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APPROACHES TO MODULATE VACCINE-INDUCED ANTIBODY RESPONSES

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Cover illustration shows “a bursting antibody whirlpool”, by Martina Soldemo.

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Approaches to modulate vaccine-induced antibody responses

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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Life is what happens, when all the ‘what if´s’ don´t.

ABSTRACT

Most successful licensed vaccines mediate protection by inducing antibody responses capable of protecting the individual from infection or disease. Despite the availability of many effective vaccines, we lack knowledge about fundamental aspects of B cell immunity and how we respond to complex real-world protein antigens. HIV-1 is a particularly difficult vaccine target since it has evolved multiple strategies to evade host antibody responses, such as conformational and glycan shielding. A consequence of this effective shielding is that the most immunogenic determinants of the envelope glycoprotein (Env) spike are those that the virus can readily mutate, which give rise to strain-specific antibodies. The conserved determinants of Env, which are targets for broadly neutralizing antibodies, tend to be less immunogenic. Another challenge has been the production of recombinant immunogens that mimic the functional HIV-1 Env spike but recent developments have improved this situation. In this thesis, I studied Env-specific immune responses induced by immunization with recombinant Env trimers to broaden our knowledge about B cell responses against this complex protein antigen.

In brief, I investigated basic B cell questions using HIV-1 Env as a model antigen in a series of mouse immunogenicity studies. In **Paper I**, we asked whether or not there is a competition between B cells that recognize distal epitopes on HIV-1 Env. The variable region 3 (V3) of Env is highly immunogenic. We therefore masked the V3 region with N-linked glycans to study if the elicited response would be re-distributed to other more conserved determinants on Env. The results indicated that there was no competition between B cells. Instead, we observed a total decrease in the antibody responses when the V3 region was shielded. Next, in **Paper II**, we manipulated the naïve B cell repertoire by pre-treating mice with recombinant BLyS to rescue additional B cells into the mature naïve B cell pool. The results showed that BLyS treatment expanded the naïve B cell population and resulted in improved neutralization capacity after Env immunization. In **Paper III**, we established protocols that would allow characterization of vaccine-induced antibody specificities. We describe staining protocols for Env-specific memory and germinal center B cells in mice. Finally, in **Paper IV**, we evaluated the effect of chemical cross-linking of HIV-1 Env trimers for the elicited antibody response after immunization. The results indicated a Th2 shift in the serum antibody isotype response compared to the response elicited in mice immunized with unfixed trimers.

In conclusion, the purpose of this thesis has been to evaluate strategies to manipulate or re-direct the antibody response induced by recombinant HIV-1 Env immunogens. The results obtained in this thesis gave insights into several fundamental B cell questions of relevance to the vaccine field and to our understanding of basic B cell immunology.

LIST OF SCIENTIFIC PAPERS

The following papers are included in the thesis:

I. Forsell MN, **Soldemo M**, Dosenovic P, Wyatt RT, Karlsson MC, Karlsson Hedestam GB. Independent expansion of epitope-specific plasma cell responses upon HIV-1 envelope glycoprotein immunization. Journal of Immunology (2013)191(1):44-51

II. Dosenovic P, **Soldemo M**, Scholz JL, O'Dell S, Grasset EK, Pelletier N, Karlsson MC, Mascola JR, Wyatt RT, Cancro MP, Karlsson Hedestam GB. BLyS-mediated modulation of naive B cell subsets impacts HIV Env-induced antibody responses. Journal of Immunology (2012) Jun 15;188(12):6018-26

III. **Soldemo M**, Pedersen G and Karlsson Hedestam GB. HIV-1 Env-specific memory and germinal center B cells in C57BL/6 mice. Viruses (Special issue on HIV vaccines) (2014) Sep 5;6(9):3400-14

IV. **Soldemo M**, Àdori M, Feng Y, Tran K, Guenaga J, Wyatt RT, Karlsson Hedestam GB. Effect of glutaraldehyde cross-linking of HIV-1 Env trimers on vaccine-induced immune responses in mice. *Manuscript*

Other relevant publications not included in the thesis:

- Dosenovic P, Chakrabarti B, **Soldemo M**, Douagi I, Forsell MN, Li Y, Phogat A, Paulie S, Hoxie J, Wyatt RT, Karlsson Hedestam GB. Selective expansion of HIV-1 envelope glycoprotein-specific B cell subsets recognizing distinct structural elements following immunization. Journal of immunology (2009) Sep 1;183(5):3373-82

- Sundling C, Martinez P, **Soldemo M**, Spångberg M, Bengtsson KL, Stertman L, Forsell MN, Karlsson Hedestam GB. Immunization of macaques with soluble HIV type 1 and influenza virus envelope glycoproteins results in a similarly rapid contraction of peripheral B-cell responses after boosting. The Journal of infectious diseases (2013) Feb 1;207(3):426-31

- **Soldemo M**, Karlsson Hedestam GB. Env-specific antibodies in chronic infection versus in vaccination. Review article, In Press in Frontiers of Immunology (2017). https://doi.org/10.3389/fimmu.2017.01057

CONTENTS

LIST OF ABBREVIATIONS

ADCC	Antibody-dependent cell-mediated cytotoxicity
AID	Activation-induced cytidine deaminase
AIDS	Acquired immune deficiency syndrome
APC	Antigen-presenting cell
ASC	Antibody secreting cell
BCR	B cell receptor
BER	Base excision repair
Blimp-1	B lymphocyte induced maturation protein-1
BLyS	B lymphocyte stimulator
BM	Bone marrow
bNAbs	Broadly neutralizing antibody
BR3	BLyS receptor 3
C	Cytidine
CD4bs	CD4 binding-site
CDR	Complementarity-determining region
CLP	Common lymphocyte progenitors
CoRbs	Co-receptor binding-site
CSR	Class-switch recombination
CTL	Cytotoxic T cell
D	Diversity
DC	Dendritic cell
DSB	Double-stranded brake
DSC	Differential scanning calorimetry
DZ	Dark zone
EM	Electron microscopy
Env	Envelope
ER	Endoplasmic reticulum
Exo1	Exonuclease-1
FDC	Follicular dendritic cell

FR	Framework
GC	Germinal center
GLA	Glutaraldehyde
gV3	Glycan masked V3 region
HEL	Hen egg lysozyme
Hib	Haemophilus influenza type B
HIV-1	Human immunodeficiency virus type-1
HSC	Hematopoietic stem cell
i.p.	Intraperitoneal
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ingLN	Inguinal LN
IRF4	Interferon regulatory factor-4
J	Joining
KI	Knock-in
L	Ligand
LN	Lymph node
LZ	Light zone
Mab	Monoclonal antibody
MALT	Mucosa associated lymphoid tissue
MHC	Major histocompatibility complex
MMR	Mismatch repair
MZ	Marginal zone
N	Asparagine
N-nucleotide	Non-templated nucleotide
NFL	Native flexibly linked
NHEJ	Non-homologous end-joining
NHP	Non-human primate
NK cell	Natural killer cell
NS-EM	Negative-stain EM

OH	Hydroxyl
PALS	Periarteriolar lymphoid sheath
PAMP	Pathogen-associated molecular pattern
PC	Plasma cell
Pre	Precursor
Pro	Progenitor
PRR	Pattern recognition receptor
RAG	Protein recombination-activating gene
RSS	Recombinational signal sequences
RT	Reverse transcriptase
S region	Switch region
s.c.	Sub cutaneous
SEC	Size-exclusive chromatography
SHM	Somatic hypermutation
SJ	Signal joint
SL	Surrogate light chain
SLO	Secondary lymphoid organ
ssDNA	Single-stranded DNA
T1-T3	Transitional stages 1-3
TCR	T cell receptor
TD	T cell dependent
TdT	Deoxynucleotidyltransferase
Tfh	Follicular helper T cell
Th	Helper T cell
TI	T cell independent
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Treg	Regulatory T cell
U	Uridine
UNG	Uracil-DNA glycosylase
V	Variable

VH	Heavy chain
VL	Light chain
WT	Wildtype

1 INTRODUCTION

The purpose of this introduction is to give the reader a broad and sometimes more detailed description of information regarding the background of this thesis. The goal is to provide background information and a survey of the most relevant literature to make the Results and Discussion sections, as well as the enclosed papers, easier to comprehend.

1.1 B CELL IMMUNOLOGY

1.1.1 Introduction to the innate and adaptive immune systems

To combat pathogens, such as viruses, bacteria, parasites and fungi, defense mechanisms throughout our body are needed. The immune system serves as our defense against foreign antigens. This highly organized machinery of different cell types and organs can be divided into two parts, the innate and adaptive immune systems. The receptors responsible for recognition of foreign structures expressed by cells of the innate immune system are germline-encoded and invariable. While the receptors responsible for recognition of antigen in the adaptive immune system, the B and T cell receptors, are highly variable and, in the case of B cells, undergo affinity maturation. Furthermore, clonal expansion and generation of immune memory are hallmarks of the adaptive immune system, while innate immune responses, unless in rare exceptions [1], do not generate memory.

1.1.1.1 Innate immunity

The innate immune system is responsible for the initial, rapid response, which takes place during the first hours to first couple of days after an infection. The very first line of defense is provided by the epithelial cells that surround most of our body surfaces and organs. If this barrier fails to block microbes from entering the body, innate immune cells and their products will immediately take over. Innate immune cells include phagocytes (i.e. neutrophils, monocytes and macrophages), natural killer cells (NK cells) and dendritic cells (DCs). These cells play different roles when it comes to host defense. The receptors expressed on innate immune cells responsible for alerting the organism to danger are called pattern recognition receptors (PRRs) [2]. PRRs recognize pathogen-associated molecular patterns (PAMPs) found on microbes. These include Toll-like receptors (TLRs) that bind to bacterial endotoxins, single- or double-stranded RNA and unmethylated DNA, and mannose receptors binding to glycoproteins or glycolipids with terminal mannose residues (reviewed in [3]). As mentioned above, these receptors have limited diversity because they are encoded in the germline.

Neutrophils and monocytes are circulating cells that can be recruited to sites of infection. Monocytes that reside in tissues for a long time will differentiate into macrophages. When a microbe has broken the epithelial barrier, local macrophages will recognize, bind and phagocytose the invading entity. In this process, the cell membrane extends and engulfs the foreign antigen bound to surface receptor of the phagocytic cell into intracellular vesicles and destroys it via lysosomal enzymes. By producing the cytokines interleukin-1 (IL-1) and

tumor necrosis factor alpha (TNFα), macrophages stimulate endothelial cells to express the adhesion molecules E-selectin and P-selectin [4, 5], which facilitate recruitment of other immune cells to the site of infection.

Natural killer (NK) cells recognize infected and stressed cells and respond by killing the target cell and by producing interferon-γ (IFN-γ), which in turn activates macrophages. NK cells express activating and inhibitory receptors (reviewed in [6]). If inhibitory receptors fail to bind to the major histocompatibility complex class I (MHC I) on the target cell, the NK cell will become activated and kill the infected cell.

Dendritic cells (DCs) belong to a group of cells that are especially capable of taking up antigen, processing and presenting fragments of it on surface MHC molecules, called antigen-presenting cells (APCs) [7]. In addition to DCs, macrophages and B cells also have antigen-presenting capacity and therefore belong to the group of APCs. Each MHC molecule can present many different peptides, but only one at the time. There are two different classes of MHC molecules, class I and II, which use two different intracellular pathways for antigen processing. In the class I pathway (MHC I), endogenously expressed antigens are processed and loaded on MHC class I molecules for presentation to CD8+ cytotoxic T cells (CTLs), whose function is to kill the infected cell. In B cells and macrophages, the class II pathway is responsible for the antigen processing of endocytosed extracellular microbes and antigens. These presented peptides are recognized by another type of T cells, the CD4+ Th cells. Due to their efficiency in taking up and presenting antigens to cells of the adaptive immunity, DCs are considered the most important linker cells between the innate and adaptive immunity [8].

1.1.1.2 Adaptive immunity

The adaptive immune system can be broadly divided into two parts; humoral and cell-mediated immunity. Compared to innate immunity, the cells of the adaptive immune system generally require at least a week to develop the functions required to effectively combat invading pathogens.

Cell-mediated immunity

T cells are responsible for cell-mediated immunity, whose main function is to clear infections caused be intracellular microbes. Different T cell subsets serve different roles in this process; CD4+ helper T cells, by activating phagocytes to destroy microbes residing in intracellular vesicles, and CD8+ T cells, by killing productively infected cells. The antiviral activity of CD8+ T cells is based on target cell killing, which induces apoptosis in the infected target cells. There are a number of different subsets that CD4+ helper T cells can differentiate into, such as Th1, Th2, Th17, regulatory T cell (Treg), follicular helper T cells (Tfh), as well as Th9 and Th22. Depending on the cytokine environment around the CD4+ T cell at the time of priming, this will influence transcription factor expression and thereby its differentiation into any of the different subsets [9]. CD4+ helper T cells that provide help to B cells during the generation of an antibody response, the Tfh cells, will be described in more detail below.

Humoral immunity

Secreted antibodies produced by B cells that have differentiated into plasma cells are the main effector molecules in humoral immunity. The B cell receptor repertoire is capable of recognizing a large variety of molecules, i.e. proteins, polysaccharides, lipids and lipid toxins of which proteins are the most immunogenic (reviewed in [10]). The effector functions of antibodies include neutralization and elimination of extracellular pathogens and microbial toxins, complement activation and antibody-dependent cell-mediated cytotoxicity (ADCC). Antibody responses are either T cell-dependent (TD) or T cell-independent (TI). In the TD response, B cells receive signals from cognate CD4+ T helper cells presenting peptides from the same antigen on its MHC class II molecules. Antibody responses elicited in TD pathway are of higher affinity and have typically undergone isotype switching (see below for more details). B cells recognizing polysaccharides, lipids and other non-protein antigens become activated in the absence of CD4+ T cell help, which is therefore named TI responses. These antibodies produced as part of a TI response have rarely undergone isotype switching and little, if any, affinity maturation [10]. Humoral immunity is the main focus of this thesis and will therefore be discussed in more detail in the following sections.

1.1.2 The development of early B cells in the bone marrow

The various stages of early B cell development take place in the bone marrow (BM) (in adults) or in the fetal liver (during embryonic life). B cells originate from hematopoietic stem cells (HSC) that differentiate to common lymphocyte progenitors (CLP) [11, 12]. During B cell development, the primary immunoglobulin (Ig) diversification takes place. At the stage of progenitor (pro) B cells and precursor (pre) B cells, DNA recombination is initiated by the first step to assemble gene segments in a stepwise process known as V(D)J recombination to generate the functional immunoglobulin/BCR repertoire (this process will be discussed in further detail later).

When the IL-7 receptor starts to be expressed at the CLP stage, it results in the induction of B cell development, first via the pro-B cells. In pro-B cells, IL-7 receptors play a central role by signaling survival [13]. Other transcription factors that are important for the CLP to pro-B cell differentiation include E2A, EBF and Pax5 [14]. V(D)J recombination starts in the pro-B cell stage by the rearrangement of D_H to J_H segments of the Ig heavy chain (IgH). Next, the pro-B cell proceeds to the pre-B cell stage where the V_H to DJ_H segment joining takes place. When a successful VDJ_H rearrangement has occurred, a pre-B cell receptor (pre-BCR) starts to be expressed on the pre-B cell surface. The pre-BCR contains the IgH and a surrogate light chain (SL), which comprises of the proteins Vpre5 and λ5. Vpre5 and λ5 make up a molecule that is structurally similar to the Ig light chain (IgL) [15, 16]. The expression of a pre-BCR indicates an important survival step in the early B development. In late pre-B cell stage, the IgL rearrangement starts. Gene segments of V_L and J_L rearrange and replace the SL, which results in BCR expression on immature/naïve B cells [17, 18]. There is an extreme turnover of B cells during development in the BM. In mice, it has been estimated that $2x10^7$ BCR expressing immature B cells are developed daily, but approximately only 10% of those will

be selected to the pool of immature/naïve B cells in the periphery [19]. This stringent filter is called negative selection, with the purpose to remove self-reactive B cells from the immature B cell pool by elimination [20-22], receptor editing [23, 24] or anergy [25, 26].

1.1.3 Architecture of secondary lymphoid organs

Secondary lymphoid organs (SLOs) are different peripheral lymphoid tissues where the lymphocytes become activated, which include spleen, lymph nodes (LNs), tonsils, Peyer's patches and mucosa associated lymphoid tissue (MALT). In humoral immunity, these organs function as a filtering system where extracellular fluids, such as blood, lymphatic- and tissue fluid, are filtered and antigens trapped. SLOs are highly qualified structures to promote antigen and cell interactions. Not only because of the capacity to filter and capture antigens, their microarchitecture also allows for different compartments where different cell types can interact to initiate immune responses and the subsequent fate of each cell type (reviewed in [27]). To optimize antigen-capture and presentation, circulating cells leave the current SLO if unsuccessful antigen capture appears to recirculate through the body to search for antigens elsewhere in other SLOs [28]. The architecture and cells located in these organs differ to some extent from each other as well as in respective counterparts in different species.

1.1.3.1 Spleen and lymph node

The spleen is the largest SLO in the body and consists of the red and the white pulp. The red pulp, which is rich in blood and therefore function as a blood filter by removing foreign material [29]. The white and the red pulp is separated by the marginal zone (MZ), which is located in the white pulp. Other structures within the white pulp are the follicle (also known as the B cell zone) and the periarteriolar lymphoid sheath (PALS). There are several differences in the architecture of the lymph node compare to the spleen [27]. One of them is that, in mice, MZ B cells are only thought to be present in the spleen, although a similar population recently was found in LN and suggested to represent MZ-like B cells [30].

1.1.4 B cell subsets in the periphery

Apart from the B cells mentioned in previous section, there are a number of other subsets of B cells that originate from the pool of IgM-surface expressing mature B cells found throughout the body. These B cell populations differ from each other by their distinct functions, where they anatomically are located, their expression of surface molecules and how they participate in an immune response. The three major subsets of mature B cells found in mice are: B-1 cells, MZ B cells and follicular B cells [31].

1.1.4.1 B-1 cells

In addition to B cell development in the bone marrow, which occurs throughout life, a distinct subset of B cells develops in the fetal liver and during early life, after which they persist throughout life via self-renewal (reviewed in [32]). B-1 cells are the dominating lymphocyte populations in pleural and peritoneal cavities. About 30-60% of all lymphocytes in these locations are B-1 cells and they respond primarily to non-protein antigens. In SLOs the

frequency of these cells is very low, 0.2-1% of all lymphocytes. B-1 cells can be further divided into B-1a and B-1b B cells distinguished by the surface expression of CD5. B-1-a cells express CD5, whereas B-1b cells do not. B-1a cells produce so called natural antibodies present at steady state which are produced in absence of exogenous stimulation. These antibodies are characterized as poly-reactive low-affinity antibodies primarily of the IgM isotype. B-1 cells are responsible for the early and rapid immune responses against TI antigens along with MZ B cells. Instead of requiring help from CD4+ T cells, co-stimulation of innate receptors activates the cell, making them a rapid responsive line of defense, within the first day of infection proliferate and differentiate into plasmablasts. This capacity of rapid response makes the B-1 cells an important compliment to the later arising extra-follicular B-2 cell responses [33, 34].

1.1.4.2 B-2 cells

The other group of mature B cells is the B-2 cells, which encompass follicular B cells and MZ B cells. B-2 cells are generated in the BM throughout life.

Follicular B cells represent the vast majority of B cells in the spleen and LNs, as well as among circulating B cells. Follicular B cells comprise up to 80-90% of all B cells in the spleen and LNs and thus represent the largest B cell subset. Their name refers to the fact that these cells resides in the follicle of lymphoid organs.

MZ B cells ($IgM^{hi}IgD^{low}CD21^{hi}CD23^{low}$) reside in the marginal zone of the spleen, located at the border of the red and white pulp. Although MZ B cells are described to be restricted to the spleen, a recent study reported nodal MZ B cells in lymph nodes of several mouse strains with a phenotype like splenic MZ B cells sharing similar functions and anatomical distribution [30]. MZ B cells function as a shuttle for antigen into the lymphoid organs and to be the first line of defense against blood-borne antigens. Their specific location helps the MZ B cells capture antigen in the MZ [35] and deliver it the follicle to follicular DCs (FDCs), also located in the white pulp. This shuttling has been shown to be CXCR5 dependent [36]. Together with B-1b cells, MZ B cells play a role in the early and rapid immune response against both TD and TI antigens, primarily the later [37].

1.1.5 Developing stages of immature/naïve B cells in the periphery

Immature/naïve B cells exit the BM via the circulation and migrate to the periphery, for further differentiation. Transitional B cells are, as the name implies, a transitional stage of B cells to differentiate from immature to mature B cells. These cells develop from the B cells leaving the BM and migrate to the spleen [38], not LNs [39]. It is unclear why transitional B cells cannot populate LNs and differentiate to mature B cells there, although suggestions regarding lack of some cell surface receptors and structural differences in that organ might be an explanation [38, 39]. The differentiation to mature/naïve B cells is a three-step process where B cells undergo the three transitional stages (T1-T3). However, it is discussed if there actually exist a third stage (T3) and therefore the transitional B cells sometimes are divided into T1 and T2/3. The different transitional B cells are distinguished by differences in surface

marker expressions, by T1 ($IgM^{hi}IgD^{-}CD21^{-}CD23^{-}AA4.1^{+}$) being similar to immature B cell phenotype whereas T2 ($IgM^{hi}IgD^{+}CD21^{+}CD23^{+}AA4.1^{+}$) and T3 ($IgM^{+}IgD^{+}CD21^{+}CD23^{+}AA4.1^{+}$) becomes more and more similar to the mature B cell phenotype ($IgM^{+}IgD^{hi}CD21^{+}CD23^{+}AA4.1^{-}$) [38, 40-42].

Like the negative selection of self-reactive B cells removes these from the B cell pool during development in the BM, a similar checkpoint takes place in the spleen among transitional B cells. In order to be able to differentiate into mature naïve B cells and MZ B cells, the transitional B cells needs to receive a survival signal [38]. A highly potent cytokine to mediate survival signals to several B cell subsets is called B lymphocyte stimulator (BLyS), also known as BAFF [43-46]. BLyS belongs to the TNF family and mediates its effect via binding to three different receptors expressed on B cells; BLyS receptor 3 (BR3; also known as BAFF-R), BCMA and TACI [43, 47-49]. Although BLyS can bind all three receptors, it is through binding to BR3 that it has been shown to have strongest association with pro-survival effect on transitional, mature/naïve and GC B cells [50]. It is worth mentioning that there is a close homologue to BLyS called APRIL, which binds the same receptors as BLyS except BR3 and does not have the same B cell survival effect as BLyS [51, 52]. Both deficiency and overexpression of BLyS impacts the final mature naïve B cell pool. In mice and humans deficient in either BLyS or BR3 shows compromised transitional and mature naïve B cell pools [53, 54], whereas hyperplasia and autoimmunity (at least signs of it) appears during overexpression of BLyS [43, 55].

1.1.5.1 Extra-follicular B cell responses

Mature/naïve B cells need two signals to become activated. The activation takes place at the T- and B cell boarder. It starts with the capturing of the cognate antigen in the B cell zone of SLOs. The first signal appears when a B cell binds the antigen to the BCR, internalize it, degrades it and finally express a peptide bound on the MHCII on its cell surface. If the B cell interacts with a CD4+ T cell that recognize the peptide expressed by binding to it with its T cell receptor (TCR), the first part of the second activation signal is received by the B cell. The interaction between CD40 ligand (CD40L) on T cells and CD40 on B cells completes the second activation signal in B cells, which also signals survival and proliferation [56]. To make it possible for the two types of lymphocytes to meet and interact, chemokines and chemokine receptors are essential for both cell types.

After encounter of cognate antigen and receiving activation signals from T cells, mature B cells become activated and can undertake two distinct pathways. Either they enter the follicle or differentiate into short-lived plasma cells (PCs) outside the follicle. The short-lived PCs leave the SLOs and produce low affinity IgM antibodies, but reports have also shown secretion of class-switched antibodies [57-59]. Although some class switching appears independently of GC reaction, the generated antibodies seem not to have undergone somatic hypermutation (SHM) or improved its antigen-affinity [60, 61]. These antibodies are the first antibody defense against TD antigens and can be measured within a few days after infection. It is not clear what decides the fate of activated B cells. It appears that B cells with higher

affinity for the cognate antigen primarily differentiate into short-lived PCs, whereas B cells expressing BCR with lower affinity (but still above a certain threshold) will enter germinal center (GC) reactions [62, 63].

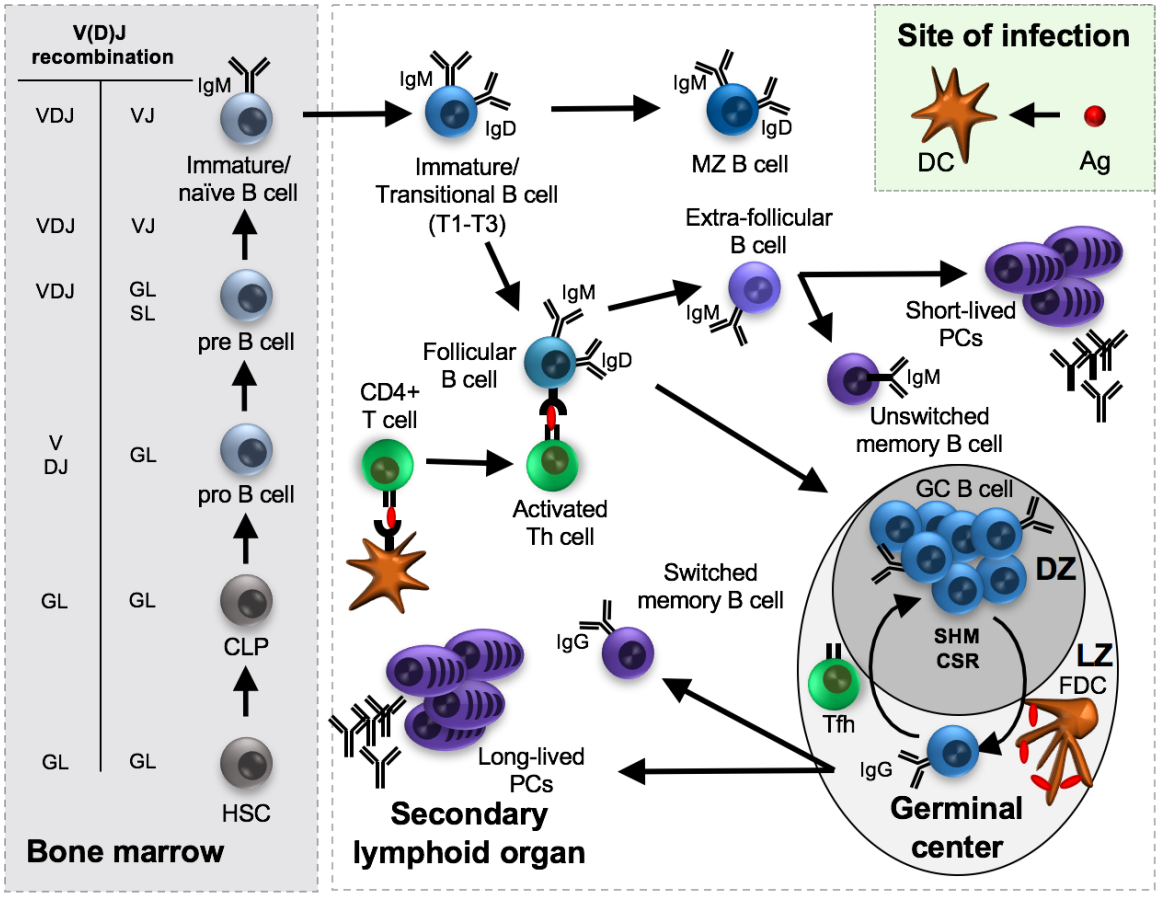

Figure 1. Overview of B cell development in BM and periphery. V(D)J recombination takes place during B cell development in the BM at several separate B cell differentiation stages. In the periphery, the immature B cell undergoes maturation by going through transitional B cell stages. B cells can then either differentiate to MZ B cells or follicular B cells. After activation, follicular B cells differentiate into extra-follicular plasma cells or enter the GC reaction, where the BCR undergoes SHM and class-switching. GC B cells that are positively selected after expansion and affinity maturation in the GC differentiate into PCs or memory B cells. *Illustration by Martina Soldemo.*

1.1.6 Germinal center reaction and affinity maturation

GCs are specialized structures in the follicle where activated B cells undergo somatic hypermutation (SHM) to increase the affinity to the cognate antigen as well as and class switching to change effector function of the antibody. These processes will be described in more details in section 1.1.8. Depending on the antigen, GCs persist between weeks to months [64-67]. Within in the follicle, GC B cells go through different stages in sub-structures, called light and dark zones (LZ and DZ). These zones are separated from each other and play different roles [68]. In the DZ, where GC B cells first end up after entering the GC, the GC B cells are called centroblasts. As centroblasts, they proliferate extensively and express CXCR4. It is in the DZ where the affinity mutation occurs of the BCRs. This process

requires that the antibody sequences undergo SHM and will be presented in more details further down The BCR may also undergo class-switching (see 1.1.8 for more details) to convert the constant region from IgM to any of the other subclasses. After this, the GC B cells entering the LZ and are now called centrocytes and express CXCR5. In the LZ, the FDCs are localized [69]. FDCs are the cell type that has antigen bound to its dendrites in order to present them to B cells [70]. They can receive antigens from MZ B cells that actively shuttle between the MZ and the GC. The surface immune complexes bind the antigens, which are later delivered to the FDCs [71]. Other factors affecting a functional GC reaction is the cytokine IL-21 produced by Tfhs, by both regulating differentiation and proliferation of GC B cells and by regulating Bcl6 expression [72, 73]. The importance of Bcl6 in GC formations has been shown by impairment of its formation, when the Bcl6 gene has been disrupted [74]. In the LZ, the mutated BCR is tested with help of the FDCs. From here, the GC B cell has two fates; if the BCR has reduced capacity to bind the antigen, or if the BCR has acquired self-reactivity, the cell will undergo apoptosis [71] and thereby be deleted from the B cell pool. But if the BCR has gained affinity to the antigen, the GC B cell will be selected to survive [75] and can leave the GC as plasmablasts (later becomes PCs) or memory B cells. The underlying mechanism for the decision of long-lived PCs or memory B cells is still not clear, but it appears that long-lived PCs to a larger extent are selected for higher affinity compared to memory B cells [76]. A recent study, suggested that a temporal switch in the GC could be the reason for the choice of becoming a memory B cell or a long-lived PC. The indication is that memory B cells are generated during early GC, whereas long-lived PCs are generated late during the GC response [77].

1.1.6.1 Memory B cell differentiation

In contrast to human memory B cells, there are few commonly accepted markers for memory B cells in mice. The marker most frequently used is CD38, which is only expressed on follicular B cells and memory B cells. As follicular B cells belong to the naïve pool of B cells, hence IgD has been used to distinguish naïve B cells from memory B cells [65, 78]. Some years ago, a number of additional memory B cell markers was suggested, which were highly expressed on memory B cells but not on naïve B cells. Different combinations of the markers PD-L2, CD80 and CD71 were shown to make up five, suggested, different subsets of memory B cells [79-81]. It is still not known though, whether these memory B cells actually are different memory B cell subsets or just phenotypic differences indicating developing stages in memory B cells.

Memory B cells can also be generated on the outside of the GC [82]. In some settings, these cells can be IgG switched and generated independent of a GC reaction or Tfh help [83, 84]. These memory B cells are similar to IgM memory B cells when comparing the SHM level in the antibody genes [85].

1.1.6.2 The generation of long-lived PCs

The process leading to the final differentiation to antibody-secreting PCs depends on several changes in gene expressions. The main genes involved are B lymphocyte induced maturation protein 1 (Blimp-1), Xbp-1, Pax5 and Bcl6. Blimp-1 is involved in regulating many of these important genes [86], hence having a central role for the generation of PCs. The expression of Xbp-1 is required for the unfolded protein response, a process that is important in PCs in order to handle the stress associated with the large production, folding and secretion of Igs. Blimp-1 is the protein that has been identified to ensure expression of Xbp-1 in PCs [87-90]. Other important genes that Blimp-1 controls by repression are Pax5, which is required to retain B cell identity, and Bcl6, which is required for B cell proliferation, both of which are not desired during PC differentiation and survival [91]. Interferon regulatory factor-4 (IRF4) also has an essential role in PC formation. IRF4 is induced by Xbp-1, which increase its expression after influence of Blimp-1 as described above [92, 93].

Before PCs are fully differentiated, but after they have gained the ability to secrete antibodies they are called plasmablasts. PCs and plasmablasts differ in several distinct ways. The main differences are that plasmablasts are migratory, they proliferate and they express MHCs on the cell surface. When it comes to the expression of chemokine receptors, plasmablasts also differ from GC B cells. Both CXCR5 and CCR7 are down-regulated, allowing plasmablasts to leave the GC [94-96]. It has been suggested that plasmablasts need to be migratory in order to reach special survival niches [97]. Long-lived PCs need survival signals in these specialized niches. The factors providing these signals have been reported to be APRIL, IL-6 and CXCL12, which are produced by eosinophils, stroma cells and megakaryocytes [98-100].

1.1.7 The role of memory B cells and long-lived plasma cells for long-term immunity

Once a memory B cell re-encounters its cognate antigen it can re-enter the GC reaction. This, so called, secondary antibody response differs from primary responses in three ways; (i) the response is more rapid, (ii) the IgG, IgA or IgE isotypes are more common than IgM, and (iii) the generated antibodies are of higher affinity [101]. Memory B cells seem to be able to persist without antigen [102, 103]. Expression of the transcription factor Bach2 differs among B cell subsets. In engineered mice (having naïve B cells expressing IgG1), IgG1 memory B cells express lower levels of Bach2 than naïve B cells, either expressing IgM or IgG1. The lower levels in IgG1 memory B cells correlated with the tendency of favoring PCs differentiation instead of re-enter the GC [104]. IgM memory B cells was not studied, but it would be interesting to evaluate if there is a mechanism behind a memory B cell's "choice" of re-entering the GC for additional rounds of SHM. Another study indicated that switched memory B cells tend to differentiate into plasmablasts rather than entering reformed GCs [105], but it remains unknown if IgM memory B cells are favored over switched memory B cells when both are present outside the GC.

As little as 10% of PCs generated after an acute infection, are estimated to become long-lived PCs. These PCs are then the main source of secreted antibodies that generates protection in

humoral memory [106]. The best example of the duration by which long-lived PCs can survive in the BM is serological studies on vaccine-induced antibodies in serum. For some vaccines, PCs can be as long-lived as the host [107].

1.1.8 Genetics and diverse repertoire of the immunoglobulin loci

To be able to recognize all possible antigens, the immune system has developed a combinatorial mechanism to produce a highly diverse antibody repertoire [108]. The Ig genes are composed of separate DNA segments, which recombine in different combinations to form antibodies with different specificities. Antibodies are glycoproteins and are found as either membrane bound (referred to as the BCR or membrane bound antibody) or in secreted forms (soluble antibody). Every antibody is composed of four polypeptides from two identical heavy (IgH) chains, expressed from the IgH locus, and two identical light (IgL) chains, expressed from either the Igκ or Igλ loci. Each Ig chain consists of one variable and one constant domain. The variable domain of IgH is encoded by three gene segments, the variable (V), diversity (D) and joining (J) segments. The variable domain of IgL is only encoded by two gene segments, the V and J segments. The variable domain of the heavy (V_H) and light (V_L) chains are composed of three hypervariable regions termed complementarity-determining regions 1-3 (CDR1-3), and four less variable framework regions 1-4 (FR1-4). The most variable region is the CDR3, where the largest part of the interactions with the antigen occur for most antibodies. The genes for the constant regions of IgH are designated μ, δ, γ, ε, and α, which after class-switching gives rise to IgM, IgD, IgG, IgE and IgA antibody subclasses, respectively [108]. Class-switching is affected by several cytokines; IFNγ induces a switch to IgG2a, IL-4 to IgG1 and IgE, and TGFβ to IgG2b and IgA [109].

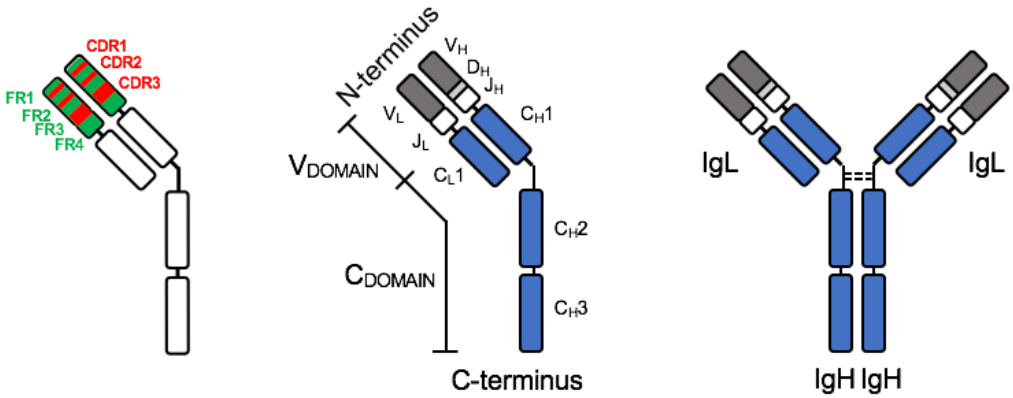

Figure 2. A schematic of the antibody genetics. *Illustration by Martina Soldemo.*

1.1.8.1 V(D)J recombination

V(D)J recombination is a controlled stepwise process. D to J recombination always precedes V to DJ recombination on the heavy chain and V(D)J recombination occurs first on IgH followed by the IgL. The V, D and J gene segments are flanked by highly conserved

recombination signal sequences (RSS) (reviewed in [110]). There are two versions of RSSs; the heptamer (5'-CACAGTG-3') and the nonamer (5'-ACAAAAACC-3') sequences separated by a less conserved spacer sequence. The DNA spacer sequence is either 12 or 23 base pair in length. To enable successful recombination between correct V gene segments the RSS plays an essential role. Efficient recombination can only occur between one 12RSS and one 23RSS, referring to the "12/23 rule". To make "12/23 rule" work, all variable gene segments of the same type, e.g. V, D or J, are flanked by one type of signal making only the joining of D-J and V-DJ possible, but not V-J joining [110, 111].

Initiation of V(D)J recombination starts with the lymphocyte-specific protein recombination-activating gene 1 and 2 (RAG1/RAG2) binding to the RSS flanking the variable gene segments. The RAGs bring the gene segments that will be recombined together via the RSS with assistance of the DNA-binding proteins HMG1 and HMG2 [112]. This complex creates a single-stranded nick between the heptamer and the coding gene segment generating a free 3'-hydroxyl (OH) group. A double-stranded break is made when the 3'-OH group links together with the opposing phosphodiester bond, creating a hairpin of the DNA at the end of the gene segment and a blunt-end at the RSS motif. To protect the RSS from degradation, RAGs remains bound to the motif as a post-cleavage complex [112].

The gene segments are brought together via the DNA double-strand break repair pathway, non-homologous end joining (NHEJ), to form a functional exon. The NHEJ proteins involved in this pathway are a heterodimer of Ku70 and Ku80 (Ku), Artemis, DNA-PKcs, and a dimer of DNA ligase/XRCC4 [113]. The RSSs are joined together to create a closed circle DNA, a signal joint (SJ), that place no further role in the recombination process. The hairpins at the end of the coding sequence are then cleaved with help of a complex of Artemis and DNA-PKcs. This follows the binding of Ku to DNA-PKcs in order to protect the broken DNA ends (reviewed in [114]).

An additional enzyme, terminal deoxynucleotidyltransferase (TdT), adds random non-templated nucleotides (N-nucleotides) to the 3' overhang (at the junctions between the V, D and J segments) to further increase the diversity of the variable region [110]. The addition of N-nucleotides makes all antibodies unique. The other enzymes in the complex will ligate together the two ends of the gene segments completing the recombination process resulting in a coding joint. After a functional V_H has been formed it is fused to a C region through alternative RNA splicing [108]. The light chain then pairs with the matching heavy chain in each B cell to form the functional BCR.

The recombination of heavy and light chain V gene segments takes place at separate stages during B cell development. D_H-J_H and V_H-DJ_H recombination occurs prior to V_L-J_L recombination. At the pre-pro-B cell and pro-B cell stage heavy chain recombination takes place whereas the light chain recombination takes place at pre-B cell prior to immature B cell stage.

As there are two alleles for each IgH chain locus, the immune system has evolved a mechanism to avoid bi-specificity of the B cell clone, the process of allelic exclusion. It is controlled that each B cell have one productive V(D)J IgH allele and one DJ_H intermediate allele. The DJ_H intermediate allele will act as a back-up if the first V(D)J IgH is non-productive after junctional diversity (described in next section). If this occurs, the DJ_H intermediate will undergo joining of V_H to DJ_H, resulting in a B cell containing one productive and one non-productive V(D)J IgH (reviewed in [115]). The allelic exclusion is controlled to some extent by the SL. As the SL binds to IgH and forms the pre-BCR, this leads to down-regulation of the expression and activities of enzymes involved in IgH gene rearrangement [116].

1.1.8.2 Combinational and junctional diversity due to V(D)J recombination

Because of the large number of ways in which the Ig gene segments can be rearranged, many different antibodies can be produced. It is estimated that approximately 10^6-10^8 different antibody combinations are possible through V(D)J recombination and is therefore referred to as combinatorial diversification. In addition to the combinatorial diversity, junctional diversity also contributes to increase the antibody diversity via insertion of non-templated nucleotides between the V, D and J segment junctions as described above. In addition to the diversity obtained after V(D)J recombination, junctional diversity is estimated to increases the diversification up to 10^{16} unique antibody specificities [108]. However, some of these may not result in functional antibodies.

1.1.8.3 Somatic hypermutation

To further diversify and increase the affinity for the cognate antigen, mutations can also be introduced through the process of SHM. Mutations are randomly introduced in the antibody genes and are mainly found in the CDR regions of IgH and IgL V genes [117]. This process takes place in the GC [118]. The reason for this is that the CDRs are the regions on the antibody that form most of the contact with the antigen whereas FRs are important for the structural integrity of the antibody [119]. CDRs were also shown to contain more so called hotspots for activation-induced cytidine deaminase (AID) than other parts of the V region [120]. These hotspots are characterized by a DGYW motif [121]. SHM is initiated by a small protein referred to AID [122], which deaminates cytidines (C) to uridines (U) in single stranded DNA (ssDNA) [123-125]. Two different repair pathways will follow to induce mutations, the base excision repair (BER) or mismatch repair (MMR) systems.

The BER repair system starts by removing the U generated by AID deamination, leaving an abasic site. The enzyme responsible for this is uracil N-glycosylase (UNG). AP endonuclease will follow and cleave the DNA backbone to create a nick at the abasic site, which will be processed into a single-nucleotide gap. The gap will, in turn, be filled by help from DNA polymerase. DNA ligase 1 or 3 then takes over to seal the nick (reviewed in [117]).

The MMR pathway normally works by maintaining genomic integrity via elimination of mutations that arise spontaneously. During SHM, the MMR pathway will contribute to the

mutation. A heterodimer of MSH2 and MSH6 will start the process by recognizing the mismatch, followed by exonuclease-1 (Exo1), which removes DNA that surrounds the mismatch. DNA polymerase then fills the gap and DNA ligase 1 seals the nick (reviewed in [117]).

1.1.8.4 Class-switch recombination

Class-switch recombination (CSR) affects the constant region of IgH by introduction of double-strand-breaks (DSB), resulting in a switch of the effector function of the antibody. This process is not concentrated only to GCs, thus it can occur both inside as well as outside of the GC [126]. AID also plays a major role in CSR. The hotspots for AID are here located in the switch (S) regions, which are long repetitive, non-coding regions. Palindromic variant (AGCT) of DGYW can be found on the complementary strand making it possible for DSB [117]. Each Ig class (Cγ, Cε and Cα) has its own S region (Sγ, Sε and Sα). AID starts the CSR by introducing a C:T mismatch in the S region located downstream of C_H genes.

After the initiation of C:T mismatch, DSB are introduced by BER and MMR pathways. The DSBs undergoes end-joining via C-NHEJ pathway (described in more details in previuos section) when Ku70 and Ku80 have bound to the DSBs. The binding to the DSBs promotes end ligations of XRCC4/ligase 4 (Lig4). This end-joining brings the S regions together and completes the CSR process (reviewed in [117]).

Different Ig isotypes play different roles in the immune system to eliminate invading pathogens; IgG works through endocytosis of antibody-coated antigens, IgE are involved in allergic reactions by triggering mast cells to degranulate and IgA works at mucosal surfaces to eliminate pathogens breaking the mucosal barrier [127-129].

1.2 VACCINES

1.2.1 General functions of vaccines and the role of B cell induced responses

The goal of vaccinations is to induce long-lasting immunity consisting of both PCs and memory B cells specific to the pathogen in question. Memory B cells are required to generate a rapid and protective recall response to antigen re-encounter and long-lived PCs are needed to provide first line defense in the form of secreted antibodies [130]. Vaccines are one of the most important medical innovations of public health to reduce morbidity and mortality to infectious diseases. “Herd immunity” is a term used to describe how a large proportion of a population needs to be vaccinated in order to protect the few that are not or cannot be vaccinated for various reasons. However, it is important to keep in mind that different vaccines require different proportion thresholds of vaccinated individuals in a society to create and maintain herd immunity [131].

The correlates of vaccine-induced immunity are mostly by antigen-specific IgG serum antibodies. For most vaccines, a correlation is also seen with mucosal IgG antibodies. A few vaccines also show correlation with mucosal IgA antibodies [130]. When it comes to vaccine-

induced T cell immunity, only one vaccine has shown T cells as the sole cause of protection. For tuberculosis, the BCG vaccine has a strong correlation of CD4+ T cells based immunity (summarized in [132]). Antibodies produced by long-lived PCs in the BM can be life-long, depending on the vaccine [91, 130]. When half-life of circulating antibodies in vaccinated individuals was tested it surprisingly showed that live-attenuated vaccines had an incredible long half-life. Measles, mumps and rubella vaccines were estimated to have half-lives of 3014 years, 542 years and 114 years, respectively. Vaccines based on bacterial toxins for tetanus and diphtheria showed, on the other hand, much shorter half-lives of 11 years and 19 years, respectively [133]. Memory B cells are the other arm of humoral immunity with an important impact on vaccine efficacy. In contrast to antibody-secreting cells, the PCs responsible for antibody titers in serum, memory B cells do not secrete any antibody. However, as they have gone through SHM, or multiple rounds of mutation, and express BCRs of high affinity for the vaccine antigen, they are capable of responding fast to a secondary encounter with the antigen [134, 135]. Whether memory B cells are important for maintaining serological titers by continuous replenishment of the PC pool is a long-standing question that awaits further and more definitive studies [133].

1.2.2 Different types of vaccines

There are several types of vaccines, depending on when they were developed and what pathogen they were designed to protect against.

Live, attenuated vaccines consist of attenuated forms of the pathogenic virus or bacteria, which are able to replicate weakly in the recipient and can therefore cause disease in persons with intact immune systems. The advantages of using live attenuated vaccines are that they are less costly, the immune response mimics natural infection and it is usually sufficient with a single dose to stimulate long-lived responses. Examples of this type of vaccine are the measles, mumps, rubella, varicella and tuberculosis (BCG) vaccines.

Inactivated vaccines use killed pathogens that are unable to replicate in the host. These vaccines consist of whole organisms that have been treated with either heat or chemicals to become inactivated. By inactivating the pathogen, the vaccine reaches a higher safety level and can therefore be administered to immunodeficient people, pregnant women and small children. The disadvantages, on the other hand, are the requirement of several immunizations and that the addition of an adjuvant (see next section for more details) is often needed. Examples of inactivated based vaccines are the cholera, hepatitis A and pertussis vaccines.

Toxoid vaccines are used when the main cause of illness is a bacterial toxin. The toxins are inactivated by formalin treatment. These “detoxified” toxins, called toxoids, are safe for usage in vaccines. Examples of vaccines based on bacterial toxins are diphtheria and tetanus.

Subunit vaccines are based on the antigens of a pathogen that best stimulate the immune system. These vaccines can contain anything from 1 to 20 or more different antigens. Using subunit vaccines lower the risk of adverse reactions since only the most essential antigens are

used. Examples of subunit vaccines are the seasonal influenza virus, hepatitis B and human papilloma virus vaccines.

Conjugated vaccines are used for immature immune systems of infants and younger children who cannot recognize the polysaccharide coating on bacteria. This has been solved by conjugating toxoids to the polysaccharide. The toxoid is recognized by the immune system and thereby helps the immune response to be directed against the conjugated polysaccharide. [136, 137]. Examples of conjugated vaccines are haemophilus influenza type B (Hib) and meningococcal infection.

1.2.3 Vaccines against variable pathogens

Currently available vaccines have largely been developed empirically, which means that the causative agent of an infectious disease has been isolated, followed by inactivation if needed and *in vivo* testing [138]. This strategy has been successful for a long time, but has given us limited understanding on how these vaccines works to activate the immune system and which specific epitopes are targeted to mediate the protective response. When comparing the antigenic variation among pathogens for which successful vaccines have been developed and those for which vaccines are still lacking, it is clear that the pathogens for which we have vaccines show no or limited variability over time [138]. Highly variable pathogens are considerably more challenging for vaccine development such as human immunodeficient virus type 1(HIV-1), which displays extreme antigenic variability. However, despite the enormous variability of circulating HIV-1 variants, some determinants are conserved suggesting that these could be targeted to induce cross-reactive antibodies [139]. Such efforts are actively pursued but have been shown to present major challenges. The influenza virus has an antigenic variability on a yearly basis, which has been solved by developing seasonal vaccines [138].

1.2.4 Adjuvants

The word adjuvant comes from the Latin word *"adjuvare"* which means *"to help"* or *"to enhance"*. A vaccine adjuvant is a component that increases the specific immune response to an antigen. Adjuvants are necessary in vaccines where non-living pathogens are used, to help triggering the immune response. Live attenuated vaccines mimic natural infection and, hence, are capable of triggering a strong immune response by themselves. More than one adjuvant may be present in the final vaccine. Although a variety of candidate adjuvants have been evaluated in the past few decades, only a limited number of vaccine adjuvants are currently available and approved to be used in humans [140]. The mode of action for most licensed adjuvants is not fully understood, but in general, the goals of adjuvants are to increase antigen uptake, induce danger signals and to recruit the right type of immune cells to the site of injection [141, 142].

1.3 HIV-1 AS AN ANTIBODY VACCINE TARGET

1.3.1 Introduction to HIV-1 structure and function

Human immunodeficiency virus type-1 (HIV-1) was first discovered in 1981 and has since then become one of the most studied viruses. HIV-1 is a lentivirus, a member of the retrovirus group, and infects human cells expressing the surface CD4 molecule and one of two co-receptors (CXCR4 and CCR5), usually CCR5, such as CD4+ T cells, macrophages and dendritic cells [143, 144]. The only viral protein exposed on the surface of the virus is the envelope (Env) glycoprotein, which is synthesized as a gp160 precursor before the processing into a trimer containing heterodimers of gp120 and gp41 subunits. During the processing, gp160 undergoes several events in the endoplasmic reticulum (ER) including signal peptide cleavage, folding, trimerization and extensive glycosylation [145]. To become a native trimer, further events takes place within the host cell. The gp160 is transported to the Golgi apparatus where it becomes cleaved with help from furin-like proteases into non-covalently linked gp120 and gp41 subunits [146]. Glycosylation of asparagine (N) residues to attach the precursor glycan is performed by glycosyl-transferases in the ER, which are then processed into high-mannose, hybrid or complex glycans in the Golgi apparatus [146]. After assembly of the mature gp120 and gp41 heterodimers, the functional trimer has been formed on the plasma cell membrane of the infected cells for incorporation into budding virions. Compared to influenza virus, HIV-1 incorporates very few Env spikes in its membrane. It is estimated that on average only 14 functional Env spike proteins are present on each HIV-1 particle along with numerous non-functional spikes [147, 148].

HIV-1 entry can be described as a three-step process. The first step, after the virus has attached to the target cell surface via various adhesion molecules, is when the primary host cell receptor CD4 binds to the CD4 binding-site (CD4bs) of the HIV-1 Env spike [149, 150]. Conformational changes in the Env structure follows this primary binding to allow Env spike binding to the co-receptor (CCR5 or CXCR4) on the host cell [151] mediated by the co-receptor-binding site (CoRbs). This interaction leads to further conformational changes in the Env spike resulting in that the fusion peptide located on the gp41 subunit binds and penetrates the host plasma membrane [152, 153], the third step of the entry process. Once this has occurred, the virus enters the host cells' cytoplasm where it uncoats and delivers its RNA content (reviewed in [154]). The RNA will then be reversed transcribed by the error-prone reverse transcriptase (RT) to become double stranded DNA, known as a provirus. The provirus is then interacting with HIV-1 integrase and other components to generate a pre-integration complex [155]. This pre-integration complex is then transported to the nucleus of the infected cell, where it integrates with the host DNA [156, 157]. By having become integrated to the host genome, the virus can become latent and survive in the host cell [158]

Although the number of new HIV-1 infections have been declining the last two decades, around 1.8 million new HIV-1 cases were estimated worldwide by UNAIDS in 2016. The number of people living with HIV-1 and the number of acquired immune deficiency syndrome (AIDS)-related deaths in 2016 was estimated to 36.7 and 1 million, respectively

(aidsinfo.unaids.org). The prevention programs have been important locally to spread light on this epidemic, but a vaccine is needed to control this global HIV-1 epidemic.

1.3.2 Mechanisms to evade the immune system

HIV-1 has unique properties making it a difficult target to develop an efficient prophylactic vaccine against. The virus has evolved several immune escape mechanisms summarized below.

1.3.2.1 Mutation rate

The extremely high mutation rate of HIV-1 has created a constant race between the human immune system and the virus itself, where the virus is several steps ahead the immune system. As a result, the elicited anti-Env antibodies are only able to neutralize virus that was generated months earlier and not concurrent circulating variants [159-161]. The error-prone RT is the major cause for the rapid genetic variability exhibit in HIV-1. It has been estimated that it introduces on average 0.2 mutations per viral genome at each replication cycle [162, 163]. This leads to an incredible high number of 10^{10} new virions in an HIV-1 infected individual every day [164].

1.3.2.2 Glycan masking

Another efficient strategy to avoid recognition of conserved epitopes by the immune system in the host, is to cover most of its surface in glycans. As a result, HIV-1 Env is a highly glycosylated protein, with approximately 50% of the gp120 molecular weight comprising glycans. Each gp120 has approximately 25 N-linked glycosylation sites, which makes it one of the most glycosylated viral proteins known [165]. These glycans are host-derived and are large, complex carbohydrate structures that effectively shield vulnerable epitopes on Env, hence referred to as the glycan shield [166, 167]. Some chronically infected HIV-1 patients are capable of generating antibodies that can penetrate this tight glycan shield and bind to otherwise hidden Env protein structures. These antibodies show features that are uncommon in healthy individuals, such as a very high level of SHM, long HCDR3, N-glycan reactivity and self-reactivity [168-171]. By removing some of these glycosylation sites it has been shown to result in an increase in neutralization sensibility of the virus [172].

1.3.2.3 Conformational masking

To protect essential structures involved in binding to the host cell, the HIV-1 virus has evolved a mechanism known as conformational masking to escape recognition of neutralizing antibodies. The CD4bs and CoRbs are protected by glycans and variable regions as well as the natural flexibility of Env. Both the CD4bs and the CoRbs are protected by conformational masking [173], minimizing the time when antibodies can reach and bind these structures.

1.3.2.4 Low Env spike density

As mentioned previously, HIV-1 incorporates surprisingly few Env proteins on its surface. This low number of available Env proteins could be an escape strategy to prevent bivalent

binding (i.e. when an antibody binds with both arms to an antigen or several antigens) [174]. Clearly, this spike density is sufficient for the virus to effectively infect cells so it is likely that the virus has evolved to require as few spikes as possible to minimize the exposure of immunogenic determinants to the immune system.

1.3.2.5 Expression of non-functional Env on the viral surface

HIV-1 exposes a mixture of Env variants on its surface. Along with the functional trimeric Env spike other forms of trimers also exist, such as those that have shed gp120, leaving only gp41 stumps and uncleaved non-functional Env spikes [147, 175]. In addition, a considerable amount of soluble gp120 is present in circulation, acting as an efficient immune decoy. Antibodies against these non-functional forms of Env dominate the total antibody response during HIV-1 infection [176], as well as in immunization studies [177], because they expose structural elements that are highly immunogenic [147].

1.3.3 Types of antibodies induced during HIV-1 infection

Under normal circumstances, highly poly- and autoreactive antibodies are removed from the B cell pool during B cell development [178]. During HIV-1 infection the situation is different as peripheral selection check-points are perturbed resulting in HIV-1 Env antibodies that are frequently poly-reactive and bind non-HIV antigens [179, 180]. During the preparation of this thesis, we wrote a review article about this subject (Soldemo and Karlsson Hedestam, Frontiers of Immunology (2017). https://doi.org/10.3389/fimmu.2017.01057), which covered some of the points discussed below.

1.3.3.1 B cell dysfunction in HIV-I infected individuals

When comparing antibodies induced during chronic HIV-1 infection and by subunit vaccination, it is important to bear in mind that the immune system in chronically HIV-1 infected individuals differ in many ways from that of healthy individuals. Prolonged antigen exposure, but also continuously antigen variability during chronic HIV-1 infection differs remarkably from an immunization situation where a relatively short immunization schedule is used in combination with, in most cases, an invariant Env protein.

During chronic HIV-1 infection, several imbalances in B cell subsets develop, affecting the capacity of chronically infected individuals to respond to vaccination and handle co-infections [181-185]. Hypergammaglobulinemia and loss of B cell memory are hallmarks of these humoral immunity alterations [186, 187]. Dysregulation of B cells is apparent relatively early after HIV-1 infection and worsens during disease progression. Early introduction of antiretroviral therapy to dampen active viremia has positive effects on preserving B cell subsets [188]. Dysregulated B cell subsets and functions are also observed in individuals repeatedly exposed to malaria [187]. Thus, B cell alterations in both HIV-1 and malaria-infected subjects is likely a consequence of prolonged inflammatory responses that occur under these conditions, rather than caused by direct pathogen-B cell interactions. The specific

B cell alterations described in chronically HIV-1-infected individuals include effects on both antigen-inexperienced cells and antigen-experienced cells as discussed below.

Antigen-inexperienced cells. HIV-1-infected individuals display increased frequencies of circulating immature transitional B cells [189]. As transitional B cells display increased sensitivity to spontaneous apoptosis, this may lead to a decreased pool of mature naïve B cells [190, 191]. Altered migratory capacity of immature transitional B cells was also observed, which could affect the distribution of these cells between blood and SLOs in HIV-1 infected individuals [192]. Further, as previously described, peripheral B cell selection is regulated by BLyS. The BLyS levels are increased during HIV-1 infection and may result from sustained IFN type 1 responses due to chronic viremia. A potential consequence of increased BLyS levels is that the threshold for B cell selection is altered, which may promote survival of B cells that otherwise would be subject to negative selection such as poly-reactive or auto-reactive clonotypes [43, 55]. Whether the naïve B cell repertoire in HIV-1-infected individuals more frequently display features associated with poly- or self-reactivity is not known but will be important to investigate, especially in relation to the generation of broadly neutralizing antibodies (bNAbs) [193].

Antigen-experienced cells. HIV-1-infected individuals also display alterations of the memory B cell compartment. Activated human memory B cells and tissue-like memory B cells are increased during persistent HIV-1 infection, whereas resting memory B cells are decreased in frequency [181, 194]. Already early on during HIV-1 infection these alternations in B cell compartments lead to consequences in the form of poor maintenance of serological antibody levels to previous vaccination (i.e. measles, tetanus and pneumococcus) [185], as well as impaired responses to new vaccinations [195]. Exhausted B cells also appear during the chronic phase of the infection. These exhausted B cells are characterized by a decreased capacity to proliferate in response to stimulation [196] and with expression of molecules that negatively regulate antigen receptor signaling or homing to sites of inflammation [197, 198]. Further, HIV-1-infected individuals display increased frequencies of circulating plasmablasts [199] consistent with non-antigen-specific differentiation of memory B cells into ASCs resulting in hypergammaglobulinemia and decreased numbers of resting memory B cells. Thus, the immune system in chronically HIV-1-infected individuals is different from that of healthy subjects in several ways, which likely affects the kinds of antibodies that are elicited.

1.3.3.2 Non-neutralizing antibodies

A majority of Env-specific antibodies generated during infection exhibit no neutralizing capacity. These non-neutralizing antibodies are the first Env-specific antibodies to be generated after acute HIV-1 infection. They appear during the first weeks of infection and are directed to non-functional forms of Env, as the unstable Env disassociates, resulting in expose of structures that would not be recognized on a closed native Env structure [147, 200]. As these antibodies lack neutralizing activity they do not have any impact on selective pressure of the virus, nor the viral load in plasma [201, 202].

1.3.3.3 Strain-specific antibodies

Strain-specific antibodies, also known as autologous antibodies, appear after a few months after infection. These antibodies are mostly directed against the highly variable and immunogenic regions of Env [203], which constantly mutate to escape the antibodies generated [204]. As a result of this, strain-specific antibodies are only capable of neutralizing the virus it was elicited for (the autologous virus) and not for any other viral variants that arose later [159, 167, 205].

1.3.3.4 Broadly neutralizing antibodies

Eventually, after years of chronic HIV-1 infection (approximately 2-4 years), around 20% of infected individuals develop antibodies that are cross-neutralizing and 1-2% develop antibodies that are capable of neutralizing a broad range of HIV-1 strains [206, 207]. These, so called, bNAbs are rare [208] and so far, despite many HIV-1 vaccine clinical trials no vaccine candidate, nor any experimental vaccine candidate tested in animal models have been able to induce such antibodies in small animals or primates with natural immune systems. There are several distinct features that differ between bNAbs and those generated during Env immunization studies. These features include extremely high levels of SHM, long HCDR3 regions, N-glycan recognition as well as, for some classes of antibodies, restricted V gene germline use [169, 170, 209, 210]. Although HIV-1 is highly variable, there are some determinants that are too important for the function of the virus to tolerate mutations, as previously described. These determinants are therefore the main targets for vaccine-induced antibodies and include the CD4bs, some V2 determinants in the trimer apex, the base of the V3 region including the "high mannose patch" and the interface region of gp120-gp41 [211]. N-glycan recognition of antibodies is uncommon in healthy individuals [212] as it is a sign of self-recognition. As chronic HIV-1 infection results in alternations in B cell populations, this might lead to that some B cells overcome the peripheral negative selection and hence express self-reactive properties [213]. The high degree of SHM in bNAbs are most likely a result of persisting antigen exposure in HIV-1 infected individuals, which drive B cells to undergo multiple rounds of affinity maturation and selection in the GC [168, 209, 214].

Due to the disease progression and establishment of extensive viral reservoirs that are the source of escape variants, it appears that bNAbs generated late during infection do not have any beneficial effect in the individual in which they arose. Instead, the value of bNAbs, if they could be induced by immunization, would be to provide protection prior to virus exposure (prophylactic vaccination). Such protective capacity of bNAbs has been demonstrated in passive immunizations studies in non-human primates (NHPs) where bNAbs were administered prior to SHIV infection [215-222].

1.3.4 The envelope glycoprotein and immunogen design

As described earlier, Env is the sole viral surface protein and hence the only available antibody target. Thus, Env is the main focus for a protein based vaccine against HIV-1. One of the major focuses within the HIV-1 immunogen design field is to engineer recombinant

native-like trimeric Env that expose as few non-neutralizing epitopes as possible to direct the induced immune response towards the desired neutralizing epitopes. The development of native-like trimeric Env can be divided into to two separate phases, early and new generation Env trimer mimetics, based on technical advances and increased knowledge.

1.3.4.1 Early generation Env trimer mimetics

In the early days of Env engineering, a stop codon was introduced to create soluble oligomers of Env. This stop codon was introduced at the C-terminus of the gp41 ectodomain just above the transmembrane domain. The problem with this approach was that the resulting Env immunogens were unstable due to the non-covalent association of gp120 and gp41 (reviewed in [223]). To improve this, the cleavage site of the gp160 precursor was deleted or mutated. This improved the stability of gp120 and gp41 association, but as it is a non-natural covalent association it created a mixture of gp140 conformations. The next step was to introduce a trimerization motif to make more stable and homogeneous trimers. The trimerization motifs included, for example, GCN4 or foldon [224-226]. As new methods were developed to study protein structural conformations, it became obvious that these Env trimers were also heterogeneous and did not assemble into a closed native-like conformation and hence they did not mimic the native functional Env trimers [227]. Nevertheless, despite the non-native conformation of these first generation Env trimers, they have contributed extensively to our knowledge of Env-specific B cell responses in immunization settings.

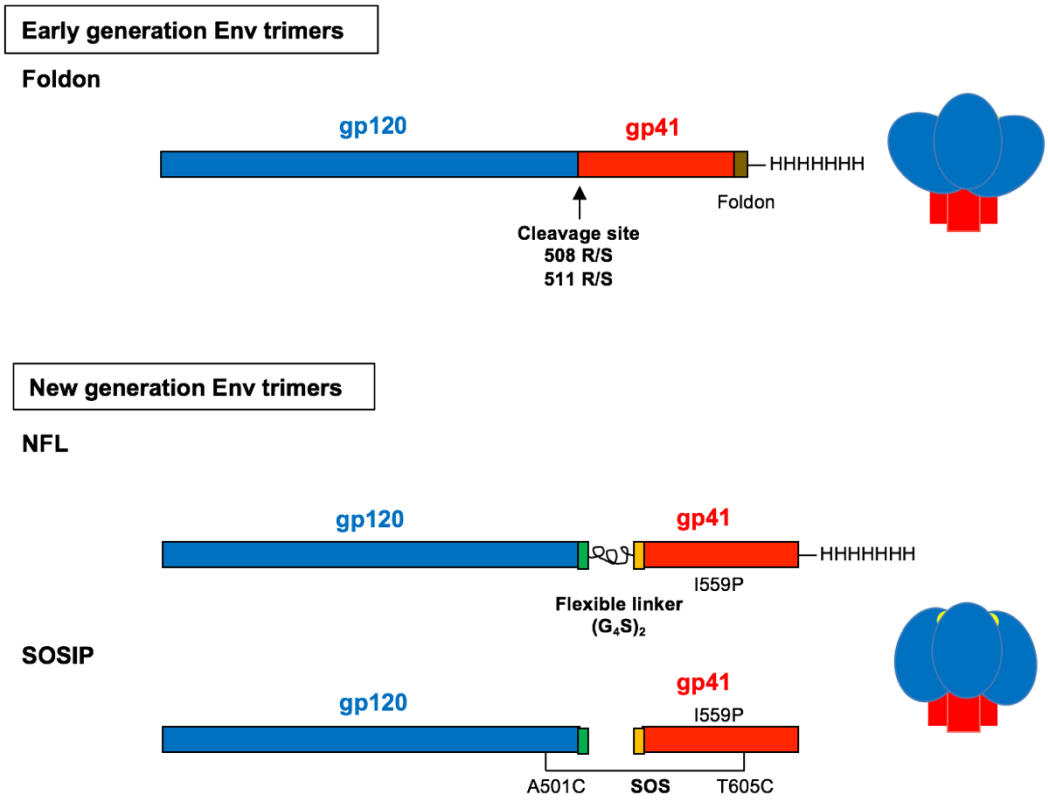

Figure 3. Examples of early and new generation Env trimers. *Illustration by Martina Soldemo.*

1.3.4.2 New generation Env trimer mimetics

One approach to generate stable trimers is to introduce one additional C residue each in specific residues of gp120 and gp41, resulting in a covalent link between the subunits but maintaining the natural gp120-gp41 cleavage site. This link is referred to as the SOS (501C to 604C) disulfide bond [228]. However, this modification alone generated only a small fraction of well-folded stable trimers. The reason for this is the spring-loaded nature of gp41 to adopt a post-fusion configuration. This tendency to go from the pre-fusion state to the post-fusion state made it challenging to increase the stability of the Env trimers. The solution for this was to introduce an amino acid substitution in gp41 (an I559P) in the SOS Env trimer structure, which created the SOSIP Env trimer [229]. An additional modification was to introduce a truncation at residue 664 on the clade A BG505 strain background, resulting in high yield of homologous trimers after purification as documented via negative-stain electron microscopy (NS EM). The BG505 virus strain was selected after screening over 50 different Env constructs [230], demonstrating that Env from different HIV-1 strains are differently prone to generate stable trimers. So far, BG505.664 is the Env that naturally shows the most favorable conformation in terms of mimicking the native Env trimer [230, 231].

Another type of new generation native-like trimers is the uncleaved native flexibly linked (NFL) trimers [232] that contain a flexible-linker of 10 residues $(G_4S)_2$ between the C-terminus of gp120 and N-terminus of gp41. When the NFL trimers were made on the BG505 background they formed well-ordered trimers consistent with that this strain has properties that are favorable for the formation of soluble trimers. When Env sequences from other strains are used the NFL trimers produced after transient transfection contains not only well-ordered trimers, but also aggregates, dimers and monomers. Additional selection steps are therefore needed to enrich for well-ordered trimers, were either bNAbs or non-NAbs are used for positive or negative selection, respectively [233, 234]. To increase the yield of well-ordered trimers further design optimizations were performed by identifying amino acid residues from the BG505 SOSIP structure that increase the propensity of Env to form well-ordered trimers, the so-called trimer derived (TD) mutations, and transferring these mutations to other Env strains [235]. Introduction of the 8 TD substitutions in the 16055 Env sequence generated mostly well-ordered trimers as determined by both size-exclusive chromatography (SEC) and EM. To stabilize the trimers in the pre-receptor bound confirmation, a cysteine-pair was also introduced into the 16055 NFL TD sequence in the bridging region (CC=201-433) to generate disulfide linkage to abolish the capacity of the trimers to undergo CD4-induced conformational changes that normally occurs when gp120 binds CD4 resulting in a trimer construct referred to as 16055 NFL TD CC [235].

Current strategies to evaluate the conformation of recombinant soluble Env trimers include 1) the use of both bNAbs and non-Nabs, where only the former recognize native Env trimer structures, 2) differential scanning calorimetry (DSC) to measure a single homogeneous melting temperature, and 3) EM to determine the well-ordered native-like trimer [232, 233, 235].

1.3.4.3 Particulate display

The immune system has evolved to recognize pathogens such as viruses, which are considerably larger than soluble proteins. In addition, many viruses display their surface antigens in a multi-valent manner, which may lower the threshold for B cell activation due to the capacity of such antigens to cross-link the BCR. Thus, it is logical to speculate that improved immunogenicity may be achieved if Env could be displayed as multi-valent arrays on some sort of particle. HIV-1 naturally has a low Env spike density as described above and the generation of HIV-1-based virus-like particles (VPLs) with sufficient amount on Env on their surface has been challenging. HIV-1 also has non-functional forms of Env on its surface. Since such forms are undesired in a vaccine context this has hampered the development of HIV-1-based VLPs as vaccine candidates. One way to enrich for functional Env spikes on VLPs is to use protease-mediated degradation of the non-functional Envs on the virion surface [236]. A strategy to develop solid-phase proteoliposomes containing native Env trimers in a physiologic membrane setting was also explored [237] but this was met with similar problems as HIV-1-based VLPs in terms of low density of Env per particle [238]. A strategy to improve VPLs is to through the concept of intrastructural help (ISH) where non-Env T helper epitopes are delivered together with Env to APCs to enhance the Env-specific T cell response ([239] and reviewed in [240]). An additional approach to present Env in a particulate manner is to couple Env at high density to synthetic liposomes [241]. One recent study from our laboratory evaluated the immunogenicity of soluble 16055 Env NFL trimers compared to the same trimers arrayed on liposomes in rhesus macaques and demonstrated that the latter induced improved neutralizing antibody responses against the autologous tier 2 virus [242].

1.3.5 The use of animal models in HIV-1 research

The use of animal models in medical research is critical for our understanding of biological processes such as infectious diseases, immunology, neurology, pathology and toxicology. Information generated from animal experiments has been fundamental when, for example, developing new vaccines, new surgery methods and designing new treatments. All achievements in the medical field are a combination of animal experiments and new cell and tissue culture techniques as well as statistical analysis and new high through-put genomic screening. Despite the need of animal experiments to drive the medical research forward, animal studies shall be performed according to the "3 R's" principle, namely to *reduce* the number of animals needed, to *refine* tests used and to *replace* the use of animals whenever possible. This principle, which was first published in 1959 by Russell and Burch as *"The Principles of Humane Experimental Techniques",* has since been embedded worldwide in legislations governing research in animal models.

1.3.5.1 Non-human primates

Non-human primates (NHPs), especially the Asian macaques and particularly rhesus macaques, are frequently used to model HIV-1 infection since they can be infected with SIV and chimeric SIV/HIV (SHIV) viruses [243]. The level of homology between rhesus

macaques and humans is high both in sequence (93%) [244], gene expression pattern [245] and immune cell composition [246, 247]. Our group also showed that there is a high homology in antibody V gene segments between humans and rhesus macaques [248, 249], but a higher variability between individual macaques than between humans [250]. Traditionally, NHPs were used primarily to evaluate serum antibody responses induced by vaccination since reagents and protocols for cell-based assays were poorly developed for NHPs. Over the past decade our group has established methodology to analyze macaque B cell populations [251-253] and to single cell sort macaque memory B cells for monoclonal antibody isolation [242, 249, 254, 255] opening up new possibilities for vaccine research in the NHP model.

1.3.5.2 Mouse strains

Studies in mice investigating immune responses to non-pathogen-derived antigens, such as hen egg lysozyme (HEL) and the hapten-carrier antigen NP-CGG, have provided the foundation for our current understanding of lymphocyte development and host responses to antigens [62, 256-259]. There are clear benefits to using mice as an experimental model because of their short breeding cycles, the relatively low maintenance cost, the ease of generating genetically manipulated strains and the comprehensive genetic data bases available. However, mice have not been commonly used in the HIV-1 vaccine research field due to concerns that they do not mimic the human immune system sufficiently well. For example, it is known that many broadly neutralizing antibodies isolated from chronically HIV-1 infected humans have long D segments allowing the generation of antibodies with long HCDR3 regions, which may be required to access certain epitopes on Env. In contrast, mice encode short D segments, which may limit their capacity to generate antibodies capable of accessing epitopes such as the CD4bs on Env. Mice are extremely useful for basic immunological studies due to the many engineered strains available. The use of mice can therefore facilitate mechanistic studies to investigate specific questions regarding immunogenic properties of HIV-1 Env immunizations where challenge studies are not required.

Various knock-in mouse systems are used trying to elicit certain classes of bNAbs. These knock-in (KI) mice are engineered systems compared to immunogenicity studies in animals with natural immune repertoire. As bNAbs are rare and it has been extremely challenging to even achieve heterologous HIV-1 specific antibodies in immunization studies, another approach has been recently developed. The question if it is possible to prime the right B cell precursor in an intact immune system by immunization has been raised. Germline-targeting immunogens have been used in genetically modified KI mice harboring BCRs with a HC of different bNAbs. This results in a hybrid BCR having human HC and mouse LC. By immunizing such mice, it was reviled that the antibody response elicited was selected for the B cell clones that had the suitable BCRs [260, 261]. The focus in this thesis has been to study immune responses to Env in mice with natural immune repertoire to ask fundamental immunological questions.

2 AIMS

The overall aims of this thesis were to improve our understanding of antibody responses elicited by immunizations with recombinant HIV-1 Env trimers in mice, and to use different approaches to modulate the responses, thereby potentially improving the outcome of vaccination.

The specific aim for each study was:

Paper I: To investigate potential competition between B cells specific for distal epitopes on the same antigen.

Paper II: To study if expanding the naïve B cell repertoire prior to immunization would improve the antibody responses to HIV-1 Env.

Paper III: To establish flow cytometry-based protocols for studying Env-specific memory B cells and GC B cells in immunized mice.

Paper IV: To evaluate how chemical cross-linking of HIV-1 Env to stabilize the trimer would influence the elicited immune responses.

3 MATERIALS AND METHODS

3.1 ANIMALS

Male or female mice, 7-9 weeks old at the beginning of each experiment, of both BALB/c and C57BL/6 strain were used (see respective paper for specific details for origin of mice).

During all studies included in this thesis, the mice were kept at the animal facility at the Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet. All experiments were pre-approved by the Committee for Animal Ethics (Stockholm, Sweden) and performed under approved conditions according to given guidelines.

3.2 IMMUNIZATIONS AND TREATMENT OF MICE

To study B cell responses against HIV-1 Env, mice were immunized (s.c. or i.p.) with 10 μg recombinant HIV-1 Env trimers (early or new generation trimers, depending on which study) in 10 μg AbISCO-100 (Isconova), also known as Matrix M-1 (Novavax), or Imject®Alum (Thermo Scientific/Pierce) adjuvant. The number of booster injections was one or two and the time interval between each one of the injections were dependent on study layout of the specific study. In **Paper II**, mice were pre-treated with injections of recombinant BLyS (10 μg per injection) or PBS for control mice, for 10 consecutive days with i.p. injections before Env immunization. Mice were sacrificed by incubation in CO_2 chamber followed by cervical dislocation.

3.3 RECOMBINANT PROTEINS

3.3.1 Recombinant human BLyS

Recombinant human BLyS was provided by Human Genome Science (Rockville, MD).

3.3.2 Production and purification of Env glycoproteins

For immunizations in **Paper I**, **II and III**, recombinant soluble Env foldon gp140 trimers (gp140-F) based on the YU2 isolate of HIV-1 was used [226]. In **Paper IV**, mice were immunized with recombinant NFL TD CC Env trimer from the HIV-1 16055 isolate. For the B cell ELISpot assay, biotinylated versions of YU2 gp140-F were used, as previously described [177]. Env-specific B cells was detected in flow cytometry by using biotinylated YU2 gp140-F trimers (**Paper III**).

YU2 gp140-F trimers for immunizations and the probes used for B cell differential ELISpot in **Paper I-III** (gp140-F-Avitag, gp120-F-Avitag, gp120-F-ΔV3-Avitag, gp120-F-ΔV1/2/3-Avitag and gp120-F-ΔV 1/2-Avitag), were previously described in [177, 226]. The Avitag sequence (GLNDIFEAQKIEWHE) was added to allow site-specific biotinylation of the probes. A foldon trimerization motif and the histidine tag $(His)_6$ was added upstream the biotinylation site. In **Paper I**, the YU2-derived gV3 Env trimer was designed as described in [262]. All YU2 Env proteins were produced transient transfection in FreeStyle HEK293F

suspension cells (Invitrogen) using the FreeStyle 293 expression system (Invitrogen). After 4-5 days, supernatant was collected and spun at 3500xg to remove cells and cell debris. This was followed by sterile filtration through a 0.22 μM filter and dissolved EDTA-free protease inhibitor tablets (Roche) and penicillin-streptomycin (Invitrogen) was added to the supernatant. Env proteins were then purified in a two-step process. First, the supernatant was passed through a lentil-lectin column where the glycans of the proteins were captured inside the column. To remove unspecific binding, the column was washed extensively in washing buffer (PBS/0.5 M NaCl). The protein bound to the column was then eluted by adding elusion buffer (1 M methyl-α-D-mannopyranoside) and the flow through was saved for the next purification step. Second, the flow through containing protein obtained from lentil-lectin (GE Healthcare) column was the loaded to the second column, the nickel (His-tag) column. The His-tag expressed on each protein binds to the nickel in the column. After extensive washing in wash buffer (PBS, 40 mM imidazole (IM), 0.5 M NaCl, 200 mMPO_4) the proteins were eluted by adding elution buffer (300 mM IM in PBS) to the column. The flow through was the concentrated by using Amicon 30-kDa cut-off centrifugation tubes (Millipore). Buffer exchange to PBS was performed be adding PBS to the concentrated protein during additional spins with the centrifugation tube. To verify that the proteins and probes have same conformation as previous batches, the proteins were checked for the recognition of several different HIV-1 mAbs (b12, 17b, 2G12 and 2F5) in a binding ELISA where new batches were compared older in-use batches. They were also analyzed with SDS-PAGE to avoid usage of degraded trimers.

The 16055 NFL TD CC trimers (**Paper IV**) were produced as previously described [232, 235]. Briefly, the trimers were expressed in 293F cells and then were isolated by affinity chromatography using GNL (Galanthus nivalis lectin-agarose; Vector Labs), followed by negative selection using the non-neutralizing mAbs, GE136, for antibody affinity chromatography, and purified by size exclusion chromatography (SEC) using SuperdexTM 200 columns (GE Healthcare Life Sciences) to isolate the predominant trimeric fractions. One part of the purified proteins was subjected to chemical cross-linking as previously described [263]. Briefly, 0.5 mg/ml of trimer was fixed with 5 mM glutaraldehyde (GLA) (ACROS Organics) at room temperature for 5 min and then the reaction was quenched by excess 50 mM glycine, pH7.5. The fixed trimers were negatively selected by GE136 antibody affinity chromatography and re-isolated by SuperdexTM 200 column SEC. The thermal melting (Tm) temperature of the trimers was determined using a Microcal VP-Capillary DSC (Malvern). Briefly, trimers were diluted in PBS pH 7.4 to 0.25 mg/ml and scanned at a rate of 1ÅC/min. Data collected were analyzed after buffer correction, normalization and baseline subtraction using the VP-Capillary DSC Automated data analysis software.

3.3.3 Enzymatic biotinylation of probes and conjugation to fluorochromes

For detection of Env-specific B cells by ELISpot analysis as well as in flow cytometry, the different Env probes were biotinylated. A biotin-ligase biotinylation kit (GeneCopia) was used for site-specific biotinylation of the Avitag sequence of each probe. The biotinylation

reaction was performed according to manufacturer's instruction. Env trimers used to detect antigen-specific GC and memory B cells via flow cytometry had to be conjugated to allophycocyanin (APC). This APC-conjugation was performed according to manufacturer's instruction. Briefly, a total of 5.3 μg (split into five incubations) Streptavidin, Allophycocyanin Conjugate (Invitrogen) was incubated at 4°C together with 10 μg Env trimers and PBS for 20 min. This process was repeated four times until all 5.3ug streptavidin-APC had been added. The resulting APC-conjugated Env trimers were then ready to use.

3.4 SINGLE CELL SUSPENSIONS OF LYMPHOID ORGANS AND *IN VITRO* STIMULATIONS

3.4.1 Single cell preparations

Single cell suspensions were performed on spleens, inguinal LNs (ingLNs) and BM (from two femurs and two tibias per mouse). Spleens and ingLNs single cells were obtained by passing the organs through a 70 μM nylon cell strainer and collected in complete RPMI 1640 medium (containing 5% FBS, 50 μM 2-mercaptoethanol, 2 mM L-glutamine, 100 U/ml penicillin and 100 μM streptomycin). BM was rinsed with complete RPMI 1640 medium using a syringe and needle to force the medium through the BM. BM cells was then collected. Red blood cell lysis buffer (hypotonic ammonium chloride solution) was added to lyse the red blood cells (this step was not performed on ingLN cells) and the reaction was stopped by addition of PBS. After spinning down the samples, the dead cells were removed by filtering the remaining cells to a new tube. These cells were then collected in complete RPMI 1640 medium and cell numbers were calculated with trypan blue stain using the automated cell counter Countess (Invitrogen) for further experiments.

3.4.2 CD4+ T cell depletion

To deplete CD4+ T cells (**Paper IV**), protocol from EasySep negative selection kit was followed (Stemcell Technologies). Briefly, splenocytes were incubated in normal rat serum (Stemcell Technologies) and biotinylated rat anti-mouse CD4 antibody (clone: RM4-5; BD Pharmingen) for 10 min and every third min the mixture was mixed. EasySep Streptavidin Rapid Sphere 50001 beads (Stemcell Technologies) were vortexed and added to the cell mixture at a concentration of 75 μl/ml of cell suspension. The tube was then inserted into a magnet and after 2.5 min the negative fraction was discarded and collected in a new tube.

3.4.3 *In vitro* stimulation

3.4.3.1 Mitogen stimulation of splenocytes

To measure IgM production in supernatant of naïve mouse splenocytes, in **Paper I**, cells were incubated in complete RPMI 1640 medium in the presence or absence of of 2 mg/ml LPS (Sigma) for 5 days at 37°C in 5% CO2. Supernatants were harvested and cleared of debris by centrifugation. The IgM binding to antigen was measured by ELISA.

3.4.3.2 T cell stimulation with peptides and proteins

In **Paper I**, splenocytes were stimulated by incubation with a pool of 15-mer peptides overlapping by 9 aa spanning all but the V3 region of the wt Ag (Env-V3), the V3 region (V3), PMA (10 ng/ml)/ionomycin (5 μM), or complete RPMI 1640 medium. After 1 hour (hr) of incubation, Brefeldin A was added to a working concentration of 10 μg/ml and the cells were incubated for another 5 hrs. Cells were then harvested for flow cytometric analysis. Protein stimulations have another incubation scheme. Splenocytes were stimulated with 0.5 μg/ml wt or gV3 trimers and incubated for 2 hrs. Brefeldin A was added and the cells were harvested 15 hrs later for flow cytometric analysis.

3.5 SEROLOGICAL ANALYSIS

3.5.1 Env-specific IgG ELISA

To detect Env-specific antibody responses in serum, ninety-six well high-protein-binding MaxiSorp (Nunc) plates were pre-coated (**Papers III and IV**) with 100 ng (1 μg/ml) *Galanthus nivalis* lectin (Sigma) diluted in PBS and incubated over-night (ON) at 4°C. Plates were then washed 6 times in washing buffer (PBS/0.05% Tween-20) followed by addition of 150 μl blocking buffer (2% fat-free milk in PBS) and incubated for 1 hr in room temperature (RT). After incubation, the blocking buffer was removed from the plates and soluble proteins was added at a concentration of 200 μg (2 μg/ml) and let to incubate in RT for 2 hrs.

In **Papers I and II**, plates were coated with 100 μl soluble proteins or peptides spanning the V3 region at a concentration of 100 or 200 ng/well diluted in PBS. The plates were incubated ON at 4°C. After the incubation, plates were washed in washing buffer (PBS/0.05% Tween-20) followed by addition of blocking buffer (2% fat-free milk in PBS). Plates were blocked for 1.5 h in RT.

Plates were then washed 6 times in washing buffer to remove access protein coat followed by another incubation of blocking buffer for 1 hr. Before sera from immunized mice were added to plates, blocking buffer was removed and replaced with new blocking buffer. Serum was added in 3-fold or 5-fold serial dilution series starting at a concentration of 1:25 and incubated for 2 hrs in RT. In **Paper I**, human-derived mAbs (b12, VCR01, F105, 48D, 447-52D and 39F) were added instead of serum at different concentrations. After washing the plates 6 times in washing buffer, secondary antibody diluted in PBS was added to each well. For total Env-specific and V3-specfic ELISA, the secondary antibody goat anti-mouse IgG-horse radish peroxidase (HRP) (Southern Biotech) was used at a concentration of 1:1000. For isotype-specific Env serum antibody detection, goat anti-mouse IgG1-HRP (Southern Biotech) diluted 1:5000, goat anti-mouse IgG2a-HRP (Southern Biotech) diluted 1:5000, goat anti-mouse IgG2b-HRP (Southern Biotech) diluted 1:5000, goat anti-mouse IgG2c-HRP (Southern Biotech) diluted 1:5000 or goat anti-mouse IgG3-HRP (Southern Biotech) diluted 1:1500 were added after dilution in PBS. Secondary antibodies were incubated in RT for 1 hr and removed by washing six times in washing buffer. To develop the plates, 100 μl of TMB

Stabilized Chromogen substrate (Invitrogen) or SigmaFAST OPD kit (Sigma-Aldrich) was added and incubated for 10 min in dark at RT. To stop the reaction 100 µl 1M H2SO4 was added to wells. The optical density (OD) was measured at 450 nm or 492 nm using an Asys Expert 96 ELISA reader (Biochrom).

3.5.2 Total IgM and Env-specific IgM ELISA

Total IgM levels were measured in serum (**Paper II**) by coating of 96-well ELISA plates (Nunc) with 100 µl polyclonal goat anti-mouse IgM (Southern Biotech) diluted to a concentration of 2 µg/ml in 0.05 M carbonate buffer. For Env-specific IgM ELISA (**Paper I**) plates were pre-coated with 200 ng/well of Env trimers. All plates were incubated ON at 4°C. The plates were the washed six times in washing buffer (PBS/ 0.05% Tween), followed by blocking for 1.5 hr in blocking buffer (PBS/ 5% FCS) at RT. After addition with fresh blocking buffer, serum was added and incubated for 2 hrs at RT. The plates were then washed six times in washing buffer and secondary anti-mouse IgM-ALP (Mabtech) or anti-mouse IgM-HRP (Southern Biotech) antibodies was added diluted in blocking buffer. After 1.5 h of incubation at RT the plates were washed six times in washing buffer. The ELISA plates were developed using either SIGMAFAST p-Nitrophenyl phosphate mix (Sigma-Aldrich), and the reaction was stopped by adding 3 N NaOH, or SureBlue TMB Microwell Peroxidase Substrate (KPL), and the reaction was stopped by adding TMB Stop Solution (KPL). The OD was measured at 405 or 450 nm, respectively, using an Asys Expert 96 ELISA reader (Biochrom).

3.5.3 Anti-dsDNA IgM ELISA

For detection of anti-dsDNA antibodies in **Paper II**, ELISA plates were pre-coated with 50 µl met-BSA (Sigma-Aldrich) diluted to a concentration of 5 µg/ml in PBS and incubated for 6 hrs at 4°C. After washing the plates four times in washing buffer (PBS/0.05% Tween) the plates were coated with 50 µl DNA (Sigma-Aldrich) diluted to a concentration of 50 µg/ml in PBS.

For detection of anti-phosphorylcholine (PC) antibodies in **Paper II**, ELISA plates were coated with 50 µl PCBSA diluted to a concentration of 2.5 µg/ml in PBS at 4°C ON. The plates were then washed four times in washing buffer.

Both sets of plates (anti-dsDNA and PC coated plates) were blocked in blocking buffer (PBS/1.5% BSA/0.1% gelatin/3 mM EDTA) for 2 hrs in RT. Fresh blocking buffer was then added to each well followed by addition of serum. The serum was incubated for 2 hrs in RT. Before addition of secondary antibodies, the plats were washed four times in washing buffer. Secondary antibodies, anti-mouse IgM-alkaline phosphatase (ALP) (Mabtech AB) or anti-mouse IgM-HRP (Southern Biotech), were added in blocking buffer and incubated for 1 hr inRT. Depending on the secondary Ab used, the ELISA was developed using either SIGMAFAST p-Nitrophenyl phosphate mix (Sigma-Aldrich), and the reaction was stopped by adding 3 N NaOH, or SureBlue TMB Microwell Peroxidase Substrate (KPL), and the

reaction was stopped by adding TMB Stop Solution (KPL). The OD was measured at 405 or 450 nm, respectively, using an Asys Expert 96 ELISA reader (Biochrom).

3.5.4 Cardiolipin-specific IgM ELISA

To measure cardiolipin in serum (**Paper II**), the 96-well ELISA plates (Nunc) were coated with 50 µl cardiolipin (Sigma-Aldrich) diluted to a concentration of 50 µg/ml in 99.5% EtOH. The coating content in the plates were allowed to evaporate completely at 4°C ON. The plates were blocked in in blocking buffer (PBS/ 10% FCS) for 1 h at 37°C. After the blocking, serum was added in fresh blocking buffer and incubated for 1 h at 37°C. The plates were then washed in PBS only. Secondary antibody anti-mouse IgM-HRP (Southern Biotech) was added in blocking buffer, and the plates were incubated for 1 h at 37°C. Before developing of the plates, they washed in PBS only. The development was performed with SIGMAFAST p-Nitrophenyl phosphate mix (Sigma-Aldrich), and the reaction was stopped by adding 3 N NaOH. The OD was measured at 405 nm, using an Asys Expert 96 ELISA reader (Biochrom).

3.6 ELISPOT ASSAYS

3.6.1 Differential B cell ELISpot

To enumerate the Env-specific and total IgG ASCs the B cell ELISPOT assay was used (**Papers I, II, III**), as previously described [177]. Briefly, 96-well MultiScreen-IP filter plates (Millipore) were pre-treated with 70% ethanol and washed three times in sterile PBS before being coated with 1 µg/well (10 mg/ml) a polyclonal goat anti-mouse IgG antibody (Mabtech). The plates were incubated ON at 4°C. Two hours before addition of the cells, the plates were washed five times in sterile PBS and blocked with complete RPMI 1640 medium at 37°C for 2 hrs. Cells were added in duplicates to the wells in three-fold serial dilutions, starting at 10^6 cells/well. For *in vitro* stimulation of memory B cells (**Paper II**), splenocytes were cultured with or without 2 µg/ml LPS for 6 days. Cells from each culture were collected, transferred to ELISpot plates and wrapped in plastic wrap before the 12 hrs incubation at 37°C.

The cells were removed by washing the plates six times in washing buffer (PBS/0.05% Tween-20) prior to the detection of spots. Total IgG-secreting cells were detected with 100 ng/well (1 µg/ml) of a biotinylated polyclonal goat anti-mouse IgG (Mabtech AB) in blocking buffer (PBS/1% FCS/0.05% Tween-20). For the detection of Env-specific B cells, 200 ng/well (2 µg/ml) of biotinylated protein (gp140-F-bio, gp120-F-bio, gp120-F-ΔV3-bio, gp120-F-ΔV1/2/3-bio and gp120-F-ΔV 1/2-bio or β-galactosidase as a control) was added diluted in blocking buffer and incubated for 2 hrs at RT. Plates were then washed six times in PBS before the addition of 100 µl ALP-conjugated streptavidin (Mabtech AB), diluted 1:1000 in PBS. Plates were incubated for 45 min in RT and then washed six times in water. To develop the plates 100 µl of pre-filtered BCIP/NBT-plus substrate (Mabtech AB) was then added and incubated for 10 min at RT. Plates were washed extensively with tap water

and air-dried. Spots were counted in an ImmunoSpot analyzer (Cellular Technology). Memory B cell-derived ASCs were calculated by subtracting the background of non–LPS-stimulated cultures from LPS-stimulated cultures. Env-specific ASCs were calculated as previously described [177].

3.6.2 T cell ELISpot

3.6.2.1 Evaluation of CD4-specific T cell ELISpot

To evaluate the specificity of the CD4+ T cell ELISpot assay (**Paper IV**), both total splenocytes and splenocytes lacking CD4+ cells due to depletion assay, were added to the ELISpot plates. The cytokines IFNγ and IL-2 production of the two samples was measured. Everything else was performed accordingly to the T cell ELISpot protocol described in detail below.

3.6.2.2 IFNγ, IL-2 and IL-4 T cell ELISpot

T cell ELISpot was performed to measure cytokine production after stimulation of total splenocytes, in **Paper IV**. First, 96-well Multiscreen-IP filter plates (Millipore) were pre-treated with 70% ethanol, washed three times in PBS followed by coating with 5 μg/well (50 μg/mL) of anti-mouse IFNγ (mAb: AN18), anti-mouse IL-2 (mAb: 1A12) or anti-mouse IL-4 (mAb: 11B11) all from Mabtech AB. Plates were incubated ON at 4°C. Before addition of splenocytes, the plates were washed six times followed by blocking with complete RPMI medium for 2 hrs at 37°C/5% CO2 in a humidified incubator. Splenocytes were the added to the wells at three different concentrations (200000, 100000 or 50000 cells) in a volume of 75 μl and stimulated with one of the following stimuli; ConA (Sigma), NFL trimer (unfixed), NFL trimer (fixed) or medium alone. ConA was added at the final concentration of 2 μg/ml and NFL trimers at 6.67 μg/ml. Final volume in the wells was 150 μl. All stimuli were diluted in blocking buffer (PBS/0.05% Tween-20/1% FBS). The *in vitro* stimulation was incubated for 20 hrs at 37°C/5% CO2 in a humidified incubator. The cells were removed from the wells and plates were washed six times in washing buffer (PBS/0.05% Tween-20) before biotinylated secondary antibodies (Mabtech AB) were added. Anti-mouse IFNγ (mAb: R4-6A2), anti-mouse IL-2 (mAb: 5H4) and anti-mouse IL-4 (BV06-24G2) antibodies were added to corresponding wells at the working concentration 1 μg/ml. After incubation in RT for 2 hrs, plates were washed six times in PBS only and strepatividin-ALP (Mabtech AB) was diluted 1:1000, added to wells and incubated in RT for 45 min. Plates were the washed in water. To develop the plates, 100 μl of filtered BCIP/NBT plus substrate (Mabtech AB) was added to wells and incubated for 10 min in RT. To stop the reaction wells were emptied and washed extensively in tap water followed by air-drying. The spots were counted in an ImmunoSpot analyzer (CTL Immunospot).

3.7 IMMUNOHISTOCHEMISTRY

For immunohistochemistry staining of spleens (**Paper II**), a part of the spleen from each mouse was first immediately immersed in O.C.T. medium (Sakura) and snap-frozen in 2-

methyl butane, which was kept cold in liquid nitrogen. The frozen sections were stored at -80°C until further use. The spleens were cryo-sectioned (6-8 μm) onto SuperFrost Ultra Plus microscope slides (VWR). For GC structure staining the following antibodies was used: APC anti-B220 (RA3-6B2; eBioscience), biotinylated MOMA-1 (Abcam) and AlexaFluor488 anti-TCRβ (H57-597; Biolegend). Streptavidin conjugated Cy3 was used to visualized the biotinylated antibody. A Leica TCS SP5X, using a 10X/0.30 magnification objective (Leica 506505 HCX FLUOTAR) was used to scan the sections. Leica Application Suite Advanced Fluorescence software was used to prepare images and ImageJ was used for final adjustment of brightness and contrast.

3.8 HIV-1 PSEUDOVIRUS NEUTRALIZATION ASSAY

Neutralization assays (**Papers II** and **III**) were kindly performed by the laboratory of John Mascola at the Vaccine Research Center at the NIH using a single round of infection HIV-1 Env pseudovirus assay and TZM-bl target cells, as previously described [264]. Serum from individual mice were analyzed for neutralization against four tier 1 viruses (MN, HXBc2, SF162, and BaL) and one tier 2 virus (6535). The results were reported as the reciprocal of the serum dilution producing 50% virus neutralization, the serum neutralization ID50. For competition-neutralization assays, in **Paper II**, test or control ligand was added to the serum 30 min prior to the addition of virus. V3-specific activation was mapped using a YU2-derived V3 peptide and a scrambled control peptide, as previously described [177, 265]. CD4bs-directed neutralizing activity was mapped using a pair of probes referred to as TriMut and TriMut368/70 [266, 267].

3.9 FLOW CYTOMETRY

3.9.1 Evaluation of binding antibodies

To detect antigen binding to the primary receptor of HIV-1 Env (**Paper I**), wt or gV3 trimers were co-incubated with purified human CD4+ T cells for 30 min. The cells were then incubated with mAb 17b for an additional 30 min at 4°C, and antigen-antibody complexes bound to the cell surface were detected by flow cytometric analysis after the addition of FITC-conjugated anti-human IgG Ab (BD Pharmingen).

3.9.2 Evaluation of splenocytes after CD4+ T cell depletion

For control of successful CD4 depletion (**Paper IV**), CD4 depleted cell fraction and total splenocytes fractions were stained with CD3e-PE (145-2C11; eBioscience), CD8a-APC (53-6.7; BD Pharmingen), CD4-FITC (H129.19; BD Pharmingen) and B220- PerCp Cy 5.5 (RA3-6B2; BD Pharmingen) and incubated on ice for 20 min. The samples were washed, fixed with Cytofix solution (BD Pharmingen) and run on FACS Calibur cytometer (BD Bioscience).

3.9.3 Intracellular cytokine production after *in vitro* stimulation

In **Paper I**, IL-2 and IFNγ–producing CD4+ T cells were detected after *in vitro* stimulation (section 3.4.3.2) by incubation with FITC-conjugated anti-CD4 (BD Pharmingen) and PE-conjugated anti-CD8 (BD Pharmingen) for 30 min at 4°C. This was followed by fixation and permeabilization of cells by Cytofix/Cytoperm, according to the manufacturer's instructions (BD Biosciences). Antibodies specific for IL-2 and IFNγ (APC-conjugated anti–IL-2 and PerCP-conjugated anti–IFNγ), both from BD Bioscience, were incubated with the cells. Cytokine-producing cells were the collected and ran on a BD FACSCalibur, and data were analyzed using FlowJo software (TreeStar).

3.9.4 Detection of Env-specific GC and memory B cells

For detection of Env-specific GC and memory B cells (**Paper III**), samples were first incubated with Fc receptor block antibody (anti-CD16/32; BD Biosciences, San Diego, CA, USA) followed by addition of biotinylated Env protein pre-coupled to APC-conjugated streptavidin (Invitrogen). For detection of GC B cells, the following antibody panel was used: Pacific Blue anti-B220 (RA3-6B2), FITC anti-GL7 (GL7) and PerCP-Cy5.5 anti-IgD (11-26c.2a). APC-conjugated streptavidin (Invitrogen) was added to visualize biotinylated Env proteins. All antibodies come from BioLegend except Live/dead AmCyan (Invitrogen). For detection of memory B cells, the following antibody panel was used: APC-eFluor780 anti-B220 (RA3-6B2; eBioscience), FITC anti-IgD (11-26c; eBioscience), FITC anti-IgM (polyclonal; Southern Biotech) PE-Cy7 anti-CD38 (Biolegend). For detection of Env-specific GC B cells the samples were first incubated with Live/Dead AmCyan followed by addition of APC-conjugated Env protein for all samples except control samples. Stained cells were fixed in fixation buffer (BD Bioscience) and analyzed on a MoFlo™ XDP (Beckman Coulter) or LSRFortessa™ cytometer (BD Biosciences). Flow cytometry data were analyzed with FlowJo (TreeStar).

3.9.5 Analysis of different B cell subsets after BLyS treatment

In **Paper II**, single-cell suspensions from spleen and LN were stained with the following antibodies: PerCP anti-B220 (RA3-62B), biotinylated anti-CD23 (B2B4), PE anti- IgM (R6-60.2), PE anti-CD95 (Jo2), allophycocyanin anti-CD8 (53-6.7) all from BD Biosciences, as well as APC anti-CD93 (AA4.1), Alexa Fluor 488 anti-GL7, PE anti-CD1d (1B1), PE anti-CD3 PE (145-2C11) and FITC anti-CD4 (RM4-4), all from eBiosciences. Biotinylated antibodies were visualized with Alexa Fluor 488-conjugated streptavidin (Invitrogen). Stained cells were fixed in fixation buffer (BD Biosciences) and analyzed on a FACSCalibur (BD Biosciences).

3.10 STATISTICAL ANALYSIS

The data was analyzed with D'Agostino and Pearson omnibus normality test. If the data passed these tests, they were analyzed by unpaired, two-tailed Student´s t-tests; data that did not pass were analyzed by the Mann–Whitney U test or the Wilcoxon matched-pairs signed-

rank test. Statistical analysis was performed using GraphPad Prism (GraphPad Software). Data were considered significant at *p=0.05, **p= 0.01, and ***p= 0.001. Statistical analysis was performed using GraphPad Prism V5.04 (GraphPad Software).

4 RESULTS AND DISCUSSION

Throughout this thesis, I have studied antibody responses elicited by immunization with recombinant HIV-1 Env trimers in mice. In **Paper I**, **II** and **III**, I used the early generation YU2 gp140-F trimers while in **Paper IV**, I used the new generation 16055 NFL TD CC trimers. Several approaches were investigated to address basic questions in vaccine immunology.

4.1 B CELL COMPETITION BETWEEN DISTAL EPITOPS ON COMPLEX ANTIGENS

Most of the knowledge regarding antigen responses comes from studies in mice, where the majority of studies have used small antigens of non-pathogen origins, such as hen egg lysozyme (HEL) or hapten-carrier antigen NP-CGG. Often mice that were transgenic for the antigen of interest were used, i.e. mice expressing a single BCR specific for either HEL or NP. Less is known about B cell responses to complex, "real world" antigens, which are more relevant for infectious diseases, including how epitope-specific responses are regulated. In this regard, HIV-1 Env has proven to be a very useful model antigen as it is extremely well characterized with multiple immunogenic determinants and specific epitopes defined, thanks to the availability of structural information and many mAbs, allowing us to ask questions of basic immunological nature.

4.1.1 Absent V3-specifc recognition of mAbs on gV3 trimer

From previous work performed in our group [177], we have shown that a large proportion of the antibody response after three immunizations with the well-characterized YU2 Env foldon trimers [226] in mice was directed to the variable region 3 (V3). Since it is known that V3 is highly immunodominant, and therefore might distract or compete with responses toward more conserved epitopes that could result in bNAbs, we asked in **Paper I** whether B cells specific for different epitopes on Env compete during a vaccine-induced response. To test this, we designed a pair of isogenic soluble Env trimers where the only difference between them was their capacity to elicit antibodies against V3. By introducing three additional N-linked glycosylation sites on each gp120 molecule of the trimers, the V3 region becomes masked by glycans. The trimers were designated as wildtype (wt) or gV3 (glycan masked V3). The antigenic properties of these trimers were characterized in Figure 1E by the recognition of six different HIV-1 specific monoclonal antibodies (mAbs). The mAbs tested were targeting the CD4bs (b12, VCR01 and F105), the CoRbs (48D) and the V3 region (447-52D and 39F) of bith Env trimers. The only difference in mAbs binding was the lack of recognition of the V3-specific mAbs to gV3.

4.1.2 Epitope-specific B cell responses

The interest in understanding the sub-specificities generated after Env immunizations stimulated us to develop a differential B cell ELISpot assays capable of detecting ASCs eliciting antibodies directed to sub-regions on Env [177]. Compared to conventional B cell

ELISpot, our optimized approach gave both clearer spots and lower background staining. With this more sensitive ELISpot assay, a number of different probes were introduced in order to dissect the cellular immune response against different epitopes of this complex antigen. Briefly, the probes were lacking one or more regions. We used gp140-F trimers, gp120-F trimers, gp120-F-ΔV1/2 (lacking variable regions 1 and 2), gp120-F-ΔV3, gp120-F-ΔV1/2/3 and gp120 monomers. By subtracting the number of ASCs recognizing one probe from another probe gives the difference in the number of ASCs between the probes. This gives information on how large the cellular response is against a certain region.

We discussed two possible outcomes before the experiment started. We knew from a previous study [177] that the gp41 response dominated after two immunizations whereas V3-specific responses only were a small portion of the total Env response and we also knew that this distribution changes after an additional immunization. After three immunizations, the V3-induced response takes up approximately 40-60% of the total Env response. We hypothesized that after three immunization we would either see a redistribution of the antigen-specific response to other than V3 in gV3 immunized mice or if the V3 masking only would lower the total Env-specific response without any re-distribution. Although, we were interested in the V3-specific response we decided to measure the polyclonal cellular response after both two and three immunizations with the differential ELISpot. It could be possible that a potential re-distribution among gV3 immunized mice was detectable already after two immunizations.

First, we enumerated the probe-specific responses. No differences in mice immunized twice with wt trimer or gV3 trimers was observed, except the slightly lower numbers of probe-specific cells in gV3 immunized mice. When the mice were boosted, a significant difference was observed between the groups of mice. Significant differences between wt and gV3 immunized mice were seen when investigating the gp140-F and gp120-F probe responses. Serological analysis with ELISA performed with two different coatings – wt or gV3 coat – indicated a difference in half-max binding titers when coated with wt protein. As no difference was seen in serum against gV3 protein, these data suggest that the difference are correlated to the V3-response.

To analyze the differences in more detail, we took advantage of the possibility to analyze the data with the differential B cell ELISpot method. By subtractive analysis, we could analyze the ASCs directed to gp41, V1/2, V3 and "other" (all responses that were not gp41 nor V1/2/3 directed). The results from the region-specific responses show that after three immunizations, there was a significant difference in the V3-specific response between wt and gV3 immunized mice. The gV3 immunized mice did not elicit V3-specific antibodies as expected due to the induced glycan masking on V3. Interestingly, the lack of V3-reponses did not re-distribute to other regions of Env that could be detected using the method in this study.

The conclusion in **Paper I** was that the V3-specific response detected after three immunizations did not out-compete other responses as no re-distribution to other epitopes on

the same antigen was seen in gV3 immunized mice. Thus, these data suggested that there was no competition between B cells specific for distal epitopes on the same antigen.

4.2 MANIPULATION OF THE NAÏVE B CELL POPULATION TO IMPACT B CELL RESPONSES TO RECOMBINANT HIV-1 ENV PROTEIN

BLyS plays a central role in B cell development, homeostasis and selection in the periphery. As mentioned previously, lack of BLyS leads to impaired populations of transitional and mature/naïve B cells [53, 54], whereas an overexpression results in B cell hyperplasia and signs of autoimmunity [43, 55]. It takes many years for the development of bNAbs in some chronically HIV-1 infected individuals. At this point, there is a dysregulation of B cell subsets, which is not seen in healthy individuals. Based on this, we asked (**Paper II**) if the treatment with recombinant exogenous BLyS would have any impact on generation of the specificities on Env-specific antibodies.

4.2.1 Effects on immune cells after recombinant BLyS treatment

First, we studied the impact of exogenous BLyS treatment in mice. The mice were injected with BLyS during 10 consecutive days before analysis on lymphocyte populations and B cell subtypes. BLyS treated mice were compared to control mice that had been injected with PBS during 10 consecutive days. The spleen size and weight increased in BLyS treated mice, which most likely is due to the increased number of B cells. The numbers of T2 and T3 B cells increased in numbers as well as the follicular and MZ B cell population, after BLyS treatment. The BLyS treatment did, however, not affect the overall architecture of GCs observed in confocal microscopy. We next wanted to study if BLyS treatment would rescue any self/autoreactive B cells. This was done by measuring if antibodies in serum had reactivity against dsDNA, phosphorylcholin (PC) and cardiolipin. The results indicated an increase of self-reactive antibodies in mice immunized with BLyS compared to control mice, which also showed some reactivity against these self-antigens. When comparing ratios between total IgM and self-antigen specific binding titers, no difference between the groups was detected. This indicates that exogenous treatment with BLyS does not induce an increase in self-reactive antibodies.

4.2.2 Pre-treatment with BLyS does not affect the overall response to Env immunization

Next, BLyS pre-treatment prior to Env immunization was investigated to analyze if any new naïve Env-specific B cells had been rescued during BLyS treatment and thereby could be part of the immunization responses initiated. At two separate time points (day 4 and 21) after the last immunization, mice were sacrificed and the GC B cell population size was first compared between BLyS and PBS treated mice. No difference in GC B cell populations was observed using flow cytometry. This suggests that GC formation is not sensitive to elevated BLyS levels. When comparing whether the BLyS treatment could have any impact on sub-regional Env responses using the differential B cell ELISpot, no differences was seen between the

groups at both time points in spleen and BM. The conclusion is that BLyS pre-treatment does not have any impact on the overall Env-induced B cell responses.

4.2.3 Elevated neutralization capacity in mice pre-treated with BLyS

When no differences were detected in Env-specific ASCs, we were interested in if the BLyS treatment could have any effect on the neutralization capacity in the serum antibodies. Sera were collected after second, third and fourth immunization. To study this, a standardized single-round of infections by HIV-1 Env pseudoviruses and TZM-bl target cells were used [264, 268]. The sera were tested against four tier 1 and one tier 2 viruses. The result showed some neutralization capacity in four mice pre-treated with BLyS after the second immunization, whereas PBS control mice showed non-neutralization capacity. The capacity to neutralize, especially against the tier 1 viruses, increased in both groups for each immunization dose, although, BLyS treated mice showed higher neutralization capacity as well as the ability to neutralize more viruses.

As BLyS has an effect on the survival of transitional B cells [269, 270] and, hence, the selection of naïve B cell repertoire, one could speculate if this altered naïve B cell pool in BLyS pre-treated mice did effect the increased neutralization capacity in those mice. As BLyS has also been reported to affect the duration of the GC responses [271], it would be interesting to perform a more detailed study on how the recombinant BLyS treatment actually effect the naïve B cell repertoire. A follow-up study is planned in rhesus macaques in Micheal Cancro's lab to determine if a similar effect of BLyS can be observed in primates.

4.3 ENV-SPECIFIC GC B CELLS AND MEMORY B CELLS IN MICE

Mouse studies in our lab were previously performed in BALB/c mice; however, most transgenic mouse strains are on the C57BL/6 background. Therefore, in **Paper III**, we compared the Env-specific B cell responses between BALB/c and C57BL/6 mouse strains. Having done so, we established protocols to detect different Env-specific B cell populations in C57BL/6 mice to allow future analysis and sorting such cells from Env immunized mice.

4.3.1 Comparable Env-specific responses in BALB/c and C57BL/6 mice

First, mice where immunized twice with Env (foldon trimers) with AbISCO-100 adjuvant. Samples were collected five days post the last immunization. In general, C57BL/6 mice had both lower Env-specific total IgG binding titers measured in ELISA and lower number of total IgG and Env-specific ASCs in B cell ELISpot. Apart from lower numbers observed in C57BL/6 mice, the two mouse strains behaved similar to Env immunizations. This made us confident to continue our studies to dissect Env-specific B cell responses in C57BL/6 mice.

4.3.2 Flow cytometry optimization to detect Env-specific B cells

Memory B cells were previously less well defined in mice compared to in humans with the exception of some recent studies [80]. In this thesis, I decided to only use the well accepted CD38 as memory B cell marker, since it is not fully understood if the newly described

markers are true memory B cell markers, if they represent different sub-populations of memory B cells or if they simply indicate memory B cells under maturation phases. For the detection of GC B cells the marker CD95 and GL7 was used, along with AA4.1 to exclude immature/naïve B cells. These markers have been used in previous experiments performed in our lab and they are also frequently used by others. To detect Env-specific memory B cells and GC B cells, mice were immunized with Env in adjuvant three times before detection of respective cell population. The individual mice differed quite a bit in the frequency of Env-specific B cells but the frequency was higher than in the control mice, which showed little or non-antigen specific memory and GC B cells.

Although these Env-specific GC and memory B cell populations are much smaller in mice compared to those detected in NHPs [249], these data still indicate that such B cell populations are possible to detect in mice. Staining panels, as those presented here, can be used for sorting either bulk Env-specific B cell populations or performing single-cell sorting for mAb cloning for further functional studies of vaccine-induced responses. Together with published protocols for antibody cloning from C57BL/6 mice [272, 273], these studies offer the possibility to define Env vaccine-induced responses in mice, which is of interest to evaluate responses in various C57BL/6-based strains where pathways of interest have been perturbed.

4.4 THE IMPACT OF CHEMICAL CROSS-LINKING OF ENV TRIMERS

Chemical fixation of viruses and antigens was used in many commercial vaccines with varying success. For RSV, the fixed vaccine was shown to be associated with a pathogenic Th2-biased response, and the vaccine was removed from the market [274-276]. GLA fixation of NFL trimers was previously investigated in guinea pigs, where the focus was on the neutralizing capacity of the induced response [263]. In **Paper IV**, we asked if GLA fixation affected other aspects of the Env-specific responses by comparing unfixed and fixed 16055 NFL TD CC Env trimers in C57BL/6 mice. We showed that the thermostability of the GLA fixed Env protein was increased, consistent with a previous report using other Env trimers [263].

4.4.1 GLA fixation impacts the isotype-specific Env antibody responses

We next immunized mice three times with either unfixed or fixed NFL trimer with a four-week interval. Serum was collected after the second and third immunization. First, the total IgG Env-specific antibody responses was measured. The binding titers in ELISA was tested against both Env trimer coatings. Overall, the Env-specific IgG titers were higher in mice immunized with unfixed trimers compared to those immunized with fixed trimers after two immunizations. This difference was much smaller after three immunizations and it was less marked when fixed Env protein was used for coating in the ELISA plates. One goal of fixate the NFL trimer is to avoid exposure of irrelevant epitopes that might be exposed in unfixed proteins. It is likely that the difference in total Env-specific binding titers represents specificities directed against irrelevant epitopes; however, this has yet to be tested.

To evaluate if the use of GLA fixed trimers has any impact of the isotype-specific Env antibody responses, an ELISA using antibodies against IgG1, IgG2b and IgG2c was used. A shift in isotype-specificity was observed between the groups of mice. Mice immunized with fixed trimers showed higher IgG1 titers and lower IgG2b and IgG2c than mice immunized with unfixed trimers. When comparing the ratio between the isotypes, there was a significant difference between IgG1/IgG2b as well as between IgG1/IgG2c. This suggests that there is a Th2 shift in the antibody response elicited after immunization with fixed trimers.

4.4.2 CD4+ T cell cytokine responses were unaffected of GLA fixation

As CD4+ T cells can influence the elicited antibody isotypes and subclasses, we stimulated splenocytes *ex vivo* with both unfixed and fixed Env proteins, separately, for 20 hrs, and the cytokine-production was measured in a T cell ELISpot assay. No significant was observed in the cytokine-production (IFNγ, IL-2 and IL-4) between the groups of mice. As no difference was seen in the number of cytokine-secreting cells, this could indicate that the uptake and processing of the two NFL trimers appears similar and that GLA fixation does not negatively impact the activation of CD4+ T cells. It would, however, be interesting to compare the proteins in another assay, where more cytokines can be evaluated. By using a bead array based flow cytometry platform (BD Bioscience), it could be possible to detect any differences in cytokine-production in greater detail.

A previous study performed in rabbits, showed a more potent neutralization capacity in animals immunized with GLA fixed Env trimers [277]. In the present study, we aimed to focus on other aspects of the B cell response than the neutralization capacity and we showed that there is a Th2 shift in the antibody serum response in mice immunized with fixed protein. This Th2 skewing was not detectable in the T cell level.

5 CONCLUDING REMARKS

The overall aim of this thesis was to use biochemically defined HIV-1 Env trimers to investigate basic B cell questions of relevance for the HIV-1 vaccine field. By using Env trimers in mouse models we wished to provide a link between more basic B cell immunology and HIV-1 vaccine research. The HIV-1 vaccine research field moves forward rapidly, especially in regards to immunogen design and structural analysis. As a result, the recombinant HIV-1 Env trimers used in **Paper I-III** were an earlier generation design compared to the trimers used in **Paper IV**.

The results in **Paper I** indicate that there is no competition between B cells that recognize distal epitopes on the same antigen. By masking the highly immunogenic V3, no re-distribution of the antibody response to other determinants on Env was detected. Instead, an overall lower response was seen in mice immunized with glycan-masked Env compared to the response observed in mice immunized with the isogenic wt trimers. In **Paper II**, the aim was to manipulate the naïve B cell pool in order to investigate if this would alter the elicited neutralizing antibody response. Pre-treatment with recombinant BLyS resulted in an increase in the number of peripheral B cells and a shift the B cell subsets and improved neutralizing responses. To be able to define the specificities of the induced response in greater detail, a staining protocol for detection of Env-specific memory and GC B cells was performed in **Paper III**. The protocols described in this paper can be used for both bulk sorting of Env-specific B cells as well as in a single-cell sorting for cloning and functional testin of the induced antibodies. In the last study, **Paper IV**, the new generation NFL trimers were used and we investigated if GLA fixation would impact the elicited Env-specific immune response. We observed a clear skewing of the Env-specific antibody isotype response toward a more Th2 response with an increased IgG1:IgG2b ratio, which merits further investigation.

In conclusion, the work included in this thesis has increased our understanding about B cell responses elicited by immunization using a highly relevant real-world antigen, HIV-1 Env.

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