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REGULATION OF B CELL FUNCTION BY PLASMACYTOID DENDRITIC CELLS

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Stockholm 2011

Published by Karolinska Institutet.
Printed by Larserics Digital Print AB, Sundbyberg.

ISBN 978-91-7457-357-2

ABSTRACT

Dendritic cells (DCs) are early sentinels of pathogen exposure and central in the initiation and orchestration of adaptive immune responses. Apart from the prominent role of DCs in the activation of T cells, DCs have been shown to influence humoral B cell mediated responses. DCs are therefore important cells for regulating immune responses to vaccines. Many of the vaccines under development today are against pathogens such as *Mycobacterium tuberculosis* and HIV-1 that likely require induction of both cellular and humoral responses to cause protection. This generates a need for new safe and effective vaccine adjuvants that can stimulate such responses. One class of adjuvants that has attracted a lot of interest over the last years is TLR (toll-like receptor)-ligands. Selected TLR-ligands can specifically activate subsets of DCs and B cells according to their cognate receptor expression and therefore represent promising candidates to shape vaccine-induced responses. The first aim of this thesis was to investigate the responsiveness of B cells to TLR stimulation to proliferate and differentiate into antibody (Ab) producing cells. Furthermore, the aims were to study, whether the distinctly different myeloid (MDCs) and plasmacytoid (PDCs) can support these responses in a T helper cell-independent or dependent manner. In addition, differences in the responses of B cells and DCs from humans versus non-human primates (NHP) were addressed.

In **paper I**, we established and refined isolation protocols for subsets of primary human PDCs, MDCs and B cells from blood and methods to examine their functions. We found that total B cells responded strongly to engagement of TLR9, less to TLR7/8 and not to engagement of TLR3. Furthermore, PDCs but not MDCs markedly enhanced B cell proliferation and differentiation into Ab producing cells in response to TLR7/8-ligand stimulation and to a lesser extent to TLR9-ligands (CpG ODN classes A, B, and C). PDCs strongly enhanced TLR7/8-ligand-induced proliferation of both memory and naive B cells but were only able to support memory cells to differentiate to CD27high plasmablasts. Type I IFN produced to high levels by PDCs was the principal mediator of the enhanced responses upon TLR7/8 stimulation. This effect may at least in part be explained by the reported upregulation of TLR7 and MyD88 by IFNα. Although MDCs expressed high levels of the known B cell growth factors IL-6, IL-10, and B cell-activating factor (BAFF) in response to TLR7/8 stimulation, they were unable to enhance B cell responses in this system. In **paper II**, we found that PDCs also had the ability to augment naïve B cell responses induced by BCR engagement and T cell help. The presence of supernatants from TLR-stimulated PDCs increased B cell proliferation, the frequency of B cells that differentiated to CD27high CD38high cells, and secretion of IgM. IFNα produced by PDCs was again instrumental in these processes and increased cell viability or proliferation were not main reasons for the improved B cell function. We found that PDC supernatants or IFNα induced upregulation of the co-stimulatory molecule CD86 on B cells. Further, these B cells showed improved ability to interact with and activate T cells. Thus, increased B cell responsiveness to T cell contact, mediated by PDCs via their production of IFNα, may facilitate B cell proliferation and differentiation into Ab producing cells.

In order to further explore the influence of IFNα and PDCs on B cell functions in vivo in humans, models such as NHPs that more closely resemble humans than rodents need to be utilized. NHPs have the advantage that they to a large extent exhibit similar subpopulations of DC and B cell subsets as well as similar TLR expression as humans. However, similarities and potential disparities between the species need to be carefully investigated to facilitate the translation of NHP studies into clinical trials. In **paper III**, we therefore examined whether the effect of activated PDCs or IFNα enhanced B cell functions was comparable in human and NHP rhesus macaque cells in response to TLR ligands. We found similar responses in human and rhesus cultures to the selected TLR ligands in terms of B cell proliferation. B cell proliferation to the TLR7/8-L and CpG class C showed a significant and comparable increase in presence of IFNα. However, upon stimulation only human B cells acquired high expression of CD27, associated with plasmablast formation, although both human and rhesus B cells produced increased levels of IgM. Instead, rhesus B cell differentiation was associated with a more prominent downregulation of CD20. This validates that rhesus macaques are relevant and appropriate in vivo models to study TLR induced B cell responses although the choice of B cell differentiation markers to measure must be considered.

In conclusion, the studies included in this thesis highlight the potential of PDCs and IFNα to shape B cell differentiation to Ab secreting cells. These studies add to the understanding on the role of DCs in modulation of B cell responses, which is crucial information for the design of novel vaccines, adjuvants and immuno-modulatory treatment formulations.

Keywords: Dendritic cell, B cell, type I Interferon, Plasmacytoid dendritic cell, Non Human Primate

LIST OF PUBLICATIONS

This thesis is based on the following original papers and manuscripts, which will be refered to in the text by their roman numerals:

I. Iyadh Douagi, **Cornelia Gujer**, Christopher Sundling, William C. Adams, Anna Smed-Sörensen, Robert A. Seder, Gunilla B. Karlsson Hedestam and Karin Loré. Human B Cell Responses to Toll-Like Receptor Ligands are Differentially Modulated by Myeloid and Plasmacytoid Dendritic Cells.

Journal of Immunology. 2009 Feb 15;182(4):1991-2001.

II. **Cornelia Gujer,** Kerrie J. Sandgren, Iyadh Douagi, William C. Adams, Christopher Sundling, Anna Smed-Sörensen, Robert A. Seder, Gunilla B. Karlsson Hedestam, Karin Loré. IFNα Produced by Plasmacytoid Dendritic Cells Enhances T Cell Dependent Naïve B cell Differentiation.

Journal of Leukocyte Biology. Epub ahead of print January 13, 2011 - doi:10.1189/jlb.0810460.

III. **Cornelia Gujer,** Christopher Sundling, Robert A. Seder, Gunilla B. Karlsson Hedestam, Karin Loré. Human and Rhesus Plasmacytoid Dendritic Cell and B Cell Responses to TLR7/8 Stimulation.

Submitted manuscript.

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LIST OF ABBREVIATIONS

Ab	Antibody
APC	Antigen presenting cell
BAFF	B cell-activating factor
BCR	B cell receptor
DC	Dendritic cell
ELISA	Enzyme-linked immunosorbent assay
GC	Germinal center
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IRF	Interferon regulatory factor
MDA5	Melanoma differentiation factor 5
MDC	Myeloid dendritic cell
MHC	Major histocompatibility complex
MyD88	Myeloid differentiation factor 88
NHP	Non-human primate
ODN	Oligodeoxynucleotides
PAMP	Pathogen-associated molecular pattern
PBMC	Peripheral blood mononuclear cell
PDC	Plasmacytoid dendritic cell
PRR	Pattern recognition receptor
SEB	Staphylococcal enterotoxin B
SLO	Secondary lymphoid organ
TCR	T cell receptor
TLR	Toll-like receptor
TSST-1	Toxic shock syndrome toxin 1

1 INTRODUCTION

1.1 INTRODUCTION TO THE IMMUNE SYSTEM

Major discoveries in the last century have increased our understanding of the immune system substantially, which has lead to the development and application of numerous medical treatment strategies. The role of the immune system is to protect the body against microbial and other environmental threats and at the same time to maintain host-microbial homeostasis with commencial microbes. These central tasks of the immune system are achieved by the recognition of microbes and the induction of anti-microbial defence against pathogens by highly sophisticated mechanisms. One important property of the immune system to direct its powerful reactions is the discrimination of self from non-self, a phenomenon called self-tolerance. Functional self-tolerance mechanisms allow for potent reactions against pathogens and the clearance of toxic and allergenic substances, but spare the body´s own tissues from such reactions [1]. The immune system in vertebrates can be divided in two branches, the innate and the adaptive immune system. This separation is based on the mechanisms and the kind of recognition molecules that are used by the respective immune cells to detect pathogens. The microbial sensing pathways employed by the innate immune system consist of genetically programmed recognition molecules that detect molecular patterns, invariant features that are shared by many microbes and that are generally not present in the mammalian host [2]. The recognition molecules that are used are expressed broadly by a large number of cells and constitute the initial host response. Upon recognition of microbial patterns exposed by an antigen (the term “antigen” is used to generally describe a foreign substance that creates an immune response), cells of the innate immune system get activated. Innate functions that are triggered upon activation include the secretion of cytokines and anti-microbial substances, phagocytosis and the transport and presentation of the invading microbes to other cells. Different cell types get subsequently activated and, in a highly orchestrated manner, generate the immune response to the invading microbe. In contrast to innate immune responses that are mounted immediately after a microbial infection, the adaptive immune response takes more time to be mounted (1-2 weeks or more). The adaptive immune system involves relatively small numbers of cells, called T and B lymphocytes, building the cellular and the humoral part respectively, of the adaptive immune system. T cells and B

cells typically recognize microbes, usually presented on capturing cells, in a highly specific way. Antigen recognition receptors of the adaptive immune system are generated de novo in each organism, which is in contrast to the innate recognition molecules that are germline encoded. Activated T cells and B cells expand and build a potent adaptive immune response that in a highly specific way contributes to the clearance of microbial infections. A key feature of the adaptive response is the development of memory by the production of long-lived cells that have the ability to mount faster specific responses upon reencounter of the same pathogen. These are very potent mechanisms that have evolved to protect the body from invading pathogens and represent the fundamental basis by which vaccines work. The innate and the adaptive immune systems collaborate to eliminate pathogens and are reciprocally regulating each other's actions. Specialized innate immune cells, including dendritic cells (DCs) and macrophages, take up and digest microbes to present them to T cells in a process called antigen presentation while antibodies (Abs) produced by plasma cells can block pathogen entry into target cells (so called neutralizing Abs), inhibit the action of soluble molecules such as bacterial toxins or flag infected cells or pathogens for destruction by innate immune mechanisms. Cells of both systems also impact each others actions by the secretion of soluble molecules such as cytokines and chemokines. In addition to the role of DCs in the presentation of antigen to T cells, DCs were also shown to impact the other arm of the adaptive immune system, i.e. the induction of Ab responses by B cells. The studies included in this thesis focus on the role of DCs in shaping humoral immune responses.

The following sections aim to introduce in more detail the aspects of immunology that are most relevant to the work included in this thesis. In particular, the sections 1.2 and 1.3 describe central concepts of innate and adaptive immunity with a focus on DC and B cell biology and pattern recognition. Section 1.4 briefly introduces to the concept of vaccine adjuvants. For adjuvant research, an increased basic understanding of how DC and B cells are influenced by adjuvants will improve vaccine design. The toll-like receptor (TLR)-ligand compounds that are potent stimulators of innate immunity that were used in this thesis and the central role of DCs in mediating the effects of vaccine adjuvants are introduced in the subsections 1.4.1 and 1.4.2 respectively. Finally, the use of non-human primates (NHP) as models in adjuvant research, in particular concerning TLR-ligands, are introduced in section 1.5.

1.2 INNATE IMMUNITY

Innate immunity is the first line of defense of an organism to invading pathogens. The innate immune system consists of specific tissues such as the skin and mucosal membranes that form a first physical barrier against pathogens and other environmental challenges. In addition, innate immunity consists of specialized cells that act together in the early onset of a response to invading microbial structures. Microbial structures are detected by membrane bound and cytosolic receptors, called pattern recognition receptors (PRRs) that are expressed in all cells although with different levels and repertoires. PRRs have the ability to bind conserved structures that are present on many microbial organisms [1], as mentioned above and introduced in more detail in the following sections 1.2.1 and 1.2.2. This initial event in the generation of an innate immune response leads to the secretion of soluble factors that attract important innate immune cells. Different kinds of phagocytic cells are attracted to the site of infection by chemotaxis and subsequently engulf, kill, digest and thereby eliminate invading microbes. Granulocytes such as neutrophils are generally the first phagocytic cells to arrive at the site of infection and they are very efficient in engulfing and eliminating microbes by different mechanisms including the production of anti-microbial peptides and reactive oxygen species. Neutrophils are relatively short lived and die at the site of infection, which leads to the release of hydrolytic enzymes and contributes to inflammation and tissue destruction. Other phagocytic cells that are recruited to the site of infection are monocytes, macrophages and DCs. Macrophages and DCs have the unique ability to present digested peptides to CD4+ T cells by major histocompatibility complex (MHC) class II molecules. This is in contrast to presentation of peptides via MHC class I to CD8+ T cells, which can be achieved by all cells upon infection. Presentation of peptides by MHC class II is exclusive for macrophages, B cells and DC and these cells are therefore called professional antigen presenting cells (APCs). Antigen presentation is essential in the initiation of T cell responses and directly links the innate and the adaptive immune system. Another type of innate immune cells is the NK cells that contribute to a successful immune response with their cytotoxic activity. NK effector functions are tightly regulated by a number of inhibiting and activating receptors and are inhibited by encounter with self-MHC molecules [3]. Ab-dependent cell-mediated cytotoxicity (ADCC) is a mechanism used by NK

cells but also neutrophils and eosinophils, in order to eliminate infected cells and pathogens respectively. ADCC is strictly cell-mediated and based on the recognition of the Fc parts of Abs (via Fc receptors) that are bound to infected cells. Soluble effector molecules such as cytokines and chemokines also represent an important part of the innate immune system. Cytokines are messenger molecules that regulate the function of other cells while chemokines attract inflammatory leukocytes. Both cytokines and chemokines are secreted immediately upon activation of cells. Lipid mediators of inflammation, reactive free radicals and bioactive amines and enzymes that can all contribute to tissue inflammation can also be secreted immediately upon activation. Other soluble molecules, such as complement proteins and anti-microbial peptides, are constitutively expressed in body fluids. The complement proteins belong to an enzyme cascade system that is involved in mediating inflammatory responses and can be triggered by antigen-Ab complexes or certain microbial products. Complement proteins also act by coating infectious agents, which may facilitate phagocytic uptake. Anti-microbial peptides possess a broad spectrum of activities and are most prevalent at mucosal surfaces and in phagocytic cells. Their concentration is increased upon microbial infection or inflammation.

1.2.1 Pattern recognition receptors

The innate immune system recognizes microbial components via distinct germline-encoded PRRs that have evolved to detect components of foreign pathogens referred to as pathogen-associated molecular patterns (PAMPs) as reviewed in [4-6]. Distinct PRRs can be broadly categorized into secreted, transmembrane, and cytosolic classes. Secreted PRRs bind to microbial cell surfaces, activate classical and lectin pathways of the complement system, and opsonize pathogens for phagocytosis by macrophages and neutrophils. The cytosolic PRRs are expressed by most cell types and include the retinoic acid–inducible gene I (RIG-I)–like receptors (RLRs) and the nucleotide-binding oligomerization domain-containing protein (Nod)-like receptors (NLRs). The RLR family consists of three members, RIG-I, melanoma differentiation factor 5 (MDA5) and LGP2, which recognize and respond to viral RNA. NLRs represent a large family of cytosolic PRRs that respond to various stimuli, including PAMPs, non-PAMP particles and cellular stresses. Among the NLRs, Nod1 and Nod2 recognize the degradation products of bacterial cell wall components, and NLR family pyrin domain containing 3 (NLRP3, formerly NALP3) responds to various stimuli to form the inflammasome

complex, which promotes the release of IL-1β and IL-18. Activation of these receptors generally requires that the cell is infected. Accordingly, these PRRs are broadly expressed because most cells can potentially be infected by pathogens, especially by viruses.

The transmembrane PRRs include the TLR family that will be described more in detail below and the C-type lectins. The expression of TLRs is cell-type specific, which allows for the distribution of recognition responsibilities to various cell types. PAMPs recognized by TLRs include lipids, lipoproteins, proteins and nucleic acids derived from a wide range of microbes such as viruses, bacteria, parasites and fungi [6]. The C-type lectin family includes Dectin-1 and -2 that detect b-glucans and mannan, respectively, on fungal cell walls. The activation of transmembrane PRRs does not require the cells to be infected and is therefore mainly mediated by specialized cells of the immune system, such as macrophages and DCs. Other sensors of bacterial components and products are likely to exist.

1.2.2 Toll like receptors

TLRs were the first PRRs to be discovered. They are evolutionarily highly conserved molecules and were discovered as homologues of the Toll-receptor in *Drosophila melanogaster* [7]. So far, 10 and 12 functional TLRs have been identified in humans and mice, respectively, with TLR1–TLR9 being conserved in both species. TLRs are largely divided into two subgroups that are either expressed on the cell-surface or in endosomal/lysosomal organelles (Figure 1). Cell-surface TLRs consist of TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11 and recognize conserved microbial membrane components such as lipids, lipoproteins and proteins. TLR3, TLR7, TLR8 and TLR9 are exclusively expressed in intracellular vesicles such as endosomes and lysosomes and recognize microbial nucleic acids as reviewed in [6]. The proper cellular localization of TLRs is thought to be important for ligand accessibility, the maintenance of tolerance to self molecules such as nucleic acids and downstream signal transduction [8].

TLRs are type I trans-membrane proteins with ectodomains containing leucine-rich repeats that mediate the recognition of PAMPs. In addition, TLRs contain intracellular Toll–interleukin 1 (IL-1) receptor (TIR) domains required for downstream signal transduction. All TLRs except TLR3 signal via the adapter

molecule myeloid differentiation factor 88 (MyD88) to activate the NFκB pathway. Instead of MyD88, TLR3 uses the adaptor TRIF, which can also be bound by TLR4 [9]. These adaptors propagate signaling by recruiting kinases that mediate activation of transcription factors such as NF-κB, interferon regulatory factor (IRF)3 and IRF7, which are all required for the production of inflammatory cytokines including type I interferons (IFNs) that will be addressed later in section 1.2.4.

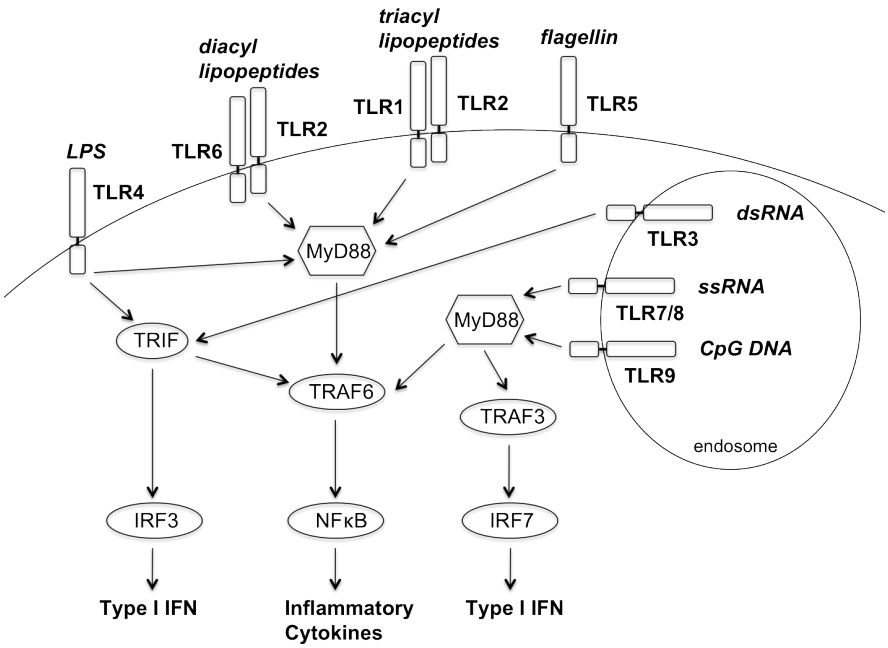

Figure 1: TLR signaling (adapted from [6])

The detection of PAMPs by TLRs is critical to alert the immune system of the presence of pathogens and foreign agents and thus need to initiate a response. Ligands binding to TLRs are therefore being exploited as vaccine components as they would help induce strong immunity. Inappropriate activation of TLRs can however cause damage to the immune system, as it is found in autoimmune situations where immune responses are directed against self-molecules. Such inappropriate TLR activation can be due to a loss of negative regulation of TLR signaling or due to the recognition of self-molecules/ endogenous ligands by TLRs and are strongly associated with the pathogenesis of inflammatory and autoimmune diseases.

1.2.3 Dendritic cells

DCs are immune cells that are central in the initiation and regulation of both innate and adaptive immune responses. They represent a highly specialized

subpopulation of leukocytes that is derived from the bone marrow. DCs have a central role in both immune tolerance and activation of adaptive immunity and are the most potent APCs.

1.2.3.1 Dendritic cell function

The ability of DCs to regulate immune responses is dependent on the maturation of the DCs. In their immature state, DCs reside in peripheral tissues, which is a strategic localization to be able to early sense and encounter antigen. Whole microbes or antigens thereof can be captured and internalized by endocytic mechanisms that do not require infection of the DC. DCs have the ability to process the internalized antigen into peptides, which are thereafter loaded onto MHC molecules for presentation to naive T cells that express the correct specificity for the given antigen [10]. This is an essential mechanism for the initiation of adaptive immunity. Following pathogen invasion, tissue damage or inflammation, as discussed earlier in this section, DCs receive additional activating signals, mediated through interaction with different PRRs. This activation induces maturation of the DCs leading to profound changes in their phenotype and function. It leads to cytokine secretion, dependent on the nature of the antigen, which then further impacts various cell types. Mature DCs are directed from peripheral tissues to regional draining lymph nodes (LNs) where they efficiently present antigen to T cells and thereby stimulate naïve T cells and initiate a primary immune response. In addition to their ability to efficiently prime T cells, DCs can also partake directly or indirectly in the activation of B cell responses. DCs were shown to impact the differentiation of B cells to Ab producing cells [11-14]. Ab responses can be initiated by DCs owing to their ability to present unprocessed antigen to B cells [15-18]. This can be achieved by a process where whole antigens can be stored in non-degrading intracellular compartments to be recycled to the cell surface [16, 18]. DCs can also transport and present unprocessed antigen using dedicated receptors such as DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DCSIGN). In addition, DCs were shown to potently modulate B cell responses by their production of soluble factors such as IL-12, IL-6, type I IFN, B cell-activating factor (BAFF), also called BLyS, and a proliferation-inducing ligand (APRIL) [11, 12, 19]. By these various mechanisms, DCs play several central roles in the orchestration and initiation of immune responses and therefore efficiently link innate and adaptive immunity [10]. This ability of DCs, to induce and shape adaptive immune responses, renders DCs important to understand

from a vaccine perspective. The studies included in this thesis address the role of distinct human DC subtypes to modulate B cell responses in context of TLR ligands that are potential components of vaccine adjuvants.

1.2.3.1 Dendritic cell subtypes

In the human immune system, two distinct subtypes of DCs are described. Myeloid DCs (MDCs, also called conventional or classical DCs (cDCs)) and plasmacytoid DCs (PDCs), characterized by their expression of CD11c and CD123 respectively. In the MDC population there is additional heterogeneity including a recently defined DNGR1+ BDCA3+ population [20]. MDCs and PDCs originate from different precursor cells, myeloid and lymphoid precursors respectively, and differ in some important histological and functional aspects. MDCs are the more frequent DC subset overall and are distributed throughout the body, being most prevalent in the skin (in the form of epidermal Langerhans cells and dermal DC subsets) and mucosal tissues. MDCs exhibit a high ability to produce IL-12 and are superior at presenting antigen and activating CD4+ T cells [10] compared to other APCs or pDCs. In contrast, PDCs are more sparsely distributed and are only found in blood, LNs, and thymus under normal conditions but are recruited to sites of inflammation or tumors in pathological conditions. PDCs are more common than MDCs in the LNs and spleen where they are located at sites in proximity to the high endothelial venules (HEVs) and T cell-rich areas through which B cells migrate [17, 21]. PDCs possess the unique ability to produce high levels of IFNα [22] in response to foreign antigen structures, particularly viruses, and may therefore play an especially important role in anti-viral immunity as reviewed in [23]. PDCs and MDCs have different TLR expression patterns and therefore respond accordingly to TLR-ligands. Of the nucleotide-sensing TLRs, TLR7 and TLR9 are expressed in PDCs, whereas TLR3 and TLR8 are expressed in MDCs [24-27] and reviewed in [28] (Table 1).

	TLR1	TLR2	**TLR3**	TLR4	TLR5	TLR6	**TLR7**	**TLR8**	**TLR9**
MDC	+	+	**+**		+	+	**+/-**	**+**	
PDC						+	**+**		**+**

Table 1: TLR expression in human DC subsets (nucleotide binding TLRs in bold)

In summary, DCs play a central role in the initiation of immune responses by their potent ability to process and present antigen and induce adaptive responses by potent cell surface co-stimulatory pathways and secretion of multiple cytokines upon activation, which shapes both innate and adaptive

immunity. By taking up or binding antigen and presenting it to T and B cells in the secondary lymphoid organs (SLOs), DCs initiate adaptive immune responses and are therefore highly interesting target cells in the development of vaccines and immunomodulatory treatments.

1.2.4 Type I IFNs

IFNs were first described more than 50 years ago as antiviral substances secreted by infected cells [29]. IFNα and IFNβ are the best characterized and most broadly expressed type I IFNs with 13 functional IFNα genes and 1 IFNβ gene found in humans. They are multifunctional innate cytokines that are produced rapidly by most cell types upon microbial infection and induce a variety of genes that can inhibit virus replication at several stages. In most pathogenic virus infections, early production of type I IFN is required to limit initial viral replication before the adaptive immune mechanisms are able to intervene [30]. Type I IFN signaling is required to control several virus infections [31, 32]. Not surprisingly, many viruses have evolved molecular mechanisms to either prevent type I IFN induction or block the signaling thorough its receptor as reviewed in [33, 34].

1.2.4.1 Induction of type I IFN

Type I IFN can be induced in TLR dependent as well as TLR independent ways. The specific TLR dependent inducers include dsRNA for TLR3, LPS for TLR4, ssRNA for TLRs 7 and 8, and unmethylated CpG-DNA for TLR9. Signaling via these TLRs leads to activation of the transcription factors AP-1, NF-κB, IRF3 and IRF7, which are all required for IFNα/β production [9]. Although signaling via these TLRs can lead to IFNα/β production in different cell types, the production of high levels of type I IFN can only be achieved by PDCs that express TLR7 and TLR9 but not TLR3, TLR4 and TLR8 [24, 26] (Table 1). PDCs, originally defined as natural IFNα producing cells [35], are the most potent producers of IFNα upon TLR stimulation. They are the only cells that constitutively express IRF7, which is required for the production of all IFNα subtypes. In most other cell types, IRF7 expression needs to be induced via IFNAR signaling. This IFNAR-mediated positive feedback loop is also operative in PDCs and may also allow IFNα secretion in the absence of viral replication [36]. However, the high IRF7 expression alone does not explain the unique ability of PDCs to produce large amounts of IFNα. The interaction of IRF7 and the TLR signaling molecule MyD88, was shown to be central for the

IFNα production by PDCs [37]. IRF7 is also required in TLR-independent production of the late IFNα subtypes [38], which is mediated by actively replicating RNA viruses and synthetic RNA that bind to the cytosolic RNA helicases, RIG-I and MDA5 [39]. Binding of RIG-I and MDA5 initiates signaling cascades that lead to IRF3, IRF7, NF-κB and AP-1 activation and IFNα/β expression. While IRF7 activation is required for the production of late IFNα subtypes, this can be achieved by a positive feedback loop mediated by the early type I IFN subtypes, IFNα4 and IFNβ, that are induced rapidly upon infection [40]. A novel TLR-independent mechanism has shown to induce type I IFN involving the NOD1 and NOD2 receptors, which are also cytosolic PRRs, possessing the ability to recognize bacterial cell wall components as described in section (1.2.1) of this thesis. It was shown that type I IFN can be induced by *Mycobacterium tuberculosis* through the activation of NOD2 in macrophages [41] and by *Helicobacter pylori* through NOD1 in epithelial cells [42].

1.2.4.2 Type I IFN signaling

After the induction of type I IFN secretion, type I IFNs execute their function via receptor signaling on various cell types. All type I IFNs signal through the same heterodimeric receptor, IFNAR, which is ubiquitously expressed on nucleated cells. It consists of two chains, IFNAR1 and IFNAR2 that are associated with the Janus (Jak) tyrosine kinases Tyk2 and Jak1. Upon receptor binding, Jak-Stat signal transduction is initiated leading to phosphorylation and activation of Stats 1 and 2 by the Jaks. These in turn form the transcription factor complexes ISGF3 and Stat1 homodimer, which leads to modulation in hundreds of genes, as shown by recent microarray data [43]. One example related to the work presented in this thesis is the upregulation of MyD88 that was shown in B cells upon IFNα exposure [44]. Elevated MyD88 expression levels may have consequences such as the amplification of TLR-mediated responses that was shown in presence of IFNα [44-46] (and paper I of this thesis). In addition, although all type I IFNs signal through the same receptor, there is evidence for differential receptor binding and signaling of IFNα and IFNβ [47].

1.2.4.3 Type I IFN modulation of DC function

Type I IFNs are of great interest for their various immunomodulatory properties, in addition to their important antiviral functions in early immune responses. Multiple reports have addressed the role of type I IFNs in the

orchestration of both innate and adaptive immunity [48-50]. IFNs were shown to have direct and indirect effects on immune cells such as NK cells, T cells, B cells, DCs and other phagocytic cells [51]. When DCs are exposed to type I IFN, they get activated, mature and exhibit increased expression of co-stimulatory molecules [52, 53], which improves their capacity to interact with other cells. Importantly, type I IFN was shown to enhance the T cell stimulatory functions of DCs including induction of their ability for cross-presentation of antigen from extracellular sources via the MHC I pathway to CD8+ T cells [54]. Type I IFN stimulated DCs also exhibit stronger migratory capacity [53]. In addition, it was shown that type I IFNs acts on DCs to become better stimulators of Ab production [55]. In these various ways, type I IFNs drive DCs to display their central function in the initiation and orchestration of immune responses. The effects of type I IFN on adaptive immunity are discussed later in more detail.

1.3 ADAPTIVE IMMUNITY

As mentioned earlier, adaptive immunity is induced after the immediate innate response. It is divided into a cellular and a humoral part that include T cell and B cell responses. Adaptive immune responses are typically highly specific and involve the receptors on T cells and B cells for antigen recognition. In contrast to innate immunity that uses broadly specific germline-encoded recognition, the highly antigen-specific receptors of the adaptive immune system are encoded by genes that are assembled by somatic rearrangement of germline gene elements, which permits the formation of millions of different antigen receptors with unique specificities for different antigens [1]. To ensure that no self-reactive responses are mounted, autoreactive B and T cells are removed during development and later at several developmental and peripheral checkpoints. A typical feature of adaptive immunity is the development of highly specific immune responses and the formation of memory that enables the body to mount specific immune responses upon reencounter with a pathogen, which is crucial for the protective effect of vaccines. For the development of new vaccines, increasing the knowledge of the factors that impact the initiation of adaptive immunity as well as the development and maintenance of memory is central.

To mount adaptive immune responses, the coordinated activation of B cells and T cells takes place in highly organized structures called SLOs. The micro-architecture of SLOs such as the LN and spleen provides the optimal

environment for the initiation of immune responses by compartmentalization of numerous cellular interactions [21]. SLOs continually sample and concentrate antigens that are circulating throughout the body. Antigens can be transported to the SLOs directly by the lymph or the blood or indirectly being transported by cells. Antigens from the local tissue segregate or get transported via the lymph to the LN and antigens from the blood get trapped in the spleen. The structure of a typical SLO can roughly be divided in an outer antigen-sampling zone, the B cell- activation zone (B cell follicle) and the T cell- activation zone [21]. In the antigen-sampling zone, e.g. the marginal zone (MZ) in the spleen and the subcapsular sinus (SCS) in the lymph node, resident macrophages that filter pathogens and antigen-loaded DCs in transition can be found. The T cell zones are densely packed with CD4+ and CD8+ T cells and DCs. In the LN and spleen, these cells are embedded in a scaffold of stromal cells that reside on collagen fibres representing conduits of the T cell zone with important transport function [21]. Naïve migratory B cells are found in the follicular zones that surround the T cell zones. Upon activation with antigen, B cells and T cells approach the border of the two zones where subsequent specific cell:cell interactions take place. B cells can then either enter extrafollicular pathways or enter germinal center (GC) reactions, where additional cell:cell interactions play important roles, which is explained in more detail below in section 1.3.2. Several specific cell:cell interactions that are crucial for the development of the adaptive immune response are dependent on the optimal microenvironment that the highly organized SLOs provide. Recent elegant studies using intravital imaging to address cell interactions in the SLOs have given many new insights [17, 21, 56].

1.3.1 T cell response

The thymus is the primary lymphoid organ that is associated with the development of T cells that represent the cellular part of the adaptive immune system. Cellular responses include the development of the two major T cell subsets, the CD4+ and CD8+ T cells. T cells generally recognize processed protein antigen that is presented on APCs on MHC molecules [1]. The successful activation of T cells is restricted by the requirement for combined recognition of a self molecule and a foreign antigenic structure [57]. Cytotoxic CD8+ T lymphocytes play an important role in killing infected cells and are thereby crucial to limit the production and spread of infectious microbes. CD8+ T cells recognize infected cells through presentation of intracellular protein antigen fragments by MHC class I. CD8+ T cells, upon activation,

secrete substances that potentially and specifically cause cell death. CD4+ T cells, also called T helper (Th) cells, play an important role in the regulation of cellular and humoral immune responses and are crucial for the activation of both CD8+ T cells and B cells. CD4+ T cells recognize processed antigen presented by MHC class II, which is constitutively expressed on APCs including B cells, DCs, monocytes and macrophages. MHC class II recognition by CD4+ T cells leads to the formation of a synapse between the two cells [58], supporting efficient cell to cell contact that involves additional interactions between co-stimulatory molecules as reviewed in [59]. This specific CD4+ T cell activation by antigen peptides presented on MHC class II to CD4+ T cells can be circumvented by activation via bacterial superantigens that link the α-chain of class II MHC molecules to the T cell receptor (TCR) [60, 61]. Upon activation, CD4+ T cells secrete a number of cytokines that potently shape the adaptive immune response [62]. Depending on the nature of cytokines present at the site of activation, CD4+ T cells differentiate into T helper (Th) cells of Th1, Th2 or Th17 types or regulatory T cells (Tregs) that in turn elicit characteristic cytokine profiles. Th1 and Th2 responses often occur combined in immune responses, although the response can become more dominant for either Th1 or Th2. Th1 cells generally support cellular responses and are therefore strived for in some vaccination formulations, while Th2 cells support humoral and allergic responses [1].

1.3.2 B cell response

Humoral immunity is characterized by the production of Abs, also called immunoglobulins (Ig), which bind with high affinity to specific areas on antigens/pathogens, called epitopes. The presence of Abs on the surface of an antigen can designate it for destruction (called opsonization) and/or neutralize an antigen and make it unable to for example infect or interact with cells. Opsonized antigen can be recognized, taken up and destroyed by phagocytes. The production of antigen specific Abs requires the proper activation and differentiation of specific B cell subsets to develop into Ab secreting cells. This includes a sequence of events that are summarized in this section below.

1.3.2.1 B cell development

Before B cells can enter the periphery and differentiate into mature naïve B cells, B cells undergo very important developmental stages that take place in the bone marrow. The main goal of B cell development is the production of

functional B cells with highly diverse specificities. The specificity of the antigen recognition by a given B cell is defined by the B cell receptor (BCR), which is expressed on the surface of the B cells and constitutes the membrane bound form of Ig. BCR recognition is an initial step in the initiation of a humoral immune response. A central part of the B cell development is therefore the assembly of the BCR by sequential heavy and light chain gene rearrangements. The variable parts of the Ig molecules are assembled by rearrangement of the variable (V), diversity (D) and junctional (J) gene segments, a process called V(D)J recombination (only VJ recombination for the light chain). V(D)J recombination is initiated by the recombinase-activating gene (RAG) 1 and RAG2 proteins, which cleave DNA near the V, D and J segments. These segments are then rejoined by a collection of DNA repair enzymes. Bone marrow stromal cells with their production of IL-7 play an important role in these processes [1]. During the development in the bone marrow, a large BCR diversity is created and, by exclusion of self-reactive B cells, cells carrying functional, non-self directed BCRs are selected. Immature naïve B cells subsequentially exit the bone marrow and enter the periphery where they have to pass two transitional developmental stages and become so called T1 and T2 B cells, before they differentiate into mature naïve B cells of different types. The majority of the mature naïve B cells circulate between the blood and the SLOs and are called follicular B cells. Two other subtypes, MZ B cells and B1 B cells are resident and often referred to as innate-like B cells that mediate rapid Ab responses [63].

1.3.2.2 B cell activation

Mature naïve B cells circulate the periphery via the blood stream and enter SLOs where they encounter specific antigens. Antigen encounter of B cells is highly facilitated within specialized areas of SLOs, such as the follicular B cell zones,. Antigen encounter involves engagement of the BCR by the antigen. The antigen can be either soluble or membrane-associated of an adjacent cell as reviewed in [15, 64, 65]. Upon antigen recognition, B cell activation is initiated leading to a chain of signaling events. The antigen is engulfed by the B cell, digested into peptides and presented on MHC class II. Depending on the nature of the antigen, the activated B cell proceeds in a T cell dependent (TD) or a T cell independent (TI) activation pathway. The studies included in this thesis address B cell responses in TD (paper II) as well as in TI (papers I and III) activation settings.

1.3.2.3 T cell independent B cell activation

TI pathways can be a consequence of activation by polymeric antigens with repeating structures that may induce strong BCR signals due to crosslinking and clustering of the BCR molecules [1]. TI pathways can be induced either by TI type 1 (TI-1) or TI type 2 (TI-2) antigens. TI-1 antigens, such as lipopolysaccharide (LPS) or flagellin at high concentrations, induce polyclonal B cell activation. TI-2 antigens consist of molecules with highly repetitive structures such as carbohydrate polymers that are present on bacterial cell membranes. TI-2 antigens are recognized by specific BCRs, especially in B-cell subsets such as MZ B cells, and activate B cells by extensive crosslinking of multiple BCRs, generating very strong BCR signaling. TI antigens also include highly organized viral capsid proteins that can be found on vesicular stomatitis virus [66, 67] and poliovirus [68]. TI pathways generally elicit no or poor memory responses and do not result in somatic hypermutation of Ab genes.

1.3.2.4 T cell dependent B cell activation

The TD pathway, which is generally associated with memory formation, includes cognate interactions between activated CD4+ T helper cells and B cells [69]. This interaction takes place in the outer T cell zone in the SLO and induces further proliferation and differentiation of the B cells. These important B cell:T cell interactions involve binding of the processed antigen, presented by the B cell on MHC class II to the cognate T cell receptor. In addition, interactions of co-stimulatory molecules take place that contribute to the subsequent activation of both B and T cells. Multiple co-stimulatory molecules have been described to be involved in B cell:T cell interactions. CD40:CD40L (CD154), CD28:CD80/CD86, CD70:CD27 and ICOS:ICOSL are best characterized as reviewed in [70-72]. CD40 signaling in B cells is essential for induction of Ig isotype switching and is associated with the formation of memory.

1.3.2.5 B cell activation signals

There are a number of other factors that can have an impact on B cell activation including soluble substances such as cytokines or microbially derived PAMPs e.g. nucleic acids that mediate signals via PRRs including TLRs [73]. This illustrates the complexity and flexibility of B cell activation settings. In vitro it was shown that in addition to engagement of the BCR (signal 1) and CD40 (signal 2) on the B cells, a third signal in the form of a TLR-

ligand was required for sufficient naïve B cell activation [74]. In vitro studies have also shown that signals of B cell activation can be replaced and that DCs are strong contributors in T cell dependent (which was the focus of paper II) as well as T cell independent systems [44, 46, 75, 76] (addressed in paper I and III). Soluble factors such as cytokines have been shown to be important in DC mediated effects and may therefore be called B cell activation signal 4 (Fig 2). The impact of DCs in particular on B cell activation will be addressed more in detail later in this section.

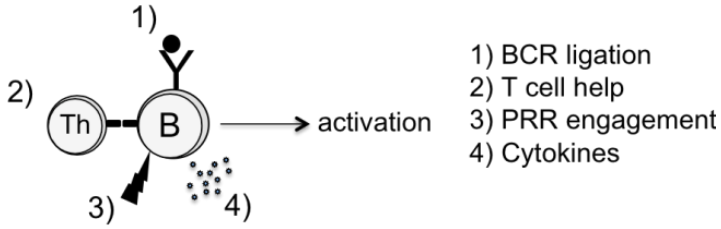

Figure 2: B cell activation signals

Another point concerning B cell activation is the synergistic engagement of the BCR and a TLR that can take place in response to immune complexes and seems to play an important role in some autoimmune scenarios [77]. In line with this, chemically linked anti-Ig and CpG-ODN that simultaneously stimulated BCR and TLR were very efficient [8, 78]. Studies addressing mechanisms involved in different B cell activation scenarios are therefore very relevant for the understanding of autoimmune diseases and responses to infection and also for the development of new vaccines.

1.3.2.6 B cell differentiation

Upon antigen encounter, activated naïve B cells can move to the B:T cell border where cognate interactions with T helper cells take place (as described in more detail above in section 1.3.2.3). Further differentiation processes can then either take place in GCs, in the follicles of SLOs or alternatively outside of the follicles (Figure 3). Follicular GC reactions include sophisticated processes that culminate in the formation of long-lived plasma and memory B cells possessing high affinity for the antigen. In contrast, extrafollicular pathways of B cell differentiation elicit rapid short-lived Ab responses with broader specificities, mediated by Ab producing cells with half-lives of 3-5 days in vivo, and are thereby contributing to an early protective immune response as reviewed in [71, 79-81]. The decision about which pathway to engage may involve the affinity of the BCR to antigen, with high affinity B cells preferentially

entering the extra follicular pathway and low affinity B cells enter the GC to undergo rounds of affinity maturation and selection [82]. TI antigen stimulation generally leads to extra follicular responses. The GC reactions include B cell expansion, class switch recombination (Isotype switching) of the BCR and several rounds of affinity maturation. In a process called somatic hypermutation, mutations are introduced in the antigen-binding portions of the heavy and light chains of the BCR [81], creating enhanced diversity. High-affinity antigen specific B cells are then selected by a process involving follicular DCs (FDCs) that present captured antigen. Using complement and Fc receptors, these FDCs (which is a non bone-marrow but stromal cell derived DC type) display whole Ab-antigen complexes on their surface, that are recognized and competed for by the antigen-specific follicular B cells [64]. The high affinity B cells enter a proliferation phase upon interaction with CD4+ helper T cells. The CD4+ helper T cells present in the GC are called follicular helper (Tfh) cells and they enter the GC after interaction with DCs and subsequently cognate B cells at the T-B border as reviewed in [83, 84]. Tfh cells produce IL-21, which is important for their own differentiation, acting in a positive feed-back loop [85]. IL-21 was also shown to support B cell differentiation and Ab production as reviewed in [86]. Using mouse models with mixed chimeras that were devoid of the IL-21 receptor, IL-21 has recently been shown to act directly on GC B cells [87, 88]. Tfh cells can promote survival signals in B cells and important cell:cell interactions between GC B cells and Tfh cells, including CD40:CD40L contact, can mediate B cell proliferation. After interaction with Tfh cells, the selected high affinity GC B cells finally develop either into long-lived plasma cells that secrete high-affinity Abs, or into memory B cells that confer long-lasting protection from secondary antigen challenge [15, 71, 81] (Figure 3). The outcome of these processes is largely influenced by the environment that supports the activation of naïve B cells. Understanding how naïve B cells are regulated by central cell and cytokine milieus during the onset of an immune response is therefore highly relevant.

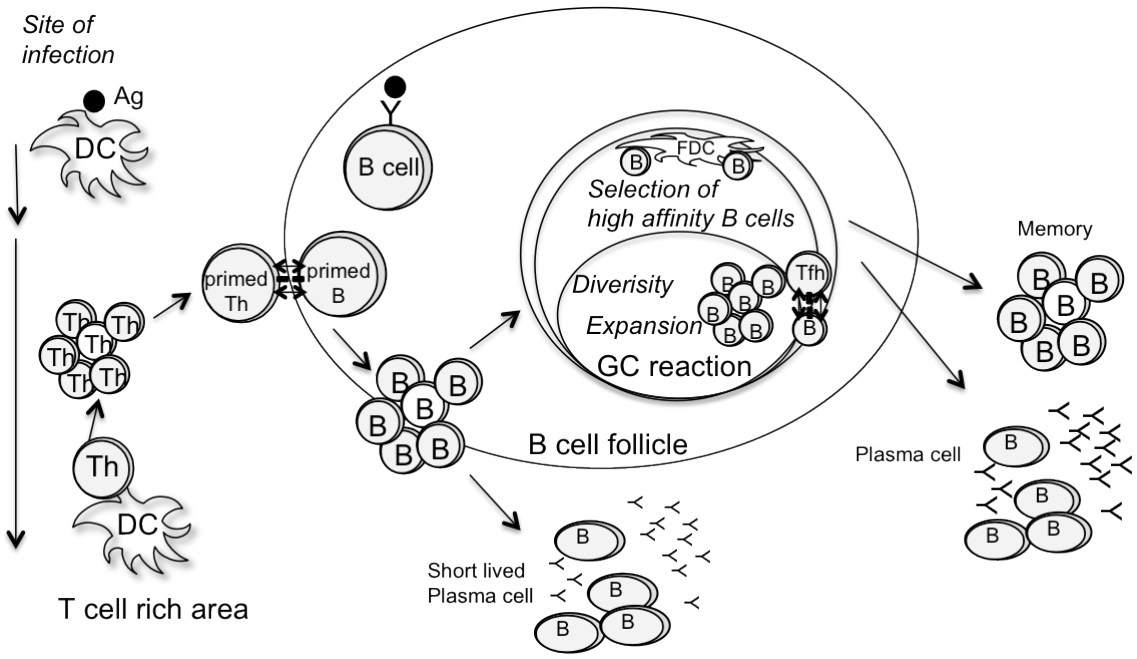

Figure 3: B cell activation and differentiation (adapted from [81])

1.3.4 The role of DCs in regulating B cell responses

By mediating the activation of T helper cells, DCs have a major indirect impact on the outcome of B cell responses. DCs also mediate the stimulation of Tfh cells, specialized to support B cells in the GC reactions as discussed above [89]. However, various studies have also reported direct DC derived signals to B cells. Bone-marrow derived DCs have been shown to enhance B cell responses in different ways. In addition to the capability of DCs to present processed antigen and thereby activate T cells, DCs also have the ability to present unprocessed antigens on their cell surface and can thereby contribute to prime B cells [16, 18]. This process has recently been investigated through elegant studies using live imaging techniques of intact LN [16, 90]. B:DC encounter is believed to take place shortly after B cell entry via HEVs and before migration into the follicle [17]. DCs that migrate from peripheral organs take up residence in the paracortex, which is close to the HEVs, after crossing the SCS of LN [17, 21]. Upon TLR stimulation, PDCs were shown to provide activation signals to B cells via cell-to-cell contact involving ICAM-1-LFA-1 interactions [91] and binding of CD70 to CD27 on the B cells [92].

Previous studies that investigated the impact of DC-derived factors on B cell activation, have shown that various DC subsets produce soluble factors, such as IL-6, IL-10, IL-12, and BAFF, which support the development of B cell responses [11, 19, 46, 91]. In vitro derived DCs, generated by culturing human

CD34+ cord blood cells, were reported to significantly support B cell responses. B cell proliferation in response to CD40 stimulation, using CD40L expressing cells, was further enhanced in the presence of in vitro derived DCs. Similarly, DC-coculture also enhanced IgM production of naïve B cells in the presence of IL-2 and upon CD40 stimulation [12]. IL-12 was shown to be an important mediator in enhancing B cell responses [11]. In the same system, DCs were shown to shape the B cell responses by skewing Ab production towards IgA [13]. In the presence of IL-2, in vitro derived DCs were also shown to enhance early T cell-dependent B cell differentiation, which was followed by a T-independent formation of plasma cells, in the presence of IL-2 and IL-10 [14]. Moreover, B cell differentiation can be enhanced through DC-derived soluble IL-6R α-chain that potentiates the biological activity of IL6 [11]. Two other DC produced factors found to have powerful effects on B cell activation and survival, are BAFF and APRIL [93]. BAFF and APRIL can act on B cells to induce CD40-independent Ig class switching [19]. An upregulated expression of BAFF and APRIL on MDCs, which was induced by PDC produced type 1 IFN, promoted Ig class switching. IFN-producing PDCs where also shown to modulate class switching by activating B cells through both T cell-dependent and T cell-independent pathways [94].

PDCs have also been shown to directly regulate B cell responses by the secretion of type I IFNs and IL-6 [44, 46, 76, 91] (and in paper I and II of this thesis). As described in paper I of this thesis and consistent with findings by other groups [44, 46], the presence of PDCs strongly enhances T cell-independent, TLR7/8-stimulated B cell responses in vitro. The production of IFNα by PDCs was in large part responsible for this effect. The observed enhancement of B cell responses to TLR7/8 stimulation may in part result from IFNα-induced upregulation of the expression of TLR7 and the adaptor molecule MyD88, rendering the cells more responsive to TLR7/8-ligand stimulation [44, 95]. Type I IFNs are highly pleiotropic cytokines with potent effects on various cell types and are discussed in the next section below.

1.3.4.1 Impact of type I IFNs on B cell responses

The importance of type I IFNs in the initiation and support of adaptive immunity was demonstrated by experiments with IFNAR deficient mice that show various degrees of deficiency for the induction of antigen-specific T cells [96, 97] and the appearance of Abs [98]. IFNα was shown in multiple studies to promote Th1 related responses [99, 100] including delayed-type

hypersensitivity [101] and IgG2a Ab production [55], when co-administrated with antigen. During specific conditions, type I IFNs were also shown to favor Th1 cytokine production by inhibiting the secretion of the Th2 cytokine IL-4 and by promoting the production of IFNγ [102]. Although type I IFNs enhance T cell and B cell responses by a variety of mechanisms including the activation of DCs and NK cells [100, 103, 104], there is also a direct effect on T cells and B cells. IFNα increased the antigen-specific Ab responses to soluble protein antigen, which required direct stimulation of both of T and B cells by IFNα [98]. This was shown using two different types of mixed chimeras, where either B cells or T cells were devoid of the IFNAR [98]. The direct type I IFN effect on T cells, particularly on CD8+ T cells, leads to extended survival during clonal expansion [97, 104, 105]. Type I IFNs also directly support survival of activated CD4+ T cells [96, 106], which was shown to be critical for increased proliferation in response to a viral but not a bacterial infection [96]. This increased survival by type I IFN, which also accounts for B cells [107-109], may contribute to the enhanced cellular and humoral responses [109].

Experiments in mouse models revealed that direct type I IFN-mediated B cell activation significantly augmented the quality and magnitude of the antiviral humoral responses [98, 110-112]. However, it was also reported that adaptive immune responses to virus-encoded antigens do not require type I IFNs [112]. Various viruses, for example sendai virus, were shown to induce potent adaptive immune responses in IFNAR-/- mice [113, 114]. In addition, immune responses against Vaccinia Virus, Theilers virus, intracellular bacteria and parasites were not type I IFN dependent [115]. In conclusion, type I IFN signaling seems to be redundant for the induction of protective responses to certain pathogens. A recent study in mice reported that type I IFN directly acted on follicular B cells by promoting their contribution to TI-2 Ab responses that are generally extrafollicular and MZ B cell associated (as described in section 1.3.2.3) [116]. In influenza virus infected mice, it was shown that type I IFNs fine-tune Ab responses by suppressing IgG1 switching and enhancing IgG2a/c switching [99]. Class switch recombination to IgG was implicated to involve the induction of the transcription factor T-bet by type I IFN [117], which may contribute to the skewing of the IgG distribution. However, the exact role of type I IFN as studied in mice, in the regulation of humoral immune responses in direct or indirect ways, remains somewhat controversial and may likely depend on the type of infection or vaccination strategy [32, 110, 118].

In early in vitro studies on human cells, type I IFN was shown to increase mitogen induced B cell activation [119, 120]. Several in vitro studies, including papers I and III of this thesis, have shown that IFNα also has the capacity to enhance B cell responses to TLR-ligands [44, 46]. In these studies, IFNα produced by PDCs was shown to dramatically modulate B cells responses to TLR7/8-ligation. As mentioned in section 1.2.4.2, IFNα was also shown to upregulate the expression of TLR7 and MyD88 [44], which at least partly may account for this effect. In line with this, impacts on the signaling via MyD88 were also reported to be involved in the IFNα mediated enhancement of naïve B cell activation in response to CpG B [45]. In paper II of this thesis we have shown that IFNα also increases the capability of B cells to respond to and interact with CD4+ T cells. In addition, type I IFNs have been shown to partially activate B cells, making them more sensitive to weak signals through the BCR [108]. It is likely by such a mechanism that IFNα can lower the activation threshold of autoreactive B cells to weak endogenous ligands [121].

In contrast to this, anti-proliferative effects of IFNα were shown in early studies, and are used in anti-tumor clinical applications, as reviewed in [122]. Type I IFNs, in particular IFNα, are also used for treatment of chronic hepatitis B [123]. Since type I IFNs are very powerful in modulating immune responses, the maintenance and function of regulatory mechanisms to keep its production under control are crucial. Aberrant IFNα production is dangerous and has been shown to be one major mediator for enhanced auto-Ab production in auto-immune diseases such as systemic lupus erythematosus (SLE) [124, 125].

These multiple scenarios illustrate the important role of IFNα in human immunology. Studying how type I IFN acts and shapes immune responses and directly and indirectly modulates immune cells, will hopefully add to a better understanding of the human immune system and contribute to the development of better vaccines and immunomodulatory treatments.

1.4 INTRODUCTION TO VACCINE ADJUVANTS

Vaccines elicit protection through induction of Abs, T cells or both. Most current vaccines confer protection by humoral immunity. However, there is a tremendous need to improve vaccines for T cell immunity and as well to improve upon the function and durability of humoral immunity. The golden

standard for inducing both Ab and T cell immunity is set by live attenuated vaccines. Indeed, several vaccines under development today target pathogens such as HIV for which live attenuated vaccines are considered unsafe. Therefore, amongst the types of vaccine formulations, protein subunit vaccines are explored, although they are usually poorly immunogenic. To increase the potential of such vaccines to induce protective immunity, efficient and safe vaccine adjuvants are required. Adjuvants are used to increase the magnitude, breadth and kinetics of a response to a vaccine so that it is most effective for protecting against a specific pathogen. Since prophylactic vaccines are often given to children in the first years of age, adjuvants need to be extremely safe and well tolerated. Thus there are only a few adjuvants approved for prophylactic vaccines in humans. The most commonly used adjuvant in human vaccines is the aluminum salt (alum), which has been in use for over 70 years. Alum is known to increase antigen uptake and stability and induce a local pro-inflammatory reaction that can increase immunogenicity [126]. Other adjuvants approved in Europe are oil in water emulsions such as MF59 (manufactured by Novartis) and AS03 (manufactured by GlaxoSmithKline) that are licensed adjuvants for influenza vaccines. AS04 is an adjuvant that is composed of monophosphoryl lipid A (MPL) adsorbed to alum. AS04 is approved for hepatitis B virus (HBV) and human papiloma virus (HPV) vaccines in Europe and was recently licenced in the USA [126]. Most adjuvants that are used today have been developed empirically and their mechanisms of action are only partly revealed. Generally, adjuvants are recognized by components of the innate immune system such as PRRs, which leads to the induction of proinflammatory cytokines and the initiation of an immune response as reviewed in [127, 128]. The mechanism of action of alum was only recently investigated and both the release of uric acid [129] and the formation of the Nalp3 inflammasome [130] were shown to be involved in mediating its adjuvant effect. Several adjuvants have also been shown to include signaling via TLRs [131] and reviewed in [132, 133], however, there is also TLR independent adjuvant activity reported [134]. One problem in the development of new vaccines is that the currently approved vaccine adjuvants do not always elicit the desired immune response against different target pathogens [126]. Alum for example predominantly induces humoral immunity while also cellular protection is likely required for protection against a number of pathogens, especially HIV, *Mycobacterium tuberculosis* and *Plasmodium falciparum*. Therefore, the identification and development of new adjuvants that are safe and well tolerated is necessary.

1.4.1 TLR-ligands as vaccine adjuvants

TLR-ligands represent one class of adjuvant components that have attracted a lot of interest over the last years [133, 135, 136]. Selected TLR ligands are under evaluation for their adjuvant effect in several human trials [137-140] with promising results. As mentioned in section 1.2.2, TLR-ligands mimic structures derived from pathogens that can specifically target and activate important APCs such as DCs and B cells by binding to their respective TLRs. TLR-stimulated DC subsets efficiently induce antigen-specific T cell responses [141-143]. In addition, recent studies, including the papers I and II of this thesis, show that TLR-stimulated PDCs significantly enhance B cell activation [44, 46]. The synthetic TLR-ligands investigated in this thesis bind to TLR3, TLR7/8 and TLR9 that are all expressed in endosomal compartments and naturally recognize nucleic acids. They will be briefly presented here:

1.4.1.1 CpG ODN classes

CpG-containing oligodeoxy-nucleotides (CpG ODNs) are a class of TLR-ligands that have been discovered only a decade ago. CpG motifs on bacterial DNA were found to bind to TLR9 [131] and CpG ODNs have then been shown to potently activate B cell responses [144, 145]. There are three main CpG classes that are described; CpG ODN class A, B and C [146, 147]. Although they all bind to TLR9, they differ substantially in structure and function. CpG A requires a palindromic CpG phosphodiester sequence with phosphorothioate G-rich ends and induces high amounts of type I IFN from PDC [148]. In PDCs, CpG A is stably retained in the early endosomes along with TLR9 and the signaling molecules MyD88 and IRF7 [37]. This mechanism is required for type I IFN production by PDCs and important to control antiviral innate immune responses. CpG B contains a phosphorothioate backbone and is known to be very potent in stimulating B cells. CpG B is rapidly transferred to late endosomes or lysosomes, which results in less activation of IRF7. The third class, CpG C, has a phosphorothioate backbone with a stimulatory CpG motif plus a palindromic sequence at the 5’ or 3’ ends. CpG C combines the effects of CpG A and CpG B [147], which makes it a very attractive adjuvant component. There are several applications of the CpG classes. Numerous studies evaluate the effects of CpG ODN as adjuvant compounds in different systems including studies in mice and NHP [143], and several clinical trials have been performed [137-140, 149]. In humans, TLR9 is only expressed on B cells and PDCs. Rodents, in contrast, express TLR9 on more cell types, most

notably mDCs, which likely has considerable impact on the potent TLR9-ligand induced effects on T cell immunity. This major difference between expression of TLR 9 in DC subsets of mice and humans may alter the predictive value of the use of rodent models for the evaluation of CpG adjuvants for use in humans related to T cell immunity. CpG B has also been used successfully for practical applications that rely on in vitro expansion of B cells [150, 151].

1.4.1.2 TLR7/8-ligands

TLR7/8-ligands mimic viral ssRNA and they are another class of adjuvant components that engage intracellular TLRs. The synthetic ligands that are used are small molecules called imidazoquinoline compounds that bind to TLR7 (e.g. imiquimod or R837) or TLR7/8 (e.g. resiquimod or R848). Imiquimod is licensed for use in the form of the cream aldara that is applied for anti-viral and anti-tumor treatments. TLR7/8-ligands are evaluated as adjuvant candidates in various studies [142, 152]. Importantly, as small molecules they dissipate quickly from the site of immunization and thus need to be formulated either in emulsion or by direct conjugation to an antigen to optimize their effect on adaptive immunity [142, 152]. TLR7/8-ligands are recognized by B cells, PDCs and MDCs.

1.4.1.3 Poly I:C

The synthetic double-stranded RNA, polyriboinosinic-polyribocytoidylic acid (Poly I:C), resembles the RNA produced during replication by some viruses. Poly I:C is recognized by TLR3 and also by the cytoplasmic RNA helicase MDA5. Binding to MDA5 results in activation of IRF-3 and represents the major pathway for TLR-independent type I IFN production [153]. Poly I:C was long known to be a potent inducer of type I IFN in mice and is under investigation as an adjuvant. It has lately attracted renewed interest as it was shown to be superior to other TLR-ligands in two recent studies [100, 154]. Especially in primate studies, Poly I:C analogues are used, which are stabilized against the serum nucleases that are present in the plasma of primates.

1.4.2 The role of DCs in mediating adjuvant activity

One main action of adjuvants is the activation of innate immunity, especially the maturation of antigen-presenting immunostimulatory DCs [92, 155, 156]. TLR-ligands efficiently stimulate DC subsets that in turn augment antigen-specific T cell responses [141-143]. TLR signaling on DCs promotes antigen

presentation on MHC I and MHC II, enhanced migration of DCs to LNs and cytokine production [157]. This is demonstrated in the highly successful yellow fever vaccine, which activates multiple TLRs to stimulate pro-inflammatory cytokines [158]. In a mouse model with a DC specific deletion of TLR signaling it was shown that MyD88-dependent signaling in DCs plays an important role in the induction of immune responses including innate cytokine production and Th1 polarization of antigen-specific CD4+ T cells [159]. Although certain TLR-ligands can also directly activate B cells, recognition of CpG by DCs was more important for Ab production than direct effects of CpG on B cells [159]. In fact, most adjuvants seem to enhance T cell and B cell responses not by direct activation but rather by engaging components of the innate immune system as reviewed in [127, 128, 132]. As described above, the ligation of PRRs including TLRs, is strongly associated with DC maturation and the subsequent induction of immune responses. However, it has been shown that this is not sufficient by itself. Instead, direct IFNAR signaling on the DCs was required for the development of their proper immunostimulatory function [100]. The importance of IFN signaling also becomes evident in a number of studies that report that direct type I IFN signaling on DCs was most important for the effective stimulation of protective responses [51, 100, 160].

1.4.3 The role of IFNα on adjuvant activity

Type I IFNs have been shown to mediate the effect of established vaccine adjuvants, for example complete Freund's adjuvant (CFA), IC31 and also different CpG ODNs. The effect of these adjuvants, all inducing signaling via TLR9, was defective or absent in mice lacking the IFNAR [51, 55, 118, 161]. Even alum, which does not depend on TLR signaling, showed impaired adjuvant activity in mice that lack the IFN receptor [118]. Also Poly I:C, when used with a protein based vaccine in a mouse model, required type I IFN production for its adjuvant activity [100]. Of note, IFNα production to Poly I:C was actually TLR-independent and mediated to a large extend by non-hematopoietic stromal cells through MDA5. Therefore, although DCs are the central cells that need to be activated for the initiation of antigen-specific adaptive immunity, the further development of adjuvants may need to consider the contributions of both hematopoietic and non-hematopoietic cells for the production of innate cytokines that work directly on DCs so that they can fulfil their functions. As discussed above, the main function of type I IFN in context of vaccine adjuvants is likely the maturation of DCs. However, type I IFNs have effects on several cell types [96, 97, 104-106] as described above in

section 1.2.4.3 and 1.3.4.1 and shown in terms of modulating B cell activation in all papers included in this thesis. Although maybe less crucial for the adjuvant effect itself, these additional effects are likely important to contribute to the optimal shaping of the required immune response or at least to the amplification of the adjuvant effect. Type I IFNs may play different roles in shaping immune responses depending on the type of vaccine antigen, the class of adjuvant that is used and maybe even the route of vaccine administration.

1.4.4 Non-human primates as model to study TLR-ligands

NHP are considered the most relevant in vivo animal model for studying human immunology including responses to infectious diseases and vaccines [162]. Their immune response to infectious agents related to human pathogens, including simian immunodeficiency virus and influenza, has made macaques the preferred model for vaccine development [163]. In contrast to rodents, several species of NHP appear to exhibit similar repertoires and functions of DC and B cell subsets as humans [164, 165]. In addition, NHP DCs and B cells have a similar TLR receptor expression as the corresponding human cells do [166], which is not the case in rodents [167]. NHPs are therefore extremely valuable for studies aimed at investigating immune responses induced by human pathogens and vaccines [162, 168]. Despite the close genetic relationship and similar phenotype of many primary cells, NHPs and humans still have inherent species differences in their immune systems which could limit the clinical utility of NHPs as vaccine models [168]. It is therefore crucial to define species differences and confirm similarities between human and NHP cells. This can be achieved by investigating key observations from studies using human cells in vitro using similar experimental procedures on NHP cells, an approach that we have followed in paper III of this thesis.

2 AIMS OF THIS THESIS

The studies in this thesis have focused on the role of DC subsets in the modulation of B cell responses. The specific aims in the individual studies were:

Paper I: To study B cell responses to selected TLR-ligands and the ability of DC subsets (MDCs and PDCs) to modulate such responses in vitro.

Paper II: To study the relative roles of the DC subsets in supporting B cell responses induced by T cell help/BCR ligation in vitro.

Paper III: To compare the responses of rhesus macaque and human B cells and DC subsets to selected TLR-ligands.

3 MATERIALS AND METHODS

The methods used for this work are described in detail in the "materials and methods" section of each paper. The main methods are described below in brief.

3.1 ISOLATION OF SUBSETS OF DCS, B CELLS AND T CELLS

To retrieve large quantities of rare cell subsets such as PDCs and MDCs and naïve B cells from human blood, peripheral blood mononuclear cells (PBMCs) were obtained from healthy donors by aphaeresis performed at the Department of Transfusion Medicine, Karolinska University Hospital. Fractions of lymphocytes and monocytes were thereafter separated by counterflow centrifugal elutriation. High yields of pure populations of BDCA-4+ CD123+ PDCs and CD1c+ CD11c+ MDCs were isolated from the monocyte population by magnetic bead separation [141, 169, 170], which is highly facilitated by the prior enrichment by the elutriation. At the same time, sufficient quantities of B cells were sorted from the lymphocyte population (Figure 4). Human B cells of memory or naïve phenotype were isolated by either flow cytometric cell sorting based on expression of CD19 and exclusion of the lineage markers CD3, CD56, CD16, plus IgG, IgA, CD27 and the dye MitoTracker (MT) (Fig 6A, paper I) or by magnetic bead isolation based on expression of CD19 and exclusion of CD27 (Fig 1A, paper II). CD4+ T cells used as T helper cells in co-culture with B cells were isolated from elutriated lymphocytes or buffy coats using a CD4+ T cell enrichment cocktail.

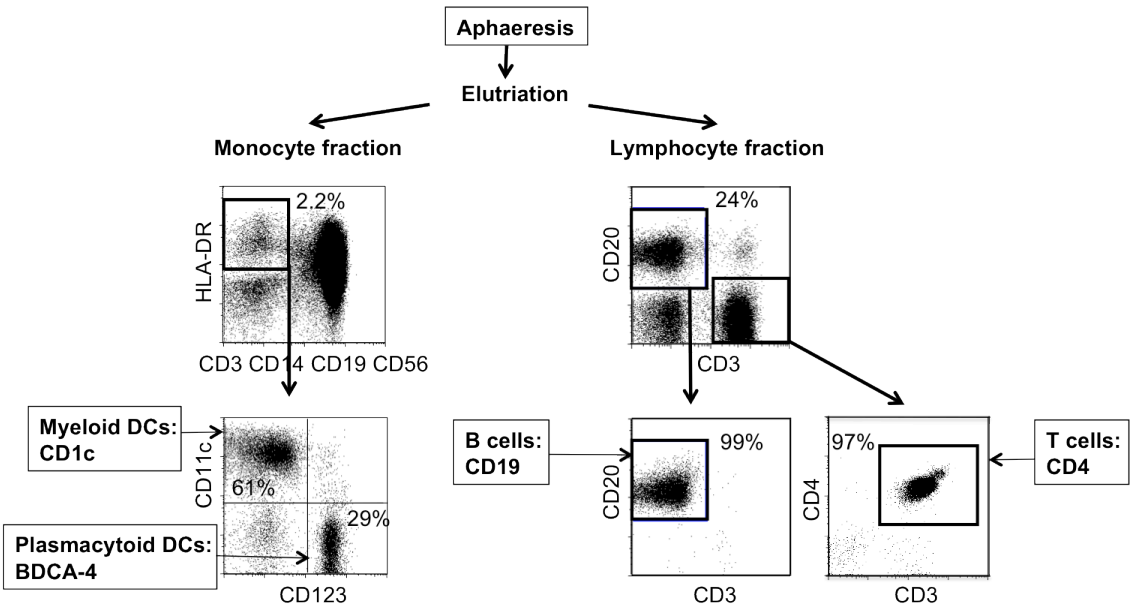

Figure 4: Isolation of DC subsets, B cells and CD4+ T cells

3.2 TLR-LIGANDS

The following TLR ligands were used in these studies. TLR3: The dsRNA complex polyinosinic:polycytidylic acid poly(I:C) (paper I-III). TLR7/8: The imidazoquinoline compound (3M-012) referred to as the TLR7/8-ligand (TLR7/8-L) (paper I-III). Imiquimod (R837; 1-(2-methylpropyl)-1*H*-imidazo[4,5-c]quinolin-4-amine, and resiquimod (R848; 4-amino-2-ethoxymethyl-α, α-dimethyl-1*H*-imidazoquinoline-1-ethanol) (paper I). TLR9: CpG ODN 2336 (CpG A), CpG ODN 10103 (CpG B), and CpG ODN 2395 (CpG C) (paper I-III).

3.3 B CELL CULTURES

To examine the role of DCs in regulating B cell responses upon TLR stimulation, cells (PDCs or MDCs) or supernatants from stimulated PDCs and MDCs were added to B cell cultures that were subsequently monitored for proliferation and differentiation into Ab producing cells (paper I). Since MDC support may contribute at a later stage in B cell differentiation, also sequential exposure of B cells to supernatants from stimulated PDCs followed by supernatants from stimulated MDCs was performed (paper II). In experiments examining the contribution of DC subsets on B cell responses in a T cell dependent manner, T cell help was provided using sorted CD4+ T cells that were γ-irradiated and pulsed with the super antigen Toxic Shock Syndrome Toxin 1 (TSST-1) to provide an initial contact between the TCR and MHC class II on the B cells [60, 61] (paper II).

To measure B cell activation and differentiation, different methods were used:

- Total levels of IgG, IgM or IFNα of all subtypes in the supernatants of cell cultures were measured using ELISA (paper I-III).
- Ig secreting cells were detected by isotype-specfic ELISPOT to IgM, IgG or IgA (paper I).
- The expression of the genes (Xbp-1, Blimp-1) that are associated with B cell differentiation was analyzed by semi-quantitative real-time PCR (paper I).
- B cell proliferation was measured by dilution of Carboxyfluorescein diacetate N-succinimidyl ester (CFSE) or by [^{3}H]thymidine incorporation.
- Phenotypic differentiation of B cells was measured by the elevated expression of the markers CD27 (paper I-III) and CD38 (paper I-II) and the upregulation of IgM and IgG (paper III) in the human B cell cultures and the downregulation of CD20 and the upregulation of IgM and IgG (paper III) in the rhesus B cell cultures.
- The role of IFNα in B cell activation was investigated by performing blocking experiments using monoclonal Abs to the IFNAR (paper I).

3.4 POLYCHROMATIC FLOW CYTOMETRY

Cells were stained with fluorochrome-conjugated Abs and analysed on a FACSCalibur. Data were analysed using FlowJo software. The Abs that were used in this thesis work include Abs against: CD3, CD4 and CD8 for defining T cell subsets and HLA-DR, CD11c and CD123 for defining DC subsets under exclusion of the lineage markers CD3 (T cells), CD14 (monocytes), CD19 (B cells) and CD56 or CD16 (NK cells). Abs against CD19, CD20, CD27, CD38, IgA, IgD, IgG and IgM were used to characeterize B cell subsets and assess B cell differentiation. Abs against the IL-2 receptor CD25 and the co-stimulatory molecules CD40, CD80, CD86 were used to measure the expression of the respective molecules on stimulated B cells. Abs against IFNα, IFNγ, IL-2 and TNFα were used to assess cytokine production in DC subtypes and T cells respectively.

3.5 RHESUS MACAQUE CELLS

Blood samples were obtained from untreated and healthy female rhesus macaques of Chinese origin. The rhesus macaques were housed in the Astrid Fagraeus laboratory at the Swedish Institute for Infectious Disease Control. Housing and care procedures were in compliance with the provisions and general guidelines of the Swedish Animal Welfare Agency.

4 RESULTS AND DISCUSSION

This thesis work has evolved around the roles of primary functionally distinct DC subsets in supporting B cell responses upon either TLR stimulation or induced by T helper cells.

4.1 PAPER I: PDCS MODULATE B CELL RESPONSES TO TLR-LIGANDS

Several prior approaches to study the role of DCs in modulating B cell responses have involved DCs generated in vitro by differentiation of monocytes or CD34+ precursor cells. Since there are several functional differences between in vitro-derived DCs and directly ex vivo-isolated DCs [171], the abilities of these various DCs to regulate B cell responses may also differ substantially. Therefore, we used an experimental approach including primary DC subsets (PDCs and MDCs) freshly isolated from blood. Since the DC subsets are rare, this was feasible since we established isolation methods using elutriated monocytes from donors that were aphaeresed. Also, isolation of B cells was facilitated by the aphaeresis and elutriation procedures.

4.1.1 B cell responses to TLR-ligand stimulation in vitro

As mentioned earlier, selected TLR-ligands can directly and specifically activate DCs and B cells according to their respective TLR expression [24, 26-28]. In **paper I**, we therefore investigated the effect of selected TLR-ligands on human primary DC and B cell subsets and explored the contribution of DC subsets on B cell function in a co-culture system. By comparing a panel of TLR-ligands binding to TLR3, TLR7/8 and TLR9 we found that total CD19+ B cells responded strongly by proliferation to the TLR9 using CpG B and CpG C while CpG A or TLR7/8-ligand induced very weak B cell proliferation (Figure 5). No B cell proliferation was found in response to TLR3 using Poly I:C which corresponds to the absence of TLR3 on B cells [24]. This response pattern was confirmed by that the expression of the differentiation marker CD38 was upregulated on the B cells and Ab production were found to the same ligands (Fig 1, paper I). This is consistent with previous reports on B cell responses to TLR-ligands [95, 147].

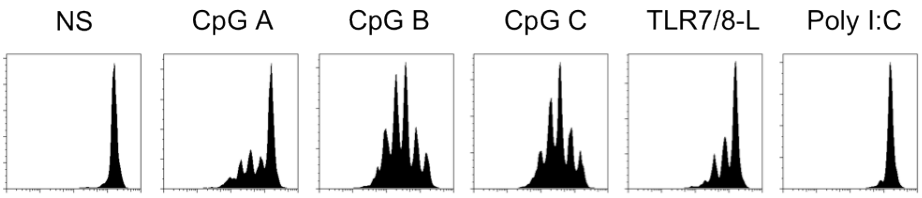

Figure 5: B cell proliferation in response to TLR-ligands (from Fig 1, paper I)

4.1.2 PDCs are superior over MDCs at supporting B cell activation

To investigate the relative roles of primary MDCs and PDCs in supporting B cell differentiation in response to these TLR-ligands, we performed co-culture experiments. Freshly isolated B cells were co-cultured with either isolated MDCs or PDCs isolated directly from blood, or supernatants thereof after TLR-stimulation. We found that the presence of PDCs but not MDCs significantly enhanced B cell proliferation in response to CpG B, CpG C and TLR7/8-ligand (Fig 2A-B, paper I). The PDC mediated enhancement was also found for B cell differentiation into Ab secreting cells after TLR7/8-ligand stimulation as measured by ELISPOT (Fig 3B, paper I). In addition, mRNA expression for Xbp-1 and Blimp-1 in B cells were upregulated in the same cultures. Since PDCs in co-culture with B cells and supernatants from stimulated PDCs showed very similar ability to enhance B cell responses, we concluded that soluble factors secreted from the PDCs likely mediated the effect. Using relative gene expression we assessed the upregulation of genes for a number of soluble factors that are associated with B cell activation. We found that MDCs upregulated significant transcript levels of IL-10, BAFF, TNFα, IL-6 whereas PDCs upregulated TNFα, IL-6 and IFNα in response to the ligands according to their respective TLR expression (Fig 4A, paper I). IFNα was exclusively produced by PDCs and no detectable levels of IFNα were found in MDCs in response to any of the TLR-ligands used. Since IFNα was produced in large amounts by PDCs stimulated by TLR7/8-ligands and the three CpG classes (Fig 4A-B, paper I), we further examined whether IFNα was involved in the enhancing effect on B cell responses. We found that the effect of stimulated PDCs or supernatants thereof on B cell proliferation and differentiation was substantially reduced when neutralizing Abs to the IFN receptor (IFNAR) were added to the co-cultures (Figure 6). In addition, the marked enhancement of TLR7/8-ligand stimulated B cell activation provided by PDCs could be reproduced using soluble IFNα.

The enhanced B cell response to TLR7/8-ligation in presence of PDCs or IFNα that we found here is consistent with data from a study where PDCs were reported to enhance specific Ig production in response to influenza virus (that signals via TLR7/8) upon production of IFNα and IL-6 [46]. In another study, an effect of PDC/IFNα enhanced B cell responses to TLR7-ligand (R848) but not CpG was reported on naïve B cells in absence of BCR engagement. In the same study it was shown that IFNα exposure of naïve B cells lead to enhancement of both TLR7 and the MyD88 [44]. This enhancement of TLR7 and MyD88 may, at least in part, be responsible for the PDC and IFNα enhanced B cell responses.

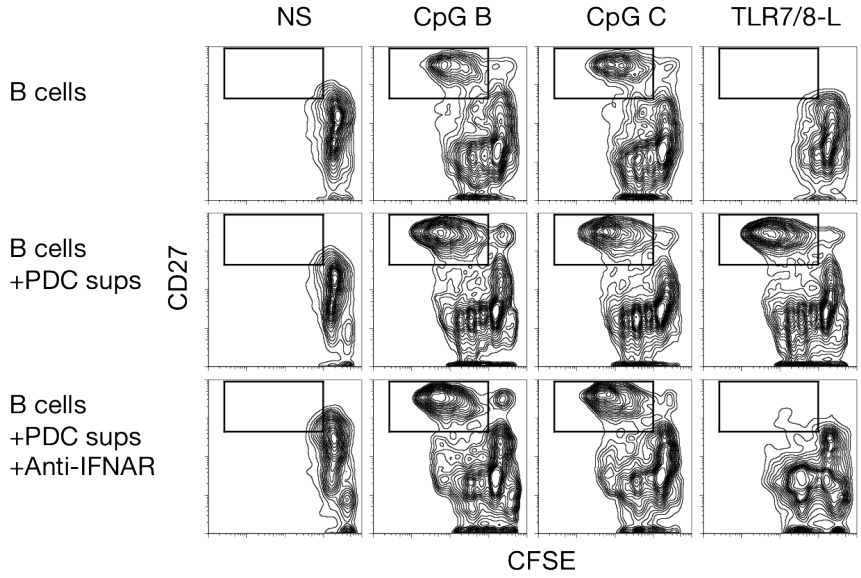

Figure 6: PDCs support B cell responses to TLR7/8-ligand via IFNα (From Fig 5, paper I)

4.1.3 DC regulation of naïve vs memory B cell activation

Since B cell subsets differ in their stimulation requirements [172], we compared the PDC or IFNα mediated enhancement on naïve vs memory B cells. We found that both B cell subsets increased their proliferative response to TLR7/8-ligand in presence of PDCs (Fig 6B, paper I), while the stimulation conditions only allowed the memory but not the naïve subset to differentiate into CD27high expressing cells (Fig 6C, paper I). In summary, this study shows that the presence of PDCs but not MDCs potently enhances B cell function to TLR7/8-ligand and that IFNα plays an important role in mediating this effect. Both memory and naïve B cell proliferation to TLR7/8-ligand are enhanced in the presence of PDCs, while differentiation is only achieved in memory but not naïve B cells under these conditions.

Naïve and memory B cell subsets possess different activation requirements and respond differently to TLR stimulation [173, 174]. Naïve and memory B cells differ in their TLR expression levels with naive B cells expressing most TLRs at low to undetectable levels while memory B cells express several TLRs at constitutively high levels [173]. In this study, we found that PDCs supported both naïve and memory B cells but only memory B cells fully differentiated to a CD27high phenotype. The presence of anti-Ig has an important impact on naïve B cell responses to TLR-ligands since it was shown to upregulate TLR9 and thereby allowing responses to CpGs [173]. Consistent with this, we found that both naïve and memory B cells proliferated in response to CpG B and CpG C in presence of anti-Ig. The differential TLR expression on B cell subsets may reflect important mechanisms to direct immune responses and avoid unwanted activation of cells. TLR activation on memory B cells was suggested to be an important mechanism in the maintenance of the memory pool [175]. Very high TLR expression is found on MZ and B-1 B cells, the B cell subsets that are responsible for early IgM production. However, as mentioned above, TLR-ligands can be upregulated in naïve B cells dependent on the local environment or stimulation.

Our data demonstrate that intrinsic signals induced by direct TLR stimulation of B cells but also signals provided by PDCs both play an important role for efficient B cell stimulation. The phenomenon that PDCs via production of IFNα can enhance B cell responses to TLR-ligands is relevant not only for the development of vaccine adjuvants and immunomodulatory treatment but also in the context of autoimmune diseases. Aberrant PDC activation and increased levels of IFNα are hallmarks of different autoimmune disorders. When clearance of apoptotic cells is insufficient, immune complexes including self nucleic acids or nucleoproteins released from dead cells can activate TLR7 and TLR9 on DCs and B cells, which can result in the development of systemic autoimmune diseases [2, 176]. Conditions associated with increased cell death and presence of innate immune factors such as upregulation of the anti-bacterial peptide LL37 have shown to lead to activation of PDCs and IFNα production [176, 177]. This could potentially shape humoral responses. A better understanding of these processes add to our knowledge about natural responses to pathogens or aberrant activation of immune responses and are important for the development on novel vaccines and immunomodulatory treatments.

4.2 PAPER II: PDCS SUPPORT T CELL HELP-INDUCED B CELL DIFFERENTIATION

4.2.1 Naïve B cell activation induced by T cell help and BCR crosslinking

It was proposed that PDCs could modulate class switching by the activation of B cells in both T cell dependent as well as T cell independent systems [94]. However, the formation of class switched Abs and B cell memory usually involve T cell help. We therefore in **paper II** proceeded with our studies by establishing a culture system where we could investigate the role of PDCs and MDCs on naïve B cell responses in absence of direct TLR-ligation but instead to anti-Ig and T cell help. Stimulation of the BCR that can be achieved by using anti-Ig is an important first signal to initiate the activation of naïve B cells [74]. In our study, the T cell help (that can be referred to as the second signal [74]) was provided in the form of irradiated CD4+ T cells that were pulsed with the superantigen TSST-1 to facilitate B cell:T cell contact. Using this co-culture system, we found that the presence of supernatants of TLR-stimulated PDCs was again unique in augmenting B cell proliferation (Fig 1B-C, paper II), phenotypic differentiation of the B cells to express high levels of CD27 (Figure 7) and CD38 (Fig 2A-D, paper II) as well as production of IgM (Fig 2E, paper II). We found a very similar enhancement in B cell responses to T cell help when IFNα was used instead of PDC supernatants (Fig 1D, 3D-E, paper II). Therefore, we concluded that the PDC enhanced effect on T cell dependent naïve B cell responses likely includes IFNα as a major mediator.

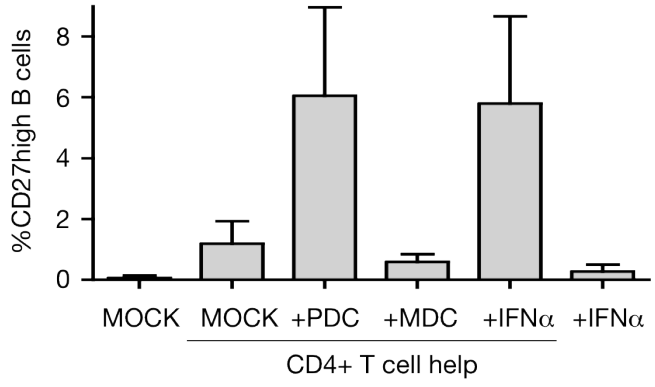

Figure 7: PDCs and IFNα enhance naïve B cell differentiation (from Fig 3, paper II)

4.2.2 PDCs but not MDCs support naïve B cell response to T cell help

In prior studies that have addressed the regulation of T cell dependent B cell activation, cytokines that can be secreted by DCs of the myeloid origin were shown be able to differentiate B cells [46]. In our studies, we also tested whether there was an effect of supernatants of TLR-stimulated MDCs, either alone or in combination with PDC supernatants, in our B cell cultures. Although we found in paper I that our TLR-ligand stimulated MDCs upregulated high levels of IL-10, BAFF and IL-6 (Fig 4A, paper I), cytokines that are known to affect B cell responses, the presence of MDC supernatants did not enhance B cell responses to TLR-ligands. It is plausible that TLR-stimulation of B cells is too strong and thus overriding potential MDC mediated effects which is why we did not notice an effect in paper I. The B cell stimulation by T cell help/BCR crosslinking used in paper II was not as strong and more subtle differences and potential effects would therefore be easier to detect. However, the presence of MDCs in the cultures did not mediate any enhancement in naïve B cell differentiation above the effect induced by T cell help and BCR ligation alone (Figure 7). Although MDCs were not shown to enhance B cell responses in the in vitro system, factors produced by MDCs may still play a role in induction and/or survival of B cells in vivo. Previous studies have found that MDCs are more potent than PDCs at presenting antigen and induce multifunctional T cell responses [141, 178]. MDCs may therefore be particularly involved in regulating cellular responses. In contrast, PDCs may be superior over MDCs at regulating B cell responses. It may also be relevant in this context to stimulate the DCs with CD40L in addition to TLR-ligation, which would substitute for a T cell signal and may alter their cytokine production profile or potency [11]. Of note, and in contrast to paper I, strictly CD27- naïve B cells were used in paper II that are known to have different stimulation requirements as compared to memory B cells [173]. For example, BAFF is reported to increase survival of memory B cells but does not support survival of naïve B cells [179].

4.2.3 PDCs or IFNα mediate CD86 upregulation in B cells

We attempted to more closely dissect why there is a PDC/IFNα mediated supportive effect on the naïve B cell differentiation to T cell help. Since in this culture system, the B cell responses are not dependent on TLR-stimulation, any upregulation of TLR expression or MyD88 is unlikely to be of impact. In the search for possible mechanisms that could explain the enhanced

responsiveness of B cells to T cell help, we monitored and found that cell viability was not substantially altered in the presence of IFNα. Thus, simply increased cell survival is not the reason. We also measured the expression levels of several co-stimulatory molecules on the surface that are known to be involved in APC:T cell interactions. We found that CD86 was upregulated on B cells upon addition of either supernatants of stimulated PDCs or IFNα (Fig 5A-C, paper II), which is also shown in (Fig 5A, paper I) and consistent with earlier reports in vitro [108] and in vivo [110, 111]. The ligation of CD86 on B cells to CD28 expressed on T cells has been shown to give a stimulatory signal to B cells leading to enhanced proliferation and IgG secretion [180-183] involving signaling via PI3K, Akt, and IκB kinase αβ [184]. The structure of CD86 that includes a cytoplasmic tail containing three potential sites for PKC phosphorylation, which are signaling-associated features, also indicates signaling potential of CD86 in B cells [70]. In contrast to CD86, we could not detect elevated levels of other surface markers involved in B cell:T cell interactions such as CD80, HLA-DR, IL-25 in the presence of IFNα. Even the expression of CD40, which is a very central molecule described in mediating signals received by T cell help (via CD40L), was not altered in our culture conditions (Fig. 5A, paper II). However, we cannot exclude that signaling through any of these molecules could be affected at a different level. From these data, we speculated that increased CD86 surface expression may render B cells more responsive to T cell help, potentiating the enhancement of B cell responses in the presence of IFNα.

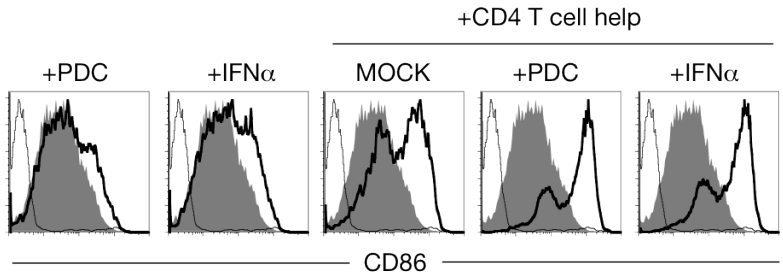

Figure 8: CD86 expression on B cells after exposure as indicated (from Fig 5, paper II)

This prompted our subsequent interest in whether B cells exposed to IFNα, in an opposite scenario, also had an increased ability to activate Th cells. Indeed, B cells preincubated with IFNα prior to superantigens showed a small but consistently increased capacity to stimulate IL-2, IFNγ, and/or TNFα production in T cells (Fig 6B-C, paper II). Although there are several prior in vivo studies in mice that have shown that IFNα can significantly enhance B

cell responses where Th cell processes are involved [98, 110-112, 121], our findings remain to be confirmed in vivo in humans.

Taken together, the data from paper II support the conclusion that PDCs have the ability to enhance B cell activation under T cell help-dependent conditions and that IFNα directly renders the B cells more receptive to T cell help. The mechanisms responsible for the observed effects may include the upregulation of the co-stimulatory molecule CD86, which could also account for the improved ability of the B cells to in turn activate CD4+ T cells. Both of these effects could shape the magnitude and quality of ensuing B cell responses.

4.2.4 The effects of IFNα on B cells

The data in paper I and II further support the notion that PDCs by their production of type I IFN exhibit a unique role to modulate B cell responses. Type I IFNs have indeed been shown in a number of studies to strongly impact the outcome of humoral responses. It was shown in mice that type I IFN-mediated B cell activation significantly affected the quality and magnitude of the antiviral humoral response [110-112]. IFNα also increased specific Ab responses to protein antigen [98], which was dependent on signaling via the IFNAR on both B and T cells. While direct IFNα signaling on B cells was shown to be required in that study, other studies point to that the major effect of type I IFNs in order to enhance humoral responses was depended on signaling of DCs [51, 100]. However, it is well established that direct type I IFN signaling has multiple potent effects on various cell types [53, 96, 97, 102, 104, 106, 110, 111], which can additionally shape the adaptive immune response.

Apart from the crucial role of T helper cells in the activation of B cell responses, B cells vice versa were shown to provide important signals for Th cell induction [185]. The Tfh in the GC have been under intense investigation in the last few years. Tfh development strongly relies on engagement of CD28 by CD80/CD86 expressed on APCs during the priming phase, as reviewed in [84]. These initial Tfh priming events by DCs are followed by interactions with cognate follicular B cells that ultimately lead to Tfh differentiation. Although CD28 engagement is not required later to maintain GC and other co-stimulatory molecules are reported to be important in the Tfh:B cell interactions [84], a regulatory involvement of CD28:CD86 interactions can not

be excluded. During various interactions with cognate B cells, the Tfh pool may be regulated according to the requirements of the overall response [84]. The central role of Tfh cells in the outcome of GC reactions renders them important cells to study further during vaccination settings. It was shown that TLR-ligand induced Type I IFN signaling on DCs could stimulate Tfh development [89]. Upon activation, Tfh secrete IL-21, which has very potent effects on GC B cells as reviewed in [86]. IL-21 acts on Tfh cells in an autocrine manner and was also shown to mediate direct signals on B cells [87]. We found that preincubation of B cells with CpG C or IFNα enhanced their capacity to induce T cell activation, presumably due to improved cell:cell interactions. This may thus also be one of the consequences that type I IFNs have on the development on Tfh cells. The increased activation of Th cells in presence of IFNα that we observed may reflect the capacity of B cells to regulate Th cell activation, dependent on the cytokine environment or their own activation status. This may then, in a more complex setting, amplify the effect of IFNα mediated enhanced B cell responses to T cell help that we have reported here.

In conclusion we showed in paper II that PDCs and IFNα significantly enhance B cell differentiation to T cell help. The mechanisms responsible for this effect may include the upregulation of the co-stimulatory molecule CD86, which could also account for the improved ability of the B cells to in turn activate CD4+ T cells. Both of these effects could be important in shaping the magnitude and quality of ensuing B cell responses. Studies of the stimulation requirements of B cells for the induction of potent Ab responses and how such responses can be regulated by DC and IFNα will increase our understanding of the complexity of immune responses to various infections and provide important information for the design of new vaccine and immunomodulatory treatment formulations.

4.3 PAPER III: HUMAN AND RHESUS PDC AND B CELL RESPONSES TO TLR STIMULATION

Further investigation of the effects of PDCs and type I IFNs on B cell responses to TLR-ligands in vivo in humans remains to be elucidated. In order to approach such investigation, NHP offer a much more physiologically relevant in vivo model than rodents do. In contrast to rodents, several species of NHP express similar DC subtypes [164, 165] and TLR repertoires [166] as compared to humans. There are numerous studies on NHP testing

the efficiencies of different vaccine adjuvants. TLR-ligands show potency as vaccine adjuvants when tested in rhesus macaques [142, 143, 186]. However, differences between the human and rhesus macaque immune systems have been reported [168], which can have direct impact on the translation of results obtained from NHP studies into clinical trials. Therefore, it is important to uncover species differences in detail and this may require the verification of key findings in both species. We therefore set out to investigate the role of PDCs and IFNα on B cell responses in rhesus cells and performed a number of comparable experiments in rhesus and human cells including phenotypic analyses of DC and B cell subsets and their responses to selected TLR-ligands and IFNα.

4.2.5 Human and rhesus B cells and PDCs respond similarly to TLR-ligands

We first showed that DC and B cell subsets in human and rhesus macaques could be identified using similar markers (Fig 1, paper III), which is consistent with previous reports [146, 164, 165, 187, 188]. We then performed side-by side comparisons of the B cell proliferation and PDC activation to our panel of TLR3, 7/8, and 9-using ligands. In total PBMCs, we found that the pattern of proliferation in response to the TLR-ligands was similar in rhesus and human B cells with a slightly different rank order. In both human and rhesus B cells, CpG B, CpG C and TLR7/8-ligand induced the strongest proliferation while CpG B was superior in the human and CpG C was superior in the rhesus cultures, which is consistent with previous reports [189] (and paper I). This may indicate small species differences in CpG recognition between rhesus and human, a concept that was supported by differences in the activities of certain CpGs between human and mice [144]. In addition, both human and rhesus PDCs were able to produce IFNα in response to CpG C and TLR7/8-ligand, although with some differences in levels and kinetics.

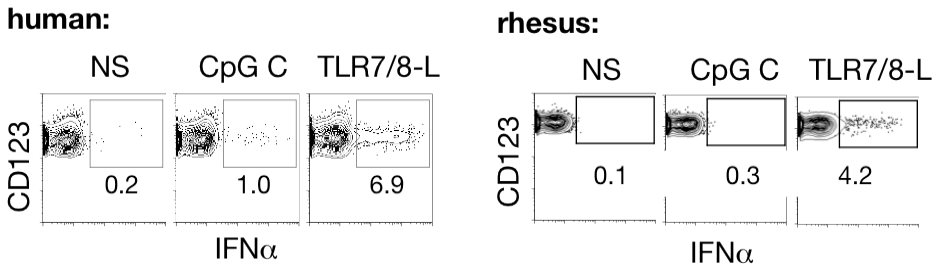

Figure 9: IFNα is produced by both human and rhesus PDCs (from Fig 3, paper III)

4.2.6 Lack of CD27 upregulation despite IgM production in rhesus B cells

The IFNα induced enhancement of TLR-mediated B cell responses as we described in paper I, was found to be reproduced in rhesus B cells in paper III. IFNα significantly enhanced human as well as rhesus B cell responses to TLR7/8-ligand and CpG C. This enhancing effect was shown for proliferation, phenotypic differentiation and induction of IgM secretion. However, we noticed that there were differences between human and rhesus B cells with regards to alterations of cell surface markers during differentiation. The distinct CD27high populations observed in human B cell cultures that is associated with plasmablast formation (paper I and paper II) [174, 190], was absent from rhesus B cell cultures under conditions when both human and rhesus B cells produced increased levels of IgM. Instead, rhesus B cells showed a distinct downregulation of CD20, which correlated with the levels of IgM production. Thus, CD20 downregulation may be a useful marker for monitoring rhesus B cell differentiation.

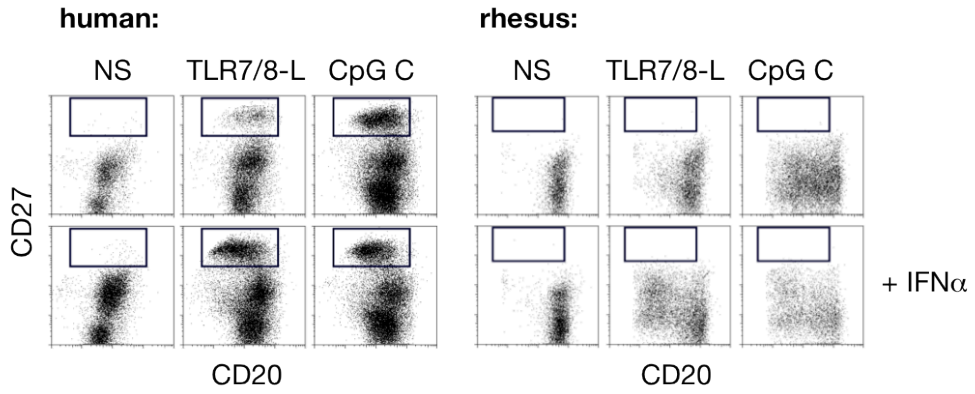

Figure 10: Differentiation stainings in human vs rhesus B cells (from Fig 5, paper III)

It is possible that these phenotypical differences between activated human and rhesus B cells may have functional impacts. CD27 can be bound by CD70, which is expressed on activated CD4+ T cells, B cells and DCs, and was reported to play a critical role in T dependent B cell responses [191]. CD27, which is widely expressed on T cells, was shown in mouse studies to have important functions in the regulation of the effector T cell pool [185]. Although CD27 is only expressed on a few percentage of memory B cell in mice, in contrast to human [192], GC formation was delayed in CD27-/- mice which was shown to be mainly due to deficient signaling on B cell CD27. However, both somatic hyper-mutation and Ig responses were normal in CD27-/- mice [193]. It may be due to the much more frequent expression of

CD27 on B cells [192] that its impact seems to be greater on human plasma cell development. Moreover, signals via CD27 were shown in human in vitro studies to promote the differentiation of plasma cells, most beneficially after a phase of initial proliferation, mediated by CD40 engagement [194]. CD27 stimulation was also shown to support development into plasma cells in combination with IL-10 [195]. In addition to that, CD27 expression on B cells was recently shown to provide a survival signal to CD8 T cells upon CD27:CD70 interaction [196]. The variation in the CD27 expression levels that we found in human vs rhesus B cells, may thus likely reflect different importance or efficiency of the CD27:CD70 interactions in human vs rhesus B cells. Another functional difference between the two species may also be attributed to the more pronounced downregulation of CD20 that we found on rhesus as compared to human B cells. Relatively little is known about the biology of CD20, although it is targeted by the anti-CD20 Ab rituximab, a successful drug that is used in lymphoma patients [197]. CD20 has no known natural ligand and CD20 knockout mice display an almost normal phenotype. However, CD20 has been shown to be resident in lipid raft domains of the plasma membrane and may function as calcium channel following ligation of the BCR [197]. The more pronounced downregulation of CD20 in activated rhesus B cells that we observed might have functional consequences including implications in experimental settings or evaluation and translation of treatment strategies that use Abs to CD20 for selective depletion of B cells.

4.2.7 The relevance of NHPs for studying the utility of TLR-ligands

In **paper III** we found that CpG A, CpG B, CpG C and TLR7/8-ligand induced proliferation while Poly I:C did not in human and rhesus macaque B cells. Both human and rhesus PDCs produce IFNα in response to TLR7/8-ligand and CpG C. IFNα significantly augmented B cell proliferative responses in particular to TLR7/8-ligand. The data presented here are highly relevant for the use of rhesus macaques as a model to study the clinical utility of TLR-ligands and can facilitate translation of data obtained from NHP studies to the design of clinical trials.

CpGs are successfully used to expand human memory B cells, which can be exploited for different applications [150, 174]. Two recent studies show the use of a stimulation cocktail for the expansion of rhesus memory B cells that consists of a combination of pokeweed mitogen (PWM), *Staphylococcus aureus Cowan* (SAC) and CpG [198, 199]. The stimulatory effect of PWM was

dependent on the presence of T cells in both human and rhesus cultures while the dependence on T cells in response to the stimulation cocktail PWM+SAC+CpG was more pronounced in the rhesus cultures. The response induced by CpG alone was lower in the rhesus as compared to human cells, which is consistent with our data [199].

CpG compounds have been used successfully as adjuvants in clinical trials for a Engerix-B HBV vaccine and an influenza vaccine [137-139]. CpG is also promising to be used as an adjuvant in immune suppressed individuals [133], since CpG successfully increased the response to therapeutic vaccination in HIV patients [149]. CpGs may favourably be packaged into virus-like particles (VLPs) to improve their efficiency [200] or directly conjugated to antigen [78]. Both CpG C and TLR7/8-ligand have shown, when administered to rhesus macaques together with a HIV Gag protein, to significantly increase Gag-specific Th1 and Ab responses, while TLR7/8-ligand was most potent when administered in a conjugated form [142, 143].

The use of well-defined TLR-ligands as vaccine adjuvants is appealing in that they can specifically activate desired cells. In contrast to several other adjuvants, TLR-ligands themselves do not disrupt conformation-sensitive epitopes of antigens, which may be a critical issue for the induction of neutralizing Abs to protein vaccines. Also, TLR-ligands are soluble and molecularly defined, which facilitates in vitro cell culture and mechanistic studies. The findings in paper I that B cells as well as DC subsets can be efficiently stimulated by TLR-ligands and the presence of PDCs can modulate the B cell response, could have implications for the clinical utility of these TLR-ligands. To this end, it is possible that the best activation of multiple cell subsets is achieved by combinations of TLR-ligands, which needs to be explored further [201].

The use of ligands targeting TLR7/8 may be promising because of their ability to directly acitvate MDCs, PDCs as well as B cells. However, indirect MDC activation via type 1 IFN production by PDCs may be as good as direct stimulation of MDCs. It has even been shown in a elegant study using mice with MyD88 lacking B cells, that TLR signaling on B cells was not required for the adjuvant effect of CpG together with a protein antigen. However, when CpG was administered in a VLP, IgG responses were significantly reduced in MyD88-/- mice [202]. CpG delivered in VLPs was also shown to

induce isotype switch on B cells via direct TLR9 engagement [203]. This may highlight the importance of different delivery systems in the contribution to induce potent immune responses to vaccine components.

Although TLR engagement was shown to be involved in the effect of several well known adjuvants [131] and reviewed in [133], the importance of TLR stimulation in context of adjuvant activity is debated [134]. While direct TLR signaling may be redundant for the potent effect of known vaccine adjuvants [134], signaling via the IFNAR was required [55] and reviewed in [51]. IFNR knockout mice were severely impaired in their successful response to vaccine adjuvants [51, 55, 118]. Type I IFNs thereby take the role of important mediators of adjuvant effects. IFNR signaling on DCs was reported to be especially crucial in the induction of adjuvant effects [51, 100] while in certain circumstances, also direct signaling on B cells and T cells was required [98]. As discussed in previous sections of this thesis, type I IFNα production can be induced by both TLR-dependent as well as TLR-independent mechanisms. Therefore, according to their differential PRR expression patterns, the contribution of both hematopoietic and non-hematopoietic cells may need to be considered in terms of type I IFN production in future adjuvant development.

In conclusion, the very similar response patterns of rhesus and human primary B cells and PDCs that we found to a number of TLR-ligands support the use of rhesus macaques as a model to study TLR-ligands. However, some differences between rhesus and human PDC and B cell responses may reflect species differences in TLR recognition or response kinetics. The partial differences in phenotypic B cell markers may also have functional consequences. These results may help to increase the awareness about similarities and disparities between the human and NHP immune systems. Studies focusing on species differences could provide important information for translating data obtained from NHP studies to human immunology as well as applying this on the design of clinical trials aimed at testing new vaccine and treatment strategies.

5 CONCLUDING REMARKS

How innate factors and cells such as DCs shape adaptive immunity is important basic immunology that would aid in the design of novel vaccines and also to understand autoimmune diseases. The purpose of this thesis work was to study the role of specific DC subsets in the development of B cell responses. Using human in vitro cultures systems, we have investigated the role of specific DC subsets, MDCs and PDCs, in B cell activation in the context of TLR stimulation. In paper I, DC:B cell interactions were studied in the presence of ligands binding to TLR3, 7/8 or 9. In paper II, we chose to examine naïve B cells in a T dependent system without direct TLR stimulation of the B cells. In both studies, PDCs but not MDCs significantly supported B cell responses. In this PDC mediated enhancement, one very central factor involved was IFNα, which can be produced by PDCs in large amounts. In paper I, PDCs/IFNα in particular supported B cell responses to TLR7/8-ligand stimulation, which may be at least in part due to upregulated TLR7 and MyD88 levels that have been reported in the presence of IFNα. The enhanced responses to TLR7/8-ligand were seen in both memory and naïve B cells in terms of proliferation but only memory B cells but not naïve B cells were able to express high levels of the differentiation marker CD27 under these stimulation conditions. In paper II we found enhanced differentiation of naïve B cells to T cell help in the presence of PDCs/IFNα. Since direct TLR stimulation on B cells was not present in this system, the enhancement is likely mediated by a different mechanism than in paper I. One possible factor that may contribute to the observed effect is the upregulated expression of the co-stimulatory molecule CD86 that we found in presence of PDCs/IFNα. We therefore speculate that the presence of PDCs/IFNα facilitates B cell:T cell interactions as supported by the enhanced capability of B cells to activate CD4+ T cells. Altogether, these studies highlight the important capacity of type I IFN, which can be produced by PDCs, in conditioning B cell responses by direct means.

Animal models including NHP are very crucial to obtain increased understanding of the in vivo impact of TLR-ligands and the role of DC subsets in the orchestration of immune responses. In order to maximize the benefit of animal models and to avoid inaccurate translation of findings from animal models to clinical trials, species differences need to be studied thoroughly. We

have therefore in paper III directly compared primary cells from human and rhesus macaques in different in vitro assays in context with TLR stimulation. We showed similarities in the responses of B cells and PDCs to selected TLR-ligands and could confirm the IFNα enhanced effect of TLR7/8 stimulation. This supports the validity of rhesus macaques to be used in studies addressing adjuvant activity of TLR-ligands. We also reported some disparities between rhesus and human B cells in differentiation markers. This information adds to the understanding of differences between the two species in context with TLR-ligation and will hopefully be helpful in the design and interpretation of future studies.

Studies that address innate factors that are involved in shaping adaptive responses and further dissect the factors that are required for the differentiation of naïve B cells into potent Ab producing cells are important. Increased knowledge about these processes will hopefully help the development of safe vaccines and immunomodulatory treatment strategies.

6 ACKNOWLEDGEMENTS

There were a number of great people around me that that have contributet to this work in different ways. I would like to express my gratitude and thank especially:

My main supervisor **Karin Loré** for accepting me as a PhD student in your group. Thank you for your guidance, encouragement, support and helpful advice. You are a great mentor and teacher and I have really enjoyed working with you. Thank you for your generosity and help in finding the right track on my PhD journey. I would also like to thank you for nice events outside the lab, especially the time we could spend in Piteå.

My co-supervisor **Gunilla Karlsson Hedestam** for support, discussions and critical help with questions. I have appreciated it a lot and thank you for running the joint KL-GKH group together with Karin and providing such a great research environment. My co-supervisor **Robert Seder** at the Vaccine Research Center, NIH, for all help arranging TLR-ligands to my studies which was an instrumental part plus all the inspiring discussions over the years.

My external mentor **Alexandra Trkola** at the University of Zurich, for guidance during my initial research encounters and useful advice for the future.

I have been priviledged to be a member of an excellent research center i.e the Center for Infectious Medicine (CIM), Department of Medicine, and would like to thank the director **Hans-Gustaf Ljunggren** and all the people at CIM for creating such a fantastic scientific environment. Great thanks for many smart questions, discussions, inputs, ideas and fun. Thank you **Ann Hellström** and **Carina Löf** for being friendly and very helpful with administrative work during my time as a PhD student.

All the colleagues and friends from the KL-GHK lab that were crucial in creating the friendly and inspiring atmosphere that made the difference and also for fun times outside the lab. I would like to thank in particular: my collaborator **Iyadh** for all your help and expertise and for introducing me to many things in the lab. **Emily** for being a very nice companion during this journey from the start. **Will** for being the perfect colleague during all these years, for your expertise and for your open minded personality. **Kerrie** for

friendliness and your help and expertise in project II. **Christopher** for being very helpful and a friendly and always interested colleague. **Pia** for sharing experience in the research and non research world and **Martina** for nice lunch time chats. Thank you **Kai** for surprises and nice talks, **Mattias, Marc**, **Thomas**, **Frank** for your help and contribution to the group. **Gerry** for enthusiasm, good science and humour and **Anna** for very helpful input. I would also like to thank **Jessica** and **Andrea** for your help and a former member of the group, **Åsa** for nice discussions.

I thank the Department for Transfusion Medicine for assistance with aphaeresis and elutriations, which was instrumental for many experiments included in this thesis.

I would like to express my gratitude to the Swedish Institute for Infectious Disease Control (Smittskyddsinstitutet, SMI) that hosted the group during most of the time of my PhD work and provided us with excellent labs and facilities. Special gratitudes go to **Hans Ottosson**, **Jan Albert** and **Rigmor Thorstensson** for making this possible and all research and non-research people that helped to create a friendly and professional working environment that I appreciated a lot. Especial thanks also to the Immunology lab in particular **Katarina Karlén** for your help. I would like to thank the Astrid Fagareus lab, especially **Mats Spångberg** and **Helene Fredlund** for the excellent NHP facility.

I would also like to thank a number of not work related people that were especially important to me in the last five years:
First of all, **Cajsa** and **Einar** for friendship and many cheerful get togethers and for generously giving me the opportunity to write major parts of this thesis in your atelier. **Pauliina**, **Tassos** and **Josephine** for being very nice friends and neighbours. **Joëlle** and **Ivan**, you contributed substantially to my social life here in Stockholm. Thank you for friendship and introducing me to such a nice group of people.

I would also like to thank **Anna** for nice walks and your patience to endure my Swedish, **Ane** for being a dear friend and introducing me to some very nice people. **Anna** and **Vandana** for good times here in Stockholm. **Eva** for your friendship and sharing important events in my life. **Parham** for friendship and being very generous in sharing your knowledge and many laughs. **Anita,**

Fabienne, Cécile, Rosette, Patricia, Eliane for long lasting friendship. Fabienne, I really appreciated our long phone calls and Anita, thank you for visiting me several times in Stockholm.

My special thanks go to my **parents** for endless support and encouragement. My **sister** and her family for taking so many trips to Stockholm during the past years and making a real effort to get to spend such wonderful times together. My **extended family** for sharing dear moments and a lot of fun. **Marlis** and **Franz**, thank you for your tremendous help during this time.

Finally **Martin** for immense support and love and everything we share, especially being parents to most wonderful little **Loris**. Thank you both for being there and making my life perfectly unique.

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