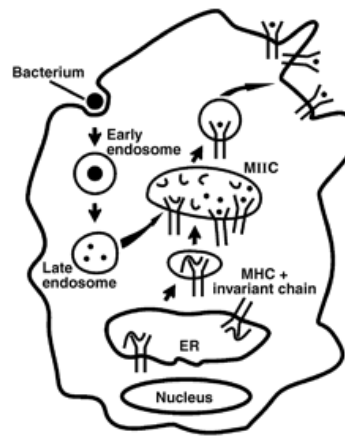
Figure 10: Mechanisms of MHC class II presentation in dendritic cells. ER: endoplasmic reticulum. Adapted from Banchereau *et al.*, Annu. Rev. Immunol. 2000, 18(1): 767-811.[16]

2.1.3 Consequences of DC maturation and activation

DC maturation is a continuous process initiated in the periphery upon antigen encounter and/or inflammatory cytokines. Following internalization and destruction of the pathogen, DCs migrate to lymph nodes where they readily display their newly antigen-loaded MHC molecules to surrounding T cells. The maturation process highly depends on the type of pathogen encountered and the molecular composition of the environment surrounding DCs and is associated with several coordinated events. Theses events can be described as the loss of endocytic and phagocytic receptors and diminished endocytosis/phagocytosis capacity; up-regulation of surface MHC class II and co-stimulatory molecules such as CD40, CD54, CD80/86; cytokine production and morphological changes (16).

The ability of DCs to respond to inflammatory and lymphoid chemokine gradients is presumably linked to their maturation state, because as DCs mature they lose responsiveness to inflammatory chemokines and gain responsiveness to lymphoid chemokines. Both human monocyte-derived immature DCs and murine CD34+ derived immature DCs express both CC and CXC chemokine receptors (CCR and CXCR), such as CCR1, CCR2, CCR5, and CXCR1, and respond to inflammatory chemokines such as macrophage inflammatory protein-1α (MIP-1α/CCL3), monocyte chemotactic protein-1 (MCP-1/CCL2), and regulated on activation normal T cell

expressed and secreted (RANTES/CCL5) chemokine (150). As immature DCs migrate toward increasing concentrations of inflammatory chemokines, they are also exposed to increasing concentrations of pro-inflammatory cytokines and the pathogen products initiating the inflammatory response. In response to these danger signals, DCs mature, and in doing so switch the usage and expression of chemokine receptors from inflammatory to lymphoid homing receptors (16). Thus, maturing DCs down-regulate the expression of CCR1, CCR5, and CXCR1 and up-regulate the expression of CXCR4, CCR4, and, in particular, CCR7, a chemokine receptor that responds to secondary lymphoid tissue chemokine (SLC/CCL21) that is produced by lymphatic endothelial cells (150, 273). The anatomic distribution of CCL21 secretion coordinately attracts DCs first from peripheral tissue to afferent lymphatics and then to T cell areas in lymphoid tissue. CCR7 is also selectively expressed on naive T and B lymphocytes, allowing these cell types to also home to lymphoid tissues. The essential role of CCR7 in DC homing to lymphoid organs is supported by the observation that in CCR7-deficient mice, maturing DCs are not able to migrate to lymph nodes (91).

DC maturation and activation can be influenced by many factors, including (*a*) pathogen-related molecules such as LPS, bacterial DNA, and double-stranded RNA; (*b*) the balance between pro-inflammatory and anti-inflammatory signals in the local microenvironment, including TNF-α, IL-1, IL-6, IL-10, TGF-β, and prostaglandins; and (*c*) T cell–derived signals (16). Recently, studies that have investigated the role of these different signals on DC maturation have revealed that certain signals can be associated with a preferential initiation of T cell polarization to a T cell helper-1 (Th1) or T cell helper-2 (Th2) phenotype. Although we have not yet addressed Th1 and Th2 immune responses, for now, it is only important to know that they drive different CD4+ T cell helper phenotypes, which have been associated to particular immunological outcomes that will be discussed in section 2.2.

Often, microbial products such as LPS, CPS, CpG and flagellin among others, are the first PAMPs recognized by DCs via TLRs and NLRs. It is becoming increasingly evident that signals received via the activation of these initial pathways are crucial and largely determine the ensuing immune response. For example, in the case of flagellin, a study demonstrated that in TLR5-dificient mice, DCs were severely impaired in inducing a flagellin-specific CD4+ T cell expansion resulting in poor Th1 and Th2 responses (146). Double stranded RNA has been shown to enhance

the surface expression of MHC and co-stimulatory molecules and to induce the release of type I IFNs, leading to the development of Th1 effector cells (23). Conversely, a soluble egg extract from the helminth *Schistosoma mansoni* (possibly acting via an unidentified TLR) promoted maturation of DCs into Th2-polarizing APCs by inducing surface expression of CD134L (65). The TLR4-ligand LPS and the TLR2-ligand PG induced comparable levels of maturation markers in human DCs but distinct cytokine and chemokines profiles: IL-12p35 was produced in response to LPS, whereas IL-8 and IFN-inducible protein 10 (IP-10/CXCL10) were secreted following stimulation with PG (207). Furthermore, *in vivo* studies revealed that LPS from *Porphyromonas gingivalis* causes DCs to prime a Th2 response, whereas LPS from *E. coli* promotes the development of IL-12-secreting DCs with Th1-polarizing activities (119, 202). Finally, the TLR2-ligand zymosan, a component of yeast cell wall, promotes the release of IL-10 and the generation of Th2 effector cells in mouse splenic DCs (79). Because of these observed tendencies, researchers have termed dendritic cells as "DC1" and "DC2" according to their naïve T cell polarization capabilities. Figure 11 below offers a potential model resuming what we have just overviewed.
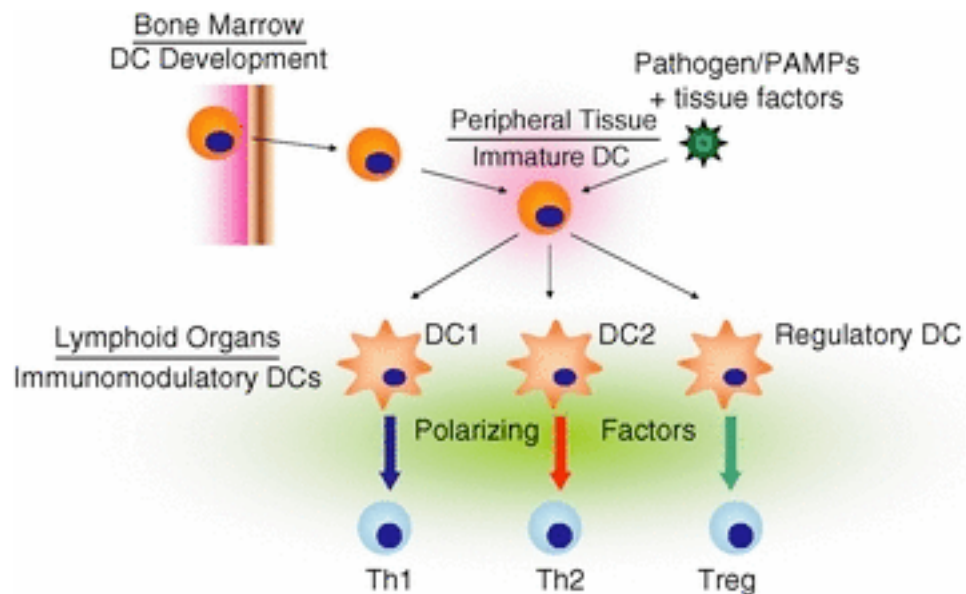

Figure 11: Pathogen-associated molecular patterns (PAMPs) and dendritic cell (DC) maturation and activation. Adapted from Kaiko *et al.*, Immunology. 2008, 123(3): 326-338.[126]

2.1.3.1 DC maturation and activation induced by GBS

Few studies have addressed the levels of DC maturation or activation following encountering GBS and the available data is limited to serotype III. It has been recently reported GBS-stimulated mouse bmDCs secreted TNF-α, IL-1β and IL-18. GBS induced the production of TNF-α and IL-1β by a MyD88-dependent mechanism that, nevertheless, does not require any of the better-characterized TLRs, such as TLR2, TLR4, TLR7 and TLR9. Interestingly, the IL-1β secretion was critically dependent on the NLRP3 inflammasome and on the production of β-H/C by GBS (57). As already mentioned, the production of type I IFNs by GBS-infected bmDCs was linked to TLR7 and TLR9 activation in phagolysosomes (160). GBS phagocytosis was shown to be significantly required for bmDC production of IL-12p70, IL-6, IL-10, IL-1β, TNF-α, CCL2, CXCL1 and CXCL10 (57, 142, 143), suggesting that phagocytosis is needed to optimally activate the production of these cytokines. In contrast, GBS induces DC-increased expression of MHC-II and the co-stimulatory molecules CD40 and CD86 in a phagocytosis-independent manner (142). The consequences of this GBS-induced DC activation status on T cell differentiation are largely unknown.

2.2 CD4+ T cells and adaptive immunity

T cells develop from progenitors that are derived from pluripotent hematopoietic stem cells in the bone marrow and migrate via the blood where they differentiate and mature in the thymus (180). T cell development is characterized by the maturation of a T-cell receptor (TCR) composed of an α:β dimer in 95% of cases or a an γ:δ dimer in 5% of cases. Each protein subunits can also be divided into a constant domain (C) and a variable domain (V) that undergoes extensive rearrangement during maturation in the thymus. The variable region defines the diversity of the T cell population and is the portion of the protein that binds the antigen-loaded MHC molecules on the surface of cells conferring the specificity of the T cell. The development of α:β T cells is also characterized by a transient double positive CD4-CD8 precursor which gives rise to a single positive for one of the two, demarking the CD4+ T cell and CD8+ T cell subsets. Before exiting the lymph nodes to re-enter circulation in the form of a naive T cell, they undergo an intense positive and negative selection eliminating potential self-reactive cells with only 2-4% making it (180). CD4+ T cells, also known as T helper cells, play a particular central role in coordinating the adaptive immune response by providing important signals in the form of cytokines and co-stimulatory molecules to effector cells of the humoral and cellular immune responses such as B cells and CD8+

T cells, respectively. T cell-derived cytokines and co-stimulatory molecules provide activation signals such as proliferation, differentiation and survival of these effector cells in an infection or inflammatory setting, but also provide inhibitory signals important for a balanced control during clearance of infection. The importance of T helper cells in the coordination of immune responses can be seen in the case of the human immunodeficiency virus which selectively depletes this cell line via the CD4 surface molecule eventually leading to the stage of infection known as the acquired immunodeficiency syndrome. When a T helper cell recognizes an Ag loaded onto MHC-II via its TCR, the T cell becomes activated and results in the acquisition of specific functions, which have been associated with different subtypes or phenotypes of activated T cells. Relatively unchallenged for almost 20 years, it was widely accepted that CD4+ T helper cells differentiate into two distinct effector populations, IFN-γ-producing Th1 cells and IL-4-producing Th2 cells. It is now customary to acknowledge at least five, if not six, CD4+ T cell subsets including Th1, Th2, Th17, T follicular helper (T Fh) and Treg cells; plus the yet to be fully accepted Th9 cells (191).

2.2.1 DC-T cell interface and cross-talk: the immunological synapse

The first DC-T interaction occurs when a naïve cognate T cell recognizes a peptide loaded in the MHC class II molecule of a DC. This initial interaction represents the first signal required for T cell activation although it is not sufficient to promote proliferation, differentiation and survival. For that to occur, the DC and T cell become particularly intimate via the formation of an immunological synapse characterized by the clustering of many important accessory proteins (180). Among the surface proteins located at the immunological synapse are the TCR; the co-stimulatory molecules CD2, CD28, CD40 and CD40L (CD154); the integrins CD11a/CD18 (also known as lymphocyte function-associated antigen-1); VLA4 (very late antigen–4); CD54 (also known as intercellular adhesion molecule–1 or ICAM-1) and ICAM-3; various cytokine receptors (INF-γR, IL-2R, IL-12R); and the chemokine receptors CCR5 and CXCR4. Integrin-ligand interactions enable the formation of tight contacts between T cells and DCs; the absence of CD54 on T cells or DCs prevents the formation of a stable immunological synapse (210). Signals received via cytokines and co-stimulatory molecules provide the extra two signals required for a complete activation of T cells (Figure 12). In contrast to the number of studies devoted to analyzing the structure and function of the immunological synapse on T cells, the DC counterpart has received much less attention. The identification of DC surface protein components of the immunological

synapse has shown, not surprisingly, that many of these molecules mirror counter-receptors on the T cell side. The DC molecules CD80 and CD86 (B7.1 and B7.2, respectively) interact with CD28 on the T cell surface. MHC class II molecules cluster on DCs in a region opposite to that of CD3 molecules on T cells (18). In addition to surface proteins, the formation of a viable immunological synapse requires intracellular cytoskeletal restructuring. Consistent with this view, is that disruption of F-actin in DCs by cytochalasin D blocks formation of the immunological synapse and activation of T cells (4).

	DC	CD4+ T Cell
Signal 1	MHC Class II/peptide	TCR and CD4
Signal 2	CD11a CD40 CD54 CD80 CD86	CD54 CD40L CD11a CD28/CTLA4 CD28/CTLA4
Signal 3	IL-12 secretion No IL-12 secretion	Facilitates Th1 response Facilitates Th2 response
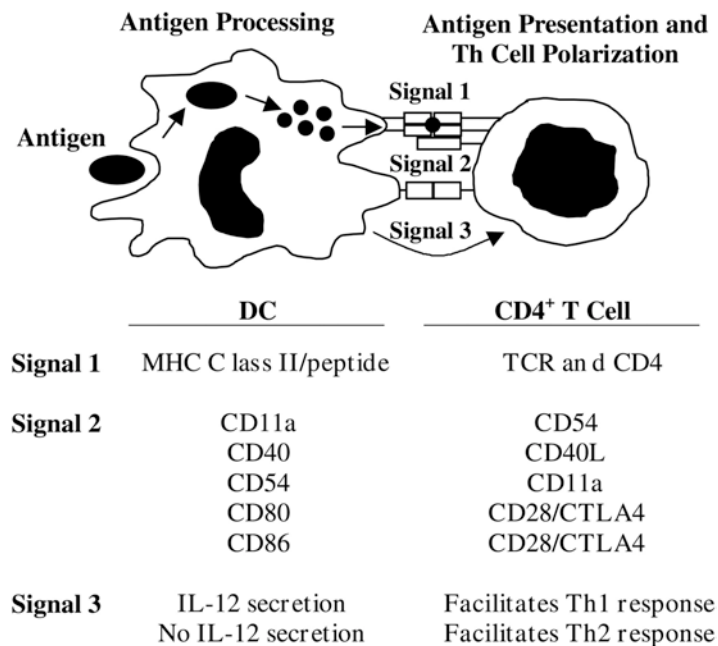
Figure 12: Example of the immunological synapse and signaling created during DC-T cell contact. DCs provide three signals to antigen-specific CD4+ T cells to initiate T cell proliferation and differentiation. DCs take up antigen and readily degrade antigens to produce antigenic peptides capable of binding to MHC class II. DCs express a high density of MHC class II/peptide complexes on their cell surface for recognition by the T cell receptor (TCR) expressed on CD4+ T cells (signal 1) and co-stimulatory molecules (signal 2) to stimulate CD4+ T cell proliferation. Secretion or lack of secretion of interleukin-12 (IL-12) by DCs (signal 3) is important in the final differentiation of CD4+ T cells into Th1 or Th2 effector T cells, respectively. All accessory molecules and cytokines are not included. Adapted from Lipscomb and Masten, Physiological Reviews. 2002, 82(1): 97-130.[150]

CD40 is expressed on APCs such as B cells and DCs, but also on activated T cells. Similarly, CD154/CD40L was initially found to be expressed on activated T cells, but is also expressed on activated human and murine DCs (150). Thus, it is important to consider the role of possible CD154–CD40 bidirectional cross-talk that occurs between DCs and lymphocytes. Indeed it was shown that CD40–CD154 interactions between T cell and DCs provide reciprocal effects that regulate both T cells and DCs (153). While it is clear that CD154 cross-linking can signal T cells, e.g. to produce IFN-γ, it is not clear how ligation of CD154 on B cells or DCs affects their functions (153).

Interestingly, it was recently proposed that prolonged DC-T cell contact could provide DCs with anti-apoptotic signals. At the final stage in the differentiation of DCs, the so-called mature stage, these cells develop various intrinsic mechanisms that make them more resistant to proapoptotic stimuli. These mechanisms include increasing the abundance of signaling molecules that confer upon DCs a reduced sensitivity to proapoptotic signals induced from death receptors, cytotoxic cells, or both (209). The high durability of the immunological synapse, which can last for up to 15 hours, raises the possibility that among the signals relayed from the DC side during this time could be those that inhibit apoptosis (49).

Cytokines are also certainly involved in DC-T cell cross-talk as their production often engages positive feeding loops reinforcing their production by the same cell and by other cells of the immune response. Cytokines also constitute a critical signal for T cell polarization, although these mechanisms will be discussed in sections 2.2.3.1 and 2.2.4.1.

2.2.2 T cell signalling cascades

The development of a T cell phenotype is the result of a balanced activation of numerous signalling cascades, which in turn impact functions of transcription factors that regulate cellular effector functions. Signalling events are mediated by three distinct entities: TCR (signal 1), co-stimulatory molecules (signal 2) and cytokines (signal 3). TCR activation in response to a recognized antigen is followed by tyrosine phosphorylation of specific ITAM domains found on CD3 that is associated to the TCR complex (116). Tyrosine phosphorylation induces GTPase activation and the recruitment of adaptor molecules to the signalling site, such as the linker for activated T cells, which then have the potential to activate serine and threonine kinases of the

MAPK pathway (275). Activation of this pathway up-regulates the transcription of factor activator protein-1 (AP-1), which promotes cell proliferation (93). Other signalling pathways also originate directly from the cell membrane after TCR stimulation by modifying its lipid composition, such as seen in the PI3K pathway. In this case, signalling induces the activation of phospholipase C which catalyzes the synthesis of phosphatidylinositol-bis-phosphate (PIP_2) from lipids of the TCR lipid-raft, to generate the second messengers inositol-1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG) (116). IP_3 diffuses into the cytoplasm and acts on its receptor located on the endoplasmic reticulum to induce internal calcium release. Fluxes of calcium lead to the translocation to the nucleus of the transcription factor, nuclear factor of activated T cells (NFAT), which is important for the expression of a number of cytokine genes for both Th1 and Th2 cells (93, 181). DAG regulates protein kinase C , which is another important mediator of T cell activation, once again through the induction of the MAPK pathway (116).

Cytokines provide the most influential signalling events that modulate T cell phenotype acquisition. This occurs when a particular cytokine binds its respective receptor causing subunits to cluster together and by extension, activates Janus kinases (JAK) and signal transducers (STAT) of the JAK-STAT pathway. There are four mammalian JAKs: JAK1, JAK2, JAK3 and tyrosine kinase 2 (tyk2) (232). When the subunits condense, the JAKs phosphorylate and activate each other and induce tyrosine phosphorylation of the cytokine receptor. Monomeric STAT proteins of the cytoplasm are recruited to the phosphorylation sites of the receptor and in turn are phosphorylated by the enzymatic action of associated JAKs. This causes STAT proteins to dimerize and translocate to the nucleus where they act as DNA transcription factors (116). Certain combinations of JAKs and STATs, alternatively spliced STATs, and STATs interacting with other signaling molecules and transcription factors, are all responsible for the specificity of cytokine signaling. Notably, IFN-γ activates JAK1 and JAK2, leading to activation of STAT1, whereas following the interaction of IL-4 with its receptor, JAK1 and JAK3 are activated, which culminates in the activation of STAT6 (89). Stimulation of the IL-12 receptor by its ligand induces the activation of JAK2 and tyk2, which in turn mediate the recruitment of STAT3 and STAT4 (239). The interaction elicited by the cytokine IL-10, upon binding to its receptor involves activation of JAK1 and tyk2 with subsequent movement of STAT1 and STAT3 to the nucleus (122).

2.2.3 Th1 polarization

2.2.3.1 Cytokines

The main criterion used to differentiate activated T cell sub-types are defined by the cytokine signals they receive during activation and the ones they produce as a result. Th1 development begins with the secretion of type I IFNs and IL-12 by APCs upon activation. As already discussed in section 2.1.3, the release of these cytokines largely depend on the engagement of TLRs as well as other PRRs by the PAMP profile of the particular pathogen at hand. IFN-γ produced by activated Th1 cells further induces APC-secretion of the priming cytokines, resulting in a positive feedback loop (126). While IFN-γ, IL-12 and type I IFNs directly induce T cells to differentiate into Th1 cells, it is mainly the IFN-γ that also acts as an inhibitor of the Th2 pathway by preventing Th2 cell proliferation (179). IFN-γ attachment to naive Th cells leads to the JAK1- and JAK2-mediated activation of the transcription factor STAT1, which then induces the expression of **T-bet**. T-bet is a member of the TATAAA-box family of transcription factors. T-bet production initiates the remodeling of the IFN-γ gene locus, production of IFN-γ, expression of the IL-12 receptor and stabilization of its own expression through the autocrine activity of IFN-γ (177). Up-regulation of IL-12R leads to its increased activation as it binds IL-12 in the surrounding environment further reinforcing Th1 differentiation (126). IL-12 signalling activates the transcription factors STAT3, STAT4 and NF-κB to promote the production of cytokines associated with the Th1 phenotype as well as allow NFAT binding to its target genes which further amplifies expression of IFN-γ (2, 126). IL-12 also acts to up-regulate IL-18 receptor expression. DC-derived IL-18 then acts to potentiate the functions of IL-12 at a later stage in the development of the Th1 phenotype (238). In addition, IL-27 is an early product of activated APCs and drives rapid clonal expansion of naive but not memory CD4+ T cells. It also strongly synergizes with IL-12 to trigger IFN-γ production by Th1 cells (194). Interestingly, prototypical type-1 and type-2 cytokines are not always autonomous, especially when they are not acting on T cells. In fact, paradoxically, Th1-inducing cytokines can stimulate Th2 cytokines from other innate immune cells. It was recently shown that IL-18 might act synergistically with IL-12 to induce the development of NK-like cells that release the Th2 cytokine, IL-13 (253).

2.2.3.2 Co-stimulatory molecules

In addition to signals received from cytokines, co-stimulatory molecules play an important role in T cell activation and their resulting phenotype. Co-stimulatory signaling is generated from DC-T cell contact that tends to occur in lymphoid organs and at the site of infection. As we have seen earlier, DC activation results in up-regulation of several membrane-associated co-stimulatory receptors such as CD40, CD54, and CD80/86 involved in T cell priming. On the T cell side, the TCR complex is clustered in the center of the synapse, together with CD4, CD2, and CD28. The CD28 interactions with the B7 family of co-stimulatory ligands (CD80/86) are essential for initiating antigen-specific T cell responses, up-regulating cytokine expression and promoting T cell expansion and differentiation. However this synapse is not exclusively related to Th1 phenotype differentiation (144). Other proposed Th1 polarizing factors include CD54 binding to its counterpart receptor CD11a/CD18 (217). In addition, it has been well demonstrated that ligation of CD40 with CD40L induces up-regulation of other co- stimulatory molecules, adhesion molecules, and the Th1-polarizing cytokine IL-12 in both mouse and human DCs (153).

2.2.4 Th2 polarization

2.2.4.1 Cytokines

The mechanisms involved in the differentiation of Th2 cells have long been more elusive than the factors driving Th1 responses but have received much attention in recent years (143, 191). The signature cytokine associated with the Th2 effector cell phenotype is IL-4, together with IL-5 and IL-13. TCR engagement, NFAT and GATA-binding protein-3 (GATA-3) may be the first signal to nudge CD4+ Th cells down a Th2 path. A low-strength TCR signal in naive CD4+ T cells induced IL-4 secretion. In contrast, higher doses promoted IFN-γ production. Mechanistically, low-strength TCR activation led to weak and transient extracellular signal-regulated kinase (ERK) activation and GATA-3 stabilization, triggering activation of IL-4. IL-2 was also induced which fed back in an autocrine manner, activating STAT-5 and providing a necessary survival and enhancing factor bypassing the requirement for exogenous IL-4. The first signal, via the TCR, during Th2 cell polarization (TCR > GATA-3 > IL-4) suggest a central role for GATA-3, a T-bet antagonist, in Th2 cell differentiation (191).

Interestingly, the initial production source of IL-4, still so crucial to for Th2 polarization, is currently unknown as DCs are incapable of secreting it. Natural killer T cells (NKT), eosinophils, basophils and mast cells have all been suggested as fulfilling this role (191, 262). Once produced, ligation of IL-4 to IL-4R on the surface of a naïve T cell induces STAT6 phosphorylation, which in turn further activates the expression of GATA-3 (86). In addition to IL-4 signaling and TCR stimulation, co-stimulation is also required to up-regulate GATA-3 transcription (276). GATA-3 mediates its activity by augmenting promoter activity of regions that are responsible for controlling Th2 cytokine gene expression resulting in secretion of IL-4, -5, -9, -10, and -13, while inhibiting the expression of IL-12R important in Th1 development (126). Once GATA-3 production reaches a certain threshold, its own gene expression is auto-activated, hence stabilizing the Th2 phenotype through an intrinsic positive-feedback loop. Furthermore, as Th2 cells mature they produce increasing levels of IL-4, which generates a paracrine loop and induces neighboring naive T cells to develop into Th2 cells (86). It is noteworthy however, that although IL-4 remains on top of the pile as a dominant Th2-promoting molecule, it was recently suggested that primary Th2 differentiation *in vivo* does not require IL-4 and STAT6 signaling but rather passes through GATA-3 dependent mechanisms further highlighting the role of this transcription factor in Th2 phenotype (90).

If Th2 responses can develop in the absence of IL-4, IL-4R and STAT-6, then which cytokine signals compensate in the absence of this pathway? Interestingly, IL-4-independent pathways have recently been identified, with cytokines such as IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) added to the list of Th2-promoting factors. IL-6 is another cytokine that is released during the early stages of a Th2 immune response. IL-6 release by macrophages, mast cells and DCs induces the Th2 phenotype through the up-regulation of IL-4 and inhibition of STAT1 phosphorylation, thereby preventing IFN-γ gene expression (69). In contrast to the Th1 phenotype, a soluble factor released by activated DC2 that is responsible for Th2 differentiation is yet to be identified. TSLP and IL-6 are potential candidates (191). Activated DC2 may also induce Th2 differentiation indirectly via the secretion of IL-10, which then inhibits IL-12 synthesis at the mRNA level and thus the Th1 pathway (132). This fact brings up much controversy as it potentially suggests that Th2 phenotype is the result of a default pathway occurring spontaneously in the absence of IL-12 (126). Whether DC2 secrete other soluble factors that promote the development of Th2 cells remains unknown.

2.2.4.2 Co-stimulatory molecules

Th2 induction via low strength TCR stimulation can by-pass the requirement for exogenous IL-4 but requires a second signal, via CD28 co-stimulation. Of particular importance, B7 molecules (CD80 and CD86) on the APC associate with CD28, and other members of the CD28 superfamily including inducible co-stimulator protein (ICOS) and cytotoxic T-lymphocyte antigen 4 (CTLA-4) on the responding T cell.

The signals elicited by the ligation CD28 with CD80/86 are considered to initiate the up-regulation of GATA-3 in conjunction with TCR providing a STAT-6 (IL-4)-independent mechanism for Th2-cell development (211). However, and as mentioned above, this pathway can also induce a robust Th1 response. CTLA-4 also interacts with B7 molecules on the APC, but unlike CD28, which provides a stimulatory signal, CTLA-4 provides an inhibitory signal for both Th1 and Th2 subsets (126).

On the other hand, ICOS, another member of the CD28 superfamily, is expressed on naive CD4+ T cells and is up-regulated on activated T cells. B7RP-1 (a third B7 family member), is expressed on B cells, macrophages, DCs and nonlymphoid tissue cells and is the ligand for ICOS (126). In the absence of CD28, ICOS can provide co-stimulation for Th2 cells, albeit at a much lower efficiency than CD28, and rescue Th2 cell development. These studies suggest a hierarchy of co-stimulation, with a critical requirement for CD28 and a less important role for ICOS (191). Immune responses in ICOS knockout mice were characterized as defective with an absence of germinal center formation, a marked impairment of T cell-derived IL-4 secretion (166). However, ICOS knockout mice were able to mount a Th1 cellular response, as noted by antigen-induced T cell IFN-γ secretion in response to immunization. Importantly, in the same study, addition of CD40 to up-regulate CD40L largely repaired the ICOS knockout defect, suggesting the role of ICOS interacting with its ligand B7RP-1 on APCs is to facilitate the downstream CD40L-CD40 interaction (166).

In addition to the CD28 superfamily, the tumour necrosis factor receptor family consists of an increasing number of receptor–ligand pairs. With regard to Th2 cell differentiation and polarization two members have received attention, CD134 and glucocorticoid-induced tumour necrosis factor receptor-related protein (GITR). CD134 is up-regulated on recently activated T cells following CD40L stimulation. CD134L-expressing DCs, but not other cells, provides a critical return

signal to the Th2 survival or expansion. However, CD134 does not appear to be required for initial priming of T cells (191).

In summary, the complete narrative regarding co-stimulation, beyond the above-mentioned interactions, for Th2 cell differentiation may never be fully realized, but so far we can certainly enhance and inhibit Th2 cell differentiation, and differentiate or disarm Th2 effector functions when necessary.

2.2.3 Th17 and Treg cells

With advancements done in the field of Th cell polarization, it became increasingly apparent that the diversity of CD4 effector T-cell responses far surpassed the Th1–Th2 paradigm and that it was insufficient to convey protection against all types of foreign pathogens. A lineage of IL-17-producing CD4+ T helper (**Th17)** cells, which are distinct from Th1 and Th2 cells, was recently discovered and shown to be crucial in autoimmune diseases and defence against extracellular bacteria (107). Th17 cells produce IL-17, IL-17F, and IL-22, thereby inducing a massive tissue reaction owing to the broad distribution of the IL-17 and IL-22 receptors. Th17 cells also secrete IL-21 to communicate with the cells of the immune system. The differentiation factors (TGF-β plus IL-6 or IL-21), the growth and stabilization factor (IL-23), and the transcription factors (STAT3, RORγt, and RORα) involved in the development of Th17 cells have been identified (134). Th17 activation results in activation of on fibroblasts, macrophages, endothelial and epithelial cells to elicit inflammatory mediators and chemokine release. The resulting environment recruits granulocytes (in particular neutrophils) and creates a general state of tissue inflammation (214).

The participation of TGF-β in the differentiation of Th17 cells places the Th17 lineage in close relationship with CD4+CD25+Foxp3+ **Tregs** (see below), as TGF-β also induces differentiation of naive T cells into Foxp3+ Tregs in the peripheral immune compartment. Central to this, IL-6, a known inhibitor of Treg development thus plays an integral part in directing the immune response from a suppressive state to an inflammatory one (126). Furthermore, IL-6 acts on naïve T cells to induce the downstream expression of IL-21, which initiates an autocrine loop that results in self-induced expression (277). TGF-β then acts in synergy with IL-21 to induce the expression of RORγt, the major Th17 transcription factor, via a STAT3-dependent mechanism. The action of RORγt induces transcription of the genes encoding IL-17 and IL-17F (277). In addition, Iezzi *et al*.

reported that CD40-CD40L co-stimulatory interactions between DCs and naïve T cells were highly important in driving Th17 differentiation as well (117).

Treg cells, as the name suggests, play an important role in the regulation of both Th1 and Th2 immune responses via suppressive immune functions. The family of Treg cells includes three sub-types. **Th3** cells are induced by oral antigen administration and exert their suppressive activity via the production of TGF-β. **Tr1** cells are induced in the presence of IL-10 and exert their suppressive activity via the production of IL-10. The last Treg sub-type comprises a subset of CD4$^+$CD25$^-$ cells that under certain condition can become CD4$^+$CD25$^+$ and acquire the expression of Foxp3 (214). Th3 and Tr1 cells do not express Foxp3 and their suppressive functions on Th1 and Th2 responses is MHC-unrestricted and non-antigen-specific suggesting an involvement in innate immunity over adaptive immune responses (260). By contrast, the mechanism of action and even the origin of adaptive CD4$^+$CD25$^+$Foxp3$^+$ cells are still a matter of debate mostly because Foxp3 is differentially expressed in mice and humans. First, in humans there is a splicing Foxp3 variant that does not exist in mice, and second, Foxp3 is constitutively expressed and cannot be up-regulated in murine T cells. However, in both models, Treg cells function by suppressing the proliferation of Ag-specific autologous effector T cell clones in an antigen-specific manner (214). Figure 13 below summarizes the major Th phenotypes described in this literature review.
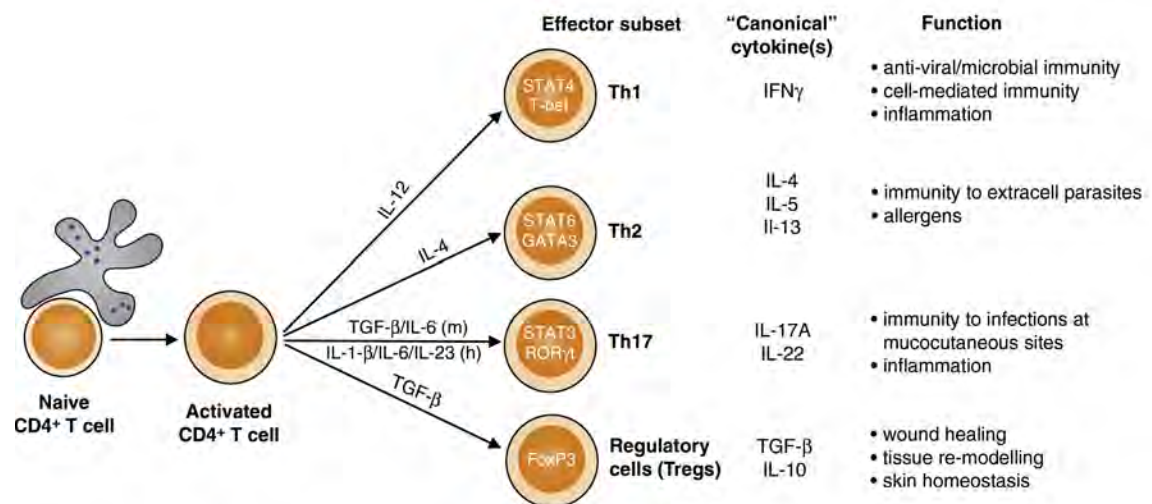

Figure 13: Activated CD4+ T helper cell phenotypes with associated transcription factors and cytokines. Adapted from Deenick *et al*., Current Opinion in Immunology, 2011, 23(1): 111-118.[66]

2.3 B cells and the humoral immune response

As it is well known, B cells are the main effector cell line of the humoral response by secreting Igs that are specifically directed to recognize a particular pathogenic epitope. Immature B cells are produced in the BM in most mammals, and maturation, like in T cells, passes by the acquisition of several surface markers and by a positive and negative selection to remove potential self-reactive B cells. Amongst these different surface markers is the B-cell receptor (BCR) composed of an IgM chain associated to Igα and Igβ which posses ITAM motifs required for intracellular signalling transduction. The IgM antibody itself is composed of two identical light and heavy chains (180). Heavy chains posses the variable region (V) which is composed of three segments; V, D and J, that recombine randomly in a process called VDJ recombination, to produce a unique variable domain which defines the B cell population diversity and specificity for particular pathogens. In addition, similar rearrangements can occur in the light chains although only the V and J segments are involved in this case (180). Following recombination of the BCR light and heavy chains, non-self reactive B cells are exported to the periphery where they circulate as immature naïve B cells. Naïve immature B cells are characterized by the expression of a mature BCR and surface IgD as well as CD45 (B220), CD93, CD23, CD138 and MHC class II (40).

The recognition of an antigen via the BCR of a cognate B cell causes the cell to rapidly proliferate resulting in the clonal expansion of a B cell population with an identical BCR that binds the exact same antigen site as the original cell (180). Many of these cells become **plasma cells**, which are short-lived antibody factories that secrete large amounts of free Ig matching the specificity of the BCR. In contrast, a small sub-group will maintain survival well after the clearance of the pathogen to become **memory B cells**. These cells can respond particularly quickly upon re-encounter of the pathogen resulting in a much more robust and efficient immune response (40). In addition to their antibody production function, B cells are also potent APCs as they can also presented antigen-derived peptides via MHC class II molecules displayed on their surface. This allows for specific engagement of the TCR on T cells and allows for intimate contact between the two partners resulting in a dynamic cross-talk that can affect both T cell and B cell functions (180).

Antigens that elicit a humoral immune response are categorised in two different groups: T cell-independent (TI) and T cell-dependent (TD). As the name suggests, TI antigens do not engage T cell help for the development of the immune response because they cannot be loaded onto

MHC molecules for presentation and depend on their interactions with B cells (180). These types of antigens are usually soluble and intact in nature, such as polysaccharides, PGs, CPS, and LPS and interact directly with the BCR leading to a polyclonal activation of this cell line (145). Often, this response is non-specific and does not elicit Ig class switching or memory formation due to lack of signalling by T cells. In contrast, TD antigens are short continuous amino acid sequences of a protein that are often buried within the native structure of the protein and are usually only accessible if the protein is unfolded and processed into peptide fragments by APCs (180). These antigens often engage a strong immune response with specific antibody production and memory responses. As this research project focuses on the T cell-dependent immune response in the context of GBS infection, the next few pages will thus focus on TD antigens and the role of T cells in B cell activation.

2.3.1 T cell-dependent B cell activation and type 1 Ags

TD-B cell activation first starts with the internalization of a pathogen by a particular APC located at the site of infection. Resulting activated APCs then travel to lymph nodes where they readily present different pathogenic epitopes via MCH class II molecules located on its surface. Secondly, a cognate T cell must recognize the presented antigen and receive cytokine and co-stimulatory signalling from the APC defining a particular phenotype in the T cell, as we have well seen in previous sections of this document. Activated T cells are then free to travel to lymph nodes where they can activate B cells presenting the same antigen via MHC class II molecules. This leads to the formation of a sophisticated compartment in the lymph node called a germinal center, which occurs at the T-cell-B-cell border of secondary lymphoid folicules (279). This structure is composed of a central zone where B cells are rapidly dividing and where a small number of DCs reside, surrounded by a mantle of antigen-specific T helper cells (180). It is also within the germinal center that activating B cells undergo Ig class switch recombination (CSR). Isotype switched germinal center B cells proliferate as centroblasts and their immunoglobulins undergo random somatic hypermutation. These centroblasts subsequently differentiate into centrocytes to undergo selection by antigen persisting on a specialized group of DCs called follicular DCs (24). Centrocytes that have successfully competed for antigen as well as for T cell help may leave the germinal center and differentiate into long-lived memory B cells or plasma cells that maintain humoral memory for a long time after immunization or infection (167).

2.3.1.1 Roles of cytokines for Ig class switch recombination

Cytokines and co-stimulatory molecules largely guide the process of Ig CSR in B cells. As we have discussed, Th1 and Th2 secrete different cytokines. They can thus solicit expression of different types of Ig classes depending on the infectious setting. However, this was not always thought to be the case. Indeed, early investigations in the role of cytokines for B cell CSR revealed a particular importance of IL-4 for driving this process, especially for IgE synthesis (156). It was later revealed that IgE secreting B cells represent a subtype of B cells that had further differentiated from IgG1 secreting activated B cells, highlighting the role of IL-4 in CSR for those two types of Ig (131). Although IL-4 plays a central role in Ig CSR, many other cytokines seem to act synergistically with IL-4 to modify or enhance resulting Ig CSR. In the same study by Maggi *et al.*, authors also observed a role of IL-2 in enhancing IL-4-dependent IgE synthesis in activated B cells, while the effects of IL-6 stimulation appeared controversial (156).

In addition to IL-4, another well studied cytokine that is particularly important in the induction and regulation of the humoral response is IL-10. Several IL-10-related molecules have been identified such as IL-19, IL-20, IL-22, IL-24, IL-26, IL-28 and IL-29 that all share an amino acid sequence which is up to 30% identical to that of IL-10 (274). In a recent study the role of these cytokines in B cell CSR was revealed *in vitro* using naïve B cells stimulated with an anti-CD40 antibody in combination with or without IL-4 or TGF-β, an important inducer of IgG2b and IgA. Authors of the study found that although none of the molecules were able to induce antibody production in B cells stimulated with an anti-CD40, IL-20, 22, 26, 28, 29 were all capable of down regulating IgG4 and capable of up regulating total IgGs when IL-4 was added to the media (115). In addition to this, all of the IL-10-related cytokines reduced IgA production of B cells stimulated with anti-CD40 and TGF-β, highlighting the role of theses cytokines in regulating antibody production by activated B cells (115).

More recently, IL-21 has emerged as a cytokine capable of mediating many different effects in responding B cells. In one study, IL-21 was reported to mediate the functions of Bcl-6, an important transcription factor in B cells, together with IL-4 to stabilize B cell class switching to IgG1 (131). The effects of IL-21 also greatly exceed those of other cytokines such as IL-2, IL-10, and IL-4. For example, IL-21 and CD40 stimulation of human naive B cells caused secretion of IgG that was up to 10-fold higher than that induced by IL-4 or IL-2 in combination with CD40 stimulation (99).

Similarly to that observed with IL-4, the outcome of IL-21-mediated Ig secretion depends on the presence of other cytokines. For example, IL-10 has been shown to synergize with IL-21 to induce the secretion of IgA by CD40L-stimulated human B cells, whereas IL-4 diminished it. Interestingly, IL-4 and IL-21 appear to possess antagonistic functions on IL-4-dependent IgE CSR. The inhibitory effect of IL-21 on IgE production was first reported in IL-21R$^{-/-}$ mice, which produced larger quantities of IgE than wild-type mice. In agreement with this observation, the administration of IL-21 in wild-type mice or the addition of IL-21 to cultures of murine splenic B cells stimulated with LPS and IL-4 prevented the production of Ag-specific IgE (240). Table 3 below briefly resumes the effects of cytokines on B cells CSR.

Table 3: Effects of cytokines on B cell class switch recombination. From Janeway *et al.*, Immunobiology. 2008, 978(4129).[180]

Cytokines	IgM	IgG3	IgG1	IgG2b	IgG2a	IgE	IgA
IL-4	Inhibits	Inhibits	Induces		Inhibits	Induces	
IL-5							Augments production
IFN-γ	Inhibits	Induces	Inhibits		Induces	Inhibits	
TGF-β	Inhibits	Inhibits		Induces			Induces

Figure 9-13 Immunobiology, 7ed. (© Garland Science 2008)

2.3.1.2 T cell help and co-stimulation for B cell activation and survival

As seen earlier, activated B cells form germinal centers within lymph nodes can secrete specific antibodies directed against the pathogenic intruder. Germinal B cells are, however, very short-lived if they do not receive proper signals and co-stimulation by T cells. Among the many signals they require, CD40 stimulation via its counter part CD40L (CD154) expressed on activated T cells, is essential for B cell survival, CSR, germinal center formation, and memory development (180). CD40 stimulation activates the TNF receptor associated factors (TRAF) signalling pathway which involves TRAF1, TRAF2, TRAF5 and TRAF6 as molecular mediators. This pathway in turn initiates transduction of genes involved in maintaining normal B cell functions (180). It is also interesting to note that the combined signals from the BCR with CD40 stimulation seem to

increase the expression of a protein called Bcl-2 which promotes B cell survival by inhibiting apoptosis (180). In addition to CD40, CD27 has been suggested to be important for B cell activation and immunoglobulin synthesis which signals via TRAF2 and TRAF5 adaptor proteins for the activation of NF-κB and MAPK8/JNK (201).

2.4 Perspectives

Throughout this text we have resumed various aspects of GBS and the T cell dependent immune response supported by scientific articles and review papers. APCs, such as DCs, and T cells play a central role in the development of adaptive immunity. As such, investigating their role in the context of GBS infection is key to understanding pathogenic mechanisms employed by this bacterium to induce disease, and to generate novel insights for the development of improved vaccines. Due to the scarceness of information available on this particular subject many questions need to be answered before understanding GBS as a whole. First, it is relevant to further characterized GBS interactions with DCs since only four papers are available on this matter. Secondly, an more importantly in the context of this thesis, is to understand how GBS-DC interactions modulate CD4+ T cell activation and the consequent development of the adaptive immune response. All in all, this research will add to the building blocks of knowledge necessary for the fight against GBS infections and even possibly against genetically related family members

III – MATERIAL, METHODS AND RESULTS

Article: Group B *Streptococcus* induces a robust IFN-γ response by CD4+ T cells in an *in vivo* and *in vitro* model

I am the first author of this article. I participated in the intellectual design of this project, performed all manipulations related to experiments, and wrote the manuscript. This manuscript has been written and edited according to the editorial guidelines of the journal of Infection and Immunity.

Group B *Streptococcus* induces a robust IFN-γ response by CD4+ T cells in an *in vivo* and *in vitro* model

Damian Clarke[1], Marie-Pier Lecours[1], Paul Lemire[1], Mariela Segura[1]

Laboratory of Immunology, Faculté de médecine vétérinaire, Université de Montréal, St-Hyacinthe, J2S 2M2 Québec, Canada[1]

Not yet submitted

ABSTRACT

Group B *Streptococcus* (GBS) serotype III is an important agent of life-threatening invasive infections and remains the leading cause of neonatal sepsis to this day. Over the years, cytokines have emerged as important players for the control of disease, in particular IFN-γ. Although potential sources of this cytokine have been proposed, no specific cell line has ever been described as specific contributors. In this study, *ex vivo*, *in vivo*, and *in vitro* approaches were developed to evaluate CD4+ T cell activation profiles in response to GBS. Results showed that total spleenocytes readily produce a type 1 pro-inflammatory response by releasing IFN-γ, TNF-α, IL-6. Spleenocytes also actively recruit T cells by producing chemokines such as CXCL9, CXCL10, and CCL3. Specifically, responding activated CD4+ T cells appeared to differentiate into Th1 cells producing large amounts of IFN-γ and TNF-α, but were not important contributors in the production of IL-2 or IL-10. In addition, the effect of GBS capsular polysaccharide (CPS) on T cell activation was evaluated *in vitro* using co-cultures of bone marrow derived dendritic cells (bmDC) and isolated splenic CD4+ T cells infected with WT GBS or an isogenic non-encapsulated mutant. Results suggest that GBS CPS improved IFN-γ production by CD4+ T cells and that CPS modulates surface expression of CD69. Globally, our results further highlight the importance of IFN-γ for the control of GBS infection, which is produce in part by responding CD4+ T cells.

INTRODUCTION

Group B *Streptococcus* (GBS) or *Streptococcus agalactiae* is a Gram-positive beta-hemolytic encapsulated bacterium and is the main cause of life-threatening invasive infections in pregnant women and newborns in North America and Western Europe (20). Recently however, GBS is becoming increasingly associated with invasive disease in non-pregnant adults, in particular the elderly and individuals with underlying chronic illnesses (13). Clinical manifestations of GBS infection are mainly associated with the development of pneumonia, septicemia, and meningitis. Type III GBS is one of the three major capsular

types associated with invasive neonatal infection and is the most common type in GBS meningitis (20, 33). Cytokines appear to play particularly important role for the control of disease, although exaggerated responses have also been described as potentially dangerous. For instance, TNF-α has been demonstrated to be an important agent in driving GBS induces sepsis (7, 32). In contrast other cytokines have demonstrated to mediate beneficial effects such as IL-10, IL-12, and IL-18 (8, 28, 32).

In particular IFN-γ has gained much interest over the years for its important beneficial role in controlling GBS infections. In fact, it was shown that IL-12 and IL-18 mediate their therapeutic effects by increasing IFN-γ production by responding immune cells (8, 28). Furthermore, IFN-γ production is severely impaired during early life and might partly explain the susceptibility of neonates to GBS infection (25, 34). Although certain immune cells have been proposed to secrete IFN-γ in response to GBS, such as NK and NKT cells (10), no specific cell line has been clearly identified as sources of this particular cytokine.

Activated CD4+ T cells can differentiate into different types of helper cells depending on the signals received from their environment. Th1 CD4+ T cells readily produce IFN-γ upon activation and could therefore represent an interesting potential source of this cytokine during the development of GBS infection. There already exist evidence that GBS induces a pro-inflammatory response that could potentially and ultimately lead to activation of T cells and production of IFN-γ. For example, GBS-infected dendritic cells (DCs) have been shown to produce large amounts of pro-inflammatory Type-1 cytokines such as TNF-α, IL-6 and IL-12(23). Furthermore DCs also produce and release large amounts of chemokines used for the recruitment of T cells such as CXCL9, CXCL10 (23). Although the evidence strongly suggests a potential involvement of T cells in the production of IFN-γ, no study has ever evaluated the relative participation of CD4+ T cells in its production.

In addition, GBS is a well-encapsulated bacterium that possesses a thick exterior capsular polysaccharide (CPS) shell as previously mentioned. The structure of type III GBS CPS is formed by the monosaccharides glucose, galactose and *N*-acetylglucosamine into an unique repeating unit that contain a side chain terminated by sialic acid α2,3 linked to galactose (3,

4). The sialylated CPS is recognized as the most important factor for GBS survival within the host (4) and has been suggested to inhibit the activation of the alternative complement pathway and to impair the bactericidal functions of neutrophils (2, 11, 27, 30). Experiments on mouse derived DCs using GBS type III and a non-encapsulated isogenic mutant showed that encapsulated GBS survives better inside DCs than its counterpart and that the CPS can engage specific endocytic pathways that mutant strains can't (23, 24). Likewise, Mancuso *et al.* found GBS antigens in two distinct DC intracellular compartments: DNA+ late endosomes/phagosomes and DNA− phagolysosomes containing partially digested GBS material (29). All together, these observations may prove to be particular important when considering CD4+ T cell activation by GBS. For instance, as the CPS confers a survival advantage to GBS, persistence of GBS within APCs such as DCs may affect their activation and thus the ensuing T cells immune response. Furthermore, it was also demonstrated on murine macrophages that the route of entry engaged by a particular pathogen influences the repertoires of epitopes presented CD4+ T cells and thus, by extension, may affect the ensuing immune response.

In this study, we investigated the role of CD4+ T cells in the development of the immune response against GBS type III using three different experimental approaches: *ex vivo*, *in vivo*, *in vitro* models. Herein, we show that activated T cells drive a Th1 type immune response by producing large amounts pro-inflammatory cytokines, in particular IFN-γ. Finally, we also evaluated DC – CD4+ T cells interactions and the effects of CPS on the modulation of T cell activation *in vitro*. We found a partial contribution of CPS for the production of IFN-γ by CD4+ T cells.

MATERIAL AND METHODS

Bacterial strains and growth conditions

Strain COH-1, a highly encapsulated type III GBS isolate extensively described in previous work (3, 29, 31), was used in this study as the WT strain. An isogenic non-encapsulated (Δ*cpsE*) mutant, constructed and characterized in our previous work was also included (22,

23). GBS growth conditions for *in vivo* and *ex vivo* experiments differed from that of *in vitro* experiments, purely for logistical purposes. For the generation of *in vivo* and *ex vivo* inoculums, GBS strain COH-1 strain was grown on sheep blood agar plates at 37°C for 18 h. Isolated colonies were inoculated in 5ml of Todd–Hewitt Broth (THB; Becton Dickinson, Mississauga, ON, Canada) and incubated for 8 hours at 37°C with shaking. Thirty ml of fresh THB were then inoculated with 10 μl of the 8h culture diluted by a factor of 10^3, with shaking for an additional 12h. GBS injections were prepared by washing 12h cultures twice with PBS, pH 7.3 before being appropriately diluted in fresh THB to desired inoculum concentrations. The number of CFU/ml in the final suspension was determined by manually plating series dilutions of working cultures on THB agar before enumerating individual colonies after overnight incubation at 37°C. *In vitro* GBS cultures were prepared by inoculating 5ml of THB with isolated colonies of either COH-1 or COH-12a strains, and cultures were incubated for 16 hours at 37°C with shaking. Working cultures for cell stimulation were obtained by inoculating 100 μl of the 16 h culture into 10 ml of THB followed by incubation for 5 h at 37°C with shaking. The number of CFU/ml were determined as described above.

Generation of bone marrow-derived dendritic cells (bmDCs)

bmDCs were generated from female C57BL/6 mice according to a technique described elsewhere (31). Briefly, after red blood cell lysis, total bone marrow cells (2.5×10^5 cells/ml) were cultured in complete medium consisting of RPMI 1640 supplemented with 5% heat-inactivated fetal bovine serum, 10 mM HEPES, 20 μg/ml gentamycin, 100 U/ml penicillin-streptomycin, 2 mM l-glutamine and 50 μM 2-ME. All reagents were from Gibco (Invitrogen: Burlington, Ontario, Canada). Complete medium was complemented with 20% GM-CSF from a mouse GM-CSF transfected cell line (Ag8653). Cells were cultured for 7 days at 37 °C with 5% CO_2. On day 7, clusters were harvested and subcultured overnight to remove adherent cells. Non-adherent cells were collected on day 8 and used as immature bmDCs for the studies.

Splenic CD4+ T cell isolation

Untouched CD4+ T cells were purified from the spleen of C57BL/6 mice by negative selection using CD4+ T cell isolation kit II according to the manufacturers instructions (Miltenyi Biotec, Auburn, CA, USA). Purity was verified by FACS at 96% (+/- 1%) of total events using FITC-labeled anti-mouse CD4 mAb (BD PharMingen, Mississauga, Ontario, Canada).

Mouse strain for *in vivo* and *ex vivo* experiments

All experiments were performed on 5 week old female C57BL/6 mice (Charles River Laboratories, Wilmington, MA, USA). All experiments involving mice were conducted in accordance with the guidelines and policies of the Canadian Council on Animal Care and the principles set forth in the Guide for the Care and Use of Laboratory Animals by the Animal Welfare Committee of the University of Montréal.

***In vivo* infection model**

C57BL/6 mice were injected I.P. with 1×10^6, 1×10^7, or 1×10^8 CFU of GBS COH-1 strain. Spleens of animals with clinical symptoms were harvested 96h post infection and CD4+ T cells were analyzed as described above. Surviving animals who had previously displayed clinical symptoms were boosted with a second shot of 1×10^6 CFU of GBS strain COH-1 two weeks after initial infection, and spleens were harvested 48h post boost for isolation of analysis of CD4+ T cells as described above.

***Ex vivo* model of infection**

C57BL/6 mice were injected I.P with a dose of 1×10^7 CFU of GBS COH-1 strain. Spleens were harvested 6h post infection, and total spleenocytes were extracted by flushing cells using PBS and mechanically disrupting spleens over sterile meshes. After red blood cell lysis, total spleenocytes were counted and suspended at a concentration of 5×10^6 cells/ml in RPMI 1640 supplemented with reagents described for bmDC generation. 1ml of cell suspension was added per well of a 24 well flat bottom plate and incubated at 37°C with 5% CO_2. Concanavalin A (ConA, 0.1µg/ml, Sigma-Aldrich, St. Louis, Missouri, USA) was added to wells used as positive controls. After 4h of additional *in vitro* infection the bacteriostatic agent chloramphenicol (CM, 12 µg/ml, Sigma–Aldrich) was added to the

culture to prevent cell toxicity. Cells were then incubated for an additional 48h at 37°C with 5% CO_2 before collecting supernatants and isolating CD4+ T cells for analysis.

In vitro model of infection

bmDCs were adjusted at a concentration of 0.8 x 10^6 cells/ml in complete RPMI 1640 and plated in wells of a 48 well flat bottom plate for 1h at 37°C with 5% CO_2. Afterwards, 1 x 10^5 CFU of GBS COH-1 or COH-12a strain (MOI:1) were added to wells for an additional hour. Extracellular GBS was killed using 100 µg/ml of gentamycin and 5 µg/ml of penicillin G (Sigma–Aldrich). After 1h of antibiotic treatment, and 3 washing steps, 5 x 10^5 freshly isolated CD4+ T cells (MOI 5:1) at concentration of 2 x 10^6 cells/ml were added to wells. For FACS analysis of surface protein expression, plates were incubated for 8h and 24h at 37°C, 5% CO_2, before T cells were collected and analyzed as described above. For T cell cytokine expression, plates were incubated for 48h after antibiotic treatment. After 48h, plates were centrifuged and replenished with fresh medium containing 10ng/ml of IL-2 (Miltenyi Biotec). Plates were placed back in the incubator at 37°C, 5% CO_2 for 3 days allowing a resting period for activated T cells. Following resting period, T cells were collected from plates, counted and plated at 1 x 10^5 cells/well in a anti-CD3 (BD PharMingen) coated 96 well flat bottom plate. Responding T cells were challenged and incubated for 48h before plates were centrifuged, and supernatants collected for ELISA testing.

Cytokine quantification by ELISA

Levels of IL-6, IL-10, IFN-γ, TNF-α, CCL3 (MIP-1α), CXCL9 (MIG), and CXCL10 (IP-10) in cell culture supernatants were measured by sandwich ELISA using pair-matched antibodies from R&D Systems (Minneapolis, MN, USA) or eBioscience (San Diego, CA, USA), according to the manufacturer's recommendations. Twofold dilutions of recombinant mouse cytokines were used to generate standard curves. Sample dilutions giving OD readings in the linear portion of the appropriate standard curve were used to quantify the levels of each cytokine.

Flow cytometry analysis

For cell surface staining, 10^6 CD4+ T cells were washed and treated for 15 min on ice with FcR-blocking reagent (FcγIII/II Rc Ab, BD PharMingen, BD Biosciences, Mississauga, Ontario, Canada) in sorting buffer (PBS-1% fetal bovine serum). Blocked cells were then incubated with FITC-labeled anti-mouse CD4 mAb (BD PharMingen) for 30 min on ice followed by washing and staining for 30 min with PE-labeled mAb against CD69 (BD PharMingen). Flow cytometry was performed using a Cell Lab Quanta^{TM} SC instrument (Beckman Coulter). Twenty thousand gated events were acquired per sample and data analysis was performed using Cell Lab Quanta Collection and Cell Lab Quanta Analysis software. Quadrants were drawn based on FITC- and PE-control stains and were plotted on logarithmic scales.

For *in vivo* intracellular staining, mice were injected I.P with 200μg of Brefeldin A (Sigma-Aldrich) diluted in 500μl of PBS. For *ex vivo* intracellular staining, a 1/1000 dilution of Brefeldin A (Sigma-Aldrich) stock solution, as prepared by manufacturers recommendations, was added to each well containing total spleenocytes. In both cases, Brefeldin A incubation times were of 5h. Afterwards, freshly isolated CD4+ T cells, obtained as described above, were washed and treated for 15 min on ice with FcR-blocking reagent in sorting buffer. FcR blocking was followed by fixation with IC Fixation Buffer 1X (eBioscience) for 20 min at RT. Cellular permeabilization was accomplished by using Permeabilization Buffer 10X (eBioscience) diluted to 1X in ddH$_2$O, followed by centrifugation at 400g, RT, for 5mins. This step was repeated twice. Permeabilized cells were then stained for 20 min at RT with PE-labeled mAbs directed against the following intracellular molecules: IFN-γ (clone XMG1.2), TFN-α (clone MP6-XT22), IL-2 (clone JES6-5H4), or IL-10 (JES5-16E3). Flow cytometry was performed using a FACSCalibur instrument (BD Biosciences). Twenty thousand gated events were acquired per sample and data analysis was performed using CellQuest software. Histograms were drawn based on PE-control stain and were plotted on logarithmic scales.

Statistical analysis

All data are expressed as mean ± SEM. Data were analyzed for significance using Student's unpaired *t*-test. A *P* value < 0.05 was used as a threshold for significance. All experiments were repeated at least three times.

RESULTS

C57BL/6 mice survival is significantly reduced with 10^7 and 10^8 CFU infection doses compared to 10^6 CFU infection dose

Inbred C57BL/6 mice were injected I.P with 3 different GBS COH-1 strain infectious doses: 1 x 10^6 CFU, 1 x 10^7 CFU, and 1 x 10^8 CFU. Mice clinical symptoms and survival rates were recorded every 6h p.i. After 18h p.i., mice injected with 10^7 CFU or 10^8 CFU doses resulted in 75% and 69% mortality respectively (Figure 1). Mice mortality continued to drop until 20h p.i to 88% and 94% respectively, and was maintained until 60h p.i. when the experiment was terminated. Mice infected with a dose of 10^6 CFU were significantly less prone to mortality as compared to mice from the other two groups. After 18h of infection, only a 13% mortality rate was observed in mice infected with 10^6 CFU, which was significantly lower than the other two groups (P < 0.05) (Figure 1). Survival continued to drop at 24h and 36h with mortality reaching 19% and 25% respectively, but remained significantly higher than mice from the other 2 groups (P < 0.05). Mortality percentage in this group did not change after 36h and was maintained until 60h p.i. when the experiment was terminated. Groups infected with 10^7 and 10^8 CFU doses quickly manifested intense clinical signs such as tousled hair, prostration, depression, and lethargy as early as 8h p.i. In groups infected with 10^6 CFU, clinical symptoms appeared later on, usually around 12h p.i., and were less intense. Based on the clinical profile and progression, all deaths were considered as related to septicemia.

In parallel to survival, bacteremia of infected mice was also evaluated. I.P infection of C57BL/6 mice with GBS COH-1 induced strong bacteremia. As highlighted by survival curves, bacteremia was similar in groups infected with 10^7 and 10^8 CFUs 18h p.i. and reached on average 3.63 x 10^8 CFU/ml and 1.34 x 10^9 CFU/ml respectively and were not

statistically different (Figure 1b). In contrast, groups infected with doses of 10^6 CFU showed significantly lower bacteremia and reached on average 6.14 x 10^6 CFU/ml 18h p.i. ($P < 0.05$) (Figure 1b). Due to the lack of individuals from 10^7 CFU and 10^8 CFU groups, no statistically viable analysis of bacteremia levels through time could be made. In groups injected with 10^6 CFU, bacteremia remained relatively high at 42h (6.38 x 10^5 CFU/ml) and 64h (1.45 x 10^5 CFU/ml) demonstrating the ability of virulent GBS to survive successfully in the host bloodstream (data not shown). In some cases however, mice were capable of completely clearing infection as early as 40h p.i.

Total spleenocytes produce type-1 pro-inflammatory cytokines and important chemokines for T cell recruitment in response to GBS COH-1 strain.

Before understanding T cell activation in response to GBS, we were interested in characterizing the contextual environment produced by total spleenocytes prior to T cell activation. To this end we used the *ex vivo* approach. ELISA tests revealed the presence of three pro-inflammatory cytokines, IFN-γ, TNF-α, and IL-6, and one anti-inflammatory cytokine, IL-10 (Figure 2). The very large amounts of IFN-γ (~ 9300 pg/ml) and presence of TNF-α (~ 2400 pg/ml) and IL-6 (4500 pg/ml) as compared to non-infected controls indicated the progression of a type-1 pro-inflammatory response. All cytokines seamed to increase at 72h except TFN-α although this difference was non significant. In addition to cytokines, it is well known that activated immune cells produce chemokines for the recruitment of accessory immune cells such as T cells. Supernatants tested by ELISA revealed the presence of three important chemokines known to recruit T cells: CXCL9, CXCL10 and MIP-1α (Figure 2).

Activated CD4+ T cells contribute to IFN-y production during infection with GBS COH-1.

CD4+ T cells were isolated from wells containing total spleenocytes from *ex vivo* experiments and were stained intracellularly for various cytokines before FACS analysis (Figure 3). Experiments showed that activated CD4+ T cells infected with GBS readily contributed in the production of IFN-γ and TNF-α and were not important contributors for the production of IL-2 and IL-10 (Figure 3). Results also indicated that CD4+ T cells are

not an important source of IL-10 which is readily produced by activated spleenocytes as seen earlier (Figure 2).

The validity of these results was further tested using an *in vivo* model of infection. Results obtained from CD4+ T cells isolated from the spleen of infected mice 96h p.i. showed very much the same tendencies as those from the *ex vivo* model (Figure 4). CD4+ T cells contributed in the production of IFN-γ and TNF-α and were not important contributors for the production of IL-2 and IL-10 (Figure 4). Surviving mice from the primary infection were used as candidates for booster shots 2 weeks later in order to determine if the infection had generated CD4+ T cells memory and to understand how this memory was manifested. FACS analysis of CD4+ T cells isolated 48h p.b displayed similar contribution in IFN-γ and TNF-α production in a non-significant manner as compared to data from 96h p.i. IL-2 and IL-10 production was significantly enhanced compared to results obtained from primary infection, with percentages resembling those from *ex vivo* experiments (Figure 4 vs. Figure 3).

The CPS of GBS contributes in the induction of IFN-γ production by activated CD4+ T cells.

As GBS COH-1 strain is a well encapsulated bacteria, we evaluated the impact of CPS on CD4+ T cell activation by working in parallel in an *in vitro* model with two strains of GBS: WT COH-1 strain and an non-encapsulated isogenic mutant, COH-12a strain. Supernatants collected from experiments were tested for the presence and quantification of cytokine by ELISA test. Results revealed the presence our expected cytokines: IFN-γ, TNF-α and IL-10 (IL-2 was non detectable) (Figure 5). In both cases, GBS COH-1 and COH-12a induced the production of large amounts of IFN-γ by responding CD4+ T cells as compared to negative controls ($P < 0.01$ and $P < 0.05$ respectively), and the loss of CPS was accompanied by a significant reduction in its production ($P < 0.05$) (Figure 5). In addition, GBS COH-1 and COH-12a also induced significant release of TNF-α ($P < 0.01$ in both cases) although the CPS did not affect its production (Figure 5). Lastly, activated CD4+ T cells release IL-10 in response to GBS COH-1 ($P < 0.05$), but failed to do so in a significant way when responding to GBS COH-12a (Figure 5).

The CPS of GBS negatively affects surface expression of CD69 on activated CD4+ T cells.

In addition to cytokine production, expression of surface molecules on CD4+ T cells is an essential event for proper T cell activation. We therefore also investigated how the CPS of GBS may affect expression of co-stimulation molecules on activated CD4+ T cells. Many surface molecules were tested, such as CD28, CD11a, CD40L, and CTLA-4 (data not shown), but the only one that appeared to be affected by the CPS was CD69 (Figure 6). Maximal significant difference in CD69 modulation was observed at 8h p.i. where an average 22% (+/- 1.36%) CD4+ T cells responding to COH-1 were stained positive for CD69 expression compared to 38% (+/- 2.77%) ($P < 0.01$) in samples infected with COH-12a (Figure 6). This difference in expression was gradually lost. At 24h p.i., CD4+ T cells responding to GBS COH-1 stained positive for CD69 on average at 16% (+/- 1.37%) compared to 25% (+/- 2.63%) in samples infected with COH-12a ($P < 0.05$) (Figure 6). At 48h significant differences in CD69 modulation were lost (data not shown).

DISCUSSION

Although information is becoming increasingly available on the interactions between GBS and cells of innate immunity, such as DCs, macrophages and neutrophils, no work to this day has focused on activation profiles of cells of adaptive immunity during GBS infection. This study addresses for the first time how CD4+ T cells contribute in the development of immune functions during GBS type III infections using 3 powerful models: *ex vivo*, *in vivo*, and *in vitro* models.

In the case of GBS, cytokines are not only important for the appropriate development of host defences but are also involved in induction of severe pathologies. Initial e*x vivo* analysis of cytokine production by total splenocytes derived from encapsulated GBS infected mice revealed the presence of IFN-γ, TNF-α, IL-6 and IL-10. Production of IFN-γ, TNF-α and IL-6 is suggestive of a type 1 pro-inflammatory response being developed shortly after infection, while IL-10 production can be related to immune-regulation. It is interesting to note that TNF-α and IL-6 have routinely been reported as

important mediators of GBS sepsis (15, 32). This observation might also highlight the particular importance of IL-10 in maintaining homeostasis as Cusumano *et al.* reported a role of IL-10 in protecting neonatal mice from developing sepsis by reducing TNF-α production (6).

Several cell types have been reported to secrete TNF-a, IL-6 and/or IL-10, including DCs, monocytes and macrophages when responding to GBS (1, 9, 12, 21, 23) although sources of IFN-γ remain unidentified. In order to determine if T cells could play a potential role in its production isolated CD4+ T cells were stained intracellularly for various cytokines. *Ex vivo* and *in vivo* analysis showed that CD4+ T cells were specifically activated by GBS and are important producers of IFN-γ and TNF-α. These antigen-specific T cells also produce low, but still significant levels of IL-2, overall suggesting the development of a Th1 response. *Ex vivo* and *in vivo* analysis of GBS-activated CD4+ T cells also showed production of IL-10 by this particular cell sub-type. The % of antigen-specific, IL-10-producing CD4+ T cells was also low, but nonetheless significant. This finding suggests that during *in vivo* infection, cells of the innate immune system are the major sources of this immune-regulatory cytokine. Similarly, cell-specific intracellular cytokine production by human mononuclear cells in response to GBS revealed less than 2% of IL-10-positive CD3+ T cells (5). Although a dual participation in the production of IFN-γ and IL-10 by CD4+ T cells may seem opposing, a study has recently demonstrated that probiotic stimulation of human PBMCs, monocyte-derived DCs, and DC-CD4+ T cell co-cultures engaged a production the aforementioned cytokines and induced simultaneous Th1 and anti-inflammatory responses (18).

The specificity of this response also proved to be well conserved and suggests the development of T cell memory. Results from surviving animals that were challenged with a booster shot two week latter showed that CD4 + T cells responded to infection by producing the same pattern of cytokines. This response was engaged much more rapidly as CD4+ T cells were isolated and stained 48h after challenge compared to 96h for initial infections (48h post initial infection revealed negative results – data not shown). As memory T cells are well known to perform their immune functions more efficiently and

faster, this observation may be indicative that CD4+ T cells developed memory to GBS. In addition, FACS histogram peaks were much sharper and representative of a homogenous T cell population, strengthening the idea that T cells had developed memory. As mentioned, IL-2 and IL-10 production was enhanced compared to results obtained from primary infection, with percentages resembling those from *ex vivo* experiments (Figure 4 vs. Figure 3). With the common denominator between results obtained from boosted mice and *ex vivo* infections being the 48h p.i. it could be suggested that IL-2 and IL-10 are important cytokines during early T cell activation and proliferation events which are no longer observed after 96h p.i. On the other hand, this might also suggest a potential shift in the T cell phenotype towards a more pronounced humoral response. However, this needs to be confirmed by measuring a potential increase of IL-4 and the antibody isotypes produced after either primary- or boost-infections.

Early chemokine release by cells of the innate immunity plays an important role in attracting various accessory immune cells to the site of infection, such as T cells. *Ex vivo* analysis of total splenocyte chemokine production suggested that T cells were actively recruited via the expression of three important chemokines: CCL3, CXCL9 and CXCL10. It is interesting to note that CXCL9 and CXCL10 are two chemokines that bind to CXCR3 receptor on the surface of T cells, and more importantly, are induced by IFN-γ (16). In the context of a systemic infection with GBS, several cells types in the spleen are potential sources of these three chemokines. GBS-stimulated spleen DCs were reported to produce CXCL9 and CXCL10 (23). Although up-regulation of *Cxcl10* gene expression was observed by DNA microarray analyses of mouse peritoneal macrophages (12) GBS was reported to be unable to induce neither CXCL10 nor CXCL9 secretion by these cells (14). On the other hand, macrophages seem to contribute to CCL3 production (12, 14).

The fact that CD4+ T cells readily produce IFN-γ in response to GBS raises an interesting point. As we have just seen, CXCL9 and CXCL10 are release in great amounts by total splenocytes and are induced by the presence of IFN-γ. We thus propose a global view where an initial production of INF-γ by cells of innate immunity could trigger the release of CXCL9 and CXCL10 by APCs, attracting T cells to the site of infection. Activation of

responding CD4+ T cells would in turn produce important quantities of IFN-γ, acting on T cells in an autocrine loop, but also acting upon APCs to further increase the chemo-attracting gradient resulting in increased T cell recruitment, including production of CCL3. This process results in the development of a Th1 response emphasized by the presence of several pro-inflammatory cytokines including TNF-α. Central to this response is IFN-γ, which acts as a master regulator of many inflammatory pathways as it is involved in regulating the production of several molecules involved in the inflammatory response.

As GBS possesses a thick exterior CPS, known to be the most important virulence factor, we were interested in understanding if it could modulate CD4+ T cell activation. To this aim, serotype III encapsulated GBS strain and its non-encapsulated isogenic mutant were used for *in vitro* infections. *In vivo* studies could not be performed as the mutant strain is rapidly eliminated from the host (data not shown). Similarly to *ex vivo* and *in vivo* results 3 cytokines were revealed: IFN-γ, TNF-α and IL-10. More importantly, IFN-γ was the only one whose level was significantly decreased in the absence CPS. Production of TNF-a was also reduced, albeit this difference did not reach statistical significance. Although it is surprising that the loss of capsule is not associated with an exaggerated immune response, studies on DC activation by GBS have shown similar trends. Indeed, it has been shown that WT GBS induces stronger cytokine production than its non-encapsulated mutant at longer incubation times (16h p.i) (23). The only exception was IL-10, which production was significantly higher in DCs infected with the non-encapsulated mutant than those infected with the WT strain (23). In this study two interrelated hypothesis are suggested to explain these observations, a) increased IL-10 production by DCs in the absence of CPS reduce the production of other cytokines; or b) higher and faster levels of non-encapsulated bacteria killing reduce or impair cytokine production by DCs. Thus, in our DC-T cell co-culture system, the consequence of this diminished DC activation by the non-encapsulated strain may be translated into lower levels of IFN-γ production by CD4+ T cells.

Lastly, *in vitro* model of infection was also used to evaluate the expression of CD4+ T cell surface molecules necessary for co-stimulation events and signaling transduction.

Different surface molecules important in T cell activation were evaluated in our study, such as CD11a, CD28, CD152, CD154 (data not shown). However, under our experimental conditions only CD69 was significantly up-regulated by T cells co-cultured with GBS-infected bmDCs. Interestingly, the non-encapsulated strain induced even higher levels of CD69 expression by T cells than the encapsulated strain. Attempting to explain modulation of CD69 expression on CD4+ T cells is quite difficult, due to the lack of information available on this particular surface marker. Although it is well known that CD69 is one of the earliest markers induced upon activation of T and B cells and acts as a signal-transmitting receptor for immune regulatory events, its cognate ligand is still unknown (26). Of the studies available on CD69 expression by T cells, a paper by Harimaya *et al.* demonstrated a dose-dependent up-regulation of CD69 on CD4+ T cells infected with *Alloiococcus otitidis*, a Gram-positive bacterium (17). Although large amounts of IFN-γ were quantified in supernatants containing total peripheral blood lymphocytes authors failed to correlate this production to CD69 surface expression (17).

Undoubtedly, IFN-γ production by T cells during GBS infection is crucial for host defence. Herein, we have identified CD4+ T cells as potent IFN-γ producers driving Th1 type immune response during GBS infection using three different models of infection. We have also identified a low, but nonetheless significant participation of this cell type in the production of IL-10. In addition, we have found significant differences in IFN-γ and IL-10 production and CD69 surface expression by CD4+ T cells co-cultured with bmDCs infected with either wild-type GBS or its non-encapsulated mutant. As T cells are a subset of cells belonging to adaptive immunity it is highly improbable that they are responsible for initiating IFN-γ production. NK or NKT cells could logically play an important role as they have routinely been identified as potent IFN-γ producers in response to GBS infection (10, 19). Although these studies offers an interesting starting point for future research, a definitive understanding of all mechanisms regulating IFN-γ production, such as the initiator cell lines and further and deeper studies on antigen-specific CD4+ T cells would improve our understanding of GBS immune-pathogenesis and contribute to develop more powerful strategies to fight GBS infections.

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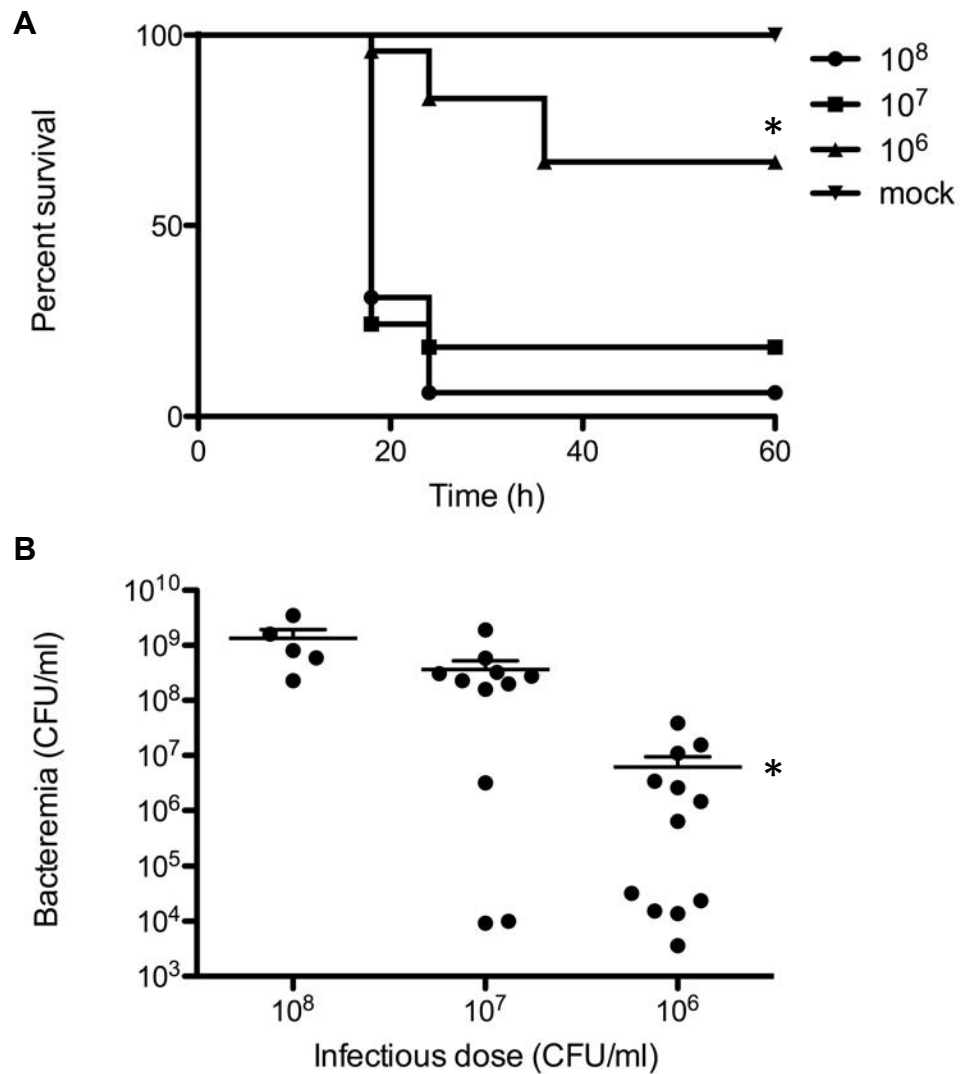
Figure 1: Survival curves and bacteremia of infected C57BL/6 mice. A) Mice were injected intra-peritoneum with different doses of encapsulated GBS serotype III strain COH-1 and clinical symptoms and survival were recorded every 6 h. Mice injected with 10^7 and 10^8 CFU were significantly more prone to mortality as compared to mice injected with 10^6 CFU (P < 0.05). Disease in mice injected with 10^7 CFU and 10^8 CFU progressed rapidly and mortality occurred between 18h and 24h of infection whereas disease in mice infected with 10^6 CFU progressed gradually and mortality could be observed up to 36h post infection. B) Bacteremia of infected mice was monitored at 18 h post-infection by diluting 5 µl of blood drawn by tail puncture in 95 µl of PBS followed by serial dilutions and plating on agar dishes. Agar plates with dilutions were incubated for 24 h at 37°C and individual colonies were counted for CFU calculations. * P < 0.05 compared to other infectious doses.
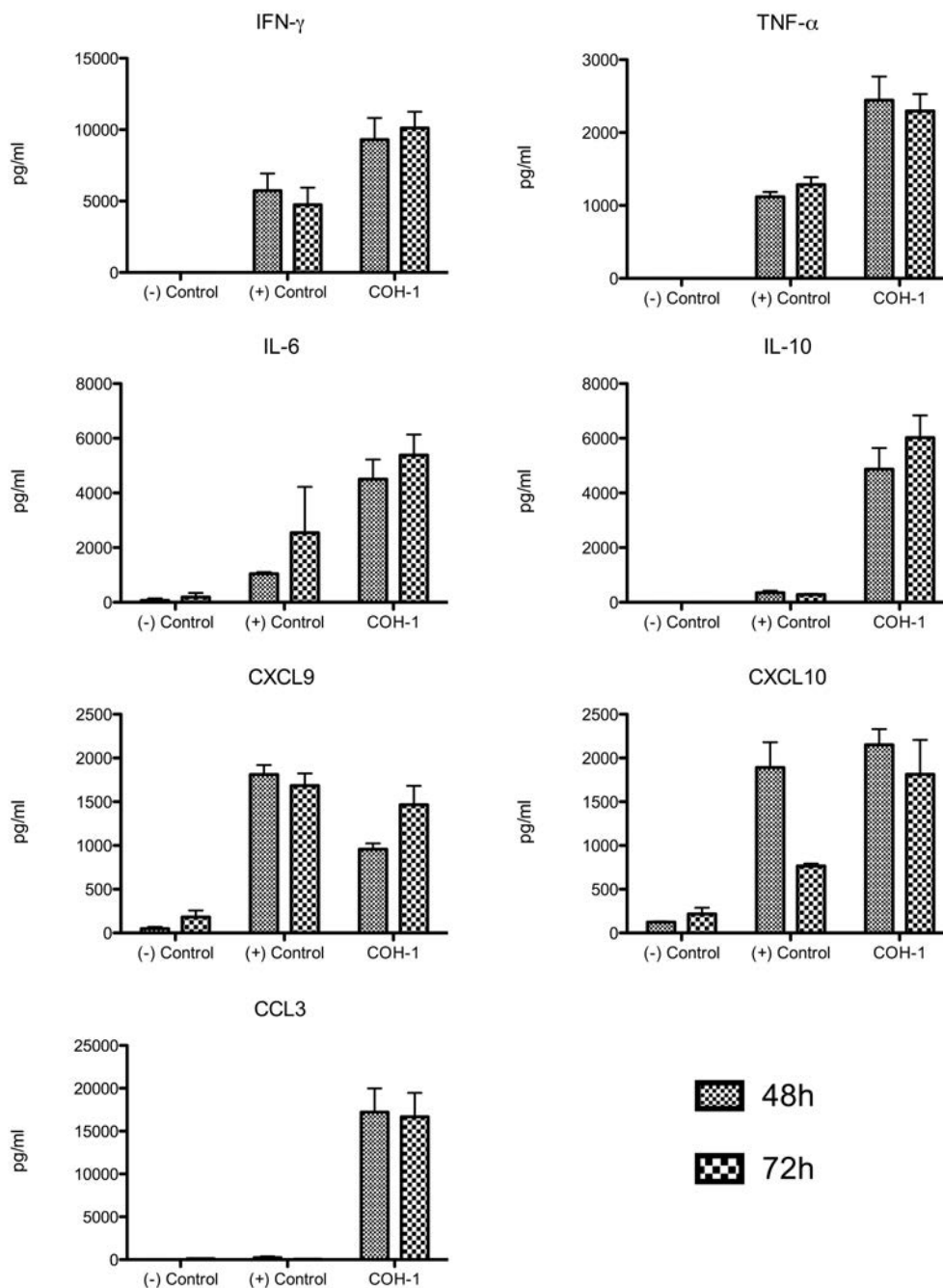
Figure 2: *Ex vivo* **cytokine and chemokine production profile by total splenocytes.** Three C57BL/6 mice per experiment were injected intra-peritoneum with a dose of 1 x 10^7 CFU of encapsulated GBS COH-1 strain. Spleens were harvested 6 h post-infection, total splenocytes were pooled and plated at 5 x 10^6 cells/well. After 4h of incubation, the bacteriostatic agent chloramphenicol (12 µg/ml) was added to the culture to prevent cell toxicity. Cells were then incubated for 48 h and 72 h and supernatants were collected. Non-stimulated cells served as negative control (- Control) for basal expression. Concanavalin A (ConA, 0.1 µg/ml) was used as positive control (+ Control). Cytokines and chemokines were quantified by ELISA. Data are expressed as means +/- SEM (in pg/ml) from 3 different experiments.

Figure 2: Clarke *et al* 2012

Figure 3: *Ex vivo* **CD4+ T cell contribution to cytokine production.** Three C57BL/6 mice per experiment were injected intra-peritoneum with a dose of 1 x 10^{7} CFU of encapsulated GBS COH-1 strain. Spleens were harvested 6 h post-infection, total splenocytes were pooled and plated at 5 x 10^{6} cells/well. After 4 h of incubation the bacteriostatic agent chloramphenicol (12 µg/ml) was added to the culture to prevent cell toxicity. Total splenocytes were then incubated for 48 h and CD4+ T cells were isolated by negative selelction. Non-stimulated cells served as negative control (- Control) for basal expression. Concanavalin A (ConA, 0.1 µg/ml) was used as positive control (+ Control). Isolated CD4+ T cells were stained intracellularly for different cytokines and analyzed by FACS. Representative data from 3 different experiments.
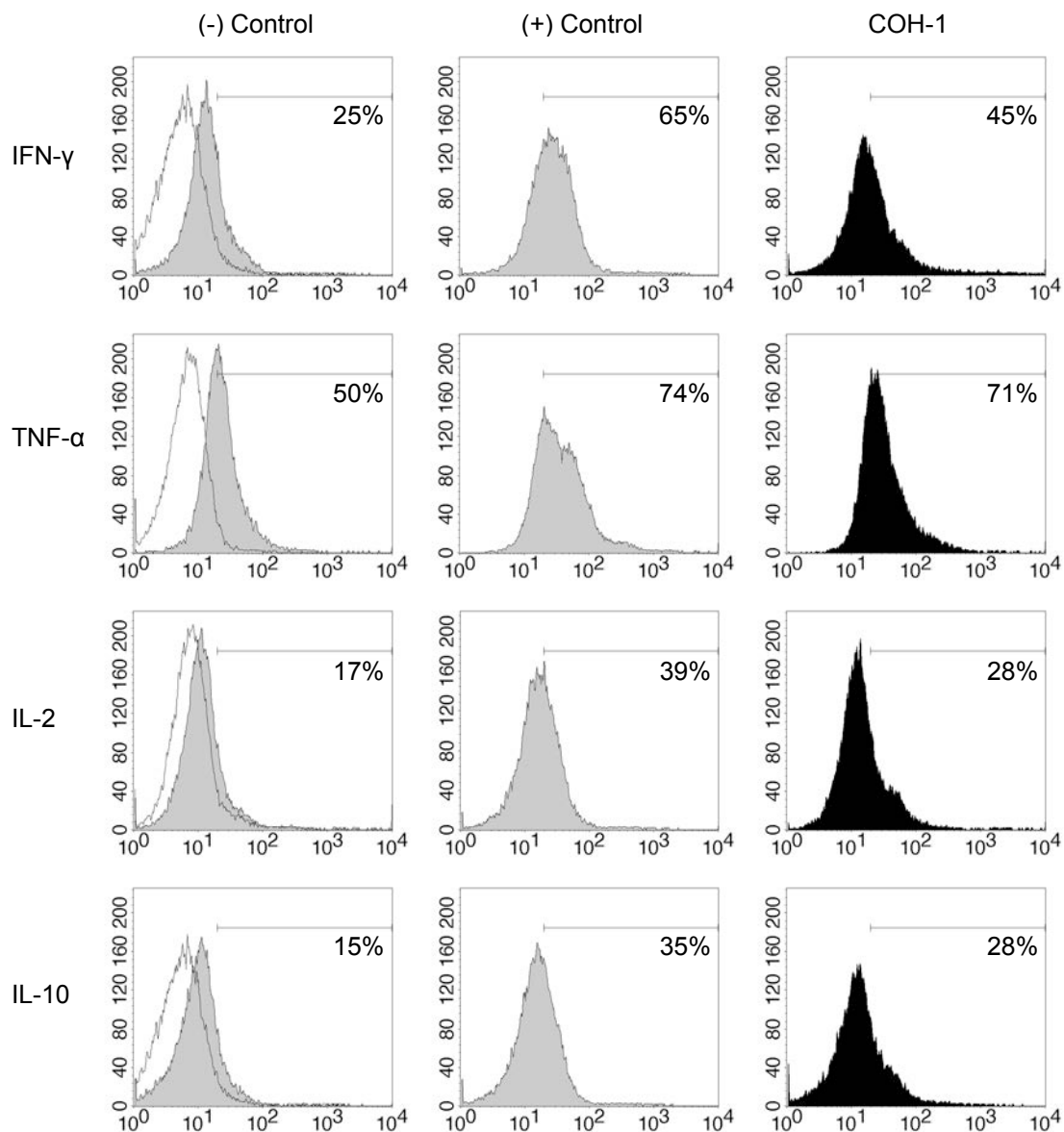
Figure 3: Clarke *et al* 2012

Figure 4: *In vivo* CD4+ T cell contribution to cytokine production. C57BL/6 mice were injected intra-peritoneum with a dose of 1×10^6 CFU of GBS COH-1 strain. Spleens of surviving candidates were harvested 96 h post-primary infection (■) , or 48 h post-boost (▨) and CD4+ T cells isolated by negative selection. Non-stimulated cells served as negative controls (▢) for basal expression. Freshly isolated CD4+ T cells were stained intracellularly for different cytokines and analyzed by FACS. Representative data from 3 different experiments.
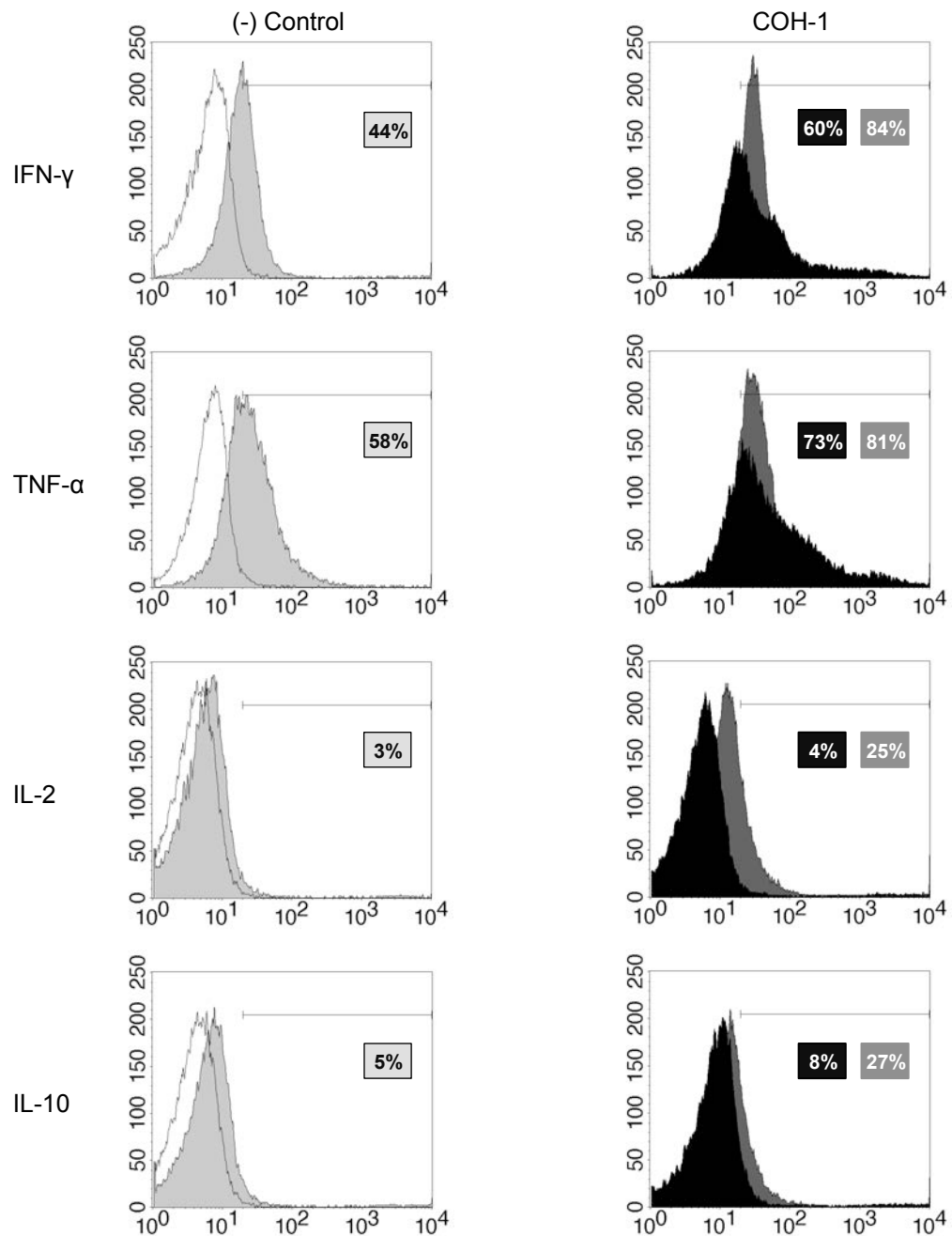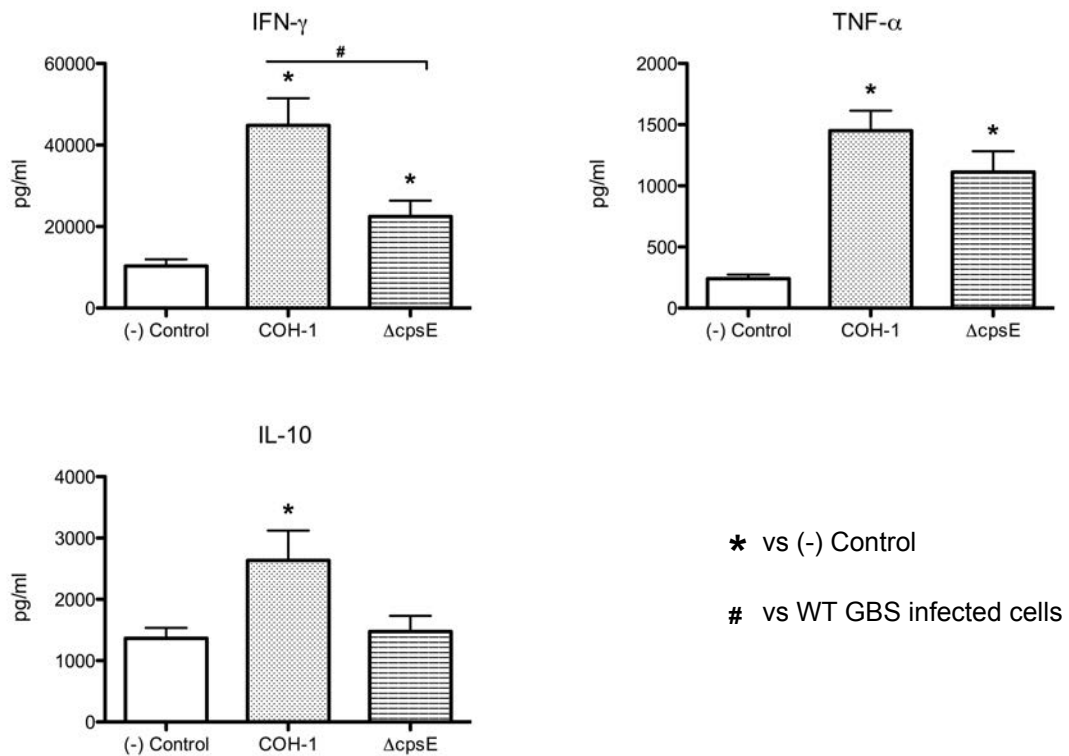
Figure 4: Clarke *et al* 2012

Figure 5: *In vitro* CD4+ T cell cytokine production. BmDC (1 x 10^5 cells) were infected with WT GBS strain COH-1 or its non-encapsulated isogenic mutant Δ*cpsE* (1 x 10^5 CFU) (MOI 1:1) for 1 h. Extracellular GBS was then killed by treatment with 100 μg/ml of gentamycin and 5 μg/ml of penicillin G for 1 h. Afterwards, freshly isolated splenic CD4+ T cells (2 x 10^6 cells) were added (MOI 5:1). Cells were incubated for 48 h before being washed and resuspended in fresh medium containing 10 ng/ml of IL-2. After 72 h of resting period, CD4+ T cells were collected and resuspended in fresh medium and were transferred to anti-CD3 coated plates for 48 h (1 x 10^5 cells/well). Supernatants were then collected and cytokines quantified by ELISA. Non-stimulated cells served as negative controls (- Control) for basal expression. Data are expressed as means +/- SEM (in pg/ml) from 3 different experiments. *P < 0.05* indicate statistically significant differences between cytokines induced by GBS vs the (-) control. #*P < 0.05* indicate statistically significant differences in cytokine induction by WT GBS COH-1 vs. its non-encapsulated mutant, Δ*cpsE*.

Figure 5: Clarke *et al* 2012
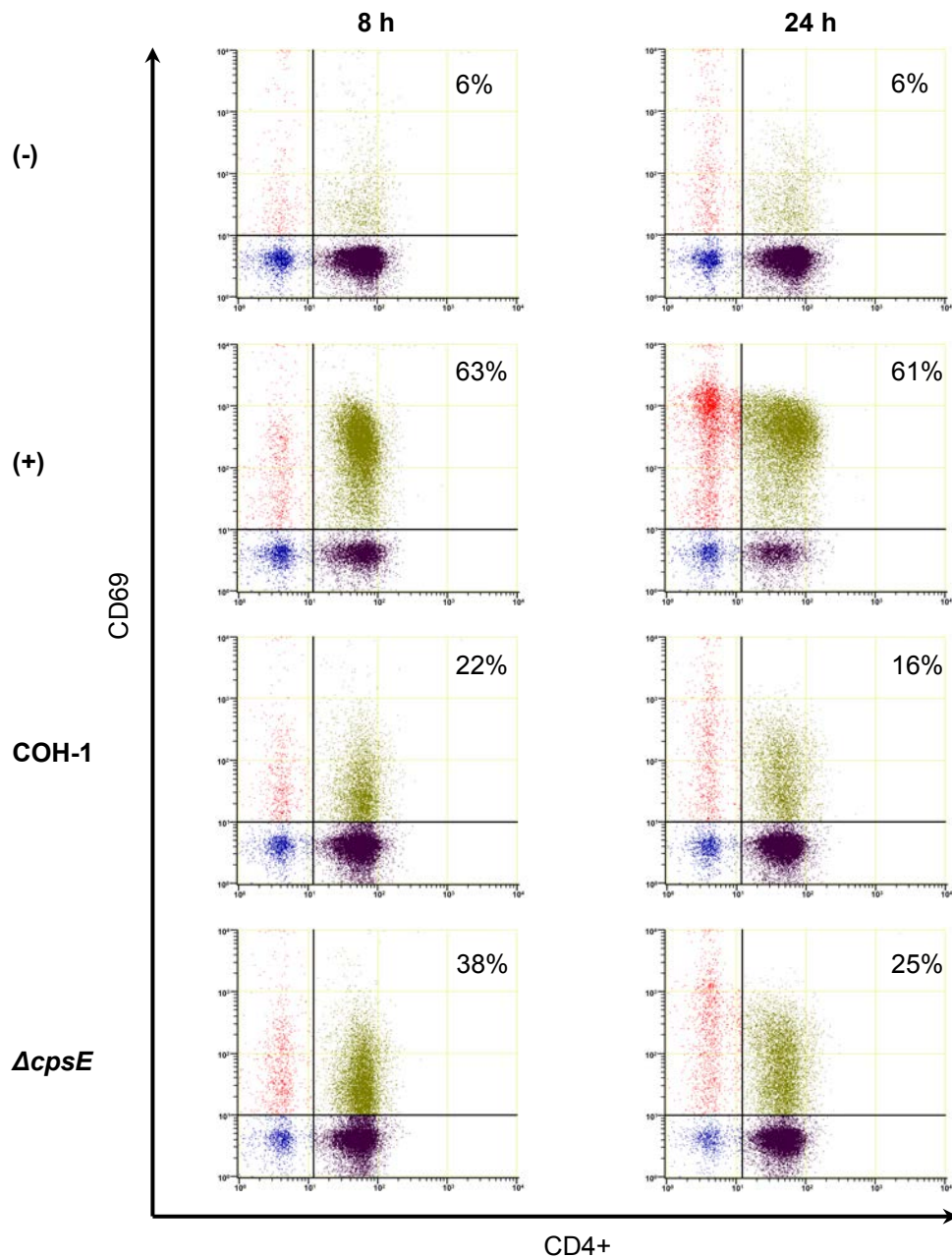
Figure 6: *In vitro* CD4+ T cell surface expression of CD69. BmDC (1 x 10^{5} cells) were infected with WT GBS strain COH-1 or its non-encapsulated isogenic mutant ΔcpsE(1 x 10^{5} CFU) (MOI 1:1) for 1 h. Extracellular GBS was killed by treatment with 100 μg/ml of g entamycin and 5 μg/ml of penicillin G for 1 h. Afterwards, freshly isolated splenic CD4+ T cells (2 x 10^{6} cells) were added (MOI 5:1). Stimulation lasted 8 h and 24 h. Cells were harvested and CD69 expression was analyzed by FACS. Histograms were obtained by gating cells based on positive CD4+ staining. Data are expressed as mean ± SEM (in %) from 3 representative experiment.

Figure 6: Clarke *et al* 2012

IV - DISCUSSION

1. Group B *Streptococcus* interactions with DCs

As we have seen throughout the scientific literature review, an important emphasis was given to DCs, their development, phenotypes and their central role in bridging innate and adaptive immunity. In our particular *in vitro* model, bmDCs were used as our APCs. As the name indicates, bmDCs are derived *in vitro* from their myeloid precursors by cultivating them in the presence of GM-CSF. The maturation process engaged in this manner renders a homogenous population of DC CD8α−. However, in reality, DC populations are much more heterogeneous and usually are composed of a mix of CD8α^+ and CD8α− DCs, with a varying ratios depending on the immune organ, such as the spleen or the thymus, and can originate from either myeloid or lymphoid precursors (9). It is therefore important to consider how these phenotypical differences of DC populations may influence the ensuing immune response.

In a study by Lemire *et al.* activation and maturation profiles of bmDCs and freshly isolated spleen DCs (sDCs) infected with GBS type III were compared. Of importance, phagocytosis and intracellular survival assays revealed that although both bmDCs and sDCs internalized GBS at similar rates, sDCs showed an enhanced ability in bactericidal functions (142). However, when cytokine production profiles were compared between counterparts, bmDCs appeared to be more reactive and produced much greater quantities of cytokines although profiles between the two DC types were very similar (142). In our *in vitro* DC-T cell co-culture model we were interested in studying downstream events from this initial contact between DCs and GBS, as such cytokines produced by DCs play a central role in determining the ensuing T cell response. Thus, in our study bmDCs were selected due to their higher activation status.

In addition to bmDCs and sDCs, a study by Mancuso *et al*. compared the production of type I IFNs by GBS-infected bmDCs to that of *in vitro* differentiated mouse pDCs. Authors demonstrated that bmDCs, but not pDCs, are the major source of type I IFNs in the context of GBS infection (160). Type I IFNs have important effects on the maturation and function of DCs and down-stream amplification of T cell responses (237). Synergy between type I IFNs and TNF-α or IL-1β resulting in enhanced pro-inflammatory chemokine expression has also been reported (259). Altogether, the few available studies on GBS interactions with DCs, indicate that myeloid CD8α− DCs seem to be major targets and players during GBS infection.

However, we cannot rule out the possibility that *in vivo* other DC sub-populations are also involved. This is suggested by the aforementioned observation of enhanced bactericidal capacity of sDCs compared to bmDCs (142). This finding might indicate that both CD8α+ and CD8α− DC sub-populations are required for efficient bacterial clearance (142), and consequently the development of the issuing adaptive immune response. Bacterial internalization and antigen degradation is critical for DC-mediated antigen presentation to T cells in the context of MHC-II. Furthermore, bacterial internalization and intracellular destruction also affect DC activation. It has been reported that GBS internalization is critical for production of IL-12p70, IL-6, IL-10, IL-1β, TNF-α, CCL2, CXCL1 and CXCL10 by bmDCs (57, 142, 143). In another study it was reported that GBS degradation in phagolysosomes is required for TLR7- and TLR9-dependent type I IFN production by infected bmDCs (160). Finally, a recent study reported that IL-1β production by GBS-infected bmDCs requires signalling thorough cytosolic NLRP3 (57).

In order to gain greater insight on how different DC sub-populations influence the T cell response, it would be interesting to repeat our *in vitro* experiments using freshly isolated sDCs as a model APC. In the case that cytokine production by GBS-infected sDCs would not be sufficient to initiate T cell activation, different approaches could be taken. As we have seen earlier, sDCs display an enhanced ability in intracellular bacterial killing, but produce less cytokines. One school of thought could be that sDCs are much more potent killers of GBS, and because of this GBS is rapidly eliminated before DCs get a chance of being sufficiently activated. In this case, it could be interesting to vary the GBS inoculum in order to obtain a cytokine production profile by sDCs similar to that of bmDCs, all the meanwhile making sure cytotoxicity levels remain low. Another school of thought could be that sDCs, isolated from a background filled with accessory immune cells, require help from a certain cell type, such as a NK cells, or a combination of cell types, such as macrophages and NK cells, to reach and fulfill their immunogenic capacities. In this case, different combination of cell types could be used for analysis. It will be important in this view, however, to stain our cells intracellularly for FACS analysis as cytokines found in the supernatant could come from a variety of cell types. Finally, it would also be interesting to analyze how CD8α+ and CD8α− fractions of total sDCs respond to GBS infection. For this, isolated sDCs could be further fractioned by MACS to obtain one fraction of sDC CD8α+ and one fraction of sDC CD8α−. Fractions could then be used for co-culture with T cells as previously performed and data could be compared to total sDCs and bmDCs.

2. CPS modulation of DC functions: impact on *in vitro* T cell activation

Now that we have seen how different types of DC populations might affect the ensuing T cell mediated response, we will now focus on an important objective of this study which was to evaluate the effect GBS CPS, considered a major virulence factor, on T cell activation. To this aim, we used a serotype III encapsulated GBS strain and its non-encapsulated isogenic mutant in our *in vitro* model. *In vivo* studies could not be performed as the mutant strain is rapidly eliminated from the host (data not shown).

We were able to identify three important cytokines produced by T cells during GBS infection *in vitro*: IFN-γ, TNF-α and IL-10. From these three cytokines, IFN-γ and TFN-α are considered as Th1 cytokines whereas IL-10 is considered anti-inflammatory and involved in immune-regulation. The fact that we observed this dual response might indicate the generation of some IL-10-producing T cells in order to maintain homeostasis. Our present analysis cannot conclude, however, on the possible Treg phenotype of these cells. More importantly, IFN-γ was the only one whose level was significantly decreased in the absence CPS. Production of TNF-α was also reduced, albeit this difference did not reach statistical significance. This is somehow surprising as the loss of capsule, which serves as a cloak to mask highly immunogenic properties found within the inner walls of GBS, would be associated with a stronger T cell activation and thus robust IFN-γ production. However, when we look back results obtained by Lemire *et al.* on DC activation by GBS and its non-encapsulated mutant, the same trend was observed as the wild-type GBS strain induces a stronger cytokine production than the non-encapsulated mutant at long incubation times (16 h post-infection) (142). The only exception was IL-10, which production was significantly higher in DCs infected with the non-encapsulated mutant than those infected with the encapsulated strain (142). In this study two interrelated hypothesis were suggested to explain these observations, a) increased IL-10 production by DCs in the absence of CPS reduce the production of other cytokines; or b) higher and faster levels of non-encapsulated bacteria killing reduce or impair cytokine production by DCs. In our DC-T cell co-culture system, the consequence of this diminished DC activation by the non-encapsulated strain translates into lower levels of IFN-γ production by CD4+ T cells.

Immune cell activation can also be determined by the presence or absence of surface molecules necessary for co-stimulation events and signaling transduction for the acquisition of

proper immune functions. On the DC side, it has been reported that encapsulated GBS and its non-encapsulated mutant induce similar levels of CD40, CD86, and MHC-II surface expression (142). Nevertheless, we cannot rule out differences in peptide-MHC-II loading between the two strains. This is suggested by the fact that intracellular survival of the non-encapsulated mutant was significantly impaired in bmDCs. On the T cell side, different surface molecules important in T cell activation were evaluated in our study, such as CD11a, CD28, CD152, CD154 (data not shown). However, under our experimental conditions only CD69 was significantly up-regulated by T cells co-cultured with GBS-infected bmDCs. Interestingly, the non-encapsulated strain induced higher levels of CD69 expression by T cells than the encapsulated strain-infected co-cultures.

Attempting to explain modulation of CD69 expression on CD4+ T cells is quite difficult, due to the lack of information available on this particular surface marker. Although it is well known that CD69 is one of the earliest markers induced upon activation of T and B cells and acts as a signal-transmitting receptor for immune regulatory events, its cognate ligand is still unknown (151). Of the studies available on CD69 expression by T cells, a paper by Harimaya *et al.* demonstrated a dose-dependent up-regulation of CD69 on CD4+ T cells infected with *Alloiococcus otitidis*, a Gram-positive bacterium (106). Although large amounts of IFN-γ were quantified in supernatants containing total peripheral blood lymphocytes authors failed to correlate this production to CD69 surface expression (106).

From data herein presented, it is difficult to draw definitive conclusions on the effects of CPS on T cell function modulation. Although we have found significant differences in IFN-γ production and CD69 surface expression by CD4+ T cells co-cultured with bmDCs infected with either wild-type GBS or its non-encapsulated mutant, it is difficult to extrapolate how these changes in T cell behaviour might be important in GBS immuno-pathogenesis. However, it is vital to understand that if the CPS indeed has immuno-modulatory functions, they would be first exerted on DCs during endocytosis events and would latter impact T cell activation by extension.

In this regard, there is a growing body of evidence showing that many encapsulated bacteria use strategic routes of entry into host cells in order to avoid clearance and to remain persistent (30, 143, 162). In addition, it was also reported that the route of bacterial uptake may

also influence the repertoire of antigenic epitopes presented to T cells, and thus by extension, the ensuing T cell response (261).

In a previous work, it was demonstrated that encapsulated GBS exploits different endocytosis mechanisms employed by bmDCs, such as clathrin-mediated and lipid raft-mediated endocytosis, whereas non-encapsulated GBS fail to engage lipid raft-mediated pathways (143). Furthermore, Mancuso *et al.* demonstrated that once GBS was internalized by bmDCs, they could be found in two types of compartments: phagosomes that contained GBS cells that stained positive for bacterial DNA and sequentially acquired early and late endosomal markers but were negative for the lysosomal marker cathepsin D; and cathepsin D-positive, DNA-negative phagolysosomes filled with amorphous GBS material, suggestive of dead bacteria (160). It is therefore very appealing to hypothesize that may exist preferential endocytosis pathways employed by encapsulated GBS for circumvention of DC bactericidal functions. Thus, as a follow-up of our studies, it could be very interesting to inhibit different DC endocytosis pathways during infection with GBS, and evaluate how this may affect T cell activation in our co-culture system.

3. Role of IFN-γ in the immune-pathogenesis of GBS

In the case of GBS, cytokines are not only important for the appropriate development of host defences but are also involved in induction of severe pathologies. Although it is well established that IFN-γ production plays a central role in the control of GBS infection (62, 137), no work has focused on the particular cell subset implicated in its production. Within this document, we disclosed for the first time a specific involvement of CD4+ T cells in IFN-γ production in response to GBS. Indeed, three types of models were developed and used to elucidate T cell contribution to the immuno-pathogenesis of systemic GBS infection: *in vivo, ex vivo, and in vitro* models.

The spleen, a specialized organ filtering blood-borne pathogens, plays an essential role in the generation of adaptive immune responses to encapsulated bacteria. In the marginal zone of the spleen, various cell types reside and interact to produce a coordinated immune response. Several cell types have a fixed position, such as the marginal zone macrophages and B cells, while other cell types, such as DCs, are migratory (169). Thus, taking into account the importance of the

spleen in the context of systemic encapsulated GBS infection, initial e*x vivo* analysis of cytokine production by total splenocytes derived from encapsulated GBS infected mice were performed. These *ex vivo* analysis revealed the presence of IFN-γ, TNF-α, IL-6 and IL-10. Production of IFN-γ, TNF-α and IL-6 is suggestive of a type 1 pro-inflammatory response being developed short after infection. IL-10 production can be related to immune-regulation, as previously discussed. It is interesting to note that TNF-α and IL-6 have routinely been reported as important mediators of GBS sepsis (57, 97, 247). This observation might also highlight the particular importance of IL-10 in maintaining homeostasis as Cusumano *et al.* reported a role of IL-10 in protecting neonatal mice from developing sepsis by reducing TNF-α production (61). In the context of GBS infection several cell types have been reported to secrete TNF-α, IL-6 and/or IL-10, including DCs, monocytes and macrophages (20, 64, 75, 136, 142). The possible source of IFN-γ was at the time of our study unknown, and will be discussed below.

In addition to cytokines, early chemokine release by cells of the innate immunity plays an important role in attracting various accessory immune cells to the site of infection, such as T cells. *Ex vivo* analysis of total splenocyte chemokine production suggested that T cells can be actively recruited via the expression of three important chemokines: CCL3, CXCL9 and CXCL10. It is interesting to note that CXCL9 and CXCL10 are two chemokines that bind to CXCR3 receptor on the surface of T cells, and more importantly, are induced by IFN-γ (102). Once more, the importance of this particular cytokine is highlighted not only for its bactericidal-inducing properties but also for its role in helping with the acquisition of adaptive immunity. Furthermore, it is also noteworthy that CCL3 production can be induced by various pro-inflammatory agents/cytokines including LPS, TNF-α, IFN-γ, IL-1α/β and others, whereas treatment with IL-4, IL-10, dexamethasone or other anti-inflammatory signals is known to down-regulate CCL3 expression (173). CCL3 has been shown to have effects on the magnitude and cytokine polarity of the T cell response by binding to the CCR1 receptor on T cells (152). In the context of a systemic infection with GBS, several cells types in the spleen are potential sources of these three chemokines. GBS-stimulated spleen DCs were reported to produce CXCL9 and CXCL10 (142). Although up-regulation of *Cxcl10* gene expression was observed by DNA microarray analyses of mouse peritoneal macrophages (75) GBS was reported to be unable to induce neither CXCL10 nor CXCL9 secretion by these cells (85). On the other hand, macrophages seem to contribute to CCL3 production (75, 85).

Now that we have a contextual understanding of total splenocyte responses to systemic GBS infection, the next few lines will focus on the specific contribution of CD4+ T cells in the development of the immune response, which was indeed the central theme of this MSc project. To this aim, CD4+ T cells were isolated either from *ex vivo* total splenocyte cultures or directly from the spleen of infected mice without further stimulation (*in vivo* model). Isolated CD4+ T cells were then stained intracellularly for various cytokines. *Ex vivo* and *in vivo* analysis showed for the first time that CD4+ T cells are specifically activated by GBS and are important producers of IFN-γ and TNF-α. These antigen-specific T cells also produce low, but still significant levels of IL-2, overall suggesting the development of a Th1 response. These results are in agreement to the aforementioned cytokine patterns produced *in vitro* by T cells co-cultured with GBS-pulsed bmDCs, although IL-2 was non-detectable in our *in vitro* supernatants, probably due to its autocrine nature.

Also in agreement to *in vitro* studies described in section 2, *ex vivo* and *in vivo* analysis of GBS-activated CD4+ T cells showed production of IL-10 by this particular cell sub-type. The % of antigen-specific, IL-10-producing CD4+ T cells was low but nonetheless significant. This finding suggests that during *in vivo* infection cells of the innate immune system are the major sources of this immune-regulatory cytokine. Similarly, cell-specific intracellular cytokine production by human mononuclear cells in response to GBS revealed less than 2% of IL-10-positive CD3+ T cells (60). Although a dual participation in the production of IFN-γ and IL-10 by CD4+ T cells may seem opposing, a study has recently demonstrated that probiotic stimulation of human PBMCs, monocyte-derived DCs, and DC-CD4+ T cell co-cultures engaged a production the aforementioned cytokines and induced a Th1 and anti-inflammatory response (Hua, 2010). In addition, as IL-10 is routinely associated with Treg cell development, it may be of interest to perform a dual intracellular stain FACS analysis for IL-10 and INF-γ within CD4+ T cells in order to determine if there is a simultaneous development of Th1/Treg immune response.

The specificity of this response also proved to be well conserved since booster GBS infection two weeks after the initial infection demonstrated the same cytokine pattern for all four cytokines in isolated CD4+ T cells, suggesting development of a memory response. The fact that CD4+ T cells readily produce IFN-γ in response to GBS raises an interesting point. As we have discussed earlier, CXCL9 and CXCL10 are release in great amounts by total splenocytes and are induced by the presence of IFN-γ. We thus propose a global model (Figure 1) where an initial

production of INF by cells of innate immunity could trigger the release of CXCL9 and CXCL10 by APCs, attracting T cells to the site of infection. Activation of responding CD4+ T cells would in turn produce important quantities of IFN-γ, acting on T cells in an autocrine loop, but also acting upon APCs to further increase the chemo-attracting gradient resulting in increased T cell recruitment, including production of CCL3. This process results in the development of a Th1 response emphasized by the presence of several pro-inflammatory cytokines including TNF-α. Central to this response is IFN-γ, which acts as a master regulator of many inflammatory pathways as it is involved in regulating the production of several molecules involved in the inflammatory response. For example, IFN-γ up-regulates the production of IL-12, TNF-α and several chemokines in macrophages and other APCs. This inflammatory response is meant to protect the host; however, the excessive production of inflammatory mediators, in some cases, leads to injury to the host. IFN-γ also contributes to increase the bactericidal functions of professional phagocytes, thus contributing to bacterial clearance (216).
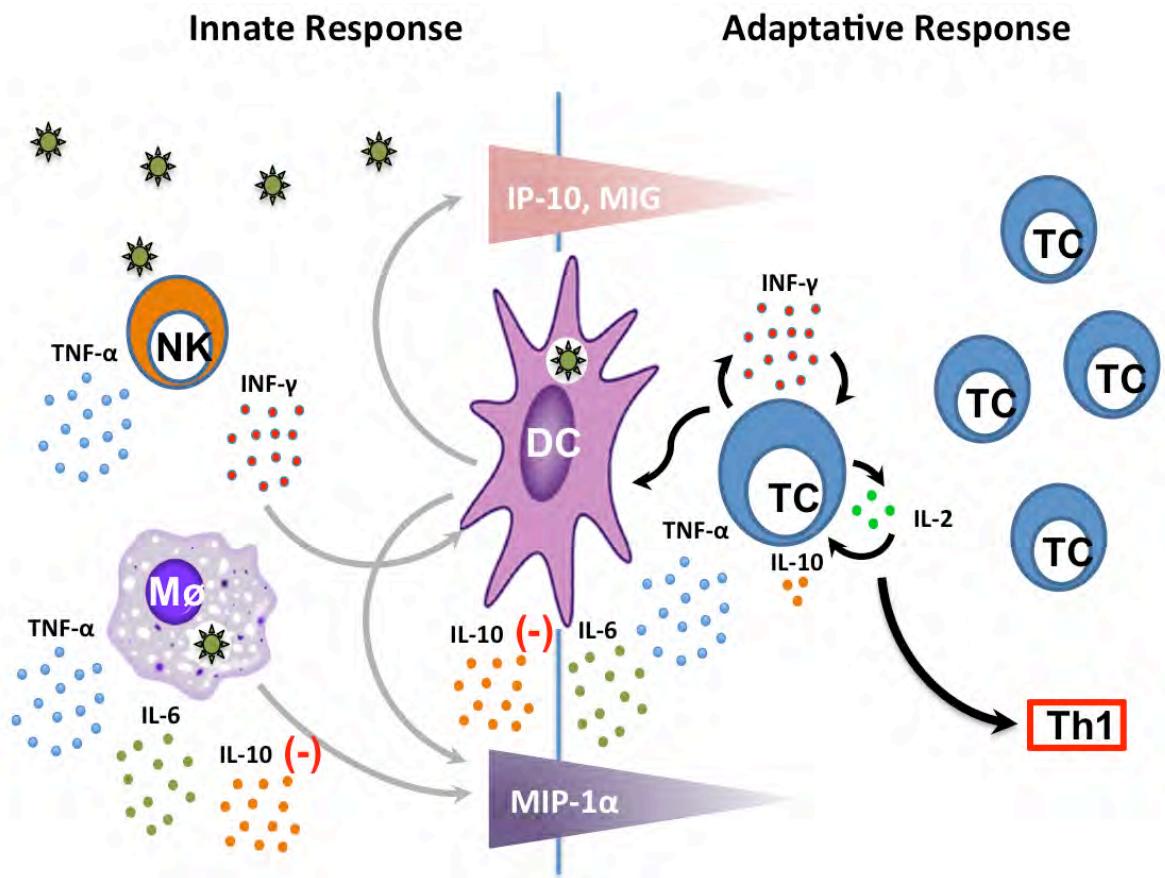
Figure 1: Proposed model of immune cell activation during GBS infection. Arrows in grey represent probable and potential immune events whereas black arrows represent identified immune events. An initial production of INF-γ by cells of innate immunity triggers the release of CXCL9 and CXCL10 by APCs, attracting T cells to the site of infection. Activation of responding CD4+ T cells in turn produce important quantities of IFN-γ, acting on T cells in an autocrine loop, but also acting upon APCs to further increase the chemo-attracting gradient resulting in increased T cell recruitment, including production of CCL3. This process results in the development of a Th1 response emphasized by the presence of several pro-inflammatory.

Undoubtedly, IFN-γ production by T cells during GBS infection is crucial for host defence. This however, brings up an important question: which immune cell population(s) initiates the production of IFN-γ? Although we have identified CD4+ T cells as potent IFN-γ producers during GBS infection, it is highly improbable that they are responsible for its initiation being a cell line belonging to adaptive immunity. Thus, the most plausible culprits could be cells of innate immunity. Indeed, NK or NKT cells could logically play an important role as they have routinely been identified as potent IFN-γ producers, although there now exists a growing body of evidence that APCs, such as DCs or macrophages, could also be involved in its synthesis (222). A study by Derrico *et al.*, seams to support that NK cells could be important initiators of IFN-γ in response to GBS infection. Their results performed on splenocytes from severe combined immunodificient mice (mice that have no functional CD3) showed that GBS induces IL-12 and TNF-α production by macrophage, which in turn promote NK cell secretion of IFN-γ, and enhances innate phagocyte resistance mechanisms (68). Recently, it was reported that NKT cells recognize GBS glycolipid antigens presented by CD1d, leading to NKT cell activation and IFN-γ production (132). Although these studies offers an interesting starting point for future research, a definitive understanding of all mechanisms regulating IFN-γ production, such as the initiator cell lines and further and deeper studies on antigen-specific CD4+ T cells would improve our understanding of GBS immune-pathogenesis and contribute to develop more powerful strategies to fight GBS infections.

V – GENERAL CONCLUSIONS AND PERSPECTIVES

Throughout this project we have developed three different models in order to analyze the response of CD4+ T cells in the context of GBS infection. In addition to T cells, *ex vivo* models of infection gave us the chance to understand the total splenocyte cytokine response to GBS, and was extremely insightful when interpreting T cell results. Our work highlights the central role of IFN-γ for the proper development of an immune response directed against GBS. We have also described for the first time the generation of antigen-specific CD4+ Th1 cells during GBS infection. As IFN-γ production is impaired in early life (Mancuso), and GBS is a neonatal pathogen, inducing an embellished IFN-γ response by CD4+ T cells may prove to be an interesting therapeutic approach. Although our work has focused on the response of CD4+ T cells to GBS, it is important to remember that other T cell subtypes may also participate to the development of immune functions. For example, recently it was demonstrated that CD8+ T cells play a particularly important role in resistance to *Streptococcus pneumonia* infection, even more important then CD4+ T cells which appeared to be dispensable (Weber). With this in mind, it could be very interesting to study CD8+ T cell activation by GBS and their role in bacterial clearance. In addition to CD8+ T cells, Th17 T cells have recently gained much attention for their pro-inflammatory functions via the production of IL-17. Research has showed that there exists an opposing duality between Th1 and Th17 T cell differentiation and that Th17 differentiation was inhibited by IFN-γ production in the case of *Streptococcus thermophilus* infection (Ogita). As we have demonstrated that CD4+ T cells readily produced IFN-γ in response to GBS, it could be interesting to abrogate its production by either using neutralizing Abs or using KO mice for this particular cytokine and observe the ensuing response.

On the other hand, we also describe a low but significant participation of CD4+ T cells in the production of IL-10. Although a dual participation in the production of IFN-γ and IL-10 by CD4+ T cells may seem opposing, a study has recently demonstrated that probiotic stimulation of human PBMCs, monocyte-derived DCs, and DC-CD4+ T cell co-cultures engaged a production the aforementioned cytokines and induced simultaneous Th1 and anti-inflammatory responses (Hua, 2010). As IL-10 production by CD4+ T cells is usually associated with Treg differentiation it may be of interest to pursue future studies in this regard in order to identify if development of such a population is indeed occurring. In addition, we found a partial modulation of this cytokine by the CPS of GBS *in vitro*. It is interesting to note that this particular cytokine seems to be very sensitive to modulation by CPS of different gram-positive bacteria. For example, WT GBS induces stronger

cytokine production by bmDCs than its non-encapsulated mutant at longer incubation times (16h p.i) except for IL-10 whose production was significantly higher in DCs infected with the non-encapsulated (142). In contrast, it was recently reported that WT *S. suis* type 2 induces strong release of IL-10 by human monocyte-derived DCs compared to its isogenic mutant with abrogated its production (171). Due to the particular importance of this cytokine during GBS infection, understanding underlying mechanism for its modulation may be of grand interest.

Finally, our work was also interested in identifying a role of GBS CPS on CD4+ T cell activation. Although we found significant differences in IFN-γ production and surface CD69 expression on T cells infected with wild-type GBS as compared to its non-encapsulated mutant, it is difficult to draw conclusions on the exact mechanisms by which GBS CPS modulates these functions. As DCs and other APCs represent the first line of defense during GBS infection, more work is required to complement our current understanding of CPS-mediated immuno-modulation, such as identifying the receptors involved in GBS recognition by APCs, the downstream endocytic and signaling pathways, and how CPS modulates these interactions.

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