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DNA vaccines for the immunotherapy of B-cell lymphoma

Thesis submitted for the Degree of

Doctor Philosophiae

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Academic Year 1999/2000

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Acknowledgements

The work described in this thesis was carried out at the International Centre for Genetic Engineering and Biotechnology (ICGEB), Trieste. I would like to thank the International School for Advanced Studies for supporting me with a predoctoral fellowship throughout the course of my PhD.

I am extremely grateful to Dr Oscar Burrone for his invaluable guidance, constant availability and warm encouragment. I'm also grateful to Dimitar Efremov for his supervision during the first part of my PhD project.

Finally, I will never forget my colleagues of the Molecular Immunology Group with whom I spent very happy years.

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Summary

Working at the International Centre for Genetic Engineering and Biotechnology, where I started my Ph.D thesis in October '96 I have directed my research activity to the immunotherapy of B-cell malignancies by DNA vaccination. I have developed an efficient vaccination strategy to induce protection in the BCL1 lymphoma model and characterized all the elements of the antigen encoding plasmid that contribute to the induction of the immune response.

In addition, I have investigated the structural requirements of the idiotypic/anti-Idiotypic interaction following scFv DNA vaccination. This analysis revealed that the polyclonal anti-Id immune response induced by scFv DNA vaccination is exclusively directed against conformational combined epitopes. Remarkably, the same immunogen delivered in a different form induced also antibodies directed against chain specific determinants suggesting that the mode of presentation to the immune system may influence the specificity of the antibody response.

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CHAPTER 1

INTRODUCTION

The human immune system is capable of specifically recognizing and responding to an enormous number of antigens. The interaction of antigens with the cells involved in the immune response is mediated by two distinct antigen specific receptor, the immunoglobulins and the T-cell receptor, expressed on the surface of B and T lymphocytes, respectively.

1. B-LYMPHOCYTES

B-lymphocytes develop from hematopoietic stem cells in specialized microenviroments provided by the fetal liver and the adult bone marrow of mammals, in an ordered process that is marked by sequential rearrangement of the immunoglobulin genes.The development of B lymphocytes can be broadly diveded into two major phases. In the first, antigen independent phase, a programmed sequence of immunoglobulin V-(D)-J gene rearrangements (pro-B cells, pre-B cells) yields a population of lymphocytes each of which makes its own characteristic immunolgobulin molecule. In the second phase, encounter with foreign antigen leads to a selective expansion of those B-cells that make a cognate antibody: they differentiate into either antibody-secreting plasma cells or memory B cells.

1.1. Immunoglobulin structure

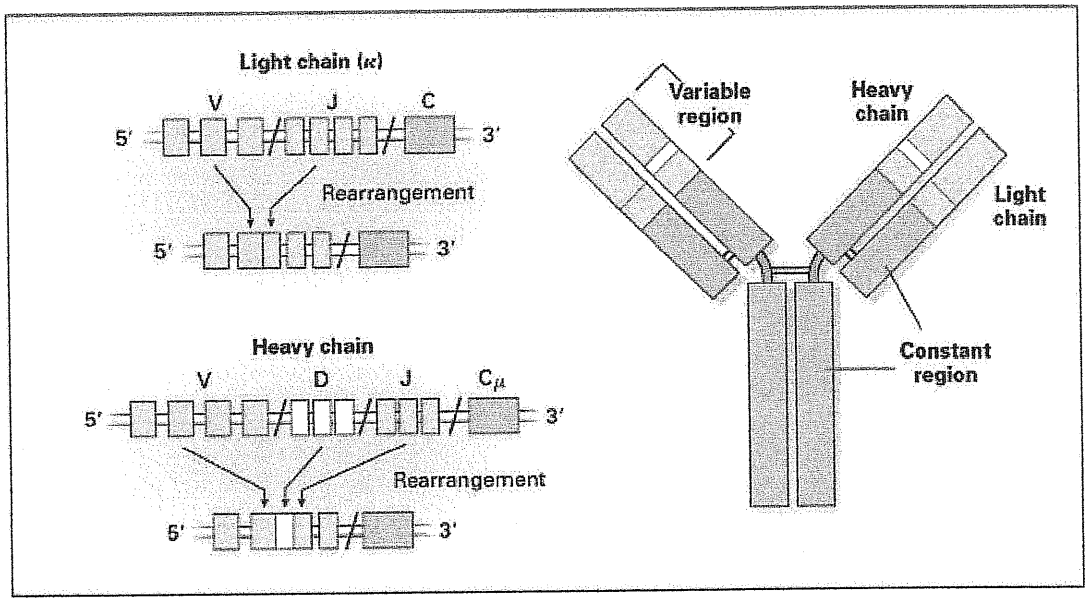
Immunoglobulins (Ig), the antigen specific membrane receptor and secreted product of B-cells, are heterodimeric molecule consisting of two identical heavy (H) and two identical light (L) chains, that are linked togheter by disulfide bonds. Both H and L chains are organized into domains that are defined by homology and that are approximately 110 amminoacid in lenght. Each domain forms a conserved structure known as the antibody fold, which is stabilized by an internal disulfide linkage that forms a loop of about 65 ammino acids. The ammino terminal domain of H and L chains comprise the variable

fold, which is stabilized by an internal disulfide linkage that forms a loop of about 65 ammino acids. The ammino terminal domain of H and L chains comprise the variable region, which varies from 112 to 136 amino acids in length (**1**). Within the variable region are three areas of greatest sequence variability (hypervariable regions) that are separated by regions of relatively constant amino acid sequence. H and L chain hypervariable regions toghether form the potential antigen binding site, and are therefore referred to as complementary determining regions (CDRs) (**2**).

Amino acid sequences within the remainder of H and L chains are relatively invariable, but deviate among different constant (C) region types (isotypes). Heavy chain C region isotype define the classes and subclasses of mammalian Igs. Five types of H-chain exist which define the corresponding immunolgobulin classes IgM, IgD, IgG, IgE and IgA, designated by their respective greeek letters (μ, δ, γ, ε, α). In humans, the IgG class is divided into subclasses 1,2,3 and 4 whereas in the mouse these subclasses are defined as IgG1, IgG2a, IgG2b, and IgG3. The C regions mediate immunological effector functions, such as complement fixation, placental transfer, and binding to cell surface Fc receptors, that are specific to particular isotypes. Functional differences have not been identified for the two isotypes of mammalian L chains, kappa (k) and lambda (λ). H chain C regions contain between two and four domains that are distinctly homologous to each other and to each L chain C region domain. Certain H chain isotype also contain a hinge region between the CH1 and CH2 domains, that may facilitate antigen binding by increasing H chain flexibility. The IgG molecule is expressed on the cell surface as a monomer, but Ig molecules can be secreted as monomers (IgG and IgE) or, in conjunction with the J chain protein, as dimers (IgA), or as pentamers (IgM).

1.2. Immunoglobulin genes

The expression of a complete immunoglobulin molecule is a consequence of a DNA recombination event that takes place independently in each lymphocyte precursor and leads to the assembly of specific DNA segment in both the H-chain and the L-chain loci.



The V region is encoded by a newly formed exon assembled upstream of the C region as a consequence of the recombination process that joins three different gene segments: a variable (V_H), a diversity (D) and a joining (J_H) segment. Each VH gene contains two exons: the first encodes a hydrophobic leader sequence that facilitates vectorial translocation of the molecule to the endoplasmic reticulum; while the second encodes the FW1, CDR1, FW2, CDR2 and FW3 amino acids of the V region H-chain. CDR3 is encoded by the D segment and by sequences at the VH-D and D-JH junctions, and a part of the JH. The reminder of a JH segment encodes the fourth framework region.

The murine IgH locus lies on chromosome 12. The number of individual VH segments vary with the strain of mouse and range from 100 to as many as several thousand (**3**), organised in 14 families defined by sequence homology (**3,4**). There are four JH segments and 13 known D segments (**5**).

In humans the IgH locus is located on chromosome 14. The region contains 123 VH segments classifiable into seven different families, of which 79 are pseudogenes. Of the 44

as mRNA, while the remaining 4 are not found in immunoglobulin cDNAs. Combinatorial diversity of VH region was calculated to be approximately 6,000. (**6**). Approximately 30 D_H gene segments have been identified, just 5' to six functional JH segments (**7,8**).

The heavy chain constant region genes are organized in clusters each encoding a different C region. In mice and humans, the μ constant region (Cμ) is located closest to the JH segment, followed by the exon clusters encoding the other H chain isotypes (**9,10**). B-lymphocytes express μ H chain first during differentiation, while the other isotypes are expressed later after the process of class switching has occurred (see later). An exception is IgD (**11,12**), which is co-expressed with IgM by alternative RNA processing and termination of transcription.

The variable region of each L chain isotype is encoded by an upstream exon that is assembled from analogous VL and JL segments which are joined directly to each other; there are no L chain D segments. The genetic segments for the assembly of functional VL proteins derived from either of two different loci: k or λ. There are at least 200 murine Vk (**4**) segment that lie 5' of five Jk segments which are in turn 5' to the single Ck gene. Lambda light chain account for only about 5% of the serum light chain in the mouse. In BALB/c, three Vλ segments and four Cλ region exons (each C region having a Jλ gene segment in close proximity to it have been identified) (**13**).

In humans, kappa light chains comprise approximately 60% of the human light chain protein. The V region is created by joining of one of the 75-80 Vk gene segments. Similar to the VH genes, each of these Vk segments has two exons, one encoding for the leader sequence and the second encoding for most of the V region. The remaining of the Vk region is encoded by one of the five functional Jk gene segments. For the lambda light chain, there are several Cλ genes, each associated with a 5' Jλ segment. Six Vλ gene segments, only one of which appears functional, are locate directly upstream of Cλ.

1.3 Ig genes expression

The process of genetic rearrangement is tightly regulated occurring in a preferred temporal order (IgH D to J joining precedes V to DJ joining) and in a lineage specific manner. The V(D)J recombination is mediated by a common type of recombination signal sequence (RSS) that flanks all recombinationally competent gene segments. Two lymphoid-specific genes, RAG1 and RAG2, recognise the RSS and operate a cleave between it and the coding sequence(**14**). Subsequent rejoining to form the mature coding segment, is mediated by a number of factors that are also involved in repair of radiation damage (**14**).

The H-chain locus is the first to be rearranged. This process occurs in two steps. The first event is a D to JH rearrangement, which invariably occurs on both H chain alleles. Precursor B cells then undergo VH to DJH rearrangement on one or both alleles, generating potentially functional VDJ H-chain genes. Additional diversity is created by the junctional imprecision of the joining events and by the deletion of nucleotides and addition of new, untemplated nucleotides between D and JH and between VH and D, forming N regions in these areas. This further increases the diversity of distinct Ig H chains that can be generated from the relatively modest amount of genetic information present in the germline. When a productive rearrangement of any one allele occurs, further changes in the second allele are inhibited (allelic exclusion) and the cell proceed to IgL rearrangement. The mechanism of allelic exclusion is still under investigation. Although it is established that this phenomenon is mediated by a feedback mechanism whereby the first productive rearrangement brings about the repression of RAG proteins which consequently prevents further rearrangment events (**15**), more recent evidences indicate that additional levels of regulation exists. During lymphoid cell development, immune system loci undergo demethylation and open their chromatin structure in anticipation of the rearrangement reaction. It has been shown that demetyhlation occurs preferentially in one allele and that this modification marks the allele for rearrangement before the initiation of the recombination reaction (**16**). However, it is not clear yet how the allele is selected and which factors regulates the demethylation process.

Control of H chain gene transcription involves both the VH promoter and a trascriptional enhancer element which is locate within the JH-Cμ intron (**17**). This enhancer element is active specifically in lymphoid cells, and functions synergistically with the VH promoter, which is also tissue specific.

The assembly of L-chain genes follows H-chain gene rearrangement. This DNA recombination event joins one of the many germline Vk regions with a particular joining (Jk) region. If a Vk and a Jk gene segment are effectively joined in a cell already possesing an effective H-chain VDJ recombination, a μ, κ surface immunolgobulin B cell results. If the rearrangement of Vk is not succesful then rearrengemnt of the Vλ locus is initiated resulting in the espression of a μ, λ bearing B cell.

L chain gene expression appear to be regulated by mechanisms analogous to those that control H chain expression. Vk and Vλ pomoter are structurally similar to VH promoters, and are most active in lymphoid cells. Activity of the promoter of a rearranged Vk segment depends on a tissue specific enhancer locate within the Jk-Ck intron (**18**).

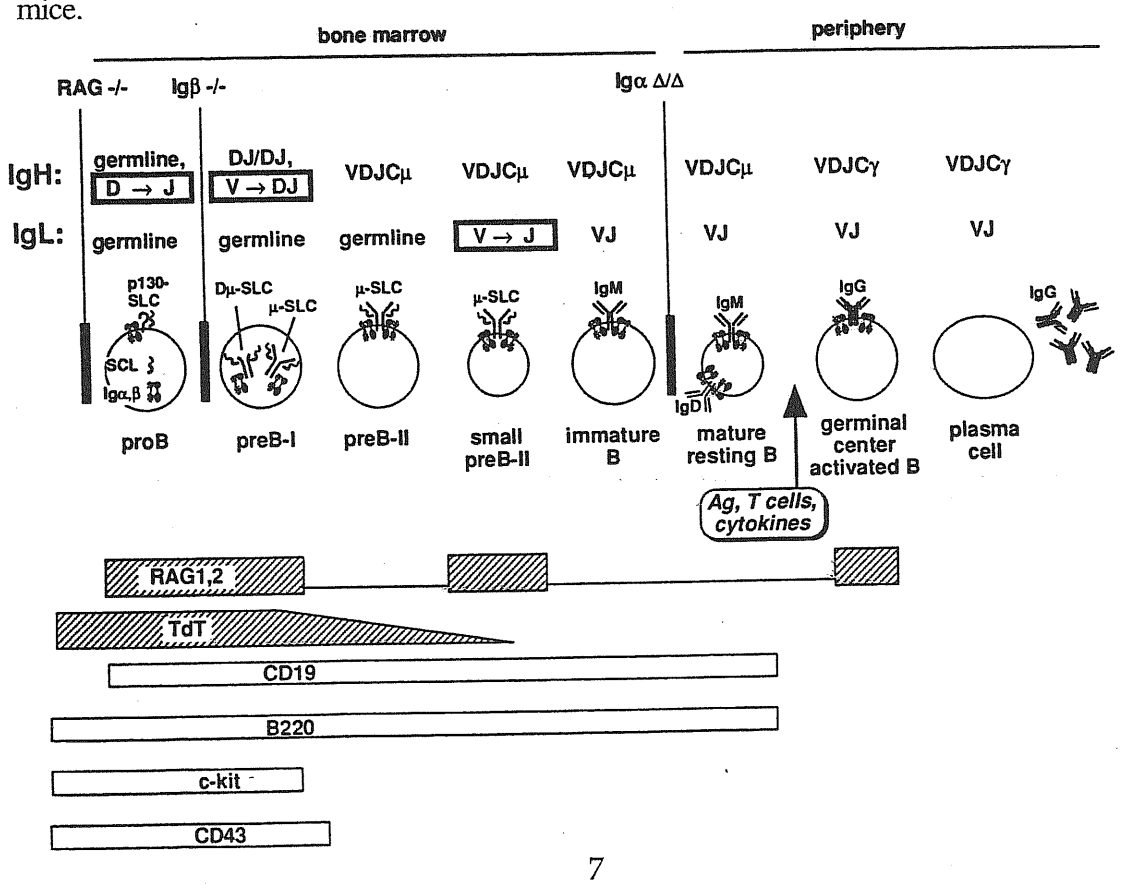
1.4 Membrane vs secreted Ig

The differentiation of the mature B cell to plasma cell is accompanied by the loss of surface immunoglobulin expression as a consequence of conversion from the synthesis of membrane to secretory immunoglobulin molecules. The secreted form of a given immunoglobulin molecule has the same VDJ segment of the membrane form, but differ at the carboxy terminal ammino acids. The two forms are encoded by mRNA species that arise by alternative splicing of transcripts derived from a single H-chain gene. It has been proposed that differential transcription termination and polyadenilation of the primary RNA transcript determines which of the two mRNA is expressed. Each immunoglobulin H-chain gene contains at least two polyadenilation sites. Usage of the promoter proximal poly A site, located immediately 3' to the stop codon of the last CH domain results in a mRNA encoding a secreted form H-chain . If however, transcriptional termination and polyadenilation occur at site located several kilobases to the 3' of the first poly A site, a

mRNA containing sequences from two additional exons (M1 and M2) is produced. These exons encode the extracellular membrane proximal domain, hydrophobic transmembrane and hydrophilic cytoplasmic carboxy-terminal portions of the membrane H-chain. RNA processing events remove the first poly A site from the membrane mRNA by splicing from a conserved donor site in the terminal CH exon (either the CH3 or CH4 exon depending on the H-chain isotype) to the M1 exon. Thus, it appears that in the case of immunoglobulin H chain gene expression, transcription termination at alternative end sites determines the expression of either the membrane or secreted protein.

1.5 B cell development

Progression of B cell along the B-cell differentiation pathway can be monitored by the expression of developmentally regulated genes (Intro, fig 1). One of the earliest expressed surface marker of B lineage cells is the Ig gene superfamily member CD19, a signal transduction molecule that is expressed throughout B cell development, in both human and mice. Other cell surface molecules defining early developmental stages of B cells and their progenitors include CD34, CD22 and CD10 in humans, B220, CD43, CD24 and BP-1 in mice.

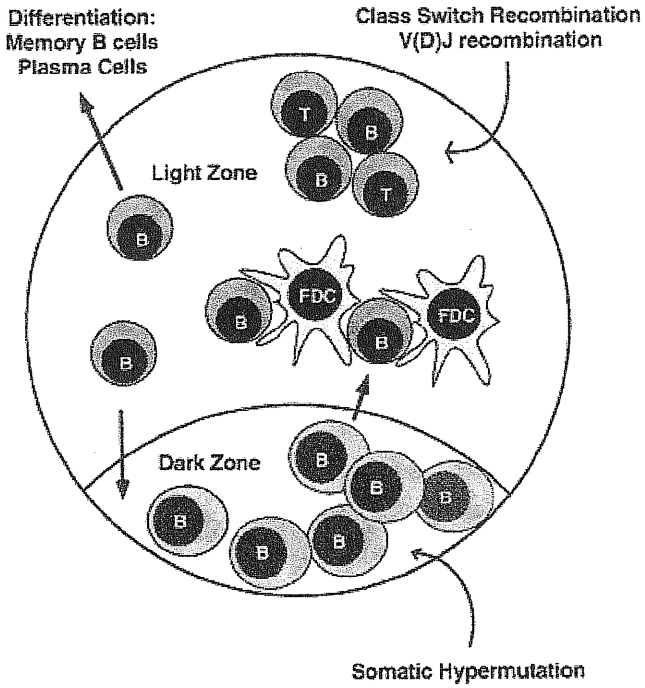


Also the rearrangement status of the immunoglobulin genes are useful developmental markers. The D to JH rearrangement is initiated in pro-B cells, that also express the Igα and Igβ component of the antigen receptor, as well as surrogate light chains (ψLC). Completion of a successful V-DJH rearrangement marks the transition to pre-B cells, with surface expression of the pre-B cell receptor (μVH/ψLC, Igα and Igβ). The pre-BCR is involved in the selection and amplification of pre-B cells by signalling the proliferative expansion of those pre-B cells that have succeeded in a functional VDJ rearrangement. As already mentioned the pre-BCR also signals for allelic exclusion at the IgH locus by preventing rearrangements in the second IgH allele. Subsequent rearrangement of a k or λ chain V gene permits cell surface expression of a conventional sIgM receptors on the immature B cell which exists the bone marrow and migrates to the periphery. Mature B cells in spleens and secondary lymphoid organs are competent to respond to cognate antigen and signalling from T cells. They circulate through the blood and lymph nodes until they contact specific antigen, tipically in the T cell area in the spleen or lymph node Some B-cells are activated in situ, proliferate to form foci, and then differentiate to the antibody secreting cell state. Other activated B-cells migrate to the follicular region, where they initiate the formation of germinal centers (see below).

1.6 The maturation of the immune response

In T-cell dependent immune responses (TD) (responses to protein antigens), the affinity of a given antibody for its cognate antigen increases from the relatively low affinity of the primary response to the high affinity antibodies predominant in a memory response. This process, referred to as affinity maturation, occurs in specialized microenviroments called germinal centers (GC)(**19**).(Intro, fig 2).Germinal centers arise from a limited number of B cells activated by antigen and migrating to primary follicles where they interact with follicular dendritic cells (FDC). The FDC carry on their surface antigen complexed to antibodies and components of the complement system, which are critical to the selection and maturation of high affinity antibody. After proliferative expansion discrete dark and

light zone are identified. The dark zone contains rapidly cycling centroblast, whereas the light zone harbors resting centrocytes derived from the centroblasts, FDC that sequester the antigen, and antigen specific T cells and macrophages.



Within GC, three processes are essential to the maturation: somatic hypermutation, selection of high affinity variants and class switching. The process of somatic hypermutation that occurs in proliferating centroblasts further diversified the Ig repertoire created by V(D)J recombination by introduction of nucleotide changes into the variable regions of the immunglobulin V genes. The hypermutation mechanism involves a mutational rate of 1 per 1000 base pairs per generation, implying that for each cell division almost one mutation takes place in either the H- or L- chain V region of an individual cell. Although the mutations can be scattered over the rearranged V genes, this process is not random, in that intrinsic hot spots can be discerned (**20**). Replacement mutations are often over represented at level of CDRs and underrepresented in the framework regions, whereas the opposite is true for silent (S) mutations. This process creates an enormous increase in antibody diversity. Although most of these mutations will either not affect the affinity with which the antibody binds its ligand or will lower that affinity, some will

increase it. Since proliferating GC cells are programmed to die unless rescued by signals involving antigen and antigen specific T-cells, only cells expressing high affinity antibody are positively selected in the light zone of the GC. Centrocytes selected by antigen may re-enter the dark zone and undergo further clonal expansion and somatic hypermutation or may exit the germinal center to differentiate into memory cells or plasma cells. Memory B-cells are long lived and more easily triggered than virgin B-cells upon re-encounter with the same antigen, contributing to render secondary immune responses highly efficient.

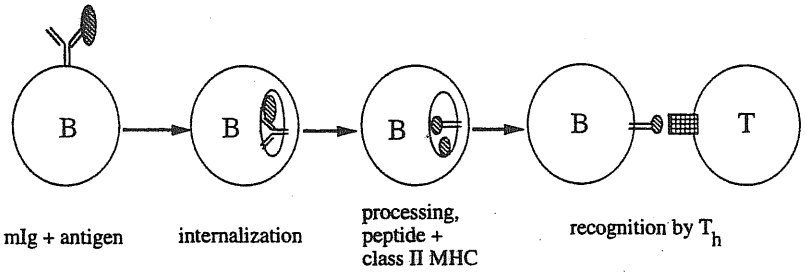
A second aspect involved in the maturation of the immune response is class switching to Ig isotypes different from the IgM and IgD characteristic of primary responses. Class switching occurs within the light zone of GC through a recombination event that produces a change in the class of antibody synthesized, hence a change in the effector function of the antibody produced, maintaining unaltered the specificity of binding. The change in antibody class is effected by a deletional DNA recombination event called switch recombination, which occurs between tandemly located sequences called switch regions (S), which are located upstream of each of the CH genes. B cells that have undergone a class switch recombination event usually have deleted all of the CH gene segments between the Sμ region and the S region of the CH gene that is expressed, since extra chromosomal circles generated by S-S mediated deletions and containing intervening DNA have been isolated (**21**). The induction of a switching process is dependent on the action of a specialized set of B-cell stimulants, like LPS and CD40L expresse on the surface of T-cells. The targeting of the C region that will be expressed as a result of switching is largely determine by cytokines. IL-4 determines that switch will be to the ε C region and to the γ4 (human) or γ1 (mouse), IFN-γ determines switch to γ2a and TGF-β to α. A variety of evidences also indicate that once a B-cell has switched to a particular CH gene, it can, upon appropriate stimulation, undergo an additional switch to a downstream CH gene (**22**). Sequential switching appears to be a physiologically relevant process, but its precise role has yet to be determined.

It was recently discovered that, in addition to somatic hypermutation and class switch recombination, germinal center B cell may also undergo receptor revision through reactivation of the V(D)J recombination machinery. It appears that Rag genes in germinal center B cells are actively suppressed when they bind avidly to an antigen but maintained or re-activated when the binding is weak. This may allow some low-affinity receptor to escape apoptotic death by the acquisition a higher affinity receptor through replacement of L-chain (or rarely the H-chain) (**23**).

1.7 Antigen presentation in B-cells

The B cell antigen receptor expressed on the surface of mature B cells consist of a membrane bound Ig (mIg) molecule and an Ig-α/Ig-β heterodimer (Intro, fig3). Ig-α and Ig-β are transmembrane proteins comprising an extracellular Ig domain and a cytoplasmic sequence of 61 and 48 amino acids respectively. These proteins are encoded by the B-cell specific genes mb-1 and B29, respectively (**24**). The association of the Ig-α/Igβ heterodimer with mIg is usually required for surface expression and is always necessary for the signalling function of the BCR complex. The B cell antigen receptor (BCR) regulates three central features of immune system function. First, it acts as a checkpoint regulator in B-cell development, controlling both allelic exclusion and early cellular transition. Second, as predicted by the clonal selection theory, cross-linking of the B-cell receptor with antigen can lead to clonal expansion and the production of specific antibodies. Third, the membrane-bound immunoglobulin functions as a receptor for antigen internalization and presentation. This event is crucial in promoting antibody responses against T-cell dependent antigen, because it provides the ligand for the binding of antigen specific helper T-cell. Upon binding, the antigen is internalized by receptor mediated endocytosis and fragmented into peptides within the enodosomal/lysosomal compartment of the cell. Some of the resultant peptides are loaded into a groove of a specialized set of cell surface proteins, the class II major hystocompatibility complex (MHC) molecules (see Intro, section 2.3). The resultant peptide/MHCII complexes are

expressed on the cell surface where they can be recognised by antigen specific CD4+ T cells.



These interaction results in the activation of the B cells through the action of cell surface molecules expressed by the T cell and cytokines produced by the T cell. In addition, signalling through the B cell receptor upon antigen binding favours the B-T cell interaction by upregulating the expression of MHC class II molecules and of costimulatory surface molecules such as B7-2, LFA-1 and ICAM-1 (25).

2.T LYMPHOCYTE

T-lymphocytes constitute the second major class of lymphocytes. They mediate a wide range of immunological functions that include the capacity to help B cells develop into antibody producing cells, the capacity to increase the microbicidial action of monocyte/macrophages, the inhibiton of certain type of immune responses, direct killing of target cells, and mobilization of the inflammatory response. These effects depend on the expression of specific cell-surface molecules and the secretion of cytokines.

T cell differ from B-cell in their mechanisms of antigen recognition. While immunoglobulins bind to antigenic epitopes on soluble molecules or on particulate surfaces, T cells invariably recognize processed peptides in association with one of the two class of MHC molecules, on the surface of other cells. Two major lineages of T cells can be distinguished, CD4+ and CD8+, that differ for the class of MHC they bind and for the different effector functions they mediate.

2.1. T-cell receptor

The T cell receptor (TCR) has many structural and functional analogies with the BCR on B cells. The TCR is a disulfide-linked heterodimer whose constituent chains are Ig supergene family members. The majority (90-95 %) of T cells express TCRs consisting of α and β chains. A small proportion of T cells carry TCR receptors made up of $\gamma\delta$ heterodimer. The role of these T cells subset in the immune response is not fully elucidated, however they recognise antigen directly, rather in association with MHC molecules (**26**).

All TCR Ig like chains consist of a variable N-terminal portion and C-terminal region, that is present as a single domain followed by a connecting peptide or hinge region, usually containing the cysteine for the disulfide linkage.

The V region of the TCR chains are assembled from a repertoire of different germline segments through recombinational mechanisms very similar to those occurring during Igs assembly. The strongest evidence of the existence of a common mechanism of

rearrangment has been that RAG knockout animals were unable to rearrange either TCR or Ig genes properly (**27**).

The V region of the TCR β chain is encoded by a gene made of three distinct genetic elements (Vβ, D, and Jβ) that are separated in the germline. Although the relative numbers of Vβ, D and Jβ genes differ from that for Ig, the strategies for creation of a very large number of distinct genes by combinatorial assembly are the same. One major difference is that TCR β has fewer V genes that IgH but much more diversity centered on the D/J region. Both junctional diversity and N-region addition further diversify the genes, and their encoded products. For TCR α only V and J segments are involved, but significant diversity is still generated from the large number of V and J segments. The trascritpional control of the TCR genes, similarly to Ig genes, depends on T-cell specific promoters and enhancers located. (**28**)

The two Ig-like TCR chains are always expressed in association with a set of non polymorphic polipeptides (γ, δ, ε, ζ) that constitute the CD3 complex. The extracellular domains of γ, δ,and ε are similar to a Ig C region domain while ζ has only a nine amino acid region extruding extracellularly.

The intracellular domain of these chains are the intracellular signaling domains of the TCR heterodimer. Each of these molecules contains a tyrosine based activation motif (ITAM) involved in signal transduction. (**29,30**).

Depending on the co-receptor associated to the TCR at the cell surface, two main lineage of T cells are distinguished, CD4+ or CD8+, that mediates different effector functions.

2.2 T lymphocyte development

To explain the restricted abilty of T-cells to react only with non-self antigen in association with self MHC molecule, it is important to describe the stages of T-lymhpocyte development. Upon entry into the tymus, T-cell precursors do not express TCR chains, the CDR3 complex, or the CD4 or CD8 molecules (triple negative thymocytes). Appropriate rearrangement at the TCR-β loci marks the transition to the double negative (DN) stage.

The pre-TCR complex at this stage is composed of a β chain associated with a pre T-α molecule assembled with the CD3 signal transducing chains. Signals transmitted through the pre-TCR complex on DN thymocytes not only cause proliferation and expansion, but also induce TCRα gene rearrangement and the expression of the CD4 and CD8 co-receptors, and therefore progression to the double positive (DP) stage.

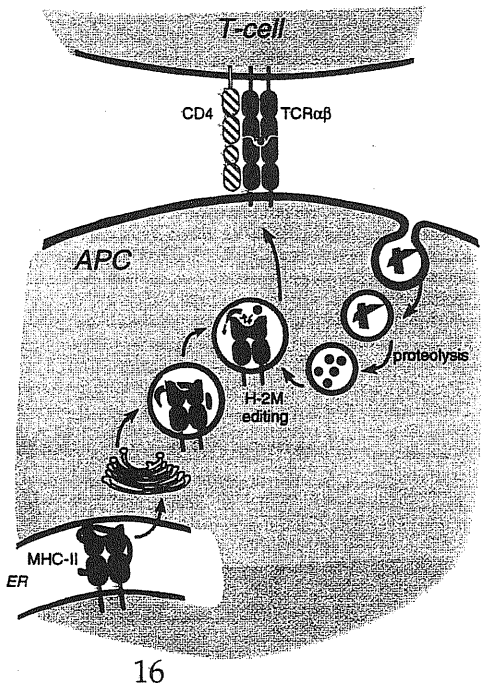
Single positive T cells that express either the CD4 or the CD8 co-receptor arise from the DP progenitors through a process of positive and negative selection. This results in a population of T cells that recognise with the proper avidity foreign peptides in association with self MHC molecules. Thymocytes with a TCR receptor specific for abundant ligand or with high affinity for peptide/MHC complexes undergo negative selection by apoptotic death. This negative selection is a major mechanism through which the T-cell compartment develops immunologic unresponsiveness to self antigens.

Positive selection ensures that only rare DP cells that express a TCR complex able to recognize self-MHC/peptide complexes with appropriate avidity will develop into single positive mature thymocytes. Studies during the last decade indicated that the CD4 and CD8 molecule contribute to this selection process by influencing both the avidity of the TCR-MHC interaction and the signaling function of the ligated TCR complex (**31**).

The molecular mechanism involved in lineage choice during the development of DP thymocytes into CD4 or CD8 SP cells remain largely uncharacterized. Examination of highly purified thymocyte sub-populations suggested that in the absence of an instructive CD8 lineage signal DP thympcytes develop by default to the CD4 lineage(**32**). Recent experiments indicate that over-expression of an activated form of Notch in thymocytes of transgenic mice favors the development of CD8 lineage cells, indicating that Notch signalling is pathway is involved in instructing the CD8 lineage choice (**33**).

2.3 $CD4^+$ T cells

$CD4^+$ T cells are the major regulatory cells of the immune system. One of their most important function is to stimulate B cells to make antibody responses to protein and other T cell-dependent antigen. CD4+ T cells respond when their TCR recognize a processed peptide in complex with a **class II major histocompatibility complex** molecule (MHC), on the surface of an antigen presenting cell. MHC class II molecules are made up of an _ and a _ chain, once again member of the Ig superfamily, with a highly polymorphic membrane distal domains responsible of binding to peptides. They are expressed in a limited set of cells, particularly professional antigen presenting cells, including B cells, macrophages, and dendritic cells. Peptides that bind to MHC class II molecules are generated in the endosomal pathway from proteins or pathogens internalized from the surrounding milieu. After their synthesis in the ER, class II α and β chains combine with the invariant chain (Ii), which has the dual function of preventing the binding of peptides residing in the ER and targeting the complex to endosomal compartments. By an active process, heterogeneous peptides generated by vesicular proteases replace the invariant chain. The newly generated peptide-class II complex is exported by an unknown mechanism to the cell surface, where it constitute the ligand for CD4+ T cells. The variable TCR chains recognise the antigen peptide and polymorphic region around the class II cleft, whereas the CD4 co-receptor binds to constant stretches along the class II α2 and β2 domains.

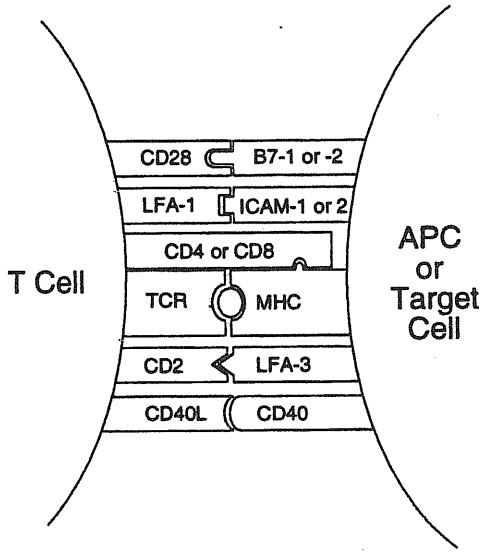


CD4+ T cells may be divided into two subcategories depending on the pattern of cytokines secreted. T_{H1} principally secrete IL-2, IFN-γ and lymphotoxin and are effective inducers of cellular immune responses. T_{H2} secretes IL-4, IL-5, IL-6 and IL-10, and are very effective in helping B-cells to develop in antibody producing cells. IL-2 enhances B-cell growth and Ig secretion. IL-4 also synergizes with signalling through CD40 to promote B-cell proliferation, but its more characteristic effect is to bias Ig class-switching, promoting the switch to the IgG1 and IgE isotypes by opening the constant region loci to transcription.(**34**).In contrast, IFN-γ enhances switching to the IgG2a. Therefore, an antibody response dominated by the IgG2a subclass indicates a T_{H1} mediated help, while IgG1 are prevalent in the case of a T_{H2} mediated help.

2.4 T-cell help in T-cell dependent antibody responses.

Induction of antibodies against protein antigens requires activation of the antigen specific B cell by means of an activated $CD4^+$ helper T-cell. In primary responses, the T cell is initially activated by antigen presented by potent professional APC like dendritic cells (DC). Immature dendritic cells reside in the peripheral tissues and are highly efficient in uptaking antigens. DC mature in response to inflammatory stimuli and migrate to secondary lymphoid organs where they become potent antigen presenting cells, thus providing the immune system with processed epitopes deriving from antigens aquired in the periphery. (**35**). The APC-T cell interaction leading to activation of T cell is very complex, involving both signals from membrane boud molecules as well as soluble signal as cytokines. Central to the activation of the CD4+T cell is the engagment of the TCR receptor by specific peptide/MHC class II complex on the surface of an APC. Many others non specific adhesion molecules, however, contribute to increase the avidity of the interaction. These include LFA-1 with ICAM-1, CD2 with LFA-3, CD4 with MHC II. Antigen presentation by dendritic cells is of crucial importance in the primary activation of T cells, because dendritic cells constitutively express costimulatory molecules necessary fo T-cell differentiation into effector helper cells. In this context, a major role is played by the

interaction between CD28 on T cells and B7-1 and B7-2 on dendritic cells. When CD28 is engaged by B7 molecules on APC it acts synergistically with signaling from the TCR to induce IL2 production by enhancing transcription of the IL2 gene and by stabilizing its mRNA, thus resulting in proliferation of specific T cell clones. Most importantly for the helper function of CD4+ T cells, this interaction induces the expression of CD40L on the T cell, a key molecule for B cell activation.



Primed CD4+ T cells deliver help primarily to B cells expressing the cognate peptide/MHC II complex. Thus, only B cells whose Ig receptor internalised the same antigen that was uptake by dendritic cells can process and present the correct peptide, and thereby receive effective help. Although B cells can internalize the antigen non specifically by pinocytosis, this process is inefficient compared to uptake mediated by the Ig receptor and is unlikely to provide sufficient processed epitopes for presentation to T cells.

Delivery of help by activated CD4+ T-cells requires direct interaction with the B cell displaying the appropriate antigen derived peptide/MHC class II molecule on the surface. The interaction is mediated by TCR and CD4 with the MHC class II plus other adhesion molecules, like those described for the interaction with dendritic cells (CD28/B7, LFA-1/ICAM-1, CD2/LFA-3). The most important interaction during the help process is

between CD40L on activated T cells and CD40 on B cells, as demonstrated by several experimental model. Blocking of such interaction result in greatly impairment of TD antibody responses. Stimulation of CD40, in association with intereleukin-4 secreted by the T cell, is required for proliferation and differentiation of B-cell into plasma cells and for the initiation of a germinal center reaction (**36**).

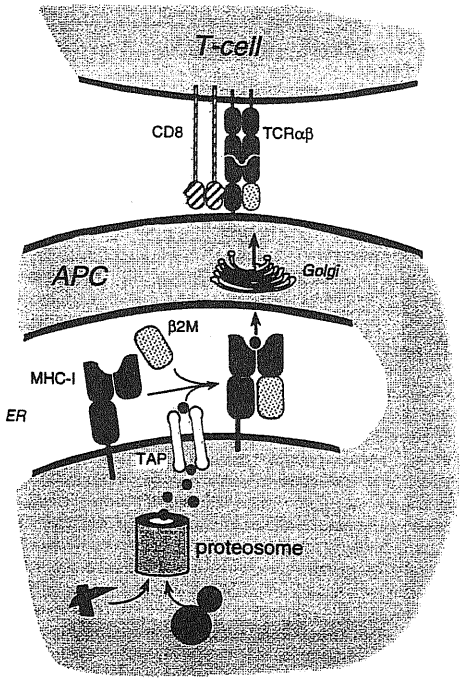
The subsequent event in the B-cell response program, including proliferation, Ig secretion and class switching either depend on or are enhanced by the action of T-cell derived cytokines. Many different cytokines have been shown to stimulate the proliferation of B-cells. Three helper T cell-derived cytokines, interleukin 2, interleukin-4 and interleukin-5 all contribute to B-cell proliferation and may act synergistically (**37**). With regard to antibody synthesis and secretion, IL-4 and IL-5 are the most potent inducers, but also IL-2 and IL-6 are required for in vivo production of antibodies against TD antigens. As mentioned in the previous section, cytokines are also involved in influencing Ig class switching. IL-4 promotes a switch to IgG1 and IgE, while INF-γ enhances switching to the IgG2a and TGF-b to the IgG2b and IgA isotpyes (**34,38**).

2.5 $CD8^+$ T cells.

$CD8^+$ T cells are primarily responsible for the elimination of target cells via direct killing, e.g., cells infected by viruses or those expressing tumor specific neoantigens. A CD8+ cells responds when its receptor recognise a particular antigen associated to an **MHC class I molecule**. The classical class I molecules are expressed throughout the body on essentially all cell types. They are composed of α heavy chain, and a light chain, $\beta 2$ microglobulin, which is not encoded in the MHC. The membrane distal $\alpha 1$ and $\alpha 2$ domains are highly polymorphic, whereas $\alpha 3$ is less polymorphic and has an Ig-like structure; $\beta 2$ also has an Ig like structure and is essentially invariable in sequence.

Peptides that bind to class I molecules are usually eight to ten amino acid long and have defined sequence motifs that vary with the particular class I isotype and alleles they are binding. These peptides are generated mostly from proteins synthesized within the target

cells, primarily those residing in the cytoplasm. Proteosomes chop the cytosolic proteins into peptide fragments, which are actively translocated into the lumen of the endoplasmic reticulum with the help of the transporter associated with antigen processing (TAP) molecule. In the ER, the peptide fragments bind to class I heavy and light chain dimers and the complex is transported to the cell surface. Here, they can be recognized by the receptor on CD8+ cells, the variable TCR chain focusing on the peptide and polymorphic residues contributing to the class I cleft, and the CD8+ molecule binding to a constant strech of sequences in the class I α3 domain .



The most striking action of activated CD8+ T cell is the lysis of the target cell. Cytotoxic T lymphocytes (CTL) may kill target cells by means of two different mechanism. The first one involves the production of perforin, a molecule that can insert into the membrane of target cells and promote the lysis of that cell. The second involves apoptotic death mediated by fas-fas ligand interaction. In fact, many active CTLs express large amount of fas ligand on their surface. The interaction of fas ligand with fas on the surface of the target cells initiate apoptosis in the target cell, leading to death of these cells.

3. B-CELL malignancies

3.1 Classification

B-cell lymphomas are clonal proliferation of neoplastic B-lymphocytes that are classified according to clinical features, morphology, immunophenotypic and genotypic analysis.

Among the broad group of lymphomas the most important distinction is between non-Hodgkin (NHL) and Hodgking (HD) lymphomas. The characteristic of the most representative groups in the NHL are listed below.

1) Chronic Lymphocytic Leukemia (CLL)

CLL is the most common leukemia of adults in the Western world. The tumor cells express the pan B-cell markers CD19 and CD20. In addition CD5, a T-cell marker that is expressed only on a smal subset of normal B cells, is present on the tumor cells. They typically also have low level surface expression of Ig heavy chain (usually IgM or IgM and IgD) and either k or λ light chain. The most common chromosomal anomalies are trisomy 12, deletion of 13q12-14, and deletion of 11q, each of which is seen in 20 to 30% of cases (**39**).

2) Follicular lymphoma

Follicular lymphoma is the most common form of NHL. The neoplastic cells resemble normal germinal center B cells, expressing CD19, CD20, CD10 and monotypic surface Ig. Follicular lymphoma cells also consistently express BCL2 protein, in distinction to normal follicular center B cells, which are BCL2 negative. The hallmark of follicular lymphoma is a (14;18) translocation, which leads to justaposition of the IgH locus on chromosome 14 and the BCL2 locus on chromosome 18. This translocation leads to overexpression of BCL2 protein. BCL-2 is an antagonist of apoptotic death and appears to promote the survival of follicular lymphoma cells. 30 to 50% of follicular lymphoma transforms to diffuse large B-cell lymphoma.

3) Diffuse large B-cell lymphoma

The diagnostic category of diffuse large B-cell lymphoma encompasses a heterogenous group of tumors that togheter constitute about 20% of all NHL. These mature B-cell

tumors express CD19 and CD20. Tumors of follicular center origin often express CD10,, most have surface Ig. Within this group of tumors, about 30% contain the t(14;18), the characteristic chromosomal abnormality of follicular lymphoma. An additional 20 to 30% of tumors contain various translocation that have in common a breakpoint involving the BCL6 locus on chromosome 3. Intact BCL6 transcripts are increased by the translocation. The BCL6 gene encodes a zinc-finger transcription factor that is expressed at high levels in germinal centers but down-regulated upon B cell activation, consistent with a role in control of somatic hypermutation and class switching.

4) Burkitt lymphoma

Within the category of Burkitt lymphoma fall: African Burkitt lymphoma (endemic), sporadic Burkitt lymphoma and a subset of aggressive lymhpomas occuring in patients infected with HIV. These are tumors of relatively mature B cells expressing surface IgM, monotypic k or l light chain, CD19, CD20, and CD10. All forms of Burkitt lymphoma are highly associated with translocation of the c-myc oncogene on chromosome 8. The partner is usually the IgH locus t(8;14), but may also be the k (t(2;8) or λ (t(8;22) light chain locus. c-myc plays a broad role in transcriptional regulation of cell growth, differentiation and apoptosis (**40,41**). A direct role for t(8;14) in the neoplasia was demonstrated by transgenic mice expressing c-myc under the control of the IgH intronic enhancer, that exhibit hyperplasia of pre-Bcells and develop agressive clonal B-lineage malignancies within several months(**42**).

5) Mantle cell lymphoma

Mantle cell lymphoma constitutes about 3% to 7% of NHL. It is a B-cell neoplasm that expresses CD19, CD20, moderately high levels of surface Ig heavy chain (usually IgM and IgD), and either k or l light chain. In 70% of cases there is a (11;14) translocation involving the IgH locus on chromosome 14 and a locus on chromosome 11 variously known as BCL1 or PRAD1. The BCL1 locus encodes cyclin D1, involved in the regulation of the G1 to S phase progression during the cell cycle. BCL1 rearrangements

lead to increased expression of cyclin D1 protein, which may contribute to tumorigenesis by causing loss of cell cycle control.

Hodgkin disease encompasses a group of disorders that differ from NHL in several respects. It is characterised morphologically by the presence of distinctive neoplastic giant cells, Reed-Stenberg cells, which induce the accumulation of reactive lymphocytes, histiocytes and granulocytes in response to cytokines secreted by RS cells. Three mayor subtypes of HD can be distinguished: nodular sclerosis, mixed cellularity, lymphocyte predominance. Recent studies indicate that RS cells in the lymhpocyte predominance subtype derive from germinal-center B cells since their V regions carry somatic mutations. However, the orign of RS cells in other subtypes still remain debated because in mixed cellularity and nodular sclerosis, RS often have features of B lymphocytes, yet they have some attributes of monocyte/ macrophages.

3.2 Transforming events as byproducts of B-Cell differentiation in the germinal center

Both in NHL and HD, analysis of the tumor derived V regions revealed, in most of the cases, the presence of somatic mutations. This indicates that neoplastic B-cells derive from cells at germinal center or post–germinal center stage of development. The high proliferative rate and gene rearrangement processes that occurs in the germinal center microenviroment may themselves contribute to the genetic alterations leading to lymphoid neoplasia. Occasional failures in the mechanism of DNA break and repair involved in the processes of class switching and somatic hypermutation appear to play a decisive part in B-cell tumorigenesis through the generation of chromosomal translocations into the immunoglobulin gene loci. In non-Hodgkin lymphomas, translocations typically place a structurally intact cellular proto-oncogene under the regulatory influence of the highly expressed Ig gene, leading to effects on cell growth, cell differentiation or apoptosis .

The well characterized t(8;14) that places the oncogene c-myc under the control of the strong IgH intronic enhancer, was linked to the process of class switching because the

chromosome 14 breakpoints occur within the switch region upstream of Cμ, Cγ or Cα. (**43**). Moreover the translocated c-myc allele often bears point mutation characteristic of somatic hypermutation that agument its transforming activity in vitro (**44**). This indicates that the somatic hypermutaion machinery occasionally targets genes outside of the Ig loci, contributing to the additional genetic changes required for progression of the malignant clone. This is further supported by the finding that the bcl-6 gene is frequently hypermutated in various type of of NHL and also in normal germinal center B cells.

The t(14;18) translocation of the bcl-2 oncogene, characteristic of follicular lymphoma, appears to be mediated by V(D)J recombinase as suggested by the fact that chromosome 14 breakpoints frequently occur at or near the RSS bordering DH or JH segments (**45**). Indeed, RAG proteins were shown to catalyse a reaction in which DNA from immunoglobulin loci invades non immunoglobulin DNA (**46**). Translocations resulting from aberrant V(D)J recombination were believed to be restricted to B-cell precursors in the bone marrow, but such rearrangement occasionally take place in germinal-center B cells (**47**)

4.IDIOTYPES

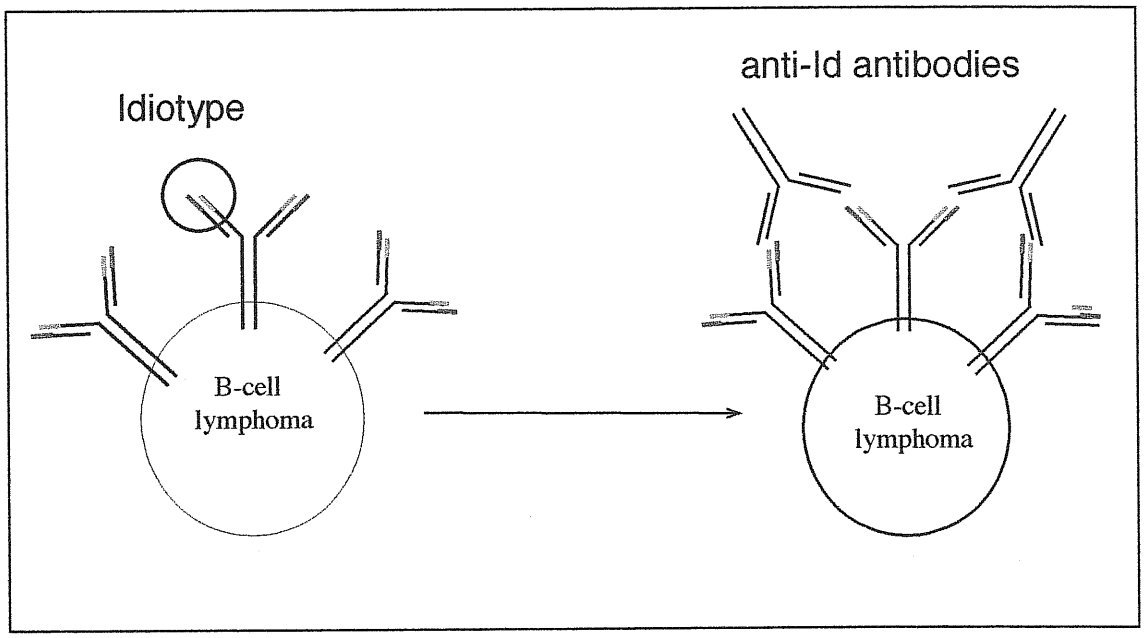
Immunoglobulin V regions are generated during the process of genetic rearrangment described in section 1.2-1.3. Consequently, each immoglobulin molecule carries at the levels of its V regions unique antigenic determinants that are called idiotopes. The combination of idiotopes on VL and VH define the idiotype of a given immunoglobulin molecule. Immune responses specific for idiotypes are called anti-idiotypic. Anti-idiotypic antibodies may be directed against private idiotypic determinants or against cross reactive idiotypes shared by different antibodies (**48**). Most anti-idiotypic antibodies bind to the antigen combining site of Ig molecule as evidenced by inhibition of binding to the specific antigen in the presence of anti-Id antibodies. However, in other instances, anti-Id antibodies were shown to bind to hypervariable region determinants close but outside of the antigen binding site. The crystal structure of one monclonal anti-idiotope bound to the corrisponding idiotope (hen egg lysozymes) showed that nearly all of the contacts are between amino acis side chains from the hypervariable regions of the idiotope and anti-idiotope (**49**).

The idiotypic network theory postulated by Jerne in 1974 proposed that idiotypic interactions play an important role in the regulation of normal immune responses.. According to this theory an antigen stimulates a specific antibody response which in turn induces a wave of anti-idiotypic responses that can recognise the original antigen-specific responding lymphocytes and inhibit or augments their activation. However, this hypothesis was never supported by quantitative data on the magnitude of this effect. A by-product of this theory that was investigated in several system implies that anti-idiotypic antibodies carry the internal image of the antigen . If x is an antigen, anti-anti-x might in some cases bear structural resemblance to the antigen x. X and anti-anti-x shares the ability to combine with hypervariable regions of anti-x. This approach has been particularly useful in the case of cell-surface receptors that are very difficult to isolate to generate anti-receptor antibodies. In these cases it was possible to use the ligand to induce anti-ligand antibodies and anti-anti-ligand antibodies that bind to the receptor because they can mimic the ligand

(**50,51**). In addition, it was demonstrated that immunization using anti-idiotpyic antibodies (anti-anti-pathogen) could induce protection against a pathogen by mimicking its structure (**52**).

5. ANTI-IDIOTYPIC IMMUNOTHERAPY OF B-CELL LYMPHOMA

Since idiotypes reflects the uniqueness of each Ig molecules, and each B-lymphocyte express a single arrange of light and heavy chain V regions, idiotypes represent clonal signature of individual B cells. In the case of surface Ig+ B-cell malignancies, such as B-cell lymphomas, idiotypes are ideal tumor specific antigen that can be used as target for immunotherapy. Although officially self antigens, idiotypic determinants are potentially immunogenic to the host.



5.1. Vaccination with idiotypic immunoglobulin protein

The ability of syngeneic tumor derived Ig to induce antibodies as well as idiotype specific resistance to tumor growth was first demonstrated in the MOPC-315 tumor system (**53, 54**). Since then the use of purified idiotypic protein for active immunotherapy of B-cell lymphomas has been documented in several mouse models.

In all this studies the protein itself resulted poorly immunogenic and it was necessary to conjugate it to a strong carrier such as keyhole limpet hemocianin (KLH) and to co-adminster it together with an adjuvant to induce high titers of anti-idiotypic antibodies and tumor protection (**55,56**). It was shown that tumor cells developed a state of tumor dormancy in the immune host (**57,58**), maintaining the ability to growth upon transfer to a

naive recipient. Further studies of one of the models, the BCL1 lymphoma, revealed that the dormant cells undergo dramatic changes in their morphology, cell cycle status and oncogene expression (**59**).

To evaluate the relative roles of cellular versus humoral immune response, passive transfer studies were performed. Transfer of immune serum but not of immune lymphocytes to naive recipients conferred protection against subsequent tumor challenge, indicating that rejection was mainly mediated by anti-Id Ab (**57**). Vaccination with idiotypic IgM generated CD4+T cells proliferating specifically in response to idiotypic antigen, but very little or none CD8+ T cells (**60**).With regard to the molecular nature of the idiotypic antigen recognised by the T cells, it was found that anti-Id T cell hybridomas established from vaccinated mice behaved like conventional CD4-positive cells, responding to processed antigen in association with MHC class II (**61**). These findings indicated that immune attack on a neoplastic B cell could be aimed at processed idiotypic peptides.

Passive immunotherapy using specific anti-Id was effective in inducing long term remission in human patients bearings B-cell malignancies in clinical remission after chemotherapy (**62,63**). However, passive immunotherapy presents two major disadvantages: the need to produce large amount of anti-Id Ad in other species, and the inability to cover point mutations in the idiotype recognised by the particular antibody that may occur during the course of the disease. The hybridoma technology allowed to overcome both these problems by providing an efficient means to produce large amount of Id specific protein from tumor biopsy material. The purified Id protein is injected as an immunogen thus actively inducing a polyclonal immune response that is more likely to cover from tumor escapes. Administration of tumor derived immunoglobulin produced by the hybridoma technology, conjugated to KLH and mixed with an immunological adjuvants, was shown to induce both antibodies and cell-mediated responses in two clinical studies (**64,65**).

5.2. The use of cytokines in immunotherapy

The pilot studies described in the previous section highlighted the necessity of improving the immunogenicity of autologous tumor derived idiotypic protein to avoid chemical coupling to immunogenic carriers and co-administration with immunological adjuvants that may have deleterious side effects. Moreover it was important to find means to activate strong cell-mediated immune responses to extend the use of Id therapy to the cases of surface negative B-cell malignacies such as myeloma.

Cytokine may influence both the magnitude and phenotype of the immune response to protein antigen. They can influence and amplify immune responses by affecting the proliferative capacity and differentiation of lymphoid and antigen presenting cells. Cytokine as immunomodulatory molecules in vaccination against cancer have been tested in several models over the past few years by transducing tumor cells with different cytokine genes. Transduced tumor cells were rejected once injected into the host and provided also protection against subsequent tumor challenge with wild type tumor cells(**66**). Several of these cell-based vaccine strategies have been shown to induce T-cell mediated systemic antitumor immunity, either by enhancing the processing and presentation of tumor antigens by host APC or by facilitating effective Ag presentation by the tumor vaccine itself. A comparative study analyzing IL-2, IL-4, IL-5, IL-6, g-IFN, IL-1 and GM-CSF (granulocyte/macrophage colony stimulating factor) revealed that GM-CSF was the most potent in the protection assay. GM-CSF has pleiotropic effect, including augmentation of antigen presentation in a variety of cells, MHC class antigen expression on monocytes and amplification of T-cell proliferation. Most remarkably GM-CSF has an important role in the differentiation and maintenance of dendritic cells (**67**), which are the most powerful class of antigen presenting cells in the initiation of immune responses (**35**). The group of Levy showed that by fusing the Id protein derived from the 38C13 mouse B-cell lymphoma to GM-CSF the Id was converted to a strong immunogen capable of inducing anti-Id antibodies without other carrier proteins or adjuvants and of protecting animals from challenge (**68**). In this study the protein was a chimera between murine,

tumor derived, V regions and human constant regions. A plasmacytoma Ig^- cell line was co-transfected with two different plasmids: one containing the 38C13 VH cloned upstream of human IgG1 heavy-chain constant region gene and the second containing the 38C13 VL cloned upstream of human Igk light chain. A genetic fragment encoding murine GM-CSF was inserted at the N terminus of the human CH3 exon. The protein was purified from the supernatants of transfected clones by protein A cromatography and tested for the maintenance of the correct idiotype structure and for GM-CSF biological activity. Mice immunized with the chimeric Id fused to GM-CSF developed high titers of anti-Id Ab, comparable to those induced by Id conjugated to KLH, while the chimeric Id protein without GM-CSF failed to induce detectable levels of anti-Id Abs. Therefore, the authors concluded that the helper effect of the xenogenic human constant region was negligible. Consistently only mice immunized with Id-GMCSF were resistant to tumor challenge and physical linkage to GM-CSF was shown to be required for the adjuvant effect.

In a later study the same group compared the effect of different idiotype-cytokine fusion proteins (**69**). Plasmids and fusion proteins were produced following the same strategy described above. The CH3 exon of the human Cγ1 gene was linked to genetic fragments encoding IL-2 and IL-4. Analysis of Id-IL2 with Id-IL4 and Id-GM-CSF in immunization experiments showed that all three fusion proteins were capable of eliciting significant levels of specific Abs against the Id without the use of carrier proteins or adjuvants. However, minor differences emerged: Id-GM-CSF was the most potent in inducing high titers of anti-Id Ab in the primary response, Id-IL2 was the only fusion protein able to evoke anti-Id Ab of IgG2a subclass whereas the others induced only IgG1 immunolgobulins. Survival against tumor challenge was comparable in all mice having significant titers of anti-Id Abs regardless of the fusion protein used. The mechanism by which the Id-cytokine fusion proteins exert their effects are discussed by the authors that suggest a cooperation between targeting of the antigen to appropriate APC via the cytokine and stimulation of cells by the presence of the cytokine in the same environment where the antigen is produced.

A different conclusion was obtained by the group of Kwak (**70**), who evaluated the effect of GM-CSF on the immunogenicity of the idiotype derived by the 38C13 lympohma. In this case, the idiotypic protein was a complete mouse IgM purified from the supernatant of a 38C13 derived hybrydoma. The protein was conjugated to KLH and injected together with recombinant GM-CSF. The results indicate that although titers of anti-Id antibodies were not significantly increased, the addition of the cytokine protected animals against tumor challenge with 100x cells the dose that was rejected by immunization with the immunogen alone. In vivo T-cell subset depletion experiments clearly demonstrated that both CD4+ and CD8+ were required. It therefore appears that the effect of free GM-CSF is different from that of a fusion protein containing GM-CSF.

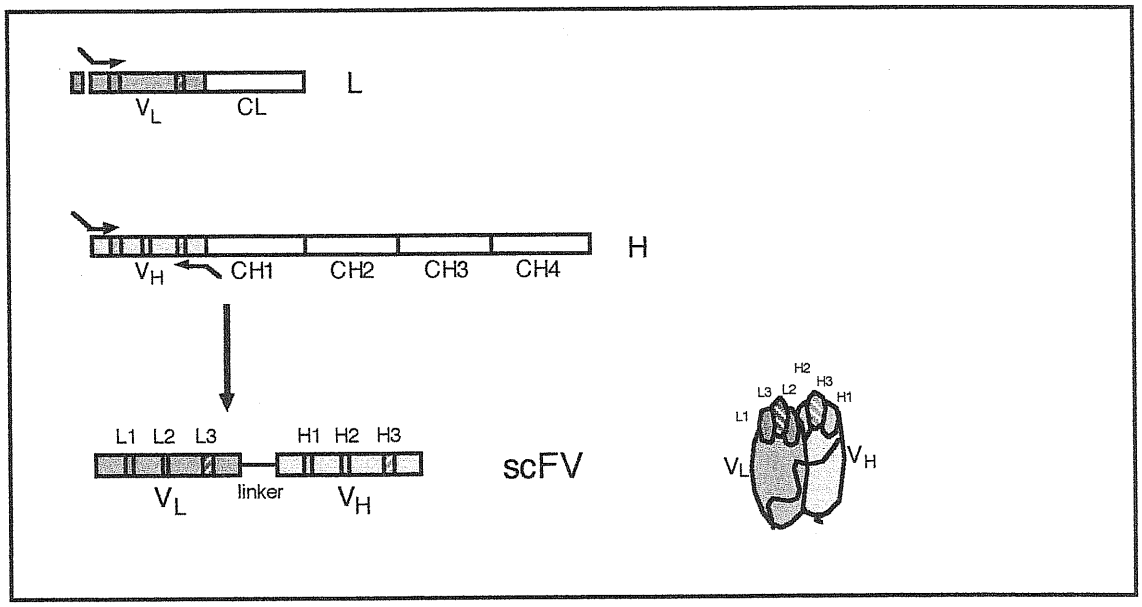
5.3. Assembly of tumor derived V regions in scFv format

Despite the encouraging results of protein vaccination against lymphoma, this approach was limited by the difficulty of producing sufficient quantities of idiotypic proteins for each patients, because the technology involving heterohybridomas and transfectomas is both expensive and time consuming. A more feasible approach relies on molecular cloning techniques and DNA-based antigen delivery system.

The extensive characterization of the molecular structure of human Ig V variable region genes and their repertoire led to investigated PCR-based approach to clone specific heavy and light chains from B-cell malignancies. V region genes of patients with lymphoma can be amplified using sets of Ig family specific oligonucleotide primers to leader and constant regions. Then V genes are sequenced and the malignant clone is readily identified based on its overrepresentation in several experiments.

Once the genes are cloned, a commonly used strategy involves the assembly of V genes into single chain Fv (scFv), consisting solely of VH and VL genes linked together in frame with a flexible linker. The scFv is the smallest fragment that shows equivalent binding affinity to the parent antibody. The most commonly used linker is a 15 amino acid residue

fragment of (Gly4Ser)3, although fully active scFvs have been produced using other linker with flexible 14 to 25 residues (**71**).



Production of scFv proteins in bacterial system has been generally succesful but problems related to the purification of the protein and consequently, to the maintenance of correct folding have been reported Like lymphoma derived Ig, scFv proteins are per se not immunogenic and need to be conjugated to a carrier. Attempts to cross link scFv to KLH failed, possibly due to the fragile conformation of scFv (**72**). A scFv molecule encoding the lymphoma derived V regions can be linked to immunoenhancing domain, produced in bacteria and used as immunogens. However, before this strategy could be fully exploited, it was overcome by the advent of DNA vaccination. DNA vaccination offers the opportunity to manipulate the antigen encoding cassette by creating chimeric immunogens and to administer cytokine encoding plasmid to modulate the immune response. As it will be described below, this strategy allowed to modify the immunogenicity of scFvs by linking them to different carriers and to elicit effective antitumor immunity in several animal models.

5.4. DNA Immunization

DNA or genetic immunization is a technique based on the use of genes encoding the protein of interest rather then the protein itself, to induce an immune response. The gene needs to be cloned into an expression cassette under the control of viral or eukaryotic regulatory elements. The plasmid is delivered to the host either by injection of a saline solution via intramuscular or intradermal route, or into the epidermis by particle mediated bombardment of DNA coated gold particles. The plasmid spontaneously transfects host cells where the encoded protein is produced.

Since the first demonstration by the group of Wolff that a foreign gene (β-gal) injected as plasmid DNA into mouse skeletal muscle could be expressed in vivo (**73**), several animal studies showed that naked DNA vaccines can induce effective immunity to a wide variety of viral bacterial and neoplastic antigens (**74**).

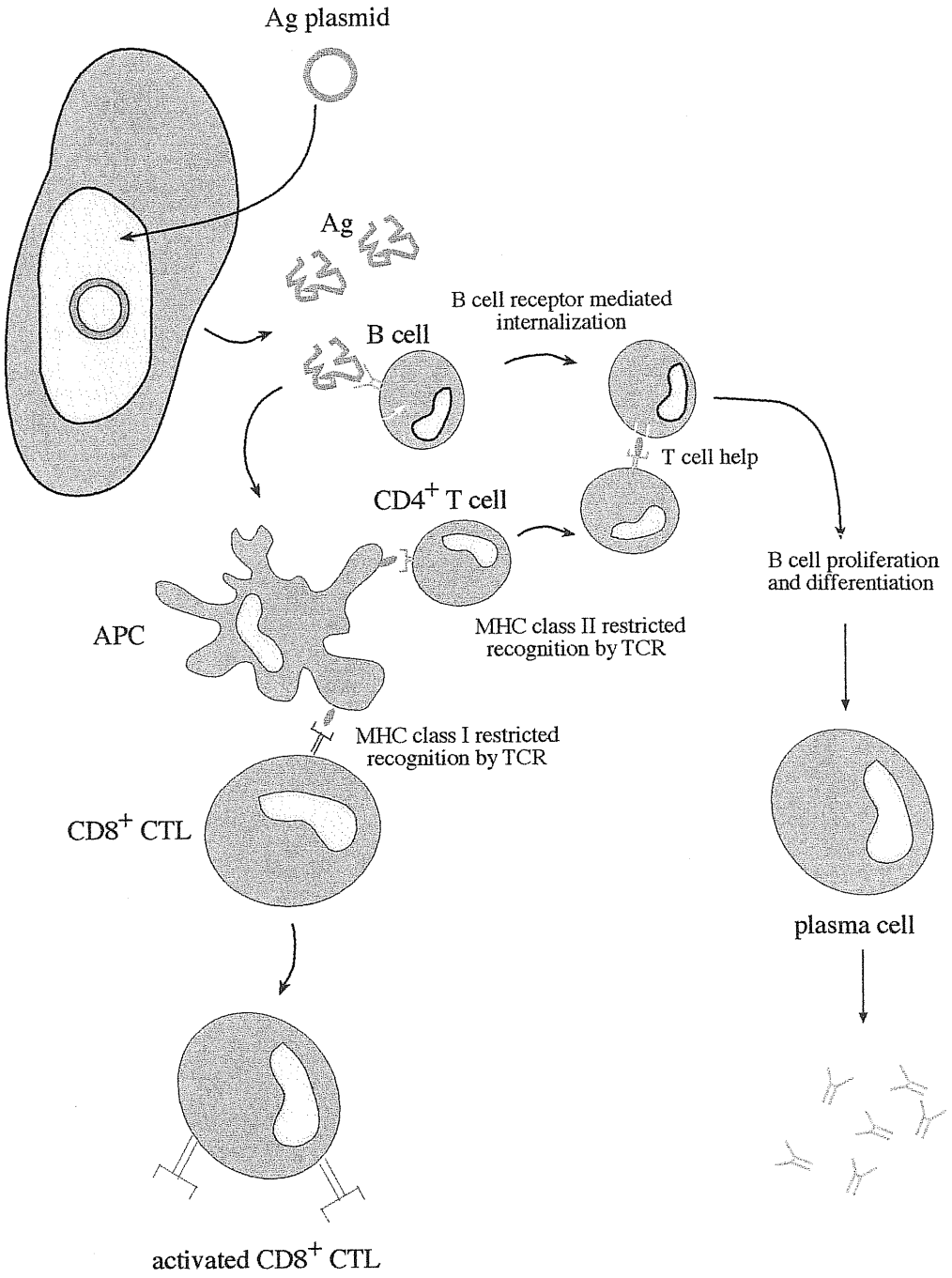
Antibody, Th cells and cytotoxic T-lymphocytes (CTL) responses have been described upon genetic immunization (**75**), but the roles of different cell types and the mechanisms of antigen presentation are not fully understood. DNA applied either intramuscularly or intradermally is mostly taken up by muscle cells or keratinocytes respectively (**73,76**), but these cells are unable to initiate primary T-cell responses. Several recent studies have provided evidence for the involvement of dendritic cells in priming naïve T-cell after DNA vaccination (**77,78**). However, it is not clear to what extent DC partecipate in stimulating an immune response after direct transfection or through taking up antigen released by other transfected cells. A study from the group of H. Tighe showed that both mechanisms appear to be involved, but the bulk of the immune response is dependent on expression of Ag by non-lymphoyd tissue and transfer to antigen presenting cells (**79**). A different group examined the response after vaccination with a plasmid encoding for a non-secreted protein, showing that although the number of transfected dendritic cell was very small, it was sufficient to provoke general activation of dendritic cells, thus providing optimal conditions for effective T-cell activation and maintenance of memory (**80**).

The immune response to certain antigens may be modulated by the route of injection. DNA immunization in saline into skin or muscle with influenza hemagglutinin (H1) gene raised a predominantly Th1 response with mostly IgG2a anti-H1 Abs, while gene gun DNA immunization produced a predominantly Th2 response with mostly IgG1 anti-H1 Abs (**81**). However others reported induction of both types of immunity, humoral and cell mediated, by gene-gun mediated DNA transfer into skin of mice, particularly the induction of efficient tumor-protective CTL to mutant p53 or from HIV gp120 (**82**).

Another interesting feature of DNA vaccine is the inherent immunogenicity of unmethylated guanosine (CpG motif), found in many plasmid and bacterial DNA, that were shown to augment both antibody and CTL response and to switch on antigen-dependent Th1 immunity (**83**).

As in the case of proteins immunization, cytokine and immunological adjuvants have been widely tested in DNA vaccination. Cytokine encoding plasmids offer the obvious advantage to overcome preparation of large amount of protein. In addition, since the cytokine is continuously produced its activity is prolonged. Also other co-stimulatory molecule have been tested in DNA vaccine for their ability to agument the immune response. A CD40L encoding vector was shown to enhance the immune response against a β-galactosidase expressing vector (**84**). This effect is probably due to constitutive activation of B-cell by CD40L, which normally would occur only when T cells are activated by APC.

Another mean to improve the immune response to a model antigen was described by J.S.Boyle, who fused the hinge-CH2-CH3 portion of a human immunoglobulin to L-selectin or cytotoxic T-lymphocyte antigen 4 (CTLA4) with the aim of targeting the antigen to sites of immune induction. In fact, L-selectin is expressed on the surface of naïve lymphocytes and directs the entry of lymphocytes into lymphonodes. CTLA4 is expressed on activated T cells and binds to B7-expressing cells, including APC, which are potent initiators of immune responses. Both fusions were shown to enhance humoral and cellular immune responses to the DNA vaccine (**85**).



DNA immunization
Schematic represention of the steps involved in the induction of the immune response folowing DNA vaccination.

5.5. Idiotypic DNA vaccines

In 1996, the group of F.Stevenson reported the first example of DNA immunization using a scFv containing lymphoma derived variable regions. The model was the BCL1 lymphoma and the scFv contained the two BCL1 V regions in VH-VL orientation separate by a 15 amino acids linker (Gly4Ser)x3. Intramuscular injection of plasmid DNA resulted in production of anti-Id Ab in half of the mice, but titers were generally low. To improve vaccine efficacy the authors co-administred a plasmid encoding either GM-CSF or IL2, whose effect was already demonstrated at the protein level (**59**). Mice co-immunized with the scFv encoding vector plus either of the two cytokine vectors showed higher titers of anti-Id Ab, but none of the responses was sufficient to confer protection against tumor challenge. (**86**).

A protective immune response by idioypic DNA immunization was successfully achieved by the group of Levy in the 38C13 lymphoma model (**87**). The construct used for immunization expressed a chimeric immunoglobulin with mouse tumor variable regions fused to human λ and k constant region. Independently from the presence of a GM-CSF encoding cassette linked to human IgG1, the plasmid generated antibodies against the tumor Id. This was surprising since the same group previously demonstrated the an identical chimeric Id protein was not immunogenic unless fused to GM-CSF. The reason for this discrepancy is discussed by the authors who attributed this effect to differences in the presentation and processing upon DNA vaccination. Two routes of immunization were compared resulting in isotype specificity differences between intradermal (predominant IgG1 isotype) and intramuscolar (predominant IgG2a isotype) injection. The contribution of the human constant region to the immunogenicity of the construct was also assessed by replacing human with murine constant region. These plasmids with or without GM-CSF failed to induce detectable titers of anti-Id Ab, indicating that the presence of a foreign constant region was essential to induce an anti-Id immune response. All the mice that had

mounted an anti-Id immune response were protected from a tumor challenge with a lethal dose of 38C13 tumor cells, indicating a major role for anti-Id antibodies in protection.

The same group tested also a scFv derived from the 38C13 lymphoma alone or fused to GM-CSF or an immunoenhancing peptide from IL1-β (**88**). The scFvs were delivered as plasmid DNA and as protein purified from bacteria. Results showed that both scFV-GMCSF and scFv-IL1–β as fusion proteins induced protective anti-Id responses, while only scFV-IL1β was protective as DNA vaccine. The scFv-GMCSF DNA vaccine may have differed from the corresponding protein formulation in proper folding of the scFv, which is essential for antibody dependent cell mediated cytotoxicity (ADCC)-mediated protection in this tumor model. The scFv fusion protein was purified by affinity cromatography using anti-Id Ab known to bind properly folded Id, thus selected for retention of proper folding.

According to the recently proposed "danger model", vertebrates have evolved innate immune mechanisms by which the immune system might distinguish, dangerous non-self from non dangerous self antigens (**89**). In this respect, King and colleagues provided Id with an "alert" signal, by fusing scFv to fragment C of tetanus toxin (FrC) (**90**). This approach has been tested as a DNA vaccines in two murine tumor models, one expressing IgMk on the surface of the B-cell lymphoma A31, and the other a myeloma cell line which secretes IgG2bk, 5T33. DNA vaccines encoding scFv-FrC promoted an anti-idiotypic antibody response and induced strong protection against the syngeneic A31 B-cell lymphoma. Similarly, DNA vaccination with scFv-Fr C fusion in the myeloma model 5T33 induced tumor protection. Although the authors described this latter model as a surface Ig-negative tumor, others have concluded that 5T33 cells express surface Ig by surface staining experiments (**91**).

The mechanism of tumor protection induced by DNA vaccination has been investigated by the group of Levy by passive transfer and depletion studies (**92**). Serum, but not adoptive transfer of spleen and lymph node cells, protected naïve recipients against tumor growth. Thus, in the model analysed, tumor-protective effects of DNA vaccination can be largely

attributed to idiotype-specifc humoral immunity. Whether this effect implies a role for Ab-dependent effector mechanism such as complement or ADCC (Ab-dependent cellular cytotoxicity), or is the result of tumor growth inhibition through direct signalling has not been established.

A novel strategy has been recently proposed, that consist in fusion of tumor antigens to chemokines, for targeting antigen presenting cells in vivo. Chemokines are secreted proteins that induce inflammatory response by orchestrating the selective migration, diapedesis and activation of blood-borne leukocytes. They act via binding to specific cell-surface receptors, which are internalized after binding with the ligand. Several chemokine receptors are expressed by dendritic cells with a pattern that varies according to their state of development (**93**).

ScFvs from two different B-cell lymphomas, (A20 and 38C13) have been cloned and fused to pro-inflammatory chemokine genes MCP-3 and IP-10. Chemokine fusion proteins retained the conformation of the respective Id, while converting scFv into a fusion protein that could bind chemokine receptors and cause chemotaxis both in vitro an in vivo. Moreover, chemokines IP-10 and MCP3 rendered the self scFv antigens potent immunogens. In both tumor models vaccination with DNA constructs encoding the scFv chemokine fusions generated superior protection against a large tumor challenge, as compared to the best available protein vaccines. The requirement for linkage of conformationally intact scFv and functionally active chemokine strongly suggested that the mechanism underlying these effect was the novel targeting of antigen presenting cell for chemokine receptor-mediated uptake of antigen, rather then simple recruitment of APC to tumor by the chemokine. In addition, these fusions were shown to induce also critical effector T cells (**94**).

CHAPTER 2

MATERIAL AND METHODS

1.Mice and cell lines.

Ten to twelve weeks old Balb/c mice were obtained from Harlan (Milan, Italy) and housed at the ICGEB animal house.

BCL1 is a spontaneous B-cell leukemia/lymphoma of BALB/c origin that expresses high levels of surface IgM/λ (**95**). The cell line exists in two variants, which express the same surface IgM idiotype. The original BCL1 clone was used in the tumor challenge experiments and was maintained through serial passages in singeneic BALB/c mice. BCL1 3B3 is a variant that unlike the original clone can be maintained in tissue culture and was used for FACS analysis and RNA extraction. Both cell lines were kindly provided by Dr. E. Vitetta (Dallas, Texas). A third variant of the BCL1 cell line ($BCL1F^+$) that grows only in vivo but with faster kinetic than the original BCL1 clone was provided by F. Stevenson (Southampton U.K.).

The hybridoma 123bcl1 secreting BCL1 IgM was kindly provided by Dr. F.K. Stevenson (Southampton, UK) as well as Mc10 4A12, a rat mAb V_H BCL1 specific, kindly provided by Dr. F.K. Stevenson, Southampton, UK (**96**).

The Ig^- non secreting mouse myeloma Sp2/0 cell line used for transfection experiments was purchased from the American Type Culture Collection (Rockville, MD. USA).

All the in vitro cell lines were mantained in RPMI (Gibco) supplemented with 10% FCS, and 50 μM 2-mercaptoethanol.

2.Cloning of DNA vaccines constructs

Total RNA was isolated from BCL1 3B3 cells using the guanidinium thiocyanate procedure and reverse transcribed using random hexamers and the GeneAmp RNA/PCR kit (Perkin Elmer Cetus, Norwalk, CT). The tumor derived VH region was amplified from the cDNA using the sense primer VH/PstI (5'-GTGCAGCTGCAGCAGTCTGG) and the antisense primer mjH/BspEI (5'-AGAGCCTCCGGAGGAGACTGTGAGAGT). The VL region was amplified using the sense primer VL/ApaLI (5'-TAGTGCACTCGCAGGCTGTTGTGACTCA) and the

antisense primer JL/SpeI (5'-GCACTAGTGCTGCCACCTAGGACAGTGACCTT). The PstI/BspEI VH fragment and the ApaLI/SpeI VL fragment were then cloned into the pUT-SEC plasmid containing a HindIII/ApaLI genomic secretion signal sequence (**97**). In the final step the HindIII/BspEI fragment (signal peptide-VL-linker-VH) was subcloned in a pcDNA3 vector containing a human IgG1 CH3 domain cloned as a HindIII/KpnI fragment resulting in plasmid **pBCL1-CH3**. Plasmid **pBCL1** was generated by substitution of the hu-CH3 coding region with a HindIII/KpnI linker containing a stop codon. The **pBCL1-SAPA** plasmid was generated by substitution of the HindIII/KpnI hu-CH3 domain with a HindIII/KpnI SAPA fragment.

The cytoplasmic **pBCL1-CH3Δsp** construct was produced by substituting the ApaLI/HindIII fragment encoding the signal peptide with a short 15 aa linker (ApaLI/Hind III).

For construct **pBCL1-CH3memb** The cDNA of the human γ1 with its EMPD, TM and cytoplasmic domains was amplified by RT-PCR from mRNA extracted from normal human lymphocytes with primers 5G1 (TACTCCGGAGGCTCTGGCGGGCAGCCCCGAGAACCACA) and 3G1 (CTGAATTCTTAGGCCCCCTCTCCGATCATGT). The amplified fragment was cloned BspEI/EcoRI into the pcBCL1 vector generating pBLC1-CH3memb.

The **p6C6-CH3** plasmid was previously built in the laboratory by amplification of VL and VH from the 6C6 hybridoma (**97**) and inserted into a vector containing the human CH3 domain, according to the same procedure used for plasmid pBCL1-CH3.

To construct plasmid **pV_L^{BCL1}/V_H^{6C6}-CH3** the fragment PstI/BspEI encoding V_H^{6C6} from p6C6 was inserted into the pBCL1-CH3 vector in place of the V_H^{BCL1}. Similarly, plasmid **pV_L^{6C6}/V_H^{BCL1}-CH3** was generated by substitution of the ApaLI/SpEI fragment encoding the V_L^{BCL1} with the one encoding V_L^{6C6}.

For the construction of **pGM-CSF** total RNA was extracted from mouse splenocytes and subjected to a RT-PCR step using the forward primer HindIII-GM (5'-CTAAGCTCCTGAGGAGGATGTGGC) and the reverse primer Xho-GM (5'-CCCTCGAGTCCTCATTTTTGGACTGGTT). The amplified fragment was then inserted in the polylinker of pcDNA3 as a HindIII/XhoI fragment.

Similarly plasmid **pCD40L** was amplified from RNA extracted from mouse spleenocytes by reverse transcription using a forward primer HindIII-CD40L (5'-

CCCAAGCTTATGGTGTCTTTGCCTCGGCTG) and a reverse primer XhoI (5'-GGCCTCGAGTCAGACCAGGGGCCTCAAGGC).

Cloning of the fusion construct pCTLA4BCL1-CH3 required two different steps. The CTLA4 coding region with its own secretion signal was first amplified by reverse transcription from mouse spleen RNA [primer HindIII-CTLA4 forward (5'-CCCAAGCTTTGCCCCCCAGCCATGGCTT), primer MscI-CTLA4 reverse (5'CCCTGGCCAGATCCGCCAGAATCCGGGCATGGTTCTG)] and cloned into a pUC vector as a HindIII-MscI fragment. Meanwhile the pBCL1-CH3 cassette was modified by substituting the Hind/ApaLI fragment encoding the secretion peptide with a HindIII/ApaLI linker containing an internal MscI site. The last step was to transfer the HindIII/MscI CTLA4 coding region to the modified pBCL1-CH3 resulting in pCTLA4BCL1-CH3. For pCTLA4-BCL1 (without CH3) a fragment HindIII/StuI from pCTLA4BCL1-CH3 that encompasses the CTLA4 and half of the VH was inserted into pBCL1 digested HindIII/StuI.

The membrane bound version of each idiotype was obtained by subcloning the HindIII-BspEI fragment (signal peptide-V_L-linker-V_H) of pBCL1, pV_L^{BCL1}/V_H^{6C6}, pV_L^{6C6}/V_H^{BCL1} and p6C6 into the **ε-mSIP** vector (Bestagno M., in preparation) that contains the C-terminal region of the human membrane IgE εH chain (εCH4, EMPD, transmembrane and cytoplamic domain).

Plasmid vectors were purified using the Qiagen Plasmid Mega kit (Qiagen, Hiden, Germany) and resuspended in saline. The ratio of OD 260/280 ranged from 1.8 to 2.0. Endotoxin assays were performed using E-Toxate (Sigma Chemical Co., St. Luis, MO) and were below 1 endotoxin U/μg of DNA.

3.Transfections and in vivo labelling

Transfections were performed by electroporation. 2 x 10^6 cells were washed, resuspended in 500 ul cold PBS, mixed with linearized DNA (15μg in 50 μl H20) and eletroshoked using the Bio-Rad Gene pulser at 250 volts and 960 μF. After 5 min on ice the cells were suspended in RPMI and transferred to five 96-well plates. Selective medium containing 400 μg/ml of G418 (Geneticin, Life Technologies, Inc., Gaithersburg, MD) was added for selection of clones.

In vitro labeling was performed by incubating $2x10^6$ cells in the presence of ^{35}S methionine at 100 μCi/ml. After four hours of incubation supernatants and cellular extracts were immunoprecipitated with anti human IgG antibodies (Sigma) and purified by protein A-sepharose (Pharmacia). The samples were analyzed by SDS/PAGE in the presence of β–mercaptoethanol.

4.Western Blot analysis

To detect the presence of scFv in the supernatants of transfectd Sp2/0 cells we performed western Blott analysis. 10 to 20 μl of supernatants of transfected cells were resuspended in reducing SDS sample buffer and loaded onto a 12% SDS-polyacrilamide gel. After electrophoresis the gel was transferred to nitrocellulose and incubated with HRP-conjugated anti-human IgG (DAKO).

5.Immunization

The standard immunization protocol implies three immunization at two weeks intervals Intradermal injection of DNA was performed using 50μg/boost of plasmid DNA. For gene gun DNA vaccination, mice were immunized intradermally by Biorad Gene delivery device (Bio-Rad, Hercules, CA). The abdominal area of mice was shaved, and 1μm gold particles carrying 1-3μg DNA were injected at 400psi.

For protein immunization, the BCL1 scFv protein was affinity purified from tissue culture supernantants of Sp2/0 cells transfected with pBCL1 using a CNBr-activated Sepharose 4B resin (Pharmacia Biotech, Uppsala, Sweden) conjugated to goat anti-human IgG Ab (Dako, Glostrup, Denmark). 50 μg/mouse of protein in CFA were injected subcutaneously three times at two weeks intervals.

Sera were collected via retro-orbital puncture two weeks after the last boost.

6. ELISA assay

Anti-idiotypic antibody levels in immune sera were measured by ELISA on plates coated with 3μg/ml of BCL1 IgM protein immunopurified from the 123bcl1 hybridoma supernatant. Reactivity against the human CH3 portion was determined using plates coated with 3μg /ml of human IgG (Sigma). Immune sera were serially diluted and bound Abs were detected with (HRPO)-conjugated goat anti mouse IgG (Kirkegaard &

Perry, Gaithersburg, MA). OD values (492nm) at 1:400 dilutions are plotted after subtracting the value obtained from a pool of non-immune sera. For the analysis of IgG subclasses sera were diluted on plates coated with 3μg/ml of BCL1 IgM and bound antibodies were detected by incubating plates with unlabeled sheep anti mouse IgG1 or IgG2a (Serotec, Oxford UK) followed by (HRPO)-conjugated anti sheep antibodies (Dako, Denmark).

7. FACS analysis

Sera from immunized mice collected two weeks after the last boost were analyzed for their ability to bind surface IgM on 3B3 BCL1 cells. The cells were incubated at 4 °C with 200ml of Id reactive sera (diluted 1:40 in 3% BSA/PBS containing 0.02%NaN3) Bound Abs were detected with goat anti mouse IgG (Kirkegaard & Perry) and FITC conjugated rabbit anti-goat Abs (Dako

To detect the expression of the ε-m SIPs, Sp2/0 transfected cells were stained with FITC conjugated goat anti-human IgE (1:200). Analysis of immune sera on BCL1 or Sp2/0 transfected cell were always performed using samples yielding comparable ELISA values against the hu-CH3 portion. BCL1 lymphoma cells or Sp2/0 transfected cells were incubated at 4 °C with different dilutions of immune sera in 3% BSA/PBS containing 0.02% NaN3. Bound antibodies were detected with FITC conjugated goat anti-mouse IgG (Kirkegaard & Perry). Mc10 4A12 is a rat mAb V_H BCL1 specific, kindly provided by Dr. F.K. Stevenson, Southampton, UK. Staining with Mc 4A12 was performed by incubation of 1hr (1:200) followed by a FITC conjugated goat anti-rat IgG (Kirkegaard & Perry). Fluorescence analysis was performed with a FACS Calibur (Becton Dickinson, CA).

8. Tumor challenge experiments

Vaccinated mice were challenged with 10^6 lymphoma cells two weeks after tumor challenge. For the variant BCL1 (F^+) mice were challenged with $5x10^5$. To performe in vivo passages of the BCL1 cell lines the spleen of animals in the terminal phase of the disease (80 to 90 % of lymphocyes were BCL cells) were collected and teased to obtain a cell suspension. The cells were washed several times in PBS and incubated in red

blood cells lysis buffer for 10 min. After two washes in PBS cells were counted and transferred to naïve recepients.

9. Ig gene fingerprinting analysis

The presence of the BCL1 clone in PBMC of challenged animals was investigated using an Ig gene fingerprinting procedure. Total cellular RNA was isolated from blood samples collected 50 days after tumor challenge. After reverse transcription cDNA was PCR amplified using a consensus mouse FW1 specific primer (mFW1: 5' GTGCAGCTGCAGCAGTCTGG 3') and a primer specific for the Cm region (mM1: 5' GGGAAGACATTTGGGAAGGA 3'). The PCR amplified fragments were labeled by primer extension with two internal ^{32}P-end-labeled oligonucleotides: mFW3, complementary to a conserved region in the mouse FW3 region (5' (CT)TGACATCTGA(GC)GA(CT)TCT 3), and BCL1sp., complementary to a sequence in the CDR3 of the BCL1 VH (5' AGATACTATGGTAACTACT 3') located 18 nts downstream from the mFW3 oligonucleotide. The reaction mixtures (20ml of the PCR samples, 10pM of ^{32}P-end-labeled oligonucleotide 200 mM dNTP, 2.5 ml of Gene Amp 10x PCR buffer and 1.25 U of Taq polymerase in a final volume of 25 ml) were subjected to denaturation at 95°C for 8 minutes, annealing at 52°C for 1 minute and extension at 72°C for 15 minutes. Two ml of each reaction were analyzed on denaturing 6M urea 6% polyacrylamide sequencing gels with sequencing reactions used as size markers.

10. Statistical analysis

Comparison of means was done with the Student's test. Survival analysis were performed using the Kaplan-Meier product-limit method and the log-rank test.

11. T-cell proliferation assay

Two weeks after the third immunization mice were sacrificed and their spleen teased to obtain a cell suspension. RBC were lysed and cells were plated at a concentration of $2x10^5$/ml in RPMI supplemented with 10% of FCS and 100µg/ml of idiotpyic protein or human IgG or control human IgM (DAKO). After 7 days spleenocytes were collected and incubated with 1:200 dilution of FITC conjugated anti-IL2R and pycoerithrin

conjugated anti-CD4 antibodies (Pharmingen). Fluorescence analysis was performed with a FACS Calibur (Becton Dickinson, CA).

CHAPTER 3

RESULTS

Section 1.

Induction of anti-Id antibodies against BCL1 lymphoma by DNA vaccination and protection from tumor challenge.

Introduction

BCL1 was the first B-cell lymphoma described in mice (**95**). It arose spontaneously in an elderly Balb/c mouse and is characterized by early spelnomagaly and later leukemia. The tumor cells express IgMλ and IgDλ; the two isotypes shares a common idiotype as defined serologically and by sequence analysis (**98**). This murine malignancy has been shown to be analogous to human chronic lymphocytic leukemia (**99**). Protein immunization using purified idiotypic BCL1 IgM has been shown to induce high titers of anti-Id antibodies and protection fron tumor challenge (**56**). Protection appeared to be mediated by anti-Id antibodies rather then by cell mediated mechanisms (**59**). Tumor cells were shown to enter a state of dormancy in immune animals characterized by changes in the cell cycle status and oncogens expression. However, DNA vaccination strategies did not succed against the BCL1 lymphoma. The authors reported induction of only low titers of anti-Id antibodies that were not protective against tumor challenge (**86**).

The following section describes the cloning of a DNA construct that contains the two BCL1 variable regions in a single chain Fv (scFv) format. This construct was used in immunization experiment and resulted very efficient in inducing high levels of anti-Id antibodies and protection from tumor challenge (**100**). We performed immunization experiments with several variants of the original construct to understand the mechanism of induction of the anti-Id antibodies and to identify the components of the idiotype encoding cassette that are crucial for the immunogenicity. We also tested the effect of co-

administering adjuvant molecules to the vaccine formulation and the effect of fusing the antigen to a signal for delivering to antigen presenting cells. In addition, to elucidate the mechanism of the initiation of the anti-Id immune response we analysed the immune response at the T-cell level.

1.Requirement of a xenogeneic domain to induce anti-Id antibodies

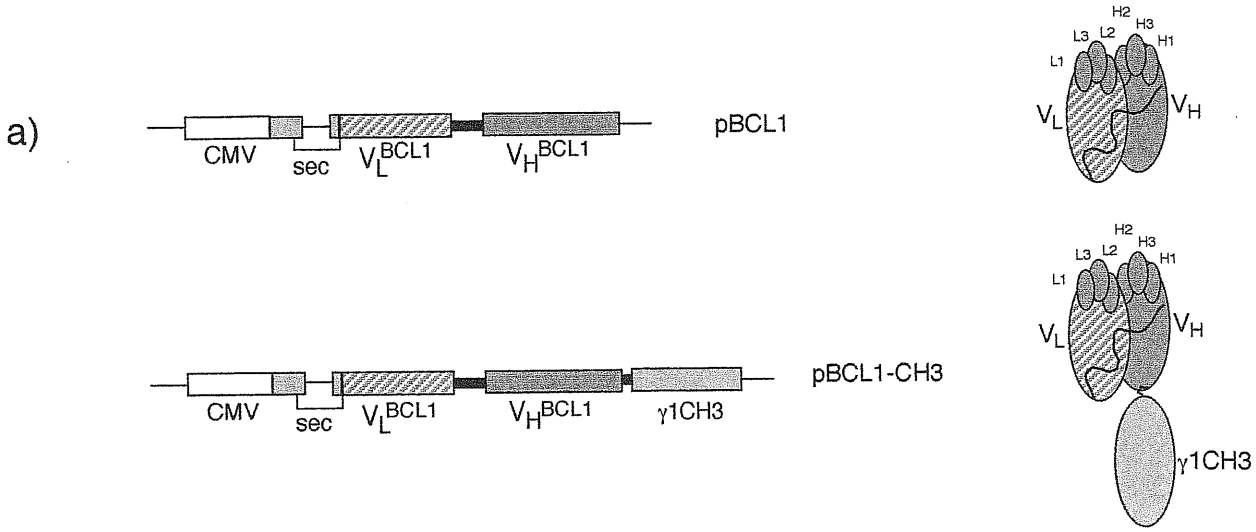
1.1 Cloning and expression of BCL1-scFv

Previous studies by our group described the construction of SIPs (small immunoproteins), containing a scFv linked to the CH3 domain of human immunoglobulin γ1 H-chain. These molecules were shown to fold correctly and to be efficiently secreted by mammalian transfected cells, retaining their antigen specificity (**97**).

The vectors for immunization against the BCL1 idiotype were generated starting from SIPs DNA cassettes.

The BCL1 V regions were amplified by RT/PCR after extraction of RNA from the in vitro 3B3 BCL1 cell line. After sequencing, the VH and VL fragments were cloned into an expression vector containing the CMV promoter. Upstream of the VL/VH coding region there is a signal secretion signal, the leader peptide required for secretion of proteins in the extracellular medium. This signal is derived from the genomic sequence of a mouse heavy chain immunoglobulin secretion signal and contains an intron that is spliced out in the mature mRNA. This leader sequence was shown to be highly efficient in driving the secretion of the downstream protein. The BCL1 variable regions were cloned in the VL-VH orientation, separated by a 18 amminoacid linker (GSTSGSGKPGSGEGSTKG) that ensures optimal folding of the scFv molecules (pBCL1).

Previous studies indicated that delivery of tumor-derived variable region in a scFv format induces only low levels of anti-Id antibodies unless the plasmid contains an additional region that aguments the immunogenicity of the idiotype (**87**). Therefore, we cloned the BCL1 scFv into the SIP vector containing the hu-CH3 region (pBCL1-CH3).



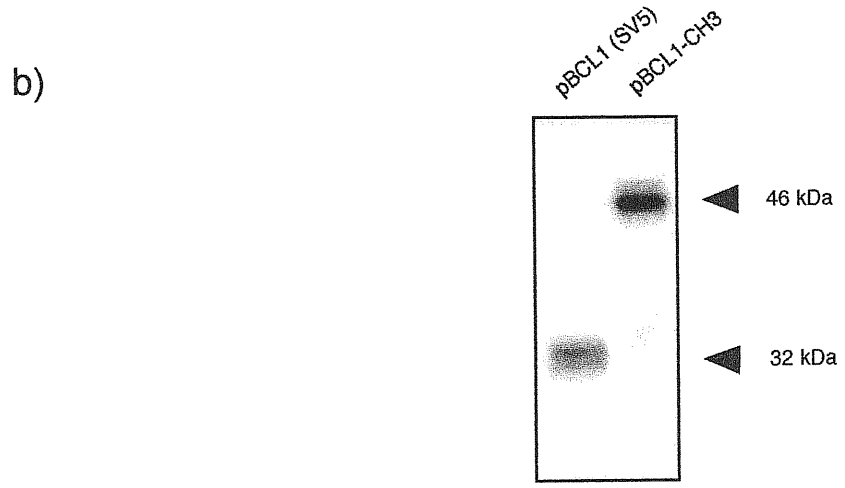


Fig 1.
a) Schematic representation of the constructs used for genetic vaccination and the corresponding protein products.
The pBCL1 plasmid was generatated by cloning the BCL1 derived heavy and light chain into a mammalian expression vector containining a genomic secretion signal sequence (sec). To generate plasmid pBCL1-CH3 a human γCH3 domain was introduced at the N- terminus of the scFv coding region.
b)Western blott analysis of supernatants of Sp2/0 cells transfected with pBCL1(SV5) and pBCL1-CH3. The 32 kDa band corresponding to BCL1 scFv was detected using anti-SV5 HRP conjugated Abs while the 46 kDa band corresponding to BCL1-CH3 scFv was detected with anti-human IgG HRP conjugated Abs.

For our goals the CH3 domain is important because is xenogeneic in the mouse, thus providing a possible adjuvant effect. Moreover CH3 is a suitable tag for detection and purification of the molecule.

To assess expression and secretion of the scFv molecules we transfected the pBCL1 and pBCL-CH3 (fig 1a) into a myeloma Sp2/0 cell line. For plasmid pBCL1 we transfected a variant that contains at the C-terminal an SV5 tag, to allow detection of the molecule. The supernatants of transfected clones were tested in Western Blotting analysis using anti-human IgG (for the pBCL1-CH3) and anti-SV5 (for pBCL1) antibodies. As shown fig1 (panel b) both BCL1 scFvs are secreted in the extracellular medium.

1.2 Analysis of the antibody response

The ability of pBCL1 and pBCL1-CH3 to induce an antibody response against the idiotype and against the human CH3 portion was analysed on sera of immunized animals. A control goup was immunized with a plasmid containing an irrelevant murine scFv (p6C6). The V regions of 6C6 were cloned from a murine monoclonal antibody obtained from a mouse immunized with human breast cancer cell membranes. 6C6 V regions share very little homology with those of BCL1. While mAb 6C6 has a κ light chain and a V_H corresponding to family VI, BCL1 light chain is λ (family I) and the V_H corresponds to family XXIV (Kabat database).

Mice received three intradermal injection of plasmid DNA at two weeks intervals. Two weeks after the last boost sera were collected and analysed by ELISA on plates coated with BCL1IgM and human IgG. As shown in fig 2 (panel a) immunization with pBCL1-CH3 induced high levels of anti-Id antibodies in 100% of cases. Immunization with the pBCL1-CH3 construct was repeated several times and the mean levels of anti-Id antibodies induced in all the groups tested were comparable in all the experiments (mean OD value of pooled sera ranged from 1.2 to 1.4). On the contrary immunization with either pBCL1 or p6C6-CH3 always failed to induce detectable levels of anti-Id antibodies.

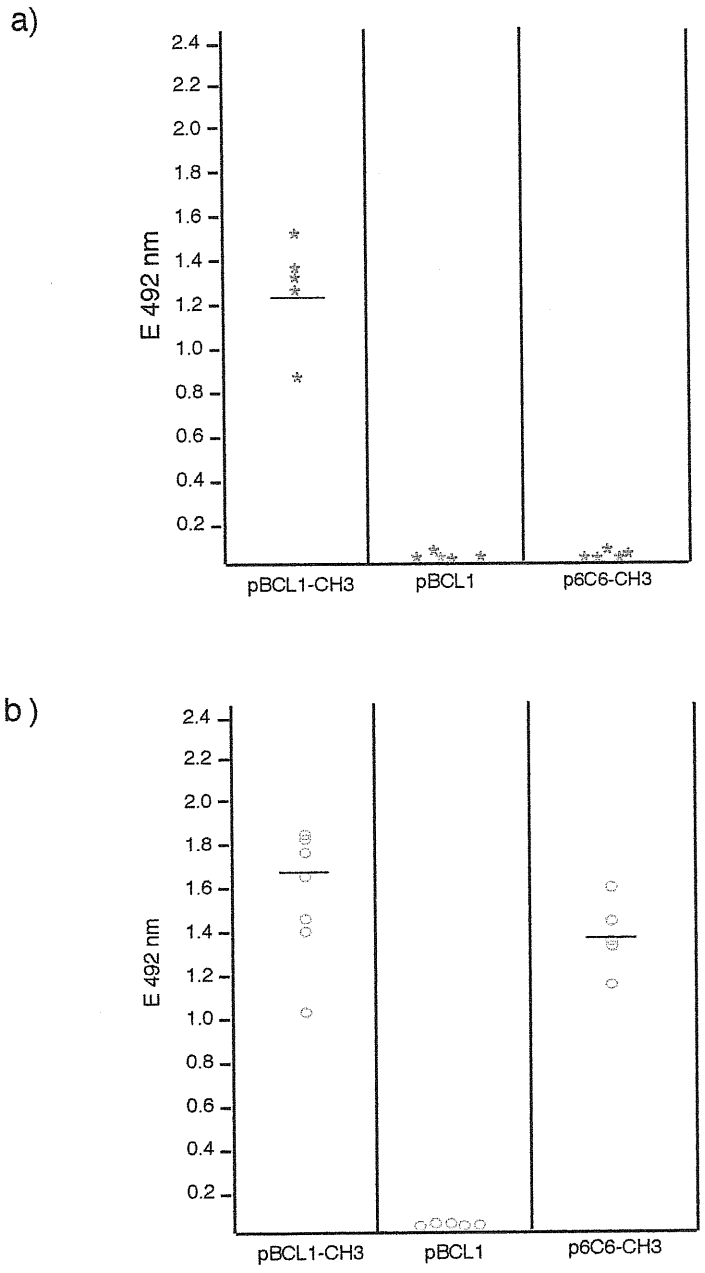


Fig 2

Antibody responses induced by DNA immunization with pBCL1, pBCL1-CH3 and p6C6-CH3. The antibody levels were measured by ELISA against BCL1 IgM (*)(panel a) or against the CH3 portion of human IgG (o) (panel b) on blood samples collected after three injections of plasmid DNA. Each symbol represents a value from a single mouse; mean group values are indicated as horizontal lines. Sera were at 1: 400 dilution. The value obtained from a pool of sera of non immunized animals was subtracted from each value before plotting. Reactivity against an unrelated mouse IgM was insignificant.

Panel b of fig 2 shows the response against the hu-CH3 region: as expected pBCL1-CH3 and p6C6-CH3 induced comparable anti CH3 antibodies while the pBCL1 construct did not. Analysis of the IgG subclass profile revealed that the anti-Id immune response is dominated by the IgG1 subclass (data not shown).

Immune sera were also tested by flow cytometry for their reactivity with the native antigen expressed on the surface of the BCL1 lymphoma cells. FACS analysis in fig. 3 shows that sera raised by pBCL1-CH3 strongly bound to tumor cells, while no binding was detected for pBCL1 and p6C6-CH3. Binding of pBCL1-CH3 sera to WEHI, a murine B-cell lymphoma expressing an unrelated surface IgM, was not significant (not shown). These results indicate that addition of a xenogeneic domain to the BCL1 scFv is essential to induce an anti-idiotypic immune response. Most likely, the human CH3 region provides T cell epitopes that break the tolerance at the T cell level, permitting the induction of a response against the idiotype.

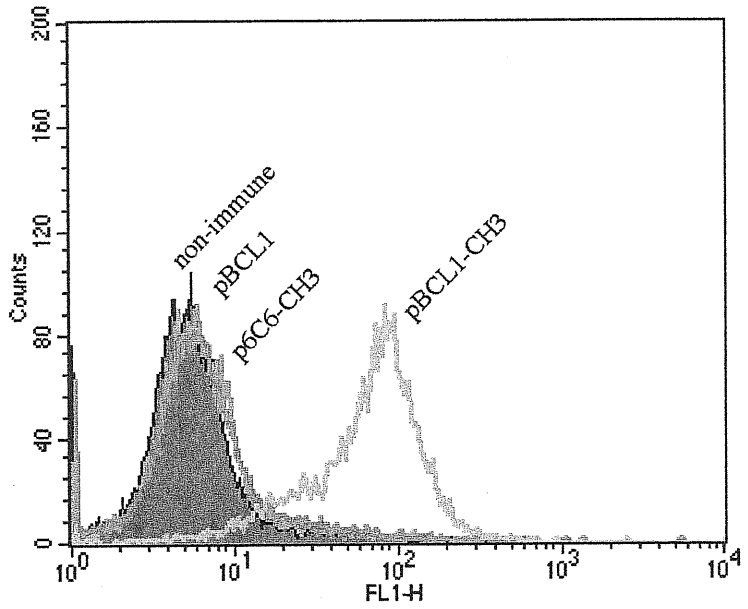


Fig 3
Reactivity of BCL1 tumor cells with anti-Id Abs induced by DNA vaccination as assessed by flowcytometry. A pool of sera from animals immunized with pBCL1, pBCL-CH3, p6C6-CH3 or a pool from a non-immunized group were incubated at 1:200 dilution with BCL1 cells. Binding to BCL1 was detected using FITC conjugated goat anti-mouse IgG.

1.3 *Route of immunization*

At this stage we investigated the effect of the route of immunization (intradermal versus intramuscolar) on the intensity of the antibody response. For intradermal delivery of plasmid DNA we used two methods: intradermal injection of plasmid DNA resuspended in saline solution or gene gun bombardment of gold particles coated with DNA (see matherials and methods). Immunization resulted more efficient when the plasmid was injected intradermally then intramuscolarly (fig 4). For intradermal delivery, injection or gene gun bombardment resulted equally efficient (fig 4). Therefore we decided to perform the rest of the experiments with the gene-gun system which is more convenient in terms of amount of DNA required. In fact, while DNA injection requires 50μg per injection, this amount is reduced to 1μg with the gene gun system. In addition, levels of anti-Id antibodies were very similar in all mice immunized with the same construct, because the efficiency of delivery is highly reproducible compared to manual injection.

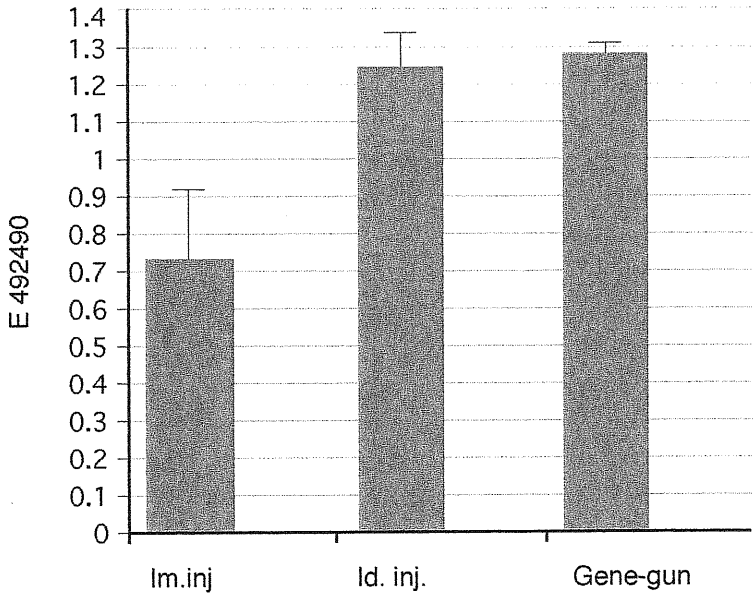


Fig4
Delivery of plasmid DNA through different routes of immunization. Three groups of 5 animals were vaccinated with pBCL1-CH3 in different ways. Immune sera were pooled for ELISA analysis on plates coated with BCL1IgM. Mean levels (E492) of anti-Id Abs induced by intramuscolar injection (Im.inj), intradermal injection (Id.inj) or gene-gun bombardment are represented as solid bars.

1.4 Cloning and expression of scFvBCL-SAPA fusion protein

In the above section we demonstrated that the addition of an immuno-enhancing sequence to the scFv construct is essential to induce anti-idiotypic Abs. Promotion of anti-Id Abs is likely to depend on delivery of cognate T-cell help to Id specific B cells by anti-hCH3 specific $CD4^+$ T-cells. In a different lymphoma model, the group of F. Stevenson showed that fusion to fragment C of tetanus toxin dramatically enhanced the response against a scFv DNA vaccine. We decided to test in our system the efficacy of an immunodominant peptide derived from the parasite Tripanosoma Cruzi (the agent of Chagas' disease) for its ability to function as immunoenhancing peptide and to compare it to hu-CH3.

The trans-sialidase enzyme derived from the parasite Tripanosoma Cruzi (the agent of Chagas' disease) is a molecule composed by two well defined region, an N-terminal domain containing the catalytic activity and a variable number of twelve amminoacid long repeated motifs known as SAPA (shed acute phase antigen) located at the C terminus (**101**). The SAPA portion is highly immunogeneic as demonstrated by high levels of anti-Sapa antibodies during cronic Tripanosoma infection in humans (**102**).

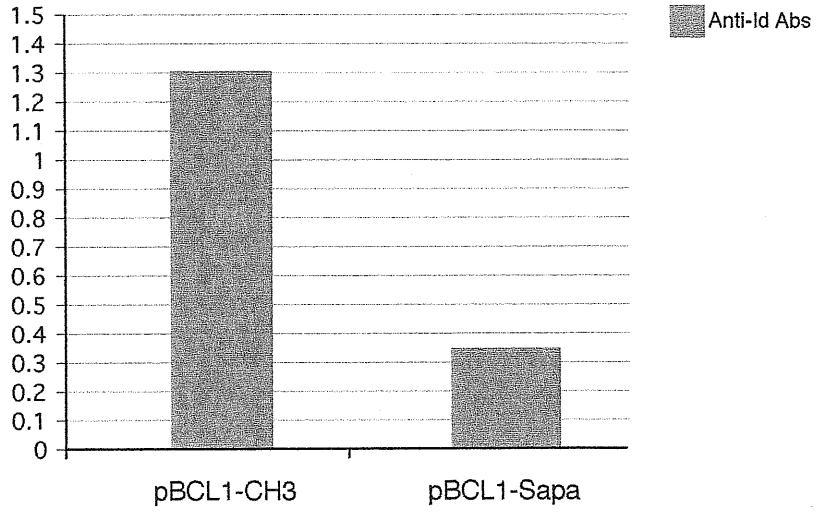
Construct BCL1-SAPA was generated by substitution of the human gamma CH3 portion with the amino acid long Sapa coding region (fig.5, panel a). Immunoprecipitation with anti Sapa antibodies of a pBCL1-Sapa transfected Sp2/0 clone showed a band of the correct size only in the cell extract and not in the supernatant indicating that the protein is not secreted and retained in the intracellular compartment (fig.5, panel b). In order to understand whether intracellular retention was due to misfolding of the scFv molecule, we immunoprecipitated a cell extract of Sp2/0 cells transfected with pBCL1-Sapa using anti-Id antibodies induced by genetic immunization with pBCL1-CH3. Since we know that anti-Id Abs induced by DNA vaccination (see Results section 2) are directed exclusively against conformational determinants, the abilty of this serum to immunoprecipitate the scFv -SAPA protein (not shown) suggest that the scFv is properly folded in the fusion protein. We therefore supposed that Sapa might act as a signal for retention in the endoplasmic reticulum.

1.5 Analysis of the antibody response

Although the pBCL1-Sapa led to the expression of a intracellular protein, we decided to evaluate the immunogenicity of the construct by immunizing a group of animals according to the protocol described in section 1.2-1.3. Immune sera were analysed by ELISA and FACS. Fig 6 shows the reactivity of sera from the pBCL1-Sapa immunized group against the BCL1 idiotype in ELISA. Intradermal delivery of pBCL1-Sapa was able to induced detectable level of anti-Id Abs, but mean values were ten times lower then those obtained with pBCL-CH3 (fig6, panel a). Although the concentration of anti-Id antibodies induced by pBCL1-Sapa was low they were able to bind the native antigen on lymphoma cells (fig 6, panel b).

Serum levels of anti-Sapa antibodies reflected those of anti-Id Abs (not shown), suggesting that the poor performance of pBCL-Sapa depends on limited availabilty of secreted antigen.

a)



b)

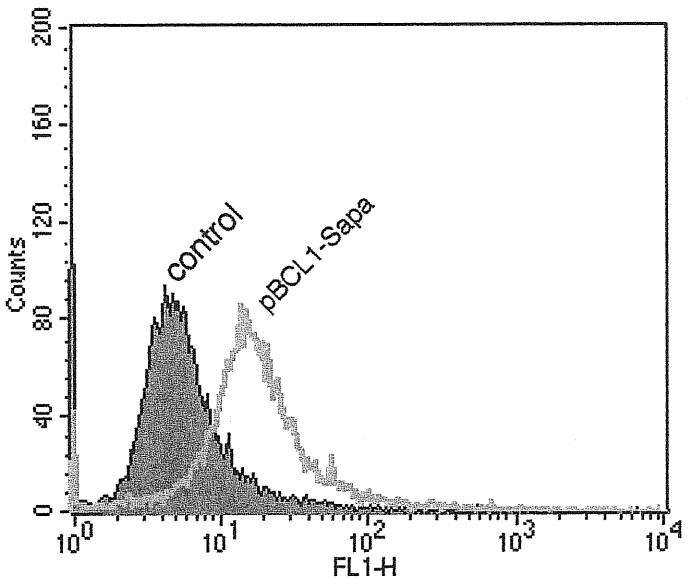


Fig 6
a)Anti-Id Abs induced by vaccination with pBCL1-Sapa. Two groups of five animals were vaccinated with pBCL-CH3 or pBCL1-Sapa as described (section 1.2). Sera were pooled for analysis on ELISA plates coated with BCL1 IgM. Mean values for each group at 1:400 dilution are represented as solid bars.
b)Sera from the pBCL1-Sapa immunized group were incubated with BCL1 lymphoma cells at 1:200 dilution. Binding was detected using anti-mouse IgG FITC. Reactivity is compared to a non immune serum.

2. Effect of antigen localization on the induction of anti-Id Abs

.2.1 Cloning and expression of localization variant of BCL1 scFv

We next decided to examine the effect of changing the cellular localization of our scFv antigen on the overall anti-Id immune response The results described in section 1 indicate that secretion of the antigen in the extracellular medium is critical for the induction of high levels of anti-Id antibodies. To address this issue more directly we generated a variant of pBCL1-CH3 that encodes for an intracellular form of the BCL1 scFv protein (pBCL1-CH3Δsp). In addition we generated a membrane version of pBCL1-CH3 to assess the effect of a third localization variant in this study.

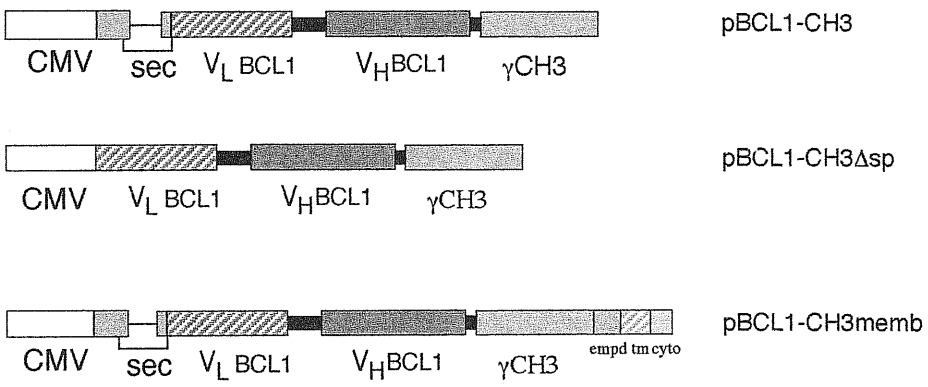
Plasmid pBCL1-CH3Δsp was generated by deletion of the signal secretion peptide. This construct therefore lacks the signal for delivery to endoplasmic reticulum and result in expression of a cytoplasmic antigen. To confirm the targeting of the scFv protein we transfectd Sp2/0 with pBCL1-Δsp and analysed the cell lysate. Immunoprecipitation using anti human IgG antibodies revealed that a band of the expected size is retained in the cytoplasm and not secreted (not shown).

To analyse the immunogenicity of a membrane bound form of the BCL1 scFv we constructed plasmid pBCL1-CH3memb. In this plasmid the CH3 domain is linked to the transmembrane and cytoplasmic domain of human IgG resulting in expression of a membrane anchored antigen. Transfection of Sp2/0 with this vector resulted in expression of a correctly folded membrane form of the idiotype as assesed by FACS analysis with anti-Id antibodies (fig 7b)

2.2 Analysis of the antibody response:

The immunogenicity of pBCL1Δsp and pBCL1-CH3memb was assessed according to the protocol standard in section 1.2. Both constructs elicited detectable levels of anti-Id antibodies, however the intensity of the immune response is weakened compared to that obtained with construct pBCL1-CH3, particularly for pBCL1-CH3memb. While serum levels of anti-Id antibodies were low and comparable for all (n=5) animals vaccinated with

a



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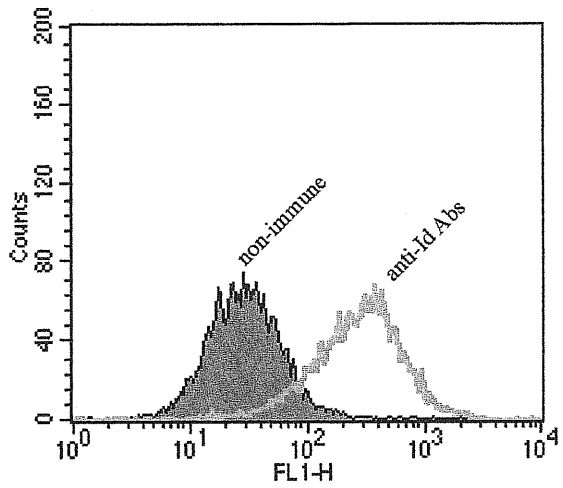
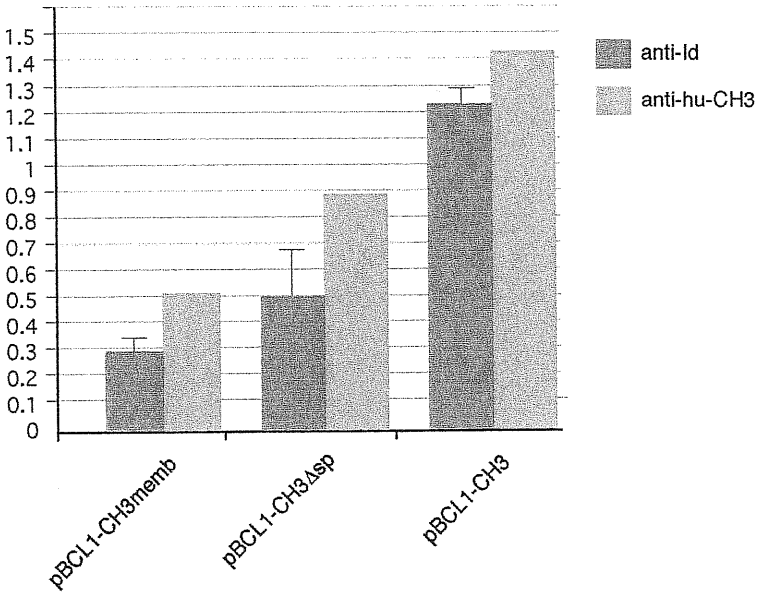


Fig 7
a) Scheme of pBCL1-CH3Δsp, pBCL1-CH3memb. and the original pBCL1-CH3. .
b) Facs analysis on Sp2/0 cell transfected with pBCL1-CH3memb. Cells were incubated with anti-Id antibodies (1:200) or pre-immune serum. Binding was detected using FITC comnjugated anti-mouse IgG .

pBCL1-CH3memb., the group of animals (n=5) vaccinated with the pBCL1-CH3Δsp showed variability among individuals.(fig 8). Immunization with pBCL-CH3Δsp was repeated two times with similar results.

Analysis of the response against the human CH3 portion showed higher mean values for the pBCL1-CH3 immunized group and lower values for pBCL-CH3Δsp and pBCL1-CH3memb. Apparently, the differential targeting of the antigen did not affect IgG subclass distribution since, for both constructs, the immune response is dominated by IgG1 as in the case of pBCL1-CH3 (not shown). Immune sera from the pBCL1-Δsp and pBCL1-CH3memb immunized groups were tested for their ability to bind the native antigen on BCL1 lymphoma cells. Consistently with ELISA values both immune sera bind to BCL1 lymphoma cells but to a lesser extent than sera from the pBCL1-CH3 immunized group. However, this indicates that anti-Id antibodies induced by an intracellular or membrane form of the idiotype recognize the structure of the native molecule. In conclusion these data indicate that both the intracellular and membrane form of the scFv idiotype are able to induce anti-Id antibodies but availabilty of secreted antigen is necessary for optimal antibody responses.

a)



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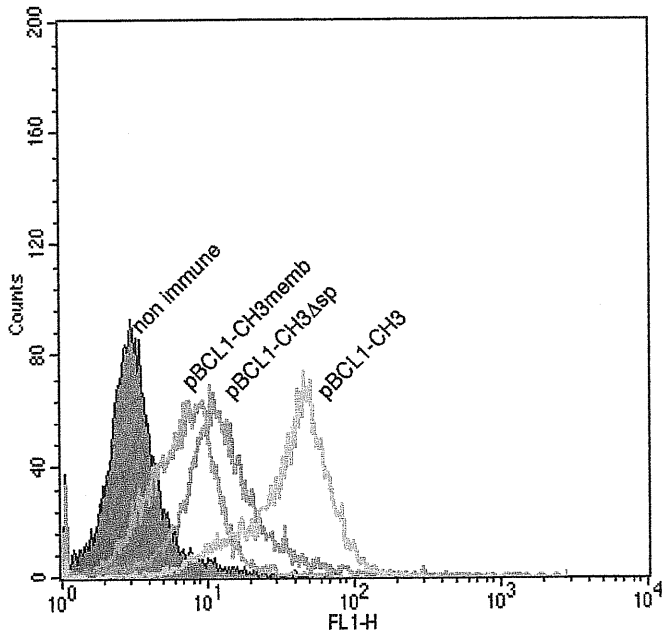


Fig8

a) Anti-Id Abs induced by vaccination with pBCL1-CH3memb and pBCL1-CH3Δsp. Sera of five animals from each group were pooled after immunization and analyzed on plates coated with BCL1IgM and hu IgG. A pool of sera from the pBCL1-CH3 group was analyzed in parallel for comparison. Mean OD values (E492) at 1:400 dilution are represented as solid bars. SDs refer to variability among individuals

b)Facs analysis of BCL1 lymphoma cells stained with anti-Id Abs induced by pBCL1-CH3, pBCL1-CH3memb and pBCL1-CH3Dsp. Pooled sera were diluted 1:200 and incubated with BCL1 cells for 1h followed by incubation with anti-mouse IgG FITC conjugated.

3. Both variable regions are required to induce anti-Id antibodies.

3.1 Cloning and expression of chimeric idiotypes

In this section of results I will present a series of immunization experiments aimed to evaluate the relative contribution of VL and VH tumor derived V regions to the overall anti-Id immune response. This aspect is important to define the general requirement of scFv DNA vaccination and also because vaccination using single V regions could provide an advantage in the preparation of customised vaccines against lymphoma

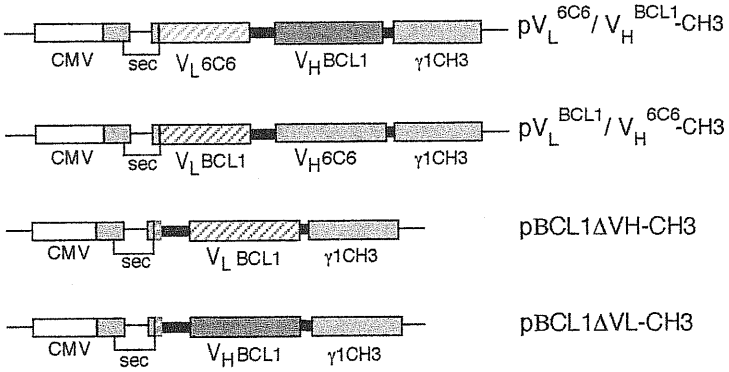
To address this issue, we generated scFv molecules containing each of the two tumor derived V regions paired to an irrelevant V region partner. These scFvs were inserted in a vector containing the elements previously shown to ensure optimal performance, i.e., leader peptide for extracellular secretion and human gamma CH3 as immuno-enhancing domain.

Plasmid pV_L^{BCL1}/V_H^{6C6}-CH3, is a chimera in which the VH from BCL1 is associated to an irrelevant (6C6, see section 1) murine V_L. Conversely, plasmid pV_L^{6C6}/V_H^{BCL1}-CH3 contains the opposite match of V regions. In addition we generated two more constructs, pBCL1ΔVL-CH3 and pBCL1ΔVH to investigate the role of VH and VL in a different configuration, i.e., unpaired and not in the context of a VL/VH pairing. Transfection into SP2/0 cells of pV_L^{6C6}/V_H^{BCL1}-CH3 and pBCL1ΔVL-CH3 indicated that while the chimeric construct leads to secretion of the encoded protein, the scFvΔVL is retained intracellularly (fig 9). Although not tested, we assume that the VL-CH3 protein encoded by construct pBCL1ΔVH-CH3 can be secreted from transfected cells. In fact it is known that light chains can be expressed in the extracellular medium, regardless of the presence of the VH.

3.2 Analysis of the antibody response

The immunogenicity of the four constructs depicted in fig 9 (panel a) was evaluated according to the standard protocol. Fig 10 (panel a) shows the levels of anti-Id and anti-huCH3 antibodies induced by immunization with the four constructs. Although in vivo expression of pV_L^{6C6}/V_H^{BCL1}-CH3 and pV_L^{BCL1}/V_H^{6C6}-CH3 was efficient, as demonstrated

a



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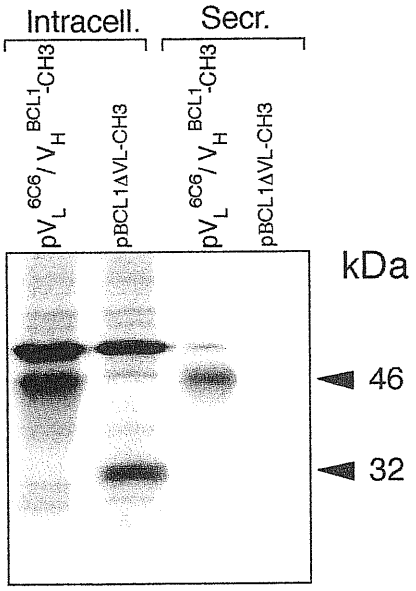
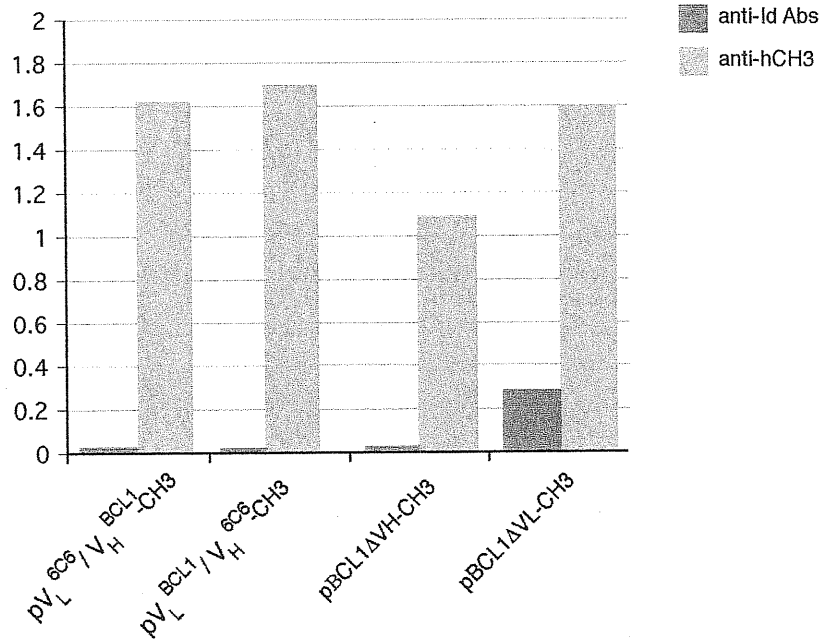


Fig 9
a) Scheme of the four constructs generated to investigate the relative role of tumor derived V regions.
b)Expression of pVL6C6VHBCL1 and pBCL1ΔVLCH3 in Sp2/0 cells. Transfected cells were in vivo labeled with ^{35}S-methionine for 4 h. Cell lysates and supernantants were immunoprecipitated with anti-human IgG antibodies and resolved in 12% SDS-PAGE.

by the levels of anti-huIgG, neither of the two constructs was able to induce anti BCL1-Id antibodies (fig 10 a). Therefore we concluded that pairing of both tumor derived variable regions in a scFv is essential to induce antibodies against the BCL1 idiotype. Concerning pΔVH and pΔVL we observed that both constructs induced antibodies against the hu-CH3 portion and pΔVL induced also a low but clearly detectable level of anti-Id Abs in two independent experiments. This is confirmed by the immunofluorence analysis on BCL1 cells (fig 10 b).

a



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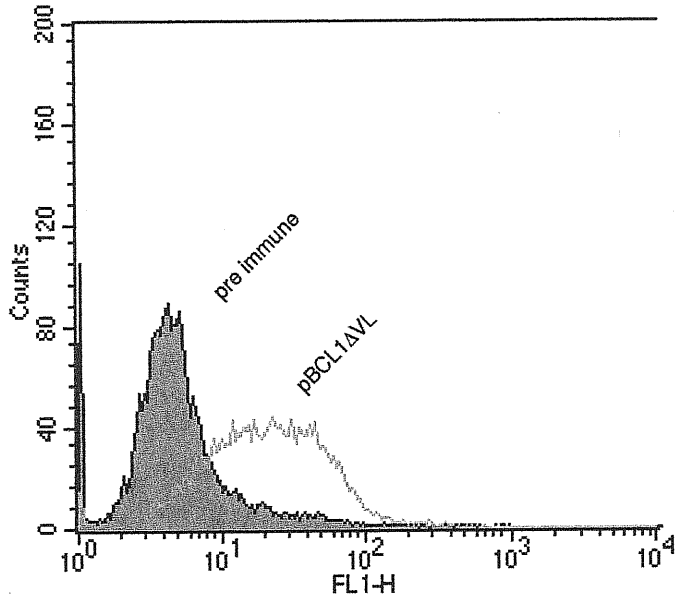


Fig 10

a) Analysis of anti-Id Abs induced by the four constructs described in fig 9. Five animals/group were vaccinated according to the standard protocol (section 1.2) and sera from each group were pooled for ELISA analysis on plates coated with BCL 1IgMor human IgG. Bars represents mean OD values (E=492) obtained incubating plates with 1:400 dilution of sera from the indicated group followed by incubation with anti-mouse IgG HRP conjugated. For pBCL1ΔVL-CH3 the mean OD value arise from two independent experiments.

b) Facs analysis of BCL1 lymphoma cells stained with anti-Id Abs induced by pBCL1ΔVL-CH3 immunization. Sera diluted 1/200 was incubated with BCL1 cells for 1hr followed

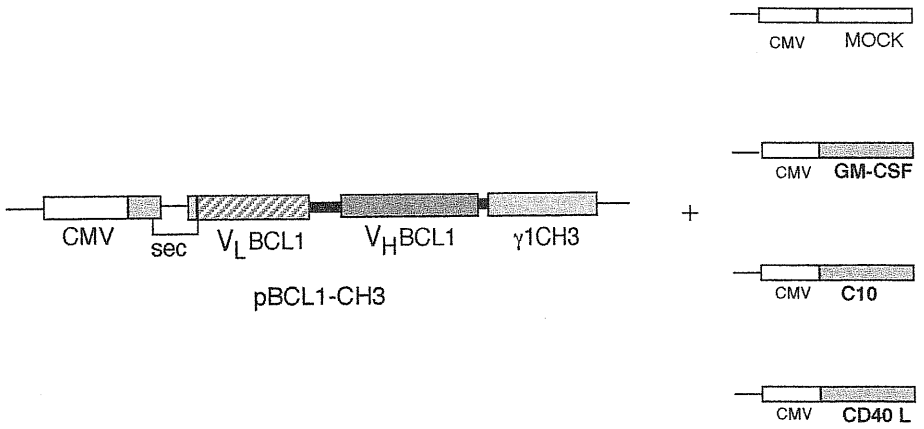
4. Comparison of different adjuvant molecules.

4.1 Cloning of GM-CSF, C10, CD40L,

As described in the introduction (Introduction, section 5.2, 5.5), the immune response against an antigen can be modulated by co-administering adjuvant molecules or by fusing the antigen with immuno-enhancing domains. Even though levels of anti-Id antibodies induced by pBCL1-CH3 were already high we were interested in further improving the potency of the vaccine. This section of the thesis describes the effects, in terms of intensity of the antibody response, exerted by 3 different adjuvant molecules co-administered togheter with pBCL1-CH3.

The ability of GM-CSF to enhance the immune response is well described (**66,69**), therefore we decided to test its effect in our system. The crucial role played by CD40-CD40L interaction in the initiation of the immune response (Introduction section 2.4), prompted us to evaluate the ability of a CD40L encoding plasmid to mimic T cell activation thus improving the production of anti-Id antibodies. A study using a model antigen (β-gal) has proved that addition of a CD40L encoding plasmid is able to enhance the Ab response (**84**). In additon a β-chemokine, C10, that was already available in the laboratory, was added in this set of experiments. This chemokine is still poorly characterized. It was shown to be produced in the bone marrow and to function as a chemoattractant for both B-cells and CD4+ T cells (**103,104**). Data from our laboratory (Mancardi & Burrone *et al.*, submitted) indicates that C10 is produced by lymphatic endothelial cells and has a potent effect in recruiting lymphocytes. For the above reasons we wanted to evaluate its in vivo ability to influence the immune response when co-admistered in association with pBCL1-CH3.

To clone murine GM-CSF we amplified by reverse transcription PCR the complete coding region from mouse spleen derived RNA. The amplified fragment was sequenced, in vitro translated and cloned in a pcDNA3 vector resulting in plasmid pGM-CSF. Following the same procedure we generated also pC10 and pCD40L using primers specific for each molecule.



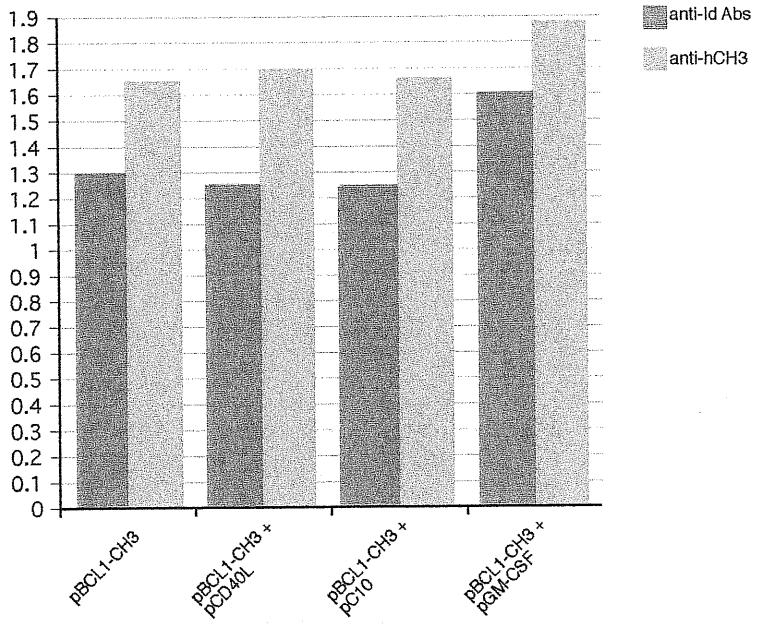


Fig 11
a) Analysis of the antibody immune response induced by pBCL1-CH3 alone or plus the adjuvant plasmids as indicated. Five animals/group were vaccinated according to the standard protocol (section 1.2) and sera from each group were pooled for ELISA analysis on plates coated with BCL 1IgM or human IgG. Mean OD values (E=492) are represented as solid bars. For pBCL1-GMCSF and pBCL1 alone mean OD values arise from three independent experiments.

4.2 Analysis of the antibody response

Three groups of five animals were vaccinated with pBCL1-CH3 plus each of the different adjuvant plasmids. ELISA values against BCL1 IgM and against hu-CH3 were measured after three shots and compared with those induced by pBCL1-CH3 alone. To make sure that animals vaccinated with pBCL1-CH3 alone received an equal amount of plasmid DNA a pcDNA3 mock plasmid was added in the preparation of the cartridges.

As shown in fig 11, co-delivery of pBCL1-CH3 with pGM-CSF slightly augmented the anti-Id and anti-huCH3 immune response. Although the increment in antibody levels was small, it was repeated in three independent experiments.

Moreover analysis of the IgG subclasses revealed that addition of GM-CSF altered the form of the immune response. While the response to all the constructs analysed showed only antibody of the IgG1 subtype addition of GM-CSF raised also a low but clearly detectable level of IgG2a (not shown). On the contrary, co-injection of pC10 and pCD40L did not affect neither the intensity nor the form of the immune response that was comparable to that induced by pBCL1-CH3 alone. Concerning plasmid pC10 the protein encoded by this plasmid was detected in the supernantants of Sp2/0 transfected cells. On the contrary pGM-CSF and CD40L were only in vitro translated but not expressed in eukariotyc cells. However, the cloning of these constructs was identical to that reported by other authors that demonstrated expression of the proteins. In particular, pGM-CSF was also used by other researchers in our lab, confirming its ability to increase the immune response in the case of a viral protein (rotavirus non-structural protein NSP5).

5. Targeting of the antigen to antigen presenting cells

5.1 Cloning and expression of BCL1-CTLA4 fusion protein.

As described in the introduction, fusion of the antigen with immunologically active molecule, like chemokines or lymphocytes stimulatory molecules, was shown to be an efficient mean to influence the immune response (**85,94**).

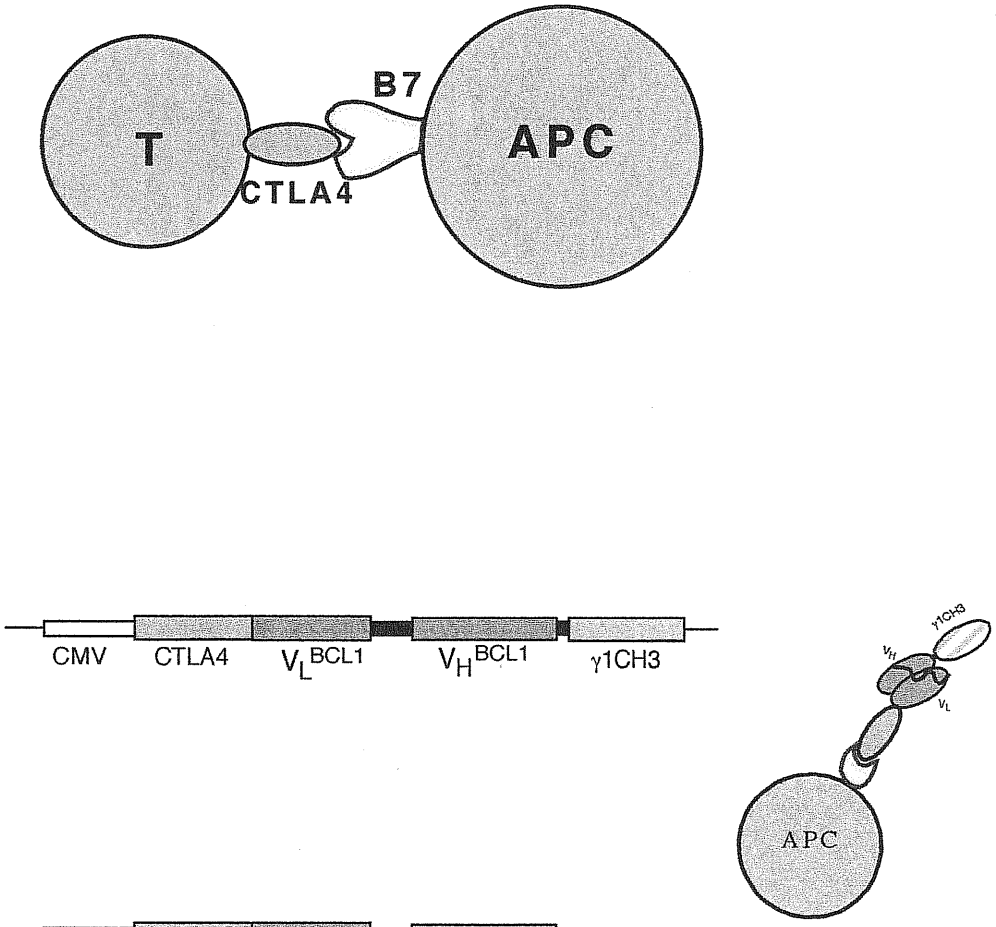
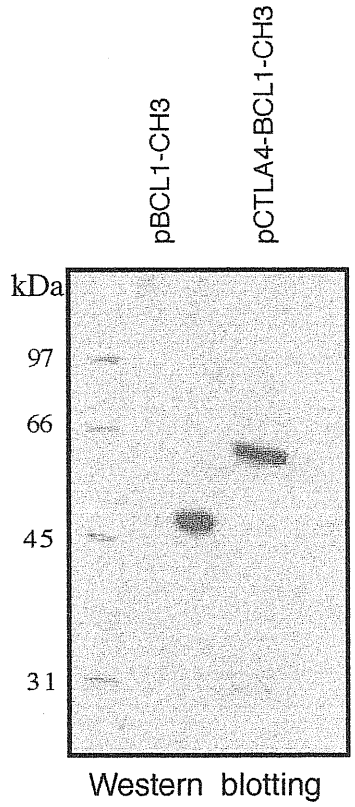


Fig 12
Scheme of the strategy designed to deliver the scFv to antigen presenting cells.
The original construct pBCL1-CH3 , the fusion construct pCTLA4 - BCL1-CH3 and the resulting proteins are depicted in the lower part of the figure.



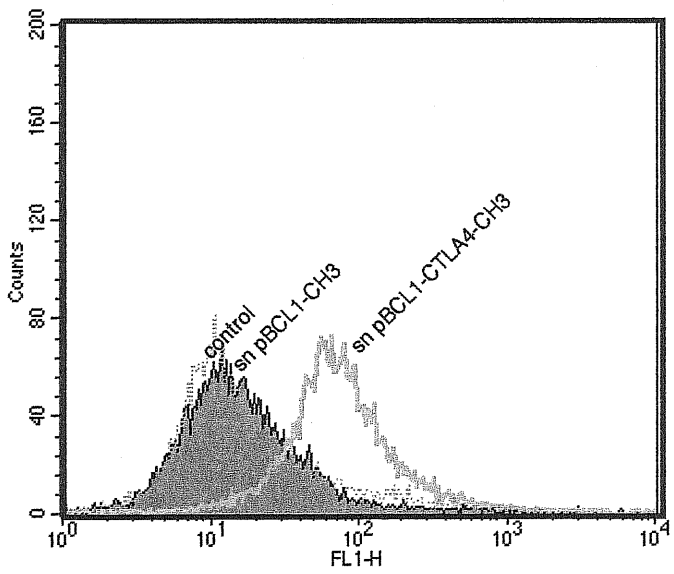


Fig 13
a)Expression and secretion of pCTLA4-BCL1-CH3 by Sp2/0 transfected cells. Supernatants of Sp2/0 cells transfected with the indicated plasmids were analysed by Western blott using HRP conjugated anti-human IgG antibodies. b) Binding of the fusion protein CTLA4-scFVBCL1-CH3 to A20 cells. A20 were crosslinked with 20 mg/ml of anti-mouse IgG and incubated with the indicated supernatants. Binding was detected using FITC conjugated anti-human IgG supernatants.

We decided to link the BCL1 scFv molecule to CTLA4, a molecule expressed on the surface of activated T-cells that binds to B7 expressing molecule on antigen presenting cells. The aim of this strategy was to specifically deliver the antigen to antigen presenting cells and to evaluate the effect of the targeting on the intensity of the anti-Id response. This design was previously shown to be very effective in inducing a dramatic increase in the antibody response against a model antigen (Fc of a human immunoglobulin).

The CTLA4 cDNA was amplified by reverse transcription from mouse spleen RNA and sequenced to verify identity to the published sequence. The CTLA4 cassette, containing the CTLA4 secretion signal, was inserted into pBCL1 and pBCL1-CH3, upstream of the VL-VH coding region generating plasmids pCTLA4-BCL1 and pCTLA4-BCL1-CH3. To verify that the fusion protein encoded by pCTLA4-BCL1-CH3 was secreted in the extracellular medium we transfected Sp2/0 cells and analyzed the supernantants by Western blot.

As shown in fig 13 (panel a), a band of the expected size was detected in the supernatant of transfected cells, indicating that the addition of the CTLA4 coding region did not affect folding and secretion of the fusion protein. Moreover, in order to demonstrate that CTLA4 linked to the scFv molecule retained its binding activity to B7-expressing cells, we incubated the supernantant containing the fusion protein with a B7 expressing B-cell line (A20). Since crosslinking of surface IgG results in upregulation of B7 molecules, we performed the experiment with both crosslinked and not crosslinked B cells. Fig 13 (panel b) shows that incubation with the supernatant of Sp2/0 cells transfected with pCTLA4-BCL1-CH3, but not with pBCL1-CH3 resulted in staining of A20 crosslinked B-cells. The shift in fluorescence was lower when the binding assay was performed with not crosslinked B-cells (not shown) further confirming the specificity of binding.

6.2 *Analysis of the antibody response*

The efficacy of pCTLA4-BCL1 and pCTLA4-BCL1-CH3 to induce anti-Id antibodies was evaluated according to the standard protocol of immunization. Immune sera were

compared with those raised by pBCL1 and pBCL1-CH3. Fig 14 shows that immunization with pCTLA4-BCL1 was totally inefficient in inducing antibodies against the BCL1 idiotype. This was confirmed also by binding to BCL1 cells (not shown). Therefore, fusion of the BCL1 scFv to CTLA4 does not render immunogenic the two V regions, further strengthening the need of a xenogeneic domain for induction of the anti-Id immune response. Comparison of pCTLA4-BCL1-C and pBCL1-CH3 showed no difference in antibody levels neither against the idiotype nor against the hu-CH3 portion (fig 14). Although immunization with pCTLA4-BCL1-CH3 was performed only once (n=5) the high reproducibilty of the system in all the previous experiments led us to conclude that this fusion does not exert any significant effect on vaccine efficacy. However, the fusion does not even negatively affects immunogenicity of the scFv, although the protein was expressed less efficiently than the BCL1-CH3 scFv in the supernatants of Sp2/0 transfected clones (not shown). Therefore, we cannot exclude that a possible adjuvant effect was masked by the lower expression of the protein. Nevertheless, we concluded that immunization with this fusion construct did not provide us with the expected results and we decided to give up this strategy.

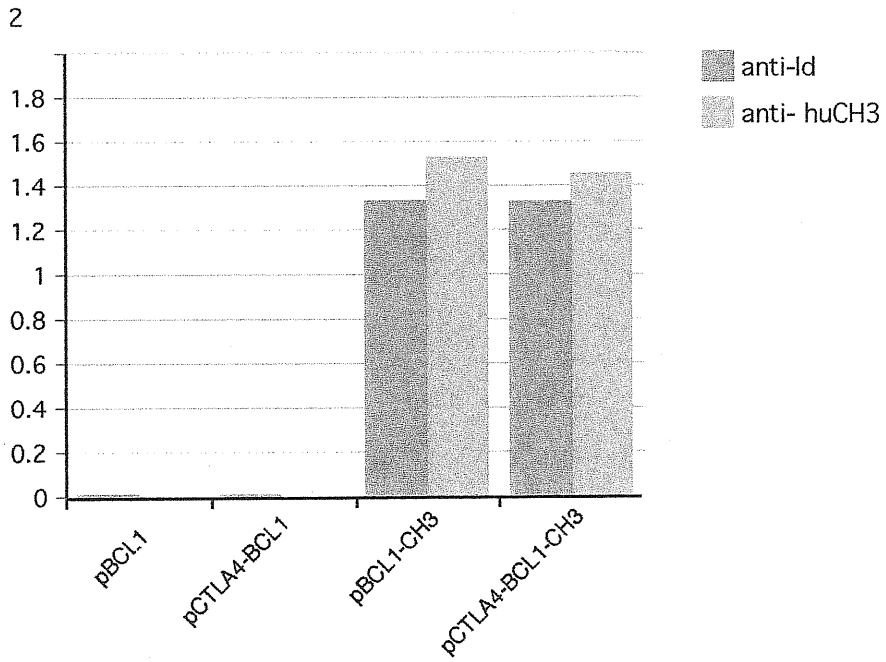


Fig 14
Comparison of titers induced by pBCL1, pCTLA4-BCL1, pBCL1-CH3 and pCTLA4-BCL1-CH3. Five animals/group were immunized according to the standard protocol and pooled immune sera were analysed by ELISA on plates coated with BCL1 IgM or human IgG.

6. Tumor growth and survival after tumor challenge

In order to assess the ability of the immune response induced by our vaccination scheme to protect animals from the growth of the BCL1 lymphoma, we performed tumor challenge experiments. In the first set of experiments we used a commercial BCL1 cell line that takes around 90 days to kill control animals. We challenged animals immunized with the secreted form of BCL1 scFv with or without the hu-CH3 portion (pBCL1 and pBCL1-CH3), and the group immunized with the construct that lacks the VL (pBCL1ΔVL-CH3). In addition we tested groups immunized with pBCL1-CH3 plus GM-CSF or CD40L. As a control we tested animals immunized with only GM-CSF or non immunized. All animals were challenged with 10^6 BCL1 lymphoma cell two weeks after the last immunization.

The kinetics of tumor growth were followed by an Ig gene fingerprinting assay, which allowed precise evaluation of the degree of expansion of the malignant clone relative to the amount of the residual normal B-cell in the peripheral blood of the challenged animals. The assay was performed on RNA extracted from PBMC on day 50 after tumor challenge. The RNA was reverse transcribed and amplified using a primer that anneals to the mouse FW1 and a second primer complementary to the first exon of the μ constant region. This PCR step allows amplification of all the rearranged μVH. transcripts. The amplified μVH transcripts are then labelled using a radioactive primer that anneals to a region in mouse FW3, just upstream of the CDR3. The CDR3s differ in length among different B lymphocytes because of imprecise joining during the process of V(D)J recombination and addition or deletion of nucleotides during this reaction. When these labelled fragments are separated on a 6% polyacrilamide gel it is possible to visualise a ladder of band corresponding to all the different CDR3 length (1 codon ladder). Thus, if all bands are equally represented it means that there is a normal, polyclonal population, in the blood of the animal tested. On the other hand, dominance of one band indicates that one clone is more represented, i.e., overgrowth of a monoclonal population. A representative fingerprinting experiment with blood samples from pBCL1-CH3, pDVL-CH3 and control non immunized animal is shown in figure 15. The pattern of a normal

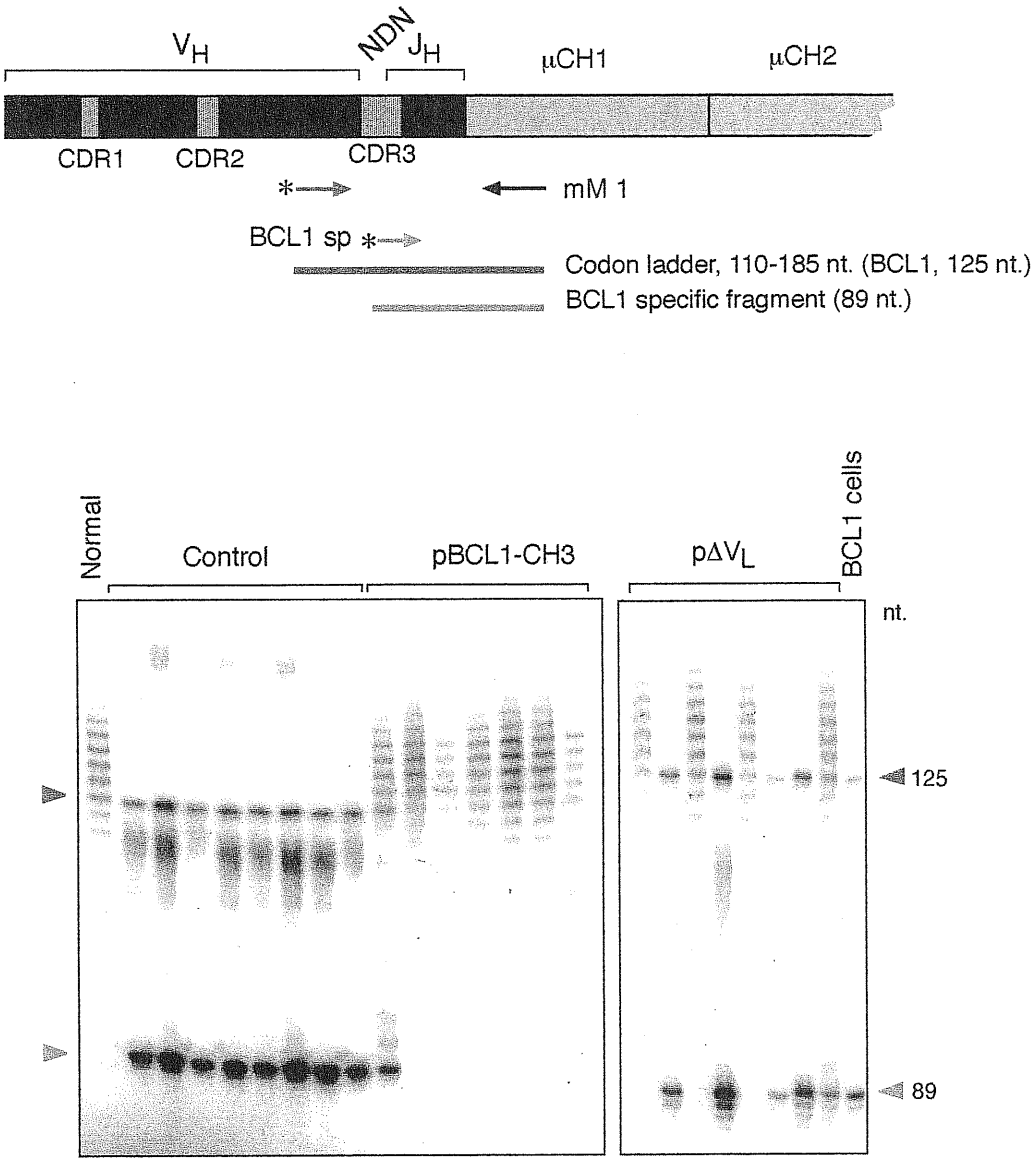


Figure 15

Assessment of tumor progression by Ig gene fingerprinting analysis of IgM expressing B-cells in challenged animals. The μVH transcripts expressed by peripheral blood B-cells were first amplified using a primer specific for Cμ (mM1) and a primer complementary to VH FW1 (indicated in the scheme at the top of the figure). Labeling of the PCR products with a FW3 specific primer (mFW3) allowed visualization of a ladder of bands corresponding to the different CDR3 lengths of the VHDJH gene rearrangements. A second labeled primer specific for the tumor derived CDR3 (BCL1sp) was included in the labeling reaction to increase the sensitivity of detection of the malignant clone. The autoradiograms show the analysis of PBMC from the indicated groups of animal. Control and immunized animals were challenged at the same time with the same number of cells. Arrowheads indicate the location of the 125 nt and 89 nt bands specific ofthe BCL1 clone.

mouse is polyclonal with a ladder of bands representing CDR3s of different lengths. Control animals challenged with BCL1 tumor cells showed a unique band corresponding to the length of the BCL1 CDR3, indicating that the malignant clone had overgrown the normal B-cells. The group of mice immunized with the pBCL1-CH3 construct showed a pattern that was identical to the one of the unchallenged animal, indicating rejection or much slower progression of the tumor. For the pΔVL group 50 % of the animals showed a polyclonal pattern while in the remaining 50% the monoclonal population of BCL1 cells was clearly detectable, but residual normal B-cells were also present, indicating that in this group the growth of the tumor is slowed down. Prediction made on the basis of the fingerprinting analysis correlated strictly with the survival time. As shown in Figure 16, unvaccinated animals as well as animals in the control pGM-CSF vaccinated group died by day 100 with a mean survival of 86 and 90 days respectively. All animals immunized with the plasmid containing the complete scFv construct linked to hu-CH3 in combination with the pGM-CSF plasmid mounted protective immune responses and were still alive after 220 days of follow-up ($p<0.001$). Immunization with pBCL1-CH3 alone led to survival of more than 220 days in 70% of the cases, while the other animals died in the same period as control animals. Mice immunized with pBCL1-CH3 plus pCD40L performed like those immunised with pBCL1-CH3 alone, indicating the lack of a significant positive effect in terms of survival. Mice immunized with the pΔVL-CH3 construct survived slightly longer than animals in the control groups (mean 118 days) but all of them still died by day 150 and the pronged survival did not reach statistical significance.

In a second set of experiments we used a variant of the BCL1 cell line that grows faster and kills control animals in 30-40 days. We compared animals vaccinated with pBCL1-CH3 and one of the chimeric constructs (pV_L^{6C6}/V_H^{BCL1}) that contains only the tumor derived VH. As a control we also tested animals immunized with the BCL1 scFv construct without the xenogenic CH3 domain and with the irrelevant p6C6 plasmid.

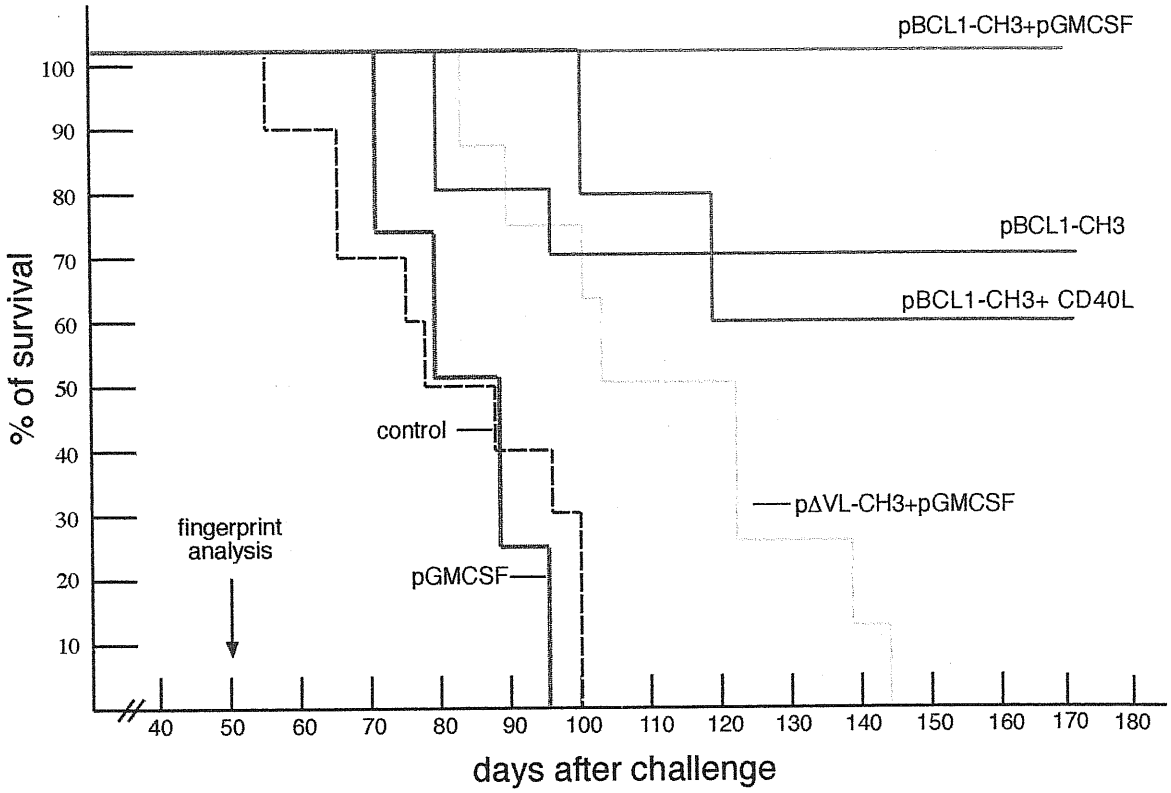


Fig 16

Survival of immunized mice after challenge with the BCL1 lymphoma. Mice were injected intradermally on days 0, 14, and 28 with pBCL1-CH3+pGM-CSF (10 mice), pBCL1-CH3 +pCD40L (5mice), pDVL-CH3 + pGM-CSF (7 mice), pBCL1-CH3 without the cytokine encoding plasmid (10 mice) or pGM-CSF alone (4 mice). Three weeks after the last vaccination they were challenged with 10^6 BCL1 lymphoma cells and their survival was compared with that of unimmunized (n=10) mice. Animals were followed until 220 days after tumor challenge.

Immunized animals were challenged with $5x10^5$ lymphoma cells two weeks after the last boost. Vaccination with pBCL1-CH3 induced protection against the BCL1 lymphoma ($p<0.01$) while all the other constructs were ineffective with mean survival that ranged from 37 to 44 days (fig 17).

The ability of our pBCL1-CH3 construct to confer protection against the BCL1 lymphoma is further supported by experiments performed in Southhampton by the group of F.Stevenson (see section 8 of results).

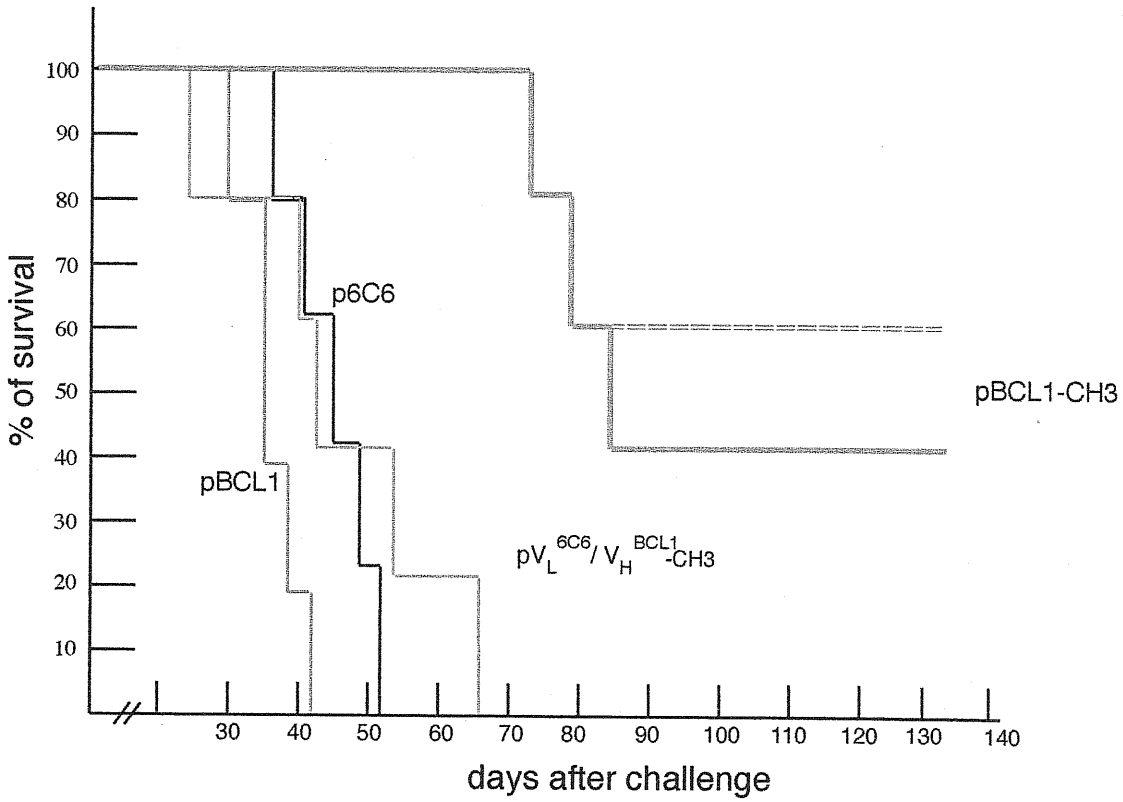


Fig 17

Survival of immunized mice after challenge with the BCL1 lymphoma (more aggresive cell line). Mice were injected intradermally on days 0, 14 and 28, with pBCL1-CH3 (5 mice), pV_L^{6C6}/V_H^{BCL1}-CH3 (5 mice), pBCL1 (5 mice) or p6C6 (5 mice). Three weeks after the last vaccination they were challenged with $5x10^5$ BCL1 lymphoma cells. Animals were followed until 130 days after tumor challenge.

7. T-cell proliferation

The ability of pBCL1-CH3 to induce high levels of anti-Id antibodies opposed to the complete failure of pBCL1 indicates that it is necessary to break the tolerance at the levels of T-cells by fusing the idiotype to a carrier that contains T-cell epitope.

Most likely CD4+ T cells carrying a TCR specific for epitopes on the idiotype have been eliminated during tymic selection. Hence, T cell help should be provided by CD4+ T cells of a different specificity. Fusion of the idiotype to a T cell epitopes carrier implies that B cells with a surface immunoglobulin specific for the idiotype will uptake, process and present epitopes from the idiotype and from the (in our case) human CH3 domain.

$CD4^+$ T cells with a TCR specific for epitopes on the hu-CH3 xenogeneic domain will recognise MHC class II/hu-CH3 peptide complexes on the surface of B cells. By this "trick" also B cells carrying a surface immunoglobulin against the idiotype will receive effective help and will differentiate into plasma cells.

To test whether the antibody response following pBCL1-CH3 immunization depends on delivery of cognate T-cell help, we have performed a $CD4^+$ T cell proliferation assay based on the expression of the IL2 receptor. Surface expression of IL2R represents a reliable marker of T-cell activation since it has been shown to be upregulated upon TCR engagement and interaction with costimulatory molecule on APC (**105**). Spleenocytes from immune (pBCL1-CH3) or control non-immunized animals were collected and incubated with purified BCL IgM or human IgG to induce proliferation of in vivo primed CD4+ T cells. After 5 days cells were harvested and double stained with anti-CD4+ (FITC conjungated) and anti-IL2R (phycoheritrin conjugated) antibodies. The result of FACS analysis in fig 18 indicates that among immune but not control splenocytes, there is a population of double positive cells (upper right panel). These cells are CD4+ cells that express IL2R in response to human IgG. On the contrary no shift was observed in the case of immune/non immune splenocytes incubated with BCL1 IgM. Controls with other Igs isotype did not exert any effect on the staining pattern of the splenocyte population.

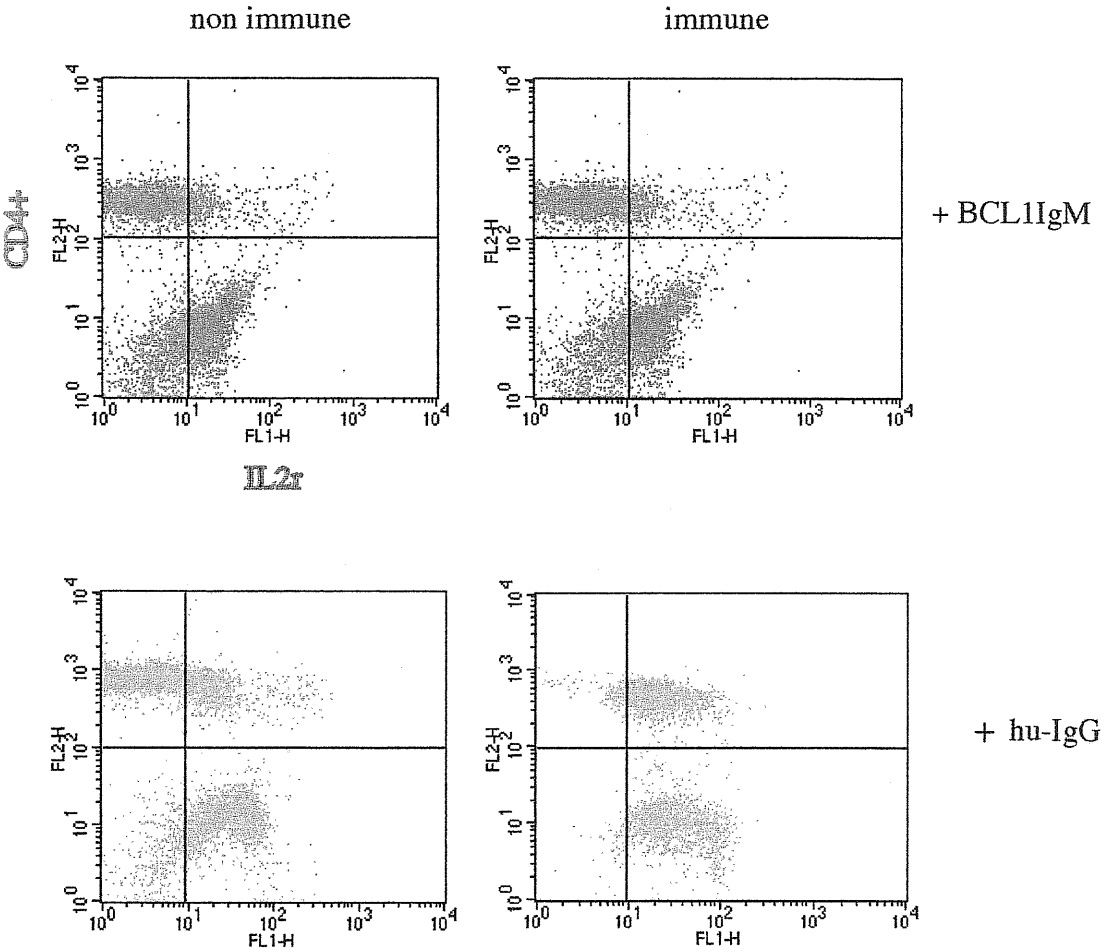


Fig 18

T-cell proliferation assay. Spleenocytes from non immune or immune (pBCL1-CH3) donors were incubated with 100ug/ml of idiotypic protein (BCL1 IgM) or human IgGfor seven days. Cell were harvested and double stained with fluoerescein labelled anti-IL2 receptor Ab and phycoeritrin ant i CD4+ Ab..

It therefore appears that there are no CD4 $^{+}$T cells specific for the idiotype. Hence, the mechanism by which idiotype specific B cells get activated is likely to depend on delivery of T cell help by hu-CH3 specific CD4^{+} T cells.

8. Role of linker sequence in the scFv design.

Thanks to the collaboration with the group of F.Stevenson in Southampton (U.K.), we have highlighted the role that the sequence of the linker between VL and VH in a scFv molecule may play in determining the structure of the scFv with regards to the structure of the original idiotype. This group was the first to demonstrate protection against the BCL1 lymphoma using purified idiotypic protein (**56,58**). However, attempts to vaccinate animals with a DNA construct encoding the BCL1 V regions in a scFv format were disappointing, therefore the group started to work on a different lymphoma model (A31). They showed that fusion of the scFv to a gene encoding fragment C of tetanus toxin substantially promotes the anti-idiotypic response and induces strong protection against the lymphoma (**90**).

The BCL1 scFv construct of F.Stevenson (pBCL1UK) differs from ours (pBCL1It) in two aspects:

1) the VH is cloned upstream of the VL (our is VH-VL)
2) it contains a 15 aa linker [(Glyx4Ser)x3] (**71**) while our is 18 amino acid long (GSTSGSGKPGSGEGSTKG) (**97**)

In order to evaluate whether these differences in design can explain the different outcome of vaccination experiments, (pBCL1-It fused to a xenogeic region is very effective in inducing anti-Id antibodies against BCL1 while pBCL1-UK is inefficient even when linked to a strong carrier such as FrC of tetanus toxin) the two scFvs were compared in the same conditions.

These experiments were performed in the laboratory of F. Stevenson. The pBCL1-It coding region (VL-linker 18-VH) was cloned into a pcDNA3 vector containing FrC (pBCL1It-FrC) and compared in the same immunization experiment with pBCL1It-CH3 (our original contruct) and pBCL1UK-FrC. ELISA values of animals immunized with three intradermal injection of each construct at two weeks intervals are shown in fig 19 (panel a). It appears that both pBCL1UkFrC and pBCL1It-FrC induce anti-FrC antibodies but the anti-Id response came up only with pBCL1It-FrC. Levels of anti-Id antibodies are

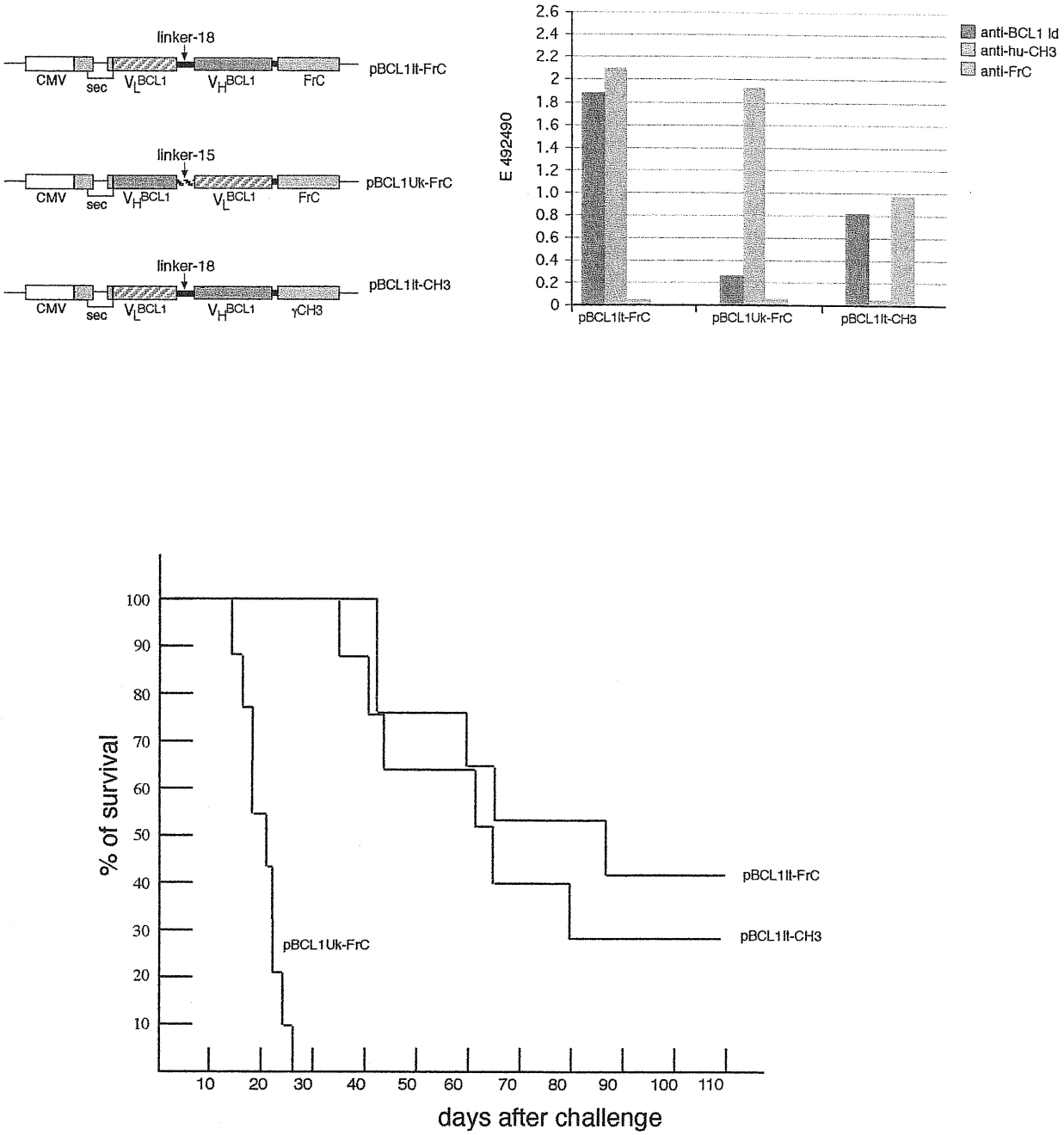


Fig 19

Comparison of two different BCL1 scFv constructs. a) Animal were immunized according to the standard protocol with the indicated constructs. Immune sera were analysed on plates coated with BCL1 IgM (anti-Id), fragment C of tetanus toxoid (FrC) or human IgG. Mead OD values for each group (1:800) are represented a solid bars. b) Survival curve of mice immunized with the indicated construct (n=8/group) and challenged with 10^5 tumor cells. The experiment was performed in the lab of F.Stevenson (Southampton, UK)

higher for the pBCL1It-FrC fusion than for pBCL1It-CH3, indicating that FrC is a more potent T cell epitopes carrier than the hu-CH3 domain. However, vaccination with both fusions containing the pBCL1-It (pBCL1It-FrC and pBCL1It-CH3) conferred protection against tumor challenge (fig 19, panel b).

Section 2.

Analysis of the idiotype structure

Introduction

In the first section of results I described the development of an efficient DNA vaccine for the BCL1 lymphoma. In the present section we explored the structural basis of the Id/anti-Id interaction upon immunization with scFv plasmid DNA. The aim of the experiments presented below was to map the binding of anti-Id antibodies on the idiotype in native like conditions. While several studies have focused on the strategies to improve the immunogenicity of the idiotype for vaccination pourposes, very little is known on the contribution of V_H and V_L variable domains to the idiotype structure in a lymphoma model. This issue was addressed in other systems mainly using monoclonal Abs induced by protein immunization. However, these studies yielded discordant results. In same cases the binding of anti-Id antibodies was shown to depend on the presence of combined epitopes deriving from VL and VH (**106-108**) while in other cases anti-Id Abs bound to VH isolated determinants regardless of the VL partner (**109-111**).

Starting from our BCL1 model system we generated four different genetic constructs, two parental (BCL1 and 6C6) and two chimeric (mismatched BCL1/6C6 V region associations), that were used both, as immunogens in a soluble SIP version (results section 1.3) as well as displayed target idiotypes in their membrane bound forms (ε-mSIP). Analysis of the polyclonal immune response on membrane dysplayed idiotypes showed that genetic immunization with plasmids encoding scFv idiotypes is exclusively directed against determinants depending on the immunizing V_L/V_H association. On the contrary, a plasmid encoding a misfolded V_H induced antibodies reacting also with the immunizing V_H, regardless of the V_L partner. Moreover, sera raised by immunization with purified scFv protein reacted with BCL1 V_L and V_H determinants associated to irrelevant V region.

These findings indicate that presentation of properly folded idiotypes results in a highly specific antibody response directed exclusively to private idiotypic determinants of the V_L/V_H combination of the immunogen.

1. Display of chimeric scFv as membrane proteins.

We investigated the overall polyclonal anti-Id antibody response raised by pBCL1, in terms of reactivity against specific V_L, V_H, or combined V_L/V_H determinants. To address this issue we developed a genetic strategy to display idiotypes arising from different V region pairings on the surface of transfected cells. We designed a membrane version of SIP (ε-mSIP), based on the human membrane IgE isotype (fig 20). This vector contains a scFv fused to the C-terminal region of the human membrane IgE H chain, from εCH4 down to the cytoplamic tail. ε–mSIP are efficiently expressed as membrane proteins (Bestagno et al, unpublished), allowing flow cytometry visualisation of anti-Id Abs reacting with idiotypic determinants displayed in native-like conformation.

DNA cassettes encoding scFvs of BCL1, 6C6 and the two BCL1/6C6 chimeras described in section 1.3 were cloned into the ε–mSIP vector (fig 21 panel a). The four ε-mSIP were transfected into the Ig^- (non-secreting) mouse myeloma cell line Sp2/0. Screening of positive clones was performed by FACS analysis using a FITC conjugated anti-human IgE antibody. Several positive clones were identified for each construct, with heterogeneous levels of expression of surface εCH4. Therefore we decided to select four clones, one for each construct, showing comparable surface expression levels (fig21, panel b).

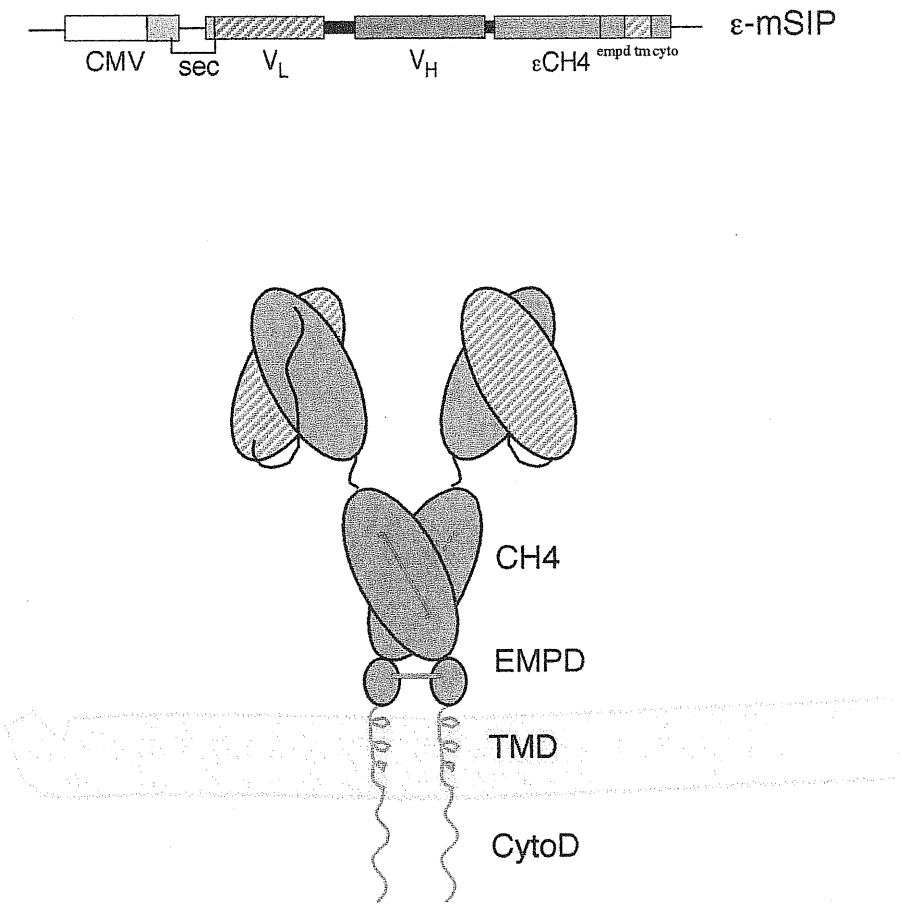
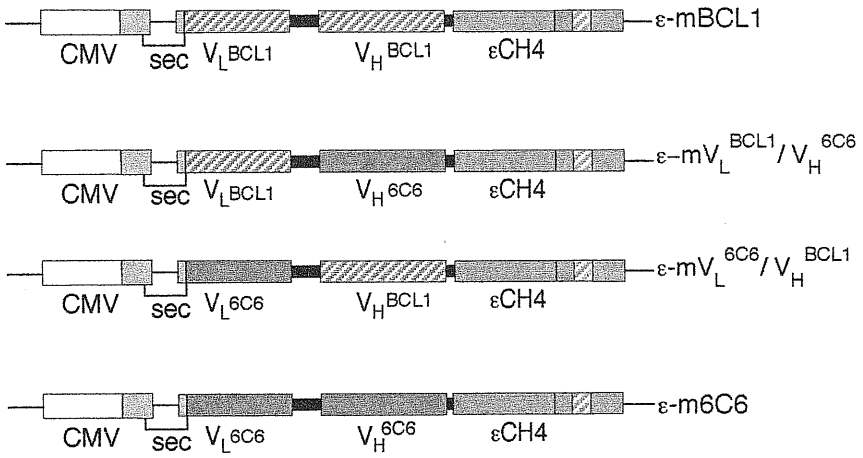


Fig 20

Scheme of the ε-mSIP. This vector contain a scFv fused to the C-terminal region of the human membrane IgE H chain. It comprises the εCH4 domain, the extramembrane proximal domain (EMPD), the transmembrane domain (TM), and the cytoplasmic tail (cyto). In the bottom part of the figure is depicted a schematic representation of the protein encoded by this vector in transfected cells.

a



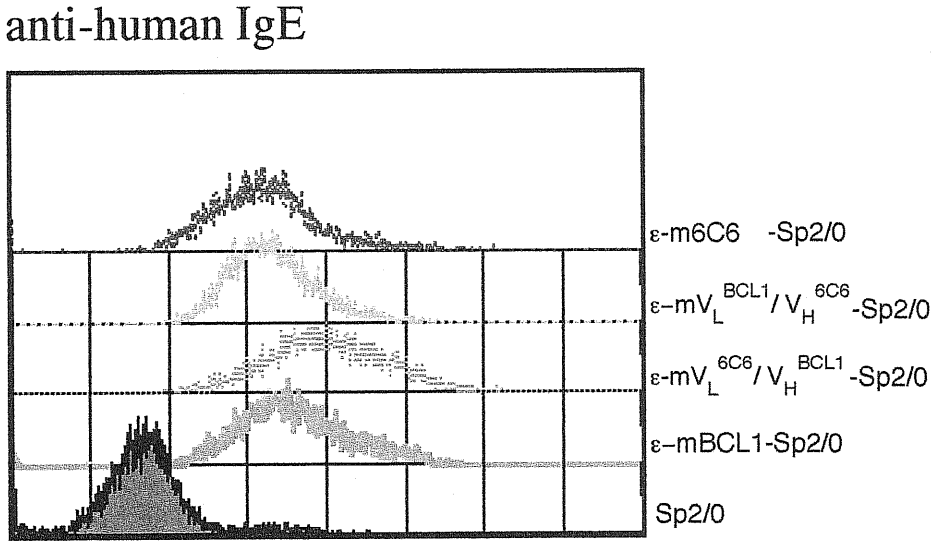


Fig 21

a) Schematic representation of the mε-SIP constructs generated to analyse chimeric idiotypes expressed in their native like confromation on the surface of transfected cells. b) Expression of membrane εCH4 domain by Sp2/0 cells transfected with the four ε-membrane scFv constructs. Sp 2/0 wild type cells (control) or transfected clones (as indicated) were incubated with FITC conjugated anti-human IgE and analysed by flow cytometry.

2. Analysis of the anti-Id antibodies raised by pBCL1 on chimeric idiotypes.

The transfected clones displaying different combination of V regions were design to analyse, individually, the response against each of the two chains. Pairing to an irrelevant VL or VH partner allow the V region investigated to be analysed independently from its own partner but folded as it would be in the original idiotype.

We first wanted to verify that the BCL1 idiotype was well displayed on the surface of Sp2/0 cells transfected with ε-mBCL1. We incubated this clone with two idiotype specific reagents. The monoclonal mAb (Mc10 4A12) was raised following immunization with BCL1 idiotypic protein and it was shown to map on the BCL1 VH (**96**). As a second reagent we used the anti-BCL1 idiotype specific sera raised by pBCL1-CH3 immunization (Results section 1, 1.2). Flow cytometry in fig 22 shows that both reagents strongly stained ε-mBCL1 Sp2/0 transfected cells. As a control we showed the sera raised by p6C6 do not bind to the BCL1 idiotype displayed in Sp2/0 cells. This control indicates that the response is highly specific. It excludes the presence of antibodies directed against the 18 aa linker between V_L and V_H, identical in the all the soluble and membrane scFvs. In addition, this result exclude any cross-reactivity of anti hu-γCH3 antibodies towards the human εCH4 domain present in the membrane SIPs.

To evaluate the ability of anti-Id antibodies raised pBCL1 to bind VL or VH of BCL1 displayed in the context of a different pairing we incubated the pBCL1 induced serum with the four membrane idiotypes. Strikingly, no staining was detected on either of the two BCL1/6C6 chimeras (ε–mV_L^{BCL1}/V_H^{6C6} and ε-mV_L^{6C6}/V_H^{BCL1}) displayed on Sp2/0 cells. This result indicates the absence, in the polyclonal anti-Id sera, of reactivity against determinants expressed on V_L^{BCL1} or V_H^{BCL1} in non parental pairing (Fig 23). As a control we showed that mAb Mc10 4A12 stained Sp2/0 cells transfected with the chimera containing only the BCL1 VH (ε-mV_L^{6C6}/$V_H^{BCL1)}$ confirming that the V_H of BCL1 is properly displayed even in the context of a non-parental pairing (fig 23, upper lane). Moreover, it was interesting to observe that sera raised by the pBCL1ΔVH-CH3 construct (see Results section 1, 3.1-3.2) bound not only to the BCL1 idiotype but also to the

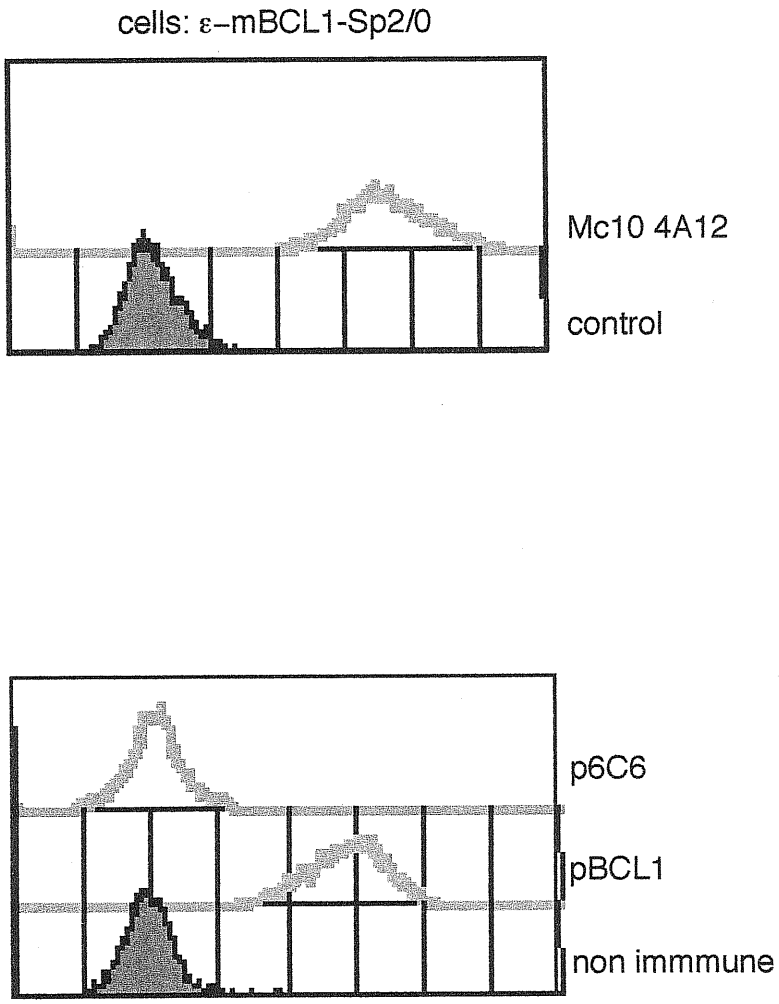


Fig 22

Display of BCL1 idiotype on Sp2/0 cells transfected with the ε–mBCL1 construct. Proper expression of the idiotype was assesed by flow cytometry using Mc 10 4A12 and immune sera raised by pBCL1 or p6C6 (1:200).

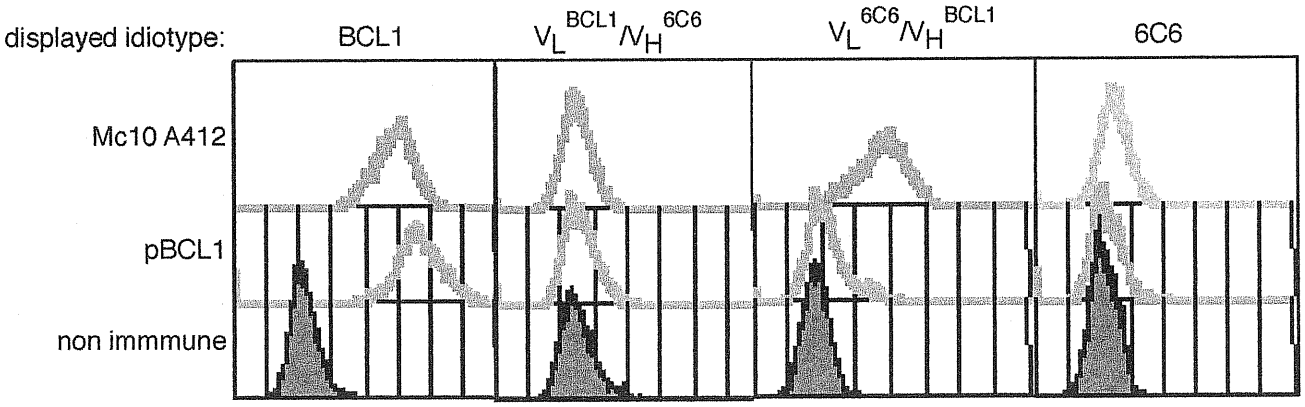


Fig 23

Reactivity on chimeric idiotypes. Different dilutions (1:50 to 1:200) of serum induced by pBCL1 were analyzed by flow cytometry on Sp2/0 cells displaying parental or chimeric idiotypes following transfection with ε-mBCL1, ε-mV_L^{BCL1}/V_H^{6C6}, ε-mV_L^{6C6}/V_H^{BCL1} and ε-m6C6. The figure shows a representative experiment at 1:200 dilution. The upper panel shows reactivity of Mc10 4A12 on the four transfectants.

chimera containing only the BCL1 VH (V_L^{6C6}/V_H^{BCL1}) (fig 24). This suggests that sera raised by pBCL1ΔVH-CH3 contain antibodies directed against determinants exposed on the VH, regardless of the VL partner. As it will be discuss later, this is probably due to the exposure of misfolded epitopes in the immunogen. In fact, the VH-CH3 protein is not secreted and retained intracellularly. It would therefore appear that this epitopes are still accessible in the folded protein. However, when the immunogen is given as folded (complete scFv), the response against folded epitopes is highly dominant.

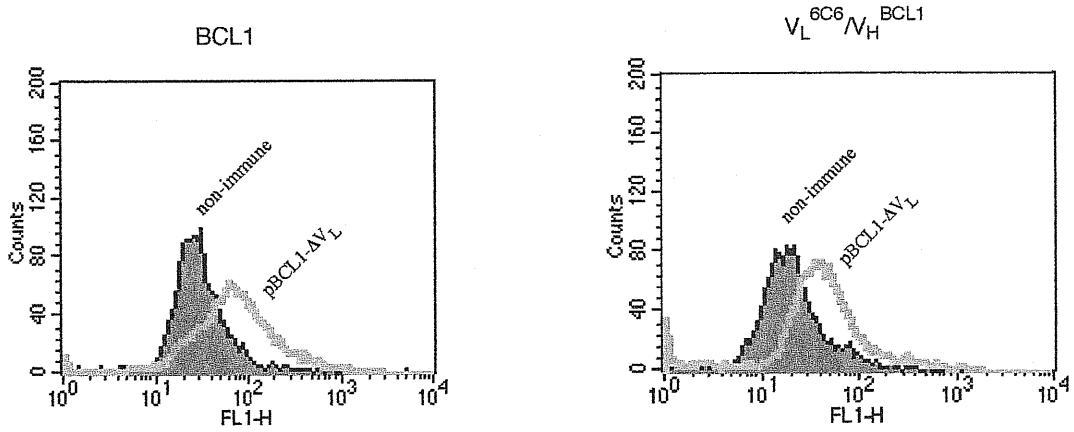


Fig 24

Analysis of sera induced by pBCL1ΔVH-CH3 immunization on Sp2/0 cells displaying th BCL1 and V_L^{6C6}/VH^{BCL1} idiotypes. Transfected cells were incubated with 1:200 dilution of immune sera (as indicated) and stained with FITC conjugated anti-mouse IgG.

3. Crossed analysis of sera induced by chimeric scFvs on chimeric displayed idiotypes.

The results obtained in the previous section were surprising because we expected at least some reactivity against "isolated" determinants. This unpredicted observations prompted us to extend the analysis to other cases. In previous experiments (Results, section 1, 3.1-3.2) we raised sera with constructs encoding the corresponding soluble scFv form of each displayed idiotype. Therefore we performed a crossed analysis of the displayed idiotypes with sera raised by pV_L^{BCL1}/V_H^{6C6}, pV_L^{6C6}/V_H^{BCL1} and p6C6. Flow cytometry analysis shown in figure 25 clearly demonstrates that each membrane displayed idiotype is only recognized by antibodies induced by the corresponding soluble scFv. Noteworthily, no detectable cross-reactivity was observed by any of the immune sera raised with scFvs containing only one of the two V regions.

All four cases presented provide strong evidence that the anti-Id immune response induced by scFv genetic vaccination is strictly confined to conformational V_L/V_H combined determinants with no reactivity against isolated V_L or V_H determinants. The inability of sera raised by pBCL1 to recognize purified BCL1 IgM protein in western immunoblotting confirms that DNA vaccination induces exclusively conformational anti-Id Ab (data not shown).

4. Comparative analysis of DNA-versus protein-induced sera

The high specificity of the antibody response towards combined V_L/V_H determinants in the four idiotypes investigated, suggested this to be a characteristic of genetic immunization. Hence, we decided to compare the reactivity of anti-Id antibodies induced by protein immunization. The BCL1 scFv protein was purified from the supernatants of Sp2/0 cells transfected with pBCL1-CH3 (Results, section 1, 1.2) by using an affinity column linked to anti-human IgG. Purified fractions were dialysed and concentrated to 1μg/μl. Following two injections of 50 μg of scFv protein, sera were collected and analysed by ELISA. The titers against the hu-CH3 were identical to those induced by three shots of plasmid DNA,

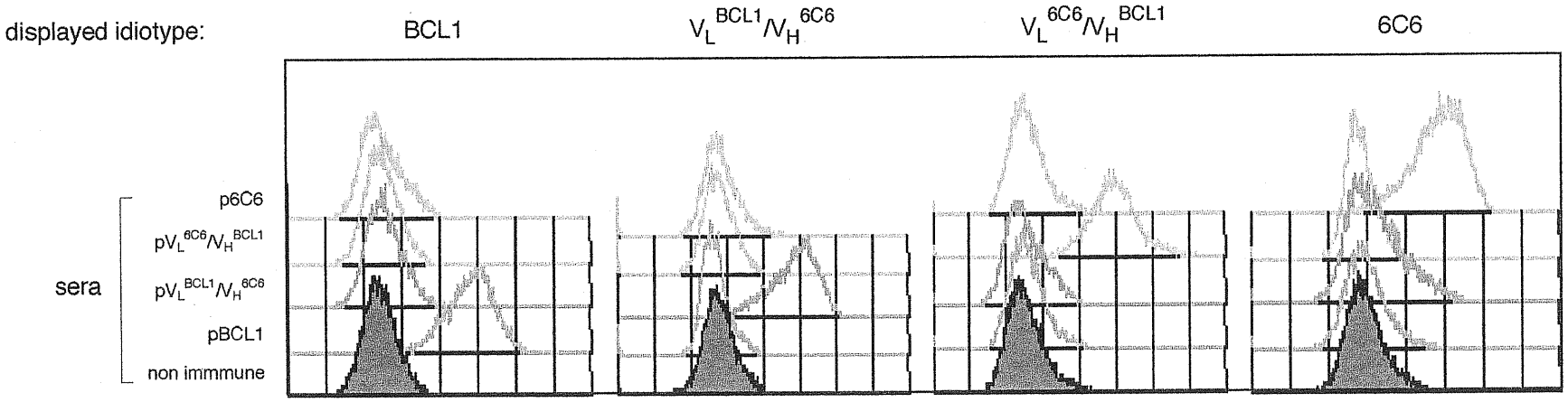


Fig 25

Crossed analysis of immune sera on chimeric idiotypes. Different dilutions of sera induced by the indicated plasmids were incubated with Sp2/0 displaying the different idiotypes and analyzed by flow cytometry. A representative experiment at 1:200 dilution is shown.

therefore we decided to terminate the immunization and test the sera on the displayed idiotypes.

Sp2/0 cells transfected with the four displayed idiotypes (BCL1, V_L^{BCL1}/V_H^{6C6}, V_L^{6C6}/V_H^{BCL1} and 6C6) were incubated with sera raised by the BCL1 scFv protein and by pBCL1 for comparison. Indeed, flow cytometry revealed that anti-Id antibodies induced by protein immunization, as opposed to those induced by DNA immunization recognized isolated V_L^{BCL1} and V_H^{BCL1} determinants (Fig 26). This result indicates that a distinct antibody response is obtained depending on the mode in which the same protein antigen is presented to the immune system.

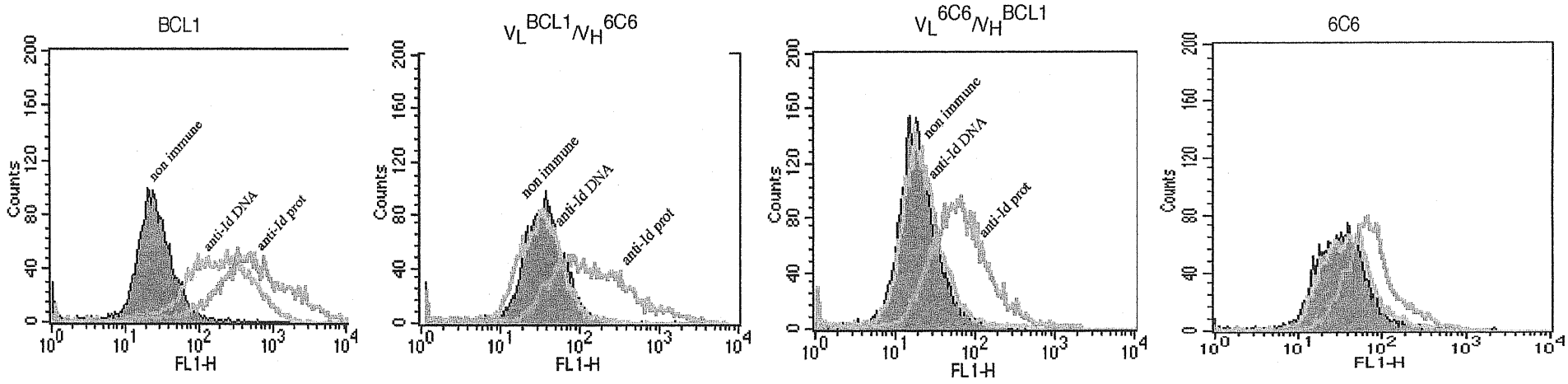


Fig 26

Comparative reactivity of DNA- versus protein-induced sera. Transfected Sp2/0 cells displaying the BCL1, V_L^{BCL1}/V_H^{6C6} and V_L^{6C6}/V_H^{BCL1} and 6C6 idiotypes were incubated with sera (1:50) induced by pBCL1 (anti-Id DNA) or by BCL1 scFv-CH3 protein (anti-Id prot) and analysed by flow cytometry.

CHAPTER 4

DISCUSSION

Although conventional chemotherapy and radiation treatments of NHL often induce tumor regression it is difficult to eradicate all tumor cells, and eventual relapse is common. The ineffectiveness of these modalities, coupled with the potentially armful effects of such treatments, has motivated the search for alternative tumor therapies. One such alternative is to utilize the immune system specifically to target and to eliminate neoplastic cells based on their expression of immunogenic surface markers. Ig^+ B cell malignancies are unique because they bear a well defined tumor specific antigen in the form of idiotypic determinants expressed by immunoglobulin V regions.

After the first demonstration that in the MOPC-315 murine model injection of idiotypic protein could induce anti-idiotypic antibodies and protection against tumor challenge (**54,55**), several vaccination strategies based on injection of idiotypic protein proved beneficial in variuos lymphoma models (**56-65**). Most recently, the protein based vaccination approach has been overtaken by molecular cloning techinques and DNA-based antigen delivery system. In this respect, tumor derived variable regions cloned in a single chain Fv format, the smallest molecule that retain the antigen binding specificity of the original antibody, were most extensively tested (**86-88,90,92,94**).

Collectively, these studies showed that the syngeneic idiotype (adminstered as protein or as DNA) needs to be linked to a T cell epitopes carrier to become imunogeneic. Concerning the mechanism of tumor protection it has emerged that rejection of tumor cells is mainly mediated by anti-idiotypic antibodies.

In this thesis we investigated in details the requirements of a DNA vaccine in the BCL1 lymphoma model. We developed an efficient strategy to induce high levels of anti-idiotypic antibodies and protection against tumor challenge (**100**). By modifying the

idiotype encoding plasmid we addressed several questions concerning the mechanism of the immune response following DNA vaccination. In particular, we analysed the contribution of each of the two tumor derived V regions both to the induction of the immune response and as target for the binding of anti-idiotypic antibodies.

Earlier studies in our laboratory focused on the production of small immunoproteins (SIPs) based on scFv fragments connected to the CH3 domain of the human immunoglobulin γ1 H-chain. The CH3 domain drives the formation of dimers, resulting in bivalent SIPs carrying a double antigen binding site. SIPs with antigen specificity for tumor antigens are very interesting as therapeutic molecules because of their enhanced tissue penetration and increased avidity, and as diagnostic tools for their rapid clearance.

We reasoned that these molecules could be suitable for our vaccination strategies, since they are efficiently expressed and secreted upon transfection in mammalian cells (97). Moreover, it is important to recall that in this setting mouse V regions represent a self-antigen. It is therefore conceivable to assume that tolerance at the levels of T $CD4^+$ cells may exist. The presence of a xenogeneic (human CH3) domain linked to our scFv could break the tolerance and allow the delivery of cognate help to B cells specific for the idiotype encoded in the scFv.

To generate a BCL1 specific scFv we cloned the BCL1 VL and VH domain in the backbone of the SIP construct containing the human CH3 domain. The linker between VL and VH in our scFv design is 18 amino acid long (71) and guarantees optimal folding of the scFv molecule. As discussed later, this linker played a crucial role in the success of our vaccination strategy, as documented by a comparative study with a BCL1 scFv containing a different linker (Results section 1, 8).

To evaluate whether the CH3 domain was indeed helping the induction of the anti-idiotypic immune response, we compared one plasmid containing only the scFv coding

region (pBCL1) to a second plasmid containing the same scFv linked to the CH3 domain of human IgG1 (pBCL1-CH3).

Immunization experiments showed that only pBCL1-CH3 was able to induce anti-idiotypic antibodies while pBCL1 was completely inefficient (Results, fig 2).

T cell proliferation experiments further supported the evidence for the need for an MHC class II, T-cell epitope to induce a response against a syngeneic idiotype. In fact, T cell from an immune spleen (pBCL1-CH3) were only able to proliferate in response to human IgG and not in response to idiotypic protein (Results, fig 18).

We also tested a second T cell epitope carrier, the SAPA amino acid repeats from Tripansoma Cruzi. This domain is the immuno-dominant portion of an enzyme essential to the parasite cell cycle. Anti-SAPA antibodies were shown to persist for years after the infection in humans (**101,102**). We linked the SAPA domain to the BCL1 scFv instead the hu-CH3 domain. However, the fusion protein encoded by pBCL1-SAPA was retained in the endoplasmic reticulum (Results, fig 5). Immunization with pBCL1-SAPA induced low but clearly detectable level of anti-Id antibodies as assessed by both ELISA and FACS analysis (Results, fig 6). This indicates that although SAPA is able to function as a MHC class II restricted T-cell provider for the activation of idiotype specific B-cells, it is less potent that human gamma CH3. The low levels of anti-Idiotypic antibodies induced by pBCL1-SAPA cannot be accounted for only by the intracellular retention. In fact, immunization with an intracellularly retained form of BCL1-CH3 still induced 2 to 4x higher titers than the BCL1-SAPA fusion.

The immunodominance of the SAPA domain in humans may depend on the presence of MHC alleles which are very efficient in loading and presenting SAPA epitopes. This may not necessarily be true in the mouse thus explaining the disappointing result we have observed in the murine model. Although for obvious reasons it is not possible to test vaccines in humans, it is worth considering that the efficacy of T cell epitopes carriers can vary among species.

Analysis of the IgG subclass isotype induced by immunization with a given antigen has been considered as a marker to distinguish between a TH_1 and TH_2 dominated immune response (Introduction, 2.3). In our case we detected only antibodies of the IgG_1 subclass, indicative of a type 2 T helper (T_H2) response. T_H2 cells are defined based on the pattern of cytokines they secrete (IL-4, IL-5, IL-6 and IL-10) and are very effective in helping B-cells to develop into plasma cells. A T_H2 predominance after DNA intradermal immunization was often reported in the literature (**87,112**). As it will be discussed later, this is consistent with the postulated mechanism of protection against lymphoma.

The mechanisms underlying the induction of immune responses after DNA immunization are unclear. It seems unlikely that keratynocytes (for intradermal) or myocytes (for intramuscular) transfected during the immunization can act as APC to present the antigen because they express only low levels of MHC class I molecules and lack constitutive expression of MHC class II and costimulatory molecules. Most likely keratinocytes and myocytes act merely as a source of antigen that is acquired by APC following active secretion or release subsequent to cell damage. Alternatively, it is possible that a few peripheral antigen presenting cells transfected at the injection site are sufficient to activate the response upon migration to lymphoid organs.

Comparison of the antibody response induced by different cellular localization of the antigen can help to discriminate between these possibilities. We generated two variants of the original construct that target the antigen to different cell compartments. Plasmid pBCL1-CH3memb was constructed to compare a membrane-bound versus a secreted antigen and plasmid pBCL1-CH3Δsp, that lacks the peptide leader sequence for extracellular secretion, to examine the effect of an intracellularly retained antigen (Results, fig 7). We evaluated the expression of the localization variants of scFvs by transfecting the two plasmids into an eukariotyc cell line. Consistently, pBCL1-CH3memb was expressed as a membrane protein while pBCL1-CH3Δsp as a non secreted, cytoplasmic protein. Immunization experiments showed that both constructs induced anti-idiotypic antibodies

but less efficiently then pBCL1-CH3 (Results, fig 8). This indicates that the availability of secreted antigen is a limiting factor for the priming of B-cells. Our results are in agreement with those reported by other groups who examined the response of plasmids encoding different forms of OVA (**113**) or viral peptides (**114**) and found the secreted form to be the most effective. From these studies and ours it would therefore appear that availability of soluble antigen to be transferred to APC is an important requisite for the induction of antibody responses upon DNA immunization However, these data are not sufficient to exclude also direct transfection and presentation by APC. Interestingly, the cytoplasmic form performed better than the membrane form in our system. A possible explanation is that accumulation of a cytoplasmic antigen may be toxic to the cell, hence increasing the amount of antigen released from the cells.

Another important issue commonly addressed in immunization studies, is the use of adjuvant molecules to augment or modify the immune response. Although our design was itself able to induce high levels of anti-Id antibodies, we were interested in testing a series of approaches that were previously described as successful by other authors. Granulocyte/macrophages colony stimulating factor (GM-CSF) has pleiotropic effects including activation of proliferation and differentiation of B and T lymphocytes. In particular it is a potent activator of antigen presenting cells like dendritic cells, thus influencing the initiation of the immune response. GM-CSF was shown to be efficient in enhancing the immune response against a variety of antigens, both in protein and DNA vaccination studies (**66, 68-70**).

Co-administration of pGM-CSF and pBCL1-CH3 resulted in a modest but clear and reproducible enhancement of the immune response both against the idiotype and against the human CH3 portion (Results, fig 11). Interestingly, addition of GM-CSF induced also IgG2a antibodies that were otherwise absent following immunization with pBCL1-CH3 alone. Switching to IgG2a is a consequence of IFN-γ and is indicative of a cytotoxic

immune response (**37**). This result is consistent with data reported by other authors that reported an enhancement of cytotoxic responses following GM-CSF administration (**70**). CD40L is a key molecule for activation of B cells and differentiation into plasma cells. It is expressed on activate T cells and binds to CD40 on the surface of B cells and APCs. One study reported that coinjection of a CD40L plasmid dramatically increased the response to a model DNA vaccine encoding β-gal (**84**). However co-administration of CD40L with pBCL1-CH3 did not exert any effect either on the extent or the form of the immune response. Levels of anti-idiotypic and anti-hu-CH3 antibodies were comparable to those obtained immunizing with pBCL1-CH3 alone (Results, fig 11). However, we cannot exclude that CD40L delivered as a protein may have effects that are negligible when it is administered in low amounts such as in the case of DNA immunization.

C10 is a β-chemokine that is still very poorly characterized. It is secreted by the bone marrow and macrophages. In our laboratory it was demonstrated that C10 is produced by lymphatic endothelial cells and is chemotatic for lymphocytes (Mancardi S. et al , submitted). The availability of this molecule in the lab. prompted us to test it in vivo for its ability to influence the response against our antigen. In fact it was shown that chemokines may have potent effects in promoting tumor immunity (**115, 116**) by recruiting lymphocytes and monocytes. It was recently demonstrated that immunization with a scFv plasmid encoding the variable regions of the 38C13 lymphoma fused to interferon inducible protein 10 (IP 10) was very potent inducing antibodies and protection against tumor challenge (**94**). Unfortunately, we did not detect any adjuvant effect (results, fig 11). We did not find a convincing explanation for the failure of these two molecular adjuvants (pCD40L, pC10). Although testing of C10 was challenging we were expecting an effect from CD40L, since it was previously described to be very effective. It might be that our pBCL1-CH3 plasmid provokes a response that is very closed to the maximal possible, thus hiding a possible adjuvant effect. However, we know from other immunization experiments that fusion of the BCL1 scFv to fragment C of tetanus toxoid (Results,,

section 1, 8) induces higher titers of anti-Id antibodies than fusion to the hu-CH3 domain. Therefore there is still a margin of improvement of the immunization efficacy in terms of levels of anti-Id antibodies. It is more reasonable to conclude that with this particular fusion to the hu-CH3 domain, the system is closed to saturation. Alternatively, it may be that some molecular adjuvants (CD40L, chemokines) that proved beneficial in other experimental systems are ineffective in our case.

We have also tested an additional strategy to modulate the anti-Id immune response by fusing the BCL1 scFv to a signal for targeting the antigen to antigen presenting cells. In fact, it was reported that a model antigen (the Fc region of human IgG) fused to CTLA4 dramatically increased the levels of anti-human IgG antibodies in mice. The effect was due to CTLA4 mediated targeting of the antigen to APC, optimizing antigen uptake. CTLA4 is a CD28 homologue expressed by activated T cells. CTLA4/CD28 both bind to B7 molecules on the surface of B cells and APCs providing co-stimulatory signal for the activation of the immune response (**117**). While CD28/B7 interaction is clearly involved in the activation of both B and T cells, the role of CTLA4 is still controversial (**118**). It appears that CTLA4 could inhibit T cell activation by competing with CD28 for the binding to B7. However, the amount of CTLA4 protein that was shown to induce suppression of the immune response in vivo (50μg) (**119**) is much higher (10.000-fold) that the amount of protein produced following protein immunization.

For the above considerations, we decided to test this approach in the case of a "well characterized" tumor antigen such as the BCL1 idiotype. We generated two APC targeting constructs by adding the CTLA4 coding region at the N-terminus of the BCL1 scFv construct containing the xenogeneic CH3 region and also to the version without hu-CH3 (Results, fig 12). For pCTLA4-BCL1-CH3 we demonstrated that the fusion molecule was secreted in the extracellular medium of mammalian transfected cells and that the CTLA4 domain retained its ability to bind B7 molecules (Results, fig 13). Nevertheless (unfortunately), immunization with this construct did not result in an increment of anti-Id

antibody levels (Results, fig 14). Surprisingly, we did not observe any effect even against the hu-CH3 domain, which is very similar to the model antigen used in the study we referred to. It is worth to mention that secretion in the extracellular medium upon transfection of Sp2/0 cells was lower for CTLA4-BCL1-CH3 scFv than for BCL1-CH3 (data not shown), but the levels of antibodies induced by the two constructs were comparable. It is therefore possible that the most efficient targeting of the antigen to APCs compensated a lower efficiency in secretion. However, we do not have any evidence at present to confirm this hypothesis. This could be addressed by using mutants of CTLA4 that lacks the ability to bind B7 molecules.

Immunization with pCTLA4BCL1 (without the xenogeneic hu-CH3 domain) was totally inefficient as in the case of pBCL1. Therefore, addition of CTLA4 did not render immunogenic the idiotype, further strenghtening the concept of tolerance at the T cell level. This result is in contrast to what reported by the group of Kwak who showed that fusion of the scFv encoding the V regions of 38C13 to the chemokine IP10 resulted in high reponses against the idiotype, otherwise not immunogeneic (**85**). According to the authors, in this study the acquired immunogenicity is due to targeting of the molecule to antigen presenting cells but the mechanism of activation of CD4+ helper T cells is not discussed.

A vast part of the experimental work described in this thesis concerns the analysis of the role played by the two tumor derived V regions in the induction of the anti-Id immune response. We also turned the question round and analysed the composition of the polyclonal anti-idiotypic immune response to dissect it in anti VL anti VH or anti VL/VH combined determinants. We first addressed the ability of chimeric constructs containing only one of the two BCL1 V regions associated to irrelevant partners (6C6) to induce specific anti-BCL1 idiotypic antibodies when used in immunization experiments (results, fig 9). Both chimeric construct induce high titers of anitbodies against the hu-CH3 portion (Results, fig 10) and against the corresponding idiotype displayed on the surface of transfected cells (Results, fig 25), but failed to induce any detectable level against the

BCL1 idiotype (Results, fig 10). These results attested that both parental, tumor derivedV regions, need to be associated to induce an anti-BCL1 specific antibody response.

We also tested a different design by creating deletion mutants that lacks either the VL or the VH ("one domain construct"). Immunization with the VL-CH3 alone did not induce anti-BCL1 idiotypic antibodies while, surprisingly, the VH-CH3 alone evoked a low but clearly detectable anti-BCL1 immune response (results, fig 10). We know from transfection experiments that the VH-CH3 is retained intracellularly and not secreted due to misfolding (Results, fig 9). As discussed later, the fact that the immunogen is presented to the immune system as a misfolded protein, may explains the apparent discrepancy of this result.

We next performed an analysis of the anti-idiotypic polyclonal sera induced by vaccination with pBCL1-CH3 to evaluate whether the antibodies presented reactivity against determinants expressed by isolated VL^{BCL1} or VH^{BCL} in addition to reactivity against combined VL^{BCL1}/VH^{BCL1}. We exploited a strategy developed in our lab., based on the use of vectors (ε-mSIP) that allow expression of scFv molecules on the surface of mammalian cells (Bestagno M., in preparation). ε-membrane SIPs contain a scFv fused to the C-terminal region of the human membrane IgE H chain from εCH4 down to the cytoplasmic domain (Results, fig 21).

We expressed as membrane bound molecules scFvs containing each of the two tumor derived V regions paired to an irrelevant partner (Results, fig 22). The chimeric scFvs displayed on the membrane were incubated with sera raised by soluble scFvs and the binding detected by FACS analysis. This strategy is particularly relevant for the analysis of the Id/anti-Id interaction in native like conditions. Reactivity against each of the two V regions is analysed individually but using folded epitopes, resembling the conditions of the immunoglobulin expressed on the surface of tumor cells. In addition, flow cytometry analysis of idiotypes displayed on the surface of cells, as opposed to ELISA tests, guarantee native conformation and homogeneity of the product, avoiding the treatments used during purification and coating of proteins that may produce unwanted alterations. In

addition, the idiotype protein displayed in the scFv format is identical to the one used to elicit the immune response.

A crossed analysis of the four different immune sera on the four membrane displayed scFv idiotypes showed that, in all cases, the anti-Id immune response was directed exclusively to the original V_L/V_H combination, with complete absence of antibodies recognizing determinants in any of the single V regions displayed in the context of a different idiotype (Results, figs 24,25).

It is likely that most antigenic determinants are formed by combined V_L/V_H sequences. However, it cannot be excluded that some Abs recognise determinants on a single V region, whose conformation are strictly dependent on a particular V region partner.

Our data suggest that epitopes formed by the V_L/V_H association are highly dominant, inducing an immune response in which antibodies against them become the major component. However, epitopes displayed on single V regions exist, as demonstrated by the reactivity of pBCL1–ΔVL and BCL1-CH3 protein induced sera. This also indicates that *in vivo* there are lymphocytes capable of reacting and produce antibodies against such epitopes. The phenomenon of dominance might be due to the higher avidity or higher representation of lymphocytes reacting with antigenic structures peculiar of V_L/V_H associations, thus resulting in sequestration of the available antigen by these specific lymphocytes. In the absence of dominant associations (immunization with pBCL–ΔVL) even determinants expressed on single V regions would reach a concentration sufficient to trigger activation of lymphocytes specific for individual chain determinants.

The same argument may also explain the results obtained with sera induced by protein immunization, that involves presentation to the immune system of larger amounts of antigen than DNA immunization. In this case, the higher availability of antigen would suffice to stimulate a broader range of idiotype-specific lymphocytes.

However, it is also possible that partial misfolding of the antigen plays a role in the differences of the anti-Id responses observed. For instance, it may be that anti-BCL1 V_H antibodies induced by pBCL1-ΔV_L (the plasmid encoding the V_H only) arose as a

consequence of exposure of misfolded epitopes in the immunogen, since the BCL1-VH-CH3 protein is retained intracellularly and not secreted (**100**). In the case of protein immunization, ex-vivo treatments of the immunogen (purification, mixing with adjuvant) might affect the folding of the protein disclosing epitopes belonging to a single V region and permitting the induction of chain-specific antibodies.

It is important to consider that genetic immunization relies on small amounts of antigen produced and assembled by cells of the host. This method provides inherent quality-control systems, by means of which only properly folded molecules are secreted and rendered available for processing by the immune system. The data presented in this report indicate that our scFv DNA vaccination design ensures a highly specific anti-idiotypic immune response that depends on the quaternary structure of the antigen. In this setting it is worth mentioning that DNA vaccination was shown to be as efficient as protein immunization in inducing protection, although levels of anti-Id antibodies against the total lymphoma immunoglobulin were much lower (**87**).

Altogheter these considerations are important for the design of proper antigenic immunogens in anti-Id immunotherapies and for the correct analysis and follow up of anti-Id immune responses in clinical trials.

We perfomed tumor challenge experiments to evaluate the ability of the immune response induced by our vaccination strategy to protect animals from the growth of BCL1 lymphoma cells. Immunization with pBCL1-CH3 was highly effective in inducing protective anti-tumor immunity (Results, figs 15, 16, 17). Seventy percent of the animals immunized with pBCL1-CH3 and 100% of the animals co-immunized with pBCL1-CH3 and pGM-CSF were still alive at the end of the study. **This was the first demonstration of a protective DNA vaccine in the BCL1 lymphoma model (100).** Both tumor derived V regions in a scFv were required to induce clinically relevant immune responses. Protection was reproducible with two different BCL1 cell lines (mean survival of control animals of 40 and 80 days) and confirmed by a set of experiments performed by the

group of F. Stevenson, who has a long-standing experience in the BCL1 lymphoma model. However it must be noted that BCL1 B cells could be detected in long term survivors animals using a sensitive RT/PCR analysis of Ig gene rearrangements, indicating that the immune response induced a state of tumor dormancy rather than complete rejection of the malignant B cells. Protection induced by the BCL1 idiotypic protein was also shown to induce a state of tumor dormancy (**57,59**). Unfortunately we were not able to performe experiments to investigate directly the mechanism that induces protection. Techinical problems in growing the BCL1 tumor cells line precluded us to test all the vaccinated groups in tumor challenge experiments. However, as far as we can conclude from the groups analized, all animals that mounted high levels of anti-idiotpyic antibodies showed resistence to tumor challenge while those with lower or absent levels were not protected.

The relative contribution of antibody versus $CD8^+$ immunity in protection against the growth of surface Ig^+ B cell malignancies has been addressed in several studies by passive transfer experiments and depletion of $CD4/8^+$ T cells subset. In the BCL1 lymphoma the mechanism was analysed following protein immunization revealing a major role for antibody in protection against the tumor (**58**). The same conclusion was achieved in the 38C13 and A31 lymphoma model following DNA immunization (**90,92**). It was shown that passive transfer of immune sera but not of immune lymphocytes to naïve recepients was able to confer protection against subsequent tumor challenge. Whether this effect implies a role for Ab-dependent effector mechanism such as complement or ADCC (Ab-dependent cellular cytotoxicity), or is the result of tumor growth inhibiton through direct signalling remains to be established. Preliminary experiments in our model indicate that anti-idiotypic antibodies do not cause direct apoptosis of tumor cells, as assessed by the annexin V staining method. On the other hand, assay of complement mediated cytotoxicity in the presence of anti-idiotypic antibodies proved to be extremely variable and did not allow to drawn conclusive results.

However, other authors reported the presence of CD8+ T cell able to lyse tumor cells in animals immunized with preparation of idiotpyic protein (**70,94**) and claim for a dominant role of cytotoxic T cells in rejecting the tumor. All together the data accumulated so far do not define a general and widely accepted mechanism to explain the rejection of lymphoma cells following idiotpyic vaccination. In considering tolerance to self antigen at the T cell level, it seems unlikely that all lymphoma V regions would carry epitopes (that should derive mainly from the CDR3s, the "less-self" portion of V regions) which can be loaded into the groove of MHC class I. While tolerance of CD4 T cells can be bypassed by using a T cell epitopes carrier linked to the idiotype, the requirements for activation of CD8+ T cells are stricter. Noteworthy, anti-idiotpyic antibodies are induced by all the strategies tested so far, while CD8+ T cells were reported only in some lymphoma models and under particular conditions.

At last, I would like to comment the results of our collaboration with the group of professor F Stevenson in Southampton. As mentioned before this group demonstrated that vaccination with the BCL1 lymphoma idiotypic protein induced high levels of anti-idiotypic antibodies that protect the mice from tumor growth. However, immunization with a plasmid containing BCL1 V regions fused to a xenogeneic region failed to induce substantial levels of anti-idiotypic antibodies. Not even fusion to fragment C of tetanus toxoid, a potent T cell epitope shown to transform into strong immunogens a variety of human and also murine scFvs (**90,120**) restored BCL1 scFv immunogenicity. They tested in the same experiment our BCL1 scFv (It) and their BCL1 scFv (UK), confirming that scFv-It (our design) fused to hu-CH3 or to FrC of TT was efficient in inducing anti-idiotypic antibodies and protection from tumor challenge. This different behaviour has been attributed to the different linker sequence between VL and VH in It and UK scFvs. While our 18 aa linker (ref) drives the correct folding of VL and VH, thus allowing the stabilization of the conformation of the native BCL1 IgM, their linker, which differs in length and sequence), creates some structural constrains that do no allow the correct

conformation to be achieved. This is supported by the fact that while our scFv is efficienlty secreted in the supernatants of transfected cells, the scFv-UK is not.

Therefore it appears that the choice of the linker is very important in the design of scFv based DNA vaccines. Although the (Gly4xSer)x3 linker present in the UK scFv has been tested in other lymphoma models, and widely used in scFv phage display libraries, it is important to consider that the linker sequence can seriously affect the VL/VH association and compromise the immunogenicity and specificity of the response.

CONCLUSIONS

In conclusion, I have developed an efficient DNA vaccination strategy to induce protection in the BCL1 lymphoma model. By analyzing several variants of the construct used for immunization I have concluded that:

1) addition of the CH3 exon from human IgG1 to the scFv encoding plasmid was essential to obtain high levels of anti-idiotypic antibodies and protection from tumor challenge;
2) the linker sequence play a crucial role in determining proper folding of the scFv molecule and thus immunogenicity;
3) both tumor derived VL and VH are essential to the induction of anti-Id antibodies: Chimeric constructs containing either of the two BCL1 variable regions associated to an irrelevant V region failed to induce any detectable levels of anti-Id Abs;
4) secretion of the scFv molecule into the extracellular medium upon transfection of host cells evokes high titer of anti-Id Abs while variants encoding either a membrane bound or an intracellular retained form of the antigen are less efficient. This indicates that availability of soluble antigen for processing by host antigen presenting cells is crucial for the outcome of the DNA induced immune response;
5) a vaccine composed of a plasmid encoding the BCL1-CH3 scFv togheter with a plasmid encoding GM-CSF improved the potency of our vaccination strategy
6) polyclonal anti-idiotypic antibodies induced by DNA immunization are exclusively directed against a combination of parental VL/VH epitopes. The binding to the idiotype is totally lost when one of the two tumor derived V regions is associated to an irrelevant partner. On the contrary the same immunogen delivered as protein induced a polyclonal response containing also antibodies directed against epitopes on VL and VH regardless of the partner.

CHAPTER 5

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List of abbreviations used in the text

Ab= antibody
Anti-Id anti-idiotypic
APC= antigen presenting cells
BCR= B-cell receptor
C= constant
CDR= complementary determing region
CTL= cytotoxic T lymphocytes
D= diversity
DC= dendritic cells
DP= double positive
ER= endoplasmic reticulum
FDC= follicular dendritic cell
FrC= fragment C
FW= framework
GC= germinal center
GM-CSF= granulocyte/macrophage colony stimulating factor
H= heavy
Id= idiotype
IFN= interferon
Ig= immunoglobulin
Ii= invariant chain
IL= interleukin
J= joining
KLH= keyhole lymphocianin
L= light
LPS= lipopolysaccaride
MHC= major histocompatibility complex
ScFv= single chain Fv
SIP= small immuno protein
SP= single positive
TCR= T-cell receptor
TD= T-cell dependent
TGF= tumor growth factor
Th= T-helper
V= variability