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PRESERVATION OF VACCINE-INDUCED LONG-TERM B CELL MEMORY AND THE EFFECTS OF IMMUNOSUPPRESSIVE TREATMENT

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Preservation of vaccine-induced long-term B cell memory and the effects of immunosuppressive treatment

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Till

Maria “Millan” Ingelman-Sundberg

1881-1971

Min inspiration

Mamma och Pappa

Min trygga bas

Martin

Min framtid

If you want to succeed with your career in research - don't think of yourself. Think of others.

Françoise Barré-Sinoussi, Nobel Prize Laureate 2008

ABSTRACT

Immune memory after vaccination is largely dependent on the combination of antibody production from long-lived plasma cells, and a supporting pool of antigen-primed memory B cells. It has been observed that individuals with certain immunosuppressive conditions or treatments have a weakened B cell memory, but the mechanisms behind remain elusive. The aim of this thesis was to evaluate B cell immunity in healthy children, and how HIV-1 infection, antineoplastic therapy, and rheumatic disease and treatment can impact on various features of B cell memory induction and maintenance.

In **paper I**, we explored the hyperactivation of B cells observed in patients carrying HIV-1 infection, and showed that it can be partly induced by ligation of soluble cleaved CD27 to CD70 on the surface of memory B cells. In **paper II**, we aimed at comparing the establishment of serum antibody titers and memory B cells after vaccination against measles and rubella in healthy children. We found that the memory B cell pool remained stable also early after vaccination, whereas the corresponding serum IgG titers decayed with time. In contrast, both the serum IgG levels and frequency of blood memory B cells in healthy young adults appeared stable. This implied that the antibody production and memory B cell compartment are two separate entities with independent regulation, and that it takes longer time to establish a stable pool of circulating antibodies. How these two parts of B cell memory are affected by immunosuppressive disease and treatment was addressed in papers III and IV. **In paper III**, we used a rhesus macaque model for high-dose Doxorubicin treatment, and concluded that the established vaccine-induced memory B cell pool was depleted, contrary to long-lived plasma cells and the resulting serum IgG titers. These observations supported the finding of independent regulation of the two B cell memory compartments, and revealed different sensitivity to chemotherapy. The bone marrow plasma cell niche was additionally studied in an *in vitro* model for plasma cell – stromal cell cross talk, where we discovered that *in vivo* relevant concentrations of Doxorubicin could hamper the output of pivotal survival factors from stromal cells. In **paper IV**, we examined memory B cells and circulating IgG titers in children with rheumatic disease, treated with low-dose Methotrexate and TNF-α inhibition. We noted that serum IgG titers against tetanus were lower in rheumatic patients than in healthy controls, and that patients who had only received one measles vaccine dose had lower levels of measles-specific memory B cells. This stresses the importance for children with rheumatic disease and treatment to follow the full vaccine schedule.

To summarize, this thesis has contributed to enhanced knowledge on how B cell memory is induced, preserved and at risk of disruption by common immune disorders and treatment. Hopefully, our findings can aid future improvement of functional vaccine regimes for immunocompromised children.

LIST OF SCIENTIFIC PAPERS

I. Dang LV, Nilsson A, **Ingelman-Sundberg H**, Cagigi A, Gelinck LB, Titanji K, De Milito A, Grutzmeier S, Hedlund J, Kroon FP, Chiodi F: *Soluble CD27 induces IgG production through activation of antigen-primed B cells.*
J Intern Med. 2011 Sep 14.

II. Kakoulidou M, **Ingelman-Sundberg H**, Johansson E, Cagigi A, Farouk SE, Nilsson A, Johansen K: *Kinetics of antibody and memory B cell responses after MMR immunization in children and young adults.*
Vaccine 2012 Nov 19.

III. **Ingelman-Sundberg HM**, Saghafian-Hedengren S, Jahnmatz M, Eksborg S, Jonker M, Nilsson A: *Selective loss of vaccine-specific memory B cells in a Rhesus Macaque model of chemotherapy – influence of Doxorubicin on immunological memory.*
Haematologica 2014 Dec 31. Epub ahead of print

IV. **Ingelman-Sundberg HM**, Laestadius Å, Chrapkowska C, Mördrup K, Magnusson B, Sundberg E, Nilsson A: *Effects of Methotrexate and TNF-alfa inhibition on long-term vaccine immunity against measles, rubella and tetanus in children with rheumatic diseases.*
(Manuscript)

CONTENTS

LIST OF ABBREVIATIONS

Ab	Antibody
Ag	Antigen
AID	Activation-induced deaminase
ALL	Acute Lymphoblastic Leukemia
APRIL	A proliferation-inducing ligand
BAFF	B cell activator of the TNF-α family
BCR	B cell receptor
BM	Bone marrow
BCMA	B cell maturation antigen
BMMC	Bone marrow mononuclear cell
BMPC	Bone marrow plasma cell
CAR cell	CXCL-12-abundant reticular cell
CD	Cluster of differentiation
DMARD	Disease-modifying antirheumatic drug
ELISA	Enzyme-Linked Immunosorbent Assay
ELISpot	Enzyme-Linked ImmunoSpot
FcRL4	Fc-receptor-like 4
FCS	Fetal Calf serum
FDC	Follicular dendritic cell
GC	Germinal center
HIV	Human Immunodeficiency Virus
ICOS	Inducible T cell co-stimulator
Ig	Immunoglobulin
IL	Interleukin
IMDM	Iscoves modified Dulbecco medium
LLPC	Long-lived plasma cell
MBC	Memory B cell
MHC	Major histocompatibility complex
MMR	Measles Mumps Rubella
MTX	Methotrexate
MZ	Marginal zone
PBMC	Peripheral blood mononuclear cell
PBS	Phosfate Buffer Solution
PC	Plasma cell
PCR	Polymerase Chain Reaction
Rag	recombination-activating gene
RPMI	Roswell Park Memorial Institute Medium
SLE	Systemic Lupus Erythematosus
SLO	Secondary Lymphoid Organ
SLPC	Short-lived plasma cell
TACI	Transmembrane activator, calcium modulator, and cyclophilin ligand interactor
TCR	T cell receptor
TD	Thymus dependent
T_{FH} cell	Follicular helper T cell
TI	Thymus independent
TLR	Toll-like receptor
TNF	Tumor necrosis factor

1 INTRODUCTION

After vaccination, host protection against pathogens is dependent on one of the cardinal features of the adaptive immune system – immunological memory. One of the major players are B lymphocytes (B cells), eliciting a specific response by means of unique gene arrangement of each cell clone, producing exact matching of the B cell receptor to its target [1]. The aim of this thesis is to dissect how B cells contribute to life-long protection after vaccination, and how this can be hampered by immunosuppressive treatment.

1.1 GENERAL FUNCTION AND DEVELOPMENT OF B CELLS

B cells can act as modulatory cytokine-producing cells in many contexts, but their main effector function is to develop into plasma cells (PCs), and produce a soluble form of their receptor – antibodies (Abs). The following chapter will explore the characteristics of Abs, and how B cells mature and are maintained.

1.1.1 B cell receptor functions

The B cell receptor (BCR) is composed by a membrane bound immunoglobulin (Ig) molecule, which consists of repeated Ig protein motifs in two larger heavy chains and two smaller light chains, and the signaling chains Igα and Igβ [2, 3] (figure 1-1). The binding part of the Ig molecule, composed by both the heavy and light chains, recognizes a specific molecular signature, defined as an antigen (Ag). Since the specificity of each BCR is created in a stochastic manner, regulating functions are needed to enhance the response to pathogenic Ags, and inhibit actions towards self-Ags or non-pathogenic external Ags (for example food-related). These mechanisms are however not discussed within this thesis.

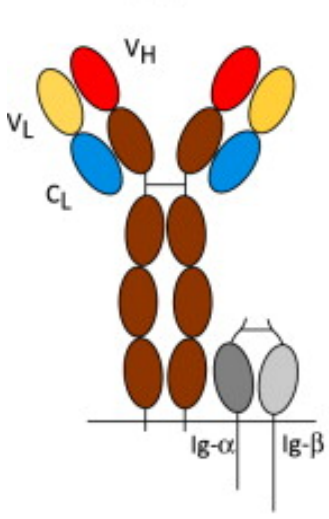

Figure 1-1. Schematic image of the B cell receptor, where each segment represents an immunoglobulin (Ig) motif. The two joined longer chains are the heavy chains, where V_H (red) is the variable region, and the brown segments the constant. The shorter chains are light chains, consisting of the yellow variable (V_L), and the blue constant (C_L) regions. The downstream signaling is initiated from the Ig-α – Ig-β complex.
Image source: [3], printed with permission from Elsevier.

Isotype	Main switch signal	Main source of switch signal	Main effector functions
IgM	Non-switched heavy chain	-	Complement activation
IgA	TGF-β	Epithelial and dendritic cells in mucosal tissue	Activation of innate cells (e.g. phagocytosis and cytotoxic effects)
IgE	IL-4	Th2 cells (also $T_{FH}2$ cells)	Mast cell degranulation
IgG	IFN-γ	Th1 cells (also $T_{FH}1$ cells)	Phagocytosis, complement activation, neutralization

Th = Helper T cell, T_{FH} = Follicular helper T cell

Table 1-1. Immunoglobulin isotypes existing as soluble antibodies, the most important factors leading to their production, and their most prominent functions. Phagocytosis is mediated through opsonization of antigens and subsequent binding to Fc receptors on innate phagocytic cells. References: [1] and [4].

As a mean of creating specificity for a large number of Ags, both light and heavy chains have regions of great variability between B cell clones (V regions), but also those more conserved (C regions) [1]. The conserved regions exist in different isotypes, named κ or λ for light chains, which are of unknown significance in humans. The heavy chain constant regions are named μ, δ, ε, α or γ, and determine the immunoglobulin isotype to IgM, IgD, IgE, IgA or IgG, respectively (including subtypes, not discussed here). Once activated, B cells can achieve the ability to produce soluble Igs, capable of different effector functions. Most of these are mediated through the Fc region of the heavy chain, which binds to various Fc receptors and elicit a variety of downstream actions (table 1-1) [1, 4]. The functions of IgA and IgE are beyond the scope of this work, which instead will focus on IgM and IgG. IgM is the first isotype produced by activated B cells, and has a limited binding strength (affinity). It is secreted as a pentamer, increasing the overall binding capacity (avidity), and mainly exerts its function through activation of the complement system. IgG is secreted as a monomer, is produced late in an Ag response and has higher affinity and capacity to neutralize pathogens, i.e. block them from further spread and pathogenic effects [5].

1.1.2 Early B cell development

B cells originate from hematopoietic precursor cells in the bone marrow (BM), where they receive signals from certain hematopoietic niches, composed of BM stromal cells expressing the ligand CXCL-12 (signaling through its receptor CXCR4) and IL-7 (signaling through the IL-7 receptor) [6]. B cells complete their maturation through several gene activation steps, of

which some of the most crucial lead to assembly of the BCR [7]. The specificity of every B cell clone is enabled through rearrangement of variable (V), diversity (D) and joining (J) segments of the germline DNA to an enormous diversity of possible heavy chains, and the similar rearrangement of V and J segments of the light chains [7, 8]. The constant regions initially contain information to code for more than one heavy or light chain, but become fixed throughout the maturation and differentiation process (discussed below). The process of gene recombination is orchestrated by the proteins *recombination-activating gene* 1 and 2 (Rag-1 and Rag-2), that together form the V(D)J recombinase complex [1].

The first recombination of Ig genes occurs in the precursor called pro-B cell, committed to the B cell lineage based on its expression of the B cell-restricted molecules CD19 and CD10 [1]. The production of the V(D)J recombinase complex induces assembly of the μ type of Ig heavy chain, which together with Igα, Igβ, and proteins similar to light chains (surrogate light chains), composes the pre-BCR and defines the pre-B cell stage [6]. Ag-independent pre-BCR signaling leads to subsequent formation of light chains, that together with the μ heavy chain constitutes a BCR of IgM type, which defines the immature B cell stage. Pre-BCR and BCR signaling are the main conveyors of survival and proliferation signals to B cells, which are mediated through *Bruton's Tyrosine Kinase* (BTK). As only self-Ags are available in the BM, too high BCR binding will result in re-editing through activation of Rag genes, or to apoptosis [1]. After leaving the BM, the immature B cells enter the process of final maturation, in which they are classified into the transitional stages T1, T2 and T3, based on phenotypic changes. This probably occurs in the peripheral blood in humans [9], and includes increase of CD21 expression, loss of CD10 and CD5, and alternative splicing of the Ig heavy chain, which leads to dual expression of IgD and IgM [1, 10-12].

After maturation, a subgroup of B cells populates the marginal zone (MZ) of the spleen and subcapsular areas of lymph nodes, and lowers its IgD expression. These are MZ B cells, and are semi-equivalent to innate cells due to their ability to respond with early and less specific Ab production [13]. The majority of mature B cells will however circulate through the blood stream and secondary lymphoid organs (SLOs), and are called follicular B cells or recirculating B cells [1, 7]. The mechanism for commitment to either lineage is not fully elucidated, but signaling through toll-like receptors (TLRs) and lower BCR affinity probably favors MZ B cell development [13]. B-1 cells are an additional type of B cell with similar function as MZ B cells, but developed through a different pathway in fetal liver, and located in the peritoneum and mucosal sites. Both B-1 and MZ B cells were earlier considered a murine feature, but there is now convincing evidence for their existence in humans [1, 13-16].

1.1.3 Homeostasis of mature B cells

The mature naïve B cells are dependent on survival signals generated by Ag binding of the BCR, and of ligation of the Tumor Necrosis Factor (TNF) family of receptors by *B cell activator of the TNF-α family* (BAFF) [7]. BAFF is produced by many cell types, most

importantly from myeloid cells such as monocytes, and can bind to three receptors. The most widely expressed, BAFFr, appear first on immature B cells in the BM, and are expressed on all B cells except for PCs [17]. The second receptor, *transmembrane activator, calcium modulator, and cyclophilin ligand interactor* (TACI), is found on all peripheral B cells, most notably on MZ B cells. The third receptor, *B cell maturation antigen* (BCMA), is upregulated at the later stages of B cell differentiation, most pronounced on PCs [18]. The latter two receptors can also be ligated by the homologous ligand *a proliferation-inducing ligand* (APRIL). Stimulation of these receptors is crucial for providing survival signals to mature B cells, and aid throughout further differentiation [7].

1.2 B CELL ACTIVATION AND ANTIBODY PRODUCTION

B cell activation is initiated through a combination of Ag encounter and auxiliary signals, leading to a cascade of events with the final goal of PC differentiation and Ab production. Later in the response, PCs produce Abs with enhanced affinity through the process of *affinity maturation*, and a variety of Ab isotypes are secreted after induction of *class switch.* The B cell response to non-pathogenic Ags is beyond the scope of this thesis, and this chapter will focus on protection from pathogens.

1.2.1 Ag encounter

Invading pathogens will encounter MZ, B-1 and follicular B cells on their respective locations in SLOs or peritoneum, where the naïve (Ag-inexperienced) B cells bind to their specific Ag. To become activated, several BCRs need to be co-localized in a process that is not completely understood, but is thought to involve either cross-linking of receptor monomers or dissociation of oligomers [2, 19]. Effective co-localization of BCRs is enhanced if the Ag has multiple binding sites (epitopes), hence classified as multivalent, compared to monovalent Ags with only one single epitope [20]. In addition, the B cells need secondary signals, traditionally classified into thymus independent (TI) and thymus dependent (TD), based on T cell involvement. Recent studies have provided evidence that TI Ag responses are more complex than previously assumed, involving activation signals from both innate-like lymphoid cells such as iNKT cells, and innate cells such as monocytes and basophils [21]. Numerous studies have also shown that the classification of Ag responses is more faceted than previously known, leading some researchers to suggest a shift of the TI versus TD paradigm [22]. However, the B cell response to Ag encounter will here be presented using the established nomenclature.

1.2.2 TI Ag responses

Non-protein Ags cannot be presented to T cells, and are therefore classified as TI Ags. MZ and B-1 B cells are the first to encounter incoming Ags, due to their location, and are the foremost executors of the TI B cell response [13, 22]. They receive secondary signals through the B cell co-receptor complex, constituted by *complement receptor 2* (CD21), CD19 and CD81 [1, 23]. This enhances the signal evoked by the Igα and Igβ parts of the BCR, together leading to

phosphorylation of the Src family of kinases [1]. Ligation of TLR5, TLR7 and TLR9, recognizing *pathogen-associated molecular patterns (PAMPs)*, can further augment the signal strength [23]. Recently, it has been shown that BCR signals are dependent on CD19, also without the co-receptor complex, to induce the clusters of receptors that are needed for signal transmission [23]. TI responses are only possible towards Ags that yield adequate TLR signals (TI-I Ags, for example viral RNA) or are multivalent, leading to ample BCR signaling (TI-II Ags, for example bacterial polysaccharides). Recently, a TI-III type of response, that is elicited towards non-protein Ags with the help of myeloid innate cells, has also been discovered [22].

Following downstream signaling of a TI response, the B cells activate the transcription factors Blimp-1 and IRF4, which in turn induces expression of XBP-1, important for handling of increased protein production. This initiates a differentiation process that also includes cell size increase and alternative processing of μ heavy chain RNA, leading to soluble IgM production [1]. The finally matured cells are called short-lived PCs (SLPCs), and produce Abs for approximately 3 days before cell death [24]. New SLPCs are produced as long as the Ab response continues, but there is also some evidence of memory induction (see chapter 1.3.3). Even though some of the cardinal features of the TI response are low affinity Abs and limitation to IgM production, it is becoming more evident that affinity maturation and class switch can be induced also without T cell help, preferentially in TI-III responses [13, 15, 22].

1.2.3 TD Ag responses

TD Ag responses are mainly attributed to follicular B cells, in a reaction that occurs in the B cell areas, follicles, of SLOs. As the B cells enter SLOs through blood vessels, they migrate towards the follicle due to a gradient of stromal cell-secreted CXCL-13, binding to CXCR5 on their surface [1]. Residing follicular dendritic cells (FDCs) capture and present incoming protein Ags to B cells, but do not provide sufficient secondary signals for full activation. A successful TD response is elicited only if the B cell engulfs the Ag for presentation to T cells, albeit TLR and CD21 signaling do have an augmenting role [25, 26]. In addition, the strength of the TD response is enhanced if the protein Ag is multivalent (see chapter 1.4.2)

T cell encounter is facilitated through expression of CCR7, which directs the B cells towards the edge of the follicle by the T cell zone chemokines CCL19 and CCL21. T cells, that are activated by the same Ag (defined as cognate T cells) and express the chemokine CXCR5, in turn migrate towards the follicle [1]. B cells present the Ag via MHC II molecules, binding to the T cell receptor (TCR), but the cross talk also involves a variety of co-stimulatory molecules, which are upregulated upon activation. Some of the most important T cell-expressed molecules are CD28, CD40 and *inducible T cell co-stimulator* (ICOS), binding in respective order to CD86, CD40-ligand and ICOS-ligand on B cells. T cells also secrete soluble IL-21, binding to the IL-21 receptor [27, 28].

The B and T cell interaction, augmented by signals from FDCs, leads to differentiation of T cells into follicular helper T cells (T_{FH} cells) through expression of the transcription factor Bcl-6 [29]. T_{FH} cells in turn drive the further B cell differentiation, either to become extrafollicular SLPCs, by expression of Blimp-1, or to proceed into a germinal center (GC). The latter requires a halt in the PC differentiation process, facilitated by B cell expression of Bcl-6, which inhibits Blimp-1. The mechanisms for up-regulation of Bcl-6 are not known, but it is thought to be dependent on a competition between B cells with high and low affinity BCRs [27, 28, 30, 31].

1.2.4 The GC reaction (figure 1-2)

The GC reaction is initiated as the B and T cells migrate back to the B cell follicle, now defined as secondary follicle. It is most commonly formed 4-7 days after initiation of the response, and can persist for weeks or months depending on the Ag [1, 32, 33]. Bcl-6 expression facilitates continuous cell cycle entry, leading to rapid B cell proliferation. This occurs within the dark zone of the GC, where abundant levels of the ligand CXCL-12 maintain B cells that express CXCR4 [34]. Cells expressing higher levels of CXCR5 migrate towards CXCL-13 in the light zone, where FDCs present the Ag and induce BCR signaling, and T_{FH} cells provide auxiliary signals [30]. B cells with low affinity BCRs and low expression of co-stimulatory receptors will eventually die by apoptosis due to lack of survival signals [27, 28], and lack of prevention from apoptosis induced by the death receptor Fas [35]. Also, signals from the receptor PD-1 on T_{FH} cells, binding to the ligands PD-L1 and PD-L2 on B cells, stimulate survival and maturation in the GC [36]. Recirculation between the light and dark zone enables an iterative process of selection, leading to gradually increased affinity of the BCRs (affinity maturation) for each round of mutations that occurs [27, 28]. This is further enhanced by induction of somatic hypermutation (discussed below), and a gradual decrease of Ag availability, strengthening the selection pressure on binding affinity.

B cells within the GC are sometimes defined as either centroblasts (proliferating cells in the dark zone), or more mature centrocytes (undergoing selection process in the light zone). However, the growing understanding of the dynamic process of cells moving between the zones, and the lack of phenotypic markers for either cell type, has led to a questioning of this terminology [28].

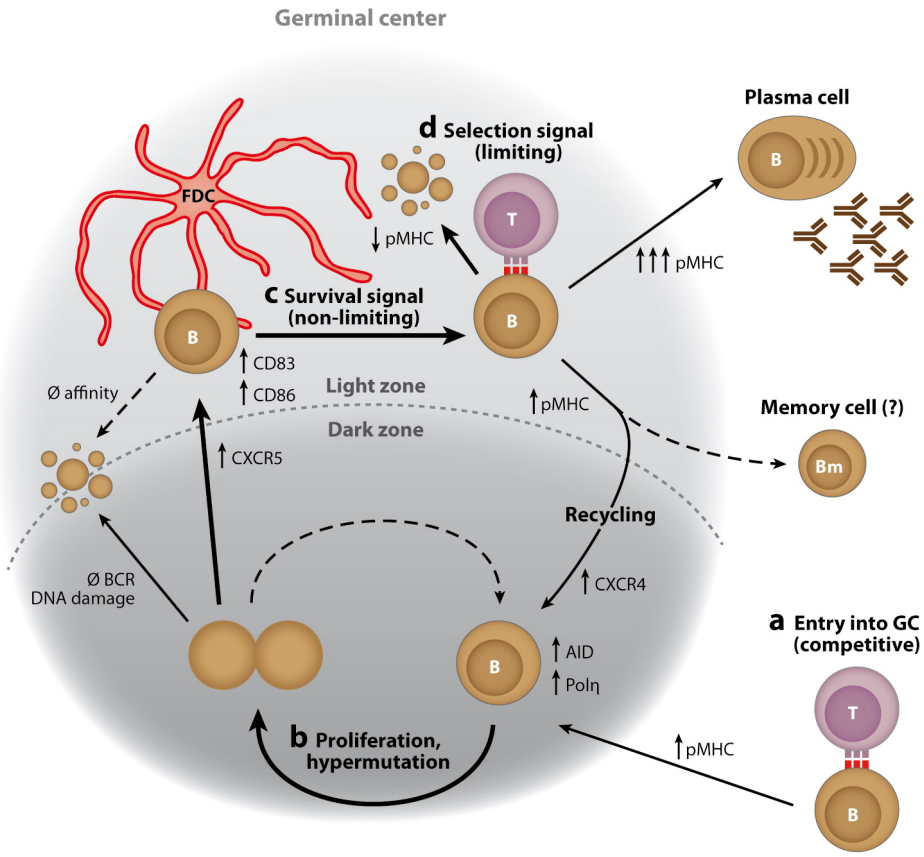

Figure 1-2. Illustrated model of the germinal center (GC) reaction. B cells enter the dark zone, based on competition for T cell interaction, where they proliferate and mutate at an extremely high rate. The cycling between the dark and light zones is guided through concentrations of the cytokines CXCL-12 and CXCL-13, and their ligands CXCR4 and CXCR5. Survival signals are provided in the light zone both from follicular dendritic cells (FDCs) and follicular helper T cells (T_{FH}), fine-tuning the B cell selection based on both B cell receptor affinity and access to T cell help. Cells that are not killed by apoptosis exit the GC as memory B cells, or as plasma blasts, later maturing to plasma cells.
Image source: [28], printed with permission from Annual Reviews.

1.2.4.1 Somatic hypermutation

Somatic hypermutation is defined as the extreme rate of mutations induced in proliferating B cells in the dark zone of the GC. It is initiated by the enzyme *activation-induced deaminase* (AID), triggered by T cell signals, most importantly CD40 [1]. AID targets recombined V regions in the Ig coding sequence, leading to deamination of deoxycytidine into deoxyuridine and a C-G mismatch [37]. Depending on the type of DNA repairing enzyme, the DNA damage can induce both point mutations into T-A base pairs, or extended mutations due to error-prone repair [37]. This series of events utterly induces mutations at a rate up to 1000 times higher than in normally replicating cells [1], providing a higher probability of generating clones with a new affinity of the BCR. The rate of somatic hypermutation in extrafollicular sites is very low, but has been shown to increase in certain autoimmune contexts [38].

1.2.4.2 Class switch recombination

Exchange of the Ig μ and δ heavy chains to α, ε or γ defines class switch from the IgM isotype, to IgA, IgE or IgG. Naïve B cells have constant DNA regions coding for each of the isotypes, and cytokines from helper T cells and other sources (table 1-1) induce transcription of one of the isotype-specific DNA loci, producing a germline transcript. This acts in an unknown way to facilitate the next rearrangement [39], in which AID targets specific switch regions adjoining both the μ and germline loci. Due to numerous tandem repeats of GC-sequences, these regions are particularly vulnerable to the deaminase activity by AID, and subsequent action by the enzyme uracil DNA glycosylase (UNG) leads to double strand breaks in the DNA code [1, 39]. The two switch regions are then joined together, deleting the intervening DNA and fixing the B cell isotype to a new heavy chain. Appearance of switched B cells before establishment of GCs [40], and the notion that prevention of GC formation did not eradicate switched cells [32], led to the conclusion that class switch is induced upon the first extrafollicular cognate contact between B and T cells, before creation of a secondary follicle. However, class switch still occurs throughout the GC reaction [24].

1.3 B CELL MEMORY

One of the major outcomes of an immune response is the generation of immunological memory, conveyed by both B and T cell specific mechanisms [41]. The details of the T cell memory compartment is however beyond the scope of this thesis, which will focus on the different layers of B cell memory.

1.3.1 Two lines of B cell memory

The first line defense of B cell memory is constituted by constantly circulating Abs against previously encountered Ags, able to elicit effector functions (table 1-1) at an early stage of pathogen invasion [42]. If the neutralizing effect is insufficient to protect the host, there is initiation of the second line defense, consisting of dwelling memory B cells (MBCs) that rapidly become activated and differentiate into Ab-producing SLPCs. The half-life of an IgG molecule is estimated to be 21-28 days and for long, the origin of circulating Abs against Ags that were encountered many years ago was an unresolved issue in immunology [1]. However, recent advances have confirmed the existence of long-lived PCs (LLPCs) located in the BM and mucosal sites, constantly secreting Abs [43]. Both LLPCs and MBCs demonstrate an extraordinary longevity independent of recurrent Ag stimulation, proven by the fact that both Abs and MBCs can be found in individuals vaccinated against smallpox more than 50 years ago, despite the impossibility of Ag re-encounter [44]. Previous hypotheses suggesting preserved Ags in various reservoirs have now been abandoned, due to accumulating evidence of Ag-independent Ab preservation [42, 45].

1.3.2 Generation of LLPCs and MBCs against TD Ags

The exit of B cells from the GC reaction gives rise to both MBCs and LLPCs, but the signals regulating which fate remain elusive [27]. The affinity to the Ag is proposed as the main determinant, since the MBC compartment contains cells with both high- and low-affinity BCRs, whereas the PCs produce uniformly high affinity Abs [46]. Selection of high-affinity GC B cells with PC potential probably occurs early in the GC reaction, given the early expression of Blimp-1 (see chapter 1.2.2) in some B cells [28, 46]. The mechanism behind this selection remains to be shown, but it is clear that increased T cell interaction through CD40 and MHC II-TCR ligation favors the PC pathway [28, 32]. Expression of Blimp-1 leads to formation of plasma blasts, which exit from the GC most probably due to T cell produced IL-21 [28, 30]. They subsequently migrate to SLOs and BM to complete their final maturation into SLPCs or LLPCs (see chapter 1.3.8).

In contrast to PCs, MBCs seem to be formed throughout the entire GC reaction, and benefit from prolonging GC sustention but less from specific T cell help [28, 30]. They continue to express Bcl-6, keeping them within the GC reaction, until they utterly leave the GC by largely unknown mechanisms. Possibly, IL-21 is involved also in MBC exit [1, 28]. The fact that the first MBCs appear in the periphery before LLPCs has also led to an alternative hypothesis of a temporal division, suggesting that MBCs are formed early, and LLPCs late, in the GC reaction. The peak LLPC production occurs 4-5 weeks after Ag encounter [47].

There is increasing evidence that MBCs and LLPCs form also in extrafollicular sites, albeit with a lower selection pressure and hence having lower affinity [32, 48]. This can possibly explain existing TD Ag-specific MBCs with limited or no hypermutation in their V regions [32], and the presence of an IgM-expressing subgroup of MBCs [49].

1.3.3 TI B cell memory

Although numerous observations in both mice and humans stress the importance of T cell help for a robust B cell memory initiation, existence of a TI B cell memory is virtually confirmed, and might be an additional source of IgM+ MBCs. Several experimental models have shown mounting of LLPCs and MBCs against TI-I and TI-II Ags [22, 50] in the absence of T cell help. Though, the most intriguing proof of TI memory in humans is the existence of somatically mutated IgM+ MBCs in patients with genetic defects in CD40-Ligand, ICOS or the adaptor molecule SAP, all three independently leading to absent GCs [51]. The mechanisms behind TI B cell memory need more exploration, but probably involve activation of MZ and B-1 B cells, and help of other innate-like lymphoid cells [22]. The resulting MBCs are sometimes classified as innate-like MBCs [52].

1.3.4 Heterogeneity of the MBC compartment (table 1-2)

The pre-conceived notion that MBCs are isotype-switched and generated from GC responses, led immunologists to define the B cell developmental stages through the expression of IgD (lost

upon switching) and the signaling molecule CD38 (generated upon activation), to the Bm1-Bm5 stages [53]. This model postulates loss of IgD to define all memory stages, and can thus not be used to identify non-switched MBCs. The discovery by Klein et al that the TNF receptor family protein CD27 is expressed on B cells that have undergone V recombination [54], resulted in a new classification based on IgD and CD27 expression, defining MBCs as CD27+, divided into an IgD+ non-switched and an IgD- switched population [53]. It was however clear that a subpopulation of B cells lacked both markers, thus not falling into the definition of either naïve or MBCs, and defined as "double negative" B cells. These were shown to have memory properties, and to be increased in patients with the autoimmune disorder Systemic Lupus Erythematous (SLE), especially in active disease [55, 56]. Characterizing this population further, Jacobi et al showed that a subpopulation of these cells displayed an activated phenotype that correlated with disease flares, and could be identified using the "death receptor" Fas (CD95) [57].

Another important classification of heterogeneous MBC subpopulations has been done in individuals infected with Human Immunodeficiency Virus 1 (HIV-1), in whom Moir and colleagues found that CD27+ MBCs down-regulated CD21 upon activation and differentiation into Ab-producing PCs, and suggested the name "activated MBCs" for the CD27+ $CD21_{low}$ B cell phenotype [58, 59]. Meanwhile, parallel investigation of human tonsils revealed CD27- B cells, showing memory properties, that lacked expression of CD21, and expressed the putative inhibitory receptor *Fc-receptor-like 4* (FcRL4) [60]. FcRL4 was also confirmed on circulating CD27- CD21- MBCs in HIV-1 infected individuals, and this subpopulation was consequently named "tissue-like MBCs" [61]. They displayed an unresponsive phenotype, expressing inhibitory receptors such as CD22 and LAIR-1, and were suggested to be a result of immune exhaustion [62], in contrast to the activated CD27- cells in SLE (lacking expression of FcRL4) [55]. CD21- CD27- MBCs are likewise enriched during primary CMV infection [63] and in individuals chronically infected with hepatitis [64], and malaria [65], but have also been found in patients with Sjögren's Syndrome [66]. Weiss et al suggested the name "atypical" instead of "tissue-like" for the CD21- CD27- phenotype in malaria, given the fact that their function is less explored and not verified as exhausted, and this terminology is used in parallel [65].

			IgD/CD38 MBC classification					CD27/IgD MBC classification					CD27/CD21 MBC classification				
	Function	**Transi-tional B cells**	**Bm1: virgin naïve**	**Bm2: activated naïve**	**Bm3-Bm4: GC cells**	**Bm5: memory**	**Plasma blasts**	**Mature naïve**	**Non-switched memory**	**Switched memory**	**Double negative**	**Plasma blasts**	**Mature naïve**	**Resting memory**	**Activated memory**	**Tissue-like/atypical memory**	**Long-lived plasma cells**
CD10	Metallo-proteinase with unknown significance	+*	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CD19	Part of BCR co-receptor complex	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+/-
CD21	Complement receptor 2, ligated by Cd3	+**	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	+	+	-/low	-	
CD27	Induces Ab secretion and differentiation	-	ND	ND	ND	ND	ND	-	+	+	-	++	-	+	+	-	++
IgD	Early BCR, not secreted	- **	+	+	-	-	-	+	+	-	-	-	+	+/-	+/-	+/-	-
IgM	Early BCR, secreted as Ab	+	+	+	+/-	+/-	+/-	+	+	-	+/-	-	+	+/-	+/-	+/-	+/-
CD38	Proliferation or inhibition depending on maturation stage	+	-	+	+	-/low	++	-	+/-	+/-	+/-	++	-	-/low	ND	+	++
CD138	Adhesion molecule	-	-	-	-	-	ND	-	-	-	-	+/-	-	-	-	-	+/-

ND = not determined, BCR = B cell receptor, MBC = memory B cell, Ab = Antibody; * decreasing with maturation, ** increasing with maturation

Table 1-2. Selected surface protein expression on the most important B cell populations. Three modes of memory B cell (MBC) classification is presented: the Bm1-5 classification, based on expression of IgD and CD38, the CD27/IgD classification, and finally the classification suggested by Moir et al, based on expression of CD27 and CD21. References: [53, 62, 67, 68]

1.3.4.1 The role of CD27 on MBCs

The ligand of CD27 is the TNF-like molecule CD70, which is transiently expressed on activated B, T and dendritic cells, and act co-stimulatory on CD27-expressing T and B cells. CD70 can also induce downstream activation on its host cell upon ligation [69]. Even before the confirmation of CD27 as a marker for V recombination, functional studies revealed that CD27+ B cells were superior in producing IgG, an effect that was more pronounced after addition of CD70 [70]. Further *in vitro* studies confirmed that soluble CD70, with addition of the cytokines IL-2 and IL-10, promoted differentiation of CD27+ MBCs into Ab-secreting cells [71]. Coherent with these findings, tissue-like MBCs lacking CD27 were proven to be less proliferative and prone to secrete Abs [61]. However, given the prominent expression of inhibitory receptors on these cells, and their parallel loss of co-stimulatory CD21, the relative contribution of CD27 down-regulation to their impaired response is yet to be determined.

An alternative way for B cells to loose CD27 surface expression is through proteolytic cleavage of the putative ligand-binding domain, producing the soluble form (sCD27). This molecule is increased in serum of HIV-1 infected individuals, as well as in and other immune disorders and malignancies [72, 73], and its role has been addressed in a number of studies. B cells from patients with the B cell malignancy Waldenström macroglobulinemia upregulated CD40-ligand upon sCD27 ligation to CD70 [74], and experimental ligation of CD70 on the surface of activated B cells has been shown to induce proliferation in mice [75]. Somewhat contradictory, another outcome from the murine model was inhibited PC differentiation and IgG production [75], suggesting a dual role for the CD27-CD70 cross talk, possibly different in mice compared to humans, and/or in normal compared to pathological conditions.

1.3.5 The pediatric MBC compartment

The majority of knowledge about B cell development and function is derived from studies in adult murine and human models, and existing differences in children is insufficiently elucidated. In addition to B cell function, changes in the T cell compartment and innate immune system contribute to changes in the overall B cell response. A full review of the development of the immune system in the growing child is beyond the scope of this thesis, but some points regarding the MBC compartment need to be addressed.

Firstly, the relative proportion B cells of the lymphocyte population is approximately 20 % at birth, and then successively declining until it reaches the adult level of 10 % at the age of 16 [76, 77]. Secondly, Ag exposure is as a rule initiated after birth. Thus, the majority of fetal B cells are of naïve type, and the MBC compartment is gradually expanded with increasing age [77, 78]. This also accounts for class switched cells, as demonstrated by the fact that IgG+ B cells are 70% and IgA+ 30 % of adult levels at the age of 1 year [78]. Furthermore, the proportion of transitional B cells out of the total B cell population is notably increased in young children (>10 % and up to 30 % in one study), reaching adult levels (< 5 %) after the age of 16 [76, 77]. In addition to quantitative changes, the TI Ag response is suboptimal in

children below 2 years of age, probably due to reduced expression of CD21 and incomplete maturation of the MZ of the spleen [78].

1.3.6 Survival of MBCs

Several mouse and human studies have aimed to localize a spatial compartment for maintenance of MBCs, but this remains elusive. Splenectomized humans have reduced, but not diminished, MBCs, suggesting redundant sites of maintenance [45]. MBCs have been found outside follicles, such as in the MZ or adjacent to GCs in the spleen [79], and in mucosal epithelium in human tonsils [80]. The number of MBCs that is found in peripheral blood is probably 100 times less than in the spleen [80]. The current consensus is that MBCs are preserved mainly in multiple SLOs, and that a proportion recirculates in the periphery [32].

In contrast to naïve B cells, MBCs are independent on both BCR and BAFF/APRIL stimulation [18, 45]. They seem to have a faster proliferation rate than naïve B cells [81], thus probably maintained through continuous turnover rather than being long-lived. Despite this, they do not express Bcl-6, known as an important inducer of proliferation [80]. The major MBC survival signal identified is the anti-apoptotic molecule Bcl-2 [32], and also telomere elongation is of pivotal importance [45]. The details behind their maintenance is however to a large extent unknown.

1.3.7 Re-activation of MBCs

To produce specific Abs, MBCs have to receive specific BCR stimulation and go through a new activation process, involving up-regulation of Blimp-1 and differentiation to SLPCs [43]. The reasons for their efficient and swift response compared to naïve B cells are several. Primarily, the clonal expansion during the GC response generate a repertoire of MBCs specific for previously encountered Ags, in contrast to the stochastic production of naïve B cell receptors. The strategic location of MBCs outside follicles promotes fast encounter with incoming Ags, and efficient downstream signals are yielded by affinity matured BCRs. Furthermore, they have a high expression of TLRs and CD21, enabling effective activation, and already express important T cell activation receptors, such as CD86 and MHC, that prepare them for a rapid response upon T cell help [80]. Ag-primed T_{FH} of memory type are feasible candidates for the T cell part of the recall response [27]. There is also recent evidence for secondary GC formation by a subgroup of IgM+ MBCs, leading to a second layer of GC response upon re-challenge with an Ag [33]. This might be one explanation for the increased formation of LLPCs that is noted after a booster immunization [27].

1.3.8 Survival of LLPCs

The most intriguing proof that MBCs are dispensable for continuous Ab secretion has been studies in both mice and humans showing that depletion of all B cells before the PC differentiation stage did not affect previously acquired Ab titers [82]. In addition, there have been numerous observations of vaccine-specific PCs in human BM [83]. After differentiation

and exit from the GC, Ab-secreting plasma blasts upregulate the surface expression of CXCR4 and migrate towards CXCL-12 secreting cells in BM and extrafollicular sites of SLOs [83-85]. Since CXCL-12 also is abundant in the GC, there are probably additional migratory factors involved [85]. As the plasma blasts have reached the final maturation sites, they stop proliferating and acquire the cell adhesion proteoglycan CD138, reaching the mature PC stage [86, 87]. PCs in SLOs are short-lived, demonstrated by high apoptosis rate, but there is some evidence of prolonged survival of PCs also in the spleen [88] and inflammatory sites in SLE [89]. Furthermore, long-lived IgA secreting PCs are partly maintained in mucosal areas [90, 91]. However, the uttermost niche for PC survival in humans is the BM [83-85], recently demonstrated in a study in which vaccine-induced serum Abs strongly correlated to corresponding BM-resident PCs (BMPCs) [92].

1.3.9 PC survival factors and composition of the BM niche

PCs generally loose expression of the BCR, with the exception of IgA-switched PCs, and are thus independent of Ag for survival signals [93]. Instead, they are dependent on a variety of nursing cells that provide necessary membrane-bound and soluble survival factors. The most pivotal intracellular signaling molecule identified for PC survival is Mcl-1, induced by binding to the BCMA receptor (see chapter 1.1.3) [32, 84, 85]. Murine studies have concluded that the major BCMA ligand in the BM is APRIL, but this remains to be shown in humans [94]. Other PC receptor-ligand pairs of importance for survival are IL-6R-IL6, CD28-CD80/CD86, and CXCL-12-CXCR4, as well as direct contact to the adhesion molecule VCAM-1, bound to VLA-4 on PCs [84, 85]. Binding of hyaluronic acid from extracellular matrix to CD44 gives a pronounced survival potential *in vitro* [95], but this finding is yet to be confirmed *in vivo* [85].The necessary signals are provided within the BMPC survival niche (figure 1-3), recently characterized in more detail as a combination of resting stromal cells, and proliferating hematopoietic cells [96]. The stromal cells in the BM constitutes < 1 % of the total BM cellularity, and include a variety of terminally differentiated mesenchymal stem cells of reticular character. It has been estimated that approximately 17 % of all BM stromal cells are specialized for PC survival, defined by the fact that they do not produce the B cell maturation cytokine IL-7, but instead express high levels of CXCL-12 and VCAM-1 [83]. These stromal cells are called *CXCL-12-abundant reticular cells* (CAR cells) [97]. The other mentioned survival factors are provided by the hematopoietic part of the niche. CD80 and CD86 are common activation ligands that are expressed on dendritic cells, including in the BM [98]. The majority of APRIL and IL-6 is estimated to be produced by megakaryocytes [99] and cells of myeloid origin, of which half are eosinophils in different developmental stages [97, 100]. Also basophils have been connected to increased BMPC survival by means of IL-6 production [101]. One model has suggested that the major role for CAR cells is to provide the overall structure that brings the essential nursing cells together [97], but this is not yet confirmed. Further research in both animal models and human samples is needed to fully understand the traits of the BMPC niche.

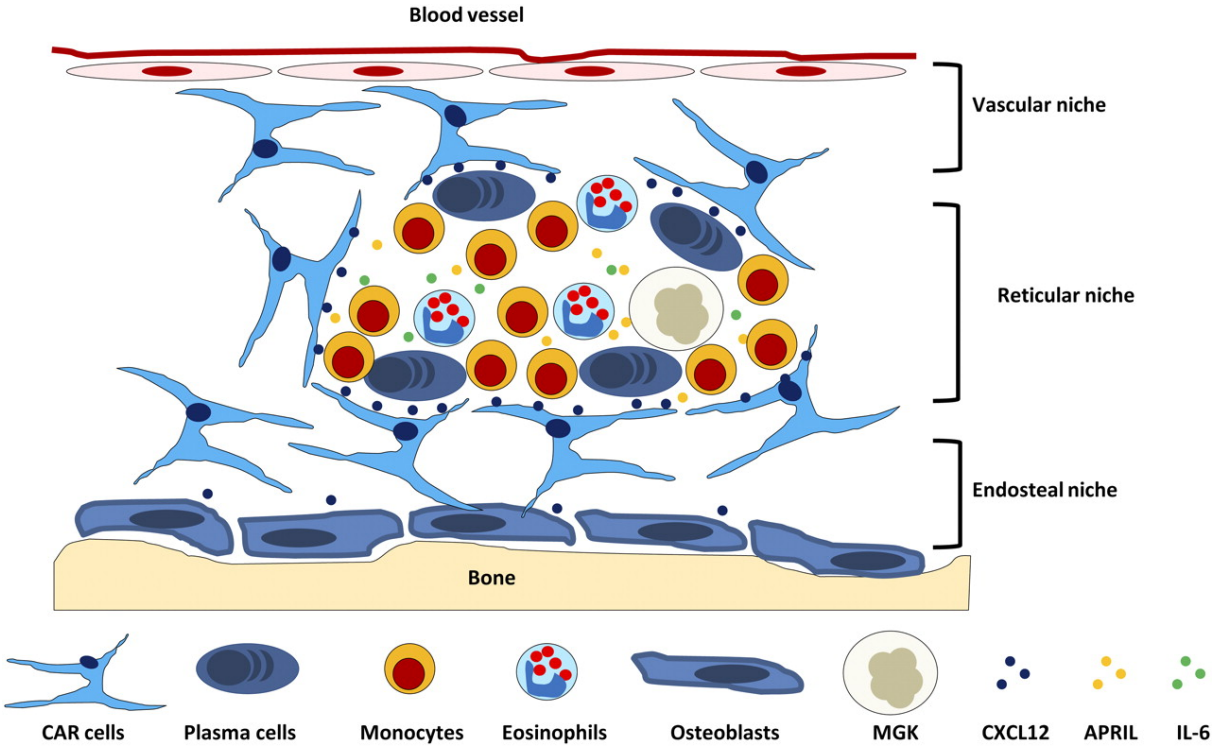

Figure 1-3. One model suggested by Belnoue et al, summarizing the current understanding of the bone marrow niche for long-lived plasma cells. Static CXCL-12-abundant reticular cells (CAR cells) of mesenchymal origin create the reticular network behind the niche structure, and provide CXCL-12. The proliferating hematopoietic cells within the niche secrete the additional soluble survival factors APRIL and IL-6. Not shown in the figure are the activation ligands CD80 and CD86, provided by dendritic cells, and the adhesion molecule VCAM-1, provided by CAR cells. MGK = megakaryocytes. Image source: [97], printed with permission from the Journal of Immunology. Copyright 2012. The American Association of Immunologists, Inc.

1.4 VACCINES

The first published description of how deliberate inoculation of an Ag induced immunity against a disease came out in 1798, when Edward Jenner described how insertion of pustule fluid with the virus Variolae Vaccinae (Cow Pox) protected from Small Pox inoculation [102]. Since then, we have begun to understand the mechanisms behind the generation of protective long-term immunity, which has enabled the design of new and more efficient vaccines against a variety of pathogens. Evaluation of vaccine efficacy has revealed that both T and B cell immunity is crucial for vaccine function [103]. However this thesis will focus on the B cell part of vaccine-induced memory.

1.4.1 Vaccine design

The three most crucial steps of inducing a long-term B cell memory are to (i) present an exposed epitope on the pathogen surface, (ii) to stimulate the innate immune system in order to provide secondary activation signals to B and T cells, and (iii) to evoke a TD immune response, which has a higher potential than TI responses to generate MBCs and LLPCs [1]. There are various strategies to reach the three goals, of which the first was attenuating or killing whole pathogens, used as soon as cultivation of viruses *in vitro* became possible [103]. Attenuated live vaccines containing replicating virus or bacteria are the most effective imitators of true infection, thereby powerfully inducing the mentioned mechanisms. In contrast, inactivated or killed pathogens fail to yield innate responses, and a general immune stimulus (adjuvant) is included in these vaccines [20, 104]. Various artificial adjuvants can be

used, such as aluminum salt that releases danger signals from host cells, but also TLR-stimulating viral proteins and bacterial subunits have been approved [104]. The discovery that the protective Abs often are directed towards selected strategic sites led to the idea of using subunits of pathogens as epitopes, which has been a successful method when using protein Ags. However, the bacterial polysaccharide capsule cannot be presented to T cells, and only induces a TI response. This lead to the development of conjugate vaccines consisting of polysaccharide epitopes with an attached immunogenic peptide. The BCR reacts against the polysaccharide, and can in addition present the peptide part to T cells and receive the auxiliary signals [1].

1.4.2 Vaccine longevity

In general, vaccination gives a limited immune activation, and it is well conceived that the immune response after infection is impossible to achieve with vaccines without compromising with safety [20]. Amanna and Slifka have suggested that the plateau of induced Ab titers after a TI response is long-lived irrespective of the strength of induction. However, a weaker immune response produces fewer LLPCs, resulting in a lower and potentially not protective Ab level [105]. A booster vaccination with the same Ag will not only induce a short-term activation of MBCs, but also generate secondary GCs and increase the number of specific LLPCs [33], setting the Ab production to a higher level [20]. The key to an extensive LLPC pool is probably coupled to the quality of the preceding GC response, and the B-T cell interactions within it. By comparing vaccines with various capacity of generating long-term response, vaccine Ags have been classified into three types based on the strength of the T-B cell interaction (figure 1-4). The most efficient inducers of cell signaling are multivalent protein Ags, which will evoke ample BCR signaling and subsequent strong T_{FH} signaling, in contrast to weaker response towards monovalent protein Ags. The third type used, multivalent Ags that only elicit a TI response, will not induce any long-term immunity [20].

Furthermore, one must not forget that Ab titers are not protective unless they target conserved and crucial parts of the pathogen, and exert a neutralizing action. These aspects have been some of the main obstacles in the development of a functional HIV vaccine [106].

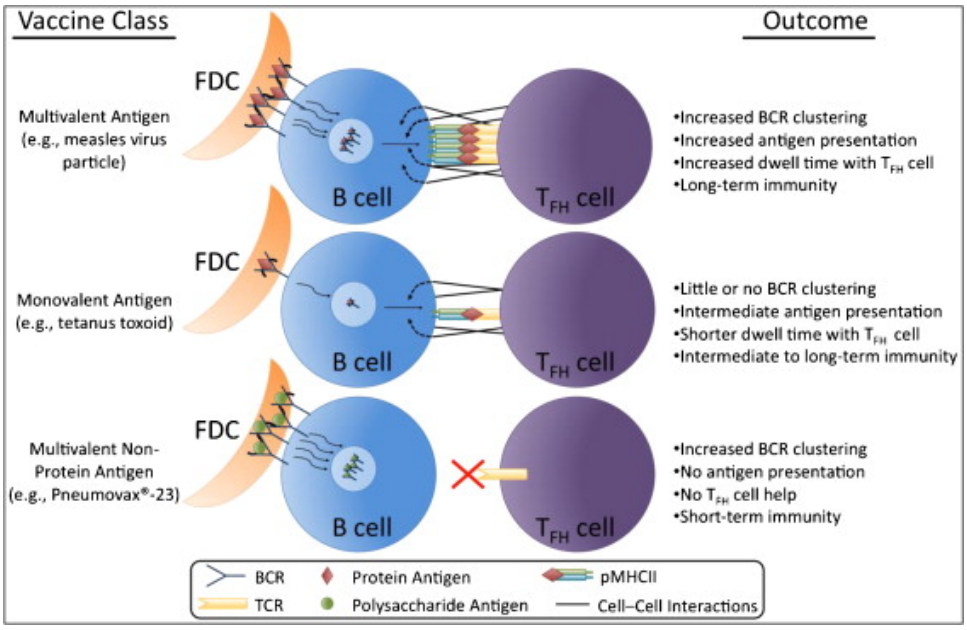

Figure 1-4. Illustration of a putative model for B cell response and memory outcome after vaccination with multivalent or monovalent protein antigens, and a multivalent polysaccharide antigen, as suggested by Slifka and Amanna. Only protein antigens will lead to engagement of T_{FH} cells, and multiple epitopes will increase the cell-cell interaction. A multivalent protein antigen is hence the most potent inducer of long-term immunity.
Image source: [20]. Printed with permission from Elsevier.

1.4.3 Measles and rubella vaccination

Measles virus belongs to the *Morbillivirus* genus of the family *Paramyxoviridae*. It is highly contagious, spread by the respiratory route, and initially causing fever, coryza, cough, and conjunctivitis, where after a maculopapulous rash appears [107]. The most severe morbidity is caused by secondary infections due to measles-induced immune suppression [108], but the virus can also lead to the serious long-term complication subacute sclerosing panencephalitis (SSPE). The overall mortality of measles is around 5-10 % in Africa, but can rise to 25 % without adequate health care [107, 108]. According to the World Health Organization, 145 700 deaths were caused worldwide by measles in 2013, mostly in low-income countries [109]. Though, the reported 2013 incidence in Europe was 10 271 cases [110], proving that the disease still is a concern also in high-income countries.

Rubella virus is the only known member of the *Rubivirus* genus of the *Togaviridae* family. It is spread through respiratory droplets, and causes a generally mild disease with fever, craniocervical lymphadenopathy and maculopapulous rash. Complications include encephalitis and arthritis, but are unusual and rarely fatal [111, 112]. However, infection of pregnant women has a high risk of causing congenital rubella syndrome (CRS), leading to severe malformations, including encephalopathy, microcephaly, hearing loss, cataracts, intrauterine growth retardation, and cardiac anomalies [111]. It is estimated that 110 000 children are born with CRS yearly, making it the largest preventable cause of congenital defects worldwide [113]. 38 847 rubella cases were reported in Europe in 2013 [110].

Both measles and rubella are prevented by vaccination with live, attenuated viral strains, most commonly administered together with mumps virus in what is known as the MMR

vaccination. Its' immunogenic potential is high, and it has an estimated protection rate of 96-100 % in a healthy population after short-term evaluation (60-70 days) [112]. A long-term evaluation in Finland 15 years after administration of a booster MMR dose showed that 95 % had measurable serum Abs against measles, and 85 % had titers above protection level. In the same cohort, 100 % had measurable Abs against rubella, and 93 % were above the protection level [114].

1.4.4 Tetanus vaccination

Tetanus is caused by the gram-positive bacillus *Clostridium Tetani*, commonly through contamination of wounds with bacterial spores. The pathogenic effect is caused by the bacterial toxin, which enters the nervous system through the neuromuscular junction, and is transported to preganglionic inhibitory interneurons. Hampering of neurotransmitter release causes disinhibition of motor neuron discharge, leading to the characteristic skeletal muscle spasms [115, 116]. Mortality caused by airway compromise and later involvement of the autonomous nervous system is estimated to be 52 % in adults and up to 88 % in neonates, in areas with insufficient hospital care [116].

Tetanus is a rare disease in Europe, with only 130 cases reported in total in 2010 [117]. The disease is mainly a challenge in neonatal and maternal care in low-income countries. Recent epidemiological data is scarce, but it is estimated that 49 000 infants died of tetanus in 2013 [118].

Vaccination against tetanus utilizes formaldehyde-inactivated toxin (toxoid) together with adjuvant, often administered together with inactivated diphtheria toxin and various preparations of subunits from Bordetella Pertussis. As is expected for subunit vaccines, Ab titers wane and a booster regimen is recommended to maintain protection [116, 119].

Age	Diphteria Tetanus Pertussis	Polio	Haemophilus Influenzae b	Pneumo-cocci	Measles Mumps Rubella
3 months	I	I	I	I	
5 months	II	II	II	II	
12 months	III	III	III	III	
18 months					I
5-6 years	IV**	IV			
6-8 years					II**
10 years	IV*				
12 years					II*
14-16 years	V**				

* Children born before 2002; **Children born 2002 and later

Table 1-3. The most recent version of the Swedish National Immunization Program, in use since 2009. Since January 1st 2010, a complementary decision recommends all girls to be vaccinated against Human Papillomavirus (HPV) at 10-12 years of age [120]. The table is a translation from publication number *2008-126-9* from *The National Board of Health and Welfare* (Socialstyrelsen) in Sweden. Printed with permission from Socialstyrelsen.

1.4.5 The Swedish National Immunization Program

Vaccination guidelines for the Swedish population are published by *The National Board of Health and Welfare* (Socialstyrelsen). The current vaccine schedule used in Sweden was updated January 1st 2009 to the current regimen (table 1-3) [121]. Children born before 2002 follow the earlier schedule (indicated in the table). Vaccine coverage is generally high, and it was estimated that > 95 % of all children in the 6th grade of school had received full vaccine protection against measles, rubella, mumps, diphtheria, tetanus, pertussis and polio in 2013 [122].

1.5 DISEASES AND PHARMACOLOGY TREATMENTS INTERFERING WITH B CELL MEMORY AND VACCINE EFFICACY

The sophisticated series of events leading to B cell memory induction and preservation is vulnerable to interference from immune disorders and chronic infections. Moreover, non-specific immunosuppressive treatment has the ability to ablate vaccine response, and as well disturb long-term preservation mechanisms. This section covers how selected diseases and treatment regimes affect vaccine-induced B cell memory.

1.5.1 HIV-1

Although the virus does not target B cells directly, a number of B cell malfunctions have been described in HIV-1 infected patients. Even before the connection was made between HIV-1 and the development of Acquired Immunodeficiency Syndrome (AIDS), it was noted that B cells in AIDS patients seemed to be hyperactivated, leading to high levels of circulating Igs (hypergammaglobulinemia) [123]. Also other signs of B cell hyperactivation were later discovered, including increase of surface expression of activation markers, cell turnover, plasma blast differentiation, autoantibody production, and occurrence of B cell malignancies [59]. Despite the increased activation, individuals infected with HIV-1 have impaired induction and preservation of vaccine-evoked B cell memory [124], which is possible to

partly restore by antiviral treatment [125]. As mentioned earlier (see chapter 1.3.4), the MBC compartment in HIV-1-infected individuals show an accumulation of unusual MBC phenotypes, probably due to exhaustion after constant immune activation [62, 126].

The reasons for the polyclonal hyperreactivity have been thoroughly investigated, but remain poorly understood. It has been shown that viral particles can act directly as polyclonal activators, that several B cell activation cytokines, such as BAFF and TNF-α, are increased in serum upon HIV-1 infection [59], and that there is an accumulation of T_{FH} cells, suggested to drive constant activation of low-affinity Abs [127]. Probably, the overall pathogenesis is multifaceted.

1.5.2 Cytotoxic therapy

Chemical agents with cell toxic effects, defined as chemotherapy, generally affect cell proliferation. They are mainly used to treat malignant conditions by targeting rapidly proliferating cells, but can also be used in autoimmune disorders, such as rheumatic disease. These pathological conditions represent two distinct modes of treatment, in which the therapy is given either in a high dose for a limited time, or in a low dose for an extended time span, often without a set date of termination.

1.5.2.1 Cytotoxic therapy in malignant diseases

High-dose chemotherapy in cancer treatment commonly causes severe immunosuppression, by means of targeting proliferating hematopoietic cells in the BM, leading to decreased output of mature immune cells [128]. In addition, numerous studies of children going through treatment of acute lymphoblastic leukemia (ALL) have revealed that pre-existing vaccine-induced circulating Abs can be lost after completion of treatment (reviewed in [129]). The same observation has been done in children after treatment for solid tumors [130], and in adult breast cancer patients [131]. The concomitant effects on specific vaccine-induced MBCs and LLPCs have not been investigated, but a general decrease of the MBC [132, 133] and BMPC [134] numbers has been observed. Moreover, children vaccinated during on-gong chemotherapy treatment have a decreased tendency to mount and/or sustain protective Ab titers [135]. Current recommendations suggest vaccination of children with relevant inactivated vaccines during chemotherapy, and re-vaccination of previously administered inactivated and live vaccines 3 months after treatment completion. The host potential to mount Ab titers shortly after treatment completion is considered adequate, but the supporting data is insecure, and more research is needed [136]. One aspect that is rarely studied is preservation of Abs for long-term, since most evaluations are done within months from the vaccination date. A study by Brodtman et al, examining 100 children that were re-vaccinated after treatment, revealed that several Ab titers were below protection level at follow-up after > 1 year [137]. Furthermore, it is not known if re-vaccination induces full recovery of the MBC compartment.

1.5.2.2 Cytotoxic therapy in rheumatic diseases

The term rheumatic disease covers a variety of conditions, divided into four main groups: inflammatory joint diseases (for example rheumatoid arthritis), degenerative joint diseases (for example osteoarthritis or spondylitis), systemic rheumatic disease (for example SLE), and finally generalized pain syndromes [138]. A full description of the various diseases is beyond the scope of this thesis, and the focus will instead be on the treatment.

The main cytotoxic agent used against rheumatic conditions is the replication inhibitor Methotrexate (MTX). As a folate analogue, it inhibits dihydrofolate reductase (DHFR) and 5-amino-imidazole-4-carboxamide ribonucleotide (AICAR)-transformylase, both important for folate metabolism and subsequent construction of methionine, purine and thymidylate, which utterly leads to disrupted DNA synthesis [139]. As a side effect, accumulated AICAR inhibits adenosine deaminase, leading to increased levels of adenosine. The replication inhibitory consequences are utilized in cancer treatment, but the exact mechanisms behind its beneficial effects in rheumatic disease are not fully elucidated. Probably, a combination of reduced immune cell proliferation and increased concentration of anti-inflammatory adenosine is the dominating cause [139]. Additionally, a skewed T cell repertoire from inflammatory-related TNF-α to regulatory-related IL-10 producing cells has been observed [140].

Both short- and long-term efficacy of vaccination in pediatric patients receiving MTX seem to be sufficient [141] [142], and MTX-treated adult rheumatoid arthritis-patients show preserved GC structure in tonsillar tissue [143]. Moreover, MTX does not seem to have any inhibiting effect on the total MBC frequency in adult [143] or pediatric patients [144]. However, MTX seems to hamper the BM output of newly formed transitional B cells [143, 144], and there are some reports of a slightly decreased total B cell number [144, 145], in both adults and children. The long-term effects of suppressing the BM B cell output during development of the immune system in children are not known.

1.5.3 Other antirheumatic drugs

MTX belongs to the group of *disease-modifying antirheumatic drugs* (DMARDs), so named because of the ability to interfere with the disease process and influence its natural course. MTX is further classified as a synthetic DMARD (sDMARD), along with other chemical compounds, in contrast to the *biological DMARDs* (bDMARDs), that includes targeted monoclonal antibodies and soluble receptors [146]. Current treatment recommendations of Juvenile Idiopathic Arthritis, the rheumatic disease examined in this thesis, suggest initial start with sDMARDs, followed by bDMARDs in case of insufficient effect [147]. The bDMARDs that are most widely used are the TNF-α-inhibtors, a group that includes both a recombinant receptor analogue (etanercept), monoclonal Abs exerting an inhibitory effect on TNF-α binding (infliximab, adalimumab and golimumab), and a humanized IgG4 Fab fragment (certolizumab) with the same mechanism [148]. TNF-α is a cytokine with both pro-inflammatory and immune regulating mechanisms, and has a prominent role in many

autoimmune disorders. Indeed, TNF-α-inhibition has shown a remarkable effect on various disease manifestations [148].

A number of studies have evaluated Ab responses to vaccination in paediatric rheumatology patients with TNF-α-inhibtors, and concluded that the short-term response (6-24 months) is adequate, but that long-term titre preservation (> 2 years) is insecure (reviewed in [141]). Furthermore, Anolik et al has found generally disrupted GC formation in adult rheumatoid arthritis-patients treated with etanercept, probably caused by cross-reactive inhibition of the GC-inducing cytokine TNF-β [143]. One knowledge gap is whether temporary intermission in treatment can boost the vaccine response. Clinical guidelines support this regime upon vaccination with live vaccines, due to the risk of inducing infection, but it has not been evaluated in any larger study [141]. In a study on 5 children with on-going anti-TNF-α treatment, no adverse events occurred when giving an MMR booster, and the Ab titers were adequate after 6 months [149]. However, more research is needed to draw relevant conclusions.

The preservation of previously acquired vaccine-induced memory has not been selectively studied in the context of rheumatic treatment, and it is not known if full-vaccinated children are at risk of loosing protective Ab titers.

1.5.4 Experimental studies on cytotoxic and bDMARD therapy on B cell memory

Selectively pinpointing chemotherapy or bDMARD effects on B cell memory is connected with methodological difficulties in the clinical context. Firstly, both cancer and rheumatic patients display a range of disease-induced immunological abnormalities that possibly interfere with immunological memory [1, 150, 151], and secondly, adjuvant treatments are used concomitantly. Hence, experimental models are needed to conduct studies with a mechanistic approach. Vaccine response and GC formation has, to our knowledge, not been investigated in any animal model for antineoplastic or antirheumatic treatment, but the BMPC compartment has been addressed in a number of studies.

One major feature of LLPCs is that they have exited the cell cycle and do not proliferate [45], putatively leading to chemotherapy resistance. In contrast, one aspect of chemotherapy-induced BM toxicity that is rarely studied is the induced damage on the PC survival niche. As mentioned in chapter 1.3.9, both proliferating hematopoietic cells and resting BM stromal cells (CAR cells) act in concert to support PC survival [96], suggesting that different parts of the niche have various receptivity to cytotoxicity. It is not known if stromal cells can compensate for survival factors that are lost after depletion of hematopoietic cells. In addition, observations in BM transplanted humans indicate that also the BM stroma can be damaged by the excruciating pre-conditioning treatment, leading to impaired support for donor hematopoietic cell survival [152, 153]. Moreover, stromal cells from BM of pediatric ALL patients on maintenance chemotherapy showed reduced capability to support survival of developing hematopoietic cells *in vitro* [154], and experimental toxicology studies in murine

models and *in vitro*-cultured stromal cells have shown reduced survival and cytokine production after exposure to several different cytotoxic agents [155-157]. However, when interpreting this data, one needs to consider that terminally differentiated CAR cells have limited proliferative potential [96], and *ex vivo* cultures of BM stroma taken from patients probably consists of the precursors to CAR cells: mesenchymal stem cells [83]. If damage to mesenchymal cells with stem-cell properties has impact on the already established stromal niche is not known.

The major contribution from the CAR cells in the PC survival niche is suggested to be the cytokine CXCL-12 [85, 158]. The CXCL-12/CXCR4 axis has been under thorough investigation in leukemia research, given its potential to attract leukemic stem cells that can cause relapse, and some specific inhibitors are in trial for therapeutic use [159]. Generalized DNA damage has been shown to induce increased levels of CXCL-12 in BM stroma [158, 160], but also decreased production after MTX treatment has been observed [161]. The reason for conflicting data can be both time and dose dependent differences.

1.6 SUMMARY

Even today, vaccines belong to one the greatest achievements in the history of science. It is thus a fascinating thought that their mechanisms of action remained largely unrevealed for more than a century, and that we still lack fine and yet crucial pieces of the puzzle. It has been said that vaccinology has done more for immunology than vice versa, and this statement is largely true for our current knowledge about how B cells encounter Ags and form a functional immune memory. Taking off from the paradigm of a first line defense of circulating Abs, and a second line defense of MBCs, the aim of this thesis has been to join immunology and vaccinology together, and shed light upon some of the remaining unanswered questions.

2 AIMS OF THE THESIS

The overall aims of this thesis are to elucidate how MBCs and LLPCs contribute to long-term vaccine-induced B cell memory:

- In healthy children and young adults
- In HIV infection and associated B cell deficiencies
- Upon high-dose chemotherapy
- Upon low-dose chemotherapy and TNF-α-inhibition

3 MATERIALS AND METHODS

3.1 ISOLATION OF PERIPHERAL BLOOD AND BM MONONUCLEAR CELLS (ALL PAPERS AND PRELIMINARY DATA)

In order to isolate mononuclear white blood cells from whole blood or BM, the high-density centrifugation technique was used. In brief, 15 ml of sodium chloride solution containing high molecular weight sucrose-polymers was poured into a 50 ml tube. The blood sample or BM aspirate was then carefully applied on top of the solution, and the tube centrifuged without brake for 20 minutes at 20.000 rotations per minute (RPM). The lightweight layer of mononuclear cells was then transferred to a new tube, using a pipette. The cells were washed once in Phosfate Buffer Solution (PBS) and once in Roswell Park Memorial Institute Medium (RPMI) before downstream assays. The RPMI used in all methods, if not otherwise stated, was of ATCC modification (including L-Glutamine, HEPES buffer, Sodium Pyruvate, and high glucose), and completed with 10 % fetal Calf Serum (FCS) and 1 % Penicillin-Streptavidin-Fungizone solution. Washed peripheral blood mononuclear cells (PBMCs) or bone marrow mononuclear cells (BMMC) were thereafter cryopreserved in FCS with 10 % Dimethylsulphoxide at -150^{o}C until further use.

3.2 CHEMOTHERAPY TREATMENT (PAPER III AND PRELIMINARY DATA)

As a model drug for experimental chemotherapy treatment, the replication inhibitor Doxorubicin was chosen. It belongs to the group of anthracyclins, and is widely used against numerous malignancies, including pediatric leukemias. The mechanisms leading to inhibition of cell proliferation remain a controversy, but involve intercalation directly to DNA, generation of free radicals and inhibition of Topoisomerase II [162, 163]. It can also induce apoptosis in non-dividing cells [164]. For paper III, the Doxorubicin dose was based on a pharmacokinetic study of the treatment analogue Daunorubicin, suggesting that a 60-minutes infusion of 30mg/m^2 was tolerable for adult rhesus macaques [165]. For the preliminary in vitro model of BM stroma – PC interplay, the dose was based on pharmacokinetic data from children treated for ALL, showing plasma concentrations of 30-900 ng/ml [166].

3.3 ENZYME-LINKED IMMUNOSORBENT ASSAY (ALL PAPERS AND PRELIMINARY DATA)

For Enzyme-Linked Immunosorbent Assay (ELISA) a 96-well plate coated with Abs specific for the protein of interest was utilized. A serum sample or cell culture supernatant containing an unknown concentration of the protein was added to the wells, and incubated for an assay-specific time. After removal of the solution, the wells were washed by filling and removal of PBS containing detergent (0.05 % Tween in the majority of assays used). A secondary Ab specific for the same protein was then added, targeting bound protein. The amount of bound secondary Ab was visualized with an enzymatic color reaction, most commonly using horseradish peroxidase, and the color change semi-quantified with an ELISA reader. The color in each well was related to a standard curve with known concentration. To quantify specific Abs in serum, the plates were instead of Abs coated with the selected Ag, and the

secondary Ab was directed towards the immunoglobulin type if interest. In the included papers herein, only IgG was detected.

3.4 TEST OF ANTIBODY AVIDITY (PAPER IV)

The overall binding capacity of specific Abs (avidity) was estimated with an Ag-coated ELISA plate, to which the serum of interest was added. Incubation of Urea solution in the first wash step allowed removal of low-avidity Abs, and comparison with wells treated with only PBS gave an estimation of the overall avidity. The data are expressed as proportional color intensity in Urea wells compared to the control wells.

3.5 POLYCLONAL ACTIVATION OF MBCS AND DIFFERENTIATION TO AB-SECRETING CELLS (PAPERS II, III AND IV)

The functionality and specificity of the MBCs in PBMC samples was determined by differentiation to Ab-secreting cells *in vitro*. In brief, the cells were incubated for 72 hours in RPMI containing activation signals for innate receptors of B cells, inducing a polyclonal activation that lead to differentiation into short-lived plasma blasts. The protocols used in paper II are given in detail within the paper. The protocol used in papers III and IV is given in detail in paper IV. BMMCs in paper III were used for ELISpot without prior activation. As BM samples from two Doxorubicin-treated rhesus macaques in paper III contained overwhelming amounts of maturing hematopoietic cells on study day 73, these samples were enriched for CD38+ cells with a magnetic bead-based method, according to manufacturer's instructions, before ELISpot assay.

3.6 ENZYME-LINKED IMMUNOSPOT ASSAY (PAPERS II, III AND IV)

The Enzyme-Linked ImmunoSpot (ELISpot) assay is similar to Ag-directed ELISA, but utilizing Ab-secreting cells instead of serum. Briefly, pre-wetted membrane plates were coated with the Ag of interest, after which PBMCs or BMMCs were added, and the Abs produced attached in clusters. After removal of the cells, the bound antibodies were visualized with an enzymatic color reaction, creating dark spots at the locations of each Ab-secreting cell. The number of spots was then used to estimate the number of specific Ab-secreting cells per added PBMC, which was related to the total number of IgG-secreting cells in the same sample, determined by using wells coated with anti-IgG Abs. The detailed protocol used in paper II is given in the paper. The detailed PBMC protocol used in papers III and IV is given in paper IV. The protocol for BMMC ELISpot in paper III was the same as for PBMC, but the plates were incubated for 6 hours instead of 16-24 hours.

3.7 FLOW CYTOMETRY (ALL PAPERS AND PRELIMINARY DATA)

Flow cytometry is a technique that, in a suspension of cells, enables quantitative evaluation of single cell characteristics based on light scattering and fluorescence. This is accomplished by a high flow in the cytometer that creates a single-cell stream of cells that pass laser beams. The size of each cell is estimated by means of how the light is scattered before quantification by a front filter (forward scatter), whereas the light scatter on a side filter (side scatter) is a

measure of cell granularity. In addition, the laser induces excitation of fluorescent parts on the cells, and the emitted light is captured and measured. By labeling specific proteins on the cell surface with Abs conjugated to fluorescent molecules, information about several markers of interest can be detected on each cell. The Abs used in this thesis were all monoclonal, and the names of the specific clones are given in each paper.

3.8 QUANTITATIVE POLYMERASE CHAIN REACTION (PAPER I)

Polymerase Chain Reaction (PCR) utilizes a DNA polymerase and complementary primer DNA sequences to multiply copies of a DNA fragment of interest. In paper I, complementary DNA was coded from extracted cell-produced RNA. The amount of DNA produced in the reaction was measured with fluorescent probes, and related to the production of the β-actin gene, known to be stably expressed regardless of treatment. The data are presented as fold-change of gene expression compared to non-treated cells. A detailed protocol is given in paper I.

3.9 IN VITRO CO-CULTURE ASSAY (PRELIMINARY DATA)

In order to study the interplay between the BM stromal cells and LLPCs in further detail, an *in vitro* co-culture assay was developed.

3.9.1 Cell line model for the stromal cell niche

As a model for BM CAR cells, the human BM-derived stromal cell line HS-27 was used, due to its known expression of VCAM-1 and CXCL-12 [167, 168]. Analysis of HS-27 in our lab also demonstrated significant production of IL-6. The cells were kept in culture flasks at 37^{o}C in 5 % CO_2, and fed every 2-3 days with RPMI. For the experiments, the cells were seeded in 24-well plates and grown to monolayers for 48 hours. The monolayers were exposed to Doxorubicin, diluted in RPMI to a concentration of 1 μg/ml, for 60 minutes, after which they were washed 3 times with RPMI. Control wells were exposed to RPMI without Doxorubicin, with the same number of medium changes. The procedure was repeated for 5 consecutive days.

3.9.2 Differentiation of PCs from MBCs in vitro

Differentiation of MBCs to LLPCs was performed according to the protocol developed by Jourdan et al [169]. In brief, CD27+ B cells were separated from PBMC from healthy blood donors, using a commercial magnetic bead separation assay. The MBCs were cultured in 6-well plates at a concentration of $1.5x10^5$ cells/ml in Iscoves modified Dulbecco medium (IMDM) supplemented with Penicillin-Streptomycin-Fungiszone, 10 % fetal bovine serum, 50μg/ml human transferrin and 5 μg/ml insulin. The medium was completed with recombinant CpG, TNF-α, IL-2, IL-6, IL-10 and IL-15 in different combinations for 10 days in total (table 4-2). Analysis with flow cytometry (see chapter 3.9.3) showed that between 30 and 50 % of the added cells completely lost expression of CD20, acquired the specific plasma cell marker CD138 and were hence classified as terminally differentiated plasma cells (figure 4-4, A). The CD138+ fraction was thereafter separated in a magnetic bead assay, and

dissolved in complete IMDM without additional recombinant cytokines.

3.9.3 Co-culture of PCs and stromal cells

After the last Doxorubicin exposure, the RPMI was carefully discarded from the stromal cell layers, and the separated PC fraction was added at a concentration of $3x10^5$ cells/ml. The co-cultures were incubated for 7 days without medium change. At harvest, the culture supernatants were frozen at -20°C, and the cells taken for flow cytometry analysis using a cell scraper. The cells were stained according to standard procedures with fluorescence-conjugated Abs against the following proteins (specific clones in brackets): CD38 (HIT2), CXCR4 (12G5), CD20 (L27) and CD138 (MI15). A fixable dead cell marker was used to exclude non-viable cells from the analysis, and a known number of fluorescent beads was added to each tube to estimate cell numbers. The supernatants were later analysed with ELISA to measure the concentrations of soluble IL-6, CXCL-12 and IgG.

Culture day	**0**	**4**	**7**	**10**
Added to medium	CpG (10 µg/ml) CD40L (50 ng/ml) anti-polyhistidine (5 µg/ml) IL-2 (100 ng/ml) IL-10 (50 ng/ml) IL-15 (10 ng/ml)	IL-2 (100 ng/ml) IL-10 (50 ng/ml) IL-6 (50ng/ml) IL-15 (10 ng/ml)	IFN-α (500 U/mL) IL-6 (50ng/ml) IL-15 (10 ng/ml)	Co-culture with HS-27, no added cytokines

Table 4-2. Protocol for *in vitro* activation and plasma cell (PC) differentiation of separated primary CD27+ memory B cells. For all steps, the cells were kept in 6 well-plates at a concentration of $1.5x10^5$ cells/ml in Iscoves modified Dulbecco medium (IMDM), supplemented with Penicillin-Streptomycin-Fungizone, 10 % fetal bovine serum, 50µg/ml human transferrin and 5 µg/ml insulin. The cells were washed in Phosfate Buffered Saline between medium changes. On culture day 10, the PC fraction was separated with magnetic beads targeting CD138, and added onto HS-27 cultures in IMDM without cytokines.

4 RESULTS

4.1 PRESERVATION OF VACCINE-INDUCED MBCS

We evaluated the survival of vaccine-induced MBCs in healthy children and young adults (paper II) and upon two distinct types of immunomodulatory treatment (papers III and IV).

In healthy children who had followed the Swedish National Immunization Schedule, we showed that the first vaccine dose against measles and rubella induced adequate numbers of functional specific MBCs, which did not vanish with time and were preserved up to 9 years after vaccination (figure 4-1, A-B). Also, in healthy young adults who had received a second vaccine dose, specific MBCs were measured up to 18 years after vaccination with no sign of decay. The general MBC compartment, quantified with flow cytometry, did not change with age in our cohort.

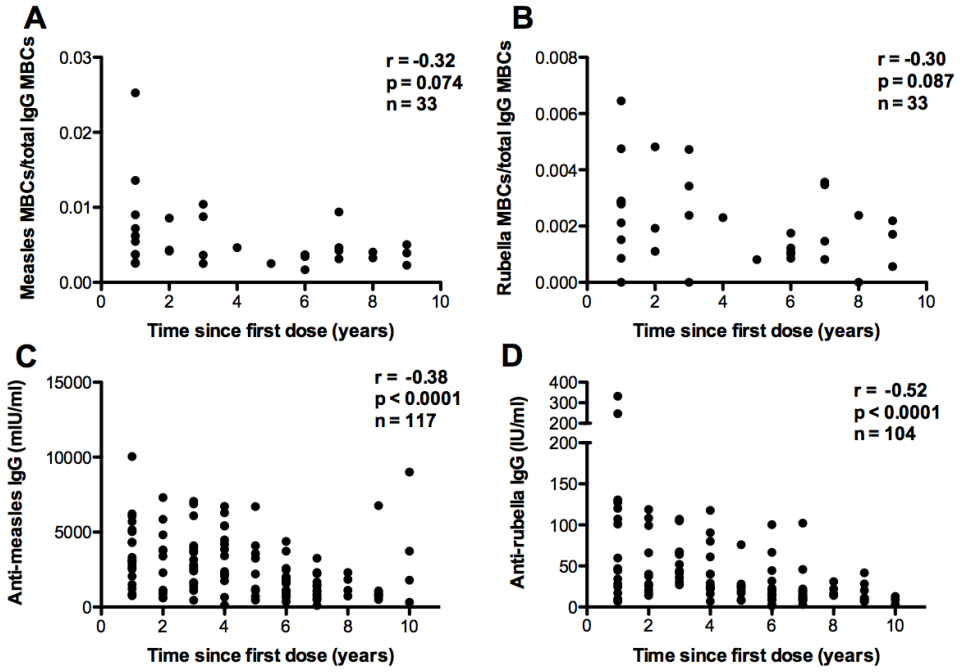

Figure 4-1. Healthy children who had received one dose measles and rubella vaccine were sampled 1-10 years after vaccination. Memory B cells specific against measles (A) and rubella (B) did not follow a trend of decrease with time, whereas serum measles (C) and rubella (D) IgG titers were markedly lower longer time after vaccination.

Next, we wanted to evaluate the survival of vaccine-induced MBCs upon chemotherapy treatment in a rhesus macaque model. Healthy measles-vaccinated rhesus macaques were given 3 infusions of Doxorubicin, at doses that were estimated to induce BM toxicity, or saline solution (5 animals were used in each group). 28 days after the last dose, all animals were re-vaccinated against measles and *de novo* vaccinated against rubella and tetanus (table 4-1). Flow cytometry and ELISpot quantification of the B cell compartment showed that both general and measles-specific resting MBCs were almost completely diminished 15 days after the last Doxorubicin dose (figure 4-2, A-B). Consecutive sampling showed adequate

recovery, and the MBC levels were comparable between the groups 43 days after cessation of treatment.

Study day	0	14	28	58	73	86	101	121	136	175	211
Handling	Doxorubicin 30 mg/m^2		Doxorubicin 50 mg/m^2	Doxorubicin 75 mg/m^2		MMR + tetanus vaccination					Euthanasia
Sampling	BMMCs PBMCs	BMMCs PBMCs			BMMCs PBMCs		BMMCs PBMCs	PBMCs	PBMCs	PBMCs	BMMCs PBMCs

BMMCs = bone marrow mononuclear cells, PBMCs = peripheral blood mononuclear cells

Table 4-1. Overview of handling and sampling of the rhesus macaques used in paper III. In addition to the outlined sampling, frequent peripheral blood samples were taken for measurement of clinical chemistry parameters, and cryopreservation of serum. Furthermore, consecutive bone marrow biopsies and whole lymph nodes were taken for cryopreservation (not used for this thesis work). The treatment group (n = 5) received the Doxorubicin doses intravenously for 1 hour. The control group (n = 5) received saline solution under equal circumstances.

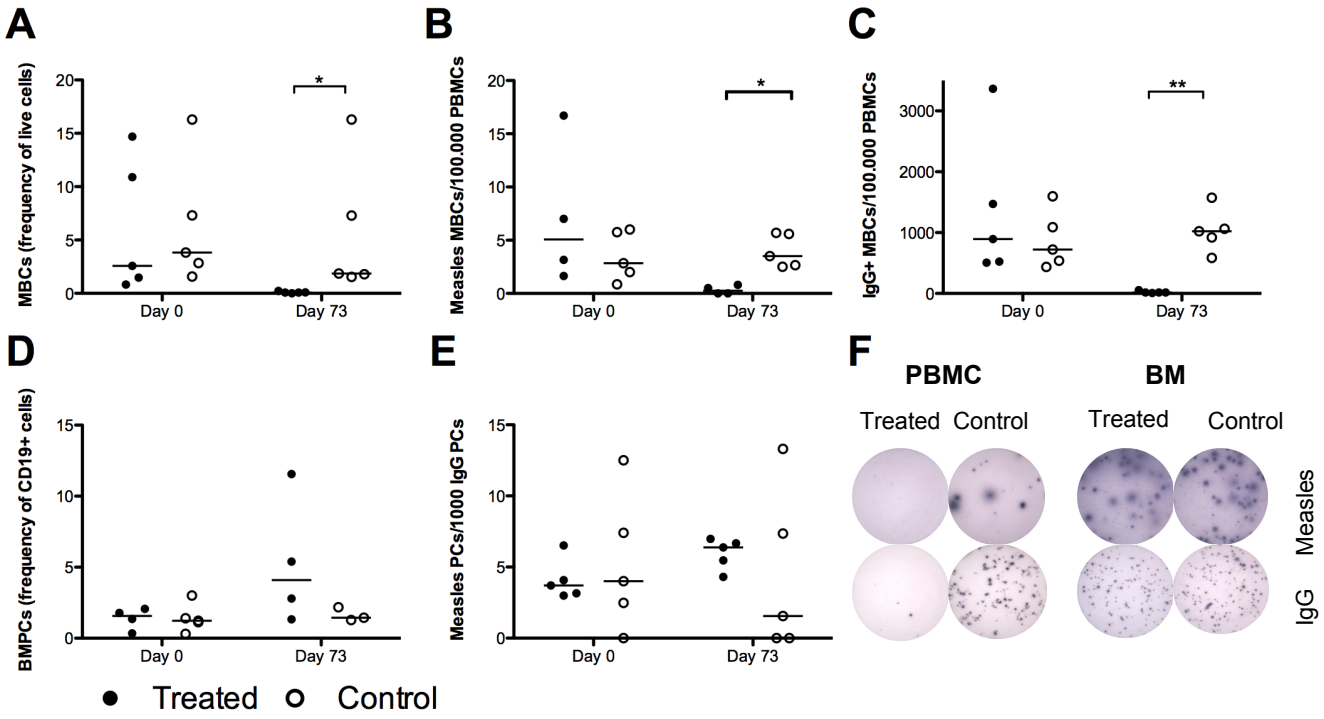

Figure 4-2. Evaluation by flow cytometry and ELISpot of pre-existing B cell memory in Doxorubicin treated and control rhesus macaques. Memory B cells (MBCs) were defined as viable CD19+ CD27+ single lymphocytes, and plasma cells (PCs) as viable CD19+, CD20+/-, CD38Hi, CD138+ single bone marrow (BM) cells. All data show a comparison before treatment (day 0) and 15 days after the last Doxorubicin dose (day 73). Horizontal lines signify median levels in each group. Both the general (A) and measles-specific (B) MBC compartments were severely decreased in the treated compared to the control group on day 73 ($p < 0.05$). Also the amount of functional IgG+ MBCs was almost completely eradicated at the same time point (C, $p < 0.01$). In contrast, the general (D) and measles-specific (E) BMPC frequencies did not differ between the groups. The findings are further demonstrated with representative ELISpot wells from study day 73 (F).

After examining high-dose short-term treatment, we wanted to evaluate whether low-dose long-term chemotherapy would have the same consequence. We thus returned to evaluate

immunity in children who had followed the Swedish National Immunization Schedule, but now compared healthy children to patients treated with the cytotoxic drug methotrexate (MTX) against rheumatic disease, in a cross-sectional study. We furthermore asked if additional treatment with TNF-α-inhibiting drugs would add to an effect, and included children from three groups: healthy controls, rheumatic patients with only MTX, and rheumatic patients with both MTX and one TNF-α-inhibitor. Flow cytometry analysis showed that the number of transitional B cells was decreased in the rheumatic patients, but that the MBC compartment was similar between all groups (figure 4-3, A-B). ELISpot analysis demonstrated that children that had received two doses of measles and rubella vaccine, and four doses of tetanus vaccine, had comparable levels of MBCs specific against measles, tetanus and rubella, irrespective of treatment. In contrast, rheumatic patients who had only received one dose of measles vaccine had significantly lower frequencies of measles-specific MBCs (figure 4-3, C).

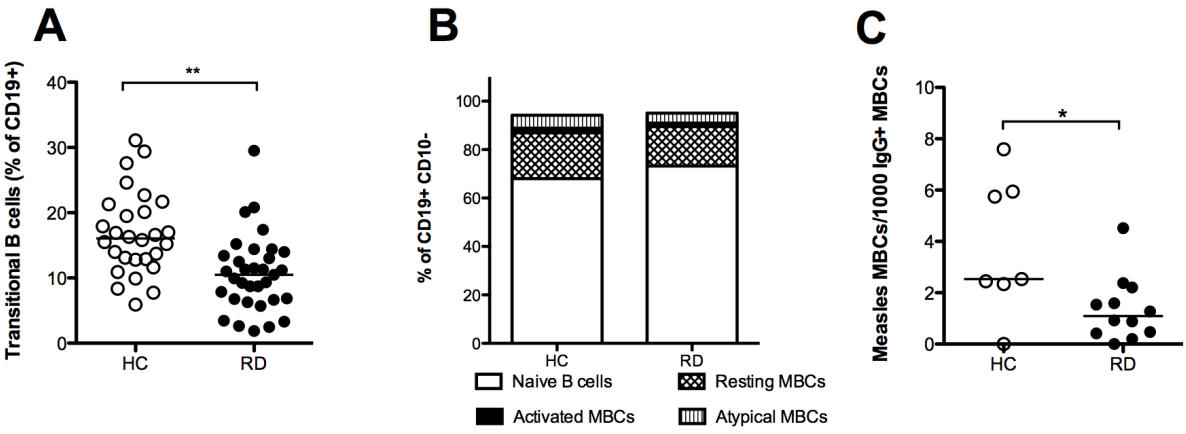

Figure 4-3. Peripheral blood mononuclear cells (PBMCs) from children with rheumatic disease and treatment (RD), and age-matched healthy controls (HC), were analyzed with flow cytometry and ELISpot. Total B cells were defined as CD19+ CD14- viable lymphocytes, mature B cells as CD10- B cells, transitional B cells as CD10+ CD21+ CD38+ B cells, naïve B cells as CD27- CD21+ mature B cells, resting memory B cells (MBCs) as CD27+ CD21+ mature B cells, activated MBCs as CD27+ CD21- mature B cells and atypical MBCs as CD27- CD21- mature B cells. The frequencies of transitional B cells of all CD19+ cells were decreased in children with rheumatic disease (A), whereas the distribution of cell populations within the mature B cell compartment did not differ significantly (B). In patients with only basic measles and rubella protection (1 MMR dose), the frequencies of measles-specific MBCs were significantly lower than in the controls (C).

4.2 FUNCTION OF MBCS IN DISEASE AND TREATMENT MODELS

The overall functionality of the MBC pool in the rhesus macaque model (paper III) and in children treated with anti-rheumatic treatment (paper IV) was evaluated by the ability to produce IgG upon polyclonal activation. In line with the flow cytometry quantified MBC frequencies, the amount of functional IgG-producing MBCs was severely diminished in the rhesus macaques 15 days after the last Doxorubicin dose (figure 4-2, C). Also coherent with flow cytometry data, children with and without antirheumatic treatment had similar frequencies of IgG producing MBCs after polyclonal activation. In both treatment models, the number of ELISpot-quantified functional IgG-producing MBCs correlated strongly to the flow-cytometry quantified frequencies of resting MBCs ($p < 0.0001$ for both studies).

In the rhesus macaque model, induction of a B cell response after vaccination shortly after cessation of chemotherapy treatment was also evaluated. We found that both rubella- and tetanus-specific MBCs were formed to the same extent as in the control group, but somewhat delayed, as the amounts were significantly lower 15 days after vaccination. Short-term evaluation of specific serum IgG, an indicator of a productive initial immune response and GC reaction, showed the same pattern. Moreover, 4 months after *de novo* or booster vaccination, both measles- and rubella-specific BMPC frequencies correlated positively to the corresponding specific serum IgG, irrespective of treatment ($p = 0.014$ for measles and $p = 0.0046$ for rubella).

In HIV-1 infected individuals, we investigated an alternative pathway for MBC activation, and its putative role in hyperactivation of MBCs that is seen in these patients (paper I). We could show that recombinant CD27 induced a dose-dependent IgG production in MBCs in vitro without BCR stimulation, exerted by the ligation of CD70 on B cells. Furthermore, incubation with sCD27 induced expression of the PC differentiation transcription factors Blimp-1 and XBP-1. MBCs from HIV-1 infected patients expressed elevated levels of CD70 compared to the healthy donors, and the IgG-producing effect of sCD27 stimulation was also more pronounced. Finally, we analyzed the patients' serum levels of IgG and CD27, and confirmed that they were higher than in healthy controls. Serum IgG correlated to sCD27 levels in both HIV-1 infected and healthy individuals, suggesting a role for the CD27-CD70 pathway of MBC activation in humans, potentially more active during HIV-1 infection.

4.3 PRESERVATION OF VACCINE-INDUCED LLPCS

We measured specific serum IgG as a secondary measure of LLPC number and function in healthy individuals (paper II) and upon two distinct types of immunomodulatory treatment (papers III and IV). In the rhesus macaque model for antineoplastic treatment, we were also able to quantify the number of total and Ag-specific BMPCs (paper III). In addition, we studied the details of how chemotherapy can interfere with PC survival in an *in vitro* model of the BM stromal cell niche (preliminary data).

In healthy children sampled 1-9 years after vaccination, we noted that specific IgG induced after one measles and rubella vaccine dose decreased with time, in contrast to the stable population of MBCs (figure 4-1, C-D). Out of the children with low Ab titers, > 50 % had high levels of MBCs. In contrast, in young adults who had received two vaccine doses, sampled at least 7 years after the last dose, the specific IgG titers showed no sign of time-related decay.

In the Doxorubicin-treated rhesus macaques, Abs against measles turned out to be stably preserved, and neither was the total and specific BMPC compartment affected (figure 4-2, D-E). This contrasted markedly against the loss of specific MBCs in the same animals. Likewise, in rheumatic children treated with low-dose MTX for long term, both measles- and rubella-specific serum IgG titers were similar to age-matched controls, irrespective of concomitant TNF-α-inhibitory treatment. Tetanus-specific serum IgG was however decreased

in the rheumatic patients compared to the controls both for individuals with basic ($p = 0.029$) and full ($p = 0.031$) tetanus vaccine protection.

We next sought to map the effects of Doxorubicin on the stromal cell – PC interplay in more detail, using an *in vitro* model. We discovered that repeated exposure to *in vivo* relevant concentrations of Doxorubicin could hamper the production of CXCL-12 and IL-6 from the stromal cell line HS-27 (figure 4-4, B-C). Accordingly, the surface expression of CXCR4 on co-cultured PCs increased (figure 4-4, D). Even though the number of surviving PCs correlated to the concentration of IL-6 and CXCL-12 in the cultures (figure 4-4, E-F), Doxorubicin-exposure decreased neither PC number nor IgG production (figure 4-4, G-H).

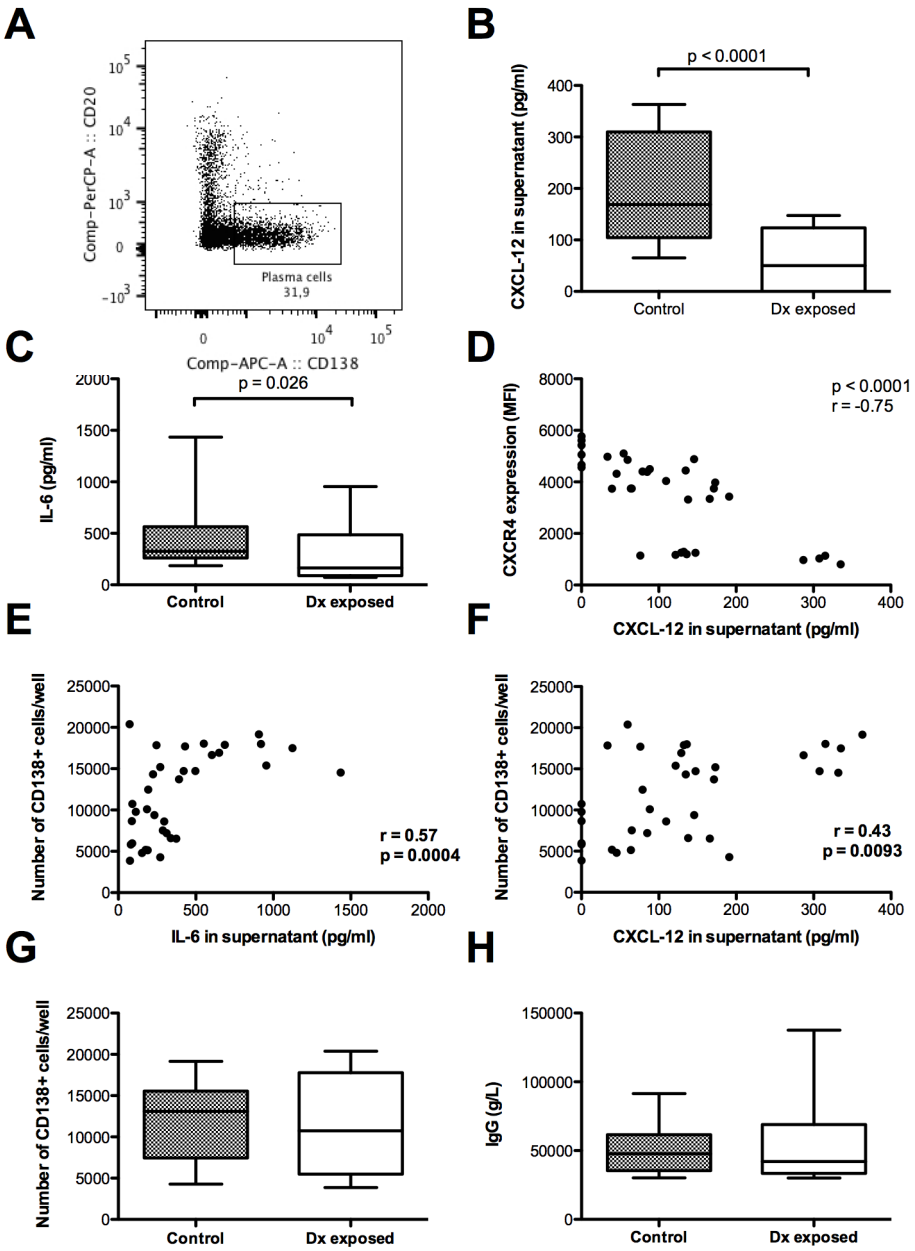

Figure 4-4. *In vitro* derived plasma cells (PCs) were kept for 7 days on the cell line HS-27, as a model for cross talk between PCs and CXCL-12-abundant reticular cells (CAR cells). Half of the wells with monolayers of HS-27 were exposed to Doxorubicin (Dx) 1 hour per day for 5 consecutive days, before addition of PCs. (A) representative FACS plot after completed PC differentiation. The CD138 fraction was purified with magnetic bead separation before addition onto the HS-27 monolayer. B-H: evaluation of co-cultured PCs with flow cytometry, and corresponding supernatants with ELISA. The concentrations of CXCL-12 (B) and IL-6 (C) in the co-cultures were significantly lower in Dx-exposed wells. The PC median fluorescence intensity (MFI) of CXCR4, the natural receptor for CXCL-12, was negatively correlated to the concentration of CXCL-12 in the supernatants (D). The number of CD138+ cells after 7 days of co-culture correlated to the supernatant concentrations of IL-6 (E) and CXCL-12 (F). Though, when comparing control wells with Dx exposed, the number of CD138+ cells (G) and the produced IgG (H) did not differ significantly. Data are from 3 independent experiments, using 6 healthy blood donors in total.

5 DISCUSSION

5.1 THE SURVIVAL AND FUNCTION OF LLPCS

The idea of a population of LLPCs resident in the BM, responsible for continuous Ab production, was first proposed by Rudolph Manz and colleagues in 1997 [170]. Since then, numerous experimental and observational studies have supported this hypothesis (summarized in chapter 1.3.8), but there are still some fundamental questions that remain to be answered.

5.1.1 Establishment of LLPCs

Firstly, when does the production of Igs shift from newly formed plasma blasts and SLPCs to the LLPC population? When evaluating IgG titers in humans, longitudinal data imply that the host reaches is a steady state when there is hardly any decay of a number of specific Abs [105], suggested as the time when the production has shifted completely to LLPCs. However, it seems to take 1-3 years before that occurs [42, 171]. It is not known if Abs produced before the steady state are from newly formed SLPCs, or if the number of LLPCs decay in the beginning or their establishment. Previously considered to be a relatively short-lived event, there is now evidence that a GC reaction can last for months after Ag encounter [33], implying that at least part of the initial Ab kinetics is due to slowly diminishing of the SLPC production. Perhaps, the "intermediate phase" of the Ab response is due to a combination of formation of new SLPCs, and LLPCs competing for their place in the BM niche (figure 5-2).

The notion that Ab titers are not stable until years after vaccination raises the question if titer quantification earlier does not represent the true long-term response. This finding was mirrored in paper II, where we concluded that there was a strong correlation between Ab titer and time since vaccination during the first years. Since sampling limitations in human patients hinder investigation of establishment of LLPCs in the BM, we utilized a rhesus macaque model for this investigation (paper III). We lack measurements later than 4 months after vaccination, but we nevertheless noticed that specific IgG correlated to the number of specific BMPCs at our latest measured time point. Furthermore, another vaccination study in cynomolgus macaques concluded that the serum IgG was mainly resulting from BM production 29 weeks after vaccination [172]. These findings contradict the model outlined above, but we cannot know, however, if the Ab-secreting population that was measured in the BM was genuinely long-lived, or consisted of recent arrivers yet to be incorporated in the niche. One suggestion is that the BM not only is a home for LLPCs, but also a short-term residence for cells with a limited life span. Possibly, there is a need to introduce the existence of a "middle-aged" PC, to explain the pattern that is observed the first years after Ag encounter.

5.1.2 Regulation of the BM niche and longevity of the Ab protection

There have been many suggestions on how the influx of new BMPCs is regulated. Given that the BM niche is spatially limited, how can the continuous encounter of new pathogens

generate PCs for a lifetime? The most recognized hypothesis is that there is an on-going competition for space, leading to an expulsion of resident PCs upon new PC formation. This would lead to a gradual decrease of Ab production with time, however sufficient for lifetime protection [173]. One observation supporting this hypothesis is the occurrence of multi-specific PCs in peripheral blood after vaccination [174]. What remains to be explained, though, is why some Ab titers show no sign of decay at all, whereas others have a relatively short half-life [105]. One proposal is that the fate of the PC is decided already in the GC reaction, where multivalent Ags engage strong T cell help and mount more LLPCs (see chapter 1.4.2). This hypothesis is strengthened by recent data on how LLPCs are formed in the GC, favoring a selection of the B cells with highest affinity and hence generated after strong signals from the BCR and T cells (see chapter 1.3.2). This would mean that some Ag responses are "destined" to wane from the beginning (applicable to both tetanus and pertussis responses, both generated from monovalent Ags) giving space for PCs generated after stronger stimulation [42]. One must however bear in mind that the Abs probably do not reach zero level, even if we do not detect them [20]. In other words, immunity to monovalent Ags can still be bona fide long-lived, but generated by too few LLPCs to be protective (figure 5-2, B)

5.1.3 Implications for patients with immunosuppressive treatment

Chemotherapy and TNF-α-inhibitory treatment hypothetically interferes negatively with mounting and preservation of a vaccine response (outlined in chapter 1.5 and summarized in figure 5-1), but the consensuses regarding treated children suggest that both groups have protective Ab response after vaccination [136]. However, assuming that the long-term preservation of Abs should be evaluated at least 1-3 years after vaccination, perhaps one needs to re-consider these conclusions. We found clearly lowered titers against tetanus in children after long-term follow up (paper IV) and coherently, two studies done on long-term assessment (> 1 year for children with cancer and > 2 years for children with rheumatic diseases) found lower Ab titers in both groups, compared to healthy controls [137, 142]. Could this be due to an inferior GC response with too few generated LLPCs, which will not be noted as long as the titer evaluation is done within the intermediate phase of Ab production (figure 5-2, C)? In paper IV, we concluded that tetanus titers in rheumatic patients were of the same avidity as in the healthy controls, thus probably generated from GC reactions and not from extrafollicular responses. However, many of the children had their diagnosis before booster vaccination, and it cannot be excluded that immune suppression caused by both the disease and the treatment, can have interfered with the number of LLPCs generated. The same can possibly be valid after antineoplastic treatment and subsequent vaccination. It is, for example, not evaluated when FDCs can re-establish and operate functionally after high-dose chemotherapy. We showed in our *in vitro* model that BM stromal cells decrease CXCL-12 production after Doxorubicin treatment (preliminary data), and a similar disturbed secretion of CXCL-12 or CXCL-13 in a GC would have devastating effects on its architecture and function. Furthermore, the broad nature of a general cytotoxic effect might also impair delicate functions of the main GC players, such as presentation of Ag by B

cells or FDCs, or expression of CD40L by T_{FH} cells. The selective decrease of tetanus titers could be explained by the already mentioned limitation of the Ag in itself, and thus an increased vulnerability to additional stress.

In addition to a suboptimal GC reaction, chemotherapy might disrupt the function of the BM niche, which we hypothesize causes the loss of pre-existing Abs after antineoplastic treatment (outlined in chapter 1.5.4 and summarized in figure 5-1). It is largely unknown how the niche is regenerated after an insult, or if the non-proliferating CAR cells at some point succumb to treatment and are replaced by maturation of mesenchymal stem cells, or – on the contrary – if surviving CAR cells exhibit a suboptimal function. As mentioned above, data from our in vitro model demonstrated that CAR cell production of the survival factors CXCL-12 and IL-6 was inhibited after Doxorubicin exposure, but if renewal of the hematopoietic part of the niche can compensate for this deficiency is hard to predict. Knowledge about the niche function after cytotoxic treatment would be an important clue to development of effective re-vaccination schedules after completed cancer treatment regimes.

Furthermore, children are at higher risk than adults to acquire side effects from proliferation inhibitory treatment. For example, their high bone remodeling rate, induced by mesenchymal stem cell activity, leads to increased sensitivity to bone cancer after irradiation therapy [175]. It can hence be speculated that cytotoxic treatment in children is able to affect other functions of mesenchymal stem cells, such as establishment of the spatial diversity of BM niches. Nevertheless, long-term effects after antineoplastic treatment on BM constitution, or specifically the LLPC niche, are largely unstudied. Finally, one must not completely rule out the possibility of chemotherapy interference directly with the PC intracellular machinery, for example proteasome activity, known to induce PC apoptosis [176].

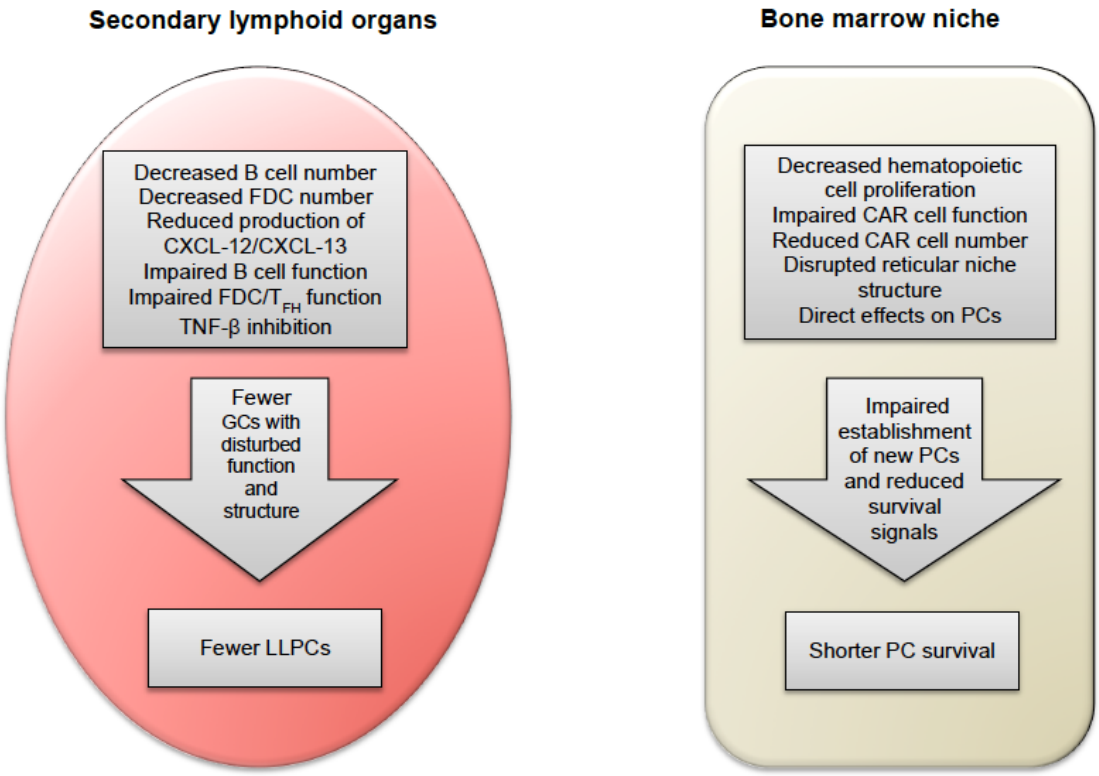

Figure 5-1. Summary of the elicited hypotheses on how chemotherapy and TNF-α-inhibitory treatment might interfere with long-lived plasma cell (LLPC) production and long-term maintenance. TNF-β inhibition most likely occurs only upon TNF-α-inhibitory treatment. FDC = follicular dendritic cell, T_{FH} = follicular helper T cell, GC = germinal center, CAR cell = CXCL-12-abundant reticular cell.

5.2 SALVAGE BY MBCS?

One of the main aims of this work has been to compare the contribution of LLPCs and MBCs to long-term immunity, and their relative susceptibility to immunosuppressive treatment. We concluded in paper II that healthy children can mount an adequate number of MBCs despite low IgG titers, and that MBC levels are stable before a steady state of Ab production. Somewhat contradictory, we saw in paper III that MBCs vanished after high-dose short-term chemotherapy treatment, whereas Ab titers were preserved, but the opposite was shown in paper IV after low-dose long-term treatment. Maintenance of MBCs is mechanistically not well mapped, and it is not known whether intrinsic or extrinsic signals play the most crucial role. However, their proliferating nature stipulates a general vulnerability for replication inhibitory drugs, and their resistance to MTX is surprising. This indicates that there is influence of other, protective signals that outweigh the cytotoxicity, possibly provided by surrounding cells. Assumingly, these signals are not enough to prevent cell death in the case of a higher chemotherapy dose.

How MBCs circulate between peripheral blood and various lymphoid organs is not fully understood, and we do not know if the evaluated MBCs from blood samples are truly representative. Perhaps the fast recovery of measles-specific MBCs that we noted in the rhesus macaque model in paper III can be explained by a preserved population in another

anatomical site, either not reached by the same drug concentrations as in peripheral blood, or equipped with more survival factors.

The most intriguing question is though which protective role the MBC compartment plays in the case of antigenic challenge, currently not known as studies on vaccine efficacy have thus far only evaluated Ab titers in terms of protection. One clue has come from studies of the Hepatitis B vaccine, known to induce serum IgG only for a limited time, but to provide protection despite titer waning. To test the protection capacity of Hepatitis B-specific MBCs, re-vaccination was used as a model for Ag re-challenge in one study on vaccinated children [177]. Indeed, children with similar MBC levels responded equally well to re-vaccination, regardless of IgG titer levels. If this conclusion is valid, also after infectious antigenic challenge, is however hard to predict.

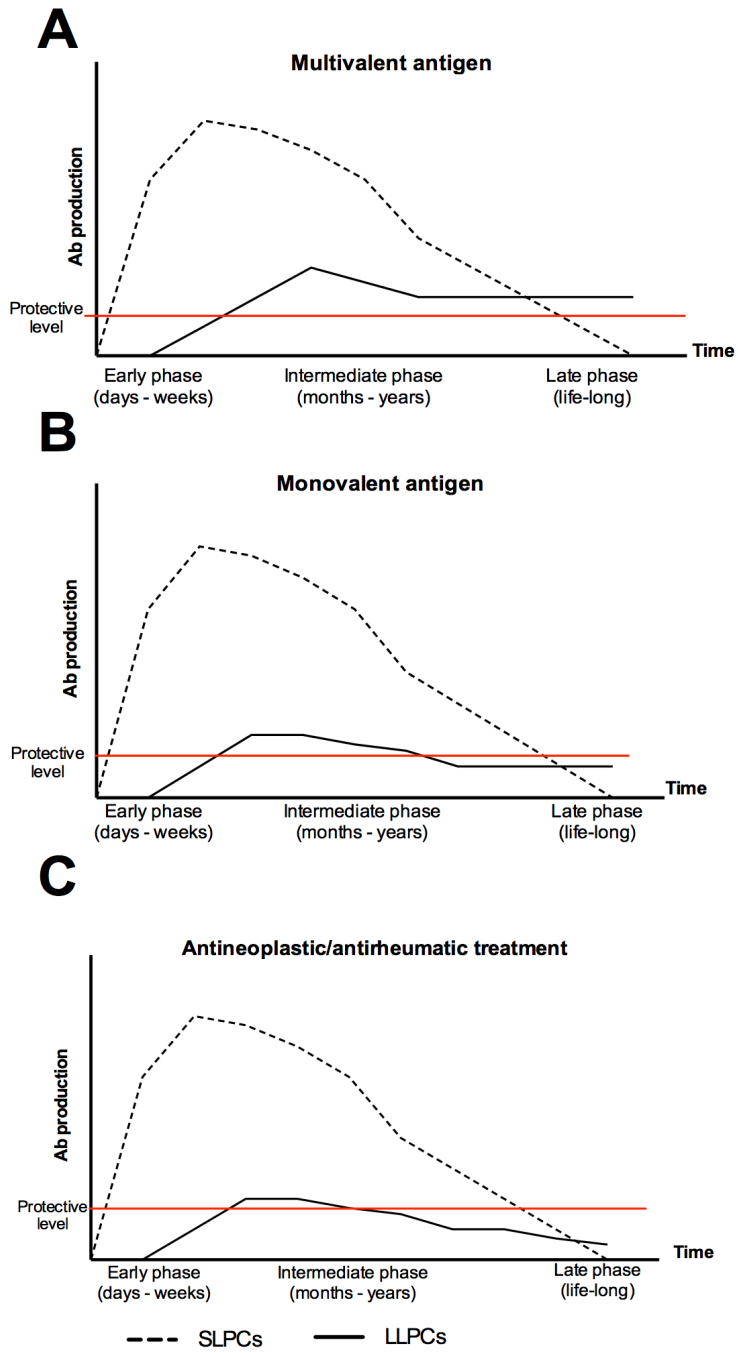

Figure 5-2. Hypothetical illustration of how the antibody (Ab) response might appear after a single vaccine dose. During the early phase, Ab production is only due to short-lived plasma cells (SLPCs, dashed line), whereas the long-lived plasma cells (LLPCs, whole line) appear later, and peak after 4-5 weeks. The Ab production in the intermediate phase comes both from a prolonged GC response, giving rise to new SLPCs, and LLPCs competing for space in the bone marrow (BM) niche. There is thus a decline of Abs throughout the intermediate phase, which hypothetically last for years according to longitudinal human data. The late phase is initiated as the SLPC response is over, and the number of LLPCs is fixed in the BM, leading to a life-long steady state. In the case of a multivalent Ag (A), the number of LLPCs is high enough to produce Abs above the protection level, whereas a monovalent Ag (B) gives rise to fewer LLPCs, setting the steady state Ab production at a lower, non-protective, level. Upon antineoplastic or antirheumatic treatment (C), the number of LLPCs is reduced due to the interference with the GC response summarized in figure 5-1, and in addition, the long-term maintenance in the BM is impaired. The late phase of Ab production is thus both lower from the beginning, and not kept in a steady state. The illustration is inspired by findings from the studies included in this thesis, and from observations reported by Amanna and Slifka [20, 42].

5.3 FUTURE DIRECTIONS

The long-term goal with our studies is to find clues to better vaccine protection of children with treatment against cancer and inflammatory diseases, but several challenges remain in this strive. Most importantly, preservation of vaccine-induced immune cells must act in concert with elimination of pathological cells. In hematological malignancies, directed therapy towards the BM niche has become of importance as this compartment has turned out to act as shelter for surviving malignant cells [178]. Similarly, treating autoreactive LLPCs has emerged as an important tool to treat refractory autoimmune disease [179]. This emphasizes the need for more targeted therapy, for example emerging immunotherapy in malignancies [180], enabling lower doses of broadly toxic drugs. For autoimmune disorders, a new application of the affinity matrix technology has recently demonstrated the potential of killing BMPCs based on antibody specificity, opening up for elimination of solely autoantibody producing PCs [181].

Mechanistic studies of the BM niche is coupled to many challenges, as the PC-preserving part is estimated to be an extremely small fraction of the entire BM [83], and cultured cells of mesenchymal origin are prone to change their original expression of surface molecules [182]. In vitro models are nevertheless needed to generate new hypotheses and look into mechanistic details, complemented by evolving in vivo imaging techniques [96], and utterly, studies on sectioned human BM biopsies.

In addition to BM-directed research, we need to further outline the protective capacity of the MBC compartment, to understand if the second line of B cell defense can compensate when the first line is impaired. We hence need better methods to study both MBC maintenance and their role in antigenic re-challenge, emphasizing the need for studies on human SLOs. Likewise, more interest should be aimed at direct effects on SLO structure and function upon generalized immunosuppressive treatment, and how this potentially hamper of vaccine efficacy.

To summarize, the optimization of vaccine protection in patients with immunosuppressive treatment is an area where vaccinology is in great need of basic immunology research, and it cannot be done without using both pre-clinical and translational approaches. Hopefully, our contribution is a small step on the road.

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