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B CELL RESPONSES AGAINST THE HIV-1 ENVELOPE GLYCOPROTEINS

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ABSTRACT

The fine specificities of vaccine-elicited B cell responses against complex proteins are not well understood, including those directed against the HIV-1 envelope glycoproteins (Env). Env is the only surface-exposed viral protein and hence a major vaccine target, in particular the conserved determinants of Env. It is now well appreciated that HIV-1 has evolved mechanisms to occlude conserved determinants of Env to limit antibody recognition of these sites. Such structural constraints are thought to contribute to the limited capacity of current Env immunogens to stimulate broadly neutralizing antibodies. Numerous strategies have been undertaken to improve the capacity of Env immunogens to stimulate effective antibody responses, but so far this has met with limited success. An improved understanding of basic B cell biology and how it relates to Env immunogenicity is therefore needed.

The primary goal of this thesis was to ask a number of basic questions regarding vaccine-induced B cell responses using an antigen that is highly relevant to human health, HIV-1 Env. To accomplish this, we developed an optimized B cell ELISpot assay, which can be used to enumerate total Env-specific B cells, as well as B cells directed against different sub-determinants of Env. In **paper I**, we immunized mice with recombinant, soluble Env trimers and we demonstrate that the relative proportion of Env sub-specificities changes significantly in response to boosting, with a robust expansion of B cells recognizing the gp41 moiety of Env after the first boost and of those recognizing variable region 3 after the second boost. In **paper II**, we designed a system to investigate how B cell responses to different elements of the same antigen affect one another. Using this system we show that different specificities develop independently of each other, suggesting that there is no or limited competition between B cell populations recognizing distal epitopes of the same antigen during the development of the immune response.

In **paper III**, we used computationally designed scaffold immunogens displaying a specific broad neutralization epitope of Env. We show that heterologous scaffold proteins could be used sequentially to boost B cell responses that were reactive with the target epitope, but that this regimen did not provide an advantage over the use of a single scaffold. In **paper IV**, we exploited recombinant B Lymphocyte Stimulating factor (BLyS) to modulate the naïve B cell repertoire prior to Env immunization. We show that a transient treatment with BLyS increased the peripheral naïve B cell pools and interestingly, qualitatively enhanced Env-specific responses as evidenced by improved neutralization of HIV-1. Our data suggests that BLyS-regulated processes can be targeted to favorably affect the quality of Env vaccine-elicited neutralizing antibody responses against HIV-1.

Collectively, the findings presented in this thesis provide new insights into the development of B cell responses to protein-based vaccines, specifically to recombinant HIV-1 Env immunogens. This information may accelerate the design of improved Env-based immunization regimens and may also be of more general use for the development of vaccines against highly variable, neutralization-resistant pathogens.

LIST OF PUBLICATIONS

I. **Pia Dosenovic**, Bimal Chakrabarti, Martina Soldemo, Iyadh Douagi, Mattias N. E. Forsell, Yuxing Li, Adhuna Phogat, Staffan Paulie, James Hoxie, Richard T. Wyatt and Gunilla B. Karlsson Hedestam. 2009. Selective expansion of HIV-1 envelope glycoprotein-specific B cell subsets recognizing distinct structural elements following immunization. *Journal of Immunology 183: 3373-3382.*

II. Mattias Forsell, Martina Soldemo, **Pia Dosenovic**, Richard T. Wyatt, Mikael C.I. Karlsson and Gunilla B. Karlsson Hedestam. Independent regulation of epitope-specific IgG responses to HIV-1 surface envelope glycoproteins. *Submitted manuscript.*

III. Javier Guenaga, **Pia Dosenovic**, Gilad Ofek, David Baker, William R. Schief, Peter D. Kwong, Gunilla B. Karlsson Hedestam and Richard T. Wyatt. 2011. Heterologous epitope-scaffold prime:boosting immuno-focuses B cell responses to the HIV-1 gp41 2F5 neutralization determinant. *PLoS One 6: e16074.*

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PUBLICATIONS NOT INCLUDED IN THE THESIS

Iyadh Douagi, Mattias N. Forsell, Christopher Sundling, Sijy O'Dell, Yu Feng, **Pia Dosenovic**, Yuxing Li, Robert Seder, Karin Loré, John R. Mascola, Richard T. Wyatt and Gunilla B. Karlsson Hedestam. 2010. Influence of novel CD4 binding-defective HIV-1 envelope glycoprotein immunogens on neutralizing antibody and T-cell responses in nonhuman primates. *Journal of Virology 84:1683-1695.*

Andreas Mörner, Iyadh Douagi, Mattias N. Forsell, Christopher Sundling, **Pia Dosenovic**, Sijy O'Dell, Barna Day, Peter D. Kwong, Gerald Voss, Rigmor Thorstensson, John R. Mascola, Richard T. Wyatt and Gunilla B. Karlsson Hedestam. 2009. Human immunodeficiency virus type-1 env trimer immunization of macaques and impact of priming with viral vector or stabilized core protein. *Journal of Virology 83: 540-551.*

Lucy Breakwell*, **Pia Dosenovic***, Gunilla B. Karlsson Hedestam, Mauro D'Amato, Peter Liljeström, John Fazakerley and Gerald M. McInerney. 2007. Semliki Forest virus nonstructural protein 2 is involved in suppression of the type I interferon response. *Journal of Virology 81:8677-8684.* *Equal contribution.

Åsa S. Hidmark, Eva K. Nordström, **Pia Dosenovic**, Mattias N. Forsell, Peter Liljeström and Gunilla B. Karlsson Hedestam. 2006. Humoral responses against coimmunized protein antigen but not against alphavirus-encoded antigens require alpha/beta interferon signaling. *Journal of Virology 80:7100-7110.*

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LIST OF ABBREVIATIONS

Abs	Antibodies
Ag	Antigen
AID	Activation-induced cytidine deaminase
AIDS	Acquired immunodeficiency syndrome
ASC	Antibody secreting cell
BCR	B cell receptor
BLyS	B Lymphocyte Stimulator
BM	Bone marrow
bNAbs	Broadly neutralizing antibodies
CD4bs	CD4 receptor-binding site
BR3	BLyS receptor 3
CDR1-3	Complementarity determining region 1-3
CLP	Common lymphoid progenitor
DC	Dendritic cell
DZ	Dark zone
ELP	Early lymphoid progenitor
Env	HIV-1 Envelope glycoprotein
FDC	Follicular dendritic cell
FR	Framework
GC	Germinal center
HCV	Hepatitis C virus
HEL	Hen egg lysozyme
HIV-1	Human immunodeficiency virus type-1
HSC	Hematopoietic stem cells
IgH	Immunoglobulin heavy chain
IgL	Immunoglobulin light chain
IL	Interleukin
LN	Lymph node
LZ	Light zone
MAb	Monoclonal antibody
MHC	Major histocompatibility complex
MPER	Membrane proximal external region

MZ	Marginal zone
MZB	Marginal zone B cells
PC	Plasma cell
SHIV	Chimeric SIV/HIV virus
SHM	Somatic hypermutation
SIV	Simian immunodeficiency virus
SLO	Secondary lymphoid organ
T1-3	Transitional B cells 1-3
TCR	T cell receptor
Tfh	Follicular helper T cell
V1-3	Variable regions 1-3
VLP	Virus like particle
WT	Wild type

1 INTRODUCTION

Neutralizing antibodies (Abs) are the correlate of protection for most vaccines in use today. Many vaccines also induce strong cytotoxic T cell responses, but their role in vaccine-induced protection against pathogens is less defined (1). Abs are produced by antibody secreting cells (ASCs), also called plasma cells (PCs), which are formed during infection or vaccination. Short-lived PCs can be found in the periphery, while long-lived PCs reside predominately in the bone marrow (BM) where they may persist for the lifetime of the individual. These PCs produce high affinity Abs, which are released into the bloodstream continuously providing first line defense against pathogens.

Even though the development of vaccines is one of the most successful achievements in medical history and many potent vaccines against harmful pathogens exist, there are still no vaccines available against pathogens such as hepatitis C virus (HCV) causing hepatitis, *Plasmodium falciparum* causing malaria and human immunodeficiency virus type-1 (HIV-1) causing acquired immunodeficiency syndrome (AIDS). Due to a number of factors, such as the extremely high genetic variability of these pathogens and the risk for infection, conventional vaccine approaches based on inactivated or attenuated preparations are not viable. Instead, strategies based on subunit vaccines using antigens (Ags) that are known Ab targets are pursued in most instances. To increase the likelihood of developing successful vaccines, the immunogenic properties of relevant Ags must be studied in pre-clinical animal models. An improved understanding of the limitations of vaccine candidates that fail to elicit the desired immune response is necessary to guide the development of improved approaches.

In the work presented here, we have used a well-characterized HIV-1 envelope glycoprotein (Env) as a model protein Ag to study the development of immunization-elicited B cell responses in mice. Env is the only relevant target Ag for an Ab-based vaccine against HIV-1 as it is the only protein that is exposed on the outside of the virus particle. It has been shown that passive immunization of broadly neutralizing Env-specific Abs elicited naturally in HIV-1 infected individuals, provides protection against challenge with chimeric HIV-SIV virus (SHIV) in non-human primates (2-7). Yet, no vaccine approach to date has successfully induced such Abs in healthy individuals. Understanding the limitations of existing vaccine candidates is a critical task on the path to develop an effective preventative vaccine against HIV-1.

2 OBJECTIVES

The overall aim of the work presented in this thesis was to study basic questions in B cell biology to help understand the development of vaccine-elicited B cell responses against protein antigens. To accomplish this, we used the HIV-1 Env glycoprotein as a model antigen and analyzed Env-specific B cell responses at both cellular and serological levels in immunized mice. The objectives of these studies were two-fold: to advance our knowledge regarding the evolution of B cell responses to large protein antigens in general and to investigate novel immunization approaches that may accelerate the development of an antibody-based vaccine against HIV-1.

The specific aims for the four individual papers were:

Paper I: To determine the evolution of total and epitope-specific B cell responses over time in Env-immunized mice using an optimized, differential B cell ELISpot assay.

Paper II: To investigate whether B cells that recognize distal epitopes on the same antigen compete with one another in response to antigen re-exposure *in vivo* (boosting).

Paper III: To investigate the capacity of computationally designed scaffold proteins, engrafted with a broadly neutralizing antibody epitope of Env, to re-elicit responses against this epitope.

Paper IV: To investigate whether modulation of the naïve B cell repertoire by exogenous BLyS treatment prior to immunization improves the outcome of Env vaccine-elicited B cell responses.

3 B CELL BIOLOGY

3.1 B CELL EVOLUTION IN THE BONE MARROW

3.1.1 Early B cell development

As with all other blood cells, B cells originate from hematopoietic stem cells (HSCs) residing in the BM. B cell hematopoiesis is a gradual process that is defined by the expression and suppression of different key transcription factors and by DNA recombination events essential for the generating functional B cell receptors (BCRs) that consists of two immunoglobulin heavy chains (IgH) and two immunoglobulin light chains (IgL) (reviewed in (8-10)). HSCs differentiate via an early lymphoid progenitor (ELP) into a common lymphoid progenitor (CLP). During the late CLP stage recombination of the IgH gene begins and the cells begin to express the IL-7 receptor. Ligand binding to this receptor provides an important survival signal and promotes differentiation of CLP into pro-B cells (11). Expression of the transcription factor Pax5 is required for the transition of late CLP into the early pro-B cell stage and its expression defines the commitment to the B cell lineage (12). IgH gene rearrangement is completed as the B cells enter the late pro-B cell phase. At this stage the cells have reached an important checkpoint since they are only allowed to mature further into pre-B cells if they display a properly expressed IgH chain in association with the surrogate light-chain components VpreB and λ5 to form the pre-BCR complex. When this complex is expressed on the surface of the cells, they are positively selected for further maturation into pre-B cells, which allows for rearrangement of the IgL gene (13, 14). The developing cells pass through another checkpoint in which only B cells with properly paired BCRs showing no to low affinity for self epitopes are selected to exit the BM for further development in the periphery (reviewed in ((15))). Self-reactive B cells or B cells with poorly paired BCR may be given a second chance to express a BCR of another specificity through a process termed receptor editing (16-18). In this process, the IgL chain of the BCR is reconstructed until the BCR does not recognize self. Failure at this step leads to B cell apoptosis (19). It has been shown that the numbers of self-reactive and poly-reactive B cells are decreased significantly at this check-point (20). This process is in general terms referred to as central tolerance (21) and is the prevailing mechanism to explain how B cells are educated to discriminate self from non-self.

3.1.2 VDJ recombination

The concept that man can produce Abs against an infinite range of pathogens may be difficult to comprehend. However, the immune system is estimated to be capable of producing Abs with over 10^{16} unique Ag-specificities (reviewed in (22)). This vast diversity is made possible through the evolution of an elegant process of DNA recombination of separate multi-gene families of immature naïve B cells. This gradual recombination event eventually forms functional individual immunoglobulin genes as the B cells mature in the BM (23). An Ab is a secreted version of the membrane bound BCR receptor. The IgH and IgL chains that build up an Ab form a

complex of approximately 160 kDa. The C-terminal domain of both IgH and IgL are constant, and the constant IgH domain defines the isotype of the Ab, which in turn defines the effector function. The N-terminal domain of both chains are variable, consisting of 3 hypervariable regions, termed complementarity determining regions 1-3 (CDR1-3) and 4 stable regions, termed framework regions (FR). Together these domains define the specificity of the Ab. The variable domain of IgH consists of three different fused gene segments termed: variable (V), diversity (D) and joining (J) segments. The variable domain of IgL consists of only V and J segments (Figure 1).

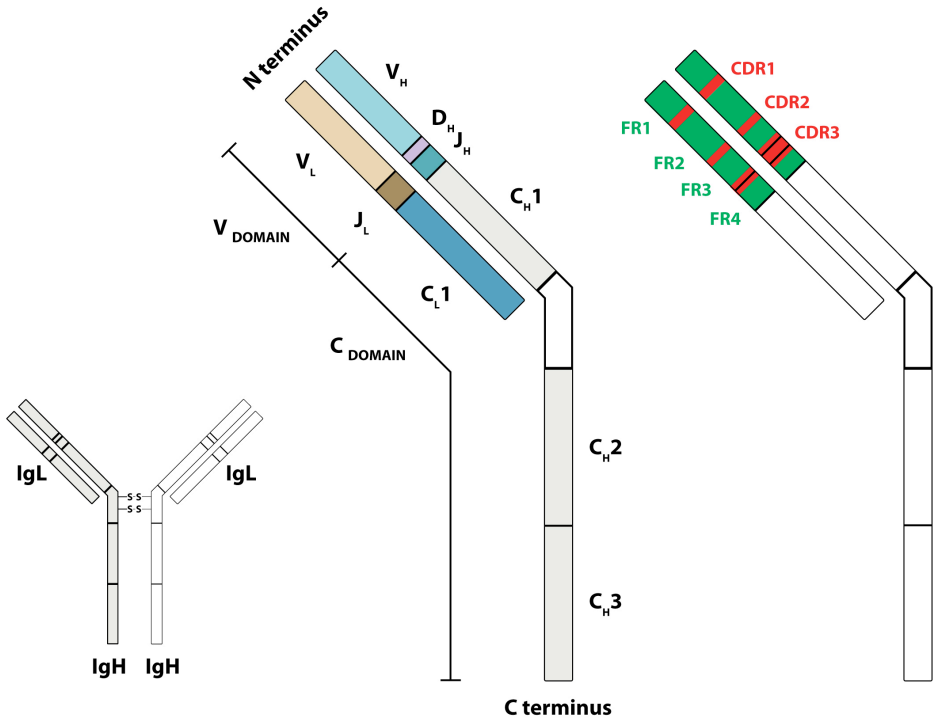


Figure 1: Schematic representation of an IgG antibody. *Left:* An antibody is composed of two IgH and two IgL chains. *Middle:* Each Ig chain consists of one variable and one constant domain. The variable domain of IgL is encoded by one V- and one J segment. The variable domain of IgH is encoded by one V-, one J- and one D segment. *Right:* Each of the variable domains is composed of three hypervariable regions called complementarity determining regions 1-3 (CDR 1-3) and four framework (FR) regions. The most variable region of the antibody is CDR3, which comprises the end of the V segment, the whole D segment and the first part of the J segment. *Illustration by Mattias Karlén.*

The number of gene segments in each locus for V, D and J varies between mouse and human and between different strains of mice though the general organization is homologous. The IgH chain locus of BALB/c mice consists of more than 100 functional V segments that are grouped into several different families, 13 D segments and 4 J segments. These segments are followed by 8 different gene segments that can build up the constant region (reviewed in (24)) (Figure 2). The IgL chain can be constructed from two different loci, the κ- and the λ-loci that are found on different chromosomes. As for the IgH locus, they also contain a large number of different V- and D segments. These segments will combine through clone-specific recombination events to form a single immunoglobulin gene for the IgH chain and a single immunoglobulin gene for the IgL chain (reviewed in (22)). The number of different segments in the immunoglobulin locus and the process through which the V(D)J

segments recombine create an enormous variability in the Ab repertoire. As each locus includes two chromosomes, each B cell could in theory produce two different Abs. This is however avoided by a process known as allelic exclusion, which ensures that each B cell only produces BCRs of one specificity (25, 26). A brief account of the VDJ recombination process is described in Figure 2 (reviewed in (22)).

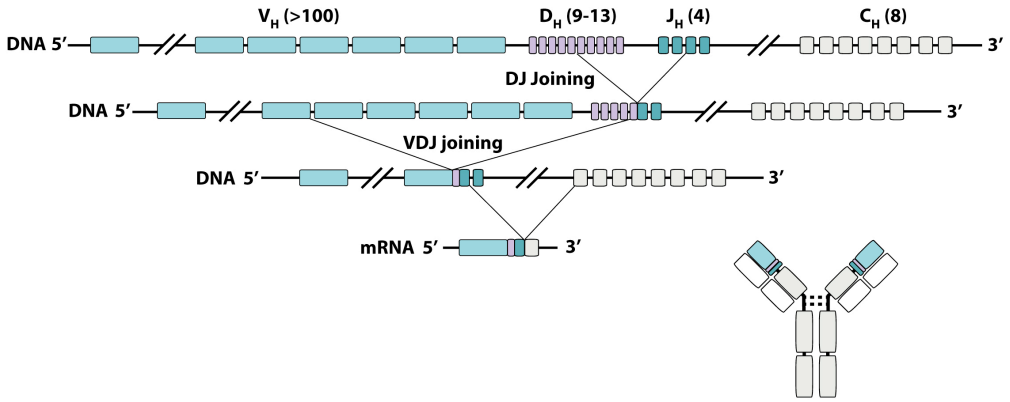


Figure 2. Gene segment layout and VDJ rearrangement of the IgH locus in mouse. The Rag-1 and Rag-2 enzymes are responsible for the recombination events of the immunoglobulin gene segments and are almost exclusively expressed in developing lymphocytes. The Rag proteins initially target a selected D-J segment of the IgH chain. Upon initiation of double-stranded breaks, the DNA repair machinery of the cell will join D and J in a non-homologous manner, and another enzyme, TdT, will add non-germline nucleotides into the sequence to make it complete. As the B cell progresses into a pro-B cell, Rag will join a single V segment with the rearranged DJ segment in the similar way. The resulting VDJ segment of the pro-B cells is then connected with a gene segment for the constant domain by splicing events at the RNA level. The variability in the N-terminal domain of the Ab provided by the different VDJ segments is then further enhanced by different mechanisms of repairing non-homologously joined DNA. *Illustration by Mattias Karlén.*

3.2 NAÏVE B CELL EVOLUTION IN THE PERIPHERY

3.2.1 B cell homeostasis in the periphery

Immature naïve B cells that exit the BM home to the spleen where they continue development into mature naïve B cells that can populate other secondary lymphoid organs (SLOs) such as the lymph nodes (LNs). In these organs, B cells can recognize and become activated by foreign Ags. The differentiation and maintenance of naïve B cells in the periphery are dependent on the expression of the BCR, as conditional deletion of the BCR on B cells was shown to induce cell death of most peripheral B cell compartments (27). In the spleen, immature naïve B cells arriving from BM will go through three developmental transitional stages termed transitional stage 1, 2 and 3 (T1-3) (28), as they differentiate into mature naïve B cells. In the mature state the cells can be further subdivided into follicular B cell and marginal zone B cell (MZB) subsets. Variants of this model have been put forward that suggest that T2 cells can be present in both the periphery and the BM (29, 30). Also, follicular B cells and MZBs may develop directly from T2 cells with the T3 stage being a dead end pathway that leads to anergy (31). B cells surviving the transitional stages will develop into follicular B cells that can populate the spleen and the LNs or to MZBs that reside in the spleen. The proportion of transitional B cells that mature into follicular B cells is significantly

greater than the proportion that mature into MZBs. The factors that regulate this fate decision are not known, but Ab specificity is thought to be critical as some specificities are prone to preferentially drive the development of one or the other population (32-34).

It has been suggested that only about one third of the transitional B cells that exit the BM survive to populate the mature B cell pools (35, 36) and the cells that persist have a skewed BCR repertoire (37, 38). Using the anti-hen egg lysozyme (HEL) mouse model in which B cells exclusively express BCRs with affinities for HEL and the HEL transgenic mouse in which HEL has been rendered a "neo-self-ag", it was further shown that self-reactive B cells are excluded from the mature naïve B cell pool within the follicles of the spleen. This process correlated to the level of self ligand that was present as well as to the affinity of the BCR for this ligand (39). In agreement with this, it has also been shown that a large number of immature B cells that exit the BM show self reactive properties and that these reactivities are significantly reduced during transitional maturation in both mice and humans (20, 40). In conclusion, these data implies that B cells undergo a second negative selection process in the periphery that is dependent on the BCR specificity. Furthermore, using the same HEL transgenic mouse models as described above, Cyster and colleagues made the intriguing finding that in the absence of a diverse repertoire of non-self B cells, self-reactive B cells are not deleted in the periphery but instead are allowed to enter B cell follicles of the SLOs as mature B cells (41-43). Since self-reactive B cells were allowed to mature in the absence of competition with non-self B cells, the authors hypothesized that B cells in the periphery not only compete for survival based on self-/non-self BCR recognition, but also for a tropic factor. This factor was suggested to be produced by cells within the SLOs. There is now growing evidence suggesting that this tropic factor is B Lymphocyte Stimulator (BLyS).

3.2.2 The role of BLyS for the survival of peripheral naïve B cells

BLyS (also called BAFF) was discovered simultaneously by several groups while searching the genome for additional members of the TNF family of ligands and receptors (44-48). BLyS is described as a survival factor for B cells in the periphery, and is produced primarily by macrophages, monocytes and dendritic cells (DCs) (49). Though BLyS can bind two receptors, called TACI and BCMA, the most profound effect of BLyS on naïve B cells is seen upon signaling through the BLyS receptor 3 (BR3) (50-52). Specifically, BLyS has been shown to have a critical role for B cell survival during the transitional stages (53-55) and increased levels of BLyS leads to a substantial increase in the population of surviving T2 cells that can enter the mature B cell pools (56). The importance of BLyS for the maintenance of mature naïve B cells has been demonstrated using two experimental approaches. First, blocking BLyS signaling with Abs led to a rapid depletion of naïve B cells, and second, mice deficient in BLyS showed significantly reduced numbers of mature naïve B cells (57, 58). Conversely, transgenic mice constitutively over-expressing BLyS show B cell hyperplasia and signs of autoimmunity (44, 59). In support of this finding, patients with certain autoimmune diseases have been shown to display elevated levels of systemic BLyS (60-62). These findings may indicate that levels of BLyS are limiting under physiological conditions since the number of B cells has not reached a maximum.

The fact that deletion of autoreactive peripheral B cells overlaps with the developmental point when B cells become responsive and dependent on BLyS to survive, suggests that BLyS could have a role in the selection of the naïve B cell repertoire. To further study this, Lesley and colleagues (63) and Thien and colleagues (40) performed the following experiments using the above mentioned HEL transgenic mice models: Lesely *et al*. could show that autoreactive B cells (anti-HEL B cells) were more susceptible to reduced levels of BLyS in the HEL transgenic mice than B cells of non-self specificities. Thien *et al.* showed that self-reactive anti-HEL B cells could indeed mature and gain access to the B cell follicles even in the presence of competing non-self B cells if the levels of BLyS were experimentally elevated. While these results support the role for BLyS in the selection of naïve B cell specificities and others have later shown similar results (64, 65), they were derived from experiments based on transgenic mice models having a restricted BCR repertoire. It will therefore be important to study the role of BLyS under more physiological conditions. The way BLyS is thought to affect and control naïve B cell survival together with BCR is not fully understood. One model that was suggested by Mackay and Browning (66) propose that self-reactive immature B cells need stronger survival signals by BLyS than non-self B cells due to the strong BCR signaling that is induced in these cells upon BCR ligation. In line with this model, crosstalk between downstream components of the BCR- and the BR3 signaling pathways was identified where the BCR signaling was proposed to provide the BR3 signaling pathway with rate-limiting substrate (67).

Since BR3 is also expressed on germinal center (GC) B cells, BLyS may also have a role in the survival of GC B cells. Less is known about this, but it was shown that the maintenance and development of high affinity Abs were dependent on BLyS signaling but the initiation of GCs was not (68, 69).

3.3 ORGANIZATION OF SECONDARY LYMPHOID ORGANS

Secondary lymphoid organs, such as the spleen and LNs, serve to substantially increase the probability that naïve B cells and helper T cells meet their cognate Ags. These organs have a highly organized dynamic structure of non hematopoietic cells that creates a meeting point for numerous immune cells required for specific B- and T cells to become activated. While LNs drain the lymphatics and can sample Ags via the lymphatics from all over the body, the spleen filters the blood and is mainly specialized to provide immune activation against blood born infections (70). A sectioned spleen displays white circular areas scattered throughout the whole interior (Figure 3A). This so-called white pulp consists of lymphocytes and various innate immune cells. The white pulp is surrounded by a much larger area, the so-called red pulp, which consists of mainly recirculating erythrocytes and macrophages. The red pulp functions primarily to filter the blood and remove old erythrocytes as well as recycle iron (70). The white pulp is the site of activation for naïve lymphocytes and thus where the adaptive immune response is initiated. The white pulp of the spleen has a very similar structure and function to the structure and function of the LNs (Figure 3B). T cells form close conjugates with conventional DCs in the center surrounded by B cell areas referred to as B cell follicles. This is where the naïve follicular B cells reside. Apart from B cells, special resident follicular dendritic cells (FDCs) are also present here. In spleen, the border between the white and the red pulp is lined by the marginal zone (MZ)

consisting of innate like MZBs. The blood drains from the central arteriole to the marginal sinus, a vessel dividing the B cell follicles from the marginal zone. From here, the blood can continue out in the red pulp or into the white pulp via specific conduit networks. The LN is surrounded by the subcapsular sinus in which the afferent lymph drains as it enters the LN. The lymph continues through the LN either via large trabecular sinuses or via conduit networks to the center of the LN, the medulla, on its way out through the efferent lymph (Figure 3) (reviewed in (71)).

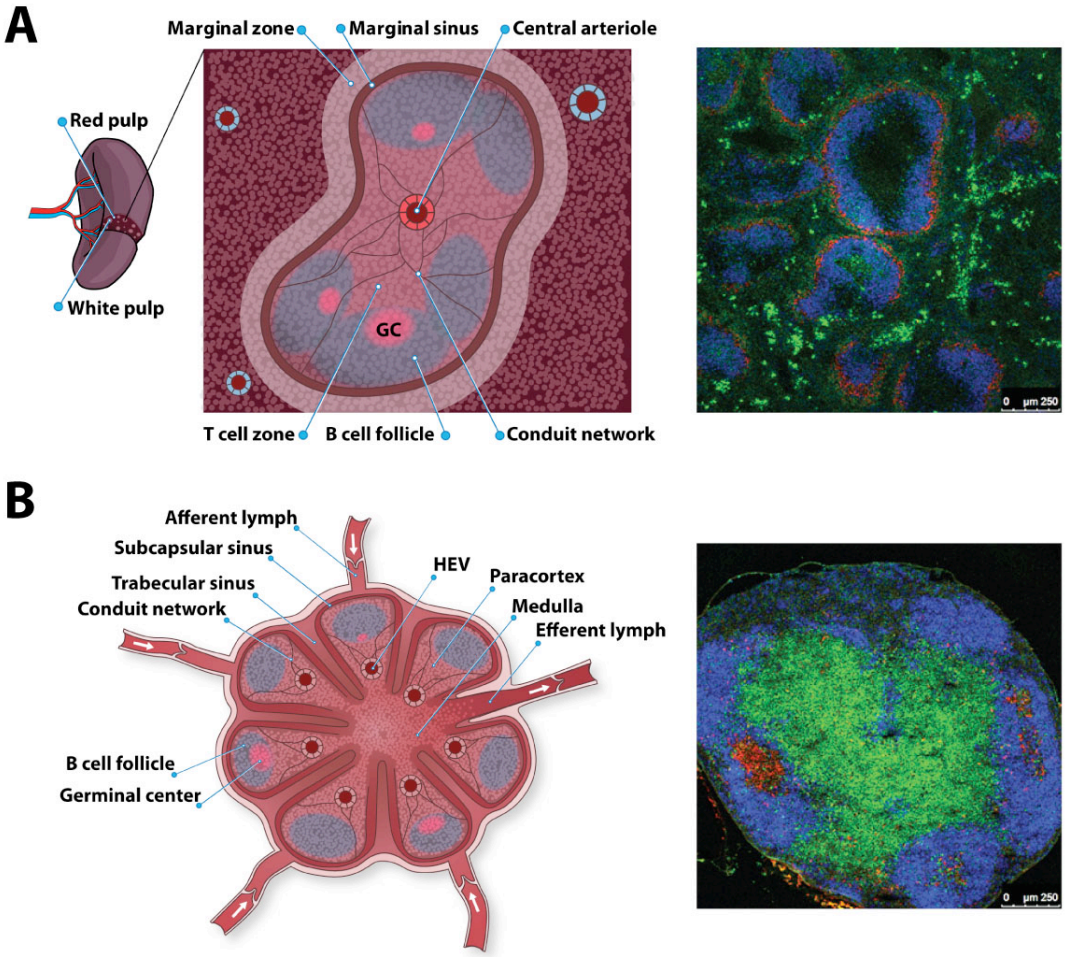


Figure 3. General structure of secondary lymphoid organs: (A) Spleen. *Left panel:* The white pulp of the spleen is embedded within the red pulp. The T cell zone is localized in the center of the white pulp, surrounded by B cell follicles, the marginal sinus and the marginal zone. Blood enters the white pulp through the central arteriole. One way for Ags to enter the white pulp is via the conduit network. *Right panel:* Confocal microscopy image of a spleen section. Blue: anti-IgD staining indicating B cell follicles within the white pulp. Red: MOMA-1 staining macrophages lining the marginal sinus. Green: anti-IgM staining IgM+ extrafollicular foci in the red pulp and MZBs surrounding the marginal sinus. **(B)** Lymph node. *Left panel:* The center of the LN is referred to the medulla, with the surrounding T cell rich paracortex and B cell follicles and the subcapsular sinus. Lymph enters the LN via afferent lymph vessels and drains into the subcapsular sinus via trabecular sinuses or via the conduit network and leaves the LN through the medulla in the efferent lymph vessel. The high endothelial venules (HEVs) allow for entry and exit of lymphocytes. *Right panel:* Confocal microscopy image of LN. Green: anti-TCRβ indicating T cell rich area in the medulla and paracortex. Blue: anti-IgD indicating B cell follicles. Red: PNA binding to germinal center lymphocytes. *Illustration by Mattias Karlén.*

3.4 ACTIVATION OF ANTIGEN-SPECIFIC B CELL RESPONSES

3.4.1 Presentation of antigen to naïve B cells

In order for an Ab response to be elicited towards an invading pathogen or a vaccine Ag, a naïve B cell that recognizes a pathogen- or vaccine-associated Ag needs to internalize the Ag and present fragments of it on major histocompatibility complex (MHC) class II for further activation by cognate helper T cells. The way Ag reaches B cells remained poorly defined for many years until recent developments in high resolution imaging techniques helped reveal these events in greater detail. Different mechanisms have been described for how Ags can enter the SLOs and each depends on the nature of the Ag. Very small Ags (<70 kD) can reach the B cell follicles directly by diffusion through pores that are present in the subcapsular sinus (72) or by travelling via a special conduit system consisting of collagenous fibres (73). Larger Ags on the other hand, reach the naïve B cells of the SLOs in a cell-associated manner via the formation of immune complexes or complement-coated immune complexes that will be recognized and captured by Fc-receptors and complement receptors on specific cells. Examples of such cells are macrophages that are present next to the subcapsular sinuses that have been shown to present Ag to cognate B cells themselves, or to hand over the Ag directly or indirectly to FDCs that can then present the Ag to cognate B cells (74-76). Unlike the classical DCs present in the rest of the body, the FDCs are not of hematopoietic origin and they are thought to lack the capacity to present peptides on MHC class II molecules (reviewed in (77)). Their main function is to present Ag to naïve and activated B cells by capturing immune complexes on Fc-receptors or complement receptors (71). In spleen, MZBs have been implicated in the transport of Ag complexes to the FDC (78). Classical re-circulating DCs expressing MHC class II are present in the T cell zone for activation of T cells, but can also present Ag to B cells that are passing on their way to the follicle. Apart from their capacity to present peptide on MHC class II to activate T cells, these DCs have been shown to internalize Ag via the FCγRIIb in the form of immune complexes in a non-degradative manner to later recirculate the Ag on their surface for presentation of the protein to B cells in its native form (79, 80).

3.4.2 Fate of activated B cells

When B cells have internalized Ag they begin to increase their expression of the receptors CCR7 and EBI-2, which together facilitate their movement to the B cell-T cell border in the SLOs where they can meet and receive help from cognate T helper cells (81-83). The B cells will at this point also up-regulate surface expression of MHC class II and increase responsiveness to CD40 signaling in order to facilitate T cell help (84). A cognate T helper cell that recognizes MHC class II with peptide on the naïve B cell via its T cell receptor (TCR) will provide further signals to the newly activated B cell by for example CD40L and ICOS (85). This leads to proliferation of the Ag-primed B cell. The proliferating B cells can after a day or two of initial proliferation at the B-T border take either of three pathways, by differentiating into extrafollicular PCs, early non-GC recirculating memory B cells or GC B cells (86-88). It is not fully understood which signals affect this fate decision though it has been suggested to depend in part on

the initial affinity of the BCR for Ag. Affinities above a certain threshold lead to the formation of extrafollicular PCs while lower affinity lead to the initiation of GC formation (89). In a recent publication it was suggested that B cells at the B-T border that were able to up-regulate the highest density of cognate MHC class II presenting peptide on their surface could compete favorably for access to T cell help and thereby enhance their chance to enter the GC compared to B cells that were not able to present as high densities of MHC class II on their surfaces (90).

Extrafollicular responses are dependent on continuing expression of EBI2 (91) and mediate the production of Ag-specific Abs very quickly after an infection. They are therefore likely to be an important part of the immune response to invading pathogens. These cells are however of relatively low affinity for the Ag and will die within a few days without forming any type of memory against the Ag. These cells are therefore not a desirable immune response in a vaccine setting, which is dependent on the formation of memory cells.

3.4.3 Follicular helper T cells

As mentioned previously, B cell survival requires cognate T cell help. The specific CD4+ T cell subset that provides this help within the SLOs is termed follicular helper T (Tfh) cells (reviewed in (92)). Tfh cells have specific attributes that separate them from other CD4 expressing T cells of the immune system. The Tfh cell program is thought to be initiated in the naïve CD4+ helper T cells present in the T cell zone of the SLOs through the initial contact with conventional DCs present in these areas. The affinity of the TCR to the MHC class II with peptide presented by the DC is thought to be of importance where high affinity has been suggested to lead to the formation of Tfh cells (93). Tfh cell differentiation is further associated with the signaling by ICOS-L on DCs and ICOS on the naïve T cell. This receptor ligation leads to up-regulation of the transcription factor Bcl-6 that is crucial for the development of Tfh cell characteristics in comparison to other CD4+ helper cells expressing the transcription factor Blimp-1 (93-96). Furthermore, Tfh cells are defined as CXCR5+ CCR7-, which is important for their movement from the T cell zone to the B cell follicular areas (97-99). After DC priming, Tfh cells are thought to receive further signals from the Ag-specific newly activated B cell at the B-T border to completely develop Tfh cell characteristics (92) and enter the GC reaction where they will continue to provide survival signals to activated and proliferating B cells. The cytokine that has been mainly associated with Tfh cell stimulation of B cells is IL21, which is a critical cytokine for optimal GC-B cell proliferation and the differentiation and survival of PCs in the SLOs (100-102). Interestingly, PCs themselves have been suggested to provide negative feedback regulation for the development of Tfh cells by acting as antigen presenting cells without providing IL-21 stimulation or up-regulation of Bcl-6 in the naïve T cell. This mechanism is thought to lead to the suppression of Tfh cell development when the appropriate level of PCs has been reached (103). The fate of Tfh cells after primary activation is not well understood but it was recently suggested that Tfh memory cells can form and that they retain the plasticity to form other T helper subset upon re-challenge (104).

3.4.4 Germinal center formation and affinity maturation

In contrast to the formation of extrafollicular short-lived B cells, the more desired outcome of a vaccination is the formation of GCs (Figure 4). These GCs will lead to the production of high affinity, isotype switched memory B cells and long-lived PC. After the B cells have been primed by Tfh cells at the B-T border, some are programmed to home back to the B cell follicle by retaining their expression of CXCR5 and down-regulating EBI2 (91), and together with other cells form the microenvironment of a GC. The GC forms upon proliferation of activated B cells and is divided into two areas, the T cell zone proximal dark zone (DZ) with highly proliferating B cells, and the light zone (LZ) where Ag-dependent B cell selection takes place (105).

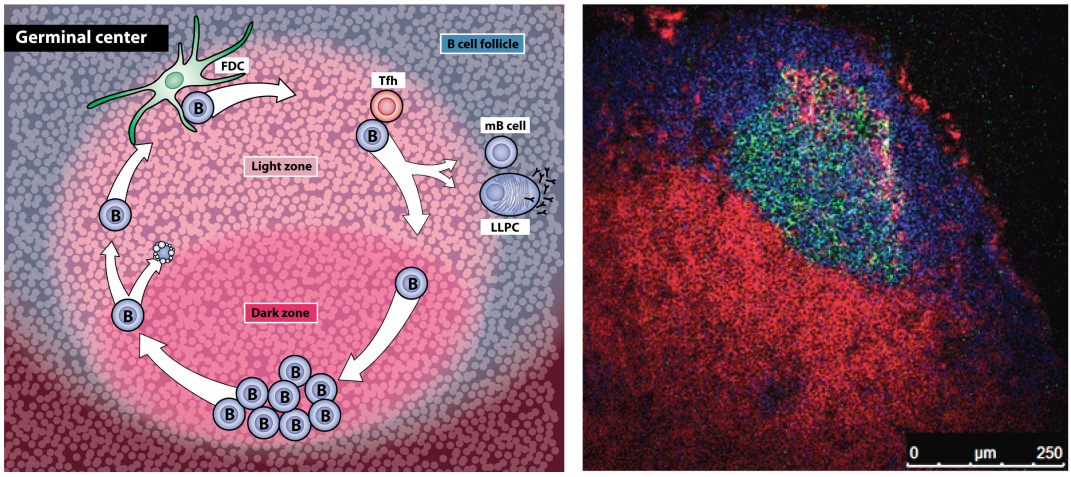


Figure 4. Structure of germinal centers. *Left:* Germinal centers form in the B cell follicles of SLOs and are divided into a dark zone and a light zone. Activated B cells proliferate within the dark zone where somatic hypermutation and isotype switching of the BCRs occur. The B cells are then directed into the light zone where the BCR affinity for the Ag is tested on Ag-presenting FDCs. B cells with high affinity are provided further survival signals from Tfh cells and either exit the germinal center as memory B cells (mB cells) or long-lived plasma cells (LLPC), or they re-enter the dark zone for further proliferation. *Right:* Confocal microscopy image of a germinal center in the inguinal LN of a mouse. Red: Anti-TCRβ indicating T cell rich area in the paracortex. Blue: Anti-B220 indicating B cell follicles. Green: GL7+ germinal center lymphocytes. *Illustration by Mattias Karlén. Adapted from: McHeyzer-Williams, M et al. 2011. Molecular programming of B cell memory. Nat Rev Immunol. 12:24-34.*

The GC consists of different cells that are important for its persistence. The most abundant cell type is the activated B cell. These B cells differ from the naïve B cells by their high expression of Fas and n-glycolylneuraminic acid (the ligand for the GL7 Ab which also binds to PNA), their loss of IgD and the fact that they are fast dividing cells (106-108). Compared to the naïve B cells, GC B cells have up-regulated levels of the transcription factor Bcl-6 (109), which among other things are important to keep the GC-B cell from apoptosis and from leaving the GC prematurely (110, 111). B cells move between the DZ and the LZ depending on their expression of CXCR4 (112). In contrast to what was believed previously (113), the B cells of the DZ and the LZ are indistinguishable in terms of size and morphology and show similar behavior in terms of movement within their compartments (114, 115). The LZ is where newly divided B cells are exposed to cognate Ag presented by the FDCs. As mentioned previously, FDCs are specialized in trapping immune-complexes on their surface primarily with complement receptors, and present Ag to B cells as they enter the LZ. In addition, they

are also producing chemoattractants and cytokines important in the formation of GCs (116). The LZ also include the Tfh cells that are essential for GC affinity maturation of B cells and GC maintenance. Another important cell within the GC is the tingible-body macrophage that is responsible for clearing the apoptotic B cells from the GC (117).

B cells proliferating in the DZ of the GC go through affinity maturation of their BCRs through a process known as somatic hypermutation (SHM). This process is dependent on the activation-induced cytidine deaminase (AID) enzyme (118). AID induces cytosine deamination of nucleotides within the variable regions of the immunoglobulin genes as the cells divide. The resulting uracil is then excised by uracil DNA glycosylase and the DNA is repaired by error prone DNA polymerases, which will introduce point mutations. These mutations hence provide B cells with non-somatic immunoglobulin genes, which further diversify the B cell repertoire. During the GC reaction, the BCR also isotype-switch from IgM to IgG, IgA or IgE in an AID-dependent process (85). At some point, the B cells will stop proliferating in the DZ and enter the LZ where they will test their newly formed BCR for binding to Ag presented on the FDCs. If the affinity for the Ag is lost, is of low affinity or have developed affinities for self-Ags, the B cells will apoptose (85). However, if the affinity for the Ag is equal or better they will acquire survival signals from Tfh cells. Low affinity for Ag has been shown to be enough for B cell survival in the GC but in the presence of high affinity B cells, B cells of lower affinity will die (119, 120), indicating that B cells may be able to compete for survival. It is unknown whether the Ag-dependent selection for survival in GCs is based on limited presence of Ag or limited presence of T cell help or if both mechanisms are in play (116). Surviving B cells can take either of three pathways, re-enter DZ for increased SHM, exit the GC as a long-lived Ab-secreting PC or as non-secreting memory B cell (Figure 4).

3.4.5 Memory B cell responses

Memory B cells are classically defined as affinity matured IgG+ B cells that are formed in the GC reaction. These cells will home to the SLOs and circulate throughout the organism until a secondary Ag encounter, when these memory B cells will rapidly differentiate into effective ASCs (121). Recently, additional subsets of memory B cells have been identified. These include somatic hypermutated GC-dependent and non-mutated GC-independent IgM+ memory B cells (86, 122, 123) as well as non-mutated non-GC dependent IgG+ memory B cells (86). However, the specific functions of these cells remain enigmatic. It is also currently debated whether or not memory B cells can re-enter GCs upon secondary encounter of the Ag. In this regard, it has been proposed that only the IgM+ memory B cells can do so whereas the IgG+ memory B cells immediately differentiate into ASCs (72, 122). In contrast to B cell survival in the GC, survival of memory B cells is thought to occur independent of Ag (124).

Long-lived PCs are generated from GCs as terminally differentiated ASCs that can be considered a distinct lineage of the memory B cell response. Long-lived PCs are thought to survive in the periphery independently of memory B cells (125) and in comparison to memory B cells, long-lived PCs express BCRs of higher affinity (126, 127). Commitment to the PC compartment requires up-regulation of the transcription factor Blimp-1, which is referred to as the master regulator of PC differentiation.

Blimp-1 was shown to be expressed in a subset of GC B cells (128), indicating that the decision of becoming a long-lived PC probably happens within the GC. Blimp-1 expression induces the expression of the transcription factor XBP-1, and both are needed for normal PC development (129). The signals that determine the fate of the surviving GC B cells are poorly understood but asymmetric cell division of the B cells could be involved (130). Furthermore, despite being well appreciated that the GCs are necessary for the development of long-lived PCs, it was recently shown conversely that long-lived PCs could arise outside of GCs (131). This finding indicates we may not fully understand the factors important for the formation of long-lived PCs.

The majority of long-lived PCs home to the BM where they may survive for the lifetime of an individual thanks to specific survival niches of stromal cells, which provide an optimal cytokine milieu for these cells. Important retention and survival signals expressed by the stromal cells include the secretion of IL-6, expression of the adhesion molecule VCAM1, and the expression of BLyS (132).

4 VACCINES

4.1 INTRODUCTION TO VACCINES

As far back as 400 B.C. the observation was made that individuals who recovered from a specific disease rarely got the same disease again. We now know this is the hallmark of vertebrate immune systems. The cells that make up our immune system have two main functions: firstly, they recognize harmful pathogens as non-self and clear them, and secondly, they develop memory for that specific pathogen to prevent disease following secondary exposure. This system can be exploited to develop vaccines with the goal to attain memory for specific pathogens without causing perturbations associated with natural infection. In general for acute pathogens the more a vaccine mimics normal infection the more efficient it is. When it comes to vaccines against chronic pathogens however, the vaccine may need to be constructed in such a way that it elicits a more potent immune response than that elicited during natural infection.

4.1.1 History of vaccination

Towards the end of the 18th century British physician Edward Jenner (1749-1823) documented experiments testing vaccination, which eventually resulted in the potent smallpox vaccine and eradication of smallpox. At this time, smallpox was a common disease with a mortality rate of about 30% (133). Jenner made the observation that milkmaids, who often became infected with a related cowpox virus only showed mild signs of infection by cowpox, and rarely became subsequently infected by smallpox-causing variola virus. Jenner hypothesized that the milder cow version of the pathogen could help protect humans against the more aggressive smallpox. He thus performed experiments in which he inoculated individuals with lesions from cowpox infections. He then challenged these individuals with lesions from variola virus-infected individuals and confirmed his theories about immunity. Remarkably, this was all done at a time when people were ignorant about infectious agents and viruses and bacteria had not yet been discovered as the etiological agent of infectious diseases. The next important milestone in the history of vaccination was marked by the discovery by Louis Pasteur (1822-1895) that attenuated pathogens could be used for vaccination.

4.1.2 Live attenuated and inactivated vaccines

Live attenuated vaccines remain in use today to protect against certain pathogens. These vaccines are potent since they moderately replicate and thus provide the immune system with innate stimuli that increase the potency of the adaptive responses (134). All ways by which this occurs are still not entirely understood. Historically, live attenuated vaccines were prepared on the principle of Jenner's smallpox vaccine, using a related virus, adopted to infect other species than humans. Guided by the experiments of Pasteur, pathogens were later attenuated by physical means. For example, the rabies vaccine was developed by drying the rabies virus. More modern ways of attenuating pathogens are to grow them *in vitro* in eggs or in cell culture or by growing the

pathogen at low temperature. These treatments weaken pathogens sufficiently to reduce pathogenesis in humans. The very efficient mumps, measles and rubella (MMR), yellow fever, varicella, oral influenza and oral polio vaccines are all examples of vaccines that have been produced using these methods (135). However, because attenuated vaccines have the risk of reverting back to their infectious form in humans, modern vaccines are often based on individual Ags cloned and expressed by molecular DNA technology as recombinant proteins or virus-like particles (VLPs), or as recombinant viruses where the Ag is inserted into a heterologous vector based on a non-pathogenic virus (135). Many of these vaccines are however still only experimental and not yet approved for human use, with the exception of the VLP-based vaccines against hepatitis B virus and human papilloma virus that are already in use.

Inactivated vaccines are, as the name implies, vaccines in which pathogens have been inactivated by different means to render them non-infectious. These vaccines include intact protein Ags for the immune system to recognize but their genomes are unable to replicate. Inactivated vaccines are commonly prepared via chemical treatment with for example formaldehyde or by irradiating the pathogen with UV light. Examples of inactivated vaccines that are used today are vaccines against tick-borne encephalitis and polio, as well as the annual influenza in which the inactivated virus is partially purified to contain Ags of interest.

For vaccines to mount a potent adaptive immune response, activation of the innate immune system is also critical. Innate immune cells can become activated through various specific pathogen recognition receptors via components that are produced during replication of pathogens. Examples of such components are bacterial and viral DNA and RNA and pathogen associated lipids and sugars. Hence, due to the lack of replication of inactivated vaccines, they will not be as potent as attenuated vaccines and therefore require the addition of an adjuvant. Many adjuvants activate innate immune cells specifically by targeting pathogen recognition receptors, but others have other or unknown mechanisms of action (136-138).

4.1.3 Vaccines based on recombinant proteins

With modern recombinant DNA technology it is possible to create vaccines based on the Ag that is the main Ab target of the pathogen (subunit vaccines). These vaccines can be formulated with appropriate adjuvants for more effective and more specific Ab elicitation. The purified proteins may be further modified to meet specific needs of a vaccine. For example, the vaccine against pertussis caused by the bacteria *Bordetella pertussis,* is based on the toxin produced by the bacteria. In this case, the toxin has been rendered non-toxic without affecting the structure of antigenic determinants required to elicit neutralizing Abs (139). Additional examples of subunit vaccines include the annual influenza vaccine and the modern rabies vaccine (140). Here, virus particles grown in vitro are inactivated, and then fractionated by detergents to only include the parts of the pathogen that are needed for the induction of neutralizing Abs, so-called split detergent vaccines. Even more sophisticated methods were used to develop the hepatitis B vaccine. In this case, the main component of the vaccine, the virus envelope protein HBsAg, was produced *in vitro* in large quantities at low cost. Previously, the only source of HBsAg was limited to the plasma of infected

people since hepatitis B virus can only infect and replicate in humans (141). Another example of a protein subunit vaccine is the recently developed vaccine against the human papilloma virus. Here, the target surface protein, L1, is produced *in vitro* to form VLPs by spontaneous self-assembly (135). It is thought that proteins presented in this form become more immunogenic than proteins that are delivered in solution (142). Some target Ags, such as the polysaccharides of certain bacterial pathogens, do normally not induce T-cell dependent responses and hence no memory response can be formed to these immunogens. To develop a vaccine based on these Ags, the polysaccharides were conjugated to proteins that included T-helper epitopes, to allow the formation of memory responses. Examples of conjugate vaccines include those against *H. influenzae*, *Neisseria meningitidis* and *Streptococcus pneumoniae* (141). Recombinant proteins can also be used as tools to investigate basic principles of vaccine-induced B cell responses to address fundamental questions in immunology, as demonstrated in this thesis.

4.2 THE ROLE OF B CELLS IN VACCINE INDUCED RESPONSES

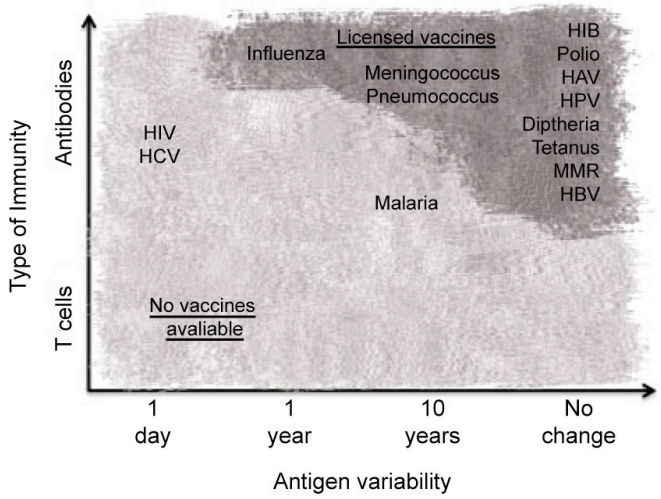
Elicitation of Abs is the correlate of protection for most existing vaccines used today. The magnitude as well as the quality of the Ab response is critical for vaccine efficacy. It has been shown that passive immunization with pathogen-specific Abs can protect from infection of specific pathogens. Vaccines that allow *in vivo* expression of Ags such as DNA vaccines, recombinant viral vector-based vaccines and live attenuated vaccines also elicit CD8+ T cell responses that may contribute to their efficacy, but the role of cellular responses in vaccine-mediated protection is less well defined (1). As discussed earlier, long-lived PCs in the BM are the source of serological Ab response that may last the life of an individual (132). For example, the half-life of the serum Ab responses to the live attenuated measles vaccine has been estimated to 3014 years, 542 years to mumps and 114 years to rubella. For protein subunit vaccines against tetanus and diphtheria the half-lives of the specific Abs were shorter and estimated to be 11 and 19 years, respectively (143). The long-lived PCs are responsible for the first line of defense against pathogen re-exposure and an important part of the immunological B cell memory. Many models have been put forward to explain the long-lived serological memory response, including memory B cell-dependent models, where it is proposed that the memory B cell pool continuously re-populates the PC pool through activation from persisting Ag in the form of immune complexes or as a result of polyclonal stimuli via toll-like receptors. In the last years however, an expanding body of data support the conclusion that memory B cells and long-lived PCs are two independent B cell populations and that long-lived PC survival, as mentioned earlier, depends on special niches in the BM where the long-lived PCs can become long-lived due to the specific cytokine milieu in the BM (125). In order to elicit as long lasting PC responses by subunit vaccines as with attenuated vaccines, it will be important to study attenuated vaccines or live pathogens in general, to identify the types of innate immune signaling these vaccines initiate at early time points of immune activation. This kind of information can give us important leads on how to formulate adjuvants and immunization regimens to generate more long-lasting B cell responses.

In contrast to long-lived PCs, memory B cells are non-Ab producing B cells that can rapidly proliferate into Ab-producing PCs upon secondary encounter of the Ag

such as a booster vaccination or an infection. This recall response will be much faster and much more potent than the initial primary response that the memory B cells originally derived from (144, 145). Both types of B cells are critical in the development of vaccines.

4.3 VACCINES AGAINST VARIABLE PATHOGENS

Most vaccines that are used today have been developed by empirical methods based on similar principles as used during the 19th century. This includes the isolation, inactivation or attenuation and injection of the specific pathogen. These vaccines offer protection against pathogens that are genetically stable or against pathogens that only vary from one year to another, like the yearly influenza viruses, but that means a new vaccine has to be generated every year. These vaccines are also efficient against pathogens that are present in a limited number of serotypes making it possible to develop a vaccine that includes Ags from all variants (Figure 5). What is also common to most pathogens for which there are vaccines today is that they cause a primary infection that humans, or at least a large percentage of the human population, are able to clear without having a pre-existing immunity. It is therefore possible to study the specific mechanisms the body uses for protection, referred to as the correlate of protection and to design vaccine candidates that induce similar immune parameters. The most common correlate of protection for the successful vaccines in use today is the induction of Abs directed against surface structures of the pathogen.



Adapted from: Rappuoli, R. 2007. Bridging the knowledge gaps in vaccine design. Nat Biotechnol 25:1361-66.

Figure 5. Implications of antigen variability on vaccine development. Most licensed vaccines are towards pathogens with little or no variability in their antigens. The vaccines against Pneumococcus (*Streptococcus pneumoniae*) and Meningococcus (*Neisseria miningitidis*) are multivalent vaccines that include immunogens from up to 14 serotypes. For influenza, for which the circulating strains vary every year, an annual vaccine has to be prepared. The correlate of protection for vaccines against all listed pathogens/diseases is elicitation of antibodies. For variable pathogens such as HIV-1 and HCV there are no vaccines available.

5 VACCINE INDUCED B CELL RESPONSES TO HIV-1 ENV

5.1 INTRODUCTION TO HIV-1

5.1.1 Classification and genetic variability

Upon the initial isolation of HIV-1 from human LNs, the virus was found to express reverse transcriptase and have similar shape and size as other retroviruses and was therefore identified as a member of the *Retroviridae* family. The cloning and analysis of the viral genome further classified the virus in the genus of *lentiviruses*. As with other lentiviruses, HIV-1 causes slow disease progression and its primary cell targets are lymphocytes and macrophages of hematopoietic origin. When phylogenetic analyses of HIV-1 viruses from different parts of the world were performed, it became clear that this was an extremely genetically variable virus. Most of the variation can be attributed to the surface Env protein. HIV-1 isolates are divided into three main groups: M (main), O (outlier) and N (non-M, non-O). The M group is further divided into the following subgroups referred to as clades: A, B, C, D, E, F, G, H, J and K, as well as 15 recombinant forms (CRF). The amino acid sequence between clades differs by approximately 30% and within one clade the difference can be up to 20% (146). Soon after the isolation of HIV-1, several distinct lentiviruses infecting primates were discovered. They were named simian immunodeficiency virus (SIV), but in contrast to HIV-1 they show asymptomatic infections in their hosts. It is now well established that HIV-1 and HIV-2, a less pathogenic variant of HIV-1 (147), originate from at least two different strains of SIV (148), and are thought to have been transmitted from primates to human some time near the early years of the 20^{th} century (149).

5.1.2 Therapy and the need for a vaccine

Today in the world, there are over 34 million people infected with HIV-1 and every year an estimate of 1.8 million people die of AIDS (numbers retrieved from the United Nations program on HIV/AIDS, UNAIDS, www.unaids.org). The development of anti-retrovirals for slowing the onset of AIDS increased during the 90s, and today, many HIV-1 infected people can live their whole life without developing AIDS. These drugs are targeting several steps of the viral life cycle and can keep the viral replication to a minimum. However, this medical treatment is primarily available for HIV-1 infected individuals in the western world. To reduce the rate of HIV-1 transmission globally, a preventative vaccine is of outmost importance. Most current HIV-1 vaccine research focuses on the use of vectors and/or DNA plasmids aimed at eliciting an effective T cell response or subunit protein-based vaccines aimed at eliciting protective Abs, or the combination of both, using heterologous prime-boost regimens.

5.1.3 The Envelope glycoproteins

Since Env is the only viral protein present on the surface of HIV-1 it is a central target for a prophylactic Ab-based vaccine. The functional Env glycoprotein is a transmembrane-spanning trimer whose main function is to bind the primary host cell receptor, CD4, and the co-receptor, CCR5 or CXCR4 to mediate viral entry (Figure 6). The Env trimer complex is formed by three heterodimers associated through non-covalent binding. Each heterodimer is initially produced as a single protein, gp160, which is then cleaved in the Golgi apparatus of the infected cell into an external glycoprotein, gp120, and a transmembrane glycoprotein, gp41. Together the trimeric heterodimers form the Env glycoprotein complex (reviewed in (150)). The complete structure of Env in its un-liganded form has so far not been solved, but truncated versions of monomeric gp120 in complex with soluble CD4 and/or specific Abs were crystallized and the structures solved (151-157). In addition, low resolution structures of the un-liganded and liganded forms of Env were determined using cryo-electron tomography (158). This information has provided important insights into the Env trimer assembly in the context of the virus. Also, using the same technique, it was shown that the HIV-1 virus has surprisingly few spike proteins present on its surface compared to other viruses, an estimated 14±7 functional spikes in addition to numerous non-functional spikes (159-161), which may affect the capacity of Abs to bind to the virus with high affinity (162).Very recently the structure of the un-liganded form of trimeric Env was resolved using single particle cryo-electron tomography at a much higher resolution than presented before (Keystone meeting on HIV Vaccines 2012: Joseph G. Sodroski. Abstract: #2).

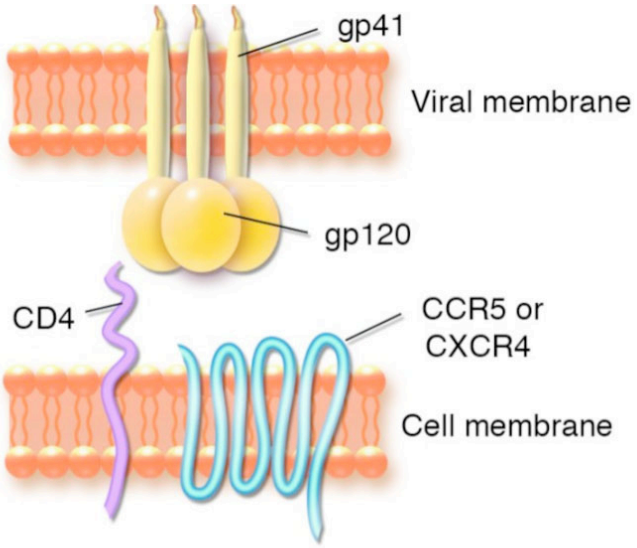


Figure reprinted with permission from Journal of Clinical Investigation: Berzofsky, J.A. et al., J. Clin. Invest. 2004 114(4):450-62.

Figure 6. Primary and secondary receptors for Env. Env is present on the surface of the virus membrane as a trimer consisting of three heterodimers. The gp41 domain of each heterodimer includes the transmembrane region and the gp120 domain includes the receptor binding sites. Upon binding of Env to its primary receptor CD4, a conformational change in gp120 forms the binding site of the secondary receptors, CCR5 or CXCR4. Binding to the secondary receptor (usually referred to as the co-receptors) allows the virus to enter target cells by fusion of the viral and cellular membranes.

5.2 TYPES OF ANTIBODIES ELICITED DURING CHRONIC HIV-1 INFECTION

Many types of Env-specific Abs with different characteristics are elicited in naturally HIV-1 infected individuals. The different Ab characteristics and the implication they have on the development of Env-based immunogens are listed below.

5.2.1 Strain specific antibodies

HIV-1 infection elicits Abs predominately directed against the highly immunogenic variable regions of Env (163). Since these regions tolerate an extreme degree of variability, escape mutants are readily selected to evade Ab recognition, resulting in circulating Abs that are not able to neutralize the most recently produced autologous viruses, but only those that were present at earlier time-points (164-167). Abs that target variable regions are usually referred to as strain-specific as they can neutralize the homologous virus (that they were elicited against), but not heterologous viruses. The extreme diversity of HIV-1 precludes the development of a "cocktail vaccine" containing different Env immunogens. Rather, it is generally appreciated that conserved determinants of HIV-1 need to be targeted, making this a particularly difficult vaccine target.

5.2.2 Non-neutralizing antibodies

A large fraction of the Ab response elicited during infection is also directed against conserved determinants of Env. However, in the majority of cases the Abs recognize conserved regions that are not exposed on the functional Env trimer present on the infectious particle. During an infection, it has been shown that soluble gp120 is readily shed from both virions and from infected cells, leaving so called "stumps" of non-functional gp41 (160, 161). Since the trimerization domain of the Env protein is located in gp41, shed gp120 dissociates into monomers resulting in the exposure of epitopes that are not visible on the functional spike, such as the N- and C-termini of gp120. Abs against these regions are therefore non-neutralizing. Shed gp120 may be considered to act as a decoy Ag diverting the immune response from more relevant targets on the functional Env trimer. Similar Abs may be generated in response to Env vaccination unless efforts are made to produce soluble Env trimers that are stabilized in a trimeric form (reviewed in (168)).

5.2.3 Broadly neutralizing antibodies

For different HIV-1 strains to infect cells with similar tropism, parts of the functional Env protein have to be kept conserved. Hypothetically, if an Ab can target such a site, it should be able to neutralize most strains of HIV-1. One of the most important conserved sites on gp120 is the CD4 receptor-binding site (CD4bs). Hence, this is a desired target for many vaccine regimens. However, most highly conserved determinants of the functional Env protein are efficiently shielded by N-linked glycans or by structural constraints that have evolved as a result of extensive immune pressure during viral evolution in multiple hosts over many years. These regions therefore tend

to be less immunogenic than the highly exposed variable regions. Interestingly, approximately 10% of infected individuals do eventually develop Abs that are specific for conserved and exposed regions of the functional Env trimer. These Abs neutralize a broad spectrum of primary HIV-1 viruses in standardized HIV-1 neutralization assays *in vitro* and are therefore referred to as broadly neutralizing antibodies (bNAbs). There is little evidence that these Abs are beneficial for the individual who develops them, probably due to the vast amounts of viral variants already seeded during chronic infection including potential escape mutants. However, in the context of a prophylactic vaccine setting, bNAbs may effectively block the infection, or if sterile protection is not achieved, blunt the acute viremia. The latter would most likely result in a lower viral set-point and a reduction in the number of viral transmission events to new individuals, which are also highly attractive goals for a prophylactic vaccine. Several bNAbs have been assessed in passive immunization studies, where they were shown to protect against challenge with chimeric SHIV viruses (2-4, 6, 7, 169-171). Another proof-of-concept for a protective role of bNAbs in HIV infection was shown using adeno-associated virus vectors for gene transfer of Ab-like molecules based on bNAbs against SIV into rhesus macaque monkeys. Vaccinated rhesus macaques monkeys showed stable production of the Ab-like molecules into the serum, and upon challenge with SIV a significant number of monkeys were protected against infection (172). Furthermore, using the same specialized adeno-associated virus vectors in humanized mice to provide a stable source of a full-length bNAb it was shown that the mice could be protected from subsequent HIV-1 infection (173). It is thus believed that bNAbs have the opportunity to neutralize virus during the short timeframe between the transmission event until the first latent reservoirs of the virus have been established. Together, these reports point to the relevance of Abs in the protection of HIV-1 and stimulates hope for the development of an Ab-based vaccine which, perhaps in combination with a T cell based vaccine, could lead to an effective prophylactic vaccine against HIV-1.

5.3 DESIGN OF IMMUNOGENS

5.3.1 Full length soluble Env constructs

The first generation of Env-based subunit immunogens were generated from full length Env generated from T cell line-adapted clade B, MN and IIIB HIV-1 strains (174-177). When tested for safety in phase I clinical trials they were shown to be safe and elicit high levels of Env-binding Abs. When these Abs were tested for neutralization activity in vitro however, they were shown to only neutralize the homologous MN virus. Only very little to no neutralization was found against primary patient isolates. Ags used in the first phase III preclinical trials to test a HIV-1 vaccine were based on gp120 monomers. The results from these trials showed that the vaccine neither protected against infection nor delayed disease progression in infected individuals (178, 179). To better mimic Env proteins as they are presented on infectious virus particles, the next generation of Env immunogens was generated as stable soluble gp140 trimers containing both the gp120 moiety and the ectodomain of gp41 with a heterologous trimerization domain. To limit gp120 dissociation, the natural cleavage site between gp41 and gp120 was mutated (180, 181) (Figure 7). Other trimers were

constructed where the cleavage was left intact and the trimers were instead stabilized by the introduction of artificial disulfide bonds between gp41 and gp120 (182, 183). A consistent improvement in the ability to elicit neutralizing Abs has been observed for trimeric Env immunogens compared to monomeric Env in several independent studies. (184-186).

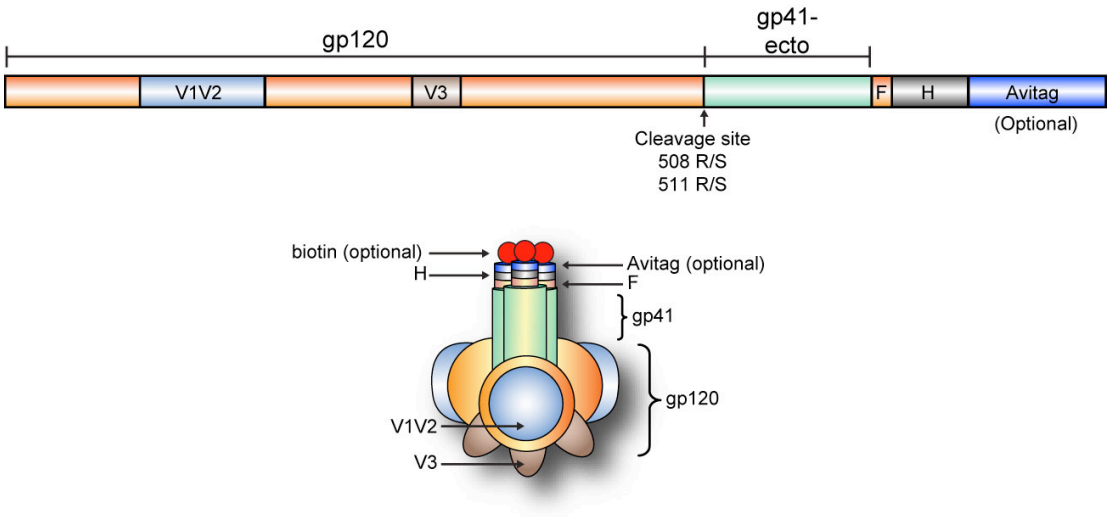


Figure 7. Model of recombinant soluble trimeric Env (gp140-F). Soluble mimetics of Env were constructed by deletion of the transmembrane region of gp41. To keep the protein soluble as an intact trimer, a heterologous trimerization domain (F) was inserted in the C-terminus of the sequence. To further stabilize the trimer, the cleavage site normally present between gp120 and gp41 was eliminated. In order to make biotinylated versions of this protein, a specific biotinylation sequence (Avitag) was inserted following the His tag (H). This allows for specific biotinylation only at the distal part of the protein. The different domains, gp41, gp120, Variable region 1 and 2 (V12) and 3 (V3) are depicted in the model.

5.3.2 Epitope scaffolds

As discussed earlier, while bNAbs are elicited in a subset of naturally HIV-1 infected individuals, they have so far not been induced by vaccination. A novel approach of immunogen design is to construct scaffold immunogens. Scaffold proteins are non-HIV proteins selected using computational methods for their ability to present a heterologous epitope of interest in its native three-dimensional conformation (http://www.rosettadesigngroup.com/index.php) (187). The rationale behind using scaffolds as immunogens is to enhance the immunogenicity of poorly immunogenic epitopes by stably exposing them in a more favorable way to facilitate immune recognition. Scaffold immunogens have been engineered to present linear gp41 bNAb epitopes referred to as the membrane proximal external region (MPER) epitopes, as well as a discontinuous CD4bs epitope (188, 189). The method for constructing a scaffold protein for a specific epitope begins with searching the Protein Data Bank to find known protein with an exposed three-dimensional structure resembling the epitope of interest. Selected proteins are then used for transplantation of the epitope of interest, or more specifically the identified homologous epitope on the selected protein is modified to resemble the epitope of interest even further. Next, additional mutations within the selected protein are inserted to keep the scaffold stable. The resulting scaffold immunogens are then characterized for binding to bNAbs specific for the

epitope of interest (190). So far, some scaffold immunogens have been shown to be capable of re-eliciting Abs with structural specificities similar to the original Abs (189, 190), but Abs capable of neutralizing HIV-1 have not been induced. This approach is under active investigation to develop novel generations of more sophisticated scaffold proteins.

5.4 ISOLATION OF BROADLY NEUTRALIZING ANTIBODIES AGAINST ENV FROM HIV-1 INFECTED INDIVIDUALS

To identify new bNAb epitopes and to understand more about known bNAb epitopes on Env, efforts are underway to isolate bNAbs from infected individuals that display broad serum neutralization activity. Until recently, only four Env-specific Abs (b12, 2F5, 2G12 and 4E10) that conferred broad neutralization in a standardized HIV-1 neutralization assay (191) were known (192-196). However, several additional broadly neutralizing monoclonal antibodies (MAbs) have been isolated recently using single cell sorting of memory B cells, or similar methods, from HIV-1 infected individuals whose serum displayed broad neutralization (197, 198). The detailed characterization of the precise epitopes of these MAbs on Env has provided valuable information. These analyses include Abs such as b12, VRC01 and NIH45-65 targeting the discontinuous CD4bs (156, 199, 200), 2F5 and 4E10 targeting a linear epitope within MPER (201, 202) and 2G12, which targets the glycans on the gp120 outer domain, which are thought to have a slightly different composition compared to those present on endogenous glycoproteins (195, 203). Furthermore, PG9 and PG16 are two clonally related Abs that target a glycan-sensitive epitope at the base of the V2 and V3 domains that is preferentially recognized on the trimeric form of Env (197, 204). To understand the genetic properties of some of these Abs, the germline V(D)J immunoglobulin genes have been determined (200, 205). Interestingly, these analyses showed that many bNAbs that target the Env CD4bs use a specific IgH V gene, namely the $IgHV_{1-2}$. Whether this is a pre-requisite for the development of bNAbs targeting the CD4bs is not known. An improved understanding of how bNAbs arise during natural infection may help guide the development of more efficient Env-based vaccine approaches.

5.5 EVASION STRATEGIES OF ENV AND ITS IMPLICATION FOR VACCINE DESIGN

HIV-1 has evolved several strategies to evade the elicitation of bNAbs. One such strategy is the high number of glycosylation sites present on Env. The gp120 monomer has approximately 20-25 sites for N-linked glycosylation (depending on strain) including both high-mannose and complex type carbohydrates. Together these sugar residues add up to as much as 50 % of the total mass of gp120 (206). Since these glycans are host-derived, they provide an effective shield that protects gp120 from Ab recognition (164, 207, 208). Another strategy the virus has evolved to evade immune recognition is the high genetic variability in exposed Env domains. The amino acid sequence of Env consists of five hypervariable domains (V1-V5) that are separated by more conserved regions. The variability arises during viral replication due to the error-prone reverse transcriptase and to evolutionary pressure from elicited Abs (165).

Another hypothesis to explain the difficulties in re-eliciting broadly neutralizing Abs to Env by vaccination is the lack of cognate specificities in the pool of naïve B cells. It is possible that Env has rendered its conserved epitopes to resemble epitopes that are selected against in the naïve B cell pool, due to for example recognition of self Ags (209, 210). In other words, it is possible that HIV-1 exploits mimicry of self-epitopes in order to evade the immune response. There are several scientific evidences that argue in favor of this hypothesis. First, although still a matter of debate (211), it has been proposed that some of the isolated bNAbs as well as the total Env-binding Abs of HIV-1 infected individuals have self-reactive properties in ELISA measurements (209, 212-217). Second, it is well established that many of the characterized bNAbs against HIV-1 have long CDR3 loops (199), which is a common characteristics of Abs with polyreactive properties (20, 218, 219). Third, when some bNAbs were experimentally mutated back to their germline parental sequence, they were shown to completely loose affinity for the Env protein, suggesting that some other Ag provided the initial activating signal for these B cells and that B cells recognizing the conserved sites of Env are not present in the naïve B cell repertoire (200, 220).

Due to the various evasion strategies mentioned, extensive efforts have been made to develop improved Env immunogens. Examples of such immunogen design includes various deglycosylation strategies (221, 222), deletion of variable regions (223, 224) hyperglycosylation of variable domains (225) and the stabilization of Env proteins in its CD4 bound site (226, 227).

6 RESULTS AND DISCUSSION

Even though soluble trimeric Env proteins are superior immunogens as compared to soluble monomeric Env proteins they are still not successful in eliciting bNAbs (184, 185). In **paper I**, we sought to investigate the limitations of the YU2-based, soluble Env trimers (Figure 7) to guide future improvements of this and other immunogens. An initial goal was to increase the resolution of our read-outs and we therefore developed a differential Env-specific B cell ELISpot assay that allows the detection and enumeration of B cells recognizing distinct sub-regions of Env. This assay provides an important complement to the serological assays that are commonly used to detect Env-specific Ab responses in vaccinated subjects today.

In brief, B cell ELISpot assays are used to enumerate Ag-specific ASCs *ex vivo* (Figure 8A). In conventional B cell ELISpot assays, 96-well plates with specifically engineered membranes that allow high binding capacity of Ag are coated with the Ag of interest. Single cell suspensions from selected organs (spleen, BM, LN or blood) are then added. During over night incubation, ASCs specific for the coated Ag will bind to the bottom of the well. Subsequently, all cells and non-specific Abs are washed away and a biotin-conjugated secondary Ab specific for the Ab species is added. The assay is developed by the addition of enzyme-linked streptavidin and the corresponding substrate. Each spot visualized in the wells is a footprint of a single Ag-specific ASC. The number of Ag-specific B cells may be compared to the total number of IgG-secreting cells in the same sample by counting spots in wells coated with anti-IgG (Figure 8C).

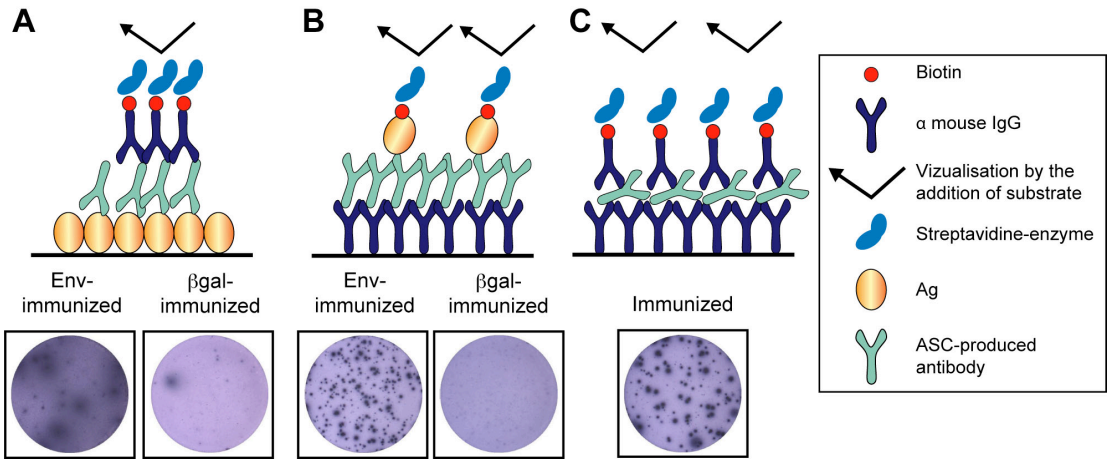


Figure 8. The principles of the B cell ELISpot assay. (A) Conventional Ag-specific B cell ELISpot assay. The Ag of interest is coated on the bottom of the well. Cells are added in single cell suspensions and Abs from Ag-specific B cells will bind to the Ag coated wells. Cells and non-specific Abs are washed away and the assay is developed by the addition of a biotinylated secondary Ab, streptavidin conjugated enzyme and substrate. **(B)** Optimized Ag-specific B cell ELISpot assay. In comparison to the conventional Ag-specific ELISpot, here, wells are coated with an unconjugated anti-mouse (for detection of Ag-specific murine B cells) IgG. All IgG Abs produced from the cells are therefore captured but only the Ag-specific Abs are visualized using a biotinylated Ag as a probe. **(C)** Total IgG assay. Wells are coated with anti-mouse IgG. All IgG Abs produced from the cells are captured and are detected using a secondary biotinylated anti-mouse IgG Ab. Lower panels show images of wells from respective ELISpot assay using Env as the Ag with cell suspensions from Env- and control (β–gal) immunized animals. Each spot represents the footprint of one single antibody-secreting B cell.

When we used the conventional ELISpot assay to detect Env-specific ASC responses, we observed large and quite undefined spots as well as relatively high background staining (Figure 8A) making the results difficult to analyze. Increasing the concentration of coating Ag had little or no effect on the quality of the spots. Therefore, to optimize the assay we coated the membranes with anti-mouse IgG Abs to first capture all IgG produced from ASCs in the suspension. Biotinylated Env was then added to visualize the Env-specific spots. Instead of chemical biotinylation, where added biotins could possibly shield important structures of the Ag, we constructed Env probes expressing a sequence for enzymatic biotinylation (avitag) at the C-terminus of the protein, resulting in biotinylation only at distal parts of the protein (Figure 7). With this improved assay format we detected well-defined Env-specific spots with very limited background staining (Figure 8B). This assay was further shown to be more sensitive than the conventional assay since we detected up to three-fold more Env-specific ASCs. Another advantage was that we could titrate down the concentration of the Env probe used for detection approximately ten-fold without decreasing the signal strength. Furthermore, we used an Env-specific hybridoma cell line to show that the sensitivity of the optimized assay was similar for Env and total IgG (Figure 9). Together, these experiments demonstrate that the optimized ELISpot assay is highly specific and more sensitive as compared to the conventional method. The optimized B cell ELISpot assay has now been used successfully to detect ASCs specific also for other Ags such as IFNγ, IL2 and IgE (228) and for B cell studies in other species such as rhesus macaques (186, 229).

Env is a complex protein displaying distinct structural domains with different antigenic properties. We therefore reasoned that it should be possible to enumerate B cells recognizing different sub-specificities of Env at the cellular level. This information may provide insights into how B cells recognize large protein Ags. Mapping studies to reveal Env sub-specificities contained within the polyclonal response were previously performed on sera using linear Env-derived peptides, chimeric HIV viruses and mutated Env proteins as competitive ligands in binding or neutralization assays (230-233). With a more sensitive B cell ELISpot assay in hand, we speculated that it would be possible to include additional probes such as Env proteins harbouring deletions of specific regions. We anticipated that by comparing the difference between the number of spots detected using different probes we could enumerate B cells that are specific for certain sub-regions of Env by subtractive analysis (we term this a differential ELISpot). We therefore generated a set of variant Env probes to be used in parallel with the trimeric gp140-F probe. The additional probes included: gp120-F (trimeric Env lacking the gp41 domain), gp120-F-ΔV1/2 (trimeric Env lacking the gp41 domain and the variable domains 1 and 2), gp120-F-ΔV3 (trimeric Env lacking the gp41 domain and the variable domain 3), gp120-F-ΔV1/2/3 (trimeric Env lacking the gp41 domain and the variable domains 1,2 and 3) and monomeric gp120 protein (Figure 9A). These probes would together allow us to determine the number of B cells that recognize the gp41 region, the variable regions 1, 2 and 3, the core (defined as non gp41, non V1/2 and V3 specific Abs), and gp120 trimer-specific Abs. Successful deletion of the various regions was assessed by binding the probes to a panel of domain-specific mAbs. Further characterization of the probes

was performed using the ELISpot for detection of Env-specific hybridoma cells with known specificities for different regions of Env (Figure 9B).

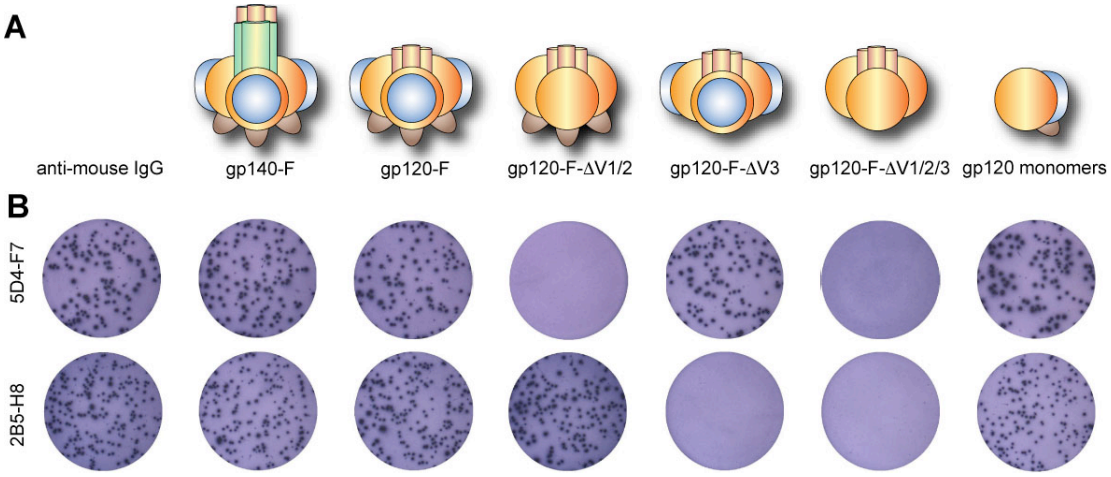


Figure 9. Differential Env-specific B cell ELISpot. (A) To enumerate B cells with specific sub-specificities of Env we constructed a panel of Env-proteins to be used for probing. Each construct lacks one or more sub-domains. Apart from the full-length Env (trimeric gp140) used for immunization we also constructed gp120-F (trimeric Env lacking gp41), gp120-F-ΔV1/2 (trimeric Env lacking the gp41 domain and the variable domains 1 and 2), gp120-F-ΔV3 (trimeric Env lacking the gp41 domain and the variable domain 3), gp120-F-ΔV1/2/3 (trimeric Env lacking the gp41 domain and the variable domains 1, 2 and 3) and monomeric gp120 protein. **(B)** The different probes were characterized in the ELISpot using two mouse hybridoma cell lines. 5D4-F7 secreting Abs specific against V2, and 2B5-H8 secreting Abs specific for V3. As expected, we saw no spots in the 5D4-F7 wells probed with proteins lacking V2 and no spots in the 2B5-H8 wells probed with proteins lacking V3.

There are a number of questions to address using the differential B cell ELISpot approach. We were first interested in enumerating the total Env-specific ASCs and characterizing the sub-specificities of Env-elicited ASCs in mice after two or three immunizations with soluble Env trimers. In this regard, we analyzed responses directly on unstimulated splenocytes harvested three days after boosting. We anticipated that the response detected at this time point would consist mainly of short-lived PCs that differentiate from Env-specific memory B cells upon Ag re-exposure *in vivo*. However, the assay may also detect non-GC derived, short-lived, low affinity ASCs elicited after each immunization.

We observed a clear difference in sub-specificities of the ASCs at different time-points. After two immunizations, the Env-specific response was dominated by gp41-specific B cells while after three immunizations, the response was primarily directed against the variable regions of Env, in particular the V3-specific region, with almost undetectable levels of gp41-specific ASCs. When we analyzed the Env-specific B cell responses in spleen at 21 days after two immunizations we could start to detect variable-region specific B cells. This finding indicated that these B cells develop with a third immunization as well as simply over time. This pattern of response to some extent resembles the response elicited after acute HIV-1 infection (234, 235). While further studies are required to understand the kinetics of the response observed for the distinct epitope regions of Env, it is possible that our results reflect differences in precursor frequencies of naïve B cells recognizing the different sub-specificities of Env, or that they arise from different types of naïve B cells. For example it is possible that the initial response to gp41 comes from naïve B cells with different characteristics such as MZBs

rather than the classical follicular B cells. Alternatively, gp41 and V3-specific B cells may induce different compositions of memory B cells that could lead to the differences in the recall response observed in our studies.

The results in **paper I** demonstrated that the V3-directed response dominated after three immunizations. This is consistent with the notion that the variable regions of Env are immunodominant (236) and may act as decoy epitopes to divert B cell responses away from conserved neutralizing Ab targets of Env. Thus in **paper II**, we sought to investigate the impact of the V3-directed response and if there is competition between B cells recognizing distinct epitope regions of Env. As in **paper I**, we took advantage of the differential B cell ELISpot assay. Our previous results showed that the V3-induced response constituted 40-60% of the total Env-specific recall response measured after three immunizations. We hypothesized that if competition were in play, immunizing mice with an Env immunogen in which the V3 region was immunologically silenced would increase B cell responses to other regions of Env. Alternatively, if there were no competition between B cells specific for distinct epitope regions, the total Env-specific response would be reduced, as the V3-specific fraction of the response would be lost.

To address this, we generated an Env immunogen in which the V3 region was masked by the addition of extra N-linked glycans. We employed this strategy instead of deleting the V3 regions as that may introduce novel Env epitopes. We referred to the V3 shielded trimers as gV3 (glycan-masked V3). The biochemical integrity of gV3 was characterized in binding studies to CD4 as well as to well-characterized monoclonal Env-specific Abs with known specificities. We found that Abs specific for non-V3 regions bound the wild type (wt) Env trimers and gV3 in a similar way, while as expected, two V3-specific Abs, 447-52D and 39F, failed to bind gV3, thereby confirming effective immune shielding of the V3 region.

We know from results in **paper I** that a very small proportion of the recall response is specific for V3 after two immunizations of wt Env. However, it was still interesting to compare the sub-specificities in the recall response of mice that were immunized twice with wt Env or gV3 to detect if the lack of V3 could have an effect on the outcome of the gp41- and core reactivities that dominate at this time point. However, the results indicated that the lack of V3 did not alter the outcome of the recall response after two immunizations. Upon three immunizations, as detected in the experiments in **paper I**, wt Env-immunized animals showed a dominating V3-specific recall response. Interestingly, when analyzing the gV3-immunized response, we saw a significantly lower number of total Env-specific B cells when using gp140-F and gp120-F as probes. Furthermore, upon using the gp120-F-Δ3 or the gp120-F-Δ123 probe, we detected similar numbers of spots between the groups, indicating that the reduced B cell response detected with gp140-F and gp120-F was due to the lack of V3 specific B cells. This data intriguingly suggest that the development of recall responses specific for one region of the Env protein does not affect the development of the responses towards other sites of the protein.

Epitopes on Env that are known to be sensitive to bNAbs are poorly recognized by the humoral immune system due to partial shielding of conserved determinants of Env. One of the first identified Abs showing broad neutralization to Env was 2F5

(192). The epitope recognized by this Ab is a linear epitope, ELDKWA, present in the MPER of gp41. This epitope was found to be conserved in a significant proportion (72%) of screened HIV-1 isolates (194). A number of attempts to re-elicit the 2F5 Ab have been undertaken by immunization with different immunogens containing the MPER peptide with only weak or no neutralization activity observed (237-241). Detailed information about the structure of the ELDKWA peptide is known (154, 239, 242-244), which has allowed the development of several different scaffold immunogens presenting this epitope for more optimal display to the immune system (189).

In **paper III,** we selected three such epitope scaffolds (ES1, ES2 and ES5) for further studies. The immunogens were designed with or without a common MHC class II T-helper epitope, PADRE. We first analyzed the immunogenicity of the ES proteins following three homologous immunizations of mice using ES1, ES2 or ES5 in adjuvant. The presence of 2F5-like Abs in the different groups was assessed by analyzing binding to a fourth 2F5 scaffold protein, ES4. The Abs binding to this scaffold should be 2F5-specific since this scaffold was not used for immunization in any of the groups. We found that 2F5-specific responses elicited with ES1 and ES2 were very low to non-detectable. In contrast, the ES5 scaffold induced high levels of 2F5-binding Abs after three homologous immunizations. The specificity of the response was confirmed by using free 2F5 peptide to successfully compete out serum binding to ES4. Inclusion of the PADRE epitope in the scaffold protein did not enhance the 25F-elicited response, probably because the scaffold proteins already included potent T-helper epitopes.

It is likely that when homologous prime-boosting is used, a large proportion of the recall response is specific for parts of the scaffold protein other than the introduced 2F5 epitope. To circumvent this problem, we tested whether a heterologous immunization regimen, using different 2F5-presenting scaffolds inoculated in a sequential manner, would lead to a more focused 2F5-specific response. We immunized mice two weeks apart with the ES5, ES1 and ES2 scaffolds with or without the PADRE epitope. Our results show that the 2F5-specific ES5 elicited primary Ab response in serum could be boosted by the heterologous epitope ES1. Furthermore, the ES2 scaffold, which only induced a very low 2F5-specific response in the homologous regimen further boosted the response suggesting that memory B cells formed by priming with ES5 effectively recognized the 2F5 epitope on the other scaffold proteins. Compared to the homologous regimen, the inclusion of PADRE was more important in the heterologous regimen, consistent with the notion that the same helper epitope needs to be present when boosting in order to activate the existing memory helper T cells.

To determine the relative frequency of 2F5-specific ASCs elicited in the homologous versus the heterologous immunization regimen we adopted the B cell ELISpot assay developed in **paper I** to detect 2F5 specific B cells. Using this assay, we showed that 2F5-specific B cells were only detected in mice immunized in a homologous regimen with ES5 or in the heterologous regimen with ES5, ES1 and ES2. There were no detectable 2F5-specific B cells in the mice immunized in a homologous regimen with ES1 and ES2. This did not depend on the lack of T cell help since only scaffolds including PADRE were used. There was no significant difference in the ratio of 2F5-specific ASCs induced by the ES5 homologous regimen compared to the

heterologous regimen. This suggests that engagement of B cell responses to the other parts of the scaffold did not impact the evolution of graft-specific responses. We do not yet know why the immunogenicity of the 2F5 epitope in the context of ES5 measured both on the plasma and cellular levels, was greater than for the other scaffolds. One possibility is that this scaffold is more flexible (189), which has been noted to correlate positively with immunogenicity for other Ags (245).

When Abs from both the homologous and the heterologous regimen were tested for neutralization capacity *in vitro*, no neutralizing activity against selected HIV-1 isolates was detected. One reason for this could be that in comparison to the scaffold induced Abs, the CDR3 loop of the original 2F5 Ab expresses several highly hydrophobic amino acids that are thought to form additional contact with the membrane of the virus. Currently, novel scaffolds are being produced where this is considered. Another reason why the scaffold elicited Ab responses were not neutralizing could be that the 2F5 epitope is not presented on the scaffolds in exactly the same three-dimensional structure as it is on the native Env trimer.

In **paper IV**, we took advantage of the effects of the B cell survival factor BLyS, to investigate the hypothesis that there are limitations in the naïve B cell pool that limit elicitation of bNAbs. BLyS has been proposed to regulate the homeostasis of self/non-self BCR reactivities in the naïve B cell pool. We hypothesized that exogenous BLyS treatment of naïve mice would alter the naïve B cell repertoire and that this would influence the outcome of Env-induced B cell responses compared to control-treated mice. Accordingly, we injected naïve mice with 10 μg of BLyS or PBS, once per day, for ten consecutive days before they were immunized with Env. To confirm the effects of BLyS and determine the kinetics of BLyS-induced effects on the naïve B cells, we first sacrificed selected mice for analysis at 1, 5, 9, 13, 17 and 21 days after the last day of BLyS treatment without Env immunization.

Upon analyzing these mice at day one after terminating BLyS-treatment, we found the total size of the spleen significantly increased in BLyS-treated mice most likely due to increased numbers of B220+ B cells detected. Specifically, it was the number of surviving transitional B cells that caused this increase since we could detect an increased number of T2 but not T1 cells in BLyS-treated mice compared to control mice, which agrees with the literature and shows that BLyS does not affect the output of immature B cells from the BM (56). Upon analysis of the mature naïve B cell pools, we determined that there were increased levels of both follicular B cells and MZBs with a more pronounced effect on the MZBs. This is consistent with the notion that MZBs are more sensitive to depletion of BLyS than follicular B cells (53). Because we planned to immunize BLyS-treated mice and study the development of the Ag-experienced B cells, we also wanted to assess the effect of BLyS on the architecture of the SLOs as the T and B cell areas were found to be disrupted in mice that were transgenic for BLyS (44). However, by immunostaining the white pulp in spleen, we demonstrated that the architecture of the B- and T cell areas was intact after ten days of BLyS treatment. Analysis of serum levels of Ab reactivities against dsDNA, phosphorylcholine (PC) and cardiolipin showed elevated levels of these reactivities in BLyS treated mice but when these levels were normalized to total IgM, there were no difference between BLyS-treated and controls indicating that there were no specific increase in self-reactive Abs. When we analyzed the BLyS-induced effects at later time

points after the last day of injection we could see that they were highly transient, and already at day five, the numbers of naïve B cells were close to baseline levels. We concluded that subsequent Env immunization should therefore be performed immediately after finalized BLyS treatment.

To analyze the effect of BLyS-treated animals on the outcome of an Env-immunization, BLyS- and PBS-treated animals were primed with Env and adjuvant one day after the last day of treatment. Mice were then boosted once or twice with Env trimers with two-weeks intarvals. We hypothesized that if any new Env-specific naïve B cells had been generated during the BLyS treatment, these B cells could get recruited into the immune response at the priming event and further proliferate with subsequent boosting. At four or 21 days after the second Env-immunization we analyzed the initiation of the B cell response by determining the level of GC formation by flow cytometry. Surprisingly, even though the total number of naïve B cells available at the priming event was increased in the BLyS-treated animals, we detected no difference in the total number of GC-cells. There were also no difference between BLyS- and control treated groups when we used the optimized B cell ELISpot to enumerate the total number of Env-specific ASCs in spleen at day four after second immunization, the Env-specific PC in BM at day 21 after second immunization or when we analyzed the presence of Env-specific memory B cells in the spleen after *in vitro* stimulation. Also, we could not detect any difference when we analyzed the total Env-binding Abs in sera by ELISA after two or three immunizations. We hypothesized that this could be due to further regulation of the total number of cells that can enter the GC. For example, it is possible that there is limited space or limitations in T cell help or the amount of Ag thereby restricting the formations of larger GCs.

In addition, we investigated whether the quality of the Env-specific B cells were different in BLyS and control-treated mice using the optimized differential Env-specific B cell ELISpot described in **paper I**. These analyses showed no consistent difference in the sub-specificities in the BLyS and control-treated mice. We further analyzed the quality of the serum Abs by determining their ability to neutralize selected strains of HIV-1 pseudoviruses *in vitro*. In brief, the neutralization assay is based on the infection by pseudo-HIV-1 viruses of reporter target cells, a well-validated assay in the HIV-1 neutralizing Ab field (191). Using this assay for analysis of the serum after two immunizations with Env trimers, we detected neutralizing activity above background in four out of twelve BLyS-treated mice compared to zero out of twelve in the control group. When this experiment was repeated using BLyS and control-treated mice immunized three times with Env trimers, we detected neutralization activity in both the BLyS- and the control-treated mice, but with significantly more responders in the BLyS-treated group. A third experiment of mice immunized four times with a longer timeframe, 60 days, between the third and the fourth immunization, gave the similar result.

BLyS has been proposed to have an effect on the selection of the naïve B cell repertoire by affecting the survival of transitional B cells (40, 63). It is thus tempting to speculate that the increased neutralizing activity we observed in the BLyS-treated animals is due to an altered naïve B cell repertoire, which led to the engagement of novel B cell reactivities into the Env-specific B cell response. It is also possible that the effect we detected was due to modulations by BLyS on the GC since it has been

suggested that BLyS has an effect on the duration of the GC response (246). However, since we only administered BLyS prior to the first Env immunization and the effect of BLyS treatment was short-lived we consider this possibility less likely. For a more complete understanding of how BLyS treatment induced these downstream effects, a more detailed analysis of the naïve B cell repertoire in BLyS and control treated non-immunized and Env-immunized animals is needed.

7 CONCLUDING REMARKS

Throughout the work presented in this thesis, we used the well-characterized, recombinant, soluble HIV-1 Env trimer as a model immunogen to study basic B cell questions relevant to vaccine development. The primary advantage of this approach is the obvious clinical relevance of Env compared to model Ags more commonly used in the basic B cell biology field such as NP, OVA and SRBC. A potential disadvantage of using HIV-1 Env as a model Ag is that it is a highly complex protein Ag, which may not be representative of smaller, less glycosylated or unglycosylated Ags. Nevertheless, we believe a more thorough and basic understanding of how organisms respond to relevant vaccine Ags is needed and this requires studies that bridge the fields of basic B cell biology and vaccine research.

The results in **paper I** confirm that different epitopes on a protein are not equally immunogenic and also show that the level of immunogenicity for a specific epitope is not static. Rather, epitope recognition seems to vary over time and with the number of times the immune system is challenged with the Ag. In many circumstances, low immunogenicity can probably be explained by steric hindrance and occlusion of the epitope. In these cases, approaches such as scaffold design may be helpful tools to increase immunogenicity of a specific epitope as was characterized in **paper III**. Yet, is it also possible that some epitopes are less immunogenic because there are other surrounding epitopes that are better at activating the immune response? The findings in **paper II** suggest that this is not the case but rather that there are undefined intrinsic regulatory features of individual epitopes. In a way, the results in **paper III** also confirm these results since we do not see increased responses to common epitopes using heterologous scaffolds upon immunization. Interestingly, in **paper IV** our results suggest a possibility for the naïve B cell repertoire to be a limiting factor for the Ab response to certain epitopes. As has been speculated elsewhere (210), it is possible that some plasticity in the naïve B cell repertoire is allowed in order to maintain a fine balance between recognizing self and foreign Ags by mechanisms such as those controlled by BLyS signaling.

In conclusion, the results presented in this thesis demonstrate how the B cell response evolves following recognition of a clinically relevant viral Ag. These results may be become important in the development of future subunit-based vaccines.

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