Combination of hu-PBL-SCID mice and scFv phage display library: an effective alternative for hu-mAb production

by

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ABSTRACT

The mammalian immune system recognizes and responds to a wide spectrum of molecules, and its diversity is facilitated by V-(D)-J recombination during ontogeny. The central function of the immune system is to protect the animal from traumas, invasion of foreign agents and aberrations within the animal itself. One of the major components, which help the immune system to fulfill its functions, is the B cell. Each B cell clone expresses or produces a specific immunoglobulin molecule (Ig) that can recognize a distinct molecule or a class of related molecules. The development of the mouse hybridoma technique to produce unlimited amounts of specific mouse antibodies (Ab) in 1975 has opened doors to a wide spectrum of Ab applications, which has facilitated scientific research. Especially, the generalization of using Ab in human immunotherapy is closer than ever. The immunogenicity of the Ab molecules has, however, made human, not mouse Ab an ideal candidate for clinical uses. Currently, four approaches are studied and used to produce specific Ab for therapeutic purposes. The first two approaches are based on the availability of a vast array of ready-made specific mouse hybridomas. Chimeric human/mouse Ab is generated by replacing mouse Ig constant (C) regions by the human counterparts. Furthermore, human framework regions (FR) are grafted into the

chimeric human/mouse Ab by polymerase chain reactions (PCR) to produce humanized Ab that retains the original specificity. Over the last 10 years, the generation of complete human Ab (hu-Ab) has been achieved by transgenic and recombinant approaches. Specific human immune responses (hu-IR) can be induced in transgenic mice that are carrying human Ig transgenes. Hybridomas can be easily generated from these mice and used to produce highly specific hu-Ab. Phage display library techniques have been developed and used to express large repertoires of hu-Ig genes, and specific hu-Ig genes can, in turn, be isolated from this library by simple panning steps.

We previously demonstrated that, with appropriate immunization procedures, both primary and secondary hu-IR could be established in severe combined immunodeficient mice that had been engrafted with human peripheral blood lymphocytes (hu-PBL-SCID mice). These results prompted us to establish an efficient system to generate specific human monoclonal Ab (hu-mAb). By combining this animal model with a single chain antibody Fv (scFv) phage display technique, we were able to generate panels of hu-scFv specific to either self-Ag (i.e. hu-TNF-α) or foreign Ag (i.e. respiratory syncytial virus). Our results show that:

(1) hu-PBL-SCID mice can be effectively used to generate large hu-Ig gene repertoires for the cloning of highly specific hu-mAb.

(2) The combination of hu-PBL-SCID mice and scFv-library can be applied widely to generate large repertoires of hu-Ab against both foreign and self-Ag.

The work presented in this thesis provides a significant step toward the generalization of specific hu-Ab production for both research and clinical purposes.

My parents, my sisters and their families, to whom I'll forever be indebted,

Hai P Nguyen

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ABBREVIATIONS

Ab	-	antibody
Ag	-	antigen
APC	-	antigen presenting cell
BCR	-	B cell antigen receptor
CDR	-	complementarity determining region
DMP	-	dimethyl pimelimidate dyhydrochloride
DNA-PK	-	DNA-dependent protein kinase
EBV	-	Epstein-Barr virus
FDC	-	follicular dendritic cell
FR	-	framework region
GC	-	germinal center
HRP	-	horseradish peroxidase
hu-Ig	-	human immunoglobulin
hu-mAb	-	human monoclonal antibody
hu-PBL	-	human peripheral blood lymphocyte
hu-PBL-SCID mice	-	hu-PBL engrafted SCID mice
hu-TNF-α	-	human tumor necrosis factor
IDC	-	interdigitating dendritic cell
Ig	-	immunoglobulin
IPTG	-	isopropylthio-β-D-galactoside
IR	-	immune response
KLH	-	keyhole limpet hemocyanin

LCL	-	lymphoblastoid cell line
mAb	-	monoclonal antibody
MHC	-	major histocompatibility complex
mo-mAb	-	mouse monoclonal antibody
PALS	-	periarteriolar lymphoid sheath
PCR	-	polymerase chain reaction
RAG-1, -2	-	recombination activating gene-1, -2
RSS	-	recombination signal sequence
RSV	-	respiratory syncytial virus
scFv	-	single chain antibody Fv
SCID	-	severe combined immunodeficient
SL	-	surrogate light chain
TCR	-	T cell antigen receptor
TdT	-	terminal deoxynucleotidyl transferase
V_H	-	variable domain of the Ig heavy chain
V_L	-	variable domain of the Ig light chain

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CHAPTER I

Introduction

I. INTRODUCTION

Kohler and Milstein first reported in 1975 that homogeneous mouse monoclonal antibodies (mo-mAb) with a selected specificity could be produced by fusing antibody-producing cells sensitized against a defined antigen (Ag) with a myeloma cell line [1]. The technique was rapidly developed, used to generate countless number of specific mo-mAb, and then extended to other species, notably rat and human. Murine mAb soon became very useful reagents for both research and *in vitro* diagnosis. As early as 1981, the first clinical therapeutic trial was initiated with OKT3, a mouse mAb (mo-mAb) directed against the CD3 cluster of the T cell receptor (TCR) complex that is expressed on virtually all circulating T cells [2]. This initial and some following clinical trials of unmodified mo-mAb in human patients were shown to be disappointing. However, a number of factors contributing to the low therapeutic efficacy of mo-mAb were identified. Mo-mAb are highly immunogenic and elicit a human anti-mouse Ab response that renders the mAb ineffective for repetitive treatments. Additionally, most mo-mAb are not cytocidal against cells in humans because they cannot activate the human complement system or facilitate cell-mediated cytotoxicity. To circumvent these difficulties, genetically modified mAb variants such as chimeric mouse/human mAb and humanized mAb that combine the human Ab backbone with murine variable or hypervariable regions respectively were produced [3]. However, in some cases, mAb variants produced by this approach exhibit reduced antigen (Ag) binding affinity comparing to that of the original mo-mAb. The drawback of this approach has been addressed in two ways. The chosen human framework should be as homologous as

possible to the original mo-mAb. Some distinct amino acids, that are outside the hypervariable or complementarity determining regions (CDR) but likely to interact with the CDR or Ag, should be retained in the humanized mAb. Even though computer modeling and other powerful molecular techniques have significantly simplified these tasks, the genetic engineering approach is complicated and somewhat uncertain. Thus far, this approach still has to be carried out on a case-by-case basis and the produced mAb retains some mouse sequences.

Another solution to the problems of immunogenicity and low effector functions characteristic of mo-mAb in humans is to produce fully human mAb (hu-mAb). hu-mAb of proper specificity and high binding affinity have been shown to be difficult to isolate using the conventional Epstein-Barr virus (EBV) immortalization and hybridoma technologies. Very often, EBV immortalization results in human lymphoblastoid cells producing Ab of IgM isotype, low Ag binding affinity and at low quantity. True human myeloma cell lines that are used as fusion partners with human B cells to generate hybridoma are almost non-existent. Moreover, the use of mouse or rat myeloma cells leads to preferential loss of human chromosomes and instability of the hybrids in long-term culture.

The recent development of transloci mice, in which the endogenous immunoglobulin (Ig) genes have been targeted, disrupted and replaced by a large section of human Ig (hu-Ig) gene loci, have brought the production of hu-mAb closer to reality than ever. The functional human humoral immune system in these transloci mice has facilitated the generation of a fully hu-Ab repertoire from which those with high binding affinity to a variety of Ag including human Ag can be selected using conventional

hybridoma technology [4]. Even though the size of hu-Ig loci, stretching over several megabases on different chromosomes, is a major challenge to this transgenic Ab technology, the establishment of transloci covering the full complement of hu-Ig gene loci is just a matter of time. At the present time, if an appropriate transloci mouse line is available, specific hu-Ab with high binding affinity can easily be selected. Thus far, patent issues have hampered the general use of these transloci mice in hu-mAb production.

Parallel with the transgenic mice technology, combinatorial antibody Fab and single chain antibody Fv (scFv) phage display library techniques have been developed and successfully exploited to generate hu-mAb. Repertoires of Fd (V_H/C_H1) and light chain cDNA are amplified from a pool of hu-Ig mRNA using the polymerase chain reaction (PCR), and cloned step-wise into phagemid vectors to generate a combinatorial Fab library. Unlike Fab, the scFv library is constructed by a single cloning step of a repertoire of scFv into phagemid vectors. Human IgV_H and V_L cDNA are randomly linked together by a DNA fragment encoding for a hydrophilic and flexible peptide to generate scFv. The encoded Ab fragments are displayed on the surface of filamentous phages M13 from which Fab and scFv clones with appropriate specificity and high binding affinity are readily isolated by rounds of simple panning over plastic plates coated with target Ag. The major advantage of the scFv over the Fab technique is its single cloning step that facilitates the construction of very large library of up to 10^{11} members. However, repeated immunization of humans with virtually any Ag to boost specific Ab response is impossible on ethical grounds. As a result, most if not all Ab

libraries have been constructed from naïve human peripheral blood lymphocytes (hu-PBL) and the presence of specific Ab with high binding affinity is very limited.

Recently, we have been able to demonstrate an effective method to engraft hu-PBL into severe combined immune deficient (SCID) mice (hu-PBL/SCID). Immunization with various Ag/adjuvant mixtures induces both primary and secondary specific Ab responses in these mice [5,6]. This thesis is based on our effort to establish a novel combined approach of using hu-PBL/SCID mice to generate specific hu-Ab gene repertoire and scFv library to isolate specific hu-mAb with high binding affinity and neutralizing activity. Respiratory syncytial virus F-protein (RSV-F) and human tumor necrosis factor alpha (hu-TNF-α) were used as model Ag. This simple approach is a significant step toward the generalization of hu-mAb production for both research and therapeutic purposes.

II. BACKGROUND

II-A. Immunoglobulin (Ig)

Ab molecules, which collectively form a family of plasma proteins known as immunoglobulins (Ig), are the Ag-specific products of B cells and the production of Ab in response to infection is the main contribution of B cells to adaptive immunity. The Ab molecule itself has two separate functions: to specifically bind to molecules from the pathogen that elicited the immune response; and to recruit and activate other cells and molecules of the immune system to destroy the pathogen once the Ab is bound to it. These functions are structurally separated in the Ab molecule: the Ag-binding or variable (V) region varies extensively among Ab molecules, and effector or constant (C) region is highly conserved among Ab molecules of a same class or subclass. All Ab molecules are constructed in the same way from four polypeptide chains, two identical heavy (H) chains of approximately 50kDa each, and two identical light (L) chains of approximately 25kDa each. The two H-chains are joined together by one or more disulfide bonds positioned at the hinge region. Each individual L-chain is covalently linked to a H-chain by a disulfide bond (**Figure 1**). There are two types of L chains, κ and λ, that can be found in all Ab isotypes. No functional difference has been found between Ab having κ or λ chains although, for some unknown reasons, the expression ratio of the two L-chains varies from species to species. The five main H-chain classes, μ, δ, γ, α, and ε, determine the isotypes or the functional activity of an Ab molecule. The five corresponding Ab isotypes are IgM, IgD, IgG, IgA and IgE. In addition to the five major classes, there are four subclasses of IgG (IgG1, IgG2, IgG3 and IgG4) and two subclasses of IgA (IgA1 and

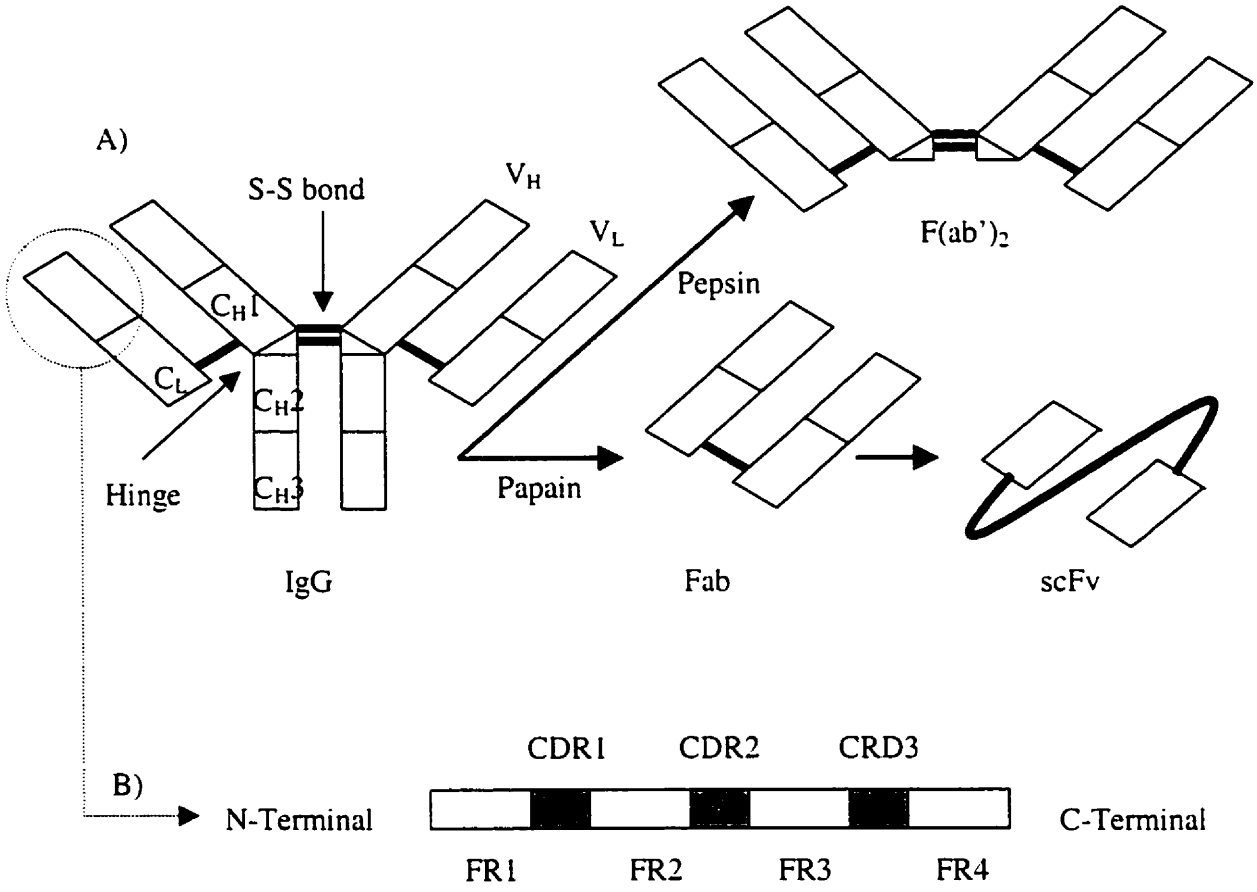

A)
S-S bond
V_H
V_L
C_H1
C_L
C_H2
C_H3
Hinge
Pepsin
Papain
$F(ab')_2$
IgG
Fab
scFv
B)
CDR1
CDR2
CRD3
N-Terminal
C-Terminal
FR1
FR2
FR3
FR4

Figure 1: Ab structure

A) Ab molecule consists of two heavy and two light chains. They are joined together by disulfide bonds. Pepsin digestion releases $F(ab')_2$ fragment. Papain digestion releases two Fab fragments. V_H and V_L can be joined together by a short polypeptide to form scFv

B) Each V region consists of three CDR and four FR

IgA2) in humans. Limited digestion with papain cleaves Ab molecules on the amino-terminal side of the disulfide bridges in the hinge region releasing three fragments, two identical Fab or Ag binding fragments, and a Fc or crystallizable fragment. Pepsin, another protease, cleaves Ab molecule on the carboxy-terminal side of the disulfide bridge producing a $F(ab')_2$ fragment and small pieces of the Fc fragment.

The idea that the V regions of an Ab can be tethered from the C-terminus of one chain to the N-terminus of the other, without disrupting Ab binding, originates from the understanding of the structure of V regions and the requirements for accurate folding and function. Each V region consists of three hypervariable or complementarity determining regions (CDR), and four less variable or structural framework regions (FR) [7]. Each domain of the V region is folded into nine strands of closely packed β-sheets. This overall folding pattern is highly preserved from one Ab to another. The FR sequences determine the folding of the V domain and the CDR sequences are found in loops at the ends of β-strands on the same side of the molecule and shown to make contacts with the Ag.

II-B. B cell antigen receptor (BCR)

i) Structure and function

It has become clear in recent years that the BCR plays a central role in signaling the fate of a B cell both before and after it encounters Ag. It was shown that progression through the pre-B cell stage of differentiation depends on signals from BCR or its precursor, preBCR. It is unknown whether a physiological ligand for the preBCR exists. However, it seems that the very existence of a preBCR is all that is needed for the pre-B

cell to progress through these maturation checkpoints due to the fact that pre-B cell lines can undergo ordered V gene rearrangement. BCR consists of a membrane-anchored isoform of the Ig sheathed by a heterodimer (Ig-α/Ig-β) responsible for mediating the signal transduction. During pre-B cell development, productive V_H-D-J_H rearrangement leads to the formation of a pre-BCR that is composed of μ and surrogate light (SL) chains sheathed by the Ig-α/Ig-β heterodimer (**Figure 2**). The preBCR instructs the cell to cease further V_H rearrangements and ensures allelic exclusion at the IgH locus. The signals driving pre-B cell development are transmitted through the Ig-α/Ig-β sheath. However, different from B cell activation, the signals from preBCR that drives progression through maturational checkpoints are transmitted in the absence of foreign Ag. When the cell has achieved productive rearrangements at both H and L chain loci, a complete BCR is assembled and this signals an end to the proliferation and rearrangements of the pre-B cell phase.

In mice, the diversity of 14 families of V_H, 13 functional D, and 4 J_H segments that randomly rearrange with each other, and the added diversity introduced by N-region insertions into the V_H-to-D and D-to-J_H joints, provide an immense potential repertoire of different μH chains in preBCR. However, the V_H, D, or J_H segments represented in pre-B cells and later in the mature sIgM/sIgD repertoire do not proportionally correlate with their presentation in germline [8,9]. V_H genes belonging to D-proximal V_H7183 and V_HQ52 families are over represented early in B cell development. In contrast, peripheral mature B cell populations in adult mice express a V_H repertoire in which the frequency of each V_H family used correlates with its germline complexity [10,11,12]. The suppression of representation of V_H7183 andV_HQ52 family in the expressed V_H repertoire, as seen in

peripheral mature B cells, is shown to be mediated by the preBCR [13]. Thus the preBCR mediates changes in V_H gene repertoire of developing precursor B cells in mouse bone marrow.

It is proposed that mature B cells need continual signaling from the BCR in order to simply stay alive [14]. The BCR can deliver different signals that lead to either persistence or activation. The distinction lies probably in the nature and extent of BCR cross-linking. *In vivo* ablation of the BCR on mature B cells by inducible IgH gene targeting leads to rapid cell death [15]. The IgH depletion leads to diminished BCR expression, down regulation of MHC class I and II expression, up regulation of Fas ligand, and apototic cell death. In the absence of IgH chains, the BCR cannot be assembled and the Ig-α/Ig-β cannot be transported to the cell surface. Therefore, *in vivo* BCR expression is needed for mature B cell maintenance. Only a small fraction of mature B cells generated everyday actually makes it into the long-lived recirculating pool (secondary B cell repertoire) (**Figure 2**). They must depend not only on the existence of a functional BCR but also on its capability to recognize specific foreign Ag with high affinity. While a mature B cell is awaiting a foreign Ag to engage its BCR and deliver a full-scale activation, the cell is continually relying on the same BCR to provide it with a weaker but crucial survival signal.

ii) V(D)J recombination

The recombination process is mediated by an enzymatic system, the V(D)J recombinase, which is shared by the T and B cell lineages (**Figure 3**). This site-specific recombinase system recognizes recombination signal sequences (RSS) identified at the 3' side of the V segments, the 5' side of the J segments, and on both sides of the D

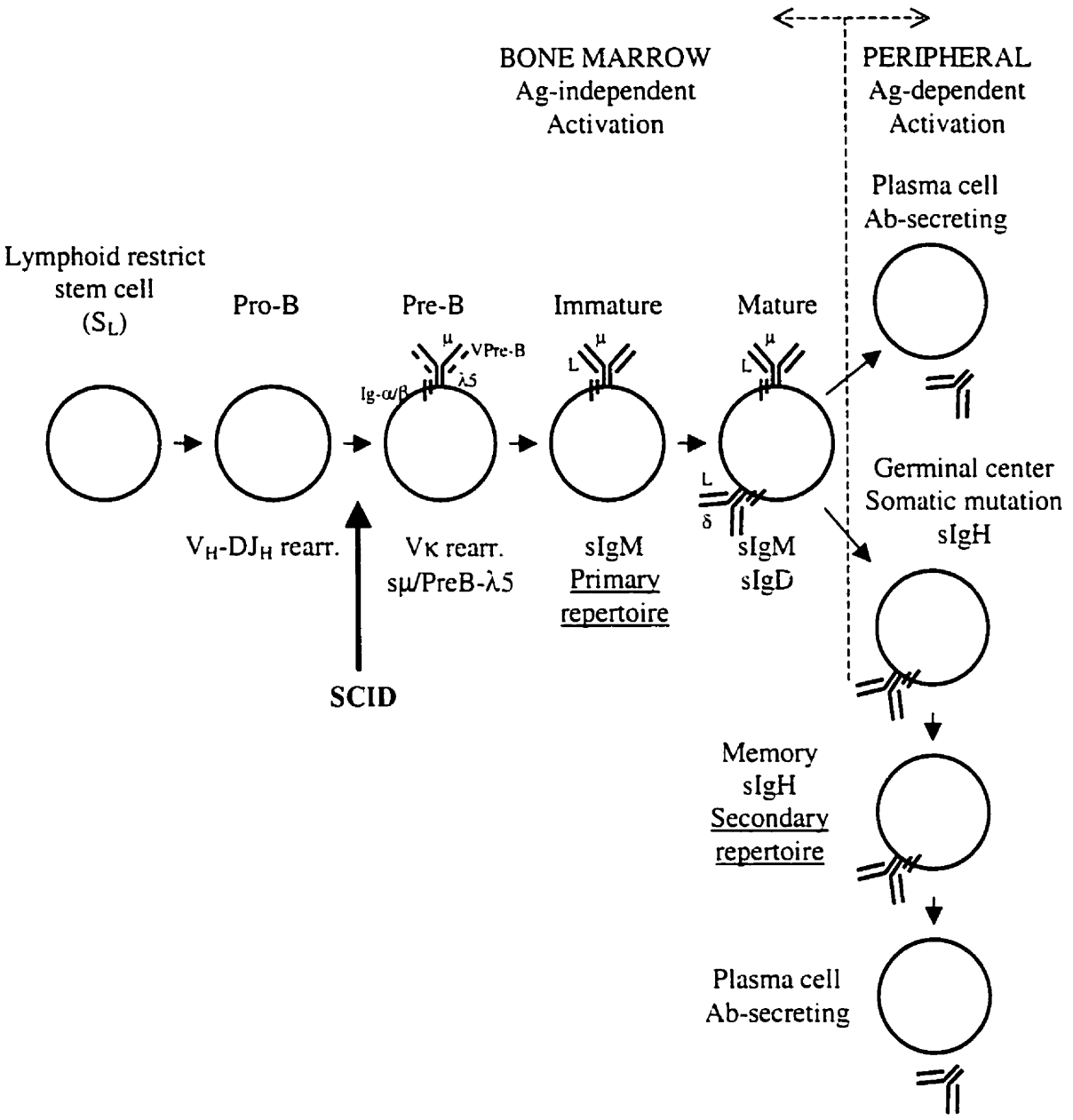

BONE MARROW
Ag-independent
Activation
PERIPHERAL
Ag-dependent
Activation
Plasma cell
Ab-secreting
Lymphoid restrict
stem cell
(S_L)
Pro-B
Pre-B
Immature
Mature
μ
VPre-B
λ5
Ig-α/β
L
δ
Germinal center
Somatic mutation
sIgH
V_H-DJ_H rearr.
Vκ rearr.
sμ/PreB-λ5
sIgM
Primary
repertoire
sIgM
sIgD
SCID
Memory
sIgH
Secondary
repertoire
Plasma cell
Ab-secreting

Figure 2: B cell differentiation

The process can be divided into two phases: Ag-independent phase and Ag-dependent phase. In Ag-independent phase, a programmed sequence of Ig V(D)J gene rearrangement yields primary repertoire of B lymphocytes, each of which expresses its own characteristic Ig molecule. In Ag-dependent phase, encounter with Ag leads to selective expansion of B cells expressing cognate Ab. They differentiate into either Ab-secreting plasma cells or into memory B cells that represent the secondary repertoire of B lymphocytes. SCID defect blocks B cell differentiation at Pro-B/Pre-B stage.

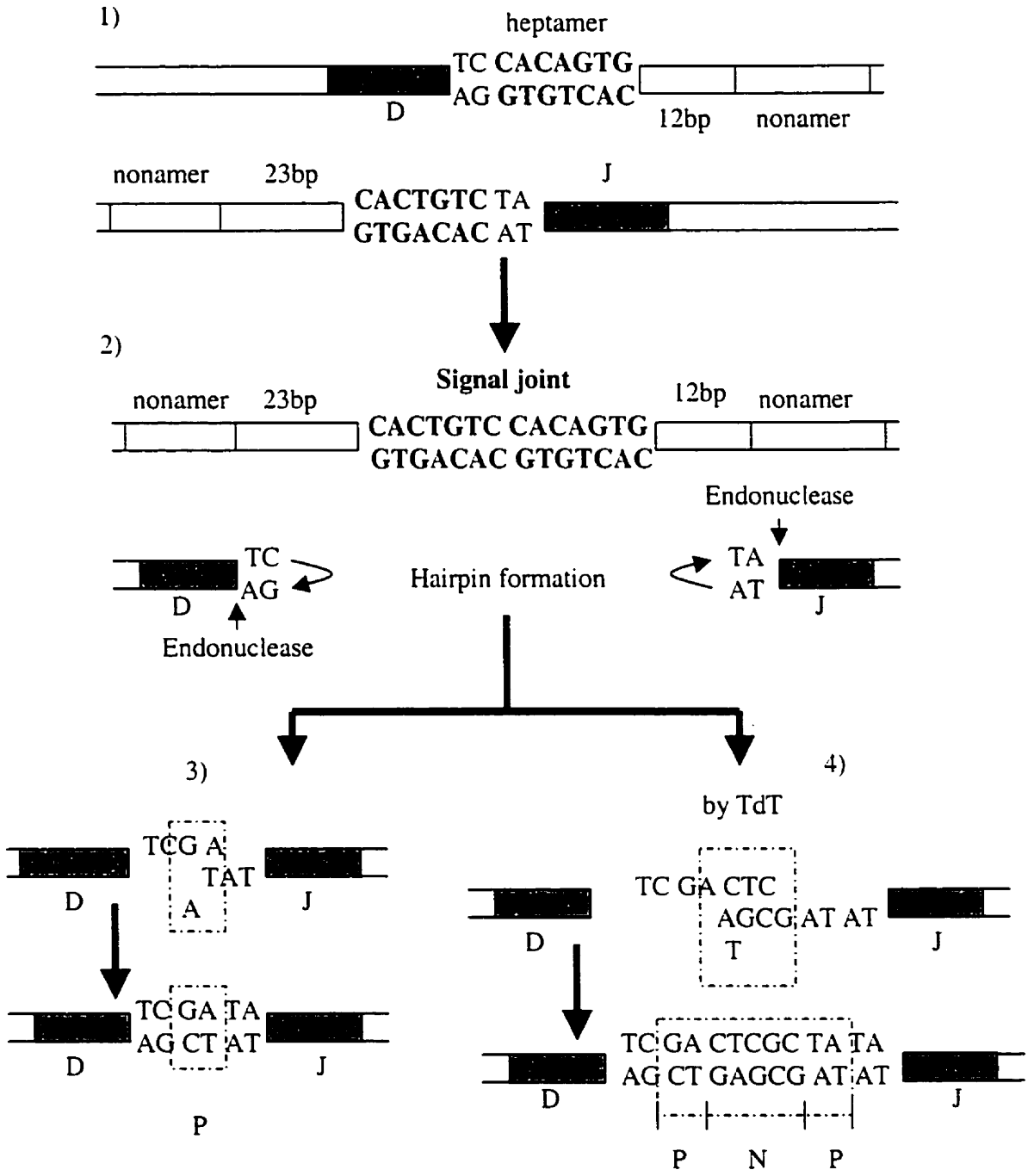

1)
heptamer
TC CACAGTG
AG GTGTCAC
D
12bp
nonamer
nonamer
23bp
CACTGTC TA
GTGACAC AT
J
2)
Signal joint
nonamer
23bp
CACTGTC CACAGTG
GTGACAC GTGTCAC
12bp
nonamer
Endonuclease
TC
AG
D
Endonuclease
Hairpin formation
TA
AT
J
3)
4)
by TdT
TCG A
TAT
A
D
J
TC GA TA
AG CT AT
D
J
P
D
TC GA CTC
AGCG AT AT
T
J
TC GA CTCGC TA TA
AG CT GAGCG AT AT
D
J
P
N
P

Figure 3: V(D)J recombination

1) The process is mediated by the V(D)J recombinase and follows strict 12/23 rule. Exonuclease recognizes RSS and initiates cutting the DNA strand precisely at the end of the heptamer sequence (bold). 2) The two heptamers are ligated together to form the signal joint, while an endonuclease cleaves the DNA hairpins at a random site. The subsequent events differ between L chain and H chain. 3) In the absence of TdT, the joining of L chain D and J segments forms a coding join with extra P-nucleotides (dash box). 4) In the present of TdT, the joining of H chain D and J segments forms a coding join with extra P- and N-nucleotides (dash box).

segments. The RSS consist of a conserved padlindromic heptamer (CACAGTG or CACTGTG) and a conserved nonamer (ACAAAAACC or GGTTTTTGT) separated by either 12 or 23 non-conserved base pairs of spacer DNA. The process follows the strict 12/23 rule that recombination only occurs between a heptamer and nonamer pair separated by a 12mer spacer and a pair separated by a 23mer spacer. This recognition results in a juxtaposition of segments to be joined followed by cleavage of the DNA strands at both coding segment/signal heptamer junctions. If the two gene segments to be jointed have the same transcriptional orientation, two products are formed, a precise signal joint between the two heptamers results in the removal of the intervening DNA, and a coding joint between the two coding segments. In some cases, the two coding segments are oriented in opposite directions, and bringing together the RSS requires a looping out of the intervening DNA. Joining the ends of the two heptamers sequences now results in the inversion of the intervening DNA. The coding joint, which is characteristically imprecise with respect to the nucleotide sequence at the junction, is important for generating of binding diversity of the Ag receptors and is therefore known as junctional diversity. During recombination, two different processes add nucleotides to the coding joint. One gives rise to P-nucleotides that depend on the endonuclease cleaves of the DNA hairpin and tends to be palidromic, and this process occurs in both H- and L-chain genes [16]. The other gives rise to N-nucleotides that are non-template encoded, and occurs only during the recombination of H-chain gene segments since this process depends on the enzyme terminal deoxynucleotidyl transferase (TdT) [17]. The hu-IgH, Igκ and Igλ loci each span over 1 megabases (Mb) on chromosome 14, 2 and 22 respectively. They consist of a large number of discrete segments encoding the variable

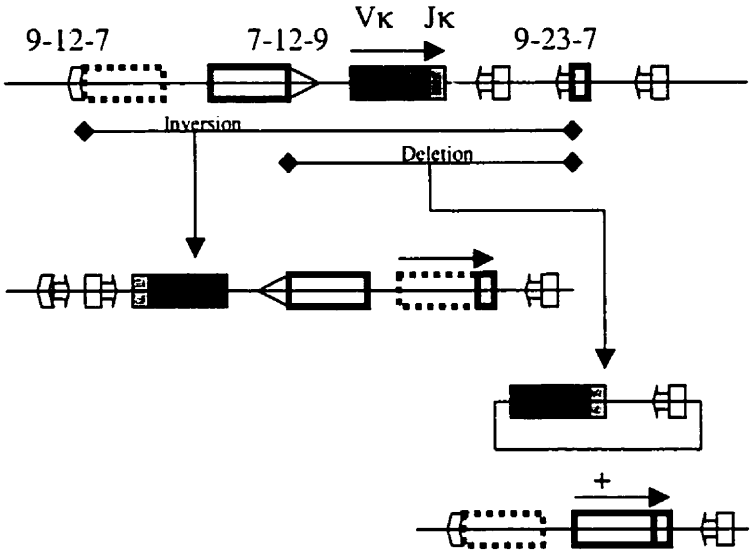
(95V_H, 76Vκ, 52Vλ), diversity (~30D_H), joining (6J_H, 5Jκ, 7Jλ) and constant domains [18-20]. Together with the junctional diversity, these exceeding large and complex hu-Ig loci facilitate broad humoral immune responses.

iii) Receptor Editing

A majority of B-lymphocytes that arise in the bone marrow do not migrate to peripheral organs to participate in an immune response but undergo apoptosis and are disposed of by resident macrophages [21]. At least three reasons can account for this phenomenon: the non-productive assembly of the V_H and V_L chain gene segments; the generation of autoreactive BCR; and the exhaustion of potential for additional V gene rearrangement. Receptor editing is unique due to the fact that it provides a mechanism for B cells to alter their $IgV_{H,L}$ gene segments and, as a consequence, to change the specificity of the BCR expressed at their surface. Therefore, receptor editing depends on the capacity of V_H and V_L genes to undergo successive rearrangements. This process not only diversifies the BCR repertoire, but also allows B cells to avoid high affinity autorecognition. Hence, it is important with regard to the maintenance of B cell tolerance and to the induction of pathogenic autoimmunity.

After primary rearrangement, V_L chain locus is well suited for secondary rearrangements because unrearranged V_L and J_L gene segments usually flank the rearranged V_LJ_L segment (**Figure 4**). Analysis of B cell tumor models demonstrates that rearranged VκJκ gene segments can be replaced by secondary rearrangements of upstream Vκ to downstream Jκ gene segments [22-25]. The remains of the primary

A) Light chain



B) Heavy chain

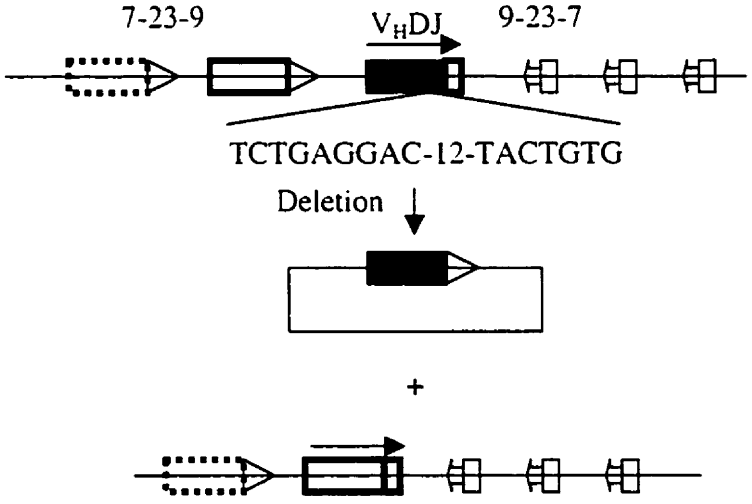


Figure 4: Mechanism of receptor editing at the L chain and H chain Ig loci

A) For L chain, a secondary rearrangement may use either inversion or deletion depending on the orientation of the Vk involved. The two different RSSs are indicated by numbers. Arrows indicate each of the expressed Vk-Jk rearrangements.

B) For H chain, a secondary rearrangement deletes the primary V_HDJ_H joint. Rearrangement uses the conserved heptamer-nonamer that is embedded within the 3' end of most V_H germline genes. The standard flanking RSS is shown. A variable amount of the original V_H-D junction may remain and N-nuleotides may be added to the new junction.

rearrangements can be recovered as extrachromosomal DNA or upstream on the same chromosome [26,27].

Due to the fact that V_HDJ_H primary rearrangement removes all unrearranged D gene segments on the same allele, the cell has to adopt a totally different strategy from that of the L-chain secondary rearrangement to undergo H-chain secondary rearrangement. Additionally, available unrearranged V_H and J_H gene segments are not compatible with the 12-23 spacer rule that governs the joining of RSS. The observation that the J_H proximal to V_H81X gene dominates the repertoire in early B cell ontogeny leads to the proposal that a primary rearrangement may serve as a substrate for subsequent V_H gene rearrangement (**Figure 4**) [28]. A novel heptamer identical to the heptamer signal sequence of D segments is found in the third framework region (FR3) of over 70% of murine V_H genes. This heptamer sequence has been proposed to participate in the arrangement between a RSS 3' of an unrearranged and upstream V_H gene and the rearranged V_HDJ_H gene segment [29,30]. The rearrangement of V_HDJ_H by an unrearranged V_H gene segment can repair a nonproductive primary rearrangement as well as replace a functional H chain [31]. These studies suggest that secondary V gene rearrangements take place even when the primary rearrangement is productive.

II-C. B cells

i) B cell ontogeny and its lineage commitment

The differentiation of B lymphocytes from their early bone-marrow precursors progresses through a series of maturation steps that are characterized by sequential rearrangement of the Ig loci and by expression of various maturation stage-specific cell

surface markers [32,33]. This process can be broadly divided into two phases. In the first phase, antigen-independent phase, a programmed sequence of Ig V-(D)-J gene rearrangements yields a population of lymphocytes, each of which makes its own characteristic Ig molecule. In the second phase, the antigen-dependent phase, encounter with foreign Ag leads to a selective expansion of those B cells that make a cognate antibody (Ab). They differentiate into either Ab-secreting plasma cells or into memory B cells.

Early B cell precursors undergo a sequential cascade of gene rearrangements for the assembly of functional Ab variable (V) regions by joining of V, D, and J elements. In normal development, V(D)J recombination is initiated at the Ig heavy (H) chain locus, and functionally rearranged H chains can be expressed at the cell surface together with SL chain [34]. First, D segments are rearranged to J_H segments on both alleles of the H chain locus [35]. In mice, B-lineage precursors, called preB-I cells, express the V_{preB} and λ5 genes, which code for the SL chain [36-38]. Next, V_H segments are rearranged to DJ_H-rearranged segments.

The productive V_HDJ_H-rearrangements are found in large cycling preB-II cells, all of which express cytoplasmic μH chains, 20% of them as μH chain/SL chain-containing preB cell receptors (preBCR) on their cell surface (**Figure2**) [39]. PreB-II cells expressing preBCR on their surface are positively selected for proliferative expansion. This selection is abolished in mice that are defective for preBCR expression, such as λ5-deficient mice [40]. Every B cell expresses either κ- or λ-light chains in association with IgH. Light chain gene assembly at the κ locus usually precedes recombination at the λ locus. Moreover, the rearrangement of the κ chain locus is independent of the presence of

any DJ_H or V_HDJ_H rearrangement, or the membrane bound or secreted μ chain, as indicated by the occurrence of VJ-joint at the κ locus [41,42]. The control of κ chain rearrangement has been addressed in mice deficient for cis-acting DNA-elements and coding segments of the κ chain gene. Mice homozygous for Cκ, JκCκ, or intronic Eκ enhancer mutations exclusively contain λ chain positive B cells, but total B cell numbers are approximately 50% of those observed in wild-type mice [43]. Even though λ-rearrangement and expression is independent of κ rearrangement or expression, it is unclear why in κ-deficient mice λ-expressing B cells cannot expand to normal numbers. Recombination is mediated by a combination enzyme system with RAG-1 and RAG-2 as key members [44,45]. Expression of functional surface Ig molecules consisting of both H and L chain products terminates the early phase of B cell development in bone marrow. Individual differentiation stages of bone marrow B cell progenitors can be distinguished by analysis of B220 (CD45), CD43 (leukosialin), heat stable Ag (HSA), and BP-1 cell surface expression [46].

After assembly and expression of functional IgH/L chain genes, B lineage cells mature in the bone marrow and then migrate into the periphery where, upon interaction with cognate Ag, they can undergo further maturation. Such activation of the B cells results in the formation of germinal centers (GC) and the activation of a somatic hypermutation process that introduces mutations into the V_H and V_L region genes giving rise to further affinity maturation of the Ab response [47]. Mice lacking major histocompatibility (MHC) class II genes contain normal numbers of B cells, but for an unknown reason fewer B cells coexpress μ and δ heavy chains. GC are missing, a reduction in basal amounts of IgG1 is observed, and T helper cell-dependent Ab response

are completely absent. These mice, however, mount normal amounts of Ab of both IgM and IgG isotypes against T cell-independent Ag [48]. The functional defects are caused by the absence of class-II restricted T cells.

ii) Allelic exclusion theory

Under the condition of allelic exclusion, a B cell expresses an IgH from only one of their IgH alleles. It is originally believed that allelic exclusion is due to a cell-autonomous developmental control mechanism by which gene rearrangements in the IgH locus are arrested once the cell expresses an H chain from one of its IgH alleles. The initial signal for this arrest results from H chain expression at the cell membrane (preBCR) [49,50]. Another view is that B cell progenitors expressing H chains from both alleles are generated, but rapidly counterselected so that they become undetectable. It is postulated that H chain expression from both alleles at an early developmental stage is toxic for the cells, resulting in rapid cell death (H chain toxicity theory) [51]. Recently, evidence has begun to emerge to modify that point of view. Transgenic mice carrying two distinct productive V_HDJ_H transgenes in their two IgH alleles have been created [52]. These mice possess normal sizes of both splenic B cell and bone marrow B cell compartments, and most of these B cells express both IgH transgenes. Moreover, B cells expressing transgenic V_L regions from several transgene copies are counterselected in the germinal center reaction in which somatic Ab mutants binding Ag with high affinity are generated and selected [53]. Therefore, it is likely that, once generated, B cells expressing H chains from both IgH alleles are not counterselected by B cell-intrinsic mechanisms such as H chain toxicity. The counterselection of B cells expressing both IgH alleles most likely relies on a failure to compete with single producers for selection by Ag.

iii) Ig class switching

Ig class switching recombination allows a B cell to express Ab with identical specificities but different effector functions that are critical for the generation of functional diversity of a humoral immune response [54,55]. Induction of germline C_H gene transcription by cytokines, B cell activators, or both is a key event for targeting the C_H gene for subsequent switch rearrangement. An increase in transcriptional activity may confer to the C_H locus a state of enhanced accessibility for the binding of additional factors important for mediating the switching. This regulatory paradigm is known as the "accessibility" model of Ig class switching. At the cellular level, switching is manifested by the transition from B cells expressing membrane IgM, IgD, or both to those expressing IgE, IgA or one of four IgG subclasses. At the molecular level, the predominant mode of Ig class switching comprises a recombination event that includes looping out and deletion of all C_H genes 5' to the C_H gene that is to be expressed. In a B cell that is initially $mIgM^+$, this recombination event occurs between Sμ and the S region of the downstream C_H gene (**Figure 5**) [56].

Prior to switch rearrangement, populations of activated B cells express one or more RNA species encoded by different C_H genes in the germline. Germline C_H RNAs are spliced products of distinct I exons, located 5' to every S region, and the immediate 3' C_H gene (**Figure 5**) [57]. Splicing out of the S region creates the final germline C_H RNA. Germline C_H RNAs lack VDJ-encoded sequence and hence cannot direct synthesis of intact Ig molecules by themselves. Upon switch rearrangement, the I and S regions are deleted and the relevant C_H gene is brought into proximity with the VDJ gene. In this

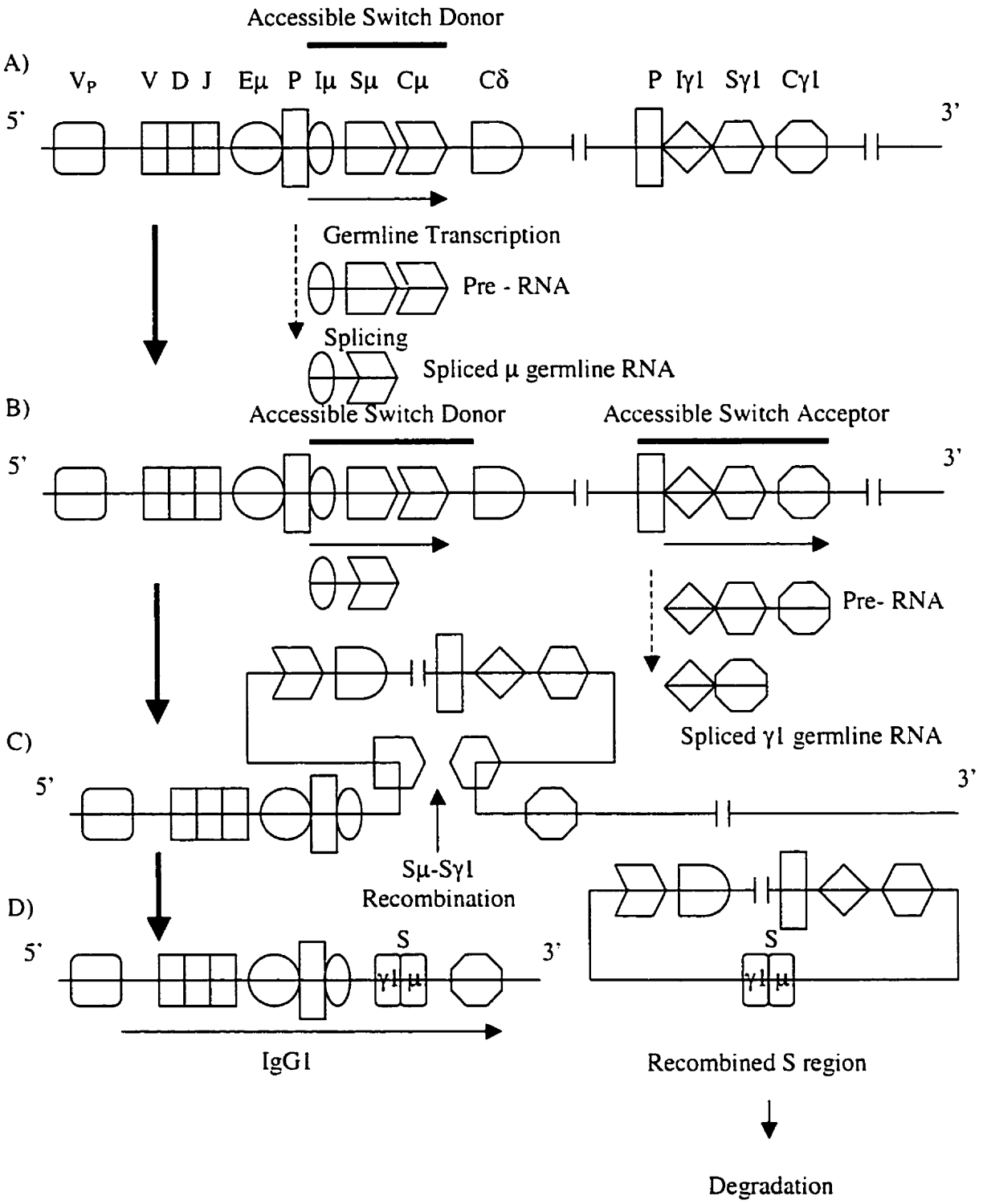

Accessible Switch Donor
A)
V_P V D J Eμ P Iμ Sμ Cμ Cδ P Iγ1 Sγ1 Cγ1
5'
3'
Germline Transcription
Pre - RNA
Splicing
Spliced μ germline RNA
B)
Accessible Switch Donor
Accessible Switch Acceptor
5'
3'
Pre- RNA
Spliced γ1 germline RNA
C)
5'
3'
Sμ-Sγ1
Recombination
D)
5'
S
3'
γ1
μ
IgG1
Recombined S region
Degradation

Figure 5: Ig class switching from IgM to IgG1

Prior to switch rearrangement, one or more RNA species encoded by different CH genes in the germline are expressed and indicated by dash arrows (A, B). Upon switch rearrangement, the DNA sequence extending from Sμ to Sγ1 is looped out and deleted (C, D). The Cγl gene is brought into proximity with the arranged VDJ gene. In this position, it encodes a productive VDJ-Cγ1 RNA for synthesis of an intact IgG1 molecule expressing the same Ag specificity but a new Ig class.

position, it encodes a productive VDJ-C_H RNA for synthesis of an intact Ig molecule expressing the same Ag specificity but a new Ig class. Target recombination also occurs when V, D, and J segments are rearranged during B cell development to form functional IgH or IgL chain gene [32]. V(D)J recombination is defective in SCID mice and deficient in RAG-1 and RAG-2 mutant mice [58-60].

The Ig class switch is influenced in both a positive and negative manner by a number of cytokines and B cell activators. The mechanism for this seems to lie in part in the ability of cytokines and activators to regulate transcription selectively, which initiates upstream of each of the I exons. Initiation of germline C_H transcriptions confers a level of accessibility to the C_H locus for the binding of additional regulatory elements that participate in switch recombination. Analyses of switch-recombinase substrates also support the accessibility model by demonstrating a requirement for substrates to be transcriptionally active in order to undergo switch rearrangement [61-64]. This transcriptional requirement is DNA-strand specific. Further, recombinase activity is not only B cell specific, but B cell-stage specific. It is restricted to cell lines representing late stage preB and mature B cells [65].

Even though both DNA synthesis and germline C_H gene transcription appear to be necessary for switch rearrangement to occur, there is evidence suggesting that these two processes are not sufficient. A number of observations imply that in addition to germline C_H transcription, other events under the controls of some cytokines, B cell activators, and transcription factors may also be central to controlling the Ig class switch. IL-4, IL-5, IL-10, INF-γ, p50/NF-κB, anti-Ig-dextran, and disodium cromoglycate selectively regulate Ig class switching in murine B cells in a manner that appears to be independent of

changes in either DNA synthesis or germline C_H transcription [66-72]. These observations lead to an expanded view of Ig class switch regulation - switch rearrangement may be regulated independently from alterations in germline C_H RNA expression.

iv) Clonal selection hypothesis and B cell tolerance

The clonal selection hypothesis evolved largely as a mean to account for the absence of immunological response to self-constituents and the related phenomena of immunological tolerance. The presence of a BCR that reflects the unique specificity of each B cell provides a convenient means for both selective antigenic stimulation and selective elimination of the cell. The hypothesis stands as a framework for understanding self-nonself discrimination. Its major premise is that immature B cells but not mature B cells are susceptible to tolerance induction [73-76]. The fundamental expectation of the hypothesis is that tolerance induction should be more dependent on the maturational status of the B cell *per se* than on either the age of the animal or the form of the Ag used to induce tolerance. Tolerance induction should also be dependent on a signal transduction cascade that is applicable to immature but not to mature B cells. Recent studies demonstrated differences in the inositol phospholipid signaling pathway, the expression of FYN, FGR and SRC tyrosine kinase families, and the induction of apoptosis in immature ($sIgD^-$) versus mature ($sIgD^+$) B cell lines [77]. These findings have led to the conclusion that: "*it is the stage of B cell development and not the ability to engage T cell help, the presence of positive or negative autocrine factors, or the relative expression of IgD that is critically important in determining the fate of the B cell upon Ag*

encounter. Differential responsiveness and tolerance susceptibility is due to intrinsic differences in BCR signal transduction" [76].

Numerous findings concerning B cell tolerance have been unanticipated and have even contradicted the expectations of the clonal selection hypothesis. These findings have led to the need for modifying the original hypothesis. It has been demonstrated that immature fetal B cells entered a maturational stage wherein they simultaneously became susceptible to both tolerance induction and stimulation [78]. B cell development is truncated at the pre-B to B cell transition in mice that express transgene-encoded anti-dsDNA Ab [79]. However, B cell development in mice expressing a transgene-encoded anti-2,4,6-trinitrophenyl-specific Ab, is truncated at a later maturational stage and not until after sIgM and B220 are up-regulated [80]. Therefore, B cells are tolerance susceptible during two maturational stages. Mature B cells of transgenic mice expressing anti-MHC class I Ab are tolerized when transferred to mice expressing the cognate MHC molecule [81]. Antigenic determinants presented on erythrocytes have been shown to tolerize mature peripheral B cells [82-83]. Thus, cell surface Ag can tolerize mature B cells. Only Ag that present multiple copies of individual epitopes are capable of inducing tolerance [84]. Monovalent Ag do not only fail to induce the tolerance trigger but can also act as antagonist to tolerance induction [84-85]. These findings seem to favor the notion that tolerance induction requires sIg receptor cross-linking. Assessment of tolerance induction by homologous versus cross-reactive Ag demonstrated that although binding and cross-linking sIg receptors of immature B cells is necessary for tolerance induction, it is not sufficient unless the affinity of the interaction exceeds a minimal threshold [85]. As currently understood, in both mice and humans, somatic mutation

primarily plays a role in the generation of memory rather than naïve B cells [86,87]. However, somatic hypermutation during the course of memory B cell generation following antigenic stimulation can potentially also generate novel anti-self specificity. Thus, the presence of a second window of tolerance susceptibility unanticipated by the early clonal selection hypothesis is required [88]. Memory B cell generation takes place mainly in germinal centers (GC) within the secondary follicles of peripheral lymphoid tissues [89]. Although, prior to stimulation, naïve progenitors of memory B cells are resistant to tolerance induction, following stimulation, the newly generating clonal progeny of these cells rapidly become tolerance susceptible [88]. *In vivo* analyses of GC cells of both normal and Ig transgenic mice have confirmed the tolerance susceptibility of newly generating memory B cells. A marked increase in apoptotic cells in GC of mice that were immunized and subsequently challenged with tolerogen has been demonstrated [90,91].

v) Germinal center

Upon immunization with a thymus-dependent Ag, a selected subset of Ag-binding B cells of the preimmune repertoire in peripheral lymphoid organs respond by the generation of plasma cells and the formation of GC (**Figure 6**). GC formation is a complex process that requires interaction of B cells with T cells, follicular dendritic cells (FDC), Ag-Ab complexes and complement [93-95]. Further, it has been suggested that not all B cells are competent to enter the GC differentiation pathway [96]. The reaction begins in the T cell zones of secondary lymphoid tissues, where T and B cells initiate Ag- and costimulus-dependent proliferation. These initial cognate interactions are essential

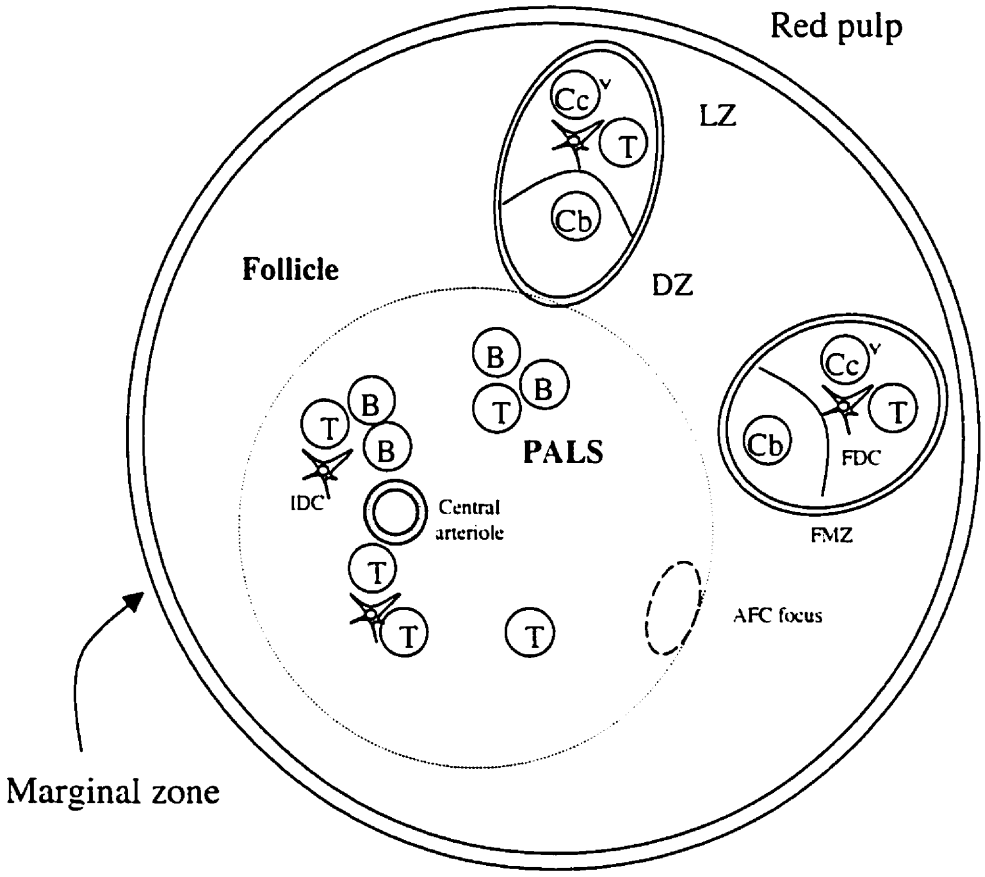

Red pulp
LZ
Cc
T
Cb
Follicle
DZ
B
B
T
B
T
B
PALS
IDC
Central
arteriole
T
T
T
Cc
T
Cb
FDC
FMZ
AFC focus
Marginal zone

Figure 6: Germinal center

After immunization, Ab-specific B cells enter the PALS and initiate costimulus-dependent interactions with specific T helper cells, which was primed and activated by interdigitating dendritic cells (IDC). The activated B cells migrate to the primary lymphoid follicle to establish GCs or remain in the periaterialar lymphoid sheath (PALS) to differentiate into foci of short-lived Ab forming cells (AFC focus). A GC surrounded by a mantle zone is polarized into a dark zone (DZ) containing rapid growing Ig-negative centroblasts (Cb) and a light zone (LZ) filled with non-dividing Ig-positive centrocytes (Cc). These centrocytes pick up Ags presented on FDCs, present it to T helper cells, and differentiate into either memory B cells or long-lived plasma cells.

for humoral immunity, but alone result in only transient and low affinity Ab responses [93,97]. Histologic studies have revealed that, in the spleen, Ag-activated B cells initially proliferate in the periarteriolar lymphoid sheath (PALS) after contact with Ag-specific T cells. This population of B cells continues to proliferate locally and produces a focus of B cells specialized for Ab production, Ab-forming cells (AFC). Abs produced by these cells are encoded by unmutated rearrangements of germline Ig V(D)J gene segments and generally have low binding affinities for the Ag. It is a subsequent set of cellular encounters, GC reaction, that drives affinity maturation by V(D)J hypermutation, B cell memory, and the continued self-tolerance of lymphocytes bearing mutated Ag receptor molecules [96,98,99]. For a long time, GC were thought to be the only site for Ab affinity maturation by somatic hypermutation. This belief has been challenged by analyzing mutant mice that lack GC formation in the spleen but are still able to manifest a high-affinity immune response after immunization with high doses of Ag [100,101]. These data convincingly indicate that affinity maturation is not absolutely dependent on the presence of GC.

Shortly after primary immunization, foci of proliferating B cells can be found in the T cell rich zones of secondary lymphoid tissues. T zones are rich in both T cells and specialized Ag-presenting cells, interdigitating dendritic cells (IDC) [102]. These IDC first contact Ag in the periphery then carry it to the T cell rich zones. IDC are known to be highly efficient at processing and presenting Ag, in association with MHC class II, to T helper cells [103]. Some B cells, with cognate T cell help, proliferate and differentiate into plasma cells secreting specific IgG and IgM with virtually no mutation

and usually low Ag-binding affinity. However, these are the main source of early circulating Ab in course of primary infections.

After their activation in the T cell rich zone (PALS), selected subsets of T and B cells migrate to primary follicles where they accumulate within the extensive processes of FDC that form a scattered network within the B cell-rich zones. FDC are known to express high level of Fc receptors (FcR) and complement receptors (CR) that help the cells to retain Ag-Ab or Ag-Ab complement complexes on their surface for long periods and act as depots for the Ag that sustains the GC reaction [104]. On the arrival of Ag-specific B and T cells from the PALS, a massive wave of clonal expansion of the B cells follows such that, within a short period of time, the entire follicular recticulum becomes filled. These T and B cells acquire novel phenotypic characteristics. B cells of GC avidly bind peanut agglutinin and most T cells of GC down-regulate expression of Thy-1 [105]. This early phase of the GC reaction compresses the surrounding uninvolved follicular cells to form a mantle zone about the newly formed GC, or secondary follicle. After this initial period of expansion, the GC polarizes to form a dark zone (DZ) proximal to the T cell area that contains rapidly dividing Ig-negative B cells called centroblasts and a distal light zone (LZ) that contains non-dividing Ig-positive centrocytes. It has been shown that B cells keep on changing their properties (centroblasts vs centrocytes) and cycling between the two zones [97]. This cycling migration helps to explain the rapid selection for high affinity B cells into the memory B cell compartment [106]. It is important to note that newly formed GC represents oligoclonal B cell populations [98]. Each mature GC is derived on average from 1 to 3 B cell clones that survive a drastic reduction in clonal diversity preceding the onset of significant V(D)J hypermutation [107]. In mice, the GC

reaction reaches its maximum by day 10 to 12 of primary responses, accounting for as much as 1 to 3% of the total splenic volume. Without further antigenic stimulation, GC wanes by day 21 post-immunization.

The GC is best known as the site of Ab maturation by Ag-driven V(D)J hypermutation and selection [98]. Random changes are introduced into the V regions especially CDR of productive rearranged Ig genes. B cells clones recovered from mice six to seven days after immunization express mutated germline Ig genes [108]. Mutations accumulate steadily at least until day 18 of the response by the step-wise introduction of 1 to 3 nucleotides substitutions, resulting in clonal genealogies that recapitulate the repeat rounds of intraclonal mutation, selection and proliferation that takes place in GC. Rare B cells with higher Ag-binding affinity are selected to dominate the late Ab response [109]. Clonal evolution proceeds independently in each GC as there is almost no B cell trafficking among GC and absence of significant convergent selection driven by circulating Ab [110]. Where and when interclonal competition among memory B cells takes place remains an important unanswered question.

The microenvironment of the GC is thought to be a requirement for the hypermutation process to be activated. However, it appears not to be sufficient to induce mutations [111]. T cells help is clearly proved to be critical for this process. T helper cells exhibit their function either by cognate cell-cell contact or by secreting cytokines that act as costimulatory signals. Prior to these processes, T helper cells have to be activated within the PALS region. T helper cell activation requires at least two distinct signals from IDC: 1) an Ag-specific signal is provided through the T cell receptor (TCR) complex that recognizes processed Ag in a complex with the MHC class II molecule and 2) a

costimulatory signal mediated by the coreceptor CD28. Mutant mice lacking either TCR or CD28 show defective T cell-dependent Ab responses and GC formation [112-116]. In these mice, the extent of defects in Ig class switching and memory B cell formation in response to T cell-dependent Ag varies depending on the type of Ag used. After immunization with KLH-DNP, only mice deficient for both CD28 and heat-stable Ag (HSA) fail to produce specific Ab [117]. This deficiency correlates with a defective induction of Ag-specific cytokine-producing T helper cells. In contrast, normal responses are seen in single-mutant (CD28 or HSA) animals. These results suggest that CD28 and HSA-mediated costimulatory pathways are redundant for some Ag and that CD28-independent induction of T helper cells and Ig class switching requires costimulation by HSA. However, costimulation of CD28 is necessary for induction of GC but that of HSA is neither necessary nor sufficient [114,117]. Following T cell activation, CD28 is down-regulated in associated with the up-regulation of CTLA-4 (CD152), a CD28-related molecule [118]. The role of CTLA-4 is controversial at the present, as it has been associated with both costimulation and inhibition of T cell activation [119,120]. Mice transgenic for a soluble form of murine CTLA-4 have been reported to be defective in GC formation and enhanced expansion of Ag-specific T helper cells [113].

Ligands for CD28 and CTLA-4 are B7-1 (CD80) and B7-2 (CD86) molecules that belong to the Ig superfamily. They both have low affinity for CD28 and high affinity for CTLA-4 [119]. Both B7-1 and 2 are expressed on APC, activated B cells and mature dendritic cells. Analyses of mouse strains lacking either or both B7 molecules demonstrate that B7-mediated signaling plays a critical role in GC formation and Ig class switching *in vivo* and that T cell help is critically diminished by the absence of B7

molecules [121]. The results also show that B7-2 has an important role in initiating Ab responses, but the induction of B7-1 by adjuvant can compensate for the absence of B7-2. Additionally, B7-deficient mice show a normal level of serum IgM, suggesting no direct influence of the B7-CD28/CTLA-4 signaling in this B cell activation pathway.

The outcome of cognate interaction between Ag-specific B and T helper cells is critically influenced by CD40-CD40L (gp39 or CD154) signaling [122]. These molecules belong to the TNF/TNFR family. CD40-mediated signals induce B cell proliferation and differentiation and are essential for Ig class switching. Absence or disruption of the CD40-CD40L interaction diminished Ig class switching and GC formation [123]. A recent study demonstrated that signals through CD40L to T cell were important for GC formation [124]. Administration of soluble CD40 to CD40 knockout mice can restore the initiation of GC formation *in vivo*, and T cells primed in the absence of CD40 fail to help normal B cells to undergo class switching or to form GC. The CD40-CD40L interaction results in the up-regulation of B7-1 and -2 [125], and B7-1 and -2 binding to T cells result in the up-regulation of CD40L expression [126]. Additionally, CD40 ligation during cognate interaction between T and B cells is apparently not essential for colonization of extrafollicular foci and differentiation to short-lived plasma cells [127]. Patients with hyper-IgM syndrome cannot make high-affinity Ig responses and they lack GC [128]. Together, these data indicate that costimulation of T cells through CD40L causes the differentiation into cells helping B cells to make mature Ab responses and to generate memory population. They also suggest that the defects in Ab responses in mice lacking B7 expression may be caused by impaired CD40L induction in T helper cells.

It is clear that correct migration of activated lymphocytes is critical for GC formation. However, very little is known about mechanism of this process. Studies of lymphocyte migration suggested potential roles for VLA-4 and LFA-1 on B cells, and ICAM-1 and VCAM-1 on cells within the primary follicles in GC formation [115]. The ability of lymphocytes to segregate into T or B cell areas is dependent on signaling through the lymphotoxin-β receptor (LT-βR) since blocking of LT-βR or targeted mutation of LT-βR gene disturbs the segregated homing of B and T cells [129,130]. Chemokine receptors that belong to G-protein-coupled receptor family have also been shown to be involved in the lymphocyte migration [131]. One member of this family that is restricted to mature B cells and a subpopulation of T helper cells is BLR1 [132]. In BRL1-deficient mice, Ab-secreting B cells and PNA^+ GC founder cells still develop, but fully formed GC are totally absent. Even though the expression levels of several adhesion molecules are normal in the mice, the activated B cells cannot migrate to appropriate sites to form functional GC [133].

It has been shown that FDC within the B cell follicles provide a source of palatable Ag and ligands for costimulation and that signals initiating from B cells are critical for organized FDC development [134]. Cytokines of the TNF/LT family are important for the establishment and maturation of the FDC network. Mice lacking any of the LT-α, LT-β, LT-βR, TNF-α, or TNFR-1 fail to form FDC clusters in their primary follicles [98,135-140]. A plethora of data has indicated that signals initiating from B cells are critically important for organized FDC development. LT-α and TNF-α production by B cells, and TNFR-1 expression by some non-bone marrow-derived cells are necessary for the development of FDC clusters in the spleen [130,141-143]. However, intracellular

pathways and targets that are regulated by the TNF/LT family and that contribute to FDC maturation driven by B cells have not yet been elucidated. Once Ab-specific B cells have entered the primary follicle and have initiated the maturation of an organized FDC network, affinity-driven selection of B cells starts to take place. B cells of GC are selected for their ability to bind an Ag retained on the surface of FDC in combination with costimulatory signals mediated by T cells of GC. This selection is both important for the development of high-affinity plasma and memory B cells, and dependent on the presence of complement receptor CD21/CR2 and CD35/CR2. These receptors, which are encoded by the murine Cr2 gene locus, are mainly expressed on B cells and FDC [144]. Target mutation of Cr2 locus revealed an impaired immune response to T cell-dependent Ag and drastically reduced size and number of GC in the spleen [145]. Moreover, analyses of B cell maturation in Cr2 deficient transgenic mice suggested that the expression of CR1 and CR2 not only provides an enhancing signal for lowering the threshold of B cell activation, but also mediates a survival signal for B cells within the GC [146,147]. Mice deficient for complement C3, whose activated products are natural ligands for CR1 and CR2, reveal similar defects in specific Ab responses [148].

II-D. Severe combined immunodeficient (SCID) mice

i) Occurrence and effect

The congenital syndrome known as severe combined immune deficiency was first recognized in human infants [149] and later in Arabian foals [150]. It is characterized by a loss of both B and T cell immunity. The *scid* mutation in mice was discovered by chance observation during the course of quantitating serum levels of IgG in pathogen-free mice of the IgH congenic strains, BALB/c and C.B-17 [58]. Four litter mates in a litter of

7 of 35 C.B-17 were unexpectedly found to lack all of the major serum Ig classes (IgM, IgG3, IgG2a, IgG2b and IgA). The defect was later shown to be heritable and under the control of a recessive gene by selective breeding. Further studies showed that C.B-17*scid/scid* mice lack both B and T cell immunity and their disorder resembled that of SCID infants and foals.

ii) The SCID mouse phenotype

The defect in SCID mice is specific and intrinsic to the lymphoid system [151]. The lack of both T and B cell immunity is accompanied by a paucity of lymphocytes and elevated proportions of granulocytes in the blood. The serum generally contains less than 0.2 μg/ml of Ig. SCID mice have a small thymus, approximately 1% normal size, that lacks a cortex and contains relatively few lymphoid cells. The spleen is about one-fifth the size of the spleen in C.B-17. Splenic and lymph node follicles as well as solitary follicles and Peyer's patches of the intestine tract are severely deficient in lymphocytes and mainly consist of stroma cell elements. The block in SCID occurs early in the development of B and T cells. Detail studies indicate that development occurs normally in both T and B lineage until the stage requiring gene rearrangement (**Figure 2**). Thus, cells appear to develop in normal numbers up to the pro-B stages in the B lineage since $B220^+$, Thy-1^- pre-B cells are undetectable in bone marrow, and up to the double-negative ($CD4^-$, $CD8^-$) cells in the thymus. The SCID defect is an intracellular defect such that SCID bone marrow or fetal liver cells cannot differentiate into mature, functional lymphocytes even in the presence of normal lymphocytes and their products. Although SCID mice are defective in both cellular and humoral immune function, they possess enhanced natural killer (NK) cell and complement activities. Lauzon et al

demonstrated that the TCR genes do not rearrange in NK cells isolated from SCID mice [152]. It was later confirmed that SCID defect involves the inability of precursor cells to join correctly the cleaved ends of the coding strands of V gene segments, since only signal joint formation but no coding joint formation could be detected in SCID pre-B and pre-T cell lines [153]

The marked lymphoid cell dysfunction of the SCID mice has facilitated reconstitution with human fetal and adult hematopoietic cells. However, it has been shown that host NK cell activity plays a major role to limited longevity and function of engrafted human lymphoid cells [154]. Complement deficiency in immunocompetent mice has been associated with prolonged survival of foreign tissue grafts [155]. The SCID mutation has been backcrossed onto the NOD/Lt strain background to develop NOD/LtSz-scid (NOD/SCID) mice with multiple defects in both adaptive and innate immune function [156]. These NOD/SCID mice show severe depletion of T and B cells, absence of complement activity, and undetectable level of NK cell activity. The synergistic effects of the SCID mutation on lymphoid development, and of NOD-specific genes on innate immunity cause this multiple immunodeficiency.

iii) The *scid* gene

The SCID mouse mutation maps to chromosome 16 lying close to the centromere and approximately 7 cM from the λ light chain locus. The *scid* gene appears to be closely linked to the B cell specific developmental genes *VpreB* and *λ5* [157]. This discovery provides an important step toward positional cloning of *scid* gene. While the predominant phenotype of the SCID mutation is the absence of a functional immune system, the basic defect is in a DNA repair gene, leading to an inability to repair double-strand DNA

(dsDNA) breaks [158]. DNA repair systems are essential in maintaining the structural integrity of genes. Cells have evolved distinct DNA repair pathways to cope with particular DNA lesions, and most of these pathways show conservation among eukaryotes. DNA double-strand breaks that confer the highest potential for genomic instability are repaired in mammalian cells by an unique recombination pathway. This pathway functions in DNA repair and in V(D)J recombination, the process of assembling the Ig and T cell receptor genes from gene segments by site-specific recombination. The inability of SCID mice to repair dsDNA breaks is consistent with their inability to produce functional rearrangements of B and T cell receptor genes. Thus, in addition to the immune deficiency, SCID mice are highly susceptible to ionizing radiation and chemicals that cause dsDNA breaks.

Applying the genetic approach of positioning cloning, Kirchgessner et al. has been able to identify the catalytic subunit of the DNA-dependent protein kinase (DNA-PK), p350, as the cause for murine SCID defect [159]. This group has shown that human chromosome 8q11 segment, coding for p350, can protect murine SCID cells from ionizing irradiation, and restore V(D)J recombination proficiency of murine SCID fibroblasts. They have also established a new synteny group between human chromosome 8q11, containing the p350 and CEBPδ (CCAAT-enhancing binding protein δ) genes, and the centromeric region of mouse chromosome 16 at the position of the SCID locus. DNA-PK is a multiprotein complex that contains a DNA-end binding component identified as the heterodimeric Ku protein and a large catalytic component designated p350 or DNA-PKcs [160,161]. DNA-PK is a serine threonine protein kinase that requires dsDNA for activity and phosphorylates substrates in vitro including transcription factor

and proteins involved in the response of cells to DNA damage. The mouse DNA-PK has been cloned and mapped in detail [162,163]. The SCID defect was later identified as a nonsense mutation on Tyr-4046 residue in the open reading frame of p350. A substitution of nucleotide A for nucleotide T, TAT to TAA (ochre stop codon), results in 83 amino acid removal from the C terminus of p350 [164,165]. Tyr 4046 seems to be an important residue that directly involves in the catalytic activity of p350 since it is just 3' to the proposed catalytic motifs of p350 and is conserved between mouse and human. Another possibility is that the nonsense mutation affects the stability or/and folding of p350 since the transcripts of p350 are normal in SCID cells, but the protein level especially kinase activity drastically decreases.

iv) hu-SCID mouse and hu-PBL-SCID mouse models

Since the discovery of the SCID mice in 1983, the ability of these mice to accept xenografts has been utilized in the search for a laboratory animal model that mimics the human immune system. Three distinct approaches for generating these models have been developed, studied in detail and proven useful. Human fetal liver or bone tissues are coimplanted with fetal thymus tissue to create the hu-SCID model. This model allows continuous differentiation of human lyphoid and myeloid lineages for about a year, and supports differentiation of injected CD34+ progenitor cells into mature lymphocytes. This model has primarily been used to study hematopoiesis and viral infectious diseases [166]. Another approach is the engraftment of purified human hematopoietic precursor cells into SCID mice to create a SCID mouse repopulating cell assay. This SCID mouse model is mainly used to study the developmental program of normal stem cells and those that initiate proliferative diseases such as leukemia [167]. The third model is established

by engrafting SCID mice with human peripheral blood lymphocytes (hu-PBL) [168]. This model, a hu-PBL-SCID mouse model, has widely been used in the studies of human diseases such as immunodeficiency, autoimmune disorders and EBV-induced lymphomagenesis. It has also been a valuable tool for vaccine studies [169]. The engraftment of mature human lymphocytes resulting in spontaneous production of human Ig readily detectable in hu-PBL-SCID mouse serum has prompted us to develop an efficient method for generating specific human monoclonal antibody (hu-mAb) with clinical potential.

II-E. Human antibody production (Figure 7)

i) Epstein-Barr virus (EBV) immortalization

EBV is a herpesvirus originally isolated in 1964 from an Burkitt's lymphoma biopsy and has been shown to be the etiological agent of infectious mononucleosis [170,171]. The immortalization/transformation of B cells by EBV infection leads to permanent stimulation of cell growth indefinitely in medium lacking exogenous growth factors, and preservation of the characteristic of the original B cell including Ig secretion [172]. B cells that grow out *in vitro* and carry EBV DNA are termed lymphoblastoid cell lines (LCL). *In vitro* transformation of B cells with EBV to obtain LCL secreting Ab to selected Ag has been exploited successfully in numerous laboratories since the first report in 1977 [173]. EBV transformation offers a simple alternative to somatic cell hybridization for the immortalization of human Ab-secreting cells. The generalization of this technique for human Ab production is, however, hampered by various inherent limitations. One of the major limitations of this technique is that EBV-transformed lines

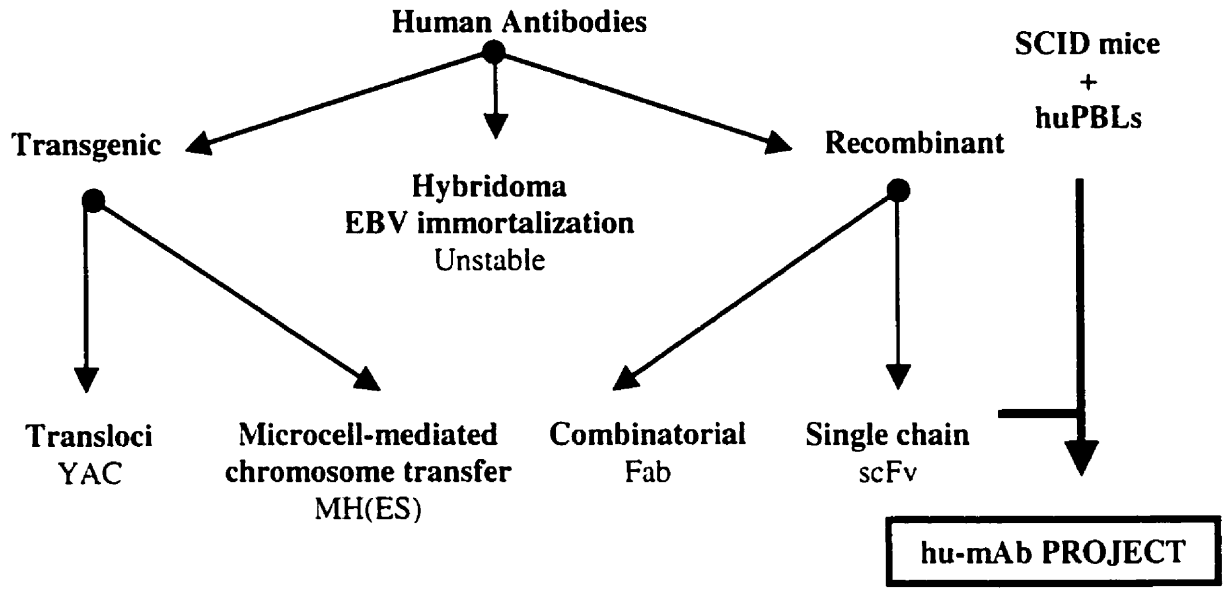

Human Antibodies
SCID mice
+
huPBLs
Transgenic
Recombinant
Hybridoma
EBV immortalization
Unstable
Transloci
YAC
Microcell-mediated
chromosome transfer
MH(ES)
Combinatorial
Fab
Single chain
scFv
hu-mAb PROJECT

Figure 7: hu-mAb production

hu-mAbs are currently produced by three different approaches: Transgenic mice, hybridoma and EBV immortalization, and DNA recombinantion. The current thesis project combines hu-PBL-SCID mouse and scFv library technologies for hu-mAb production.

lose their ability to produce specific Ab after long-term culture [174]. B cells expressing surface IgM generally have a high density of EBV receptors, which are closely associated with C3 receptors.

ii) Hybridomas

Human Ab-producing hybridomas can be created by somatic fusion of lymphocyte or plasma cells with established lymphoblastoid or myeloma cell lines. The lymphocyte provides the genetic information for the synthesis of specific Ab, and the malignancy confers immortality. The requirements for the establishment of hu-Ab secreting hybridomas are basically the same as in murine system [176,177]. The fusion partners must have similar phenotypes or the differentiated functions of both partners will not be maintained. For example, the fusion of lymphocytes with fibroblasts permits only transient Ig mRNA synthesis.

The original technique developed by Littlefield et al is still the most common way to select myeloma partners for somatic fusion [178]. The parental tumor cell lines are deficient in hypoxanthine-guanine phosphoribosyltransferase (HGPRT). In selective medium containing hypoxanthine-aminopterin-thymidine (HAT), only hybridomas produced by fusion of myeloma cells and lymphocytes can utilize exogenous hypoxanthine to satisfy purine requirement. The parental myelomas are selected out by aminopterin, a purine synthesis inhibitor. No selection pressure is necessary to eliminate the residual normal lymphocytes after hybridization since they will not proliferate indefinitely in tissue culture. However, EBV-transformed human B lymphocytes can grow in HAT medium. It is necessary to be sure that hu-mAb secreting cell lines are hybridomas rather than EBV-transformed lymphoblasts. A method has been devised to

eliminate EBV-transformed human B lymphocytes that survive the hybridization procedure. Secondary mutants resistant to micromolar concentration of ouabain, an inhibitor of Na^+, K^+-ATPase, can be selected with relative ease from HGPRT-deficient human myeloma cell lines [179]. HAT sensitive, ouabain-resistant human myelomas have become universal hybridization partners. Therefore, in HAT medium, only hybridomas that have acquired a functional HGPRT gene from B lymphocyte and a mutant ATPase from the malignant parent can proliferate. Lymphoblastoid and plastocytoma cell lines from human, mouse and rat have been used as fusion partners of human B lymphocytes to generate hu-Ab secreting hybridomas. In brief, a preferred cell line for fusion with normal human B lymphocytes should 1) support Ab production and not secrete Ig by itself, 2) have a high fusion frequency, 3) clone efficiently and grow quickly, 4) possess easily selectable markers, and 5) yield hybridomas with stable karyotypes and phenotypes. To date, none of the isolated cell lines used as fusion partners to generate hu-Ab secreting hybridomas possesses all these favorable characteristics [180]. Most of the isolated human cell lines are not true myelomas and do not support high level of hu-Ab production. Additionally, most of these cell lines are EBV positive; therefore, the B lymphocytes used in fusion may be infected with EBV rather than fused to form a true hybridoma.

Thus far, a major problem that has hampered hu-mAb development has been the source of B cells. Repeat immunizations of humans with Ag to increase the B cell population programmed to make specific Ab are not possible for ethical reasons. Moreover, except in very rare experimental settings, most available protocols for developing hu-mAb depend on hu-PBL or draining lymph node cells. They are a poor

source of memory B cells compared to splenocytes [181]. Only 5-10% of hu-PBL have surface Ig and most of them are in a resting (G_0) state. Most circulating plasma cells are terminally differentiated with limited growth potential. The fusion of these cells with dividing lymphoblasts commonly yields non-dividing, multinucleate, cytoplasmic hybridomas [180].

iii) Chimeric and humanized Ab

The vast array of available mouse monoclonal antibodies (mo-mAb) has triggered the development of several Ab engineering techniques to generate "more human" mAb that are more suitable for clinical use. These techniques can be summed up into two related approaches, constructing mouse/human chimeric mAb (m/h-mAb) and humanized mAb. The constant regions of mo-mAb are replaced by the human counterparts to construct m/h-mAb [182]. Even though the immunogenicity of these chimeric Ab to human recipients has been shown to be significantly reduced, their mouse contents are still too high and, as a general consensus, too immunogenic for any clinical use. In 1988, the development of humanization procedures has fostered intent in the clinical potential of available rodent mAb [3]. Humanization is achieved by combining the CDR of a murine mAb, which fold to form an Ag binding site of unique structure, with human FR, which appear to be conserved in sequence throughout all races [183]. The resulting molecule retains the specificity of the mo-mAb, but the substituted human sequences confer a longer *in vivo* half-life and nearly eliminate immunogenic side effects [184]. The availability of universal V-region specific primers for PCR have made possible the development of chimeric and humanized Ab that retain approximately 30% and 3% of the residues constituting the original Ab respectively [185]. Humanizing mo-mAb by

CDR-grafting is now a standard procedure for reducing immunogenicity and recruiting human effector functions. The most common method is to directly graft the CDR of a donor mo-mAb into human acceptor variable regions that have high sequence identity to the original mo-mAb sequence. Other workers prefer to first construct a chimeric Ab. The CDR-grafting is initiated only after the chimeric Ab has been shown to preserve the binding affinity of the original mo-mAb [186].

Although the engineering of chimeric and humanized Ab is relatively straightforward using the available techniques of molecular biology, the design is quite often a tedious and difficult task [187]. Humanization is a relatively complicated and uncertain process. Sometimes, even the crystal structure of the mo-mAb cannot make the task easier [188]. Out of numerous attempts, there are less than a hundred successful examples of humanized Ab in literature (the World Wide Web: http://mathbio.nimr.mrc.ac.uk/jsaldan). Unlike the common belief that chimerization of mo-mAb is a simple replacement of the constant regions of the mo-mAb molecule with human counterparts, the process sometimes is a complicate task. The choice of human constant regions is a critical step to preserve the specificity of the original mo-mAb. Even though the development of powerful computer software and other technologies that help to predict and choose suitable human antibody framework regions have improved the speed as well as the reliability of the whole humanization process, the end result is still, so far, based on trial and error approaches. In addition, these effort have to be applied to each and every single antibody, and the resulting mAb still retains some mouse sequence.

iv) Transgenic mice

The goal of producing fully hu-mAb with desired specificity for therapeutic use has been a major challenge for immunologists ever since the first mo-mAb were created. In 1994, two groups successfully created mice that had been engineered to produce specific hu-Ab in response to antigenic challenge [189,190]. This result has been accomplished by using either microinjection or yeast artificial chromosome (YAC) techniques to insert hundred kilobase (kb) DNA fragments of the hu-IgH and IgL-chain locus into mice. These mice were then crossed with IgH, L knock-out mice to create a strain of hu-Ig transloci mice that primarily produces fully hu-Ab and can be used to generate mouse hybridomas secreting hu-mAb. The YAC transfer technique has gradually become a general procedure for inserting large hu-Ig gene segments into mice. YAC carrying large segments of hu-Ig genes are introduced into the mouse germlline via fusion of yeast spheroplasts with mouse embryonic stem (ES) cells. The resulting transloci mice produce large repertoire of hu-Ig and capable of giving rise to Ag-specific hu-Ab upon immunization. Human chromosomes 2, 14 and 22, or their fragments derived from normal human fibroblasts have been introduced into mouse ES cells via microcell-mediated chromosome transfer (MMCT) and stable chimeric mouse strain is successfully generated [191]. The transferred chromosome appears to be retained as an independent chromosome in cells of adult chimeric mice and the corresponding transferred genes including hu-Ig genes can be expressed under normal tissue-specific regulation. Moreover, the human chromosome can pass through meiosis in both male and female to generate functional gametes, and transmission of human chromosome down to the F4 generation has been observed. At the present time, the generation of these chimeric mice for specific hu-mAb production has not yet been materialized since the transfer of

chromosome 14 or its fragments containing hu-IgH genes results in high mortality and sterility rates.

The large and complex hu-Ig locus is required to facilitate broad humoral responses. The hu-IgH and Igκ loci each span over 1.5 megabases (Mb) on chromosome 14 and 2 respectively. The hu-Igλ locus spans 1.14Mb on chromosome 22. Together, they consist of a large number of discrete segments encoding the variable ($95V_H$, 76Vκ, 52Vλ), diversity (~$30D_H$), joining ($6J_H$, 5Jκ, 7Jλ) and constant domains [18-20]. These loci also contain the interspersed regulatory elements that control Ab expression, allelic exclusion, class switching and affinity maturation [192]. Exploiting the ability to transfer large YAC into ES cells, Mendez et al. have created a hu-Ig transloci mouse strain (Xenomouse II) carrying 1020 kb heavy and 800 kb κ light chain loci, some of the largest transloci today [193]. These mice have exhibited a highly diverse hu-Ab response, and been used to generate multiple high affinity Ab to a variety of Ag. It is important to note that the proportion of Ab containing κ or λ light chain varies considerably among different species; the κ/λ ratio is 95/5 in mice and 60/40 in human. Therefore, for a transloci mouse to fully perform human humoral immune response, it should carry all of the human IgH, κ and λ transgenes. Mice carrying hu-Igλ transgenes of kilobase sizes (>500kb) have recently been created and studied. [194,195]. These hu-Igλ transgenes have been shown to behave normally in these mice. In the foreseeable future, we expect to see transloci mice carrying the full complement of hu-IgH and IgL genes including all the regulatory elements. Any hu-Ab, against either self- or nonself-Ag, can be generated using these mice. At the present time, the advantage of this approach is that, if a suitable transloci mouse line is available, highly specific hu-mAb can be generated using common

mouse hybridoma technology. Thus far, it is still a challenge to create mice carrying a complete set of hu-Ig genes due to their enormous sizes and complexity. The techniques involved in this transgenic technology are very complicated and the use of the available transloci mice for hu-mAb production is very limited due to the patent issue.

v) Antibody phage display library

Parallel with the development of the transgenic approach, molecular technologies have been developed and exploited to generate a repertoire of hu-mAb from which those with specific binding sites can be selected [196]. hu-mAb are successfully isolated using two related molecular technologies, combinatorial Ab (Fab) and single chain antibody Fv (scFv) phage display libraries [196,197].

In general, combinatorial Fab library relies on the ability to synthesize repertoires of Fd (V_HC_H1) fragments and light chains independently by RT-PCR, and to randomly recombine them into functional Fab (Fd/L) regions in a system that allows the specificity of binding to be probed. Heavy chains and light chains are sequentially inserted into a pComb3 phagemid in which expression of Fd/g3p fusion and light chain proteins on the surface of filamentous phage M13 are controlled by separate *lac* promoter/operator sequences, and directed to the periplasmic space by a *pelB* leader sequence for functional assembly on the membrane. Phage clones carrying Fab/g3p fusion protein with specific binding are selected by several rounds of biopanning over plate coated with target Ag. The g3p gene is excised from the phagemid DNA of positive clones and the resulting g3p negative phagemid DNA is used to transform *E. coli* XL1-Blue to produce soluble Fab for further analysis [198]. The stepwise cloning of Fd and light chain repertoires into the

expression phagemids, however, limits the size of the Fab library that is commonly in the range of 10^6 members.

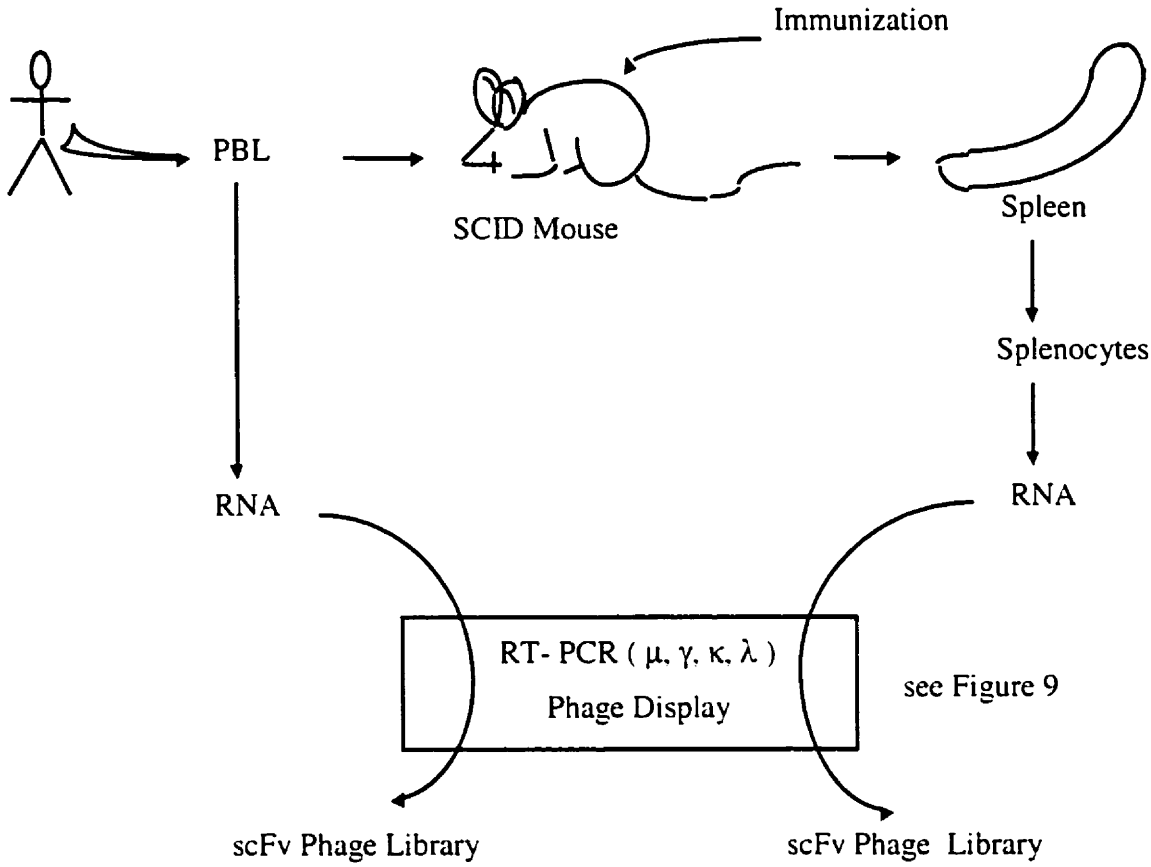
Single chain antibody Fv phage display library is another gene technology of tapping the Ab repertoire of human. Recent understanding of hu-Ig genes has facilitated the design of PCR primer sets capable of tapping the whole Fv (V_H/V_L) gene repertoire [199]. Human IgV_H and V_L gene repertoires are synthesized by RT-PCR, and randomly linked together by a short DNA sequence encoding for a hydrophilic and flexible peptide linker. The resulting scFv repertoire is cloned into the phagemid vector M13 and expressed on the surface of the filamentous phage as an scFv/g3p fusion protein. The most common peptide linker used to assemble scFv is $(Gly_4Ser)_3$ [200]. The insertion of an amber translational stop codon at the junction between the g3p sequence and the cloned scFv has significantly simplified the production of soluble scFv proteins. When a *sup*E strain of *E. coli*, such as TG1, is transformed with the recombinant vector, translation continues through the amber codon to produce the scFv/g3p fusion protein displayed on the phage tip. In nonsuppressor strains, such as HB2151, the amber stop codon is recognized, protein synthesis is aborted at the end of the scFv gene, and only scFv protein is made. The simple single cloning step involved in this technology has facilitated the construction of scFv libraries of enormous sizes of up to 10^{11} members.

III. THE PRESENT INVESTIGATION

(Some information from this study has been reported in the review publication entitled "Production of human monoclonal antibodies in SCID mouse" by Nguyen H et al. Microbiol Immunol. 1997; 41(12):901-907.)

The goal of this study was to develop a widely accessible and highly efficient system for the generation of specific hu-mAb. For decades, *ex vivo* production of hu-mAb for the materialization of Ab therapy in human disease has been a major challenge, owning to the extremely complex multicellular interactions underlying the humoral response. The growing understanding of the immune system and its mode of function have led to some progresses in the ability to utilize *in vitro* immunization. Especially, the discovery of SCID mouse and subsequently the development of hu-PBL-SCID mice have led us and others to the idea that this mouse model could serve as a platform for the generation of hu-mAb. The whole project is summarized in **Figure 8**.

Since the human immune system transferred to SCID mice could be manipulated with minimal biohazardous and ethical constrains, we first set up a series of experiments to investigate the basic immune parameters of hu-PBL-SCID mice. These experiments helped to unravel some critical adjustments needed to improve the level of hu-PBL engraftment and to optimize the induction of specific hu-Ab responses. Pretreatment with low dose γ-radiation (300rad) and anti-asialo GM1 were found to be required for achieving the highest level of engraftment as previously reported by Shpitz et al. and Sandhu et al. [5,6]. We found that optimal level of engraftment was achieved when



Panning, screening for Ag binding clones

Figure 8: Thesis project - combination of hu-PBL-SCID mice and scFv library for hu-mAb production

SCID mice were pretreated with 300rad of γ-radiation and anti-asialo GM1 rabbit antiserum. Each pretreated SCID mouse was reconstituted with $3x10^7$ freshly isolated hu-PBL followed by immunization with Ag-CFA/IFA mixture on the next day. Serum and spleen were collected on day 14 post-immunization for further analyses. scFv libraries of γκ, γλ, μκ and μλ isotypes were constructed from donor's PBL and mouse splenocytes for cloning of Ag-specific scFv.

$3.0x10^7$ freshly isolated hu-PBL were used. After immunizing with an Ag-CFA/IFA mixture, over 90% of these mice survived for more than 3 weeks post-immunization. At 14 days post immunization, specific Ab titres reached high levels and up to 80% of the splenocytes in these mice expressed the human CD45 marker. It was important to induce specific hu-Ab responses within the first 16 days of hu-PBL engraftment since we tried to minimize the skewing of human immune response toward mouse Ag [201].

We were able to induce hu-Ab responses to the fusion protein F of respiratory syncytial virus (RSV-F, a foreign Ag) and hu-TNF-α, a self-Ag. Since immunization with purified RSV-F or with formalin-inactivated RSV produced an unbalanced immune response, with high Ab titre but low neutralizing Ab titre [202-204], a mixture of whole RSV lysate-CFA/IFA was used to immunize hu-PBL-SCID mice in our investigation. In another experiment, hu-PBL-SCID mice were immunized with KLH-TNF conjugate-CFA/IFA mixture. KLH, a strong T cell immunogen, was used as carrier to boost hu-Ab response to hu-TNF-α in a non-specific manner [5]. Significant increases of specific IgG titers and decreases of specific IgM titers to both RSV-F and TNF-α implicated that specific hu-Ab responses had successfully been induced in these mice. Their splenocytes were prepared for scFv library construction.

We decided to use scFv phage display to clone hu-Ab. The whole procedure is summarized in **Figure 9**. Various scFv libraries of γκ, γλ, μκ, μλ classes were constructed following a previously described protocol [199]. Briefly, repertoires of V_H and V_L were separately amplified by RT-PCR and then randomly linked together by linkers coding for a flexible and hydrophilic peptide $(Gly_4Ser)_3$ to generate repertoires of

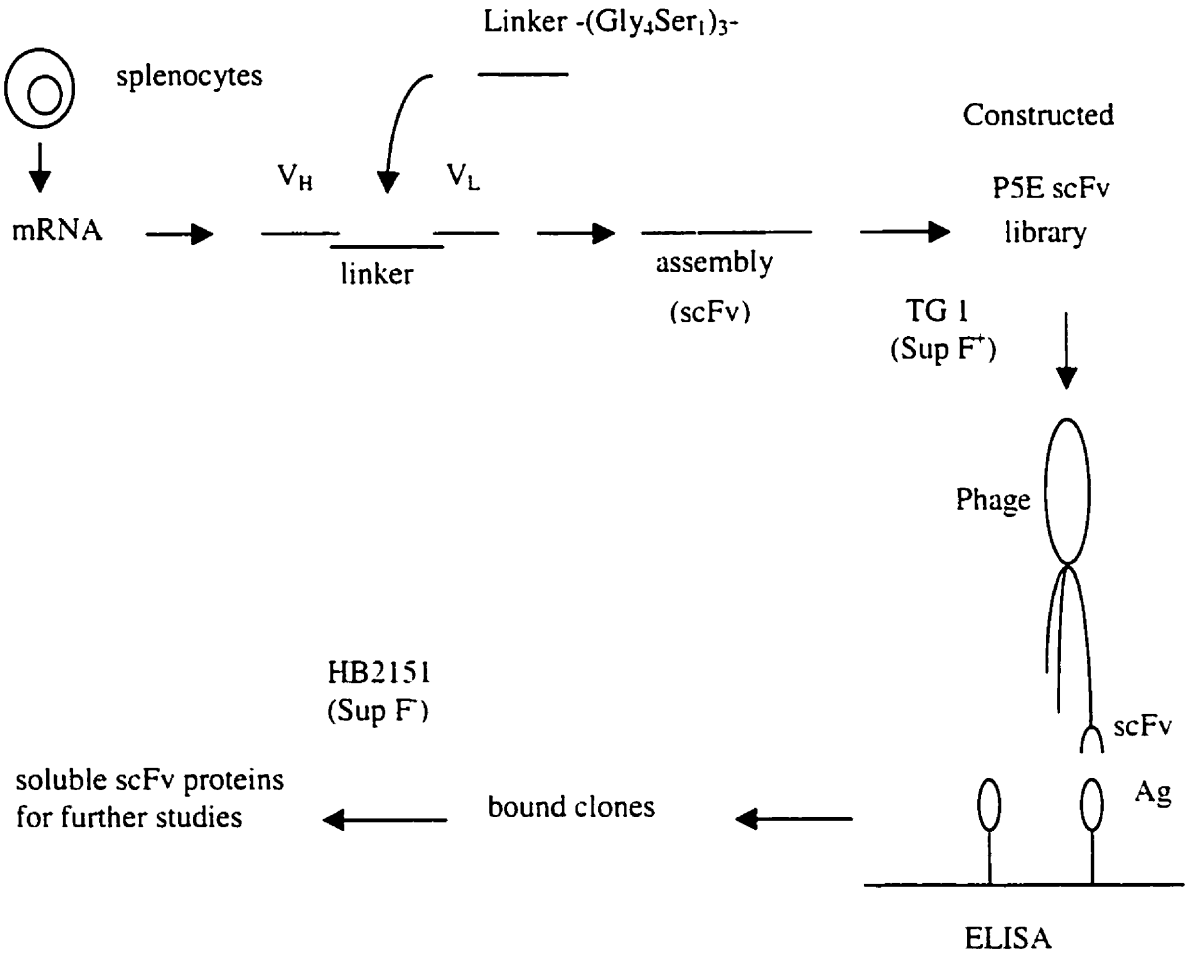

Linker -(Gly4Ser1)3-
splenocytes
Constructed
P5E scFv
library
mRNA
VH
VL
linker
assembly
(scFv)
TG 1
(Sup F+)
Phage
scFv
Ag
HB2151
(Sup F-)
soluble scFv proteins
for further studies
bound clones
ELISA

Figure 9: scFv phage display library - construction and screening

Repertoires of IgV_H and V_L were amplified from RNAs isolated from either donor's PBLs or splenocytes by RT-PCR, and randomly linked together by linker sequences encoding polypeptide $(Gly_4Ser)_3$ to generate scFv repertoires. scFvs were inserted into PCANTAB 5E phage vectors. scFv proteins were expressed as g3p-scFv fusion proteins at the tip of M13 phages. Ag-specific phages were screened by standard ELISA based panning assays. Soluble Ag-specific scFv proteins were isolated from periplasm of phage infected *E. coli* strain HB2151.

scFv. The constructed scFv were cloned into the phagemid PCANTAB 5E. Due to its unique construction, this single vector could be used in conjunction with different host *E. coli* strains to produce both phage-displayed recombinant scFv for affinity selection on immobilized Ag and soluble scFv proteins for use as immunological reagents. When *E. coli* TGI ($SupE^+$) was used as host for the scFv containing phagemid, scFv-g3p fusion proteins were produced and expressed on filaments of M13 phages. The switch to soluble scFv production was accomplished by infecting *E. coli* HB2151 ($SupE^-$) with phage from an Ag-positive clone. See Index page 158 of this thesis for detail scFv construction procedure.

IV. HYPOTHESIS AND THE THESIS PROJECT

It has been clearly demonstrated that hu-mAb possess desirable therapeutic potentials for a wide spectrum of major pathological conditions, such as autoimmune diseases, infectious diseases, cancer and intoxication, that are currently unsolved by the presently available chemical drugs. We have demonstrated that specific human humoral immune response can be induced in our hu-PBL/SCID mouse model. With appropriate immunization protocols, we can generate large and diverse repertoires of Ag-specific hu-mAb in these hu-PBL/SCID mice. hu-mAb exhibiting both high binding affinity and neutralizing activity can be selected with ease and efficiency using scFv phage display library technology. Using RSV-F and hu-TNF-α as model antigens, **we hypothesize that the combination of hu-PBL/SCID mice and scFv phage display library would be a highly efficient method for generating hu-mAb with therapeutic potentials**. The following issues are addressed in this thesis:

1) Can specific hu-Ab responses to both self- and nonself-proteins be induced in our hu-PBL-SCID mouse model?

2) Can the scFv library be used to select specific hu-mAb from immunized hu-PBL-SCID mice?

The results of these studies are described in the following chapters II - IV. They support our notion that this combined approach can be used to select panels of specific hu-mAb against either foreign Ag (i.e RSV-F) or self-Ag (hu-TNF-α). These hu-mAb exhibit both high Ag-binding affinity and neutralizing activity *in vitro*.

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CHAPTER II

Title

Efficient generation of RSV neutralizing human mAbs via hu-PBL-SCID mice and scFv phage-display libraries

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I. Summary

Respiratory syncytial virus (RSV) is one of the major causes of pneumonia and bronchiolitis in infants and young children and is associated with high mortality. RSV neutralizing human antibody (hu-Ab) is known to mediate resistance to viral infection as well as to be an effective treatment for severe lower respiratory tract RSV infection. We have previously demonstrated that human primary and secondary immune responses can be established in severe combined immunodeficient mice engrafted with human peripheral blood lymphocytes (hu-PBL-SCID). By combining this animal model with the single chain Fv antibody (scFv) phage display library technique, we were able to further investigate its clinical potential by generating a panel of human scFvs that exhibit both high F glycoprotein (RSV-F) binding affinities (~10^8 M^{-1}) and strong neutralizing activities against RSV infection *in vitro*. Sequencing analysis of the randomly isolated anti-RSV-F scFv clones revealed that they were derived from different V_H families with mutations in the complementarity determining region 1 (CDR1). The results suggest that: i) RSV-F-specific human immune responses and affinity maturation can be induced in hu-PBL-SCID mice; and ii) this approach can be applied to generate large numbers of human scFvs with therapeutic potential. Despite the fact that hu-PBL-SCID mouse and human scFv phage-display libraries have been established individually, our approach contributes a simple and significant step toward the generalization of antigen specific human monoclonal antibody (hu-mAb) production and their clinical applications.

I. Introduction

RSV is a major cause of lower respiratory tract infection resulting in brochiolitis and pneumonia in infants and young children [1,2]. There is an infection rate up to 69% during the first year of life, and almost all children have been infected at least once by two years of age [3]. Children with congenital heart disease, chronic lung disease or those born prematurely are at high risk of severe lower respiratory tract disease caused by RSV. The respiratory epithelium is the primary site of infection, replication and spread of the virus [4]. Bronchiolitis is the result of both direct virus-induced cytopathic damages and destructive inflammatory immune responses. Even though several RSV encoded proteins can induce protective immune responses, highly conserved RSV-F has been shown to be the most important target for immunotherapy by the humoral and cell mediated immunity studies [5-7]. Vaccination against RSV has been unsuccessful due to the immature immune system of young children [8,9]. Instead, intravenous injection of immunoglobulins (Igs) and Palivizumab, a "humanized" anti-RSV-F mAb, are currently used for treatment of high-risk infants with RSV infection [10]. Therefore, the generation of RSV-specific neutralizing hu-mAbs would be clinically important.

Phage display technology, developed a decade ago, has been successfully used to screen human scFv libraries. Due to biohazardous and ethical constrains, scFv libraries were usually constructed from hu-PBL samples or/and human lymphoid tissues without advance boosting of Ab responses with appropriate Ags. The generation of highly specific hu-mAbs with neutralization capability has been shown to be laborious and unpredictable. We previously demonstrated an effective method to achieve a high level

engraftment (up to 80%) of hu-PBL into SCID mice [11,12]. Further, human primary and secondary immune responses against various antigens (Ags) detected in these hu-PBL-SCID mice after having been immunized with Ag-adjuvant mixtures suggest that hu-Ab maturation may take place *in vivo*. We report here a combined effort of applying hu-PBL-SCID mice and scFv phage display library to boost human immune responses against RSV, and to clone a panel of RSV neutralizing scFvs, respectively. The resulting scFvs were shown to be derived from different human V_H families with mutated CDR 1 nucleotide sequences and some of them exhibited high RSV-F binding affinity associated with RSV neutralizing activity *in vitro*. These data suggest that the generated scFvs have clinical potential to treat severe RSV infection and that our combined approach may be widely applicable for cloning highly Ag specific and neutralizing hu-mAbs.

III. Materials and Methods

Mice and materials. Homozygous C.B.-17 scid/scid (SCID) mice were bred and maintained at the Samuel Lunenfeld Research Institute, in Toronto. FCS, complete and incomplete Freund's adjuvants (CFA, IFA), and reverse transcriptase (RT) were obtained from Gibco-BRL (Gaithersburg, MD). All oligonucleotide primers were obtained from Dalton Chemical Lab. Inc. (Toronto, Ont., Canada). Ficoll-Hypaque, Superdex^{R} 75 HR10/30 and Recombinant Phage Antibody System (RPAS) were obtained from Pharmacia (Piscataway, NJ). Anti-asialo GM1 antibody was obtained from Wako Chemicals (Dalas, USA). Anti-human CD3-PE, CD20-FITC and CD45-PE mouse mAbs were purchased from Serotec Ltd., (Kidlingto, England). Restriction enzymes were obtained from New England Biolabs (Beverly, MA). HRP-conjugted goat anti-human IgM and IgG antibodies were obtained from Jackson Immunoresearch Lab. Inc.(Westgrove, PA). KLH, whole molecule human IgG and IgM were obtained from Pierce (Rockford, Il). Mouse anti-human IgM and IgG were obtained from Pharmingen (SanDiego, CA). IPTG and dimethyl pimelimidate dyhydrochloride (DMP) and other chemicals were purchased from Sigma Chemical Co. (St. Louise, MO). Mouse anti-RSV-F mAbs 18B2 and 23A3 were obtained from Argene Inc. (North Massapequa, NY). VERO cells (ATCC CCL-81) and RSV long strain A2 (ATCC VR-26) were obtained from American Type Culture Collection (Rockville, MD). Whole RSV proteins and purified RSV-F were kindly provided by Pasteur Merieux Connaught Canada (Toronto, Ont., Canada). Millipore HPLC system was used in this study. The system consists of Waters 600E System Controller, Waters 650E Advance Protein Purification System, Waters 486 Tunable Absorbance Detector, Waters 746 Data Module, and Waters

Fraction Collector. Experimental animals and protocols were approved by the subcommittees of the University of Toronto and Mount Sinai Hospital, Toronto.

RSV-immunized hu-PBL-SCID mice. Fresh hu-PBLs were isolated from five consenting donors and subjected to Ficoll-Hypaque gradient. Isolated hu-PBLs from each donor were engrafted into six pretreated SCID mice (n=30) on day two. Each mouse was immunized i.p. with 100µl CFA/IFA (v/v=1/10) adjuvant mixture containing 50µg of whole RSV protein on day three (RSV-hu-PBL-SCID) following the previously described procedure [11]. Mouse sera and spleens were collected and pooled into 5 corresponding groups on day 16. mRNAs were prepared individually from the donor's PBLs and from the splenocytes of the corresponding group of RSV-hu-PBL-SCID mice and were used to construct the human scFv libraries.

Single-chain antibody phage display libraries. The recombinant phage antibody system obtained from Pharmacia Biotech was used to clone scFv. In brief, human Ig heavy and light chain (V_H & V_L) variable region genes were amplified by RT-PCR using the primer sets described by Marks et al. [13]. V_H & V_L were randomly linked together by oligonucleotide linkers: 5'-TC TCC/T TCA GGT GGC GGC GGT TCG GGC GGA GGA GGC TCT GGC GGT GGC GGA TCG GA-3' which encoded flexible and hydrophilic peptides $(\text{-Gly}_4\text{Ser-})_3$. The resulting scFvs were then cloned into the pCANTAB 5 E phage vector following the manufacturer's instructions.

Biopanning. Direct panning was conducted as previously described by Marks et al. (13) using purified RSV-F protein. Briefly, wells in an Immulon I 12-well strip (Dynatech, VA, USA) were coated overnight at 4^0C with the RSV-F (100µl of 1µg/ml in 0.1M sodium bicarbonate buffer, pH 9.6) and blocked with blotto (3% skim milk in

PBS). A phage library (10^8 plaque-forming units (pfu)/well in 0.1ml of blotto) was added into the well and left rocking mildly for 2 hrs. Wells were then washed 60x with PBSTT (PBS + 0.05%Tween 20 + 0.01%Triton X-100) and 5x with sodium bicarbonate buffer, pH9.6. The bound phages were eluted with 100 µl of 0.1M triethylamine pH 11.5 and rocked for 5 min before neutralization with 10µl of 3M Tris-HCl (pH7.5). Eluted phages were used to infect 2ml of growing *E. coli* TG1 (Suppressor F^+ strain) for 30 min at 37^0C and plated on 5 of 100mm LB plus Amp agar plates (150µg/ml ampicillin). Binding phages were picked and used to infect *E. coli* HB2151 (Suppressor F^- strain) to produce soluble scFv proteins.

ELISA. Immulon I 96-well plates were coated with 100µl of 1µg/ml RSV-F in 100 mM sodium bicarbonate buffer, pH9.6 overnight at 4^0C, then blocked with 0.3ml of blotto (PBS + 3% skim milk). 0.1ml of supernatant containing either phages (~ 10^9 phages) or soluble scFv plus 3% skim milk and 0.05% Tween 20 was added into each well and incubated at room temperature for 2h with shaking. 0.1ml of either HRP-conjugated mouse anti-M13 Ab or HRP-conjugated anti-E Tag Ab was added into each well and incubated for 1h with shaking. Binding was detected using an HRP substrate kit (Bio-Rad Lab.,CA) following the manufacturer's instructions. Wells directly coated with 10^9 phages were used as positive controls. Both 10^9 phage and soluble scFv protein samples of randomly picked RSV-F binding clones were subjected to ELISA for selecting strong binding clones. Clones exhibited both phage signals of at least 50% of those of the positive controls and strong soluble scFv signals were arbitrarily called strong binders.

RSV-F binding affinity, BIAcore. scFv protein samples were purified from periplasmic extracts of selected clones by affinity chromatography using anti-E taq antibody conjugated, protein G sepharose 4B column. The purified scFv samples were then subjected to size-exclusion chromatography on Superdex 75 HR10/30 to isolate scFv monomers. All buffers and samples had been filtered through 0.22μm filters before use. Binding affinity of soluble scFv to RSF-F was measured using BIA-core, Biosensor system (Pharmacia) following the manufacturer's manual. In brief, a solution of 250μg of RSV-F in 1ml of 10mM acetate buffer pH 4.0 was used in the immobilization process. The RSV-F solution was injected onto each sensor cell at a continuous flow rate of 2μl/minute for 10 minutes. This condition resulted in 4000 to 5000 response units (RU) of immobilized RSV-F. Solutions of scFv (in PBS) were injected onto the chip under continuous flow conditions of 20μl/minute for 1 minute. The association (k_a) and dissociation (k_d) rates of the scFv on the chip were detected and formulated by BIA Evaluation software version 2.1 provided in Biosensor system model BIAcore Upgrade (Pharmacia Biosensor AB). The collected data were fitted to homogeneous single-site interaction between two molecules (homogeneous kinetics). Dissociation model name AB = A + B was used to calculate the dissociation rate constant kd by fitting data to the equation: $R = R_0 e^{-k_d (t-t_0)}$ (t: time in seconds; k_d: dissociation rate constant; R_0: response at the start of dissociation; t_0: start time for the dissociation). Association model name A + B = AB type 1 was used to calculate the association rate constant k_a and the steady state response level R_{eq} by fitting data to the equation: $R = R_{eq} [1-e^{-(k_a Cn+k_d)(t-t_0)}]$ (t: time in seconds; k_a: association rate constant; R_{eq}: steady state response level; C: molar

concentration of analyte; n: steric interference factor; t_0: start time for the association; k_d: dissociation rate constant).

RSV neutralizing assay – plaque reduction assay. The ability of control mAbs and isolated scFvs to inhibit RSV infection was investigated by a modified micro-neutralization assay developed by Prince et al. [14]. In brief, 1 x 10^5 VERO cells in 1ml of complete medium 199 (medium199 plus 10% anti-RSV Ab free FCS) was added into each well of a 24 well plate (Falcon) and incubated overnight. To prepare a test Ab mixture, approximately 50pfu of RSV long strain A2 was mixed with dilutions of either test scFv or RSV neutralizing 18B2 mouse mAb, or RSV non-neutralizing 23A3 mouse mAbs as controls, in 0.2 ml of test medium 199 (medium 199 + 2% FCS), and incubated for 1h at 37^0C. The culture medium was then replaced by the Ab mixture and incubated for 1h at 37^0C. The Ab mixture was replaced by 1ml of overlay medium (medium 199 + 2% FCS + 1% methylcellulose) and the test plate was incubated for 6 days. The overlay medium was discarded and cells were fixed with 1ml of ice-cold 80% methanol for 30 min at 4^0C and blocked for 1h with blotto. 0.2ml of 0.5 μg/ml 18B2 Ab in blotto was added into each well and incubated for 1h with shaking. 0.2ml of 1/2000 dilution of HRP-conjugated anti-mouse IgG Ab was added into each well and incubated for 1h. 0.2 ml of HRP substrate (0.6 mg/ml of 3,3-diaminobenzidin tetrahydrochloride + 0.03% of $NiCl_2$. + 1μl/ml of 30% H_2O_2) was added into each well and incubated for 20 min. Plaques appeared as dark blue spots.

IV. Results

Enhanced hu-Ab responses to RSV-F in hu-PBL-SCID mice. In accordance with our previously published data [9,10], the RSV-hu-PBL-SCID mice had similar levels of serum human Igs to those of the donors (data not shown). FACS analysis of splenocytes confirmed that these cells expressed human lymphocyte markers: 15% to 23% expressed human CD20; 34% to 42% expressed human CD3; and 66% to 84% expressed human CD45. These markers were not expressed on splenocytes of RSV-immunized SCID mice. Further, serum human anti-RSV-F IgG titres in RSV-hu-PBL-SCID mice averaged from 90 to 165 fold higher than those of the corresponding donor's sera, while IgM titres decreased from 12 to 30 fold. Duchosal et al. reported similar hu-Ab responses to tetanus toxoid in hu-PBL-SCID mice [15]. Since all five groups of RSV-hu-PBL-SCID mice showed consistently high levels of hu-PBL engraftment and Ab responses to RSV-F, the results were therefore highly reproducible. Any of these five groups of mice could be further characterized as a representative of the results.

Only the mouse derived γκ library contained anti-RSV-F scFv clones. The splenocytes of group 3 mice with the highest increase of serum human anti-RSV-F IgG titres (165 fold) and PBLs from the corresponding donor were used to construct individual scFv libraries (γκ, γλ) of more than 10^8 members ($2.7x10^8 - 3.8x10^8$) each and screened for RSV-F binding scFvs. Stringent washing conditions with both strong detergent (Triton-X) and increasing pH (up to pH9.6) were used to ensure that the number of clones recovered from the negative control wells would be less than 1/1000 of those of the test wells. The numbers of RSV-F binding clones were found not to differ

between the two γκ libraries, while the two γλ libraries did not contain any RSV-F binding clones after the biopanning. Binding clones were randomly picked to check for strong binders that showed both phages' ELISA signals of at least 50% of those of the positive control and also strong soluble scFv's ELISA signals. Ten percent of the randomly picked clones (6/60) from the mouse derived γκ library were positive, while none were found in the other γκ library (Table 1). The fact that standardized number of phages (10^9) of every clone was used in this assay suggested that the strong binders might express scFvs with higher RSV-F binding affinities or avidities than those of the other clones tested. Furthermore, soluble scFv's ELISA signals likely indicated that soluble scFv proteins were effectively produced by these clones. These results were consistent with the likelihood that the human donor had been exposed to RSV. They also suggested that circulating anti-RSV-F IgG with high binding affinity were more abundant in the RSV-hu-PBL-SCID mice than in the corresponding human donor. With this information, we selected the strong binders for further characterization.

Distinct scFv clones exhibited RSV-F binding affinities in $10^8 M^{-1}$ range. We performed sequencing analyses to determine the clonality of the selected anti-RSV-F scFv clones. The results showed that V_H DNA sequences of the six clones were different from one another and that they were derived from various human germline V_H families (Figure 1). These data suggest the presence of a relatively diverse repertoire of anti-RSV-F Abs in the γκ library. In addition, mutations were found in CDR1. Unlike CDR3, which is VDJ region, CDR1 is adequate for determining if the V region has been mutated. The isolated scFvs exhibited RSV-F binding affinities in the range of high 10^7 to 10^8 M^{-1} based on the BIAcore assay (Table 2). It is important to note that a scFv, generated from

any given Ab, usually has a binding affinity of at least 2 to 30 fold lower than that of the original whole Ab [16]. Thus, it is anticipated that if whole hu-IgG molecules have been constructed from these isolated scFvs, they likely exhibit RSV-F binding affinity in the range of 10^9 M^{-1} or even higher.

RSV neutralizing activity in vitro. RSV neutralizing mouse mAb (18B2) and non-neutralizing mouse mAb (23A3) were used as the positive and negative control Ab in a RSV neutralization assay, respectively. Clone R5C showed a very high RSV neutralizing activity, where RSV infectivity was completely blocked by 30ng/0.2ml (~1nmole) of R5C protein. The positive control mAb, 18B2, showed total inhibition of RSV at a concentration of 240ng/0.2ml (~1.6nmole) (Table 3). These data indicated that the neutralizing capability of clone R5C was at least comparable to that of 18B2 on a molar basis, since the molecular weight of a scFv (~ 30 KDa) is about 5 times smaller than that of the complete IgG (150 KDa). Clone R1C also showed some neutralizing activity within the concentration range tested; however, it was about 16 fold lower than that of clone R5C. Our estimation is that this library contains from 14 to 41 distinct anti-RSV scFv clones with both high RSV-F binding affinity and strong virus neutralizing activity.

V. Discussion

In this study, by combining our previously published hu-PBL-SCID mouse model with the scFv phage display library technique, we demonstrated that RSV neutralizing hu-mAbs of therapeutic potential could be cloned with reasonable ease and high efficiency. The use of RSV-hu-PBL-SCID mice to boost hu-Ab response *in vivo* enabled us to obtain a large repertoire of distinct RSV-F specific B cells expressing mutated V_H sequences that, in turn, simplified the cloning of RSV neutralizing scFvs. We were able to bypass the laborious hybridoma route [16] as well as to avoid the skewing of hu-Ab responses toward the dominant, non-neutralizing epitopes as has been noted by the cloning of virus neutralizing hu-mAbs directly from human donors [18-20]. The work we report here has some favorable characteristics directly applicable to the cloning of specific and neutralizing hu-mAbs. We have consistently been able to enhance specific hu-Ab responses and to induce Ab affinity maturation in the hu-PBL-SCID mice within the first 16 days of hu-PBL engraftment, thus avoiding the progression of hu-Ab responses toward mouse Ags [21,22]. The use of RSV-hu-PBL-SCID mice to boost the human anti-RSV Ab response was so effective that only a single round of highly stringent biopanning was needed to isolate RSV neutralizing scFv clones.

Recently, several protocols have been developed to enhance human immune responses in hu-PBL-SCID mouse to clone complete human Ig molecules [23-27]. However, in general, virus neutralizing hu-mAb production has not been utilized. In this study, scFv libraries derived directly from the donors did not yield any clones with high

binding signals, despite the fact that serum anti-RSV IgG titres were detected clinically. It is worth noting that donor serum exhibits an undetectable level of RSV neutralizing activity *in vitro* (data not shown). Of the four constructed libraries, only the mouse derived γκ library contains strong RSV-F binding clones that exhibit strong RSV neutralizing activity *in vitro* (Table1, 2 & 3). Further, the results of DNA sequencing analysis showed that these clones were derived from different V_H families (Figure 1). Together with those results, mutations detected at CDR1s strongly favors the idea that both RSV-F specific human Ab response and normal affinity maturation may have taken place *in vivo*, consistent with the findings made by Brams et al. and Smithson et al. [23,24]. One possible explanation for the discrepancy between our study and those of Tsui et al. and Sakurai et al. is that the hu-PBL-SCID mice may lack some regulatory mechanisms that direct Ab response toward an immunodominant epitope of the RSV-F during the course of natural RSV infection in human [18,19]. As a result, engrafted hu-PBLs can indiscriminately recognize and respond to a number of RSV-F's epitopes, followed by Ab affinity maturation. In addition, different immunization protocols have been shown to strongly influence the outcome of Ab responses, and our use of Freund's adjuvant in immunization might very well be responsible for the discrepancy. More studies are needed to resolve this question. Recently, by employing a similar protocol, we have been able to clone scFvs against a self-Ag, human TNF-α, with binding affinity in the range of $10^8 M^{-1}$ (unpublished data). These clones are being characterized for their neutralizing activity both *in vitro* and *in vivo*.

Since RSV-F is responsible for the fusion of RSV into respiratory epithelial cells [28], it is conceivable that R5C-scFv may operate by disrupting the entry of RSV into the

host cell and/or its subsequent uncoating. scFv is monovalent, therefore, crosslinking of RSV-F, aggregation of virus particles or effector Fc regions cannot explain the RSV neutralizing activity of R5C-scFv. It was previously reported that $F(ab')_2$ derived from human RSV neutralizing Abs were as effective as the whole IgG on a molar basis when they were delivered into the lungs of RSV-infected cotton rats. RSV titres in the lungs did not rebound. [29]. The same results have been reported when aerosolized monoclonal Fab was delivered into the lungs of RSV infected mice. Monoclonal Ab was 25 to 200 times more effective than pooled polyclonal Abs [30,31]. Therefore, the Fc region does not seem to be a requirement for local passive immunotherapy of pulmonary RSV infection.

Aerosolized immunoglobulin has been used in a clinical trial for treatment of RSV infection in infants [32]. The trial was, however, largely unsuccessful for various reasons. A whole IgG fraction of pooled human serum with a relatively low RSV neutralizing titre of 1:789 was used in this clinical trial. This neutralizing titre might have been too low since the effectiveness of aerosolized IgG has been shown to be dose-dependent [33]. The IgG preparation is subtype A specific while both subtype A and B were detected in some patients later. However, the study did show that a single dose of 100mg of aerosolized IgG per kg of body weight could be delivered within 60 minutes and this dose was well-tolerated by the infants enrolled in the study. Being small, highly specific, and monoclonal, RSV neutralizing scFvs may have a greater potential as an aerosol for severe lower respiratory tract RSV infections. Additionally, a number of distinct RSV neutralizing scFv clones that carry different human Ig variable regions recognizing different antigenic epitopes of RSV-F can be cloned from our scFv library

with relative ease. These scFvs can be used either in combination or in an alternating regiment to counteract both anti-idiotypic Ab responses and the emergence of RSV antigenic escape mutants.

Human mAbs have also been cloned from human Ig transloci mice [34]. The advantage of this transgenic approach is that, if a suitable transgenic mouse line is available, specific hu-mAbs can be cloned using hybridoma techniques. The techniques involved in the development of these mice are, however, still difficult and their availability is limited. Chimeric mouse/human and humanized mAbs have also been constructed and used in clinical trials [1,10,35-38] but the number remains limited. In fact, Palivizumab is the only humanized mAb available for treatment of severe RSV infection [2]. Although no adverse effects specifically related to Palivizumab have been reported, possible anti-idiotypic Ab responses and the emergence of RSV antigenic escape mutants may raise questions about its long-term therapeutic efficacy.

In summary, our results suggest that RSV-immunized hu-PBL-SCID mice and phage display libraries can effectively be used to generate human scFvs with therapeutic potential, bypassing ethical and hazardous concerns of immunizing human subjects with various deleterious Ags and adjuvants. Our combined approach may not be limited to foreign Ags since we, by applying the same protocol, have been able to clone anti-human TNF-α scFvs with high binding affinity. Further studies are currently underway to test the therapeutic potentials of anti-RSV-F and anti-human TNF-α scFvs *in vivo*. It is concluded that the current approach represents a simple and highly efficient model for the generation of human mAbs with potential clinical applications.

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Legend:

Figure 1: Comparision of DNA and amino acid sequences of the isolated scFvs to the corresponding human Ig germlines.

DNA and amino acid sequences of six RSV-F binding scFv clones were compared to those of the corresponding V_H germlines that have identical framework region 1 and 2 [39]. V_H sequences were compared to a chosen master germline sequence of the family. scFv sequences are aligned to the corresponding germline sequence, and nucleotides identical to the germline sequence are shown as dots. Mutated nucleotides and the corresponding amino acid are shown in bold and underlined. Amino acid sequences are shown in single letter amino acid code. Primer sequences at the 5' end of FR1 were underlined.

Table 1: Screening for RSV-F binding scFv clones by ELISA

Library	γλ		γκ	
	# of binders [A]	Strong binders [B]	# of binders	Strong binders
Donor's PBLs	0	NA	1.0×10^3	0 / 60
RSV-hu-PBL-SCID mice	0	NA	1.4×10^3	6 / 60

Wells of 96 well plates were coated overnight with 100 µl of 1µg/ml RSV-F protein in 50mM of sodium bicarbonate buffer, pH 9.6 and then blocked with blotto. Phages (10^8 cfu in blotto) of each library were added into a well and incubated for two hours at room temperature with gentle shaking. Bound phages were eluted, infected into *E. coli* TG1 and plated in 5 LB + Amp plates. The numbers of colonies were reported as # of binders. Uncoated and blotto blocked wells were used as negative controls. No phages bound to these wells and the ELISA signal was at background level. The average of three independent experiments is reported here.

[A] # of binders: Number of scFv clones recovered after panning against RSV-F. Only γκ libraries contained RSV-F binding scFv clones. [B] Strong binders: Randomly picked RSV-F binding scFv clones (from the previous panning step) were tested by ELISA. Clones that showed both phages signals of at least 50% of positive control signals and strong soluble scFv signals were reported as strong binders. Wells directly coated with 10^9 phage particles were used as a positive control. NA: Not applicable.

Table 2: RSV-F binding affinities.

	R1C[A]	R2C	R3C	R4C	R5C	R6C
k_a ($M^{-1}. s^{-1}$)[B]	$4.39x10^5$	$2.73x10^5$	$3.11x10^5$	$2.95x10^5$	$2.73x10^5$	$5.11x10^5$
k_d (s^{-1})[C]	$4.75x10^{-3}$	$2.10x10^{-3}$	$2.40x10^{-3}$	$4.40x10^{-3}$	$3.87x10^{-3}$	$2.88x10^{-3}$
$K_A=k_a/k_d$ (M^{-1})[D]	$9.24x10^7$	$1.03x10^8$	$1.30x10^8$	$6.70x10^7$	$7.10x10^7$	$2.77x10^8$

RSV-F binding affinities of scFv clones (R1C-R6C). BIAcore system was used to measure binding affinity. 5μg of RSV-F was immobilized on each cell (4 cells/chip) of the CM5 sensor chip. A solution of each scFv clone was injected on to the cell under continuous flow conditions (40μl/min.). Association rate, dissociation rate and binding affinity were measured and calculated automatically.

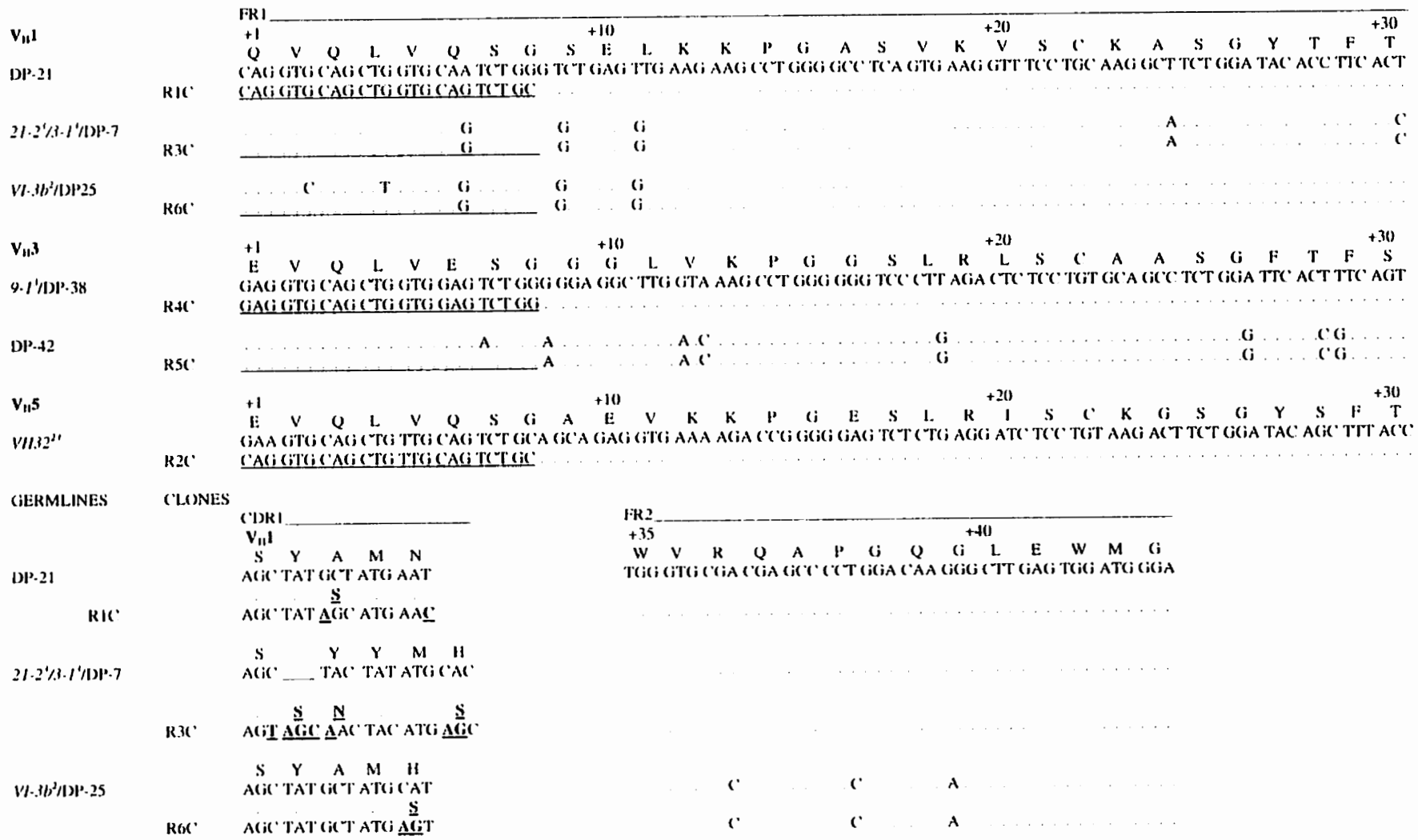
[A] scFv clone (R1C, R2C, R3C, R4C, R5C and R6C). [B] Association rate. [C] Dissociation rate. [D] Binding affinity

Table 3: Neutralization assay - plaque reduction assay

Conc. ng/0.2 ml	18B2 [A] +ve control	23A3 [B] -ve control	R1C + [C]	R2C -	R3C -	R4C -	R5C +	R6C -
0	57 [D]	55	50	54	48	45	52	55
5	53	69	49	49	56	50	31	54
10	62	54	56	53	58	56	27	57
20	49	45	61	51	48	37	17	62
30	42	51	38	51	50	64	0	41
60	25	57	49	53	39	52	5	43
120	20	45	19	61	47	42	1	55
240	1	54	5	49	52	55	0	61
480	2	47	2	56	48	54	3	52

VERO cells (1 x 10^5) were added in each well of 24 well plates and incubated overnight. The media was replaced with 0.2ml of tested scFv/RSV mixtures and incubated for 1hr with gentle shaking. Tested mixtures were replaced with 1ml of overlay media and cultured for 6 days. Plaques were visualized by HRP conjugated Ab.

[A] RSV netralizing mouse mAb control. [B] RSV non-neutralizing mouse m Ab control. [C] Plus sign (+) means neutralizing activity was detected and minus sign (-) means no neutralizing activity was detected within the test concentration range. [D] Plaque number is an average of three independent assays.


GERMLINES
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FR1
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DP-21
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R3C
VI-3b'/DP25
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9-1'/DP-38
R4C
DP-42
R5C
V_H5
VH32''
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VI-3b'/DP-25
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Figure 1

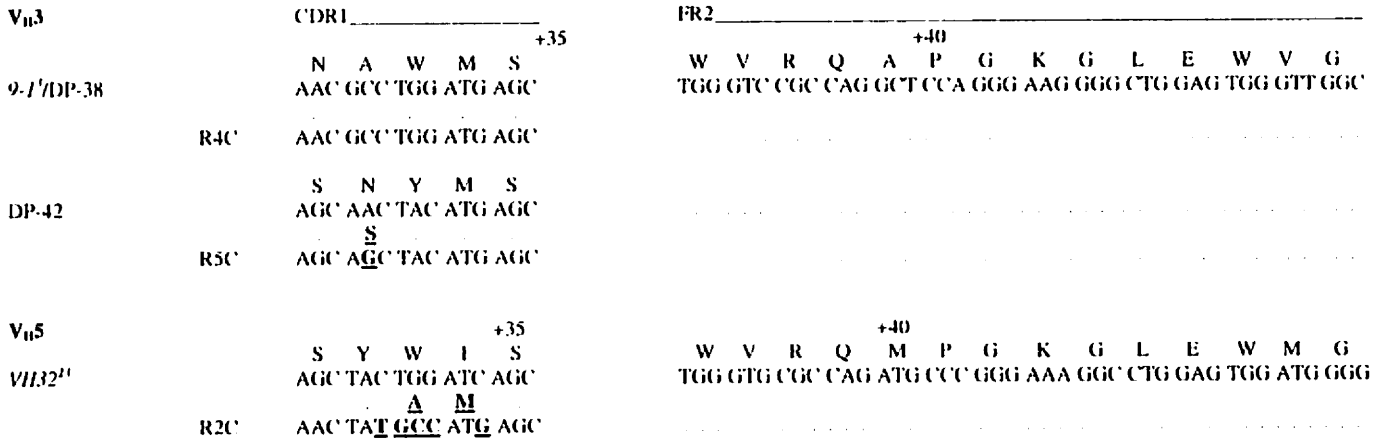

Figure 1 (continue)

CHAPTER III

Title

Human single chain antibody (scFv) against hu-TNF-α: Isolation and characterization

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Data presented in this chapter will be part of the manuscript submitted for future publication

Jul. 2000

I. SUMMARY

By developing an appropriate immunization protocol for hu-PBL-SCID mice to work in combination with the available single chain antibody Fv (scFv) phage display library technology, we were able to establish an efficient strategy to clone and select human monoclonal scFv against a human self-antigen, human tumor necrosis factor (TNF-α). Human antibody response to TNF-α has been induced in hu-PBL-SCID mice by immunization with a mixture of keyhole limpet hemocyanin conjugated human TNF-α (KLH-TNF-α) and Freund's adjuvant. Three distinct anti-TNF-α scFv clones with binding affinity in the range of 10^7-10^8 M^{-1} from a γκ scFv library, which had been constructed from amplified human Igγ and Igκ gene repertoires isolated from the TNF-α immunized hu-PBL-SCID mice, were isolated for further characterization. Here we describe a widely accessible and highly efficient method for the rapid selection of specific human scFv against human TNF-α. Together with our previously published work on the isolation of respiratory syncytial virus (RSV) neutralizing monoclonal scFv, the results of this study have implicated that this combined approach is one of the effective alternatives for the cloning of human monoclonal antibodies specific to a wide range of antigens of interest.

I. INTRODUCTION

TNF-α, a pleiotropic proinflammatory cytokine of 17kDa, was original identified as an endotoxin-induced factor that caused wasting syndrome and hemorrhagic necrosis of tumors in tumor-bearing mice [1]. TNF-α is mainly produced by activated monocytes and macrophages. It is initially synthesized and expressed as a membrane-anchored precursor of 233 amino acid. The cleavage of an Ala-Val bond between residues 76-77 of the precursor by a matrix metalloproteinase-like (MMP) TNF convertase, a unique Zn^{2+} dependent endopeptidase, gives rise to a secreted 17kDa mature form [2, 3, 4]. With an ability to induce the expression of inflammatory and cytotoxic mediators, TNF-α is a potent effector molecule of central importance to the pathogenesis of many disorders such as Crohn's disease, multiple sclerosis, rheumatoid arthritis and the cachexia associated with cancer or human immunodeficiency virus (HIV) infection [5]. TNF-α is now known to play important roles in promoting both host defense and pathologic processes. It takes part in maintaining the host homeostatic state by influencing cell proliferation and differentiation as well as inducing apoptotic cell death in various cell types.

Clinical trials of cA2, a chimeric mouse/human anti TNF-α monoclonal antibody (mAb), showed that a single infusion was effective short-term treatment in many patients with moderate-to-severe Crohn's disease that was resistant to glucocorticoid treatment [6]. Similar therapeutic effects of cA2 were reported in patients with rheumatoid arthritis[7, 8]. Long-term treatments were however ineffective due to the development of anti-cA2 responses that can occur within two weeks of the initiation of the treatment. Fully hu-mAb are expected to minimize the immunogenic and allergic responses intrinsic to mouse or mouse-derived mAb and thus to optimize the efficacy and safety of the

administered Ab. The general consensus is that long-term passive Ab therapy is most effective with fully human mAb (hu-mAb), and that a number of distinct mAb should be available and used either in combination or in an alternating regimen to counteract anti-idiotypic Ab responses. The enticing goal of producing large number of fully human mAb with desired specificities and at reasonable ease to fulfill the promise of Ab therapy in human has, however, remained elusive.

Recent developments in transgenic mouse and DNA recombinant technologies have brought large-scale selection and production of hu-mAb closer to reality than ever before. Transloci mice bearing large portion of human immunoglobulin (hu-Ig) loci capable of mounting highly diverse hu-Ab responses have been created and used to generate high affinity hu-Ab to a variety of antigens (Ag) [9]. Human chromosomes 2, 14, 22 or their fragments, which include hu-Ig heavy, λ or κ genes respectively, have been introduced into mouse embryonic stem (ES) cells *via* microcell-mediated chromosome transfer technique [10]. Both studies have demonstrated that hu-Ig transgenes are properly exploited by the mouse machinery for diverse Ab responses. However, generation of transgenic mice carrying the whole complement of hu-Ig is still the goal for the future. At the present time, the generation and use of transloci mice for hu-mAb production is limited due to patent issues.

We previously demonstrated the relative ease of cloning specific hu-mAb with high binding affinity and neutralizing activity using our combined approach of hu-PBL-SCID mice and scFv phage display library. Specific hu-Ab response to fusion protein F of respiratory syncytial virus (RSV-F) was induced in hu-PBL-SCID mice and RSV-neutralizing hu-mAb were selected using scFv phage display library [11]. It was our belief that, due to the lack of immunological tolerance to human proteins of the hu-PBL-

SCID mice, hu-Ab responses to hu-Ag could be induced in these mice if appropriate immunization protocol was applied. We here report the effectiveness of this approach in selection of human scFv to hu-TNF-α. Thus far, the results of this study have strengthened our notion that this widely accessible, relatively simple and highly effective approach is an alternative toward the generalization of Ab therapy in human disease.

III. MATERIALS AND METHODS

Materials. Homozygous C.B.-17 scid/scid (SCID) mice were bred and maintained at the Samuel Lunenfeld Research Institute, Toronto, Canada. Anti-asialo GM1 Ab was purchased from Wako Chemicals (Dallas, Texas). Other antibodies were purchased from Serotec Ltd. (Kidlingto, UK). All oligonucleotide primers were purchased from Gibco-BRL (Gaithersburg, MD). Restriction enzymes and Vent^R DNA polymerase were purchased from New England Biolabs (Beverly, MA). Superdex^R 75 and Recombinant Phage Antibody System were obtained from Pharmacia (Piscataway, NJ). All other chemicals were purchased from Sigma Chemicals Co. (St. Louise, MO). Human TNF-α and cA2 monoclonal Ab were kindly provided by Centocor (Malvern, PA). Millipore HPLC system was used in this study to isolate scFv monomers. Experimental animals and protocols were approved by the subcommittees of the University of Toronto and Mount Sinai Hospital, Toronto.

TNF-immunized hu-PBL-SCID mice. Human TNF-α was conjugated to KLH at a ratio of 1/1 (w/w) and the resulting KLH-TNF in PBS was stored at -72^{0}C until use. hu-PBL-SCID mice were prepared following previously described protocol [12]. In brief, SCID mice were irradiated with 300 rad of γ-radiation and injected i.p. with 100μl of anti-asialo GM1 antiserum/saline (1/9) on day one. Fresh blood from a consenting donor was collected and subjected to Ficoll-Hypaque gradient. $3x10^7$ freshly isolated hu-PBL were engrafted into each pretreated SCID mouse (n = 15) on day two. Each hu-PBL-SCID mouse of the first group of five was immunized i.p. with 100μl of complete/incomplete Freund's adjivant (CFA/IFA) (v/v =1/10) mixture containing 10μg of KLH-TNF on day three (TNF-hu-PBL-SCID mouse). Five of hu-PBL-SCID mice were immunized with 100μl of the CFA/IFA emulsion without KLH-TNF. This second

group of five mice was named C-hu-PBL-SCID. The last five hu-PBL-SCID mice were immunized with 100μl of PBS and called P-hu-PBL-SCID. The later two groups and a group of five KLH-TNF immunized SCID mice were used as negative controls. Mouse sera and spleens were collected and pooled together on day sixteen for further analyses. Total RNA were seperately prepared from the donor's PBL and from the splenocytes of TNF-hu-PBL-SCID mice and were used to construct human scFv libraries of γκ and γλ classes.

Single chain antibody Fv phage display (scFv) libraries. The recombinant phage antibody system obtained from Pharmacia Biotech was used to construct scFv library following the manufacturer's instructions. Briefly, variable regions of hu-Ig heavy, κ and λ (V_H, V_L) were amplified by RT-PCR using the primer set described by Marks et al.[13, 14]. V_H and V_L repertoires were randomly linked together by oligonucleotide linkers, 5'-TC TCC/T TCA GGT GGC GGC GGT TCG GGC GGA GGA GGC TCT GGC GGT GGC GGA TCG GA-3', that encoded a flexible and hydrophilic peptide $(\text{-Gly}_4\text{Ser-})_3$ [11]. The resulting scFv repertoires were then cloned into pCANTAB 5 E phagemid vector.

Biopanning. Direct panning was conducted as previously described by Marks et al. and Nguyen et al. using recombinant hu-TNF-α [11, 13]. Briefly, wells in an Immulon I 12-well strip (Dynatech, VA, USA) were coated overnight at 4^0C with hu-TNF-α (100μl of 1μg/ml in 0.1M sodium bicarbonate buffer, pH 9.6) and blocked with blotto (3% skim milk in PBS). Wells directly blocked with blotto without TNF-a coating were used as negative controls. A phage library (10^8 plaque-forming units (pfu)/well in 0.1ml of blotto) was added into the well and left rocking mildly for 2 hrs. Wells were then washed 60x with PBSTT (PBS + 0.05%Tween 20 + 0.01%Triton X-100), 5x with sodium bicarbonate buffer, pH9.6, and then 1x with PBS. The bound phages were eluted

with 100 µl of 0.1M triethylamine pH 11.5 and rocked for 5 min before neutralization with 10µl of 3M Tris-HCl (pH7.5). Eluted phages were used to infect 2ml of growing *E. coli* TG1 (Suppressor F^+ strain) for 30 min at 37^0C and plated on 5 of 100mm LB plus Amp agar plates (150µg/ml ampicillin). 90 binding phages were randomly picked and used to infect *E. coli* HB2151 (Suppressor F^- strain) to produce soluble scFv proteins. Culture supernatants were subjected to electrophoresis on 12 % polyacrylamine gel. The presence of soluble scFv proteins was detected by directly staining with basic fucin solution. Test samples containing similar concentration of soluble scFv proteins were prepared for ELISA.

ELISA. Immulon I 96-well plates were coated with 100µl of 1µg/ml hu-TNF-α in 100 mM sodium bicarbonate buffer, pH9.6 overnight at 4^0C, then blocked with 0.3ml of blotto (PBS + 3% skim milk). 0.1ml of supernatant containing either phages (~ 10^9 phages) or soluble scFv proteins plus 3% skim milk and 0.05% Tween 20 was added into each well and incubated at room temperature for 2h with shaking. 0.1ml of either HRP-conjugated mouse anti-M13 Ab or HRP-conjugated anti-E Tag Ab was added into each well and incubated for 1h with shaking. Binding was detected using an HRP substrate kit (Bio-Rad Lab.,CA) following the manufacturer's instructions. Wells directly coated with 10^9 phages were used as positive controls. Both 10^9 phage particles and soluble scFv protein samples of the randomly picked hu-TNF-α binding clones were subjected to ELISA for selecting strong binding clones. Clones exhibited both phage signals of at least 50% of those of the positive controls and strong soluble scFv signals were arbitrarily called strong binders. DNA samples of 14 strong binders were prepared and digested with BstN1 restriction enzyme. Three clones exhibited distinct BstN1 digestion patterns were selected and subjected to DNA sequencing analysis.

TNF-α binding affinity, BIAcore. scFv protein samples were purified from periplasmic extracts of selected clones (n = 3) by affinity chromatography using anti-E taq antibody conjugated, protein G sepharose 4B column. The purified scFv samples were then subjected to size-exclusion chromatography on Superdex^R 75 HR10/30 to isolate scFv monomers. All buffers and samples had been filtered through 0.22μm filters before use. Binding affinity of soluble scFv to TNF-α was measured using BIA-core, Biosensor system (Pharmacia) following our previously described protocol [13]. In brief, a solution of 50μg of TNF-α in 1ml of 10mM acetate buffer pH 5.5 was used in the immobilization process. The TNF-α solution was injected onto each activated sensor cell at a continuous flow rate of 2μl/minute for 10 minutes. This condition resulted in 1000 to 1500 response units (RU) of immobilized TNF-α. Solutions of scFv (in PBS) were injected onto the chip under continuous flow conditions of 20μl/minute for 1 minute. The association (k_a) and dissociation (k_d) rates of the scFv on the chip were detected and formulated by BIA Evaluation software version 2.1 provided in Biosensor system model BIAcore Upgrade (Pharmacia Biosensor AB). The collected data were well fitted to homogeneous single-site interaction between two molecules (homogeneous kinetics). Dissociation model AB = A + B was used to calculate the dissociation rate constant kd by fitting data to the equation: $R = R_0 e^{-k_d(t-t_0)}$ (t: time in seconds; k_d: dissociation rate constant; R_0: response at the start of dissociation; t_0: start time for the dissociation). Association model A + B = AB type 1 was used to calculate the association rate constant k_a and the steady state response level R_{eq} by fitting data to the equation: $R = R_{eq}[1-e^{-(k_a Cn+k_d)(t-t_0)}]$ (t: time in seconds; k_a: association rate constant; R_{eq}: steady state response

level; C: molar concentration of analyte; n: steric interference factor; t_0: start time for the association; k_d: dissociation rate constant).

IV. RESULTS

Increased hu-IgG response to TNF-α in TNF-hu-PBL-SCID mice. We have previously demonstrated the high degree of hu-PBL engraftment in SCID mice pretreated with low dose of γ-radiation and anti-asialo GM1 rabbit antiserum [11, 12, 14]. The same results were achieved in this study. FACS analyses of splenocytes from all three groups of hu-PBL-SCID mice confirmed that over 80% of these cells expressed the human CD45 marker. The pooled sera of the two mouse groups, TNF-hu-PBL-SCID and C-hu-PBL-SCID mice, contained similar levels of hu-IgG and IgM as those of the human donor - about 7mg/ml and 0.5mg/ml respectively. Pooled serum of the P-hu-PBL-SCID mice contained only 1mg/ml of hu-IgG and 0.3mg/ml of hu-IgM. Splenocytes of KLH-TNF immunized SCID mice did not express the human CD45 marker and hu-Ig are completely absent in their sera. The results verified our previous finding that the pretreatment with γ-radiation and anti-asialo GM1 is crucial for high degree of hu-PBL engraftment into SCID mice and that adjuvant is required to activate the engrafted hu-PBL.

ELISA was used to measure serum anti-TNF-α hu-Ab titres. In agreement with the general consensus that anti-TNF-α Ab in donor is mainly IgM isotypes, the anti-TNF-α IgG in the donor's serum could only detected by ELISA down to 1/2 dilution. Based on unit of hu-Ig, serum human IgG and IgM titres to TNF-α in the pooled serum of TNF-hu-PBL-SCID mice were 60 fold higher and 238 fold lower than those of the donor's serum respectively. Even though the serum hu-Ig levels in C-hu-PBL-SCID mice were similar to those of the donor's serum, both hu-IgG and IgM anti-TNF-α levels were undetectable by this assay. The anti-TNF-α Ab levels were also undetectable in the pooled serum of P-

hu-PBL-SCID. These data showed that adjuvant was necessary for the activation of the engrafted hu-PBL in the pretreated SCID mice and that specific hu-Ab response to hu-TNF-α was successfully induced in the TNF-hu-PBL-SCID.

scFv clones of γκ isotype that exhibited hu-TNF-α binding were isolated from TNF-hu-PBL-SCID mice. Generally, it is believed that specific IgG produced during an Ab response usually possesses higher binding affinity than IgM does. Therefore, we constructed scFv libraries of γκ and γλ classes from the donor's PBL and splenocytes of TNF-hu-PBL-SCID mice. These libraries contained more than 10^8 members each ($2.7x10^8$ to $3.8x10^8$). About $1x10^8$ phage particles from each of the four libraries were used to screen for TNF-α binding by panning over TNF-α coated wells of a 96F well plate. High stringent washing conditions with both strong detergent (Triton-X) and increasing pH (up to pH9.6) were applied to minimized non-specific binding. Under these washing conditions, less than 10 clones were recovered from each of the negative control wells. Both γλ libraries did not produce any binding clone while γκ libraries derived from the donor's PBL and from splenocytes of TNF-hu-PBL-SCID resulted in 2 and $4.6x10^3$ binding clones respectively (Table1). The results reported here were the averages of three independent panning assays. At first, the strikingly different numbers of the binding clones of the two γκ libraries seemed not to correlate well with the reported titres since the IgG titre in the serum of TNF-hu-PBL-SCID mice increased by only 60 times. One explanation for this discrepancy is likely related to the binding affinity/avidity and the applied stringent washing conditions. Under normal physiological conditions, autoreactive B cells produce IgG with very low binding affinity to self-Ag such as TNF-α, and the γκ library constructed from the donor's PBL mainly contains TNF-α weak

binding clones. These weakly bound clones were washed off during the panning. Only clones with binding affinity/avidity higher than one specific value could sustain the applied stringent washing. These results implied that either the autoreactive B cells to TNF-α were enriched by the TNF-hu-PBL-SCID mice, or human anti-TNF-α Ab maturation actually occurred in these mice. The shifting of anti-TNF-α titre from IgM to IgG and panning results seemed favor the latter.

Randomly picked binding clones were tested by ELISA to look for 'strong binders'. We arbitrarily defined 'strong binders' as clones that exhibited both phages' ELISA signals of at least 50% of those of the positive control and strong soluble scFv's ELISA signals. Fifteen percent of the randomly picked clones from the mouse derived γκ library were positive (14/90), while none were found in the other γκ library (0/2) (Table 1). It should be mentioned that only 6 binding clones were found in the hu-PBL derived γκ library. The standardized number of phages and amount of soluble scFv proteins used in this assay suggested that the strong binders expressed scFv with higher TNF-α binding affinities or avidities than those expressed by the other clones. It also showed that soluble scFv proteins were properly produced by these clones. We selected the strong binders for further analyses.

Distinct scFv clones with hu-TNF-α binding affinities in $10^{-8}M^{-1}$ range were isolated from TNF-hu-PBL-SCID derived γκ library. To find out the diversity of the library, DNA was prepared from the 14 isolated strong binders and subjected to BstN1 restriction enzyme digestion. Three different digestion patterns were found from this group. Representative clones were selected and subjected to DNA sequencing analysis to determine their clonality. Clones 2 and 14 contained a same V_H but different V_L

segments, while clone 20 carried a totally different scFv sequence (Figure 1). These utilized V_H and V_L sequences were derived from different germline IgV families. The results suggested that a relatively large repertoire of anti-TNF-α clones were present in the TNF-hu-PBL-SCID mouse derived γκ library. BIAcore assay was then applied to measure binding affinities of these clones. All the three isolated clones exhibited hu-TNF-α binding affinities in the range of 10^8 M^{-1}; they were $9.42x10^7$, $1.50x10^8$ and $3.24x10^8$ for clones 2, 14 and 20 respectively (Table 2). If complete Ab molecules were constructed from any of these three scFv clones, we expected binding affinities of 10^9M^{-1} or higher could be achieved since scFv usually exhibited much lower binding affinity than that of the complete Ab molecule from which the scFv was derived [15].

It was worth noting that a healthy human donor was chosen for this study. Therefore, it was reasonably safe to say that every one of these clones was not derived from a specific autoreactive B cell clone present in the donor. The presence of these strong binders in the library could simply explained as a result of library construction process. By random combination of V_H and V_L, 'favorable' gene segments were brought together producing high binding scFv clones. In the same direction, the redundancy of strong binders in the mouse-derived library could be explained as an expansion of the autoreactive B cell clones by the KLH-TNF immunization. We expected that high binders could be found from the donor-derived library if multiple panning steps were applied [13].

The fact that the engrafted hu-PBL was exposed to a sea of mouse Ag beside the KLH-TNF conjugates seemed, however, to be unfavorable to the above explanation. We believed that the redundancy of the strong binders in the mouse-derived library was more than a result of the expansion of the autoreactive B cell clones. The shifting of specific

Ab titre from IgM to IgG and the presence of a number of high binders in the mouse-derived γκ library seemed to work together toward the explanation that specific on-going hu-Ab response to hu-TNF-α occurred in the TNF-hu-PBL-SCID mice.

V. DISCUSSION

Human immunoglobulins are widely used as both prophylactic and microbicidal agents, but it would be far better to have available hu-mAb of desired specificities [16]. The use of hu-mAb should minimize the anti-globulin responses during therapy by avoiding anti-isotypic Ab [17]. A large body of studies has shown the effectiveness of hu-mAb as a therapeutic agent. However, the lack of a simple and efficient system to clone specific hu-mAb has been one of the major setbacks for fulfilling the promise of antibody therapy in human disease for decades.

To continue on our effort to generalize specific hu-Ab production, we describe here the generation of high binding affinity and autoreactive hu-scFv using our previously established procedure of combining hu-PBL-SCID mice and scFv phage display library [11]. The presence of autoantibodies in mice and in humans has been extensively documented and autoreactive Ab against well-defined hu-Ag have been detected in serum of healthy individuals. Under normal physiological condition, ten to thirty percent of B cells are committed to produce autologous Ab of mainly IgM class with very low binding affinities. It has been suggested that autologous IgM contributes to regulate expression of autoreactive IgG through V region-dependent interactions, resulting in low levels of serum autoreactive IgG, and that this regulation mechanism is missing in hu-PBL-SCID mice [18]. Therefore, we hypothesize that it is possible to induce hu-Ab response to human self-Ag in hu-PBL-SCID mice. We set out to test this hypothesis by immunizing our hu-PBL-SCID mice with KLH-TNF and Freund's adjuvant mixture.

The induction of specific hu-Ab responses in hu-PBL-SCID mice has been subjected to some drawbacks and debate especially when the hu-PBL-SCID mouse

model established by Mosier et al. was used [19-23]. Our hu-PBL-SCID mouse model was designed to partially avoid and overcome those drawbacks. The pretreatment of SCID mice with γ-radiation and anti-asialo GM1 helped to further reduce the residual innate immunity of the mice in order to improve hu-PBL engraftment. By using CFA in immunization and limiting the whole procedure within the first two weeks of hu-PBL engraftment, we were able to induce specific Ab response avoiding the skewing of human immune response toward mouse Ag [11, 12, 14]. High degree of hu-PBL engraftment in the spleens, high serum hu-Ab level as well as the shifting of serum hu-Ab titre from IgM to IgG implied that specific anti-TNF-α hu-Ab response occurred in TNF-hu-PBL-SCID.

A number of mouse/human chimeric and humanized Ab specific to hu-TNF-α such as cA2, Infliximab and CDP 571 has been produced and used in clinical trials for treatments of rheumatoid arthritis, Crohn's disease, septic shock, and multiple sclerosis [7, 8, 24-29]. The beneficial effects of anti-TNF-α mAb therapy, at least in the short term, have clearly been demonstrated by these studies. In one study, up to four cycles of treatment with cA2, an anti-TNF-α chimeric mAb, have been shown to be well tolerized by the enrolled patients, and the beneficial effects was extended up to 60 weeks [8]. However, a single mAb was used in every one of these trials. Patients finally developed anti-idiotypic response rendering long-term treatments ineffective.

It has been suggested that multiple distinct mAb with desired specificity are required for long-term Ab therapy. These Ab can be used either in combination or in an alternating regimen to counteract the development of anti-idiotypic Ab responses. We estimated that the mouse-derived γκ library would contain hundreds of distinct anti-TNF-α scFv clones with high binding affinity since we were able to isolate three clones from

the first 90 'strong binders' tested (Table 1). One of the advantages of scFv phage display library technique over the conventional EBV immortalization and hybridoma techniques is that the whole library can indefinitely be preserved and scFv clones with desired specificity can be isolated when needed. Work is in progress to construct complete hu-IgG1 from the three isolated anti-TNF-α scFv clones for further characterization, including their neutralizing activity both *in vitro* and *in vivo*.

The results of this study have demonstrated that the combination of hu-PBL-SCID mice and scFv phage display library can effectively be used to clone multiple distinct scFv exhibiting high binding affinity to a self-Ag, hu-TNF-α. Together with our previously reported work of isolating neutralizing, anti-respiratory syncytial virus hu-scFv, this study has further affirmed our notion that this combined procedure is an effective alternative for cloning multiple hu-mAb against any Ag of interest [11]. This procedure may represent a significant step toward fulfilling the promise of Ab therapy in human disease.

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VII. LEGEND:

Figure 1: Amino acid sequences of the isolated scFv clones (2, 14 and 20).

The sequences are shown in single-letter amino acid code.

Table 1: Screening for hu-TNF-α binding scFv clones by ELISA

Library	γλ		γκ	
	# of binders [A]	Strong binders [B]	# of binders	Strong binders
Donor's PBLs	0	NA	2	0/2
TNF-hu-PBL-SCID mice	0	NA	4.6×10^3	14/90

Wells of 96 well plates were coated overnight with 100 μl of 1μg/ml hu-TNF-α in 50mM of sodium bicarbonate buffer, pH 9.6 and then blocked with blotto. Phages (10^8 cfu in blotto) of each library were added into a well and incubated for two hours at room temperature with gentle shaking. Bound phages were eluted, infected into *E. coli* TG1 and plated in 5 LB + Amp plates. The numbers of colonies were reported as # of binders. Uncoated and blotto blocked wells were used as negative controls. No phages bound to these wells and the ELISA signal was at background level. The average of three independent experiments is reported here.

[A] # of binders: Number of scFv clones recovered after panning against RSV-F. Only γκ libraries contained RSV-F binding scFv clones. [B] Strong binders: Randomly picked TNF-α binding scFv clones (from the previous panning step) were tested by ELISA. Clones that showed both phage signals of at least 50% of positive control signals and strong soluble scFv signals were reported as strong binders. Wells directly coated with 10^9 phage particles were used as a positive control. NA: Not applicable.

Table2: TNF-α binding affinities

	2[A]	14	20
k_a ($M^{-1}.s^{-1}$)[B]	3.73×10^5	3.55×10^5	4.76×10^5
k_d (s^{-1})[C]	3.91×10^{-3}	2.37×10^{-3}	1.47×10^{-3}
$K_A=k_a/k_d$ (M^{-1})[D]	9.54×10^7	1.50×10^8	3.24×10^8

BIAcore[R] system was used to measure TNF-α binding affinities of selected scFv clones. hu-TNF-α was immobilized on the CM5 sensor chip. A solution of scFv protein was injected on to the cell under continuous flow conditions (40µl/min). Dissociation rate, association rate and binding affinity were measured and automatically calculated.

A scFv clones - 2, 14 and 20.

B Association rate.

C Dissociation rate.

D Binding affinity.

V_H amino acid sequence

Clones	FR1	CDR1	FR2
2	EVQLVESGGDLVQPGGSLRLSCAASGITVS	SSYMS	WVRQAPGKGLEWVS
14	EVQLVESGGDLVQPGGSLRLSCAASGITVS	SSYMS	WVRQAPGKGLEWVS
20	EVQLVESGAEVKKPGESLRITCKGSGYSFT	SYWIT	WVRQMPGKGLEWMG

	CDR2	FR3
2	VIYSGGSTYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR
14	VIYSGGSTYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR
20	IIYPGDSDTRYSPSFQG	QVTISADKSISTAYLQWSSLKASDTAMYYCAR

	CDR3	FR4
2	EGHTGMDV	WGQGTTVTVSS
14	EGHTGMDV	WGQGTTVTVSS
20	AGALYYYGSGSYYNDDYYYGMDV	WGQGTTVTVSS

V_L amino acid sequence

Clones	FR1	CRR1	FR2
2	DIVMTQSPDSLAVSLGERATINC	KSSQSILYSSDNKNYLA	WYQQKPGQPPKLLIY
14	DIQMTQSPSSLSASVGDRVTITC	RASQGISNALA	WYQQKAGNPPKLLLY
20	DIVMTQSPDSLAVSLGERATINC	KSSQSVLYSSNNKNYLA	WYQQKPGQPPKLLIY

	CDR2	FR3
2	CASTRES	GVPDRFSG SGSGTDFTLTISSLQAEDVAVYYC
14	AASRLES	GVPSRFTGSGSGTDYTLTISSLQPEDFATYSC
20	WASTRES	GVPDRFSGSGSGTDFTLTISSLQAEDVAVYYC

	CDR3	FR4
2	QQSYNLPWT	FGQGTKVEIKR
14	QQYYSIPLT	FGGGTKVEIKR
20	QQYYSTPWT	FGQGTKVDIKR

Figure 1:

CHAPTER IV

Discussion and conclusion

DISCUSSION

I. Brief review of hu-mAb production and Ab therapy

The mammalian immune system recognizes and responds to a wide spectrum of foreign molecules. At the same time, it has to develop a state of tolerance toward self-constituents. The generation of specific Ab to fight infections, toxic substances and aberration within the host is the major contribution of B cells to the adaptive immunity. Ab diversity sufficient to enable the selective recognition of innumerable antigenic determinants is created during B cell ontogeny in the adult bone marrow. Quite often, responsiveness to many antigenic determinants is started by a small number of B cells whose BCR bind Ag with low affinity [1]. Both the frequency and BCR affinity of responsive cells can be drastically increased subsequent to immunization by the generation of memory B cells whose BCR recognize determinants of the immunizing Ag [2]. Our understanding of the events occurring during the humoral arm of the immune response has significantly built on the information generated by a large number of investigations during previous decades [3]. The information has provide valuable insight into when, where and even how V(D)J recombination, Ig class switching and Ig hypermutation events occur. Stepwise processes of these events and their involvement in the ontogeny and function of B cells have been demonstrated [4-8].

The idea of Ab therapy dated back a century ago with Paul Ehrlich's envision of the "magic bullets" for treatment of virtually any illness [9]. This vision has become a reachable goal following the discoveries and developments of EBV immortalization and hybridoma technologies of the 1970's [10,11]. mAb have significantly increased the therapeutic expectations in Ab therapy. These technologies have successfully been used to generate innumerable specific mouse mAb with relative ease. Despite a significant

effort, the application of these two technologies in hu-mAb production has been unsatisfactory. The keys to successful hu-mAb production by both hybridoma and EBV transformation techniques are the quality, purity and state of activation of the donor lymphocytes. These cells are exceedingly difficult to obtain since the routine immunization of human with many Ags is impossible on ethical and safety grounds. As a result, the therapeutic efficacy of mAbs has only been proven in a minority of clinical studies since most mAbs used in those studies have been of mouse origin. The elicitation of human anti-mouse Ab renders these mAb ineffective.

The availability of a vast array of specific mo-mAb has triggered the development of techniques to modify these mAb for therapeutic purposes. The mo-mAbs should be modified in a way that the resulting mAb retains the original specificity and becomes less immunogenic to human recipients. Chimeric mouse/human mAb with mouse variable domains and human constant domains were successfully created in the 1980's [12]. In the same period, the technique of "*reshaping human antibodies for therapy*" by engrafting CDR of a specific mouse mAb into hu-Ab framework was developed to further reduce the mouse content of the resulting humanized Ab [13]. These Ab molecules have the same specificity as the original mouse mAb, but the substitution of human sequences confers a longer *in vivo* half-life and minimizes immunogenic site effects [14]. Humanization of mouse mAbs by CDR-grafting has become a standard procedure for reducing immunogenicity and recruiting human effector functions. Although the engineering of humanized mAb is technically straightforward, the result is uncertain. A decrease of binding affinity and even total loss of specificity is often reported [15-17]. Thus far, chimerization and humanization are still case-by-case, trial and error approaches.

Transloci mice whose Ig gene loci were replaced by a large portion of human Ig genes have been created and shown to generate large and diverse repertoire of hu-Ig, and to be capable of mounting some Ag-specific hu-Ab response upon immunization [18]. One of the major advantages of these transloci mice is their capability to mount hu-Ab responses to hu-Ags since the mice do not recognize these Ag as "self". If a transloci mouse strain carrying the full complement of hu-Ig genes is available, hu-mAb specific to virtually any Ag can be generated with relative ease. At the present time, the transfer of the whole hu-Ig genes into mouse ES cells is still a goal to reach.

Molecular technologies for tapping the hu-Ab gene repertoire have also been developed and used to isolate specific hu-mAb [19,20]. Antibody phage display library has been shown to be one of the most powerful technologies for generation of specific hu-mAb. It is possible to construct an Ab library that truly represents the whole donor Ab repertoire. Moreover, randomly linking (pairing) V_H and V_L during the construction of the library can theoretically create new useful binding specificities that are not present in the original hu-Ab repertoire. This logic has led to idea of one large library constructed from normal hu-PBL can be used to isolate Ab against virtually any Ag of interest [21]. In our hands, however it is not yet proven possible; like hybridoma and EBV immortalization techniques, the success rate is heavily dependent on the quality of hu-PBL.

II. The impact of Ab engineering on Ab-based diagnostics and therapies

The development of mo-mAb, as described in 1975 by Kohler and Milstein, allowed us to treat Ab more like a well-characterized chemical than a variable biological serum product since hybridomas could continuously be cultured, producing the same Ig molecules for long periods of time [11]. Hybridomas themselves can be stored

indefinitely and Ig have also been shown to exhibit excellent shelf-life of up to several decades. Over the past few decades, advance assay formats based either on competitive or noncompetitive principles have drastically improved specificity as well as sensitivity of Ab-based immunoassays. Today, mAb have become the predominant immunoreagent reaching over 50% market share in diagnostics [22].

The advent of Ab phage display library and Ab engineering will likely have a strong impact on the development of Ab for immunoassays since it permits the selection of rare specificities such as Ab against carbohydrates and conserved epitopes [23]. Immunoassay technology based on microtiter plate format is presently limited to the analysis of up to a few thousand assays per day. Proteomic maps of a large number of different cell types and a rapid global analysis of proteome are likely dependent on the currently developing protein microarray technology. Differences between proteomic maps of healthy and disease cells will reveal detail maps of cell signaling and metabolic pathways, and will establish milestones for rapid development of future therapeutic reagents. One of the most obvious choices for protein probes used in protein microarrays is Ab molecules due to their high specificity. In fact, a small protein microarray using Ab probes has already been developed and used to analyze cellular changes occurring in cultured human cells [24]. The use of Ab phage display library technology to quickly produce large numbers of recombinant Ab with desired specificity and affinity for protein chip development will signify impacts that Ab have in the development of future protein analysis.

Since the beginning of the 1990's, fueled by the development of Ab phage display technology and hu-Ig transloci mice, development of mAb for therapeutic purposes has become one of the most active biomedical fields. More than seventy mAb are currently in

clinical trials beyond phase I and phase II, and more than a quarter of all new biological products currently undergoing clinical development are Ab-based [25]. A large number of mAb against a wide spectrum of pathological conditions such as cancers, transplantation rejections, autoimmune diseases, bacterial infections and viral infections are being in development [26-29]. Mechanisms of action of the developing mAb are more diverse than ever. Some such as anti-RSV and anti-TNF-α mAb act by blocking either a receptor-ligand interaction or a key cytokine [30, 31]. Some such as Campath1H mAb act by recruiting effector cells of the host immune system to eliminate target cells [32]. Others such as anti-Her-2/neu mAb act by inducing a number of signaling events that may play a role in controlling tumor growth [33]. In the foreseeable future, we will likely see an expansion of hu-mAb, made either by Ab phage display or in hu-Ig transloci mice, to act as therapeutic agents for a wide range of human pathological conditions.

III. Review of the data generated by the present project

The ultimate goal of this project was to establish a widely accessible and highly efficient system to generate specific hu-mAb for both research and clinical purposes. The project was designed to fulfill the following criteria: 1) to develop a simple procedure, using well established techniques; 2) the approach should be accessible to both research and clinical communities; 3) the procedure should be highly efficient for cloning hu-mAb of useful specificities even when target Ag is of human origin; 4) it should be reliable and reproducible.

In brief, the project was divided in to two parts: induction of specific hu-Ab responses in hu-PBL-SCID mice, and using scFv phage display library technology to isolate specific hu-mAb. The first series of experiments were designed to optimize the engraftment of hu-PBL into SCID mice as well as the induction of specific hu-Ab

responses in hu-PBL-SCID mice. RSV-F and hu-TNF-α were chosen to be the model nonself- and self-Ag respectively for this study since hu-mAb against either of these Ag are highly valuable for both research and therapeutic purposes. Pretreatment of SCID mice with 300rad of γ-radiation and anti-asialo GM1 rabbit antiserum to further reduce residual immune system of the SCID mice was found to be a must to ensure high level engraftment of hu-PBL. We also found that up to 80% of splenocytes isolated from hu-PBL-SCID mice are of human origin if freshly isolated hu-PBL were used. Moreover, immunization with mixture of Ag-CFA/IFA produces both high levels of serum hu-Ab and increases of specific Ab titres. The use of fresh hu-PBL and pretreated SCID mice has enabled us to induce high hu-Ab responses to both RSV-F and hu-TNF-α. Serum specific IgG titres increased significantly for both model Ag while IgM tires decreased drastically. These results implicated that specific on-going hu-Ab responses occurred in the mice. It also suggested that hu-Ab maturation might happen in these mice.

Using scFv library techniques, we were able to isolate a number of highly specific hu-mAbs with relative ease. Six out of the first sixty 'strong binders' isolated from γκ library derived from RSV-hu-PBL-SCID mice exhibiting binding affinity in range of 10^8M^{-1} were selected after a single round of high stringent panning. Importantly, two of them exhibited RSV neutralizing activity without the effector functions of Fc region. It is worth noting that neutralizing activity is undetectable in the donor serum. Even though donor's serum exhibited high anti-RSV-F IgG titre, we could not isolate any 'strong binder' from the donor's PBL-derived γκ library. Almost the same results were obtained for hu-TNF-α experiments. Only a mouse-derived γκ library produced three distinct anti-TNF-α scFv with binding affinities in range of 10^8M^{-1}. DNA sequence analyses revealed

that these scFv derived from different germline V_H and V_L families. The results indicated that large numbers of distinct hu-mAb exhibiting high binding affinity could be isolated from these specific scFv libraries. It is a general consensus that a number of distinct hu-mAb carrying different variable regions is needed for counteracting anti-idiotypic Ab response in long term Ab therapy.

The presence of autoreactive B cells in healthy human has been well documented. These B cells however use to express germline Ig variable sequences of IgM isotype with very low binding affinities [22]. The sequence analyses revealed that CDR of the isolated anti-hu-TNF-α scFv had been mutated. The fact that these scFvs were of γκ isotype and exhibited high binding affinities indicated that specific hu-Ab response accompanied by class switching and hypermutation had successfully been induced in these hu-PBL-SCID mice. It also showed that scFv phage display library technique was effectively used to clone highly specific hu-mAb.

IV. Conclusion

The work presented in this thesis has established the foundation for a simple and efficient method to generate hu-mAb to virtually any Ag of interest. This method will have a major impact in both medicine and economy at large without the investment of large sum of capital and efforts. Above all, it is our wish that this method will be used and further developed by any interested individual. In conclusion, the combination of the hu-PBL-SCID mouse model and scFv phage display library technology is one of the most effective alternatives for generating multiple specific hu-mAb. This thesis may represent a significant step toward the materialization of Paul Erhlich' s "*magic bullet*".

V. References

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Detail scFv library construction procedure

Four first strand cDNA syntheses corresponding to Vγ, Vμ, Vκ and Vλ were made from the isolated RNA using either Igγ or Igμ or Igκ or Igλ constant region primer as described by Marks et al (**Table 1-I, Figure 1-1**) [1]. V_H genes were amplified using a V_H back primer and an equimolar mixture of the appropriated family-based forward primers (**Table 1-II, Figure 1-2**). Twelve reaction mixtures of 100μl were prepared containing 5 μl of the supernatant from a H-chain cDNA synthesis (μ-chain or γ-chain), 20pmol of a V_H back primer, mixture of 20pmol each of four J_H forward primers, 400μM of dNTPs, 1x Vent^R DNA polymerase reaction buffer (10mM KCl, 10mM $(NH_4)_2SO_4$, 20mM Tris-HCl pH 8.8, 2mM $MgSO_4$ 0.1% Triton X-100 and 100μg of BSA) and 1 unit of Vent DNA polymerase (New England Biolabs). The reaction mixture was overlaid with light mineral oil and subjected to 30 cycles of amplification using a RoboCycler Gradient 96^R (Stratagene). The cycle was 95^{0}C for 1 min, 57^{0}C for 1 min and 72^{0}C for 1 min. This condition was used for all PCR amplifications in this study excepted when noted. The products were purified on a 2% (w/v) agarose gel and extracted from the gel by QIEX^R (Qiagen). Vκ genes were amplified using an equimolar mixture of 20pmol each of the six Vκ back primers and the five Vκ forward primers. Reaction mixture (100μl) was prepared containing 5μl of the supernatant from the κ-chain first strand cDNA synthesis, a mixture of 20pmol each of the six Vκ back primers and a mixture of 20pmol each of the five Vκ forward primers. The reaction mixture was subjected to the same amplification cycles as those used to amplify the V_H genes. Similarly, Vλ genes were amplified using equimolar mixtures of 20pmol each of the appropriate family-based back and forward primers.

Fifty-two separate 100μl PCR reactions were performed to synthesize scFv linker DNA using each of the 4 reverse J_H primers in combination with each of 13 reverse Vκ and Vλ primers (**Table 1-III, Figure 1-3**). The template was 1ng of 5'-TC TCC/T TCA GGT GGC GGC GGT TCG GGC GGA GGA GGC TCT GGC GGT GGC GGA TCG GA-3' which encoded a flexible and hydrophilic peptide $(\text{-Gly}_4\text{Ser-})_3$. The PCR reaction reagents were as described above and the cycle was 95°C for 1min, 57°C for 1min and 72°C for 0.5min. The linkers were purified on 12% acrylamine gel and extracted from the gel by QIEX. The purified linkers were pooled together in an equimolar fashion to make 2 linker mixtures, a mixture of 24 heavy chain - κ (H-κ) linkers and a mixture of 28 heavy chain - λ (H-λ) linkers.

Approximately 1μg of each of the 6 primary H-chain amplification (Vμ or Vγ) and 1μg of the primary L-chain amplification (Vκ or Vλ) were combined with approximately 300ng of an appropriate linker mixture (H-κ or H-λ linker mixture) in a 70μl PCR reaction mixture for PCR assembly of scFv repertoires. The reaction mixtures (6Vμ-Vκ, 6Vγ-Vκ, 6Vμ-Vλ and 6Vγ-Vλ) were cycled 10 times (95°C for 1min, 62°C for 2.5 min and 72°C for 1.5min) to join the fragments (**Table 1-II, Figure 1-3**). The reactions were held at 95°C and 20pmol of appropriate back and forward outer primer mixture in 30μl volume was added into each reaction mixture. The reaction mixtures containing V_H1, V_H3 and V_H6 were subjected to 20 PCR cycles of 95°C for 1min, 67°C for 3min and 72°C for 2min, while the reaction mixtures containing V_H2, V_H4 and V_H5 were subjected to 20 PCR cycles of 95°C for 1min, 65°C for 3min and 72°C for 2min. The assembled products were purified on 1% agarose gel and extracted by QIEX and reamplified for 25 cycles of 95°C for 1min, 62°C for 2min and 72°C for 2min with the

flanking primers containing the appended restriction sites (*Sfi1* or *Not1*) (**Table 1-IV, Figure 1-4**). Appropriate products were pooled together into mixtures that represented μκ, μλ, γκ and γλ scFv gene repertoires, and were purified by selective precipitation (100mM NaCl, 20mM Tris-HCl pH7.5, 10mM EDTA, 2M ammonium acetate, 70% ethanol and spin at 10,000g for 20min at room temperature to pellet the DNA). The purified DNA of the scFv gene repertoires (5μg) were digested with 20 units of *Sfi1* (New England Biolabs) (50mM NaCl, 10mM Tris-HCl pH7.9, 10mM $MgCl_2$ and 1mM DTT) at 50^0C for 1hr. The buffer was adjusted to 100mM NaCl, 50mM Tris-HCl pH7.9, 10mM $MgCl_2$ and 1mM DTT. Twenty units of *Not1* (New England Biolabs) were added and the mixtures were incubated at 37^0C for another 1hr. The digested DNA fragments were purified by selective precipitation, and ligated into PCANTAB 5E vector (Pharmacia) that had been digested with *Sfi1* and *Not1*, and treated with calf intestine phosphatase. The ligated DNA was electroporated into *E. coli* TG1 (0.2cm gap, 2.5Kv, 25μF). The transformed bacteria were overnight grown in 2x YT broth containing 100μg ampicillin/ml. The culture was stored at -70^0C as a library stock after 15% glycerol had been added.

Table 1

Primers for PCR of human Ig genes

I. First strand cDNA synthesis

C_H primers

IgG C_H1 For	5'-GTC CAC CTT GGT GTT GCT GGG CTT-3'
IgM C_H1 For	5'-TGG AAG AGG CAC GTT CTT TTC TTT-3'

C_L primers

Cκ For	5'-AGG CTC TCC CCT GTT GAA GCT CTT-3'
Cλ For	5'-TGA AGA TTC TGT AGG GGC CAC TGT CTT-3'

II. Primary PCR

V_H back primers

V_H1 Back	5'-CAG GTG CAG CTG GTG CAG TCT GG-3'
V_H2 Back	5'-CAG GTG AAC TTA AGG GAG TCT GG-3'
V_H3 Back	5'-GAG GTG CAG CTG GTG GAG TCT GG-3'
V_H4 Back	5'-CAG GTG CAG CTG CAG GAG TCG GG-3'
V_H5 Back	5'-GAG GTG CAG CTG TTG CAG TCT GC-3'
V_H6 Back	5'-CAG GTA CAG CTG CAG CAG TCA GG-3'

JH Forward primers

J_H1-2 For	5'-TGA GGA GAC GGT GAC CAG GGT GCC-3'
J_H3 For	5'-TGA AGA GAC GGT GAC CAT TGT CCC-3'
J_H4-5 For	5'-TGA GGA GAC GGT GAC CAG GGT TCC-3'
J_H6 For	5'-TGA GGA GAC GGT GAC CGT GGT CCC-3'

Vκ back primers

Vκ1 Back	5'-GAC ATC CAG ATG ACC CAG TCT CC-3'
Vκ2 Back	5'-GAT GTT GTG ATG ACT CAG TCT CC-3'
Vκ3 Back	5'-GAA ATT CTG TTG ACG CAG TCT CC-3'
Vκ4 Back	5'-GAC ATC GTG ATG ACC CAG TCT CC-3'
Vκ5 Back	5'-GAA ACG ACA CTC ACG CAG TCT CC-3'
Vκ6 Back	5'-GAA ATT GTG CTG ACT CAG TCT CC-3'

Jκ forward primers

Jκ1 For	5'-ACG TTT GAT TTC CAC CTT GGT CCC-3'
Jκ2 For	5'-ACG TTT GAT CTC CAG CTT GGT CCC-3'
Jκ3 For	5'-ACG TTT GAT ATC CAC TTT GGT CCC-3'
Jκ4 For	5'-ACG TTT GAT CTC CAC CTT GGT CCC-3'
Jκ5 For	5'-ACG TTT AAT CTC CAG TCG TGT CCC-3'

Vλ back primers

Vλ1 Back	5'-CAG TCT GTG TTG ACG CAG CCG CC-3'
Vλ2 Back	5'-CAG TCT GCC CTG ACT CAG CCT GC-3'
Vλ3a Back	5'-TCC TAT GTG CTG ACT CAG CCA CC-3'
Vλ3b Back	5'-TCT TCT GAG CTG ACT CAG GAC CC-3'
Vλ4 Back	5'-CAG GTT ATA CTG ACT CAA CCG CC-3'
Vλ5 Back	5'-CAG GCT GTG CTC ACT CAG CCG TC-3'
Vλ6 Back	5'-AAT TTT ATG CTG ACT CAG CCC CA-3'

Vλ forward primers

Jλ1 For	5'-ACC TAG GAC GGT GAC CTT GGT CCC-3'
Jλ1 For	5'-ACC TAG GAC GGT CAC CTT GGT CCC-3
Jλ1 For	5'-ACC TAA AAC GGT GAG CTG GGT CCC-3'

III. PCR assembly

Template for scFv linker synthesis

5'-TC TCC/T TCA GGT GGC GGC GGT TCG GGC GGA GGA GGC TCT GGC GGT GGC GGA TCG GA-3'

Reverse J_H for linker

RJ_H1-2	5'-GCA CCC TGG TCA CCG TCT CCT CAG GTG G-3'
RJ_H3	5'-GGA CAA TGG TCA CCG TCT CTT CAG GTG G-3'
RJ_H4-5	5'-GAA CCC TGG TCA CCG TCT CCT CAG GTG G-3'
RJ_H6	5'-GGA CCA CGG TCA CCG TCT CCT CAG GTG C-3'

Reverse Vκ for linker

RVκ1 Back	5'-GGA GAC TGG GTC ATC TGG ATG TCC GAT CCG CC-3'
RVκ2 Back	5'-GGA GAC TGA GTC ATC ACA ACA TCC GAT CCG CC-3'
RVκ3 Back	5'-GGA GAC TGC GTC AAC ACA ATT TCC GAT CCG CC-3'
RVκ4 Back	5'-GGA GAC TGG GTC ATC ACG ATG TCC GAT CCG CC-3'
RVκ5 Back	5'-GGA GAC TGC GTG AGT GTC GTT TCC GAT CCG CC-3'
RVκ6 Back	5'-GGA GAC TGA GTC AGC ACA ATT TCC GAT CCG CC-3'

Reverse Vλ for linker

RVλ1 Back 5'-GGC GGC TGC GTC AAC ACA GAC TGC GAT CCG CCA CCG CCA GAG-3'

RVλ2 Back 5'-GCA GGC TGA GTC AGA GCA GAC TGC GAT CCG CCA CCG CCA GAG-3'

RVλ3a Back 5'-GGT GGC TGA GTC AGC ACA GAC TGC GAT CCG CCA CCG CCA GAG-3'

RVλ3b Back 5'-GGG TCC TGA GTC AGC TCA GAA GAC GAT CCG CCA CCG CCA GAG-3'

RVλ4 Back 5'-GGC GGT TGA GTC AGT ATA ACG TGC GAT CCG CCA CCG CCA GAG-3'

RVλ5 Back 5'-GAC GGC TGA GTC AGC ACA GAC TGC GAT CCG CCA CCG CCA GAG-3'

RVλ6 Back 5'-TGG GGC TGA GTC AGC ATA AAA TTC GAT CCG CCA CCG CCA GAG-3'

IV. Reamplification with primers containing restriction sites

V_H back primers

V_H1 Back Sfi 5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCG CAG GTG CAG CTG GTG CAG TCT GG-3'

V_H2 Back Sfi 5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCG CAG GTC AAC TTA AGG GAG TCT GG-3'

V_H3 Back Sfi 5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCG GAG GTG CAG CTG GTG GAG TCT GG-3'

V_H4 Back Sfi 5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCG CAG GTG CAG CTG CAG GAG TCG GG-3'

V_H5 Back Sfi 5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCG CAG GTG CAG CTG TTG CAG TCT GC-3'

V_H6 Back Sfi 5'-GTC CTC GCA ACT GCG GCC CAG CCG GCC ATG GCG CAG GTA CAG CTG CAG CAG TCA GG-3'

Jκ forward primers

Jκ1 For Not 5'-GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT GAT TTC CAC CTT GGT CCC-3'

Jκ2 For Not 5'-GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT GAT CTC CAG CTT GGT CCC-3'

Jκ3 For Not 5'-GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT GAT ATC CAC TTT GGT CCC-3'

Jκ4 For Not 5'-GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT GAT CTC CAC CTT GGT CCC-3'

Jκ5 For Not 5'-GAG TCA TTC TCG ACT TGC GGC CGC ACG TTT AAT CTC CAG TCG TGT CCC-3'

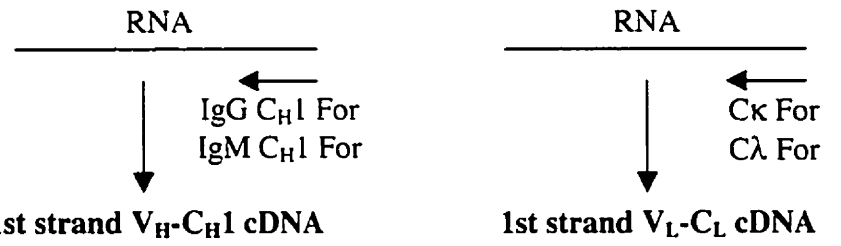
Jλ forward primers

Jλ1 For Not 5'-GAG TCA TTC TCG ACT TGC GGC CGC ACC TAG GAC GGT GAC CTT GGT CCC-3'

Jλ2-3 For Not 5'-GAG TCA TTC TCG ACT TGC GGC CGC ACC TAG GAC GGT CAG CTT GGT CCC-3'

Jλ4-5 For Not 5'-GAG TCA TTC TCG ACT TGC GGC CGC ACC TAA AAC GGT GAG CTG GGT CCC-3'

1) First strand cDNA synthesis



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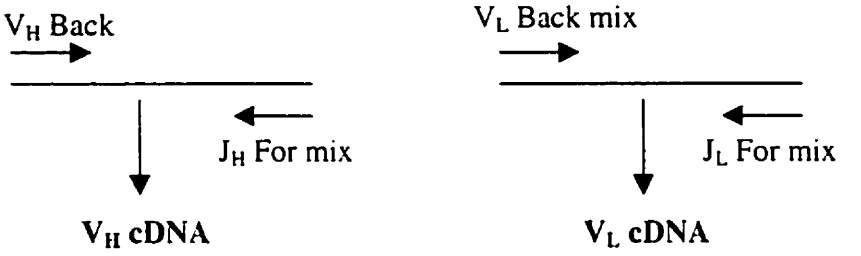
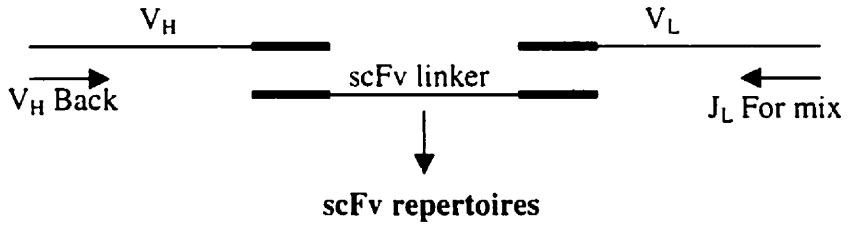


3) PCR assembly



4) Reamplification with primers containing restriction sites

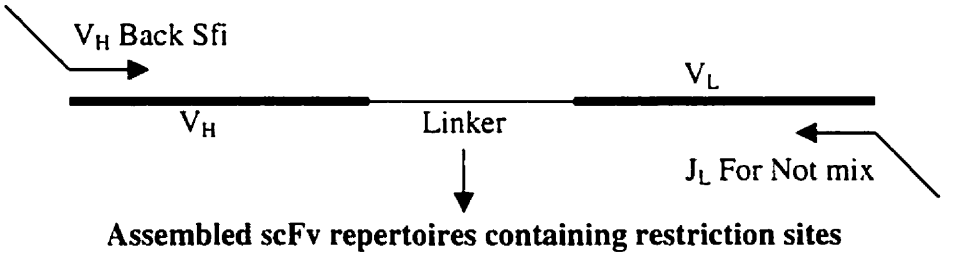


Figure 1

Figure 1

Construction of scFv gene repertoires.

1) Total RNA (5mg each) was used as template for the first strand cDNA syntheses of both IgH and IgL chains (4 reactions). 2) Vμ and Vγ genes were amplified using a V_H back primer (6 primers) and an equimolar mixture of the J_H For primers (12 reactions). Vκ and Vλ genes were amplified using an equimolar mixture of the appropriate family-based back and forward primers (2 reactions). 3) PCR was used to assemble scFv repertoires. The assembled scFv was then amplified using a V_H back primer (6 primers) and an equimolar mixture of the appropriate J_L For primers (Jκ or Jλ For primer mixture) (24 reactions). 4) The products of step 3 were reamplified using the appropriate V_H Back primer and J_L for primer mixture containing appended restriction sites (24 reactions).

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