Mucosal immunizations in a humanized transgenic mouse model and development of novel multimeric tools for detection of cellular immunity towards an HIV vaccine

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ABSTRACT

Viral vector-based vaccines represent an effective means of *in vivo* antigen expression and the ensuing generation of a sustained immune response in the host. These new generation vaccines are deemed promising against pathogens for which researchers have so far failed to put forth preventive strategies and/or effective, accessible, treatment modalities. HIV-1 stands at the foremost of this list. In the current study, we have evaluated the use of two different viral vector-based vaccines against Clade A of HIV-1, namely recombinant modified vesicular stomatitis virus (VSV-AV3) and Adenovirus serotype 5 (Ad5) expressing the Gag protein from subtype A. These viral vectors, which are also inherently endowed with adjuvant properties, were delivered via a mucosal immunization strategy in a humanized transgenic mouse model. Transgenic mice expressing both HLA-A*0201 and HLA-DR*0101 represent a versatile model in which HIV-specific immunogenic epitopes and the resulting T cell receptor (TCR) specificity can be determined. We show that following mucosal delivery of vaccine, there is induction of antigen-specific systemic T cells against epitopes which were previously shown to be immunogenic in humans. We next developed novel multimeric reagents for the detection of $CD4^+$ T cells, namely dodecameric HLA-DR1 molecules using a murine immunoglobulin M (IgM) scaffold. These reagents aim at increasing the overall avidity of peptide-MHC Class II complexes to detect low-affinity TCRs. These multimers were able to activate *in vitro* a Jurkat T cell line in an antigen-specific manner. The identification and characterization of the molecular requirements boosting the qualitative and quantitative features of the immune response to HIV vaccines, in addition to the development of novel state-of-the-art immune monitoring tools, will be crucial in better understanding of the mechanisms of interaction between the virus and the host immune system, leading to rational strategies in the fight against the AIDS epidemic.

ABRÉGÉ

Les vaccins à base de vecteurs viraux représentent un moyen efficace d'exprimer des antigènes *in vivo* et d'induire une réponse immunitaire persistante chez l'hôte. Cette nouvelle génération de vaccins s'avère prometteuse pour la prévention et/ou le traitement des maladies infectieuses pour lesquelles aucune stratégie thérapeutique efficace et abordable existe, le meilleur exemple d'une telle maladie étant l'infection par le virus de l'immunodéficience humaine (VIH). La présente étude porte sur l'utilisation de deux différents vaccins à base de vecteurs viraux dirigés contre le VIH-1 de sous-type A, soient un virus de la stomatite vésiculaire (VSV-AV3) modifié recombinant ainsi qu'un Adénovirus de sérotype 5 (Ad5), exprimant la protéine Gag de sous-type A du VIH-1. Ces deux vecteurs viraux, qui possèdent des propriétés adjuvantes intrinsèques, ont été testés dans le cadre d'une approche d'immunisation mucosale en utilisant un modèle de souris transgénique humanisée que nous avons développé. Les souris transgéniques exprimant les molécules du complexe majeur d'histocompatibilité (CMH) humain HLA-A*0201 et HLA-DR*0101 constituent un modèle idéal permettant l'identification d'épitopes immunogènes dérivés du VIH et la caractérisation qualitative et quantitative de la réponse des lymphocytes T contre les antigènes du VIH. Nos résultats démontrent que suite à une immunisation intranasale de ces souris avec les vecteurs VSV-AV3 et Ad5 exprimant la protéine Gag, il y a induction d'une réponse immunitaire spécifique dirigée contre des antigènes viraux préalablement caractérisés chez l'humain. Nous avons ensuite développé une nouvelle forme de multimères de CMH de classe II (HLA-DR1) basée sur la structure dodécamérique de l'immumnoglobine M (IgM) de souris. Nous avons démontré que ces dodécamères de HLA-DR1, lorsque chargés avec un peptide, activent une lignée cellulaire de lymphocytes T Jurkat possédant un récepteur de cellules T (TCR) spécifique à ce peptide présenté par la molécule HLA-DR1. L'identification et la caractérisation des déterminants moléculaires qui contrôlent l'induction de la réponse immunitaire par les vaccins contre le VIH, en combinaison avec l'émergence de nouvelles technologies permettant un

meilleur suivi de la réponse immunitaire, permettront de mieux comprendre l'interaction entre le VIH et la machinerie cellulaire de l'hôte et faciliteront le développement de nouvelles stratégies préventives et thérapeutiques dans le combat contre l'épidémie du VIH/SIDA.

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Chapter 3: Activation of human CD4+ T cells by dodecameric human MHC Class II chimeras and their implication in immune therapy.

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TO THE ONE WHO KNOWS...

FOREWORD

Many years of work, effort, but above all, a drive, a spark, to know what's next, what's unknown, have led to this day. A day where I stand here bewildered, with more questions at hand than answers...

Along the way, many people dear to heart have brought their share of presence and support -some still do, some have gone to walk their own path.

Too many faces, too many names, to put down in print. You know who you are.

To all of you, I take this moment and look back in gratitude and awe.

Thank you for making this worthwhile.

To my parents, for their selfless support and love throughout the years.

To my sisters, for their friendship and trust.

To my friends, for putting up with me through it all.

To my colleagues and lab-mates, for sharing all the moments, the happy ones, but above all, the more challenging ones.

To all Isha Foundation volunteers, my amazing partners in compassion, for touching and transforming the core of who I am.

To all the rural children of Tamil Nadu, south India - those I've met and those who I am still to meet - for embracing me, humbling me, and inspiring me to take the leap.

This goes to you...

1 Introduction

1.1 The Immune System: an Overview

The ability of multi-cellular organisms to defend themselves against invasion by pathogens depends on their ability to mount immune responses. While all metazoans have inborn defense mechanisms which constitute the *innate immunity,* jawed vertebrates, in addition, are able to mount specific defense mechanisms collectively known as *adaptive immunity.*

1.1.1 Innate Immunity

Through the innate immunity, pathogens are recognized by a set of receptors encoded in the germline. These receptors known as PRRs (**p**attern **r**ecognition **r**eceptors) have a broad specificity and recognize many related molecular structures called PAMPs (**p**athogen-**a**ssociated **m**olecular **p**atterns). PAMPs are essential polysaccharides and polynucleotides that differ little amongst pathogens but are not found in the host. Different defense mechanisms have evolved for eons in eukaryotic organisms with two evolutionarily conserved modalities being the complement component C3 and the Toll receptor pathway [1, 109].

Thiolester-containing molecules with highest similarity to C3 have been detected to the level of the diploblastic (two-germ layer) corals and molecules known as TEPs (**t**hiolester-containing proteins) capable of making covalent linkages to pathogens inducing opsonization have been also detected in mosquitoes and other insects [2,3-5]. Of the three functions of the complement system - opsonization, direct lysis of pathogens, and inflammation - the very first might be the evolutionarily oldest mechanism. The classical pathway and the membrane-attack complex (MAC) seem to have arisen in the jawed vertebrate lineage, while the alternative pathway and lectin pathways are older mechanisms [6].

Toll receptors have been observed in invertebrates and in plants. The detection of the signal-transduction mechanisms induced by bacterial and fungal pathogens in insects and the crucial involvement of this pathway in embryological development [7] led to the discovery of Toll-like receptors (TLR) in mammals, thus opening up an entirely new field of immunity [8, 9, 10].

On the other hand, cytokines, which are studied extensively in mammals, and also present in jawed vertebrates, appear to be absent in invertebrates. In the recent analysis of a urochordate genome, only a tumor necrosis factor (TNF) and IL-8 homologues were found. This argues that indeed, with the emergence of adaptive immunity in the jawed vertebrates, there seems to be an emergence of a host of supporting molecules and machinary required for function, maintenance, and regulation of the adaptive system [11,109].

1.1.2 Adaptive Immunity

A remarkable event in the evolution of the vertebrate immune system was the seemingly abrupt emergence of adaptive immunity at the jawed vertebrate stage. While every major component of the mammalian adaptive immune system, such as the MHC, TCR, and Ig molecules, as well as the genes directly involved in somatic generation of diversity, such as RAG1 and 2, have been identified in the cartilaginous fish, all attempts to isolate these genes from more distant organisms to bony vertebrate than cartilaginous vertebrate (i.e., the jawless fish and below) have not been successful [12,109].

Even though some recent results hint at the presence of an ancestral adaptive immune system, such as the presence of lymphocyte-like cells and orthologues to mammalian lymphocyte-specific genes reported in lamprey [13, 14] and a possible proto- MHC region identified in amphioxus [15], direct predecessors of any component of adaptive immunity have never been identified. However, one should be mindful that these results were based on techniques such as low-stringency cloning which are not infallible. A systematic search for adaptive immune-related genes or their precursors has not yet been possible in jawless

vertebrates or invertebrate deuterostomes, due to the absence of a complete genome sequence of a suitable organism.

In higher vertebrates, the adaptive immune system is defined by the clonally distributed antigen receptors (immunoglobulins (Ig) and T cell receptors), recombination-activating gene (RAG)-mediated gene rearrangement, primary and secondary lymphoid tissues, major histocompatibility complex (MHC)-encoded class I and class II molecules, and somatic hypermutation, which is activation-induced cytidine deaminase (AID)-dependent in every species so far examined. This system is further identified by the functional attributes of highly-diverse, clonally-distributed antigen receptors and by memory responses. These features are found in all jawed vertebrates, with cartilaginous fish being the phylogenetically oldest group [16].

Until recently, none of these structural, mechanistic, or functional characteristics were detected in invertebrates or in jawless vertebrates (lamprey and hagfish). Thus, the emergence of adaptive immunity is believed to have been made possible by the invasion of a transposable element into an immunoglobulin (Ig) superfamily (sf) gene sometime early during the evolution of the jawed vertebrates (possibly a placoderm).This initiating event probably resulted in a mechanism to somatically generate diversity in antigen-receptor genes, and it was the innovation propelling the "Big Bang" that resulted in the emergence of adaptive system [16, 104].

1.1.3 The Major Histocompatibility Complex.

In order to distinguish 'self' from 'non-self', the T cell receptor (TCR) on T cells must be presented with a relevant epitope cleaved from a given antigen. Class I and Class II Major Histocompatibility Complex (MHC) molecules provide the molecular cradle for peptide presentation. The MHC glycoproteins are encoded by a cluster of genes first identified due to their involvement in potent immune responses to transplanted tissues, thence the term *Major Histocompatibility Complex*. We now know that within this region of the genome, in addition to those genes encoding the MHC molecules, are many

genes expressing accessory proteins involved in the generation of MHC:peptide complexes [16, 17,18].

In humans, the MHC genes are located on chromosome 6 and in mice, on chromosome 17. The organization of the MHC genes is roughly similar in both species with separate clusters for MHC class I and class II genes, although in the mouse an MHC class I gene (H-2K) appears to have translocated relative to the human MHC, so that the class I region in mice is split in two. The genes encoding the α chains of MHC class I molecules and the α and β chains of MHC class II molecules are linked within the complex; however, the genes for β_2-microglobulin and the invariant chain are on separate chromosomes (chromosomes 15 and 5, respectively, in humans and chromosomes 2 and 18, in the mouse). MHC loci are *polygenic* containing several different MHC class I and MHC class II genes, in such a way that every individual possesses a unique set of MHC molecules with different scopes of peptide-binding specificities. Moreover, the MHC is highly *polymorphic*, i.e. there are multiple variants of each gene within a given population. In fact, the MHC genes are the most polymorphic genes known to date [19].

1.1.3.1 MHC Class I

Class I MHC molecules are present on all nucleated cells, and are mainly involved in the recognition of 'self' antigens. Also known as HLA class I (Human Leukocyte Antigen) in humans and H-2 Class I Complex in mice, the MHC class I molecule consists of two polypeptide chains, namely a variable heavy chain (α1-α2-α3) and a constant light chain (β-2-microglobulin). An antigenic peptide of 9 amino acids (AA) is presented in the cleft formed by the heavy chain. Class I molecules are involved mainly in the presentation of endogenous antigens to CD8 positive ($CD8^+$) cytolytic or NK cells [19,24].

In humans, the classical MHC class I consists of the highly polymorphic HLA-A, HLA-B and HLA-C, and the so-called non-classical, class I - like molecules, which include HLA-E, F, G, and CD1. In the mouse, there are also

3 main class I genes, known as H2-K, -D, and -L. Many haplotypes of the mouse MHC, *H2*, are still known through the names given to them by American geneticist and Nobel prize co-recipient, Dr. George Snell, starting in 1948, including *d* for *dilute* of DBA/2, *b* for *black* of C57BL, *p* for *pink-eyed* of P, *k* for *Kinky* of K8, and *s* of A.SW, among others [20].

1.1.3.2 MHC Class II

The class II region (of chr. 6 in human and chr. 17 in mouse) includes the genes for the α and β chains of the classical antigen-presenting MHC class II molecules HLA-DR, -DP, and -DQ in the human, and H-2A and H-2E in the mouse. Both the α and the β chains of MHC II exhibit a high degree of polymorphism (except for the monomorphic DR α chain). In addition, both alleles of each locus are co-dominantly expressed, thus fully heterozygous individuals express 6 different MHC class II molecules [18, 19].

The non-classical MHC class II (HLA-DM, HLA-DO in human; H-2M and H-2O in mouse), which show only limited polymorphism, are also present in this region. In addition, the genes for the TAP1-TAP2 peptide transporter, the LMP (Low molecular Mass Polypeptide) genes encoding proteasome subunits, and the gene for tapasin (TAPBP) are also in the MHC class II region in humans. In the mouse, the TAPBP gene is present in the Class I region [21].

The classical MHC class II molecules are involved in presenting antigenic peptides to CD4 positive ($CD4^+$) T lymphocytes. They are expressed on the surface of professional antigen presenting cells (APC) (i.e. B cells, macrophages, dendritic cells); epithelial cells of the thymus (both in the medulla and cortex); on fibroblasts, mast cells and epithelial cells following induction by IFN-γ; and on human activated T-cells.

The non-classical MHC class II molecules are not found on the cell surface. These molecules are involved in modulating proper transport and binding of antigenic peptides to the classical MHC class II molecules. HLA-DM is expressed by the same cell types as the classical MHC molecules while HLA-

DO expression is believed to be restricted to B lymphocytes, subset(s) of dendritic cells, and to both cortical and medullary epithelial cells of the thymus [22, 23].

The classical MHC Class II molecule is composed of two non-covalently associated membrane spanning glycoproteins, an alpha chain of 33kDa and beta chain of 28kDa. Each chain has two extracellular globular domains (α1, α2, β1 and β2), in addition to transmembrane and cytoplasmic domains. Together, the α1 and β1 domains form the peptide binding groove with open ends, thus being capable of binding peptides of variable length (12-30aa) [24, 25].

Antigens presented in the context of MHC class II molecules derive from extracellular proteins that access the endocytic route where they are processed and bind to MHC class II molecules. It is noteworthy however, that in normal circumstances, most of the proteins found in the endocytic/lysosomal compartments consist of membrane proteins being internalized and degraded as part of their due turnover. Consequently, most of the peptides eluted from the cell surface MHC class II molecules are endogenous proteins. Even in the presence of high concentrations of an exogenous antigen, it was observed that only a minor fraction of cell surface MHC class II proteins are loaded with peptides derived from that antigen. Interestingly, immature dendritic cells are very efficient in capturing small amounts of antigen, and following inflammation-induced maturation, DCs can present these captured antigens at very high levels for a prolonged period of time [26].

1.1.3.3 MHC Class III

The so-called MHC class III genes, which map within the MHC region, encode various proteins with crucial functions in immunity. These include some of the complement system components (e.g. C2, C4, and factor B), cytokines (e.g. tumor necrosis factor-α, TNF-α), and lymphotoxin (TNF-β) [27].

1.1.4 Mucosal Immunity

1.1.4.1 Overview

Regulating a critical symbiotic environment between the host and commensal organisms, while acting as a first line of physical and immunological defense against pathogenic insult, the mucosal immune system, long understated, has gained rightful attention during recent years, especially in the context of mucosal vaccines. Functioning through both its innate and acquired components, the mucosal immune system regulates homeostasis along an extensive epithelial surface area, covering the oral and nasal cavities till the respiratory, digestive and genito-urinary tracts.

Antigen-specific immune responses are initiated at special sites of the mucosa-associated lymphoid tissue (MALT). Microfold (M) cells, which are structurally and functionally specialized for transepithelial transport, are found in the epithelium overlying follicles of the MALT and deliver foreign antigens and microorganisms to organized lymphoid tissues within the mucosae of the small and large intestines, tonsils, adenoids, and airways[5, 28, 29].

Moreover, follicles localized in MALT contain a host of immune cells required for the generation of an effective immune response, including APC's, T cells, and B cells. Two major components of the MALT are the Peyer's patches in the gut and the nasopharynx-associated lymphoid tissue (NALT). The relative distribution of these secondary lymphoid structures is however greatly species-dependent. In humans, near 1000 Peyer's patches can be found in the terminal ileum, while mice or rats possess 2-11 larger patches evenly distributed throughout the small intestine. Likewise, bronchus-associated secondary lymphoid tissue is prominent in rabbits but not humans. These species-specific differences have been attributed to varied environmental set-ups which, in consequence, dictate a diverse antigen exposure in different species.

On the other hand, the Common Mucosal Immune System (CMIS) connects antigen exposure and inductive sites of the mucosal surfaces with effector sites of the lamina propria or glandular tissue. Antigen-specific T helper 2 (Th2) - dependent IgA responses, as well as Th1 and cytotoxic T lymphocyte (CTL)-dependent immune responses function as the first line of defense at mucosal surfaces.

Thorough understanding of the biology, tissue genesis, and immunological function of different mucosal sites is critical for the appropriate manipulation of the MALT immune system towards the generation of much awaited for mucosal adjuvants and vaccines, in the context of a host of infectious diseases.

1.1.4.2 NALT vs GALT Organogenesis

Even though NALT and gut associated mucosal tissue (GALT) demonstrate functional similarities as antigen encounter and mucosal inductive sites, they have different characteristics in terms of both kinetics and cytokine requirement in their programmes of lymphoid organogenesis. *Kiyono H.* and *Fukuyama S.* provide an in depth review of these distinct features in the light of mucosal vaccine development [30].

Remarkably, different homing receptors are expressed in NALT vs. GALT. While high endothelial venules (HEVs) associated with Peyer's patches express mucosal vascular addressin cell-adhesion molecule-1 (MADCAM1), HEVs associated with NALT express peripheral-node addressin (PNAD). In terms of tissue genesis, vascular cell adhesion molecule-1 (VCAM-1) expression was shown to be associated with the development of Peyer's patches at 2 weeks following coitus, while formation of mature NALT tissue was completed only 5-8 weeks following birth, and the postnatal initiation of NALT formation is believed to be facilitated through exposure to environmental antigens and mitogens. In addition, cytokine-mediated organogenesis mechanisms differ between NALT, Peyer's patch, and peripheral lymph node development. Lymphotoxin (LT), tumour necrosis factor (TNF), interleukin-7 (IL-7), and their corresponding receptors are

crucial to the development of secondary lymphoid tissue genesis, including Peyer's patch development. Moreover, a subset of mononuclear cells with $IL7R^+CD3^-CD4^+CD45^+$ phenotype, which also express CXCR5, has been implicated in the development of Peyer's patches and are known as Peyer's patch inducer cells. In contrast, NALT formation seems to be independent of the signalling cascade which involves LT and IL-7 receptors. In addition to the accumulation of $CD3^-CD4^+CD45^+$ in the intestinal tract during embryonic stages of development, this subset of cells was also observed at the site of NALT formation postnatally, and their involvement in NALT organogenesis was proven through adoptive transfer experiments [30].

1.1.4.3 The Common Mucosal Immune System

The common mucosal immune system (CMIS) is an integrated pathway which enables generation of antigen-specific immune responses, by concerting signals from inductive sites of MALT with the more distant effector sites found in the diffuse mucosal tissues. Antigens encountered in the nasopharyngeal or GI lumen are transported to NALT or Peyer's patches via M cells. Dendritic cells uptake and present these antigens to T cells of these organized lymphoid tissues, or neighbouring cervical and mesenteric lymph nodes.

Sensitized $CD4^+$ T cells help induce IgA-committed B cell development in the germinal centre of lymphoid follicles. Following IgA class switching and affinity maturation, B cells migrate to the mucosal effector sites through the thoracic duct and blood circulation. Similarly, antigen-specific activated or memory T cells emigrate from the initial environment via lymphatic drainage, circulate through blood, and home to mucosal effector sites. Indeed, in a healthy human, the mucosal immune system integrates around 80% of all immunocytes. In addition to the conventional $CD4^+$ and $CD8^+$ $\alpha\beta$ T cells, mucosal tissues also contain T cells with a more limited repertoire diversity, including $\gamma\delta$ T cells and NKT cells.

The presence of antigen-specific s-IgA and CTL at mucosal surfaces further from inductive sites where the initial antigen encounter takes place, is a concrete evidence of CMIS, and supports the rationale that mucosal vaccine administration through either NALT or GALT is a convenient approach to induce efficient immune responses in various mucosal effector tissues [30,31].

1.1.4.4 Mucosal Effector Sites

Mucosal effector sites include the nasopharyngeal passages and intestinal lamina propria, as well as the reproductive tract and secretory glandular tissues such as mammary, salivary, and lacrimal glands. These are the sites where IgA^+ B cells and plasmablasts differentiate into IgA-producing plasma cells in the presence of appropriate cytokine signals such as IL-5 and IL-6 produced by $CD4^+$ Th2 cells. This in turn leads to production and secretion of dimeric IgA, which upon binding to polymeric Ig receptors on epithelial cells lining the mucosa are transported and released in the respiratory passages and intestinal tract. The mucosal effector sites are also a niche where antigen-specific T cells perform their cytotoxic or regulatory functions towards protection of mucosal surfaces.

However, it is noteworthy that mucosal effector sites, which serve as a barrier against a host of environmental antigens and mucosal pathogens, are endowed with mechanisms of protection that are significantly different from that of inductive sites. These include increased populations of IgA^+ plasma cells, and large numbers of B and T lymphocytes in the intestinal lamina propria. In the mouse, several studies have observed that the $CD4^+$ T cells of the lamina propria contain high numbers of IL-5 secreting Th2 type cells, indicating that the effector sites of mucosal immune system are biased toward a Th2 phenotype, which is also a key component of the mechanism involved in the induction of 'oral tolerance'. IFN-γ-producing $CD4^+$ cells have been shown to be important in the mucosal immune defense, although details of their protective mechanism(s) need to be elucidated [31,105].

In addition to its barrier and mechanical cleansing function, mucosal tissues are endowed with different chemical antimicrobial factors, including defensins provided by the lining epithelial cells or underlying components of the innate immune system. All in all, mucosal surfaces present a complex environment which requires well-concerted mechanisms of immune surveillance.

1.2 HIV and Current Status of AIDS Vaccines

1.2.1 Overview

1.2.1.1 The HIV virus

HIV-1 was first isolated in 1983, concurrently by three independent groups[106, 107,108]. Three years later, a genetically related virus was identified and named HIV-2. Both HIV-1 and -2 are known today to be related to distinct families of the Simian Immunodeficiency Virus (SIV), and it is believed that multiple zoonotic infections with SIV have led to the evolution of HIV species. HIV-1 is a retrovirus belonging to the genus *Lentivirus*. Its genome consists of two copies of single-stranded RNA and its life cycle is dependent on a RNA-dependent DNA polymerase, namely, the reverse transcriptase (RT). RT is a markedly error-prone enzyme, because it lacks the 3' exonuclease activity.

Particular mutations resulting from this defective proofreading ability lead sometimes to a survival advantage of the virus in the host. This inherent characteristic of RT is also responsible for the tremendous genetic diversity of HIV-1 within an individual, leading to a broad diversity of viral quasispecies.

RT reverse transcribes the viral RNA into a linear dsDNA which is subsequently integrated into the host DNA genome in a non-site-specific manner. It is this integrated form of 'proviral' DNA that is later transcribed through the host cell machinery to generate viral particles.

The retroviral gene products consist of structural proteins, including Gag, Pol, and Env, the proviral sequence being flanked on both ends by two U3-R-U5

long terminal repeats (LTRs). Sequences in the LTR are responsible for the promoter, enhancer, and modulatory machinery, in addition to containing RNA regulatory sequences. Moreover, HIV-1 genome possesses nine open reading frames (ORFs). The *gag* gene encodes for a single ORF, giving rise to the p55 polyprotein. The latter is subsequently processed into matrix (MA, p17), capsid (CA, p24), p2, nucleocapsid (NC, p7), p1 and p6 proteins, involved in the structural association of the virion. The *pol* ORF yields RT and its RNAse H component, in addition to the integrase enzyme (which leads to viral DNA insertion into the host genome), and protease enzyme (which is crucial for processing viral polyproteins). The *env* gene encodes for the envelope gp120 and trans-membrane gp41 proteins. gp120 is responsible for viral cell-specific tropism, while gp41 assists viral fusion.

In addition, HIV-1 contains six non-structural genes. These include *tat*, which encodes a trans-activating element crucial in viral RNA transcription and elongation, and *rev* the product of which serves as a post-translational transactivator involved in proper slicing, shuttling, and translation of viral RNA. The other non-structural genes consist of *vif, vpr, vpu* and *nef*. Vif and Nef are particularly important in enhancing viral infectivity and immune evasion. The exact purpose and detailed mechanism(s) of action of the accessory gene products are an ongoing subject of many studies and remain to be fully elucidated [32].

1.2.1.2 HIV-1 Clades and Subtype Diversity

HIV-1 isolates are classified into three clades: M, N and O. Clade M is the main clade and comprises subtypes A-H and J-K. Globally, one observes a heterogeneous demographic and geographical distribution of individuals infected with different clades or subtypes of HIV-1. For example, subtype A and A/G recombinant variants predominate in west and central Africa, while subtype B has been the main species encountered in Europe and the Americas. However, due to increasing immigration and globalization, about 25% of new infections in Europe currently stem from non-B African and Asian variants.

On the other hand, subtype C is mainly encountered in southern and eastern Africa, India, and Nepal. This subtype has created the recent epicentres of the HIV pandemic by its uncontrolled spread through different regions of Africa and Asia. Subtype D is generally encountered in east and central Africa, with sporadic cases occurring in southern and western Africa. Subtype E manifests as an A/E mosaic detected in Thailand, the Philippines, China, and Central Africa, while subtype F has been reported in central Africa, south America and eastern Europe. G and A/G recombinant viruses have been observed in western and eastern Africa as well as in central Europe. H has only been detected in central Africa. J has been reported exclusively in central America. K has recently been identified in the Democratic Republic of Congo and Cameroon.

Additional subtypes are constantly being discovered, and migrating populations are shaping new patterns of subtype distribution. HIV-1 clades C and A, as well as the A/G and A/E recombinant forms, are nowadays of special concern, because these represent the predominant subtypes in Africa and Asia where HIV disease is spreading most rapidly.

Classification of HIV-1 subtypes springs from the 20–50% discrepancy that exists in envelope (*env*) nucleotide sequences. Moreover, within the M subgroups, interclade *env* variations is around 20–30% in addition to intraclade variation of 10–15%. Divergence in the *pol* region of HIV-1 is less prominent than that of *env* since, as mentioned above, this region encodes for the crucial RT, integrase, and protease enzymes. If the latter are excessively mutated, they affect adversely the infectivity of the virion. *gag* sequences are even less prone to mutations, since they yield the relatively stable core proteins. Thus, vaccines against Gag epitopes may be promising due to conservation of intra- and inter-clade regions [33, 34].

1.2.2 Identification of Correlates of Protection

A hallmark of HIV-1 disease progression is CD4 T cell dysfunction. In primary HIV infection, there is a sharp decrease in the number of circulating

CD4 T lymphocytes. Low CD4 counts are attained by patients in chronic HIV infection leading eventually to Acquired Immunodeficiency Syndrome (AIDS). In addition, the ratio of circulating CD4 to CD8 T cells is inversed in chronic stages of HIV infection. Complex viral and host factors contribute to disease progression and susceptibility to AIDS. A specific group of HIV infected individuals known as long term non progressors (LTNP), control viremia for extended periods of time and do not progress to AIDS. This is thought to be partly related to host-factor variability, including genetic make-up, and studies of the immunogenetic profile of these individuals may lead to a better understanding of correlates of protection during the course of a natural infection with HIV-1, as well as to the development of tailored therapeutic strategies in HIV-infected persons [35,118].

In chronic viral infections, virus-specific T-cells have been shown to be crucial for effective control of virus replication and prevention of disease. While EBV and CMV infections are prototypes whereby cell mediated immunity plays a major role in controlling infection, it is evident that HIV-1-specific cell-mediated responses often fail in controlling infection and ensuing pathogenicity. Recently, and in order to better understand correlates of protection and heterogeneity of cellular responses, a number of phenotypic markers have been identified and functional readouts put forth to characterize virus-specific $CD4^+$ and $CD8^+$ T cell responses [10].

These signatures of protective antiviral cellular immunity indicate that polyfunctional (i.e. IL-2 and INF-γ secretion, and proliferation), and not monofunctional (i.e. IFN-γ secretion alone) $CD4^+$ and $CD8^+$ T-cell responses represent correlates of protective antiviral immunity in chronic viral infections [36, 10].

It has also been observed that both antigen load and persistence are associated with functionally distinct types of T cell subsets. For instance, predominance of IL-2 secreting cells alone was associated with antigen clearance while IFN-γ secreting cells alone are typical during antigen persistence and elevated antigen levels. On the other hand, a polyfunctional IL-2 and INF-γ response

reflects a protracted antigen exposure and low antigen load. It is noteworthy that in subjects with nonprogressive HIV-1 infection, virus-specific CD4 cells are polyfunctional. Similarly, high frequencies of monofunctional HIV-1 specific $CD8^+$ cells secreting IFN-γ fail to control viral replication and there is skewing of memory $CD8^+$ T cells toward IFN-γ secretion with impaired capacity to proliferate. On the other hand, in LTNP individuals carrying HIV-1, as well as in CMV and EBV infections, the presence of antigen-specific $CD8^+$ cells with the ability to proliferate and secrete IL-2 is believed to be associated with low viral loads and effective viral control [37].

With regard to humoral immunity, and despite substantial research on the function of neutralizing antibodies, their role in conferring 'sterilizing immunity' and impact on the control of established, chronic HIV-1 infection remain to be elucidated, especially if one considers the very rapid generation of viral escape variants.

Moreover, one should bear in mind that correlates of protection during the natural course of HIV-1 infection might not necessarily represent responses induced in the presence of pre-existing vaccine-related immunity. Therefore, rational and carefully crafted clinical trials and appropriate state-of-the-art immune monitoring tools are crucial to determine how pre-existing immunity can shape the outcome of HIV-1 infection [4].

1.2.3 Current HIV Vaccines

Two major obstacles to the advancement of vaccine development are the latent persistence of the virus in the host genome and the high error rate of RT. Neutralizing antibodies block the viral entry process by binding to surface gp120, however, the generation of the latter against primary viral isolates has been hindered due to poor accessibility of the receptor binding site and gp120's heavy glycosylation.

Two major targets for a potential HIV vaccine involve, on one level, the virus entry, and on another, the replication process. Concurrent advances in our

understanding of the details of immune mechanisms following HIV infection, and the development of novel quantitative immune monitoring tools dissecting the Ag-specific cellular response, have assisted explore a new generation of vaccines which induce T cell responses. Several experimental studies have yielded promising results in primate models, yet these data show that, overall, novel T-cell-based vaccines do not prevent, but rather control challenge. A potential problem in such a scenario is that of viral escape from immune control [38].

To summarize, in the course of HIV infection, protective mechanisms of $CD8^+$ T lymphocytes include: cytolotic function against infected cells, suppression of viral replication through soluble cellular factors, and prevention of viral entry by blockade of chemokine co-receptors by secretion of chemokines such as MIP-1α, MIP-1β, and RANTES. More than 200 HIV-1 specific T cell epitopes have been mapped to date and despite an overwhelming viral genomic diversity, there is a fair amount of intra- and inter- subtype conservation. The vast majority of current vaccines candidates target the induction of cell-mediated immunity. These vaccines are now known as T cell vaccines. Among these, two types of vector-based vaccines have shown considerable potential, and have entered clinical evaluation, namely viral-vector and DNA-based vectors [39].

The viral vector-based vaccines under assessment include poxvirus (such as MVA and NYVAC) and adenovirus (Ad) based vaccines. In addition, canarypox based vaccines such as ALVAC are being tested. A variety of prime-boost regimens, in conjunction with heterologous viral, DNA, or protein based vaccines, have been widely undertaken. Results show that DNA and poxvirus based vaccines, when used alone, result in limited immunogenicity characterized by transient T cell responses, but are effective when used in prime-boost combinations [40, 41, 137].

When compared to recombinant adenoviral (Ad5) based vectors, DNA and poxvirus vaccine candidates have shown a dominant CD4 T cell response with minimal CD8 T cell responses, while Ad5 based vaccines result in both CD4

and CD8 responses. Moreover, the latter were shown to have the highest immunogenicity and long-lasting cellular responses. However, a major drawback of adenovirus based vaccines in humans is the generation of anti-vector antibodies or pre-existing immunity to adenovirus in the population (50% in the case of US population). This has been suggested to be overcome by immunization with higher doses of immunogen and/or priming with heterologous vector that is not subject to neutralizing antibodies (i.e. DNA vectors) [39].

There are still several challenges which hinder the development of successful AIDS vaccines. Neutralizing antibodies cannot readily access their targets, since conserved epitopes are thermostably concealed, while exposed sites are highly variable. In addition, these sites are camouflaged by heavy glycosylation. On the other hand, latent proviral DNA establishes in niches early during the course of infection and has a long half-life. Moreover, and as described earlier, HIV has high variability, with 12 known subtypes, inter-subtype recombinants, and high error rate RT enzyme [38].

The complex array of the above parameters, including different viral pathologic and host defense mechanisms during the course of the infection, should be taken into account toward adopting a rational approach in the ongoing quest of vaccine design [42].

1.2.4 Need for mucosal vaccines

Perhaps the most important example to highlight the need and importance of targeting the mucosal immune system is the fact that over 80% of HIV infections occur via sexual transmission. Mucosal tissues are the primary site of natural HIV infection, and represent a major reservoir for viral replication. Enhancing immune responses at mucosal surfaces would prevent viral dissemination, as well as clear viral reservoirs of replication [79, 114]. In this context, it has been shown that CTL cells should be present in mucosal sites to prevent viral transmission, while systemic CTL alone are not sufficient [38]. A number of delivery routes have been used in experimental mucosal AIDS

vaccines, including intranasal, intrarectal, intravaginal, as well as transcutaneous and iliac lymph node targeted vaccines, to enhance the mucosal immune response [115].

An ideal HIV vaccine should induce a broad array of cross-reactive neutralizing antibodies and increased levels of antigen specific effector and memory T lymphocytes, specifically targeting the generation of long-term responses at mucosal surfaces [43,110].

1.3 Mucosal Vaccines and Immunization Strategies

1.3.1 Mucosal Vaccines

In the development of mucosal vaccines, it is critical to carefully select both an effective delivery route and vehicle. Most common strategies and routes target the natural encounter of mucosal sites with the antigen in question. Mucosal immunizations most commonly are performed through either oral or intranasal administration. A key and much sought-after advantage of mucosal immunization is the fact that this approach results in the induction of both mucosal and systemic arms of the immune response in the host.

1.3.2 Intranasal Vaccines

As described in section 1.1.4.2, NALT is an important component of the organized lymphoid tissue. In rodents, NALT is located on both sides of the nasopharyngeal duct and corresponds to the Waldeyer's Ring in humans, which includes the palatine tonsils and adenoids. Moreover, structures similar to NALT, in forms of follicles, have been identified in the middle concha of children less than two years of age.

Endowed with all necessary elements for the generation of a successful immune response upon encounter of antigen, NALT represents an attractive

target as a site for immunogen delivery and uptake. This route of vaccine administration has proven to elicit both humoral and cell-meditated immune responses. In addition, when compared to oral vaccines, intranasal (i.n.) vaccines require a smaller antigen dose and less adjuvant, since antigens are not exposed to the degrading effect of enzymes and low pH as in the digestive tract. Importantly, intranasal administration of the vaccine results in the induction of both mucosal and systemic antigen-specific immune responses [30].

To increase the efficiency of NALT targeted immunogens in stimulating effector cells associated with NALT, there is a need to develop safe and potent mucosal adjuvants as a component of nasal vaccines.

1.3.3 Mucosal Adjuvants

Mucosal adjuvants have been widely studied in the context of oral vaccines. Among these, the cholera toxin (CT) of *Vibrio cholerae* and the heat-labile enterotoxin (ET) of *Escherichia coli*, have been successfully used in experimental systems with the aim of generating toxicologically acceptable derivatives for human use. The B subunits of these toxins act by binding to ganglioside GM receptors of intestinal epithelial cells, and translocate the A subunit into their cytosol, leading eventually to activation of adenyl cyclase and the subsequent elevation of cyclic adenosine monophosphate [44].

Recombinant CTB (subunit B of CT), used in combination with different antigens, is able to either induce mucosal sIgA responses to pathogens or result in peripheral anti-inflammatory tolerance to self-antigens. Both CT and ET are potent adjuvants when administered by oral, intranasal, or even parenteral route. However, it is well known that, in humans, both result in severe diarrhea and clinical symptoms of enteritis. Several studies have attempted to reduce the toxicity of these molecules while retaining their adjuvanticity through the generation of mutant counterparts [44,45] .

Other mucosal adjuvants include hybrid molecules such as CTA1-DD, whereby the A1 subunit of CT is linked to an APC-binding protein derived

from the bacterium *Staphylococcus aureus* (Protein A). This molecule helps specifically target antigens to B cells, and has been used safely in intranasal immunizations [45].

On the other hand, bacterial DNA or synthetic oligonucleotides which contain unmethylated CpG motifs, have been also shown to act as effective mucosal adjuvants through the stimulation of TLR-9, thus initiating an immune-modulatory cascade [46].

When rationally designed, mucosal adjuvants can help successfully direct the mucosal system towards a desired response. Besides, mucosal adjuvants are needed not only to boost mucosal and systemic immunity, but also to prevent the generation of mucosally induced tolerance.

1.3.4 Mucosal Tolerance

In addition to the induction of antigen-specific IgA and serum IgG responses following mucosal immunization, the introduction of proteins at mucosal inductive sites often results in the induction of systemic unresponsiveness or tolerance. This approach is useful in the context of preventing allergic responses to food proteins and allergens, and in the treatment of autoimmune diseases through administration of self-antigens.

Oral intake of a single high dose or repeated low doses of proteins was shown to result in peripheral tolerance along the generation of mucosal IgA responses. Systemic unresponsiveness was also observed following intranasal delivery of proteins, still the majority of the data and immune mechanisms involved therein emanate from studies involving oral administration of antigen. The major mechanisms believed to be involved in the induction of tolerance appear to be mediated by T cells, through active suppression, clonal anergy or deletion. This mucosal intervention has become an increasingly interesting approach to prevent development of illnesses resulting from adverse immunologic reactions against antigens encountered or auto-antigens found in non-mucosal tissues [47].

1.3.5. Mucosal Delivery Systems

In addition to adjuvants, mucosal delivery systems are key vehicles which assist in improving vaccine efficiency. Important parameters that need to be considered in the design of mucosal vaccines should support the immunization by:

(a) preventing degradation of antigen (i.e. due to enzymatic digestion);

(b) enhancing antigen uptake by M cells or mucosal epithelial cells;

(c) stimulating the innate immune system towards creating a desirable immune milieu for the development of antigen-specific adaptive response; and

(d) generation of immunological memory.

Depending on the pathogen or disease at hand, different mucosal delivery systems and vectors have been developed to tailor the local and systemic antigen-specific response.

These include [46]:

1.3.5.1 Biodegradable Microparticles

Biocompatible PLG polymers, long used in humans as a drug delivery system, are now being tested as microparticle vaccine delivery vehicles. Microparticle delivery facilitates uptake of the antigen in question by M cells, and possibly by mucosal epithelial cells, and enhance targeting of Peyer's patches following oral intake. Microparticles are also used as a delivery vehicle through intranasal route. PLG particles have been shown to result in a more protective Th1 type response, as opposed to a mainly antibody-mediated, Th2-biased, conventional mucosal microenvironment.

1.3.5.2 Liposomes

Liposomes consist of a combination of different proportions of lipids and are combined with antigens to increase their mucosal delivery efficiency. These formulations protect the antigen from degradation in acidic GI environment, including bile and pancreatin secretions. Liposomes used in intranasal formulations have also been successful in generating enhanced antibody

responses, such as in the case of the bacterial *Yersinia pestis* and *Streptococcus mutans* vaccines.

1.3.5.3. Immune-stimulating agents

Saponins (extracted from soap bark tree, *Quillaja saponaria*) are used in veterinary vaccines and function through intercalation with cell membranes. They form pores in the cell membrane by interacting with its cholesterol content and thus facilitate antigen uptake and processing by APCs. Saponins contain also an immunestimulatory component, which in conjunction with lipid ISCOMs (immunostimulating complexes), form a safe vehicle to target antigens to APCs. ISCOMs have been used effectively in both oral and intranasal formulations, leading to $CD8^+$ and Th1 based responses, along with the induction of IL-12 production by innate immune system cells.

1.3.5.4.Chitosan

Derived by deacetylation of chitin, chitosan and other chitin derivatives induce transient openings of tight junctions of epithelial layer, and thus enhance mucosal absorption of protein antigens. In addition to their mechanical action, these agents have been shown to stimulate non-specific immune responses to pathogens, such as Sendai virus and *E. coli* bacterium, highlighting their additional immunemodulatory role.

1.3.5.5.DNA vaccines

Over the past decade, DNA vaccines have been used to generate immunity to a host of pathologic agents. Developed initially as an intramuscular formulation, DNA vaccines have been tested through a variety of delivery routes, including mucosal surfaces. Intranasal, oral, genitourinary, and ocular routes have been used to enhance local mucosal immunity to encoded antigens. Systemic responses, in addition to mucosal responses, further from the inductive site have been observed following mucosal DNA immunizations. Several techniques have been developed since to enhance efficiency of mucosal delivery of these formulations, including intra-oral jet injection or use of biodegradable microparticles, liposomes and bioadhesive polymers, such as macroaggregated albumin complexes.

1.3.5.6.Edible vaccines

One promising avenue of delivering vaccines via the oral route is being investigated through the incorporation of antigens from pathogenic organisms

in transgenic plants. This would potentially allow production of antigen on a large scale. Studies in mice have incorporated antigens from bacteria, viruses or parasites in transgenic potatoes, tomatoes, and tobacco. These antigens were shown to result in a specific immune response without the need for adjuvant. However, a major obstacle yet to be overcome in this practical system, is the potential risk of breaching tolerance to food antigens.

1.3.5.7.Live attenuated vectors

Bacterial and viral vectors have been extensively explored as efficient mucosal delivery and/or expression systems for heterologous antigens. Among live bacterial vectors, there are those based on attenuated bacterial vehicles including *Salmonella typhi* or *paratyphi*, BCG, and *Bordetella pertussis.* Another category consists of commensal bacteria such as lactobacilli or some species of streptococci and staphylococci. In addition, several attenuated viral vectors have been used as vehicles of antigen delivery and expression. Vaccinia virus has been widely used towards this end. More recently, other candidate vectors among poxviruses have replaced vaccinia vectors; these include the canarypox virus and recombinant adenoviruses. VSV based vectors have been used recently as successful mucosal delivery agents and are endowed with inherent adjuvant features. Highly immunogenic virus-like particles (VLPs), also known as pseudoviruses, are also being tested as combined carrier/adjuvant systems. VLPs are self-assembling, non-replicating, recombinant viral core structures which are produced *in vitro.* Antigens can be expressed on the surface of VLPs or carried within their core, as in the case of DNA vaccines. Studies have shown promising data in the use of VLPs as mucosal delivery vehicles resulting in effective sIgA and CTL responses on mucosal surfaces. The safety and efficiency of some of the above vectors are currently being tested in preliminary clinical trials [46].

1.4 Transgenic Small Animal Models

1.4.1. 'Of Mice and Not Men'

- inter-species differences in immune system components and correlates.

Over the past several decades mice have become *The* experimental model of choice for many immunologists. It is indeed undeniable the tremendous insight that such experimentations have brought in elucidating immune mechanisms involved in pathologic conditions or following immunization in murine models assisting in the development of therapeutic agents, vaccines or diagnostic tools for human application. Yet, despite genetic similarities and common biological mechanisms, one should bear in mind that those two species diverged somewhere between 65 and 75 million years ago, and since have obviously not shared ecological niches and face significantly different antigen encounters.

Due to many similarities and paradigms that have translated successfully into human clinical trials, we often ignore this divergence, and tend to assume that what is true for mice will naturally be true for humans. The many inter-species immunological discrepancies have been extensively described in the literature [48] (also Table 1), however, it is noteworthy to summarize key differences herein, in order to put the development of a transgenic animal model in the current study in better perspective and recognize the importance of avoiding straightforward assumptions in extrapolating immune correlates among species. In this regard, and though there are general structural similarities between murine and human immune systems, some significant differences do exist.

It is noteworthy that, in contrast to healthy humans, mice have a considerable bronchus-associated lymphoid tissue. This can be explained as an adaptation to the higher breathable antigen load for rodents whose habitat is closer to ground. This difference should be kept in mind, especially in the context of the design of intranasaly administered vaccines and mechanisms of protection involved therein. Another structural difference points at the number and organization of various lymph nodes. Murine lymph nodes are few in number and organized in simple chains, with smaller lymphatic trunks and nodes. In contrast, lymph nodes from larger species are numerous and organized into more complex chains individually draining proportionately smaller areas of tissue.

On the other hand, in rodents, lymph nodes retain some residual haematopoietic activity which is absent in humans. With regards to the spleen, the periarteriolar lymphocyte sheath and marginal zone observed in mice are also absent in humans. Throughout the animal's life, the mouse spleen represents major haematopoietic activity (especially lymphopoiesis), while in humans minimal activity is observed during embryonic life which is completely absent in adult life, except in pathologic conditions. One reflects upon the impact of this activity while using rodent splenic cells in an array of immunologic and toxicologic assays in lieu of circulating leukocytes. The location, size and appearance of GALT nodules also varies, however corresponding functional differences have not been described yet. Mice have Payer's patch follicles that are uniform in size throughout the small intestine with 6-12 follicles present in aggregates. Humans have duodenal patches with few follicles, these increase in size and number more distally in the gut, with the terminal ileal Payer's patches containing up to 1000 individual follicles. The human appendix contains many IgG plasma cells in its dome area, while mice lack an appendix, possessing instead large aggregates of lymphoid cells in their cecal walls.

On the other hand, while assessing the cellular composition of peripheral blood, the neutrophil to lymphocyte ratio is significantly different between the two species: 50-70% of neutrophils and 20-40% of lymphocytes constitute adult human blood, while murine blood exhibits a predominant lymphocyte ratio of 75-90% with only 10-25% neutrophils.

With regards to innate immunity and Toll-like receptors (TLR), some species differences are also prominent: TLR2, which is constitutively expressed on murine thymus and T cells, is poorly expressed on circulating lymphocytes except when induced by LPS. In contrast, TLR2 is not constitutively expressed on human thymocytes, but it is on circulating leukocytes, however this is not induced by LPS. Another example is TLR3, which is strongly induced on murine macrophages following LPS induction. In humans, TLR3 is expressed on myeloid dendritic cells only [116].

Studies of the biology of murine NK cells show that their activity in the spleen and blood peaks early in life (4-10 weeks of age), while in humans NK cell activity is stable throughout life. In addition, in mice this activity is high in the lung, which is not the case in humans. Fc receptors (FcR) are readily detected on human, but to a lesser extent on murine NK cells.

FcR also represent a link between the innate and adaptive arms of the immune system. Mice lack expression of FcRI (CD89) which is an important IgA receptor expressed by several types of cells in humans. It is presumed that mice use alternative receptors such as the transferrin receptor (CD71) which also binds IgM. Besides mice lack two IgG receptors expressed in humans, namely, FcγRIIA and FcγRIIC. Moreover, as shown in Table 1, there is a major difference in the expression of immunoglobulin isotypes and subtypes between the two species as well as in the factors involved in the induction of class switching. Differences in B- and T-cell development and regulation have also been observed. For example, mature murine B cells exclusively express either CD5 or CD33 while human plasma cells express CD38.

An important feature of adaptive immunity is often the skewing of T cells toward a Th1 or Th2 phenotype. This polarization is readily observed in mice, yet the paradigm has not been so obvious in humans, whereby in many diseases both subsets can be generated simultaneously. While in mice IL-10 is considered to be a Th2 cytokine, both Th1 and Th2 cells secrete IL-10 in humans. In addition, in humans, IFN-α is secreted in response to viral infection by several cell types, leading to a Th1 response through STAT4 activation. In contrast, in mice, IFN- α does not induce Th1 cells nor activates STAT4.

Other major interspecies discrepancies within the adaptive immune system involve the costimulatory and activation pathways. One such example is the fact that human T cells express MHC Class II molecules upon activation while murine T cells don't. Another example is expression of the costimulatory molecule CD28. This receptor is expressed by nearly 100% of murine CD4

and CD8 T cells, while in humans 80% of CD4 and only 50% of CD8 cells express CD28.

While it is yet early to draw global conclusions about the significance of the above divergences, species differences in structure and function of the immune system should be kept in mind especially when designing preclinical experiments to be extrapolated to humans.

Alternatively, in immunological investigation, careful selection of the appropriate species and strains undoubtedly enhances the value of experimental data and facilitates the transition from bench to bed, and species/strain differences can be useful in delineating immunologic mechanisms and therapeutic efficacy. In this context, the availability of various mouse stains with well-defined immunologic features, including knockouts and transgenics, are vital in the development of vaccines and immunotherapeutics and the understanding of underlying mechanisms of diseases pathology [48, 49, 50].

Table I. Summary of some known immunological differences between mouse and human

	Mouse	Human	Notes
Hemotopoiesis in spleen	Active into adulthood	Ends before birth	
Presence of BALT	Significant	Largely absent in healthy tissue	
Neutrophils in periph. blood	10–25%	50–70%	
Lymphocytes in periph. blood	75–90%	30–50%	
Hemotopoietic stem cells	c-kit^{high}, flt-3^{-}	c-kit^{low}, flt-3^{+}	
TLR2 expression on PBL	Low (induced on many cells including T cells)	Constitutive (but not on T cells)	Binds lipopeptides
TLR3	Expressed on DC, Mac. Induced by LPS	Expressed by DC. No LPS induction	Binds dsRNA
TLR9	Expressed on all myeloid cells, plasmacytoid DC and B cells	Expressed only on B cells, plasmacytoid DC and N	Binds CpG
TLR10	Pseudogene	Widely expressed	
Sialic acid Neu5GC expression	Widespread	Absent	Binds pathogens
CD33	Expressed on granulocytes	Expressed on monocytes	Binds sialic acids
Leukocyte defensins	Absent	Present	Neutrophils
Paneth cell defensins	Processed by MMP7. Stored preprocessed	Stored as pro-form. Processed by trypsin	
Paneth cell defensins	At least 20	Two	
Macrophage NO	Induced by IFN-γ and LPS	Induced by IFN-α/β, IL-4^{+} anti-CD23	
CD4 on macrophages	Absent	Present	
Predominant T cells in skin and mucosa	γ/δ TCR (dendritic epidermal T cells—DETC)	α/β TCR	
γ/δ T cells respond to phosphoantigens	No	Yes	
CD1 genes	CD1d	CD1a,b,c,d	
NK inhibitory Rs for MHC 1	Ly49 family (except Ly49D and H)	KIR	
NKG2D ligands	H-60, Rae1β	MIC A, MIC B, ULBP	NK activating Rs
fMLP receptor affinity	Low	High	
FcαRI	Absent	Present	
FcγRIIA, C	Absent	Present	
Serum IgA	Mostly polymeric	Mostly monomeric	
Ig classes	IgA, IgD, IgE, IgG1, IgG2a*, IgG2b, IgG3, IgM * absent in C57BL/6,/10, SJL and NOD mice, which have IgG2c	IgA1, IgA2, IgD, IgE, IgG1, IgG2, IgG3, IgG4, IgM	
Ig CDR-H3 region	Shorter, less diverse	Longer, more diverse	
BLNK deficiency	IgM^{high} B cells in periphery	No peripheral B cells	
Btk deficiency	Normal pre-B and immature B	Blocks pro-B to pre-B transition	
λ5 deficiency	"leaky" block at pro-B to pre-B transition	Blocks pro-B to pre-B transition	
CD38 expression on B cells	Low on GC B cells, off in plasma cells	High on GC B cells and plasma cells	
B cell CD5 and CD23 expression	Mutually exclusive	Co-expression	
IL-13 effect on B cells	None	Induces switch to IgE	
Thy 1 expression	Thymocytes, peripheral T cells	Absent from all T cells, expressed on neurons	
Effect of γ_c deficiency	Loss of T, NK, and B cells	Loss of T, NK, but B cell numbers normal	
Effect of Jak3 deficiency	Phenocopies γ_c deficiency	Phenocopies γ_c deficiency	
Effect of IL-7R deficiency	Blocks T and B cell development	Only blocks T cell development	
ZAP70 deficiency	No CD4+ or CD8+ T cells	No CD8+ T but many nonfunctional CD4+	Related to syk level?
Caspase 8 deficiency	Embryonic lethal	Viable—immunodeficiency	
Caspase 10	Absent	Present	

(table continues)

Table I. *continued*

	Mouse	Human	Notes
IFN-α promotes Th1 differentiation	No	Yes	Mutant stat2 in mice
Th expression of IL-10	Th2	Th1 and Th2	
IL-4 and IFN-γ expression by cultured Th	Either/or	Sometimes both	
CD28 expression on T cells	On 100% of CD4+ and CD8+	On 80% of CD4+ 50% of CD8+	
ICOS deficiency	Normal B cell numbers and function, normal IgM levels	B cells immature and severely reduced in number, low IgM	Possibly age-related
B7-H3 effects on T cells	Inhibits activation	Promotes activation	
ICAM3	Absent	Present	DC-SIGN ligand
P-selectin promoter	Activated by TNF and LPS	Unresponsive to inflammation	
GlyCAM	Present	Absent	
MHC II expression on T cells	Absent	Present	
Kv1.3 K+ channel on T cells	Absent	Present	Regulates Ca flux
MUC1 on T cells	Absent	Present	Regulates migration?
Granulysin	Absent	Present	In CTL
CXCR1	Absent	Present	
IL-8, NAP-2, ITAC, MCP-4, HCC-1, HCC-2, MPIF-1, PARC, eotaxin-2/3	Absent	Present	Chemokines
MRP-1/2, lungkine, MCP-5	Present	Absent	Chemokines
IFN-γ effects in demyelinating disease	Protective in EAE	Exacerbates MS	
DTH lesions	Neutrophil-rich	Lymphocyte-rich	
Constitutive MHC II on EC	Absent	Present	
EC present Ag to CD4+ T	No	Yes	Memory T only
CD58 (LFA-3)	Absent	Present	CD2 ligand
T cell dependence on CD2-ligand interactions	Low	High	
CD2-ligand interaction	Lower affinity, with CD48	Higher affinity, with CD58	
CD40 on EC	Absent	Present	
Vascularized grafts tolerogenic?	Yes	No	
Microchimerism induces graft tolerance?	High success rate	Low success (expts. in non-human primates)	
Passenger leukocytes	Account for graft immunogenicity	Do not account for graft immunogenicity	

Table 1: Differences between Mouse and Human Immune System.

1.4.2. Generation of Small Animal Models

The discrepancy between immunogenicity and bias of T cell response among different vaccines is not fully elucidated. Several factors may be involved, including the route of delivery of the immunogen, which in turn may dictate the initial anatomical target and further development of ensuing immune response in distant lymphoid sites. Furthermore, studies have demonstrated that the initial antigen dose resulting from the vaccine dose, as well as the persistence of the antigen, critically influence the magnitude of the primary response and the subsequent development of memory response, respectively. The nature of the antigen and its processing and presentation mechanisms impact the inclination of the T cell response towards CD8 or CD4, with either a Th1 or Th2 bias of the latter. Delineating these factors in vaccine development is key to the development of rational vaccines, however such studies are often hampered due to lack of practical small animal models of disease and immunotherapy.

Since the first gene transfers into mice were successfully executed in 1980, transgenic mice have become models for studying human diseases and their treatment. Among the numerous genes examined for their role in disease development, polymorphism within MHC Class I and Class II loci have been shown to be critically involved in predisposition to certain pathologic conditions. This polymorphism also determines the specificity and affinity of peptide binding and ensuing T cell recognition.

More specifically, the development of transgenic mice expressing human MHC Class II molecules has been of great value in studying the immunopathology of human MHC class II-associated autoimmune diseases including rheumatoid arthritis, multiple sclerosis, and insulin-dependent diabetes mellitus. These mice are useful in the identification of target antigens that are involved in disease initiation and provide an *in vivo* disease model since they develop aspects of the respective human disease, either spontaneously or following immunization with a relevant antigen. This has

helped in better understanding of autoimmune disease mechanisms and testing novel immunotherapies.

HLA transgenic mice play a key role in the development of vaccines and the study of immunogenetic models of human disease. Individual haplotypes dictate the response to a given antigen and antigen specificity of human CD4 T cells is largely controlled by the widespread Class II HLA polymorphism. Hence the need to carefully define both the immunogenicity and efficacy of candidate vaccines in this context [138].

To study the interactions involved between class II molecules (DQ and DR) and define the immunologic mechanisms in various diseases, several groups have generated HLA-DR and DQ transgenic mice with or without the presence of the endogenous class II molecules [52,53,88,89,92,111]. The HLA molecules in these mice were shown to positively select $CD4^+$ T cells expressing various Vβ T-cell receptors (TCR). In addition, a peripheral tolerance is maintained to transgenic HLA molecules thus indicating that these molecules act as self and that mouse co-stimulatory and accessory molecules are able to interact with the HLA-peptide-TCR complex leading to efficient T-cell activation. HLA class II transgene-restricted T cells recognize the immunodominant antigens and peptide epitopes, similar to HLA class II-restricted human T cells. Therefore, these mice provide powerful tools to understand the role of HLA class II molecules in predisposition, onset, and prognosis of human diseases [51, 52, 53].

1.5 Immune Monitoring Tools

The development of reliable, sensitive *in vitro* assays that allow the quantitation and characterization of antigen-specific T cell responses is crucial in monitoring the immune response both in the context of infectious or non-infectious diseases, and in assessing outcomes of immunization strategies. With recent advances in our understanding of the intricacies of the molecular basis of the generation of adaptive immune responses, sensitive assays which

would dissect ensuing antigen-specific immune responses are imperative to the assist in depicting a clear picture of *in vivo* scenarios.

Traditional assays such as thymidine incorporation, CTL, ELISpot, and immunoscope are being complemented or at times replaced by an array of novel tools, such a flow cytometry-based techniques (ICS, CBA, proliferation assays), HTA, and microarray analyses. Below is a summary of some of the assays that are currently used as immune monitoring tools in a variety of applications from basic research to clinical diagnosis and immune monitoring. The need of sensitive, accurate, specific, and standardized immunologic monitoring is imperative towards the better understanding of often elusive immunepathologic and protective mechanisms.

For example, recent advances in the methods used for the characterization of HIV-1-specific cellular immune responses, including quantification of antigen-specific IFN-γ production by ELISpot assay and flow-cytometry-based intracellular cytokine quantification, have allowed for a much more comprehensive assessment of virus-specific immune responses. Emerging data show that the regulatory and accessory proteins serve as important targets for HIV-1-specific T cell responses, and multiple CTL epitopes have been identified in functionally important regions of these proteins [54].

Moreover, the use of autologous peptides have allowed for the detection of significantly stronger HIV-1-specific T cell responses in the more variable regulatory and accessory HIV-1 proteins Tat and Vpr. These data indicate that despite the small size of these proteins, regulatory and accessory proteins are targeted by cellular immune responses in natural HIV-1 infection and contribute importantly to the total HIV-1-specific $CD8^+$ T cell response. This may ultimately lead to the development of a multi-component vaccine, with the inclusion of these proteins plus structural proteins as a promising option towards an effective AIDS vaccine [55].

In recent years, CD4 and CD8 T cell responses to HIV and SIV infection have been increasingly measured with the use of single-cell assays such as ELISpot,

MHC-peptide oligomers, and cytokine flow cytometry. The results of these assays have been compared to those obtained with traditional bulk assays such as lymphoproliferation (by ^{3}H-thymidine incorporation) and cytotoxicity (by ^{51}Cr release). Such comparisons have led to some general understanding of the T cell responses that characterize progressive disease, long-term non-progressors, and individuals with viral suppression achieved by anti-retroviral therapy. Prophylactic and therapeutic vaccine trials have also begun to use these assays of T cell immunity to gauge the immunogenicity of the vaccines.

Whether such analyses will allow us to pick the best vaccine constructs, and whether they will provide us with an improved understanding of what constitutes protective cellular immunity to HIV, are major questions for the field. Today it is becoming clearer that sophisticated multiparametric assays are required to sort out the factors relevant for the generation of protective immune responses and identification of functional signatures [55,56].

1.5.1. Existing Tools:

Functional assays (ELISpot, Intracellular Cytokine Staining), detection of precursor cells ([3H]thymidine incorporation assay, CFSE or PKH26 labeling), and structural assays (HTA, Immunoscope, Class I and II Tetramers) are commonly used in a host of applications. Below we present an overview of these techniques and pros and cons of their application.

1.5.1.1.ELISPOT

Enzyme-linked immunospot, or ELISpot, assay allows the detection of low frequencies of cells secreting various molecules. ELISpot has been used in many areas of research and, because of its sensitivity and relative simplicity, has the potential to become a valuable diagnostic tool. Based on the same "sandwich" immunochemical principles as enzyme-linked immunosorbent assay, ELISpot is easy to perform. At the same time ELISpot remains a sensitive technique that requires accuracy, thorough selection of antibodies and detection reagents, and an understanding of the principles of data analysis.

ELISpot appears to be 200 times more sensitive than ELISA in detecting secreted cytokines, with as many as 10–100 cells per well being sufficient for the detection of cytokine-releasing cells. Such sensitivity makes ELISpot a technique of choice for the detection of spontaneous and antigen-induced secretion of cytokines (e.g., interferon [IFN]-γ, tumor necrosis factor [TNF]-α, interleukin [IL]-2, IL-4) from peripheral blood lymphocytes. ELISpot is widely used for vaccine development, AIDS research, cancer research, infectious disease monitoring, autoimmune disease studies, and allergy and transplantation research [57].

Even though the ELISpot technique has a host of advantages which include detection of cytokine secretion by Ag-specific cells at the single cell level, generation of results within 24hrs, performance doesn't require use of sophisticated equipment, can be used to assess response to a panel of peptides, however, it requires *in vitro* stimulation with Ag, and high background is often a problem while looking at low responders. Also, this technique will not distinguish CD4 from CD8 responders without prior cell separation, and cells can not be further manipulated following the assay. Besides, production of one cytokine might not be the sole marker of an activated cell. As some of the other common assays, ELISpot looks only at the functional aspect of T-cells and fails to detect some subsets of cells, including anergic cells.

The performance of this assay is greatly dependent on the quality of assay components including capture and detection antibodies, enzyme conjugates, chromogenic substrates, and membrane-backed plates. Careful optimization of all these parameters and appropriate controls are necessary toward the best interpretation of results [54, 57].

1.5.1.2.Intracellular Cytokine Staining (ICS)

Upon treatment with inhibitors of secretion such as Brefeldin A or Monensin (protein transport inhibitors), T cells accumulate cytokines in their cytoplasm following *in vitro* activation. ICS is a modification of the basic immunofluorescence staining and flow cytometric analysis protocol allowing

the simultaneous analysis of surface molecules and intracellular cytokines at the single-cell level.

In this approach, following *in vitro* activation, cells are stained for surface antigens, then fixed with paraformaldehyde to stabilize the cell membrane and permeabilized with the detergent saponin to allow anti-cytokine antibodies to stain intracellularly. The *in vitro* stimulation of cells is usually required for detection of cytokines by flow cytometry since cytokine levels are typically too low in resting cells. Stimulation of cells with the appropriate reagent will depend on the cell type and experimental conditions. A host of T cell activators have been used as positive control in ICS protocols depending on targeted cytokine, including a combination of PMA (phorbol myristate acetate - a phorbol ester / PKC activator) and Ionomycin (a calcium ionophore) or anti-CD3 antibodies, and in some cases lipopolysaccharide (LPS).

When compared to ELISpot, intracellular cytokine measurement necessitates larger sample volumes, still ICS is one of the commonly used assays which allows the measurement of cytokine production on a single cell level along the simultaneous determination of the cell's phenotype using a combination of surface markers. Some of the drawbacks of ICS include: requirement of *in vitro* Ag-stimulation; background levels in staining which should be carefully compensated by using isotype controls; and the fact that the protocol involves fixation and subsequent permeabilization during staining, which prevents further manipulation or growth of cells [54,58].

1.5.1.3.Cytometric Bead Array (CBA) Assay

The recent BD™ CBA analytical tool makes use of the flow cytometry principal and employs a series of particles with discrete sizes and fluorescence intensities to simultaneously detect multiple soluble analytes from a single biological sample. The BD CBA, combined with flow cytometry, creates a powerful multiple analyte (multiplex) assay system to measure soluble analytes with a particle-based immunoassay. The combined advantages of the broad dynamic range of fluorescence detection via flow cytometry, and the

efficient capturing of analytes via suspended particles coated with distinct capture antibodies enable the BD CBA to use fewer sample dilutions to determine analyte concentration in substantially less time (compared to conventional ELISA).

The specific capture beads are mixed with fluorochrome conjugated detection antibodies and then incubated with recombinant protein standards or test samples to form sandwich complexes. Following acquisition of sample data using the flow cytometer, the sample results are generated in graphical and tabular format using the BD CBA Analysis Software.

This assay allows acquisition of multiple parameters from a single small-volume sample and avoids artifacts associated with enzyme-dependent signal generation. Ready-to-use kits allow generation of quantitative results with less time and labor. For large volumes of experimental samples, the acquisition can be automated with the platebased BD FACSArray bioanalyzer or other BD flow cytometers equipped with the high throughput sampler (HTS) option. CBA allows the measurement of secreted form of cytokines in supernatants, and live cells can be further analysed or cultured. However, this method requires Ag-stimulation *in vitro* (some populations of T-cells might have limited growth and division potential *in vitro*) and looks only at cells which are functional based on their cytokine secretion capacity (effector function).

Different bioassays and immunoassays are used throughout many laboratories around the world for the measurement and report of cytokine levels in a variety of biological samples. Recently, the availability of international standard preparations of proteins has become essential to allow accurate analyses and comparison of results among different laboratories. These reagents are known as 'primary' (or aka, gold) standards and are frequently used to calibrate biological activities and protein concentrations between different secondary assay standards [59].

1.5.1.4.*in vitro* Proliferation Assays

Assays to detect precursor T cells, which are not endowed with an effector function but proliferate upon exposure to antigen, include the traditional thymidine incorporation assay and the more recent, flow cytometry-based methods.

The incorporation of radioactive [^{3}H] thymidine into the cellular DNA, gives an overall picture of the total amount of synthesized DNA, yet fails to indicate the actual frequency of specific T cells. Another approach is the use of the dye bromodeoxyuridine (BrdU) which is incorporated in replicating chromosomes, yet has limited application and sensitivity because of basal T-cell proliferation. Moreover, this approach does not allow further purification of Ag-specific live cells.

More recent proliferation assays make use of fluorescent dyes which stain either the cell membrane or cytoplasm. In this protocol, cells are incubated in the presence of a fluorescent dye, such as carboxy-fluorescein diacetate succinimidyl ester – CFSE or PKH26 which is a long chain aliphatic dye. PKH26 stains the cell membrane by integrating into the lipid bilayer, while CFSE binds to amino groups of cytoplasmic proteins. With each division, the cell becomes half as fluorescent, and the number of respective cell cycles can thus be directly deduced from intensity of fluorescence and the initial frequency of precursor T cells can be calculated. CFSE (or PKH26) labelling can also be combined with detection of other cell surface markers or tetramer staining. However, anergic T cells or T cells incapable of undergoing proliferation cannot be detected using the above mentioned methods [54].

1.5.1.5. HTA

The migration of double-stranded DNA molecules through a non-denaturing electrophoretic gel is determined by the degree of base-pairing within the double helix. The heteroduplex mobility assay (HMA), also known as heteroduplex tracking assay (HTA), allows the comparison of PCR amplicons.

Amplicons comprising of exactly complementary single strands, with only A-T and G-C base pairs, will migrate more quickly than identically sized amplicons that have mismatched bases.

Due to mismatches, typical `bubbles' in the `rod-like' amplicons occur which delay their movement through the gel matrix. If one of the two strands of the amplicon has an insertion or deletion then a `bulge' is formed that results in an even greater perturbation of the structure of the DNA and thus affects its electrophoretic mobility. Based on the generation of homoduplexes vs. heteroduplexes, HTA counters the need for extensive gene sequencing [60].

Among their many applications, specific HTAs known as T-cell receptor (TCR) heteroduplex mobility shift assays, are often used to analyze clonal expansion of T cells and assess TCR repertoire diversity in a variety of infectious diseases, such as HIV infection [61].

1.5.1.6. Immunoscope

This technique is a reverse transcriptase-polymerase chain reaction (RT-PCR)-based method, which subdivides any bulk population of T cells into approximately 2800 categories based upon different VβJβ gene combinations. Due to the varied lengths of N (non-genomic) and D (diversity) insertions within the junctional area of the TCR gene, at least 10 discrete CDR-3 lengths can be found within each VβJβ pairing. A cDNA sample for immunoscope is first amplified with forward and reverse primers to the variable and constant region segments, respectively (for the mouse this would be 24 reactions, one for each Vβ segment). Next fluoresceinated primers to each of the joining segments are used for "run-off" reactions. If there is no induced T cell expansion within the spectra, a Gaussian distribution of CDR-3 lengths is observed. In contrast, clonal expansions are observed as a perturbation of this Gaussian distribution. For a less extensive subdivision, a fluoresceinated primer to the constant region can be used for the "run-off" reaction, or Vβ primers can be designed which bind multiple Vβ gene segments [62, 63].

1.5.1.7.Genomics & Proteomics

It is well established that to sustain health, it is critical that a vast number of genes is expressed in a concordant manner and at normal levels. Many pathologic conditions today are proved to stem from disruptions or variations in such expression. Recent advances, both in knowledge and technology, have made possible the quantitative study of gene expression and thence the identification of the involvement of specific genes in the pathophysiology and prognosis of given diseases. Importantly, the completion of the Human Genome Project has generated a massive amount of information regarding our genome, therefore facilitating the task of researchers in identifying a host of previously unknown genes. On the other hand, the concurrent advancements in genomic biotechnology through the 'DNA microarray technology' is allowing the identification and classification of the above DNA sequence information and assignment of functions to these new genes.

Briefly, a microarray uses the ability of mRNA to hybridize to a DNA template from which it originated. By using an array of several predetermined DNA samples arranged in a regular pattern on a membrane or glass slide, the expression levels of hundreds or thousands of genes within a cell can be determined by measuring the amount of mRNA bound to each spot on the microarray. Using specially designed softwares, the amount of bound mRNA is measured accurately and generates a profile of gene expression in the cell at hand. (NCBI - http://www.ncbi.nlm.nih.gov/About/primer/microarrays.html) The challenge currently facing scientists in this field is to find a way to organize and catalog this vast amount of information into a usable form [64].

Immunotherapies for human immune-mediated diseases are proliferating rapidly. With these changes comes the need to monitor patients for immune responses to therapy based on early surrogate markers for clinical responses. The development of fast and sensitive bioassays is central towards this end. Over the past decade, novel state-of-the-art technologies have been developed.

These tools allow the detection of T cells based on the Ag-specificity of their TCR.

1.5.2. Oligomeric tools for detection of cellular immunity and their application

The T-cell receptor (TCR) solely recognizes a specific peptide presented in the context of a particular MHC molecule. This has been successfully used to develop technologies that allow the detection and isolation of distinct T-cell populations, in which all of the T cells carry the same specific TCR. However, the fairly low affinity between the monomeric MHC complex and the TCR does not easily permit efficient binding or detection. By the introduction of fluorescent-labeled multimeric MHC reagents, it has become feasible to detect and quantify the TCRs [65].

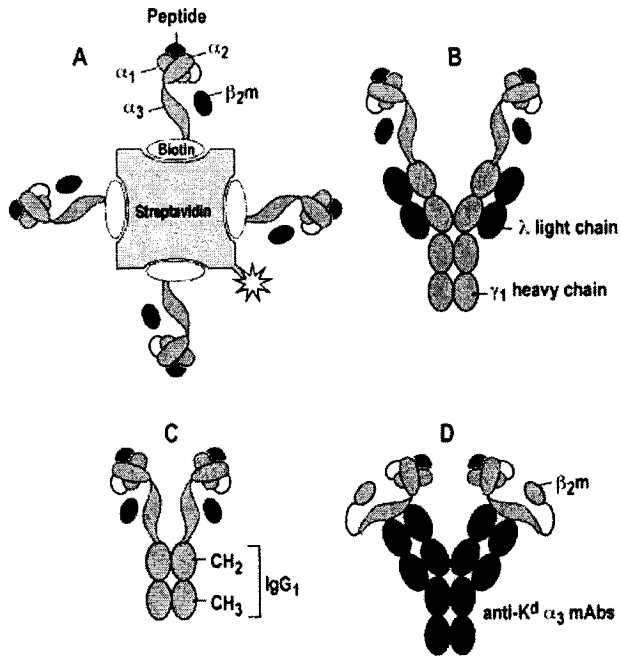

Figure 1 Schematic representations of multivalent MHC class I molecules.

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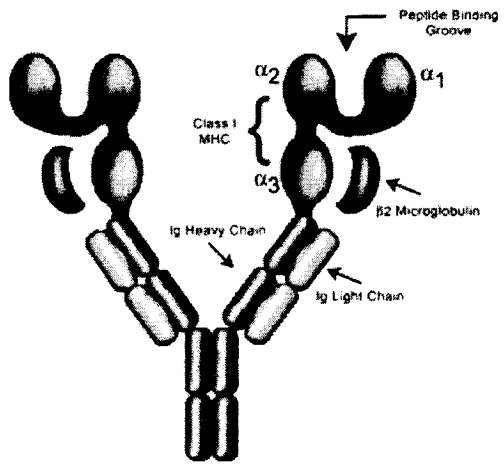

Figure 2 Schematic of a dimeric class I MHC, MHC–Ig (BD™ Dimer X).

Reprinted from the **Journal of Immunological Methods**, vol 268, Fahmy T.M., Joan G. Bieler J.G., Schneck J.P., *Probing T cell membrane organization using dimeric MHC–Ig complexes*, pp. 93-106, Copyright (2002), with permission from Elsevier.

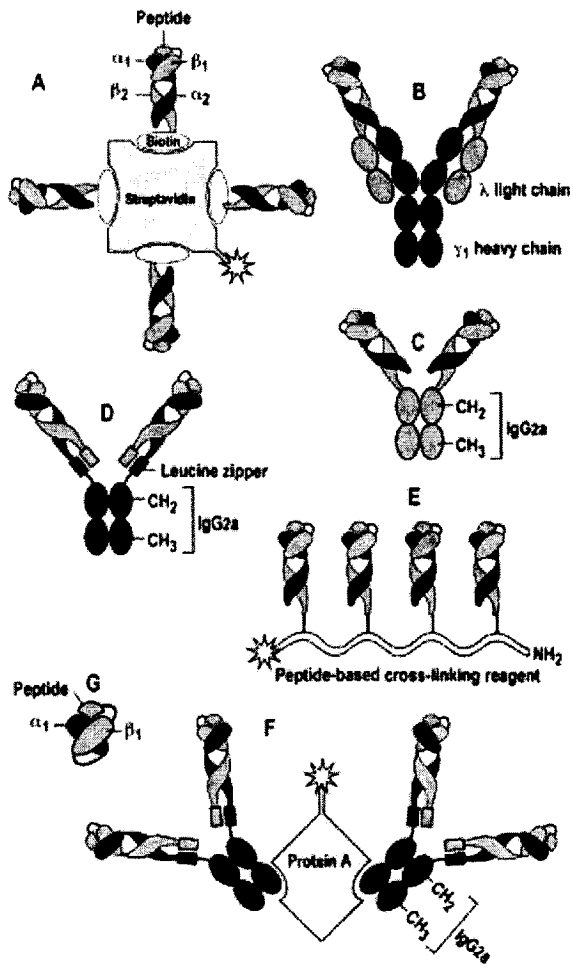

Figure 3 Schematic representations of monovalent and multivalent MHC class II molecules.

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1.5.2.1. Tetramers

MHC tetramers are oligomeric forms of soluble recombinant MHC molecules associated with specific peptide epitopes. The recombinant MHC molecules are assembled into oligomers to provide multiple ligands for enhanced interaction with antigen-specific T cell receptors (TCRs). These multimers are surrogates for the MHC–peptide ligand that is expressed on the surface of an antigen-presenting cell during a normal immune response. By binding to the TCR, a labeled tetramer molecule becomes a specific marker, identifying T cells by virtue of their antigen specificity.

The standard procedure in preparation of MHC tetramers involves production of recombinant soluble MHC class I or class II molecules bound to a specific peptide epitope. These molecules are later biotinylated at a specific biotinalation site introduced on one of the MHC chains. The biotinylated molecules are then incubated with fluorochrome labeled commercial streptavidin (or modified counterpart) to produce tetramers. Tetramers are used to stain CD4 or CD8 T cells expressing a TCR specific to the peptide epitope bound to the MHC molecule [65, 66].

Tetramer technology has proved to be a valuable tool and has the potential to serve as a powerful marker for immune monitoring thereby assisting with patient management, therapy selection, and improved outcomes. However, important issues of TCR avidity require resolution, because much is still unknown regarding location, quantitation, and characterization of the human T cell responses in a host of infectious and non-infectious conditions. Opportunities for application of tetramer technologies would enable both clinical progress and bring new insights into *in vivo* T cell biology.

1.5.2.1.1. Class I Tetramers

Originally described in 1996 [67], MHC class I tetramers have become widely used for quantitation of antigen-specific CD8+ T cell responses. MHC class I

tetramers contain HLA–A or HLA–B molecules assembled with peptides from pathogen-associated or cellular targets, and have been used to enumerate $CD8^+$ T cells to those targets, and to study $CD8^+$ T cell specificity, avidity, and phenotype. The range of antigen-specific cells detected by tetramers varies depending both on the pathogen at hand and on the clinical setting. Indeed, fluctuating levels of tetramer-positive $CD8^+$ T cells can be used as a clinical correlate of disease activity or immune response to administered vaccine. When coupled with methods that predict peptide binding to MHC class I molecules, tetramer analysis can be extremely useful for identifying T cell epitopes [112,113].

The application of class I tetramers to study self antigens has been most extensively developed in studies of tumor antigens. For example, peptides from melanoma-associated antigens loaded into HLA–A2 tetramers have been used for patient monitoring, phenotyping, and clinical correlations in patients with melanoma and in cancer vaccine trials. Insights based on the strength of tetramer binding have been used to infer the overall avidity of the TCR–MHC–peptide recognition complex, information that may help direct the design and use of candidate vaccines for inducing tumor-specific immunity [68].

1.5.2.1.2. Class II Tetramers

In contrast to many successful examples of the application of class I tetramers, success with widespread use of MHC class II tetramers has lagged. In fact, structural differences between class I and class II molecules necessitate different approaches to tetramer design and use. The single-chain class I molecule can be efficiently renatured in the presence of antigenic peptides to yield a stable β2-microglobulin–associated class I monomer, while engineering a soluble form carrying a biotinylation tag allows tetramerization with avidin. In contrast, class II molecules are noncovalent dimers of α and β chains, which have a variable range of stability and solubility in solution. Peptides bind into the α / β chain groove of class II molecules based on specific interactions with amino acid side chains, which confer specificity, determining which peptides will bind which class II molecules [69].

Detection of individual $CD4^+$ T cells based on their antigen specificity has been hampered by 2 significant barriers: the low frequency of such T cells in human peripheral blood and the low avidity of binding between the TCR and the MHC–peptide complex [70].

To circumvent these problems, different approaches to producing class II tetramers have been attempted. One is the covalent synthesis of single-chain class II– peptide complexes, directed by engineering peptide-specific complementary DNA (cDNA) sequences proximal to the β chain cDNA. In this strategy, the resulting polypeptide refolds with the peptide sequence extended from the amino terminus of the class II molecule. A tethering linker sequence in the peptide allows enough flexibility for the peptide to occupy the peptide binding groove in the mature class II molecule. A presumed advantage of this methodology is that every class II molecule produced contains the same peptide sequence; a potential disadvantage is that a separate molecular construct must be produced for every class II–peptide tetramer designed [69].

The second approach that has been used is to load the biotinylated class II dimer with exogenous peptides, followed by multimerization with avidin . The principal advantage of this technique is that a single MHC molecule can be loaded with a large number of distinct peptides, greatly expanding the number of T cell epitopes that can be studied. Recombinant class II molecules that incorporate "leucine zipper" motifs were also produced, replacing the native transmembrane and cytoplasmic portion of the molecules. The addition of these leucine zipper sequences provides both stability and solubility to the complex, which is produced in stably transfected *Drosophila* cells and subsequently purified by affinity chromatography [71].

By using fluorochrome-labeled streptavidin molecules, T cells that bind specific tetramers can be readily distinguished from those that do not, using flow cytometry. However, the low number of antigen-specific $CD4^+$ cells in peripheral blood poses challenges for the sensitivity of flow cytometry. It is indeed difficult to detect cells at frequencies below 0.2% (1:500) since

background staining using tetramer loaded with an irrelevant, nonspecific peptide may approach 0.1% in these types of assays. To overcome this, tetramer-positive antigen-specific cells are expanded following *in vitro* activation leading to both the accumulation of proliferating antigen-specific cells and the loss of unrelated T cells [72, 73].

Furthermore, a combined technique has been set forth which allows determining the frequency of antigen-specific cells in peripheral blood, in which cell divisions and tetramer staining are determined simultaneously. In this system cells are labeled with CFSE, as described in section 1.5.1.4. Cells are then incubated with antigen-presenting cells and specific antigen, and cultured *in vitro* for 4–7 days. Cells are stained with tetramer prior to analysis, and separate fluorescent wavelengths are used to detect tetramer binding and CFSE dye fluorescence, simultaneously. This method allows for the straightforward calculation of precursor frequencies of cells that respond to specific antigens. This approach is particularly useful when the original frequency of such antigen-specific cells is exceedingly low in peripheral blood, below the threshold for detection in the unamplified sample [73].

However, this requirement for *in vitro* amplification prior to tetramer detection poses some problems for analysis. Changes in T cell phenotype, differing rates of cell death, and selected expansion of higher-avidity cells are likely to occur during culture, potentially influencing quantitation of the tetramer-positive cells. Nevertheless, it is likely that some sort of *in vitro* amplification is necessary for routine clinical use, since direct detection of antigen-specific cells, both in peripheral blood and in synovial tissue, has so far yielded very low or absent tetramer-binding populations. By performing simultaneous analysis of tetramer fluorescence alongside other T cell parameters, information about T cell specificity and phenotype can be directly gauged, even within very complex samples.

On the other hand, it has been observed that activation markers, such as CD69, and production of interferon-γ by a clonal population of $CD4^+$ T cells, increase proportionately with the addition of increasing concentrations of specific

tetramer for staining. Thus, tetramer binding, which indicates a certain level of TCR–MHC–peptide avidity, can itself lead to T cell activation. Other recent studies have compared activation profiles among T cells stimulated with dimers (bivalent class II–peptide complexes), trimers, or tetramers. These studies indicate that multimerization of TCRs occurs early after interaction with MHC oligomers, followed by rapid T cell activation. Fluorescent staining with the multimers was enhanced by this activation, suggesting that clustering of receptors and/or endocytosis occurs, which augments the fluorescent tetramer signal detection. There is thus a relationship between avidity, activation, and tetramer binding. It appears that, while high-avidity T cells are readily detected using MHC tetramer fluorescence technology, detection of low-avidity T cells may require simultaneous measurement of activation markers or methods to facilitate enhanced tetramer staining by improving TCR clustering and/or internalization [72,73,117].

These studies highlight important issues of T cell frequency, avidity, and activation that have implications for analysis of rare autoreactive T cells in patients with autoimmune disease. The current methodology detects cells that are present in blood at frequencies above 0.1%, or 1/1,000. This is below the frequency of autoreactive $CD4^+$ cells circulating in blood, and direct detection of such rare T cells remains elusive. It is reasonable to attempt direct detection strategies in human studies when the antigen challenge is robust, such as in, for instance, vaccine trials, but it is unlikely that this approach will suffice for studies of disease pathogenesis if peripheral blood is the only source of material available for analysis.

On the other hand, avidity of the TCR–MHC–peptide interaction depends not only on the affinity of each TCR for its MHC–peptide ligand, but also on the density and clustering of TCR on the T cell surface. Together with factors such as the density of the MHC on the surface of the antigen-presenting cells, the concentration of peptide, and the type of costimulatory ligands available, TCR signaling leads to variable outcomes influenced by the strength of the interaction.

In clinical studies of patients with autoimmunity, most T cell staining with tetramers is of low-to-moderate avidity, suggesting either that the specific MHC–peptides used in the tetramers are not the most avid ligands for autoreactive T cells (i.e., we are using the wrong antigens) or, more likely, that circulating autoreactive T cells are indeed of low-to-moderate avidity and that this is intrinsic to their role in immunity. If the latter argument is correct, detection and monitoring of low-avidity T cells is an important challenge for future clinical applications. Approaches based on enhancing the clustering and/or internalization of TCR–tetramer complexes are one likely direction, as foreshadowed by the observation that higher temperatures give better staining. It will be worthwhile to search for additional molecular tools to facilitate TCR multimerization on the cell surface and increase the overall avidity of interaction achieved in large complexes.

Another promising direction is to take advantage of the association between tetramer binding and T cell activation. Even in the absence of antigen-presenting cells, soluble tetramers of sufficient avidity can activate T cell clones, as determined by both surface activation markers and cytokine secretion. Thus, analogous to interaction with anti-CD3–activating antibodies such as OKT3, addition of MHC–peptide tetramers to $CD4^+$ T cells can elicit partial activation responses that may be useful as markers for detection of specific cells. One such tetramer-facilitated activation profile is the up-regulation of CD4 on antigen responsive $CD4^+$ T cells. This $CD4^{high}$, $CD25^+$ population has been previously noted to include recently activated T cells, and is a hallmark of *in vitro*–stimulated antigen-specific $CD4^+$ T cells . For example, in studies of autoimmune diabetes, the activated, tetramer-positive phenotype was correlated with recent onset of disease, suggesting that detection of these cells may be a useful marker for disease progression and response to therapy [65].

Detection and monitoring of patients' T cell specificity and phenotype are only some of the clinically relevant uses for tetramers. There are a multitude of applications for this technology in clinical medicine and translational research. In addition, the ability to load the class II dimer with a variety of peptide

antigens enables the combination of tetramer analysis with peptide array strategies for epitope identification.

Peptide-MHC tetrameric complexes described above have undoubtedly many advantages. However, one should bear in mind that the use of tetramer technology depends primarily on the intimate knowledge of the individual peptides presented in the context of a particular MHC class I or class II molecule and that T cells with low affinity TCR may not be detected using these tools.

1.5.2.2.Novel oligomers

The absence of natural soluble TCR molecules in the blood and the low affinity of the TCR-MHC peptide interaction have been the main barriers to developing simple detection methods. The multimerization of MHC-peptide complexes answers partially this latter limitation by allowing an avidity effect and the staining of Ag-specific T cells. However, this method, largely used for the detection of cytotoxic $CD8^+$ T cells, has limitations. The first one of them is simply the geometry of display. Streptavidin is a rigid structure and the four biotin binding sites are positioned such that the MHC molecules are projected in the four cardinal directions. These structural constraints render almost impossible the simultaneous engagement of the four MHC molecules. Interestingly, a linear display of MHC molecules on synthetic polypeptides should lessen these steric problems.

The second limitation of tetramers is still the low affinity or the resulting low avidity. Indeed, the enhancement of binding, provided by multiple binding sites, is highly variable and sometimes of limited impact. Finally, additional limitations come from the T cell itself and a complex set of parameters linked to T cell activation, such as cell cycle, membrane fluidity, microdomain organization, receptor density, and receptor accessibility, all of which influence tetramer binding positively or negatively. This complexity explains most of the unusual observations that have been made for MHC class II tetramers and their low success rate. In all cases, systems of low functional

affinity cannot be detected. Thence the critical need of alternative Ag-specific detection systems.

An ideal system of T cell detection should be endowed with high specificity, high reproducibility, high sensitivity, and should work within the 4–25°C range. This association should be ideally reversible, should not result in activation, induce programmed cell death, or impair long-term cell viability. We summarize below development alternative oligomers of peptide-MHC complexes (see also Figure 1-3) [74].

1.5.2.2.1. Dimer X (BD Biosciences)

BD™ DimerX reagents are MHC-immunoglobulin fusion proteins developed to detect antigen-specific $CD8^+$ T-cells. Three extracellular domains of MHC class I molecules are fused to the N terminal of VH region of the mouse IgG1 through recombinant DNA technology. The expression vector containing the fusion protein is then co-transfected with genes containing human β2-microglobulin (β2m) into a myeloma cell line which is deficient in immunoglobulin heavy chain but retains the expression of immunoglobulin lambda (λ) light chain. The secreted product is a three-chain complex molecule consisting of a recombinant heavy chain of MHC-Ig fusion chain, an immunoglobulin light chain disulphide-bonded to the heavy chain, and a non-covalently associated human β2m molecule. The bivalent nature of peptide-binding sites of the DimerX molecules increases the avidity of MHC-peptide complexes to TCR and results in stable binding to antigen-specific $CD8^+$ T-cells. Furthermore, the hinge region in the immunoglobulin scaffold of DimerX provides a more flexible access for T-cell binding [75]. (*www.bdbiosciences.com/pharmingen* and Figure 2).

1.5.2.2.2. Dextramers

DakoCytomation™ MHC Dextramers consist of a dextran polymer backbone carrying an optimized number of peptide-loaded class I MHC and

fluorochrome molecules. The MHC molecules are aligned as pearls on a string on the dextran backbone. The biotinylated MHC moieties are attached via streptavidins to the dextran backbone carrying a fluorochrome of interest. Avidin-biotin bonds ascertain the anchoring of the MHC moieties to the dextran backbone that carries the fluorochromes.

These MHC Dextramers are multimeric reagents that have an apparent higher TCR-binding affinity compared to single MHCs. The TCRs experience numerous peptide-loaded MHCs and an evidently higher binding affinity. The higher binding affinity of the MHC-Dextramers is caused by the increased avidity, which is the sum of the individual affinities of the multiple MHC and TCR interactions. To date, the standard MHC Dextramers allow only monitoring of $CD8^+$ T-cell responses [76]. (http://www.dakocytomation.us)

1.5.2.2.3. Liposomes as Artificial Antigen Presenting Cells

Liposomes that display recombinant MHC class II molecules and can bind in an Ag-specific manner to $CD4^+$ T cells have been developed in an attempt to track cells which could not be detected using class II tetramers. As mentioned earlier, a strong correlation exists between binding of MHC class II-peptide multimers to $CD4^+$ T cells, the affinity of TCR, and the temperature used for multimer binding. Whereas low-affinity TCR can only bind MHC class II-peptide complexes at 37°C, high-affinity TCR also stain at 4°C. Fluorescently labeled liposomes added to $CD4^+$ T cells can co-localize with markers for endocytotic compartments, indicating that these T cells have the potential to internalize multimers.

These liposomes, also known as artificial APC (aAPC) were shown to mimic physiological interactions with T cells by allowing migration of molecules whose accumulation is an essential requirement and by inducing T-cell activation, through formation of immunological synapse. The composition of these aAPC allows free movement of the MHC–peptide complexes in the artificial membrane leading to active clustering of TCR–MHC molecules at

the immune synapse after interaction between aAPC and T cells. To date, multimer based methods for the detection of antigen-specific T cells rely mainly on absolute affinity between the ligands and do not allow the physiologic phenomenon of capping. This may limit the use of these tools to detection, not manipulation, of antigen-specific T cells [77].

1.6 Project Rational and Research Objectives

The pathogenesis of HIV infection is complex and multifactorial in humans, with genetic susceptibility, host responses, and viral factors all playing significant roles. The development of small animal models addressing some of these variables is paramount in assessing the impact of different vaccine strategies. Such a model will also be very useful in the pre-clinical validation of the functionality of immune monitoring tools.

On the other hand, to date, detection of Class II-restricted antigen-specific T cells by oligomeric MHC–peptide complexes has been faced with a host of physiological and biochemical challenges. Staining intensity being a direct function of T - cell receptor affinity, low-affinity TCR-bearing $CD4^+$ T lymphocytes might not be recognized by existing tools, such as tetramers. In order to bring new insights for the quantification and dynamic studies of $CD4^+$ T cell responses, we undertook the development of novel multivalent molecules of peptide-MHC Class II complexes for the identification and stimulation of antigen-specific T cells.

The specific research objectives that lead to the undertaking of this work were:

I. To use double transgenic humanized mice expressing chimeric murine – human MHC Class I and Class II molecules (CHAD) as a small animal model for studying functional signatures of cellular immune responses in the context of HIV vaccines.

II. To characterize the immunogenicity of recombinant Vesicular Stomatitis Virus (VSV-AV3) and Adenovirus serotype 5 (Ad5)

vectors expressing HIV-1 Gag A antigen inoculated in CHAD mice via a mucosal route.

III. To study the specificity and potency of systemic immune responses following mucosal immunizations using the above viral-vector based vaccines.

IV. To map epitopes within Gag subtype A in transgenic mice using synthetic peptide pools and matrices towards the identification of HLA-restricted responses and their comparison to immunogenic epitopes observed in HLA-A2 and HLA-DR individuals infected by HIV-1.

V. To generate novel multimeric reagents with increased avidity for the detection and manipulation of the intensity and quality of scant numbers of antigen-specific CD4 T lymphocytes.

2 Generation of a chimeric human A2DR transgenic mouse model: a unique platform for validation of immunization and immune monitoring strategies

GENERATION OF A CHIMERIC HUMAN A2DR TRANSGENIC MOUSE MODEL: A UNIQUE PLATFORM FOR VALIDATION OF IMMUNIZATION AND IMMUNE MONITORING STRATEGIES.

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SUMMARY

In the context of development of immune-based treatment against a variety of infectious or non-infectious diseases, the availability of a valid small animal model is crucial for the preclinical evaluation of novel prophylactic and therapeutic vaccines and immunotherapies. In fact, successful progress towards a promising HIV vaccine has been largely hindered over the past two decades due to lack of one such model. We have generated a humanized, HLA transgenic (Chimeric Human A2DR) mouse model. These mice crossed on a DBA/2 background express high levels of human MHC Class I A*0201 and MHC Class II DR*0101 molecules. This expression is systematically screened for by flow cytometry using anti-HLA antibodies on peripheral blood and by PCR on tail tissue using allele-specific primers. We extensively immune-characterized these mice using established phenotypic markers and functional assays. When compared to the wild type strain, these mice show comparable frequencies of CD3, CD4, CD8, B220, CD11b and CD11c positive cells in peripheral organs. In the thymus, different stages of T cell development are conserved. Naïve and effector T cell populations are also maintained or generated in the context of immunization assays. In addition, when stimulated with different polyclonal T cell stimulators or antigen-specific peptide pools in the context of mucosal immunization experiments, splenocytes and lymph node cells from these mice show normal proliferative and functional capacity. Our data show evidence of HIV immunodominant epitopes, originally mapped in humans, result in antigen-specific responses in this mouse model following mucosal immunization with two distinct viral vectors expressing HIV Gag from Clade A, namely VSV AV3 and Adenovirus serotype 5. This transgenic mouse model promises to be an important tool in testing novel vaccine strategies and elucidating protective mechanisms involved therein. Moreover, this model constitutes an excellent platform for the identification of new epitopes and for the validation of emerging immune monitoring tools in preclinical stages.

INTRODUCTION

A major limitation in advancing the study of a host of chronic viral infections, autoimmune disorders or tumors is the lack of suitable small animal models which allow accurate, quantitative monitoring of candidate vaccines and therapeutic regimens. Deciphering putative mechanisms of immunologic events following immunization are difficult in human subjects and require further elucidation in order to define important parameters for improving vaccine design and efficacy.

The Major Histocompatibility Complex (MHC), a set of polymorphic genes expressed on the surface of host cells, is involved in antigen presentation to immune cells. Since the discovery of HLA genes, several groups have developed HLA Class I and II transgenic mice, created to facilitate the study of HLA-restricted CTL and helper responses that may be crucial in generation of therapeutic targets against viral disease, cancer and autoimmune diseases. This has been extensively reviewed elsewhere [1-4,21].

CD8+ cytotoxic T cells, through their TCR, recognize MHC Class I molecules complexed with peptides derived from endogenously processed proteins. One of the largest common HLA Class I alleles, HLA-A*0201 (HLA-A2.1) represents 28% of the Caucasians in North America, and has been used as an ideal target transgene for transgenic mice systems. It is now established that the α1 and α2 domains of the MHC Class I molecule restrict the ensuing response and play a dominant role in the generation of specific CTL repertoire across species. In the case of HLA Class I transgenic mice, and in order to improve the efficiency of interaction of the mouse CD8 co-receptor with the HLA Class I molecules, the third domain of the human Class I heavy chain was substituted by the α3 to cytoplasmic domain of its murine counterpart [20]. These transgenic mice are considered the 'classic' A2.1/H-2 transgenics, which express heterodimeric HLA-A2.1 molecules in an H-2 Class I wild-type context. Transgenics expressing chimeric molecules have been widely used for the study of HLA-A2.1-restricted

responses [5-7]. On the other hand, transgenic mice expressing human MHC Class II molecules have also been generated and used as a model for studying HLA Class II-restricted patterns of immune response. More specifically, the HLA Class II DRB*0101 allele displays an allelic frequency of 9.3% in the North American Caucasian population, and plays an important role in many infectious and non-infectious diseases including Rheumatoid Arthritis, HIV, Hepatitis B, diabetes, and Goodpasture's disease. In this chimeric model, and to counter the inefficiency of interaction of mouse CD4 co-receptors with HLA Class II molecules, the peptide-binding region of the MHC is derived from the human HLA-DR sequence whereas the CD4-binding domain conserves the murine Class II sequences [8, 9, 10].

It is nowadays evident that an effective and potent CD4 help is crucial both to priming and maintenance of CD8 mediated responses [11]. In this study, we generated a double transgenic murine line that expresses chimeric human-mouse MHC Class I and Class II molecules by crossing transgenic HLA-A*0201 and HLA-DR*0101 mice which co-express normal levels of endogenous murine MHC. This system offers a convenient small animal model for the pre-clinical evaluation of vaccine regimens and other therapeutic interventions in the context of specific alleles without compromising the endogenous murine T cell numbers and other cell populations involved in antigen processing and presentation. We thoroughly immune-characterized these mice and validated this model in primary immune response scenarios using two different viral vector-based systems, namely a modified vesicular stomatitis virus (VSV-AV3) and Adenovirus serotype 5 (Ad5) expressing Clade A Gag of HIV-1.

MATERIALS & METHODS

Generation of HLA transgenic mice

To generate HLA-HLA-A*0201/HLA-DR*0101 H-2D^d/ I-A^d double transgenic mice (referred herein as HLA-A2.1/DR1), HLA-A2.1/H-2D^d transgenic mice were crossed with HLA-DR1/I-A^d mice and progeny were screened for the presence of HLA-A2.1 and HLA-DR1 chimeric transgenes until double transgenic animals were obtained. These mice express a transgenic monochain MHC Class I molecule in which the C-terminus of the human β2-microglobulin (β2m) is covalently linked to the N-terminus of a chimeric heavy chain (HLA-A2.1 α1-α2; H-2D^b α3, transmembrane, and intracytoplasmic domains), and a chimeric MHC Class II molecule consisting of the recombinant MHC class II molecule in which the α1 and β1 domains of mouse I-E^d were replaced by the corresponding domains of human DR*0101 molecule [8, 10]. PCR on tail tissue was performed using primers previously described [12]. Flow cytometry on tail blood using anti-A2-FITC and anti-DR-PE labeled antibodies allowed selection of mice that were high expressors of transgene. In addition, these mice express a full set of endogenous H-2D^d and I-A^d molecules.

Mice were bred and maintained at the Animal Care Facilities of the CHUM-Hôpital Notre-Dame, under specified pathogen-free (SPF) conditions, and sentinel mice were routinely tested for absence of mouse pathogens. All animal procedures described herein were approved by the institution's ethical review committee CIPA.

Harvesting of cells

Cells from lymphoid organs were isolated by mechanical disruption, homogenized into single cell suspensions and treated with Red Blood Cell (RBC) lysis buffer (0.15 M NH_4Cl, 10.0 mM $KHCO_3$, 0.1 mM Na_2EDTA, pH 7.4). For immune compartment analysis, blood was collected by heart puncture. Blood from tail vein was collected using EDTA containing disposable Sarstedt

Microvette®200 blood collection microcapillary tubes. RBC were lysed as described above.

Phenotyping, immune compartments and Flow Cytometry

$1x10^6$ cells from either the thymus, Lymph Nodes, spleen, or blood where labelled with a panel of fluorochrome conjugated antibodies [see *Antibodies* below] and respective isotype controls. The assessment of the levels of expression of endogenous and transgenic MHCs, and the phenotypic characterization of immune cell subsets were performed using a BD LSR II flow cytometer. Data analysis was performed using either CellQuest™ or DiVa™ analysis software (BD Biosciences, San Jose, CA).

Antibodies

The following antibodies from BD Pharmingen and BD Biosciences were used: PE-Cy7 anti-mouse CD4 (L3T4) (RM 4-5); APC anti-mouse CD3e chain (145-2C11); Alexa-700 anti-mouse CD3e chain (500A2); APC-Cy7 anti-mouse CD8a (53 – 6.7); R-PE anti-mouse CD11c (HL3); R-PE anti-mouse CD11b (M1/70); APC anti-mouse CD25 (PC61); R-PE anti-mouse CD44 (IM7); APC anti-mouse CD62L (MEL-14); PE-Cy5 CD45R/B220 (RA3-6B2); R-PE anti-mouse H-$2D^d$ (34-2-12); R-PE anti-mouse I-A^d (AMS-32.1); R-PE anti-HLA-DR (G46-6, L243); FITC anti-HLA-A2 (BB7.2).

Viral Vectors

VSV AV3-Gag (Consensus Subtype A) and VSV AV3-GFP control vectors were generously gifted by Dr J. Hiscott (McGill University, Montreal) and subsequently propagated and purified from *vero* cells in Biosafety Level 2 laboratory. The VSV-AV3 vector has been previously described by Stojdl DF et al [13].

The Clade A Gag-expressing Adenovirus serotype 5 (Ad5) vector was generated in our laboratory using the Clade A Gag insert of the VSV intermediary cloning vector. Briefly, the 1550 bp fragment of Gag Clade A (Gene Bank U51190) was

inserted into the *Eco*RI site of the Ad5 shuttle vector pDC315 (Microbix) and screened for correct orientation. 293 cells were co-transfected with pDC315GagA$^+$ and the adenoviral genomic vector pBHGloxdelE1cre (Microbix) (Figure S11 in Appendix) and successful recombinant viruses were picked and tested for production of Gag protein (Figures S12 & S13 in Appendix). Positive clones were propagated on 293N3S cells, in spinner flasks. The recombinant Ad5 viruses were purified by double cesium chloride gradient ultracentrifugation and concentrated by means of Centricon® centrifugal filter devices (Millipore). Viral stock titration was performed by lytic plaque assays on 293 cell monolayers. Expression of Gag was confirmed by western blot. Control vectors in the immunization protocol consisted of the virus containing the empty vectors (Ad5CTL) and were prepared similarly.

Immunizations

Groups of 6 transgenic mice aged between 6-8 weeks were immunized intranasaly (i.n.) through both nostrils with 10 µl of VSV AV3-GagA diluted to $5x10^6$ pfu/mouse/10 µl in LPS free PBS (Sigma) just prior to inoculation and kept on ice or with $1x10^8$ pfu/mouse/10 µl of AdGag. Control mice received respective amounts of AV3-GFP, AdCTL, or PBS. Immunizations were carried out under general gas anesthesia using isofluorane/oxygen mixture. Splenocytes were harvested 7 days following immunization. All immunizations were carried out according to animal experimentation protocols approved by the institutional animals ethics committee. Immunized animals were maintained under microisolator-barrier conditions in a Biohazard Class II (P2) –equipped facility and given food and water ad libitum.

Peptides

Peptide pools consisting of 15 amino acid (AA) length peptides, overlapping by 11 AAs and spanning the entire sequence of HIV-1 Consensus Subtype A Gag, were obtained from the NIH AIDS Research and Reference Reagent Program (MD, USA). Individual peptides were dissolved in DMSO at 10 mg/mL, aliquoted

into matrix pools, and used at a final concentration of 1 μg per peptide per stimulation.

Stimulation Assays

Briefly, splenocytes ($20x10^6$/mL in PBS) were labeled with a predetermined concentration of 5-(and-6)-carboxyfluorescein diacetate-succinimidyl ester (CFSE; Molecular Probes, Eugene, OR) freshly diluted solution in PBS, incubated 8 minutes at RT in dark, washed twice with PBS supplemented with 2%FCS to quench the dye, and plated at 2×10^6 cells per well during 5 days in 96-1mL-deep-well-plates (Nunc) in complete DMEM culture medium (SIGMA) supplemented with 10% FCS, 10 mM HEPES (Gibco), 1% Penstrep (Gibco), and $5x10^{-5}$ M 2-mercaptoethanol (Sigma). CFSE-labeled cells were cultured in medium alone for negative control (NS) or in presence of 2.5μg/mL of Concavalin A (ConA) for positive control. Peptide pools or individual peptides were used at a concentration of 1μg per peptide per stimulation.

MHC Class II Blocking Assay

Splenocytes from vaccinated and control mice were labeled with CFSE and cultured during 5 days (as described above) in the presence of purified anti-HLA-DR L243 antibody at 1 μg/mL and 10 μg/mL. L243 antibody was added 1hr prior to addition of Gag peptide pool and again 48hrs following stimulation.

HLA-A2 Tetramer Staining

Direct *ex vivo* staining using HLA-A2/Gag-specific (SLYNTVATL p17; FLGKIWPSYK p15, epitopes) and CMV (GILGFVTFL epitope) control tetramers was performed as previously described [14]. The tetramers were obtained from the Tetramer Core Facility, Canadian Network for Vaccines and Immunotherapeutics (CANVAC), Montreal, Canada.

Statistical Analysis

Probability levels and p values were calculated using two-tailed Student T-test.

RESULTS

Expression of MHC endogenes and transgenes in periphery and thymus

Blood collected from tail vein was marked with antibodies against HLA-A2 and HLA-DR1, as well as endogenous H-2D^d and I-A^d molecules. As illustrated in Figure 1, CHAD mice express high levels of both chimeric human MHC Class I (93.40% of cells in M1) and Class II (43.57% of cells in M1) molecules in periphery. In addition, when compared to wild-type DBA/2 mice, CHAD mice maintain comparable levels of endogenous H-2D^d (91.29% for CHAD vs. 99.23% for DBA/2 in M1) and I-A^d molecules (49.28% for CHAD vs. 43.65% for DBA/2 in M1) (Fig 1A). In the thymus, expression of the transgene in CHAD mice did not hamper expression of the endogenes as shown in Fig. 1B by nearly overlapping histograms of H-2D^d and I-A^d expression on CHAD vs DBA/2 thymocytes. Parallel screening through Polymerase Chain Reaction (PCR) using previously described allele-specific primers[12] allowed amplification of both HLA-A2 (lane 2, band at 813bp) and HLA-DR1 (lane 3, band at 315bp) from tail tissue (Fig 1C).

T cell maturation in the thymus

To assess thymocyte differentiation, we looked at the phenotypic stages which correspond to developmental checkpoints in the mouse, namely, Double Negative (DN CD4$^-$CD8$^-$: mean 2.80±0.36% vs. 2.23±0.15% of total thymocytes in CHAD vs. DBA/2), mouse Immature Single Positive (ISP, CD8$^+$CD3$^-$), Double Positive (DP CD8$^+$CD4$^+$: mean 67.73±4.61% in CHAD vs. 81.53 ± 2.06% in DBA/2 of total thymocytes) and Single Positive (SP, CD4$^+$CD3$^+$: mean 18.37±4.23% in CHAD vs. 9.60±0.95% in DBA/2 and SP CD8$^+$CD3$^+$: mean 2.80±0.26% in CHAD vs. 1.70±0.2% in DBA/2) populations. Furthermore, we looked at the DN subsets based on surface CD44 and CD25 expression, numbers indicating the percentage of each subset within the total DN population: DN1 (CD44$^+$25$^-$: mean 17.10±0.72% in CHAD vs. 18.10±1.21% in DBA/2), DN2 (CD44$^+$25$^+$: mean 9.60±0.30% in CHAD vs. 5.87±0.64% in DBA/2), DN3 (CD44$^-$25$^+$: mean

43.63±7.77% in CHAD vs. 57.70±0.72% in DBA/2), DN4 ($CD44^{-}25^{-}$: mean 29.70±7.22% in CHAD vs. 18.33±1.24% in DBA/2, p=0.05), as shown in Figure 2A. The total $CD3^{+}$ population in the thymus was represented by a mean of 23.70±4.71% in CHAD and 16.00±1.35% in DBA/2 of total thymocytes (p=0.05). The different populations described above showed normal distribution in CHAD and DBA/2 mice, with p values > 0.05, except in the DN4 and CD3 populations, whereby the p-value was equal to 0.05.

Distribution of Lymphoid Subsets in Periphery

Subsets of different lymphoid compartments were analyzed in peripheral tissues of CHAD vs. wild type DBA/2 mice using multiparameter flow cytometry. These compartments included: $CD3^{+}$, $CD4^{+}$, $CD8^{+}$, DP, and $CD62L^{hi}CD44^{lo}$, $CD62L^{hi}CD44^{hi}$, $CD62L^{lo}CD44^{lo}$, $CD62L^{lo}CD44^{hi}$ populations within $CD3^{+}CD4^{+}$ and $CD3^{+}CD8^{+}$ cells, in Lymph Nodes (Fig 2B), Blood (Fig2C), and Spleen (Fig 2D), in addition to the $B220^{+}$ compartment (Figure S2.1 supplementary data). Our results indicate that the transgenic CHAD mice overall have conserved basic immune cell compartments and subsets relative to wild-type DBA/2 mice. Other compartments that were also tested included $CD11b^{+}$, $CD11c^{+}$ and $CD3^{-}CD11c^{+}$ cells in the spleen and lymph nodes [data not shown]. The p-values were > 0.05 unless otherwise specified, indicating that there was no considerable statistical difference between the studied cell populations in CHAD vs. wild type mice.

Peripheral Lymph Nodes. In the LN the relative distribution of the following subsets was observed in CHAD vs. DBA/2. 78.10±3.41% vs. 82.10±1.08% of cells were $CD3^{+}$. Within the $CD3^{+}$ compartment, 22.83±2.95% vs. 24.27±1.62% of cells were $CD8^{+}$, 69.50±2.52% vs. 67.77±1.52% were $CD4^{+}$, and 5.27±0.96% vs. 6.27±0.58% were $CD4^{+}CD8^{+}$ (Fig 2B). Of the $CD3^{+}CD8^{+}$ cells 68.57±6.00% vs. 66.17±6.55% were $CD62L^{hi}CD44^{lo}$; 18.70±3.66% vs. 18.33±3.61% were $CD62L^{hi}CD44^{hi}$; 7.20±4.16% vs. 9.80±4.33% were $CD62L^{lo}CD44^{lo}$; and 1.97±0.55% vs. 3.33±1.45% were $CD62L^{lo}CD44^{hi}$. Similarly, of the $CD3^{+}CD4^{+}$ cells 49.87±9.65% vs. 55.77±10.29% were $CD62L^{hi}CD44^{lo}$; 34.63±5.02% vs.

24.30±6.61% were $CD62L^{hi}CD44^{hi}$; 5.17±1.95% vs. 5.53±0.71% were $CD62L^{lo}CD44^{lo}$; and 6.50±0.17% vs. 11.43±4.58% expressed a $CD62L^{lo}CD44^{hi}$ phenotype (Fig 2B, p>0.05). B220+ cells in peripheral lymph nodes were represented by 33.20±4.83% in CHAD vs. 24.83±1.58% in DBA/2 mice (p=0.05) (Fig. S2.1 supplementary data).

Blood. Blood was collected through heart puncture and displayed the below distribution following RBC lysis. 41.23±12.06% in CHAD vs. 31.67±5.20% of DBA/2 blood cells were $CD3^+$. Within the CD3+ compartment, 21.80±2.88% vs. 18.93±0.96% of cells were $CD8^+$, and 68.53±2.52% vs. 67.77±1.52% were $CD4^+$ (Fig 2C). Of the $CD3^+CD8^+$ cells 78.30±5.79% vs. 72.10±2.80% were $CD62L^{hi}CD44^{lo}$; 16.23±4.31% vs. 15.53±1.70% were $CD62L^{hi}CD44^{hi}$; 2.10±0.70% vs. 5.33±2.02% $CD62L^{lo}CD44^{lo}$; and 0.73±0.35% vs. 2.20±0.30% $CD62L^{lo}CD44^{hi}$. On the other hand, of the $CD3^+CD4^+$ blood cells, 80.43±2.86% vs. 78.43±3.57% were $CD62L^{hi}CD44^{lo}$; 12.33±1.76% vs. 9.27±2.17% were $CD62L^{hi}CD44^{hi}$; 3.03±0.64% vs. 2.80±1.45% were $CD62L^{lo}CD44^{lo}$; and 2.00±1.08% vs. 8.07±3.55% expressed a $CD62L^{lo}CD44^{hi}$ phenotype (Fig 2C). The p values were greater than 0.05 except for the CD4+ $CD62L^{lo}CD44^{hi}$ (p=0.05) and the CD8 $CD62L^{lo}CD44^{hi}$ (p=0.01) populations. 42.07±9.05% of CHAD vs. 45.60±4.11% of DBA/2 blood cells expressed B220 (p>0.05) (Fig. S2.1 suppl. data).

Spleen. In the spleen 22.57±5.67% vs. 16.43±1.38% of cells were $CD3^+$. Within the CD3+ compartment, 19.83±2.06% vs. 26.20±0.40% of cells were $CD8^+$ (p=0.01), 69.97±3.91% vs. 60.63±1.27% were $CD4^+$ (p=0.02), and 3.77±0.35% vs. 3.20±0.44% $CD4^+CD8^+$ (Fig 2D). In $CD3^+CD8^+$ splenocytes 49.93±4.70% vs. 42.97±4.77% were $CD62L^{hi}CD44^{lo}$; 41.47±6.31% vs. 47.90±4.33% were $CD62L^{hi}CD44^{hi}$; 3.03±1.19% vs. 3.10±1.21% $CD62L^{lo}CD44^{lo}$; and 3.67±0.25% vs. 3.90±0.10% $CD62L^{lo}CD44^{hi}$. In the $CD3^+CD4^+$ compartment 49.07±3.45% vs. 49.93±6.51% were $CD62L^{hi}CD44^{lo}$; 31.93±3.69% vs. 28.50±4.58% were $CD62L^{hi}CD44^{hi}$; 6.07±2.01% vs. 7.40±0.69% were $CD62L^{lo}CD44^{lo}$; and 10.50±0.53% vs. 11.50±2.91% expressed a $CD62L^{lo}CD44^{hi}$ phenotype (p>0.05).

$B220^+$ cells constituted 51.00±3.30% of CHAD vs. 62.37±2.40% of DBA/2 splenocytes (p=0.01) (Fig. S2.1 suppl. data).

Gag-specific primary $CD4^+$ and $CD8^+$ responses following VSV AV3-GagA and Ad5GagA mucosal immunizations

To determine whether transgenic CHAD mice represent a useful model in testing vaccine strategies, their immune competency was tested in two separate viral vector based mucosal immunizations. Groups of 5 to 6 mice were inoculated intranasaly (i.n.) with either VSV-AV3/Gag or Ad5/Gag (derived from consensus subtype A) and corresponding viral vector controls (CTL). Splenocytes from immunized mice were tested using an *in vitro* CFSE proliferation assay against pools of Gag Clade A peptides. Immunization of CHAD mice with viral constructs expressing Clade A Gag led to significant and reproducible proliferative CD4 responses against Gag peptides from a previously tested pool of 10 immunodominant peptides in the VSV-AV3/Gag immunized group (Fig 3A, Fig 4A, and 4B, Gag pool). Following 5 days of *in vitro* stimulation with the above-mentioned peptide pool, 17.6% of $CD3^+CD4^+$ splenocytes were $CFSE^{lo}$ over a background of 0.5% $CFSE^{lo}$ cells in negative control samples (i.e. unstimulated, NS), while splenocytes from mice immunized with VSV AV3 vector alone immunized group showed a 4.5% of $CD3^+CD4^+$ $CFSE^{lo}$ cells following stimulation with the peptide pool (Fig 3A). $CD3^+CD8^+$ splenocytes proliferated concomitantly implying shared CD4 and CD8 epitopes among the 15-mer Gag peptide pool used in stimulations. 32.3% of $CD3^+CD8^+$ cells were $CFSE^{lo}$ in VSV-AV3 Gag group following 5-day *in vitro* stimulation compared to a vector-induced 6.1% $CD3^+CD8^+$ $CFSE^{lo}$ cells (Fig 3B). Similarly, Ad5/GagA immunized mice showed antigen-specific $CD4^+$ cell proliferation in the spleen following *in vitro* stimulation with Gag peptide pool. 13.5% of $CD3^+CD4^+$ splenocytes were $CFSE^{lo}$ compared to 5.2% in the Ad5 vector immunized group, over a background proliferation of 0.2% for Ad5Gag vs. 3.6% for Ad5 vector control in media control samples (Fig 4C, Gag pool NS samples). Furthermore, a specific CD4 proliferative response was observed when individual Gag peptides

constituting the above pool were tested in a similar stimulation assay (Fig 3C and S2.2A in suppl. data). Gag peptides G066, G065, G057 and G042 yielded the strongest response, resulting in 7%, 4.6%, 4.5% and 3.8% of $CD3^+CD4^+$ $CFSE^{lo}$ cells (Fig 3C). A similar response was also observed when individual mice from each group were tested against individual peptides (Fig. S2.2B in suppl. data). Low levels of background proliferation were detected after control vector immunization, however this was significantly lower than the response in immunogen immunized groups. Splenocytes from all groups did respond strongly to a polyclonal stimulation with concanavalin A (Con A) (Fig 3A, 3B and Fig 4B, 4C, Con A).

In vitro blocking of HLA-DR molecules results in a reduction of antigen-specific $CD4^+$ proliferation

Based on the observation that mucosal immunization of CHAD mice with Clade A Gag expressing viral vectors resulted in significant proliferation of peptide-specific $CD4^+$ T cells, we next tested whether this response was restricted to the transgenic human MHC Class II molecules. As described in Materials and Methods, we performed peptide stimulations of splenocytes from vaccinated vs. control mice in the presence of two concentrations of anti-HLA-DR (L243) antibody at 1 μg/mL/$2x10^6$ vs. 10 μg/mL/2x106 splenocytes. A second aliquot of L243 was further added following 48hrs of culture to block *de novo* expression of Class II molecules on the surface of APCs as much as possible. Proliferative $CD4^+$ cell responses were considerably reduced following the addition of 10 μg of L243 (twice during a 48hr interval) and to a lesser extent following the addition of 1 μg of L243 (Fig 4A). The following blocking effect was observed in VSV-AV3 Gag immunized group, 16.4% of $CD3^+CD4^+$ cells were $CFSE^{lo}$ following stimulation with Gag peptide pool without any blocking antibody, this number decreased to 13% and 9.7% following the addition of 1 μg and 10 μg of L243, respectively (Fig 4A and 4B). In the Ad5 Gag group, the numbers of $CD3^+CD4^+$ $CFSE^{lo}$ cells were 13.5%, 8.2% and 5.6% when no amount of antibody, or 1μg, and 10μg of L243 were added, respectively (Fig 4A and 4C). The residual level of

proliferation observed in these stimulations might be attributed to endogenous IA^d presentation as well as, on some level, to background activation observed due to reactivity to viral vector (5.6% in VSV-AV3 CTL and 4.2% in Ad5 CTL control vectors in 10 μg/mL L243 samples, Fig 4B and 4C). These cells were also brighter in CFSE, suggesting blockade at early stages of proliferation. On the other hand, the proliferation of CD8 cells was also impaired to some extent following addition of anti-HLA-DR antibodies (data not shown), implying a role of CD4 help for effective CD8 T cell proliferation. This involvement in promoting the quality of CD8 response, their clonal expansion and survival, as well as possible mechanisms involved therein have been addressed elsewhere [11,15].

Primary HLA-A2-restricted CD8 T cell response following VSV-AV3/Gag immunization

We measured HLA-A2-restricted CD8+ T cells responses by direct *ex-vivo* HLA-A2 Class I Tetramer staining of splenocytes from vaccinated and control animals, 7-days post immunization with VSV-AV3/Gag, using HLA-A2 tetramers recognizing either Gag p15 (FLGKIWPSY[S]K), Gag p17 (SLYNTVATL) or control pp65 CMV (GILGFVTFL), which are previously described epitopes in humans (Fig. 5). 1.3% of CD8+ cells were HLA-A2/ p15 Tetramer positive, implying that epitopes previously identified in humans and restricted by HLA-A2 are presented and recognized in the CHAD model. Tetramer staining on splenocytes from groups that received vector alone or PBS controls was below 0.2% with some values lower than background levels. We did not observe any response to the Gag p17 epitope through A2 p17 Tetramer staining. Lack of staining with HLA-A2 tetramers in HLA-A*0201 mice has been observed previously and attributed to the relatively poor interaction between muCD8+ receptor and the α3 domain of the tetramer Class I heavy chain. This has been circumvented through the generation and use of chimeric murine-HLA-A2 tetramers [16,19].

DISCUSSION

In the present study, we developed a chimeric human HLA-A2.1/DR1 MHC transgenic mouse model and validated it as a platform for testing vaccine strategies using two separate viral vector based mucosal vaccines against HIV-1, namely, recombinant VSV-AV3 and Adenovirus serotype 5 expressing Clade A Gag. Although MHC transgenic mouse models expressing A2.1 and/or DR1 have been developed previously, to our knowledge, this is the first study testing the immunogenicity of mucosal HIV vaccines in such a model. We demonstrate here that mucosal immunization with either VSV- or Adenovirus-based vectors results in primary antigen-specific $CD4^+$ and $CD8^+$ systemic T cell responses that could be detected directly from the spleen 7 days post-immunization. The use of *in vitro* stimulation assay of CFSE labeled splenocytes in combination with synthetic HIV-1 derived peptide pools allowed mapping of T-cell epitopes which were previously identified as being immunogenic in HIV-1 infected humans. Moreover, some of these epitopes are conserved across HIV-1 clades (A, B, and C), indicating that these mucosal vaccines can be important in eliciting cross-clade systemic cell mediated responses. Whether these vaccines induce mucosal immune responses and lead to protection against challenge in this transgenic model remains to be tested.

First, we describe the expression of endogenous and transgenic MHC molecules in this model. We then look at the different developmental stages of T cells in the thymus and immune compartments in the periphery of naïve CHAD mice, using wild type DBA/2 mice as parental control for endogenous MHC expression. Our results show that CHAD mice express high levels of transgenic Class I and Class II molecules in periphery, and that expression of the endogenous $H\text{-}2D^d$ and $I\text{-}A^d$ molecules is conserved both in periphery and thymus (Figure 1). In addition, we studied the different thymocytes differentiation stages, including DN1 to DN4 populations which showed a normal distribution (Figure 2A) as did the different cell subsets in periphery including cells of the lymph nodes, blood and spleen

(Figure 2B, C, & D), including CD3, CD4, CD8 populations and subsets delineated by the CD44 and CD62L markers. Therefore, the presence of human MHC molecules did not cause abnormal immune cell differentiation and distribution in these mice.

Next, in order to study primary immune responses elicited in mice vaccinated intranasaly with either recombinant VSV-AV3 or Ad5 vectors expressing Gag from Clade A HIV-1, we used several pools of 15-mer Gag peptides overlapping by 11 amino acids. We identified a pool of 10 peptides (Table 1) which resulted in considerable systemic $CD4^+$ and $CD8^+$ responses and used this pool in further stimulation assays. These experiments did validate the concept that T-cell epitopes previously defined in HIV-1-infected HLA-A2 or HLA-DR humans induce significant antigen-specific T cell responses in HLA-A2/DR1 transgenic mice. This in turn infers the likelihood of identifying important epitopes through epitope mapping in the current transgenic model, which might be immunogenic in humans.

We quantitated the primary response to these peptides through an *in vitro* proliferation assay alongside CFSE labeling of bulk splenocytes (Figures 3 & 4). Mice from either VSV-AV3/Gag or Ad5/Gag immunized groups showed antigen-specific proliferation of $CD4^+$ and $CD8^+$ cells. Remarkably, in the VSV-AV3/Gag immunized group, and after 5 days of *in vitro* stimulation, 17.6% of $CD3^+CD4^+$ and 32.3% of $CD3^+CD8^+$ cells were $CFSE^{lo}$, while vector alone immunized group did not show any significant response. Background levels of proliferation of splenocytes cultured in media alone were negligible (Figures 3A & 3B, NS). We further stimulated splenocytes with individual peptides constituting the above pool which led to the identification of single Gag epitopes (Figure 3C & suppl. Fig. S2.2A). Stimulation with individual peptides in some cases resulted in up to 7% $CD4^+CFSE^{lo}$ cells, such as with the G066 epitope previously identified in humans[17]. The above data were reproduced in subsequent independent experiments undertaken in parallel to adenoviral immunizations, whereby 16.4%

of $CD3^+CD4^+$ cells were $CFSE^{lo}$ in the VSV-AV3/Gag immunized group (Figure 4A & 4B). In addition, 13.5% of $CD3^+CD4^+$ cells were $CFSE^{lo}$ in the Ad5 Gag counterpart (Figure 4A & 4C). Vector alone immunized groups displayed a proliferation of 4.3% and 5.2% of $CD3^+CD4^+$ cells in VSV-AV3 CTL and Ad5 CTL groups, respectively, while cells cultured in media alone (NS) showed a background proliferation of 1.5% and 0.2%, respectively (Fig. 4B & C). Our results demonstrate that HLA double transgenic mice mount potent systemic antigen-specific T cell responses following mucosal immunization with viral vectors expressing HIV antigens.

To study HLA-DR restriction of antigen presentation in these mice, we further performed the *in vitro* stimulation assays in the presence of anti-DR antibodies (Fig 4A, B & C). The addition of anti-HLA-DR1 L243 antibody, one hour prior to - and 48 hours post- stimulation, resulted in a significant reduction of the number of $CD4^+CFSE^{lo}$ cells. As expected, this effect was more significant when a higher concentration of antibody was added. No consistent effect was observed in wells where splenocytes from vector alone immunized groups were cultured (AV3 CTL, Ad5CTL) and background levels of proliferation (NS) were minimal (Fig. 4A, B, C). The demonstration of antigen-specific HLA-DR1-restricted responses in our study suggests that a putative murine MHC Class II restricted response did not prevent generation of systemic $CD4^+$ responses to epitopes presented through the chimeric murine-human Class II molecule.

Previous studies have demonstrated that the co-expression of murine MHC genes can hinder development of HLA-A2-restricted T cell responses in HLA-A2 transgenic mice and this might be compounded by the immunodominance of the $H\text{-}2D^d$-restricted epitopes [18]. Here we used two HLA-A2 Gag tetramers, namely Tet-A2/p15 and Tet-A2/p17, to demonstrate HLA-A2 restricted primary responses in the VSV-AV3/Gag immunized groups (Fig. 5). Direct *ex-vivo* staining was performed on splenocytes 7 days post-mucosal immunization. We observed Gag p15 specific $CD8^+$ responses, but not Tet-A2/p17 staining. The

absence of the latter could be either due to low frequency of p17 restricted CD8 T cells in the absence of *in vitro* expansion of splenocytes by peptide stimulation, and/or due to the inherent biochemical characteristics of the HLA-A2 tetramers. To circumvent the problem of low affinity of interaction between muCD8 cells and α3 domain of HLA-A2, other groups have developed chimeric murine-HLA-A2 reagents [16,19]. Staining with negative control Tet-A2/CMVpp65 and staining of splenocytes from vector immunized group yielded equivalent or lower values to background levels of fluorochrome-labeled streptavidin staining.

To date, a substantial focus of immunization studies in mice has been dedicated to the delineation of murine CD8 responses while murine CD4 responses and subsets remain poorly defined. We are confidant that double transgenic mouse models such as this will be an important tool in identifying T cell subsets and mechanisms involved in correlates of immunity and shed light on the generation of memory T cells. In addition, this model can be a valuable instrument in the quality control and assessment of diagnostic tools, such as MHC Class II tetramers and epitope mapping through synthetic peptide matrices. Our results reinforce the advantages of using pools and overlapping peptide matrices alongside CFSE labeling, as this allows unbiased screening of either HLA-A2.1 and DR1 or murine MHC Class I and II restricted responses.

On the other hand, it is well established nowadays that the mucosal route of vaccine administration is an important consideration in the development of a prophylactic HIV-1 immunization regimen. The two viral vectors used in this study represent excellent candidates for prime-boost approaches in HIV vaccine development. Pre-existing immunity to vaccine vectors, such as Adenovirus based vectors, is a major concern. Since there is little seroprevalence to VSV in the human population, it would be interesting to use these vectors in combination, as heterologous prime-boost strategies. It is clear that further exploration of the T cell responses against the herein identified peptides is required in this mouse model, as the current study did not look at the faith of these antigen-specific cells

longitudinally. Currently, different prime-boost regimens using these viral vectors are underway to track these cells, and study the generation, maintenance, and role of memory cells in conferring protection.

The generation of relevant small animal models, which preserve the ability of generation of primary and memory immune responses without compromising endogenous CD4 and CD8 T cell numbers, would undoubtedly facilitate the development and pre-clinical evaluation of much awaited for vaccines and therapeutic strategies in a host of disease scenarios.

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LEGENDS TO FIGURES

FIGURE 1. Surface expression of endogenous and transgenic MHC Class I and Class II molecules in CHAD vs DBA/2 mice. Peripheral blood cells following RBC lysis **(A)** and bulk thymocytes **(B)** were stained with anti-HLA DR PE, anti-HLA A2 FITC, anti-$H2D^d$ PE, and anti-IA^d PE antibodies, followed by flow cytometry analysis. White histograms with black contour represent DBA/2, grey histograms represent expression in CHAD mice. Data representative of several routinely performed screening results. M1 depicts region positive relative to negative isotype control. **(C)** Screening by PCR amplification of HLA transgenes from tail tissue. Lane 2: HLA-A2 amplicon, expected band size 813bp, Lane 3: HLA-DR amplicon, expected band size 315bp. Products were analyzed on a 1% agarose gel against a Lambda DNA PstI digest ladder (Lane 1).

FIGURE 2. (A) T cell maturation in thymus. Anti-CD3, CD4, CD8, CD25, and CD44 antibodies were used in multi-parameter flow cytometry to delineate different maturation stages of T cells in the thymus. (SP single positive, DP double positive, and DN1-4 double negative populations). **(B-D) Distribution of T cell subsets in periphery.** Anti-CD3, CD4, CD8, CD62L, and CD44 antibodies were used to assess the relative distribution of peripheral lymphoid cell populations. Surface staining was performed on freshly isolated single cell suspensions from peripheral tissues, namely, Lymph Nodes (B), Blood (C), and Spleen (D). White bars correspond to CHAD, dark bars to DBA/2 mice. The results represent the geometrical mean of 3 mice, and y-error bars represent standard deviation within a group.

FIGURE 3. T cell proliferation assay. Splenocytes from mice immunized i.n. with $5x10^6$ pfu of either VSV AV3 Gag or AV3 Vector control were harvested 7-days post-immunization and stimulated *in vitro* with 15mer individual Gag peptides or peptide pools for a period of 5 days following CFSE labeling. 2.5ug (per $2x10^6$ cells) of ConA was used as a positive control; negative control samples

were cultured in medium alone (NS). **(A)** Antigen-specific proliferation of CD4+ cells following stimulation with a pool of 10 Gag peptides in VSV AV3 Gag immunized group (17.6% CD4+CFSE^{lo} cells) vs. vector control group (4.5%). **(B)** Antigen-specific proliferation of CD8+ cells following stimulation with above Gag pool in VSV AV3 Gag immunized group (32.3% CD8+CFSE^{lo} cells) vs. control group (6.1%). **(C)** Antigen-specific proliferation of CD3+CD4+ cells following stimulation of splenocytes with individual Gag peptides constituting above Gag pool (the vertical y-axis represents the percentage of CD3+CD4+CFSE^{lo} cells). All above results are representative of 3 independent immunization experiments. These data were also reproduced using samples from individual mice in lieu of pooled samples from a group of mice (Fig. S2.2B).

FIGURE 4. HLA DR blocking assay. Splenocytes from mice immunized i.n. with $5x10^6$ pfu of either VSV AV3 or $1x10^8$ pfu of Adenovirus (Ad5) vectors expressing Gag or respective control vectors were harvested 7-days post-immunization and stimulated with Gag pool in the absence or presence of anti-HLA Class II DR antibody (L243) at a concentration of either 1μg or 10μg/mL/$2x10^6$ cells. A second aliquot of the same concentration was added to wells 48hrs following stimulation to block *de novo* surface expression of DR molecules. The percentage of CD3+CD4+ CFSE^{lo} cells was analyzed by flow cytometry as described in Materials & Methods and the blocking effect of anti-DR antibody on proliferation is represented hereby in bar graphs (4A) and dot plots (4B & 4C).

FIGURE 5. Direct *ex-vivo* HLA A2 tetramer staining. Splenocytes from mice immunized i.n. with $5x10^6$ pfu of either VSV AV3 Gag and respective control vector, or from mice that received endotoxin-free PBS solution, were harvested 7-days post-immunization and stained with HLA-A2 Tetramers bearing either HIV Gag p17 or p15 peptides and control CMV peptide, in an direct ex-vivo assay.
(*) values below background levels.

References

1. Taneja,V. and C.S.David. 1998. HLA Transgenic Mice as Humanized Mouse Models of Disease and Immunity. *J.Clin.Invest.* 101:921-926.

2. Taneja,V. and C.S.David. 1999. HLA class II transgenic mice as models of human diseases. *Immunol.Rev.* 169:67-79.

3. Boyton,R.J. and D.M.Altmann. 2002. Transgenic models of autoimmune disease. *Clinical and Experimental Immunology* 127:4-11.

4. Gregersen,J.W., S.Holmes, and L.Fugger. 2004. Humanized animal models for autoimmune diseases. *Tissue Antigens* 63:383-394.

5. Vitiello,A., D.Marchesini, J.Furze, L.A.Sherman, and R.W.Chesnut. 1991. Analysis of the HLA-restricted influenza-specific cytotoxic T lymphocyte response in transgenic mice carrying a chimeric human-mouse class I major histocompatibility complex. *The Journal of Experimental Medicine* 173:1007-1015.

6. Ureta-Vidal,A., H.Firat, B.Perarnau, and F.A.Lemonnier. 1999. Phenotypical and Functional Characterization of the CD8+ T Cell Repertoire of HLA-A2.1 Transgenic, H-2K^{b}D^{b} Double Knockout Mice. *The Journal of Immunology* 163:2555-2560.

7. Cheuk,E., C.D'Souza, N.Hu, Y.Liu, H.Lang, and J.W.Chamberlain. 2002. Human MHC Class I Transgenic Mice Deficient for H2 Class I Expression Facilitate Identification and Characterization of New HLA Class I-Restricted Viral T Cell Epitopes. *The Journal of Immunology* 169:5571-5580.

8. Woods,A., H.Y.Chen, M.E.Trumbauer, A.Sirotina, R.Cummings, and D.M.Zaller. 1994. Human major histocompatibility complex class II-

restricted T cell responses in transgenic mice. *The Journal of Experimental Medicine* 180:173-181.

9. Yamamoto,K., Y.Fukui, Y.Esaki, T.Inamitsu, T.Sudo, K.Yamane, N.Kamikawaji, A.Kimura, and T.Sasazuki. 1994. Functional interaction between human histocompatibility leukocyte antigen (HLA) class II and mouse CD4 molecule in antigen recognition by T cells in HLA-DR and DQ transgenic mice. *The Journal of Experimental Medicine* 180:165-171.

10. Rosloniec,E.F., D.D.Brand, L.K.Myers, K.B.Whittington, M.Gumanovskaya, D.M.Zaller, A.Woods, D.M.Altmann, J.M.Stuart, and A.H.Kang. 1997. An HLA-DR1 transgene confers susceptibility to collagen-induced arthritis elicited with human type II collagen. *The Journal of Experimental Medicine* 185:1113-1122.

11. Bevan,M.J. 2004. Helping the CD8+ T-Cell Response. *Nat Rev Immunol* 4:595-602.

12. BenMohamed,L., R.Krishnan, J.Longmate, C.Auge, L.Low, J.Primus, and D.J.Diamond. 2000. Induction of CTL response by a minimal epitope vaccine in HLA A*0201/DR1 transgenic mice: dependence on HLA class II restricted TH response. *Human Immunology* 61:764-779.

13. Stojdl,D.F., B.D.Lichty, B.R.tenOever, J.M.Paterson, A.T.Power, S.Knowles, R.Marius, J.Reynard, L.Poliquin, H.Atkins, et al. 2003. VSV strains with defects in their ability to shutdown innate immunity are potent systemic anti-cancer agents. *Cancer Cell* 4:263-275.

14. Altman,J.D., P.A.H.Moss, P.J.R.Goulder, D.H.Barouch, M.G.McHeyzer-Williams, J.I.Bell, A.J.McMichael, and M.M.Davis. 1996. Phenotypic analysis of antigen-specific T lymphocytes. *Science* 274:94-96.

15. Beuneu,H., Z.Garcia, and P.Bousso. 2006. Cutting Edge: Cognate CD4 Help Promotes Recruitment of Antigen-Specific CD8 T Cells around Dendritic Cells. *The Journal of Immunology* 177:1406-1410.

16. Choi,E.M., M.Palmowski, J.Chen, and V.Cerundolo. 2002. The use of chimeric A2Kb tetramers to monitor HLA A2 immune responses in HLA A2 transgenic mice. *Journal of Immunological Methods* 268:35-41.

17. Younes, S.A., Yassine-Diab,B., Dumont,A.R., Boulassel,M.R., Grossman,Z., Routy,J.P., & Sekaly,R.P. 2003. HIV-1 viremia prevents the establishment of Interleukin 2-producing HIV-specific Memory CD4+ T Cells Endowed with Proliferative Capacity. *The Journal of Experimental Medicine* 198: 1909-1922.

18. Cheuk E, D'Souza C, Hu N, Liu Y, Lang H, Chamberlain JW. 2002. Human MHC class I transgenic mice deficient for H2 class I expression facilitate identification and characterization of new HLA class I-restricted viral T cell epitopes. *J Immunol.* 169(10):5571-5580.

19. Dunbar PR, Ogg GS. 2002. Oligomeric MHC molecules and their homologues: state of the art. *J Immunol Methods.* 268(1):3-7.

20. Irwin,M.J., Heath,W.R., & Sherman,L.A. 1989. Species-restricted interactions between CD8 and the alpha 3 domain of class I influence the magnitude of the xenogeneic response. *The Journal of Experimental Medicine* 170: 1091-1101.

21. Pajot A., Michel ML, Fazilleau,N., Pancre V., Auriault C., Ojcius,D.M., Lemonnier F.A., & Lone,Y.-C. 2004. A mouse model of human adaptive immune functions: HLA-A2.1-/HLA-DR1-transgenic H-2 class I-/class II-knockout mice. *Eur. J. Immunol.* 34: 3060-3069.

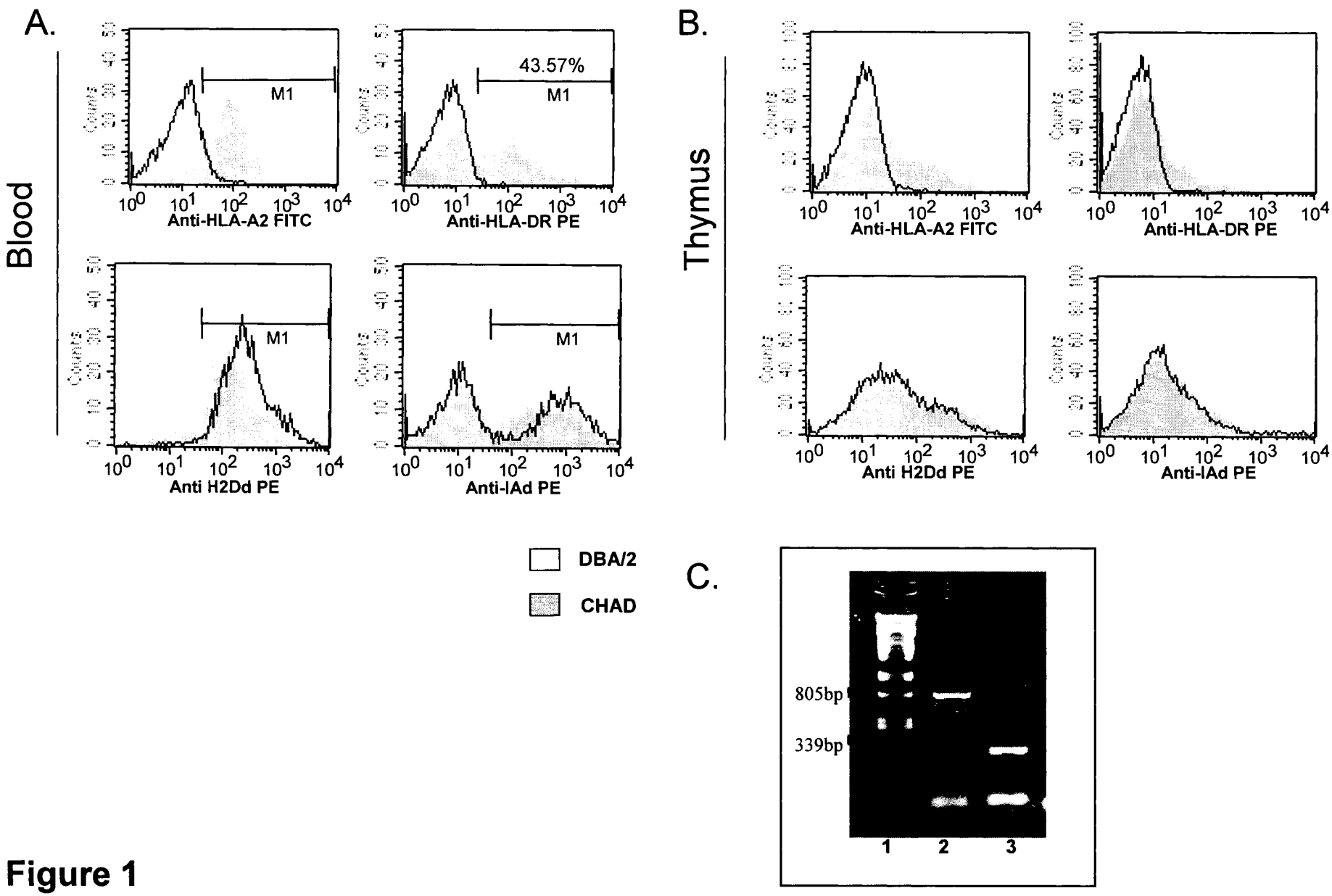
A.
Blood
M1
43.57%
Anti-HLA-A2 FITC
Anti-HLA-DR PE
Anti H2Dd PE
Anti-IAd PE
B.
Thymus
C.
805bp
339bp
1
2
3
DBA/2
CHAD

Figure 1

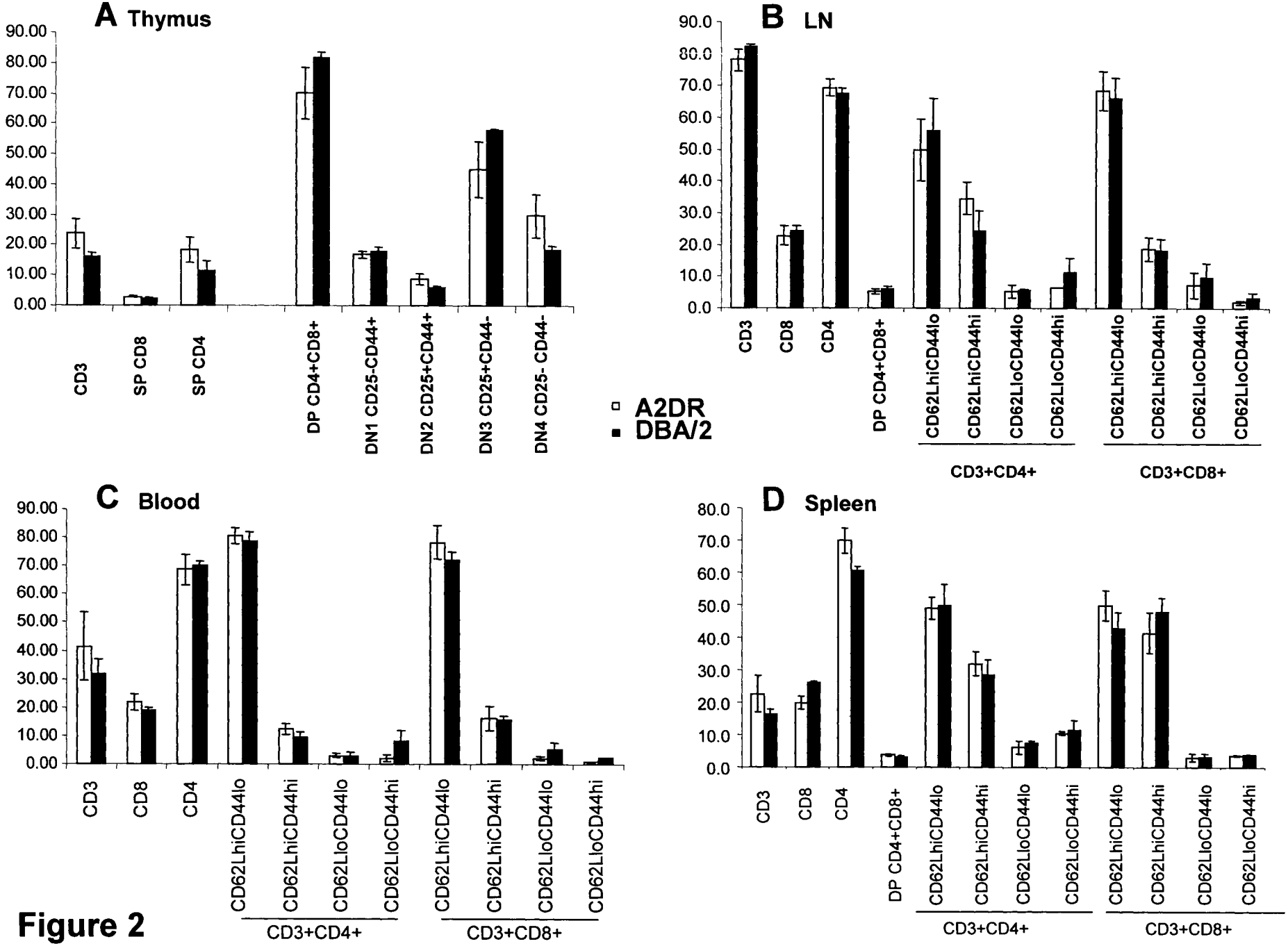
A Thymus
90.00
80.00
70.00
60.00
50.00
40.00
30.00
20.00
10.00
0.00
CD3
SP CD8
SP CD4
DP CD4+CD8+
DN1 CD25-CD44+
DN2 CD25+CD44+
DN3 CD25+CD44-
DN4 CD25- CD44-
B LN
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
CD3
CD8
CD4
DP CD4+CD8+
CD62LhiCD44lo
CD62LhiCD44hi
CD62LloCD44lo
CD62LloCD44hi
CD3+CD4+
CD3+CD8+
A2DR
DBA/2
C Blood
D Spleen

Figure 2

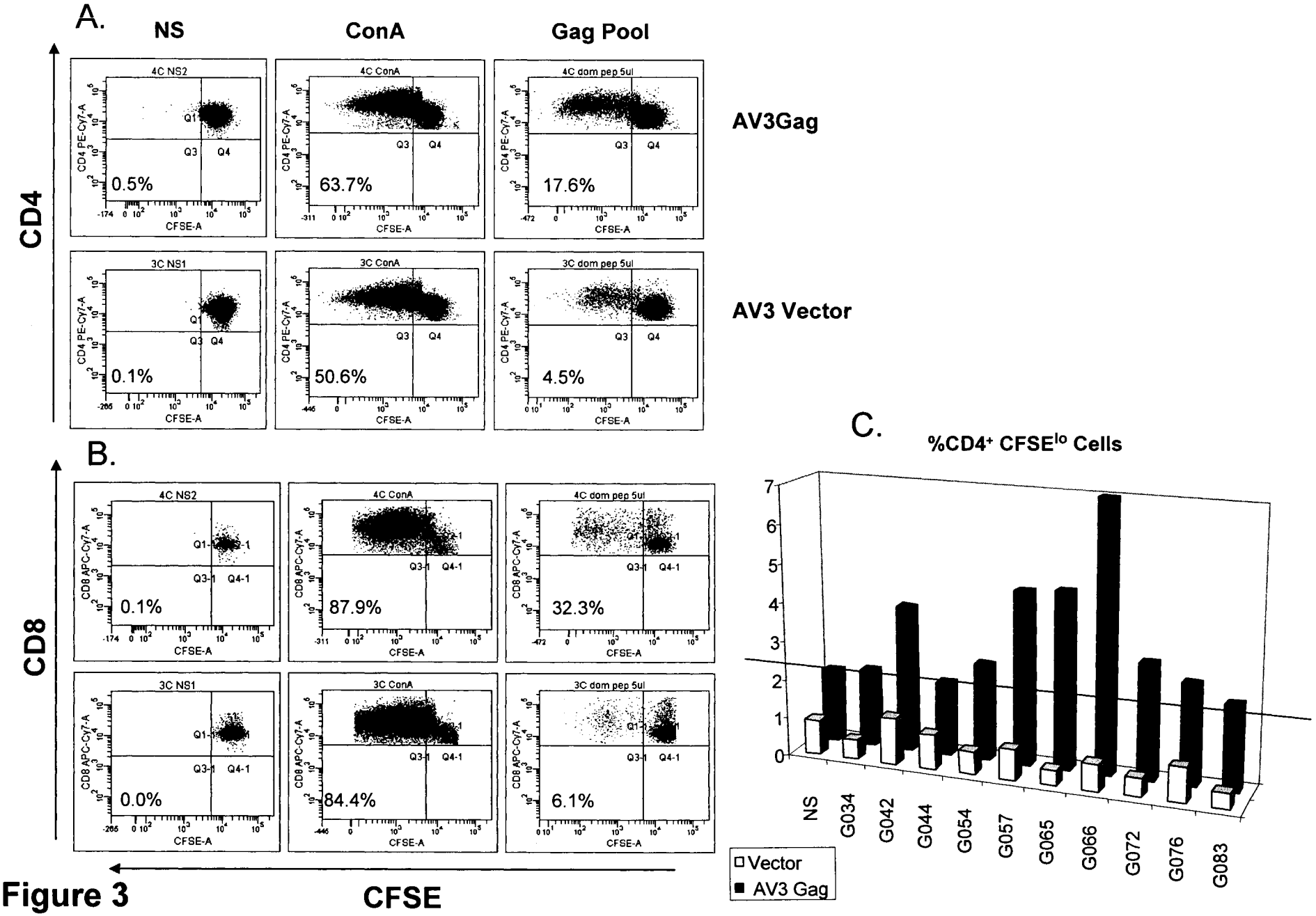
A.
NS
ConA
Gag Pool
4C NS2
4C ConA
4C dom pep 5ul
3C NS1
3C ConA
3C dom pep 5ul
CD4 PE-Cy7-A
CFSE-A
0.5%
63.7%
17.6%
0.1%
50.6%
4.5%
AV3Gag
AV3 Vector
CD4
B.
CD8
CD8 APC-Cy7-A
0.1%
87.9%
32.3%
0.0%
84.4%
6.1%
CFSE
C.
%CD4+ CFSElo Cells
NS
G034
G042
G044
G054
G057
G065
G066
G072
G076
G083
Vector
AV3 Gag

Figure 3

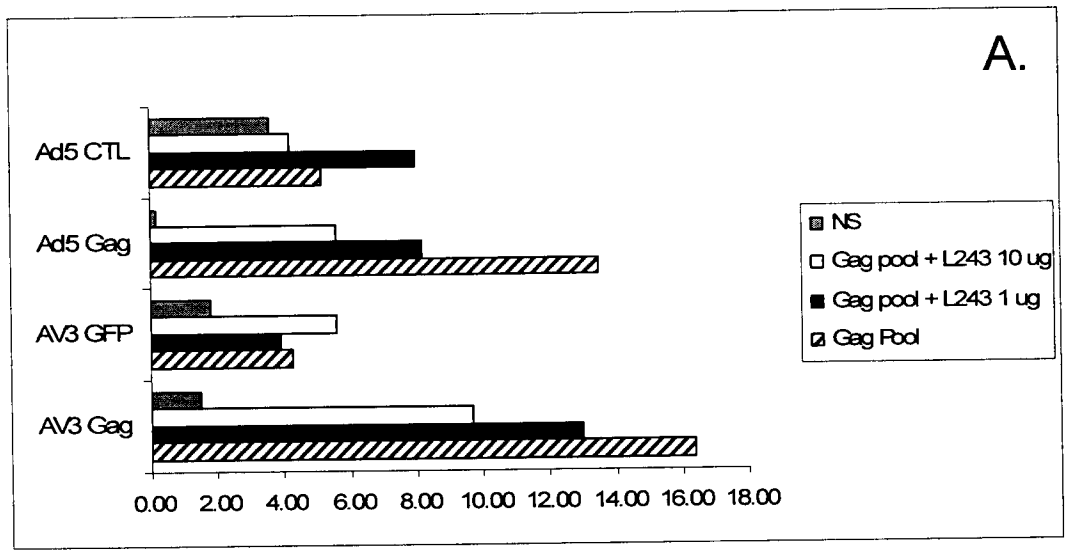
A.
Ad5 CTL
Ad5 Gag
AV3 GFP
AV3 Gag
0.00
2.00
4.00
6.00
8.00
10.00
12.00
14.00
16.00
18.00
NS
Gag pool + L243 10 ug
Gag pool + L243 1 ug
Gag Pool

Figure 4 A

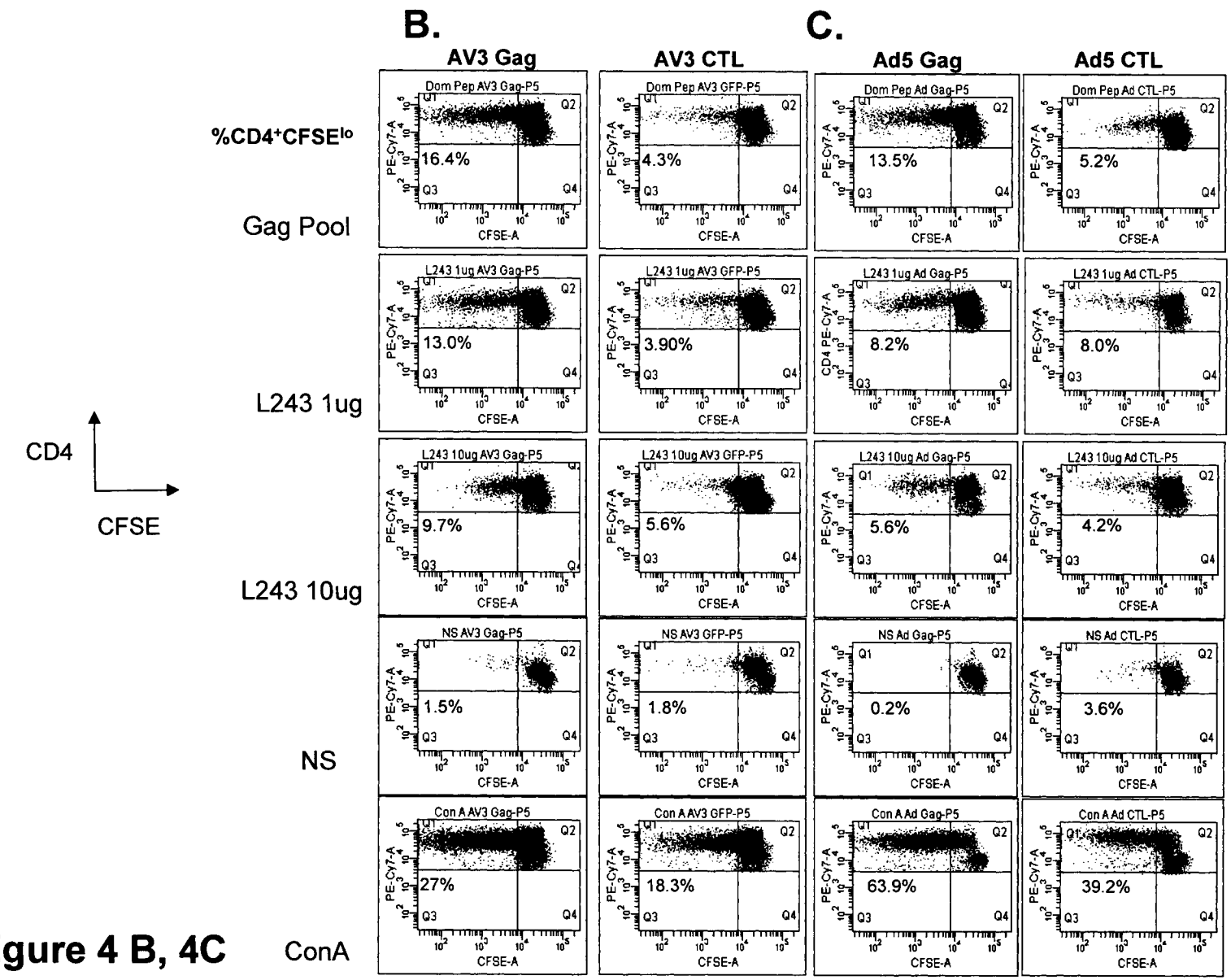
B.
C.
AV3 Gag
AV3 CTL
Ad5 Gag
Ad5 CTL
%CD4+CFSElo
Gag Pool
L243 1ug
L243 10ug
NS
ConA
CD4
CFSE
Dom Pep AV3 Gag-P5
Dom Pep AV3 GFP-P5
Dom Pep Ad Gag-P5
Dom Pep Ad CTL-P5
16.4%
4.3%
13.5%
5.2%
L243 1ug AV3 Gag-P5
L243 1ug AV3 GFP-P5
L243 1ug Ad Gag-P5
L243 1ug Ad CTL-P5
13.0%
3.90%
8.2%
8.0%
L243 10ug AV3 Gag-P5
L243 10ug AV3 GFP-P5
L243 10ug Ad Gag-P5
L243 10ug Ad CTL-P5
9.7%
5.6%
5.6%
4.2%
NS AV3 Gag-P5
NS AV3 GFP-P5
NS Ad Gag-P5
NS Ad CTL-P5
1.5%
1.8%
0.2%
3.6%
Con A AV3 Gag-P5
Con A AV3 GFP-P5
Con A Ad Gag-P5
Con A Ad CTL-P5
27%
18.3%
63.9%
39.2%
PE-Cy7-A
CFSE-A
Q1
Q2
Q3
Q4

Figure 4 B, 4C

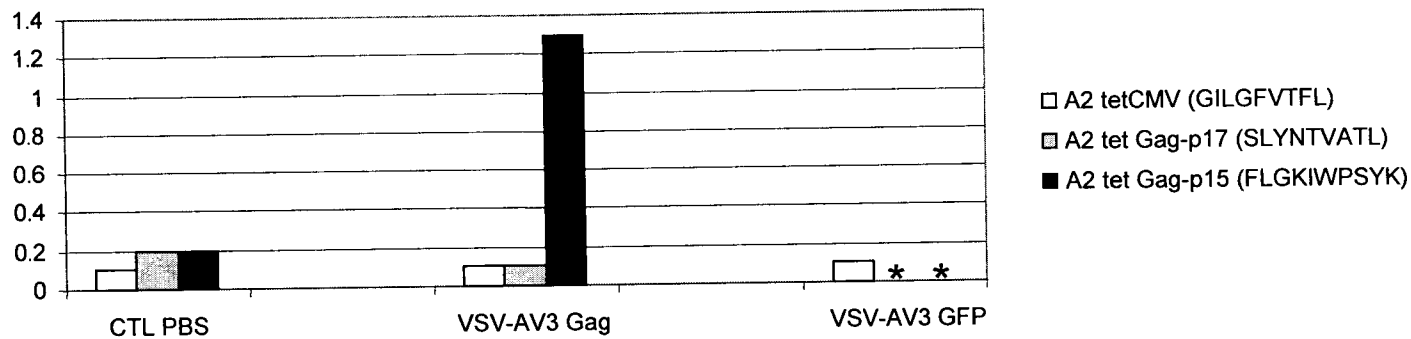
1.4
1.2
1
0.8
0.6
0.4
0.2
0
CTL PBS
VSV-AV3 Gag
VSV-AV3 GFP
* *
A2 tetCMV (GILGFVTFL)
A2 tet Gag-p17 (SLYNTVATL)
A2 tet Gag-p15 (FLGKIWPSYK)

Figure 5

Table 1. Amino Acid sequence of HIV-1 Consensus A Gag 15mer peptides used in stimulation assays.

Peptide	Sequence
G034	PIVQNAQGQMVHQSL
G042	SPEVIPMFSALSEGA
G044	SALSEGATPQDLNMM
G054	DRLHPVHAGPIPPGQ
G057	PGQMREPRGSDIAGT
G065	PVGDIYKRWIILGLN
G066	IYKRWIILGLNKIVR
G072	IKQGPKEPFRDYVDR
G076	FKTLRAEQATQDVKN
G083	DCKSILRALGAGATL

CHAPTER 2 SUPPLEMENTARY DATA

Table S 2.1: T Cell Maturation in the Thymus, data from individual mice.

	A2DR-A	A2DR-B	A2DR-C	A2DR Mean	*SD*	DBA/2-D	DBA/2-E	DBA/2-F	DBA/2 Mean	*SD*
CD3	18.80	28.20	24.10	**23.70**	*4.71*	16.40	17.10	14.50	**16.00**	*1.35*
SP CD8	2.50	3.00	2.90	**2.80**	*0.26*	1.70	1.90	2.80	**2.13**	*0.59*
SP CD4	14.70	23.00	17.40	**18.37**	*4.23*	10.20	15.30	8.50	**11.33**	*3.54*
DN % of total Thy	2.70	3.20	2.50	**2.80**	*0.36*	2.20	2.40	2.10	**2.23**	*0.15*
DP CD4+CD8+	79.50	63.30	67.40	**70.07**	*8.42*	81.30	79.60	83.70	**81.53**	*2.06*
DN1 CD25-CD44+	15.90	16.50	17.90	**16.77**	*1.03*	19.40	17.90	17.00	**18.10**	*1.21*
DN2 CD25+CD44+	9.90	6.60	9.30	**8.60**	*1.76*	6.60	5.50	5.50	**5.87**	*0.64*
DN3 CD25+CD44-	45.10	54.10	35.60	**44.93**	*9.25*	57.10	57.50	58.50	**57.70**	*0.72*
DN4 CD25-CD44-	29.10	22.80	37.20	**29.70**	*7.22*	16.90	19.10	19.00	**18.33**	*1.24*

Table S 2.2: T Cell Subsets in Peripheral Lymph Nodes, data from individual mice.

	A2DR-A	A2DR-B	A2DR-C	A2DR Mean	*SD*	DBA/2-D	DBA/2-E	DBA/2-F	DBA/2 Mean	*SD*
CD3	76.60	75.70	82.00	**78.10**	*3.41*	83.00	82.40	80.90	**82.10**	*1.08*
CD3+CD8+	23.90	25.10	19.50	**22.83**	*2.95*	22.40	25.10	25.30	**24.27**	*1.62*
CD3+CD4+	69.10	67.20	72.20	**69.50**	*2.52*	69.40	67.50	66.40	**67.77**	*1.52*
CD4+CD8+	4.40	5.10	6.30	**5.27**	*0.96*	6.60	5.60	6.60	**6.27**	*0.58*
CD4 CD62LhiCD44lo	52.10	58.20	39.30	**49.87**	*9.65*	66.90	46.60	53.80	**55.77**	*10.29*
CD4 CD62LhiCD44hi	35.90	29.10	38.90	**34.63**	*5.02*	17.10	30.10	25.70	**24.30**	*6.61*
CD4 CD62LloCD44lo	3.80	4.30	7.40	**5.17**	*1.95*	4.90	5.40	6.30	**5.53**	*0.71*
CD4 CD62LloCD44hi	6.60	6.60	6.30	**6.50**	*0.17*	9.20	16.70	8.40	**11.43**	*4.58*
CD8 CD62LhiCD44lo	68.30	74.70	62.70	**68.57**	*6.00*	72.80	59.70	66.00	**66.17**	*6.55*
CD8 CD62LhiCD44hi	22.90	17.00	16.20	**18.70**	*3.66*	18.00	22.10	14.90	**18.33**	*3.61*
CD8 CD62LloCD44lo	4.70	4.90	12.00	**7.20**	*4.16*	5.20	10.40	13.80	**9.80**	*4.33*
CD8 CD62LloCD44hi	2.00	1.40	2.50	**1.97**	*0.55*	1.90	4.80	3.30	**3.33**	*1.45*

Table S 2.3: T Cell Subsets in Peripheral Blood, data from individual mice.

	A2DR-A	A2DR-B	A2DR-C	A2DR Mean	*SD*	DBA/2-D	DBA/2-E	DBA/2-F	DBA/2 Mean	*SD*
CD3	45.60	27.60	50.50	**41.23**	*12.06*	35.70	33.50	25.80	**31.67**	*5.20*
CD3+CD8+	21.30	24.90	19.20	**21.80**	*2.88*	19.80	19.10	17.90	**18.93**	*0.96*
CD3+CD4+	72.40	62.20	71.00	**68.53**	*5.53*	69.70	71.70	69.60	**70.33**	*1.18*
CD4 CD62LhiCD44lo	80.20	77.70	83.40	**80.43**	*2.86*	77.00	75.80	82.50	**78.43**	*3.57*
CD4 CD62LhiCD44hi	13.30	13.40	10.30	**12.33**	*1.76*	8.90	11.60	7.30	**9.27**	*2.17*
CD4 CD62LloCD44lo	2.30	3.40	3.40	**3.03**	*0.64*	1.30	2.90	4.20	**2.80**	*1.45*
CD4 CD62LloCD44hi	1.70	3.20	1.10	**2.00**	*1.08*	11.60	8.10	4.50	**8.07**	*3.55*
CD8 CD62LhiCD44lo	73.20	77.10	84.60	**78.30**	*5.79*	74.90	72.10	69.30	**72.10**	*2.80*
CD8 CD44hiCD62Lhi	19.30	18.10	11.30	**16.23**	*4.31*	13.80	15.60	17.20	**15.53**	*1.70*
CD8 CD44loCD62Llo	2.90	1.60	1.80	**2.10**	*0.70*	6.40	6.60	3.00	**5.33**	*2.02*
CD8 CD44hiCD62Llo	1.10	0.70	0.40	**0.73**	*0.35*	2.20	1.90	2.50	**2.20**	*0.30*

Table S 2.4: T Cell Subsets in Spleen, data from individual mice.

	A2DR-A	A2DR-B	A2DR-C	A2DR Mean	*SD*	DBA/2-D	DBA/2-E	DBA/2-F	DBA/2 Mean	*SD*
CD3	29.10	19.00	19.60	**22.57**	*5.67*	15.40	15.90	18.00	**16.43**	*1.38*
CD3+CD8+	18.40	22.20	18.90	**19.83**	*2.06*	26.20	25.80	26.60	**26.20**	*0.40*
CD3+CD4+	72.80	65.50	71.60	**69.97**	*3.91*	61.60	61.10	59.20	**60.63**	*1.27*
CD4+CD8+	3.40	4.10	3.80	**3.77**	*0.35*	3.40	3.50	2.70	**3.20**	*0.44*
CD4 CD62LhiCD44lo	52.50	45.60	49.10	**49.07**	*3.45*	43.20	50.40	56.20	**49.93**	*6.51*
CD4 CD62LhiCD44hi	31.00	36.00	28.80	**31.93**	*3.69*	33.50	27.50	24.50	**28.50**	*4.58*
CD4 CD62LloCD44lo	4.20	5.80	8.20	**6.07**	*2.01*	8.20	7.00	7.00	**7.40**	*0.69*
CD4 CD62LloCD44hi	10.10	10.30	11.10	**10.50**	*0.53*	12.20	14.00	8.30	**11.50**	*2.91*
CD8 CD62LhiCD44lo	48.80	45.90	55.10	**49.93**	*4.70*	45.10	37.50	46.30	**42.97**	*4.77*
CD8 CD62LhiCD44hi	43.90	46.20	34.30	**41.47**	*6.31*	44.60	52.80	46.30	**47.90**	*4.33*
CD8 CD62LloCD44lo	2.20	2.50	4.40	**3.03**	*1.19*	4.40	2.90	2.00	**3.10**	*1.21*
CD8 CD62LloCD44hi	3.40	3.70	3.90	**3.67**	*0.25*	3.90	4.00	3.80	**3.90**	*0.10*

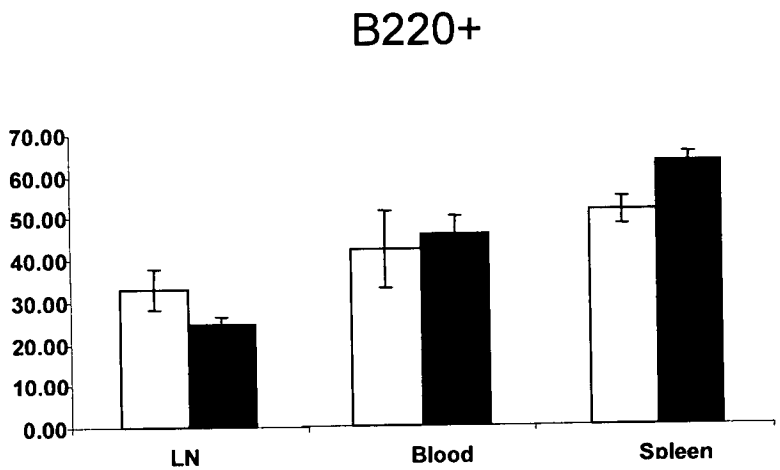

Figure S 2.1: B lymphocyte (B220+) population in lymph nodes (LN), blood, and spleen of A2DR (clear bars) vs. DBA/2 (black bars) mice.

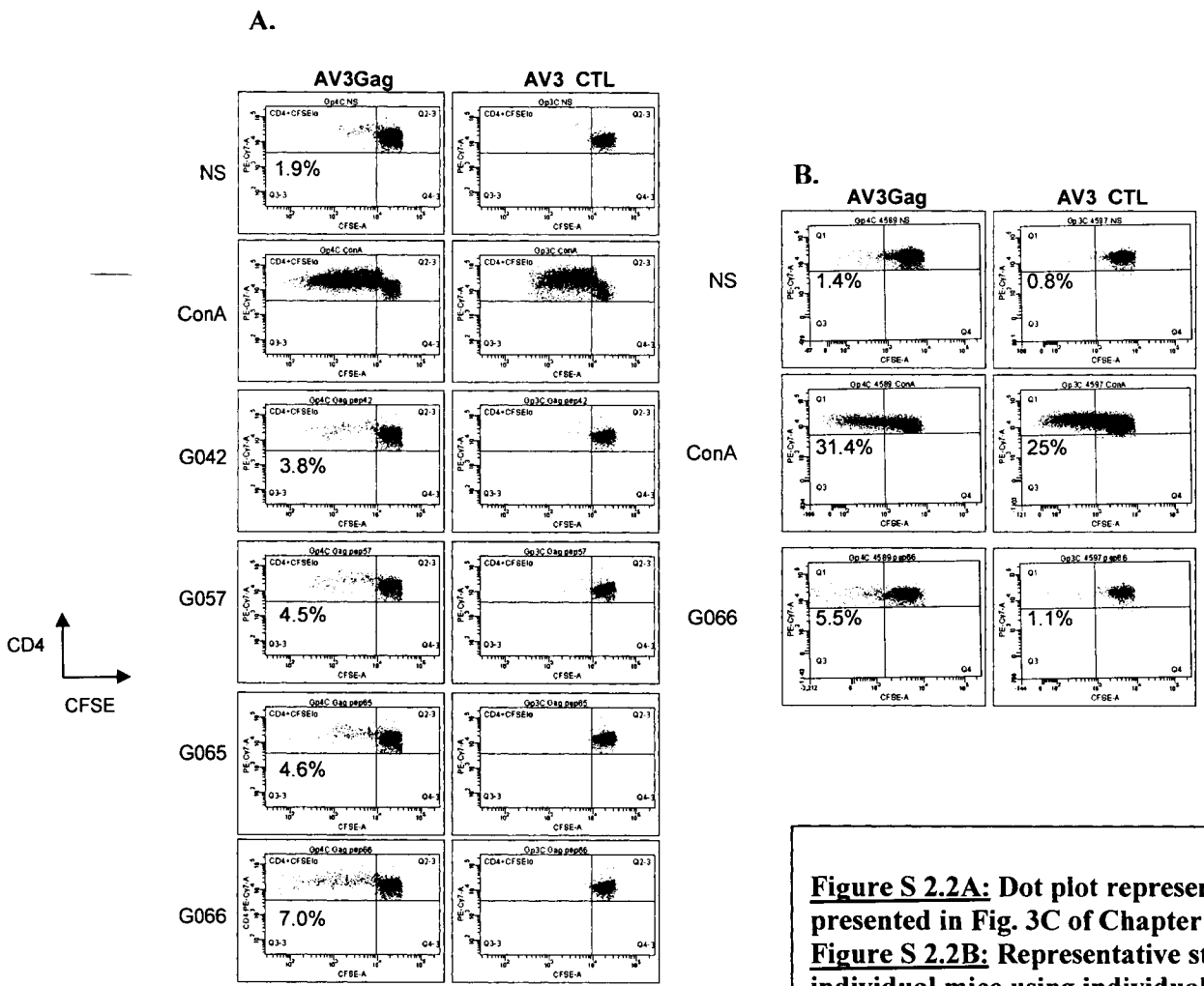

Figure S 2.2A: Dot plot representation of stimulations presented in Fig. 3C of Chapter 2.
Figure S 2.2B: Representative stimulation of splenocytes from individual mice using individual peptide.

3 Activation of human CD4+ T cells by dodecameric human MHC Class II chimeras and their implication in vaccine development and immune therapy

In an era where most therapeutic modalities and immunization strategies have become highly disease-specific, the concurrent development of targeted and sensitive immune-monitoring tools, and their subsequent standardization becomes imperative. Soluble peptide-MHC oligomers have been used in our laboratory and elsewhere for detection and tracking of antigen-specific $CD8^+$ and $CD4^+$ T lymphocytes in a host of applications. Despite the increased sensitivity of these reagents, it has been however observed that low-affinity TCR-bearing T lymphocytes are often undetected and that staining intensity reflects the overall avidity of these oligomeric molecules towards their respective TCR. In an attempt to increase the overall avidity of these reagents to scant numbers of CD4+ T cells, we undertook the design of multimeric MHC Class II molecules using murine immunoglobulin M lacking the joining (J) chain as a molecular scaffold. These novel reagents are targeted both towards the detection and *ex-vivo* expansion of antigen-specific CD4+ T lymphocytes.

ACTIVATION OF HUMAN CD4+ T CELLS BY DODECAMERIC HUMAN MHC CLASS II CHIMERAS AND THEIR IMPLICATION IN VACCINE DEVELOPMENT AND IMMUNE THERAPY

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Key words: Multimer; MHC Class II; Activation; CD4+ T cells; IgM.

SUMMARY

In the past decade, the qualitative and quantitative assessment of antigen-specific cellular immune responses has proved to be of utmost importance in understanding the clinical outcome in several diseases, specifically in tumors, acute and chronic viral infections, such as AIDS and hepatitis, and in the evaluation of immunization strategies. Many of the commonly used assays, starting with the conventional chromium release to ELISPOT or intracellular cytokine staining, are somehow biased relying mainly on the effector function of the cells to be detected. More recently, oligomeric tools (such as MHC Class I and Class II Tetramers) have been successfully developed in our laboratory and elsewhere and attested to be valuable tools in assessing antigen-specific cellular immune responses. These versatile tools are sensitive and specific, allowing direct *ex-vivo* analysis of T cells without the need of an *in vitro* amplification in most cases, and without being limited by the functionality of the cell, thus allowing visualization of all specific cells, whether precursors or effectors, functional or anergized. These reagents can be further combined with functional or phenotypic assays. Seeking to augment the overall avidity of these tools to scant numbers of antigen-specific T-lymphocytes, and thus improve the overall sensitivity of the reagents, we engaged in the development of novel MHC ClassII-chimeras of higher oligomerizing capacities, namely the dodecavalent MHC Class II DR1*IgM multimer. Here we describe the production of these reagents using an insect expression system. We then demonstrate the capacity of these molecules to activate Jurkat CD4+ T cell line in an antigen-specific and HLA-DR1 restricted manner.

INTRODUCTION

The recognition of antigenic peptides bound to MHC molecules (pMHC) at the surface of antigen presenting cells (APCs) by T lymphocytes bearing specific T-cell receptors (TCRs) is a central event in the development of the adaptive immune response. After the initial priming, the pathogen-specific T cell population undergoes a series of characteristic processes including proliferation, up-/down-regulation of adhesion and co-stimulatory molecules as well as acquisition of effector functions such as cytotoxicity and/or cytokine production.

Several reports have shown that some features of $CD4^+$ T cell activation, including activation marker (CD25 and CD69) up-regulation and TCR internalization, can be triggered by soluble reagents that multivalently engage TCRs, including anti-TCR antibodies[1] and oligomeric pMHC complexes [2,3,4]. For soluble MHC class II oligomers, it appears that dimers are sufficient to initiate signalling in $CD4^+$ T cells but that higher valency MHC oligomers, such as trimers and tetramers, are more potent than dimers at inducing CD4 T-cell activation [2,4]. These data suggest that higher order oligomeric forms of soluble pMHC molecules might prove useful at inducing potent antigen-specific $CD4^+$ T cell activation in the context of immunotherapeutic strategies.

$CD4^+$ T cells are at the center of immune responses. After being activated, naive $CD4^+$ T cells differentiate into functional subsets called T helper type 1 (T_H1) and T helper type 2 (T_H2) cells, based on their production of cytokine interferon (IFN)-γ and interleukin (IL)-4, respectively [5]. T_H1 cells secrete pro-inflammatory cytokines and are essential for protection against a variety of intracellular pathogens and viruses, whereas T_H2 cells promote the generation of high affinity antibody responses and can be protective against extracellular pathogens. In addition, $CD4^+$ T cells are an essential component of effective $CD8^+$ T cell responses, being involved in both the development and maintenance of memory $CD8^+$ T cells [6-10]. Therefore, the elaboration of methods to activate and expand

antigen-specific $CD4^+$ T cells *ex vivo* is crucial for the development of effective T cell-based immunotherapies.

Current immunotherapeutic strategies against various diseases, like cancer, involve the adoptive transfer of antigen-specific T cells (mostly $CD8^+$) that have been expanded *ex vivo* [11]. However, given the recognized role of $CD4^+$ T cells in the generation of optimal immune responses against viruses and tumours, strategies to activate and expand $CD4^+$ T cells in an antigen-specific manner are much needed, and might significantly improve the efficiency of T cell-based immunotherapies. The approaches currently used to expand these T cells include culturing T cells in the presence of allogeneic feeder cells, EBV-transformed B cells or autologous dendritic cells in the presence of antigens (in the form of whole protein or peptide). However, these techniques are time-consuming, necessitate the culture of allogeneic or virally-infected cells for antigen presentation and, especially in the case of dendritic cells, require a large number of allogeneic cells. Moreover, the use of co-culture systems might greatly limit the reproducibility and consistency of T cell expansion over time and over multiple donors.

In this paper, we describe the development of novel human MHC Class II chimeras of higher oligomerizing capacities: the dodecavalent MHC Class II DR1*IgM multimers. We demonstrate that these chimeric molecules are conformationally active when expressed in Drosophila S2 cells and that they have the capacity to potently stimulate human $CD4^+$ T cells in an antigen-specific manner.

MATERIALS AND METHODS

Generation of expression vectors

The constant regions of murine IgM (mIgM) heavy (μ) and light (κ) chains were amplified from cDNA library by PCR using forward 5′ CCG CTC GAG GCT AGC GAG AGT CAG TCC TTC CCA AAT 3′ (μ Ct), 5′ CCG GAA TTC GCT AGC GCT GAT GCT GCA CCA ACT GTA 3′ (κ Ct) and reverse 5′ CCC AAG CTT TGG TCA ATA GCA GGT GCC GCC TGT 3′ (μ Ct), 5′ CCC AAG CTT GGA CCT TTG TCT CTA ACA CTC ATT CCT GTT GAA 3′ (κ Ct) primers, respectively. The amplicons were inserted into the intermediate pGEM-T (Promega, Madison, WI) and pBS KS(+) (Invitrogen) vectors to generate subclones. The C terminus alpha-linker-μ chain and the C terminus beta-linker-κ chain segments were then cloned into the custom made, methallothionine inducible (pMT), double promoter insect cell expression pCV vector. This vector contains the extracellular portion of the DRA1*0101 (DRα) and DRB1*0101 (DRβ) chains, with their respective leader sequences, as well as a constitutively expressed eGFP gene. The heavy chain (μ) of mIgM was fused in frame at the C-terminus of the HLA-DRα gene via a GGGSAS linker. Similarly, the light chain (κ) of mIgM was fused in frame at the C-terminus of the HLA-DRβ gene via a GGGSAS linker. In the parental vector pCVDR1-$HA_{306-318}$ the N-terminus of the HLA-DRβ gene is in turn covalently linked to the hemagglutinin (HA) peptide ($HA_{306-318}$:PKYVKQNTLKLAT)-encoding sequence via a flexible linker (GGGGSLVGGGSGGGGS). The generated vector was designated pCV-MHC-DR1/$HA_{306-318}$ IgM and was later modified to generate pCV-MHC-DR1/p24-5 IgM expression vector by peptide encoding sequence substitution with Gag peptide p24-5 (WIILGLNKIVRMYS) of HIV-1. The sequences of fusion genes were confirmed by DNA sequencing.

Transfection and selection of S2 cells

*Drosophila melanogaster S*2 cells were seeded ($5x10^6$/mL) overnight in $25cm^2$ vented cell culture flasks in 4ml of S&S medium (Shields and Sang M3 Insect Medium, Sigma-Aldrich) supplemented with 1% FCS and 1% antibiotic-antimycotic reagent (Gibco). The transfection mixture consisted of 5μg of expression vector and 0.5μg of the selection vector pCOhygro in a final volume of 100μl of S&S medium. Subsequently, 100μl of S&S containing 20μl of Lipofectin reagent (Invitrogen) was added to the transfection mixture and incubated for 15-20 minutes at room temperature. 1.8ml of S&S medium was then added and the final volume of 2ml transfection mixture was carefully overlaid on the S2 cells from which the overnight culture media was aspirated. No FCS was added at this stage. After an overnight incubation at 26°C, 8ml of fresh S&S containing 10% FCS was added to the flasks. To establish stable expression, cells were treated with 315U of hygromycin B (Calbiochem, San Diego, CA) 48 hours following transfection. Culture medium was subsequently replaced once a week until stable clones were established. The cells were maintained at 26°C in S&S, 10% FCS medium containing hygromycin B for 1–2 weeks until clones could be spotted and expanded in the flasks. Constitutive eGFP expression was monitored by flow cytometry from this point onward. The cells were then adapted to and transferred into a serum free Baculo Gold MAX XP medium (BD Biosciences) for expansion and heavy metal induction of protein expression.

Expression and purification of HLA-DR*0101 IgM multimers

Stable transfectants were expanded in Baculo Gold MAX XP containing 3.15U of hygromycin B (Calbiochem) per ml, up to a maximum volume of 400mL in 1L Corning sterile bottles at 26°C, on a rotary shaker at 140 rpm. Once a density of 2.5×10^7 cells/ml was reached, cells were split at a ratio of 1:2 and induced with 1 mM $CuSO_4$ for 4 days. Cells and debris were removed by centrifugation at 4°C, 3500rpm for 20 minutes in conical bottles. Supernatants were passed through 0.8μm followed by 0.2μm low protein binding filter (Millipore, Billerica, MA). The clarified medium was passed through a monoclonal L243 anti-HLA-DR (BD

Biosciences) or rabbit anti-IgM heavy chain (Jackson ImmunoResearch Laboratories) affinity column, at a flow rate of 1ml/min at 4°C. The column was then washed with at least 10 bed volumes of phosphate-buffered saline (PBS), pH 7.4. The HLA-IgM multimer was eluted with 0.1M Glycine HCl, 0.15M NaCl elution buffer (pH 2.5), and immediately neutralized with 1M Tris-HCl buffer, pH 7.5. Fractions containing proteins were determined using the Bio-Rad Protein Detection Reagent (Bio-Rad). The fractions with higher density were pooled, buffer-exchanged with 20mM HEPES, 0.5M NaCl buffer (pH 8.1), and concentrated till 1–2 mg/ml by centrifugation through a 30-kDa molecular weight cutoff Centricon Plus-20 device (Millipore, Bedford, MA).

Western Blot analysis

Following induction, 10ml of culture supernatant from each transfection was immunoprecipitated with 10μl of LB243 monoclonal or rabbit anti-μ chain antibody-conjugated CNBR-activated Sepharose-4B beads (Amersham Biosciences, Piscataway, NJ), by head-to-head rotation overnight at 4°C. The beads were then washed with PBS and resuspended in 30μl of sample buffer containing β-mercaptoethanol. The samples were incubated at 100°C for 2 minutes and pelleted by brief centrifugation. Twenty μl of sample was loaded on a 5-10% Tris-glycine SDS-polyacrylamide gel and separated in running buffer (25 mM Tris, 192 mM Glycine, 0.1% SDS) by electrophoresis. The separated proteins were transferred onto a Hybond-C super nitrocellulose membrane (Amersham Biosciences) and blocked with 5% skim milk overnight at 4°C. The membrane was incubated for 1hr at room temperature in 10ml 5% milk-PBS-0.05% Tween-20 containing anti-μ chain (Jackson ImmunoResearch Laboratories) and anti-κ chain (PharMingen) monoclonal antibodies conjugated to peroxidase, then washed three times with PBS 0.05% (v/v) Tween-20, and once with PBS alone. Bands were revealed and detected using the ECL Plus chemiluminescence reagent (Amersham Biosciences). Expression of HLA-DR1 α and β chains was detected using rabbit polyclonal anti-DRα and anti-DRβ primary antibodies, followed by

addition of goat anti-rabbit peroxidase-conjugated (GARP) secondary antibody (Molecular Probes).

Gel Filtration analysis

A Superose 6 HR10/30 gel filtration column (Amersham Pharmacia) was used to determine the valance of purified MHC-IgM. The sample was passed at a flow rate of 0.2ml/min through an HR-10 cell pre-equilibrated with PBS, connected to a GP-250 Programmer Plus FPLC instrument (Amersham Biosciences).

T cell lines

Immortalized human T lymphoid Jurkat A14 (generously gifted by Dr. Oreste Acuto, Institut Pasteur, Paris, France) and E6.1 (ATCC: TIB-152™) lines were used in the stimulation experiments. A14 cells specifically recognize the HA peptide in the context of DR1 molecules, through expression of HA-specific TCR, in addition they express CD28. E6-1 cells express an irrelevant TCR and CD28. LG-2 (DR1/DR1) B lymphoblastoid cell line (B-EBV) was provided by Larry Stern (Massachussets Institute of Technology) and was used as APC for activation control of JKT A14 cells in the presence of the HA peptide.

T cell activation assays

96-well u-bottom microtiter plates (Falcon) were coated in wells designated for positive control samples with 1μg of anti-human CD3 (OKT3) antibody in 200μl PBS and incubated overnight at 4°C. Jurkat A14 and E6.1 cells were seeded at $1x10^5$/well, in duplicate. Stimulations were performed with 200ng of MHC*IgM multimer per well, for 16hr at 37°C. 2.5μg of soluble CD28 was added per stimulation.

Staining and Flow Cytometry

CD3 Alexa700, TCR FITC, CD25 PE and CD69 APC (BD Biosciences, San Jose, CA) antibodies were used to detect activation status of Jurkat cells. Briefly, following stimulation, cells were transferred to v-bottom 96-well plates, washed

once with PBS-2% FCS, and stained for 30min at 4°C for surface markers with corresponding fluorescent antibodies, followed by 2x wash with 200µl of PBS-2%FCS. Events were acquired on a BD LSR II and analyzed by Flow Jo software.

Antibodies

anti-HLA-DR clone L243 (BD Biosciences, San Jose, CA) and affiniPure rabbit anti-mouse IgM µ chain specific (Jackson ImmunoResearch Laboratories, West Grove, PA) were used in immunoprecipitation reactions and affinity columns. Peroxidase-conjugated rabbit anti-mouse IgM µ chain specific (Jackson ImmunoResearch Laboratories, West Grove, PA), HRP-conjugated rat anti-mouse Ig, κ light chain monoclonal antibody (Pharmingen, San Diego, CA), rabbit polyclonal anti-DRα, rabbit polyclonal anti-DRβ sera (in-house production), and goat anti-rabbit peroxidise-conjugated (GARP) secondary antibody (Molecular Probes, Eugene, OR) were used in Western Blot experiments. CD28 pure, clone L293 (BD Biosciences, San Jose, CA) and purified anti-human CD3 (OKT3) were used in stimulation assays. For control murine IgM (κ chain): purified mouse IgM, κ isotype control (anti-TNP) (Pharmingen, San Diego, CA) and purified mouse IgM CD57 (HNK-1) (Pharmingen, San Diego, CA) were used.

RESULTS

Vector generation and protein expression

To produce dodecameric complexes of the human MHC class II protein (soluble extracellular domain), the latter was covalently linked to a murine immunoglobulin M (IgM) molecular scaffold lacking the variable regions of both the heavy and light chains (Figure 1B). In the absence of the secretory J chain, IgM tends to produce higher levels of hexamers in lieu of its physiologic pentameric structure [12,13] . Using a short GGGSAS linker, the heavy chain of mIgM was fused in frame at the C-terminus of the MHC DR1 α gene, while the

light chain was fused in frame at the C-terminus of the MHC DR1 β gene (Figure 1). The N-terminus of the HLA-DRβ gene was covalently linked to the hemagglutinin (HA) peptide ($HA_{306-318}$: PKYVKQNTLKLAT) encoding sequence via a flexible linker (GGGGSLVGGGSGGGGS). The pCV-MHC-DR1/$HA_{306-318}$ IgM was later modified to generate pCV-MHC-DR1/p24-5 IgM expression vector by substituting the HA peptide encoding sequence with that of Gag p24-5 peptide from HIV-1.

To verify whether the insect expression system supports production of MHCII-IgM multimers, stable transfectants of Drosophila S2 cells were generated under Hygromycin B selection. The constitutive expression of GFP protein under the control of a pCopia promoter was used to screen for high expressors (Figure 2). In addition, western blot analysis of expression was performed on small scale culture supernatants to select positive clones. Cultures were then upscaled on an optimized shaker culture system, and expression of protein of interest was induced using CuSO4 which targets the double methallothionine promoters (pMT) of the pCV vector (Figure 1A).

Biochemical assessment of MHC-IgM multimer

Supernatants cleared from debris by centrifugation followed by filtration were run through affinity columns (either L243- or anti-μ- coupled Sepharose matrix). To determine whether the expressed protein exhibited the expected molecular weight, the purified protein was run on a 10% SDS-PAGE under reducing conditions. Coomassie staining of purified samples demonstrated a shift in molecular weight corresponding to the expected molecular weight of chimeric chains (Figure 3A). To assess the conformational quality of the purified protein, equal amounts of culture supernatant were purified concurrently with L243 *vs* anti-μ coupled beads followed by western blot analysis. Figure 4C demonstrates that both anti- μ or conformational L243 antibodies were capable of efficiently binding and purifying the protein at hand. To determine the oligomeric valence of the molecule of interest, a gel filtration assay on Superose 6 column showed a peak corresponding

to a molecular size of > 1400 kDa when compared to pentameric murine IgM and run against a panel of controls (suppl. Figure S3 & S4 in Appendix and data not shown).

T cell activation by MHC-IgM multimer

Jurkat A14 (expressing HA- peptide-specific TCR and CD28) and irrelevant Jurkat E6.1 (expressing an irrelevant TCR and CD28) cell lines were used to assess the ability of the MHC-IgM multimer to activate T cells. Activation of T cells was evaluated by measuring the upregulation of the early T cell activation marker CD69 and upregulation of the low-affinity IL-2 receptor CD25. The multimeric MHC-HA-DR1-IgM induced upregulation of both CD69 and CD25, as measured by flow cytometry (Fig. 4). CD69 and CD25 upregulation was measured 16 hr following stimulation. MHC-HA- DR1- IgM complexes did not result in any significant activation in the E6.1 clone, and expression of activation markers were comparable to those observed at basal level. For positive control wells, anti- CD3 (+/-) CD28 were immobilized on plastic microtiter plates.

Upregulation of CD69 and CD25 by Jurkat A14 cells following stimulation with HA-DR1 IgM *but not* p24-5-DR1-IgM mutimer.

Stimulation assays were performed concurrently on A14 cells using specific MHC HA-DR1-IgM and non-specific p24-5-DR1-IgM multimers (Figure 6). p24-5-DR1-IgM multimers did not result in any specific stimulation of JKT A14 cells beyond background stimulation levels when looking at the CD69 and CD25 activation profiles, implying that activation resulting from HA-DR1-IgM multimers in the presence of soluble CD28 is inherent to MHC restriction of the HA peptide multimerized on an immunoglobulin M scaffold, and that the latter Ig backbone does not result in non-specific activation of cells.

DISCUSSION

Several methods have been described for the detection of antigen-specific T cells over the past two decades. Most of these essays rely on the functional characteristics of T cells, including proliferation and lytic activity. More recently, soluble MHC multimers have been used successfully in the detection and enumeration of both CD4+ and CD8+ T cells. Some reports have also described the involvement of these molecules in activation of antigen specific T cells *in vitro*. It is well known that T cell receptors have transient and low binding affinity to peptide-MHC complexes. To this end, several groups have developed different forms of multivalent peptide-MHC complexes to study T cell specificity, and to increase the overall avidity of these tools to respective TCRs. The most widely used multimeric molecules include the Class I and Class II tetramers which use streptavidin as a molecular scaffold. In addition, immunoglobulin fusion proteins, using mainly IgG, have been used to produce multimeric MHC molecules. These have been reviewed extensively elsewhere [14,15].

It has been previously shown that Drosophila cells possess a homologue (hsc72) of the immunoglobulin binding chaperone protein (BiP) and are capable of efficiently secreting physiologically active IgG with substrate binding activity indistinguishable from that produced from vertebrate cell lines [16]. In addition, results from our laboratory and other groups have supported the Drosophila expression system as feasible for the production of conformationally active MHC Class II molecules. Moreover, in the absence of the joining (J)-chain, IgM assembles into hexamers in lieu of its physiologic pentameric structure [12,13].

In this paper we described the generation of a novel multimeric MHC Class II fusion protein, namely the dodecameric MHC Class II-IgM (Figure 1B). We showed that Drosophila cells were capable of secreting multimers of MHC DR1 molecules which were conformationally active as evidenced by immunoprecipitation and affinity purification using the anti-DR1 confomational

antibody L243 (Figure 3 and 4). By Western Blot analysis we confirmed the presence of Ig heavy chain – μ – DRα and Ig light chain – κ – DRβ chimeric polypeptides, using monoclonal anti- μ and anti- κ or polyclonal anti-DRα and anti- DRβ antibodies (Figure 3 and 4). These blots showed the expected shift in molecular weight of the polypeptides due to respective fusion of DRα and DRβ chains with immunoglobulin domains when compared to the position of control murine IgM μ and κ chains. This was also observed on Coomassie stained polyacrylamide gels of purified samples. In addition, we tested the multimerization of the chimeric molecule, by Superose 6 Gel Filtration.

In the context of other oligomeric reagents (dimers, trimers, tetramers), studies have shown that even in the absence of antigen-presenting cells, soluble molecules of sufficient avidity activate T cell clones [17]. Indeed, DR1-IgM multimers bearing an epitope from the HA antigen of influenza virus resulted in the activation of Jurkat A14 T cell line which expresses a TCR that recognizes the above epitope in the context of DR1 molecules. This was evidenced by the upregulation of surface activation markers such as CD25 and CD69, following incubation with HA-DR1-IgM multimers. We observed that this activation necessitated the concurrent addition of soluble anti-CD28 antibody [18]. In addition, we showed that this interaction was specific, since neither the addition of an irrelevant p24-5-DR1-IgM multimer on A14 cells, nor the stimulation of irrelevant Jurkat E6.1 cells by HA-DR1-IgM resulted in any significant upregulation of activation markers.

The overall increased functional avidity and the stability of the complex formed with corresponding TCR's are key factors in identifying specific populations of T-cells in clinical as well as basic research laboratories. These properties are also crucial for improving the qualitative and quantitative value of multimer-based molecular monitoring of clinical trials of vaccination with ClassII-defined immunogens, and the isolation of those populations for further phenotypic and functional assays. However, the avidity of the TCR-MHC-peptide interaction

depends not only on the affinity of each TCR for its peptide-MHC ligand, but also on the density and clustering of TCR on the T cell surface. Thus, approaches that enhance the clustering and/or internalization of TCR-peptide/MHC complexes are prime towards the *ex-vivo* manipulation of scant number and/or low affinity antigen-specific T cells [21].

In its physiologic milieu IgM is known to 'staple' upon antigen binding [19,20]. In contrast to rigid Streptavidin moieties in multimerization of MHC monomers, using IgM as a molecular scaffold offers a more flexible access for TCR binding due to the inherent characteristics of the immunoglobulin hinge region. Moreover, preliminary Fluorescence Microscopy studies using the multimeric DR1-IgM molecules showed evidence of TCR capping. Further experiments are underway to delineate the specific molecular mechanisms involved therein and the impact of experimental conditions, such as temperature, on staining. These novel reagents are promising as a multivalent system for identification, stimulation, and *ex-vivo* expansion of T cells, ultimately allowing the manipulation of the intensity and quality of T cell responses.

Acknowledgements

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LEGENDS TO FIGURES

FIGURE 1. A. Insect Expression Vector. pCV DR1 IgM is a methallothionine inducible (pMT), double promoter insect cell expression vector. This vector contains the extracellular portion of the DRA1*0101 (DRα) chain covalently linked to the heavy chain (μ) of mIgM via a GGGSAS linker; and the DRB1*0101 (DRβ) chain covalently linked to the light chain (κ) of mIgM. The vector also contains the constitutively expressed eGFP gene. In the parental vector pCVDR1-$HA_{306-318}$ the N-terminus of the HLA-DRβ gene is covalently linked to the hemagglutinin (HA) peptide ($HA_{306-318}$:PKYVKQNTLKLAT) encoding sequence via a flexible linker. **B. Cartoon of the dodecameric IgM*MHCII fusion protein, without the immunoglobulin variable domain.** In the absence of J chain, immunoglubin M molecules tend to assemble into hexamers. Here, the MHC DR1 alpha chain was covalently linked via a GGGSAS linker to the constant Cμ1 domain of the IgM heavy chain, while the MHC DR1 beta chain, carrying an antigenic peptide of interest, was covalently linked to the constant domain of the IgM κ light chain.

FIGURE 2. Constitutive eGFP expression in transfected *Drosophila* insect S2 cells. Efficiency of transfection and selection of positive clones is monitored by assessing the constitutive expression of the egfp gene. Autofluorescence from untransfected S2 cells alone serves as negative control and in-house pCV DR1 HA WT clones established for production of MHC Class II tetramers serve as positive control for MHC Class II DR1*IgM construct transfection.

FIGURE 3. Biochemical assessment of purified protein. A. Following L243 affinity column purification, the purity of the MHC II DR1*IgM fusion protein was assessed through Coomassie Blue staining of proteins separated on a 10% SDS PAGE. Lane 1. 0.2μg BSA; Lane 2. 0.5μg BSA; Lane 3. 1μg BSA; Lane 4. 0.5μg control mIgM; Lane 5. 0.5μg control mIgM; Lane 6. 1μg HA-DR1-IgM; Lane 7. 2μg HA-DR1-IgM; Lane 8. Sample 'flow through' after affinity column

elution. **B.** Following transfer on nitrocellulose membrane, samples were blotted with primary rabbit polyclonal anti-DRα and anti-DRβ antibodies, followed by secondary goat anti-rabbit HRP antibody.

FIGURE 4. Immunoprecipitation and Western Blot analysis of fusion protein. To assess protein conformation following production in insect S2 cells, culture supernatants were tested by immunoprecipitation using either the conformational anti-DR L243 or anti-IgM heavy chain antibody-coupled Sepharose beads followed by Western Blot. **A.** Supernatant from S2 cells were immunoprecipitated (IP) with L243, beads were washed, boiled in sample buffer and run on SDS-PAGE. Following transfer, membrane blotted with monoclonal anti-κ light chain antibody. Lane 1. Control mIgM; Lane 2. Untransfected induced S2 negative control; Lane 3. DR1-IgM-p24-5 (5μg of transfected DNA); Lane 4. DR1-IgMp24-5 (7.5μg of transfected DNA); Lane 5. DR1-IgM HA (7.5μg of transfected DNA). Kappa light chain originating from the L243 Ab used in immunoprecipitation is visible at 25KDa in lanes 3,4, and 5, due to loading of Sepharose beads coupled to L243 antibody (IgG κ) . **B. L243 IP**. Blot by rabbit polyclonal anti-DRβ antibody (upper panel), and monoclonal anti-IgM μ and anti-IgM κ chain antibodies (lower panel). Lane 1. Control mIgM; Lane 2. DR1-IgM-p24-5 (5μg of transfected DNA); Lane 3. DR1-IgM-p24-5 (7.5μg of transfected DNA); Lane 4. DR1-IgM-HA (7.5μg of transfected DNA). **C. Comparison of Immunoprecipitation with anti-mouse IgM heavy chain vs. anti-DR L243 antibodies.** Membrane blotted with monoclonal anti-μ chain antibody. Lane 1. Control mIgM; Lane 2. Untransfected induced S2 negative control; Lane 3. DR1-IgMp24-5; Lane 4. DR1-IgMp24-5 Lane 5. DR-1-IgM HA (7.5μg of transfected DNA). Lane 6. untransfected induced S2 negative control; Lane 7. DR1-IgMp24-5; Lane 8. DR1-IgMp24-5; Lane 9. DR1-IgM HA (7.5μg of transfected DNA).

FIGURE 5.

5A. Upregulation of CD69 and CD25 activation markers and blast formation following stimulation of A14 cells with DR1-IgM-HA multimer. A14 cells expressing HA-specific TCR and CD28 were stimulated *in vitro* for 16hr in the presence of HA-DR1-IgM multimer and soluble anti-CD28 antibody. Stimulation with anti-CD3/anti-CD28 was used as positive control. Jurkat A14 cells did not display any significant basal level of CD69 and CD25 expression when cultured in medium alone. Following stimulation with HA-DR1-IgM, in addition to upregulation of CD69 and CD25 markers, blast formation characterized by increased side and forward scatters could also be observed (cells delineated by an oval in SSC/FSC dot plot).

5B. Absence of CD69 and CD25 upregulation following stimulation of E6.1 cells with DR1-IgM-HA multimer. Control Jurkat E6.1 cells expressing an irrelevant TCR and CD28 were concurrently stimulated *in vitro* for 16hr in the presence of HA-DR1-IgM multimer and soluble anti-CD28 antibody. Stimulation with anti-CD3/anti-CD28 was used as positive control. E6.1 cells had minimal basal levels of CD69 and CD 25 expression when cultured in medium alone, which was more significant when soluble anti-CD28 was added to the medium (Figure 7).

5C. Background control. A14 and E6.1 cells were stimulated with soluble anti-CD28 alone, to determine the effect of the latter on non-specific expression of CD25 and CD69. There was no effect of addition of anti-CD28 to A14 cells, since the expression levels were comparable to basal levels of expression as observed in Figure 5A. However, the addition of soluble anti-CD28 to E6.1 cells increased CD69 expression to levels comparable to that observed in stimulation with HA-DR1-IgM + CD28 stimulation, indicating that the latter expression is due to CD28 and hence non-specific.

FIGURE 6. Irrelevant peptide MHC*IgM multimer does not stimulate A14 cells. A14 cells were stimulated with either HA-DR1-IgM (panel B) or p24-5-DR1-IgM (panel C) multimer, in conjunction with soluble anti-CD28, to determine the effect of non-specific activation of HA specific T cells due to an irrelevant MHC IgM multimer. Stimulation of A14 cells with HA-DR1-IgM alone (panel A) failed to result in significant activation, indicating the need of soluble CD28.

References

1. Yoon, S. T. et al. Both high and low avidity antibodies to the T cell receptor can have agonist or antagonist activity. *Immunity* **1.7**, 563-69 (1994).

2. Boniface, J. J. et al. Initiation of signal transduction through the T cell receptor requires the peptide multivalent engagement of MHC ligands. *Immunity.* **9.4,** 459-66 (1998).

3. Cochran, J. R. et al. Receptor clustering and transmembrane signaling in T cells. *Trends Biochem.Sci* **26.5**, 304-10 (2001).

4. Cochran, J. R., T. O. Cameron, and L. J. Stern. The relationship of MHC-peptide binding and T cell activation probed using chemically defined MHC class II oligomers. *Immunity* **12.3**, 241-50 (2000).

5. O'Garra, A. Cytokines induce the development of functionally heterogeneous T helper cell subsets. *Immunity* **8.3**, 275-83 (1998).

6. Bourgeois, C. et al. CD8 lethargy in the absence of CD4 help. *Eur.J.Immunol.* **32.8,** 2199-207 (2002).

7. Janssen, E. M. et al. CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. *Nature* **421.6925**, 852-56 (2003).

8. Shedlock, D. J. and H. Shen. Requirement for CD4 T cell help in generating functional CD8 T cell memory. *Science* **300.5617**, 337-39 (2003).

9. Sun, J. C. and M. J. Bevan. Defective CD8 T cell memory following acute infection without CD4 T cell help. *Science* **300.5617**, 339-42 (2003).

10. Sun, J. C., M. A. Williams, and M. J. Bevan. CD4+ T cells are required for the maintenance, not programming, of memory CD8+ T cells after acute infection. *Nat.Immunol.* **5.9**, 927-33 (2004).

11. Maher, J. and E. T. Davies. Targeting cytotoxic T lymphocytes for cancer immunotherapy. *Br.J Cancer* **91.5**, 817-21(2004).

12. Niles, M. J., L. Matsuuchi, and M. E. Koshland. Polymer IgM Assembly and Secretion in Lymphoid and Nonlymphoid Cell Lines: Evidence that J Chain is Required for Pentamer IgM Synthesis. *Proceedings of the National Academy of Sciences* **92.7,** 2884-88 (1995).

13. Wiersma, Erik J. et al. Structural and Functional Analysis of J Chain-Deficient IgM. *The Journal of Immunology* **160.12**, 5979-89 (1998).

14. Dunbar,P.R. & Ogg,G.S. Oligomeric MHC molecules and their homologues: state of the art. *Journal of Immunological Methods* **268**, 3-7 (2002).

15. Hugues,S., Malherbe,L., Filippi,C., & Glaichenhaus,N. Generation and use of alternative multimers of peptide/MHC complexes. *Journal of Immunological Methods* **268**, 83-92 (2002).

16. Kirkpatrick, Robert B. et al. Heavy Chain Dimers as Well as Complete Antibodies Are Efficiently Formed and Secreted from Drosophila via a BiP-mediated Pathway. *Journal of Biological Chemistry* **270.34**, 19800-05 (1995).

17. Kwok, William W. et al. "Use of class II tetramers for identification of CD4+ T cells." *Journal of Immunological Methods* **268.1**, 71-81(2002).

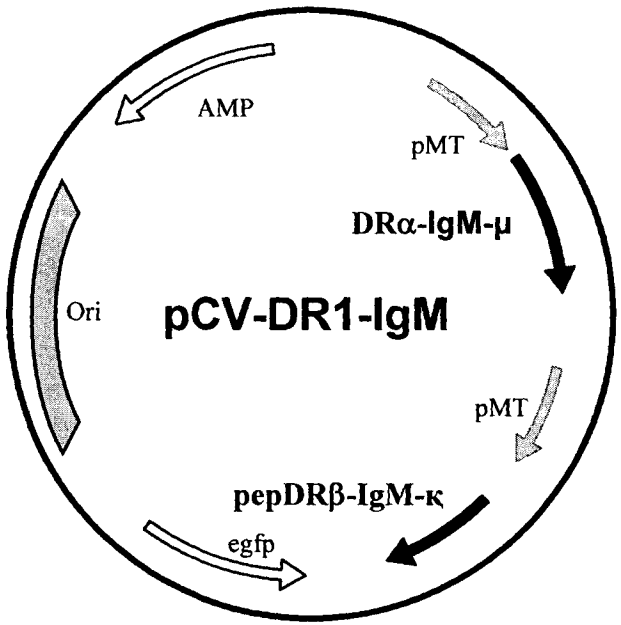
18. Buckner J.H. et al. Identification of type II collagen peptide 261-273-specific T cell clones in a patient with relapsing polychondritis. *Arthritis Rheum.* **46.1,** 238-44 (2002).

19. Armstrong SJ, Outlow MC, Dimmock NJ. Morphological studies of the neutralization of influenza virus by IgM. *J Gen Virol* **71 (Pt 10)**, 2313-19 (1990).

20. Weiner EM. On the interaction of the first complement component C1 and its subunit C1q with solid-phase IgM immune complexes. *Scand J Immunol* **28.4**, 425-30 (1988).

21. Ge Q, Stone JD, Thompson MT, et al. Soluble peptide-MHC monomers cause activation of CD8+ T cells through transfer of the peptide to T cell MHC molecules. *Proc Natl Acad Sci U S A.* **99(21)**, 13729-34 (2002).

A.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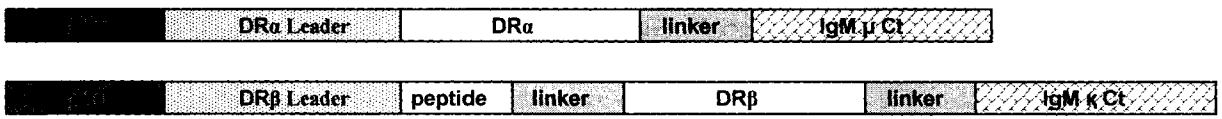
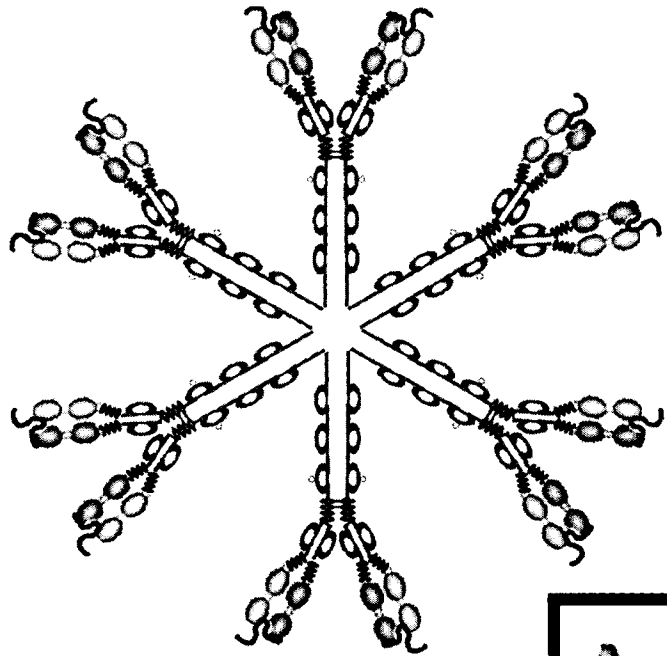
DRα-IgM-μ
Ori
pCV-DR1-IgM
pMT
pepDRβ-IgM-κ
egfp


DRα Leader
DRα
linker
IgM μ Ct
DRβ Leader
peptide
linker
DRβ
linker
IgM κ Ct

B.



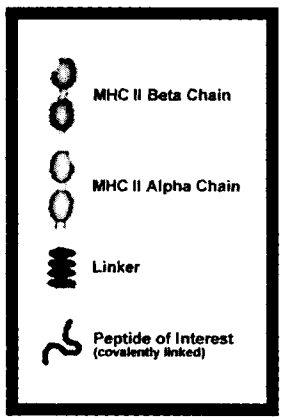
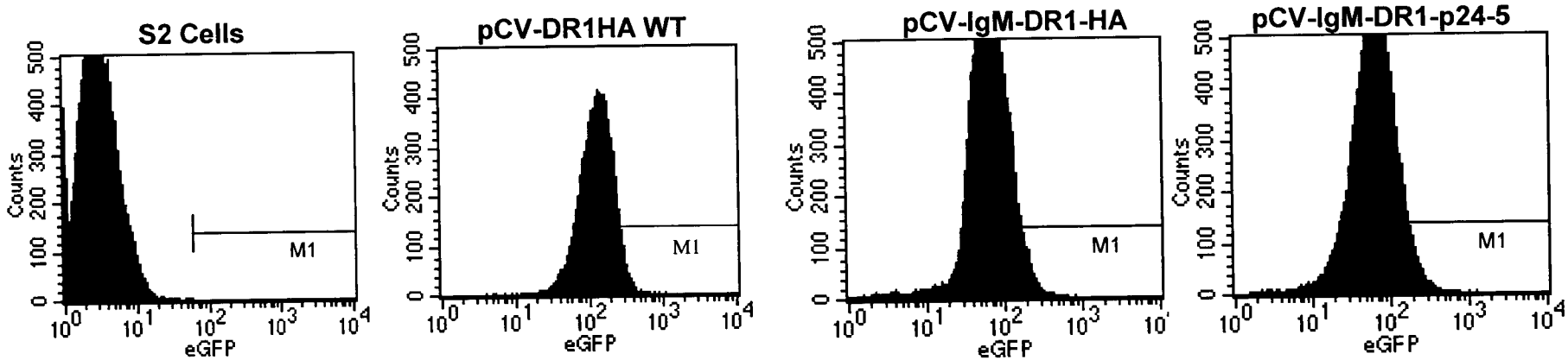

MHC II Beta Chain
MHC II Alpha Chain
Linker
Peptide of Interest
(covalently linked)

Figure 1



Marker	% Total	Mean
All	90.08	3.54
M1	0.00	***

Marker	% Total	Mean
All	53.48	130.90
M1	53.09	131.71

Marker	% Total	Mean
All	93.14	72.06
M1	53.64	95.38

Marker	% Total	Mean
All	93.24	70.98
M1	50.40	99.25

Figure 2

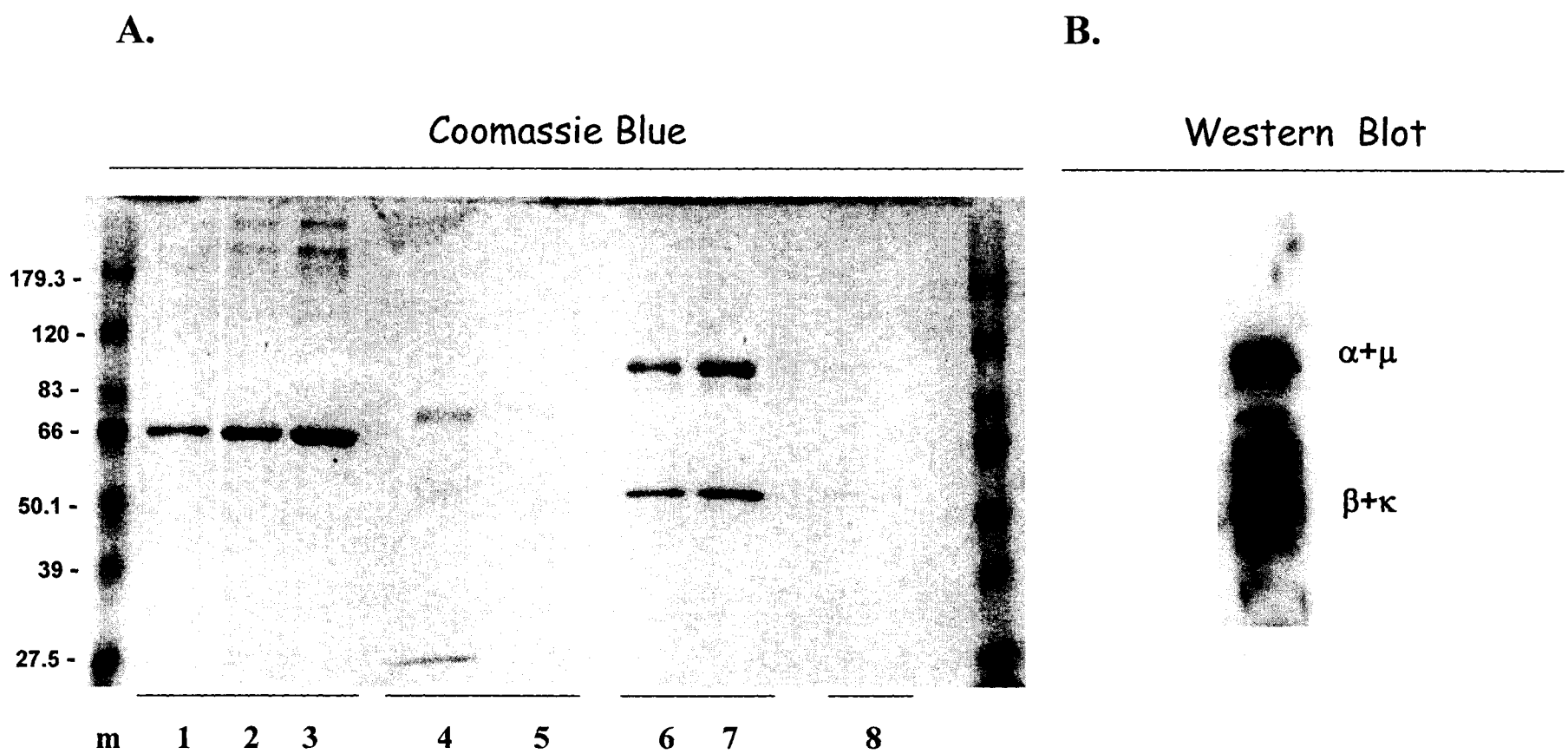

A.
B.
Coomassie Blue
Western Blot
179.3 -
120 -
83 -
66 -
50.1 -
39 -
27.5 -
α+μ
β+κ
m
1
2
3
4
5
6
7
8

Figure 3

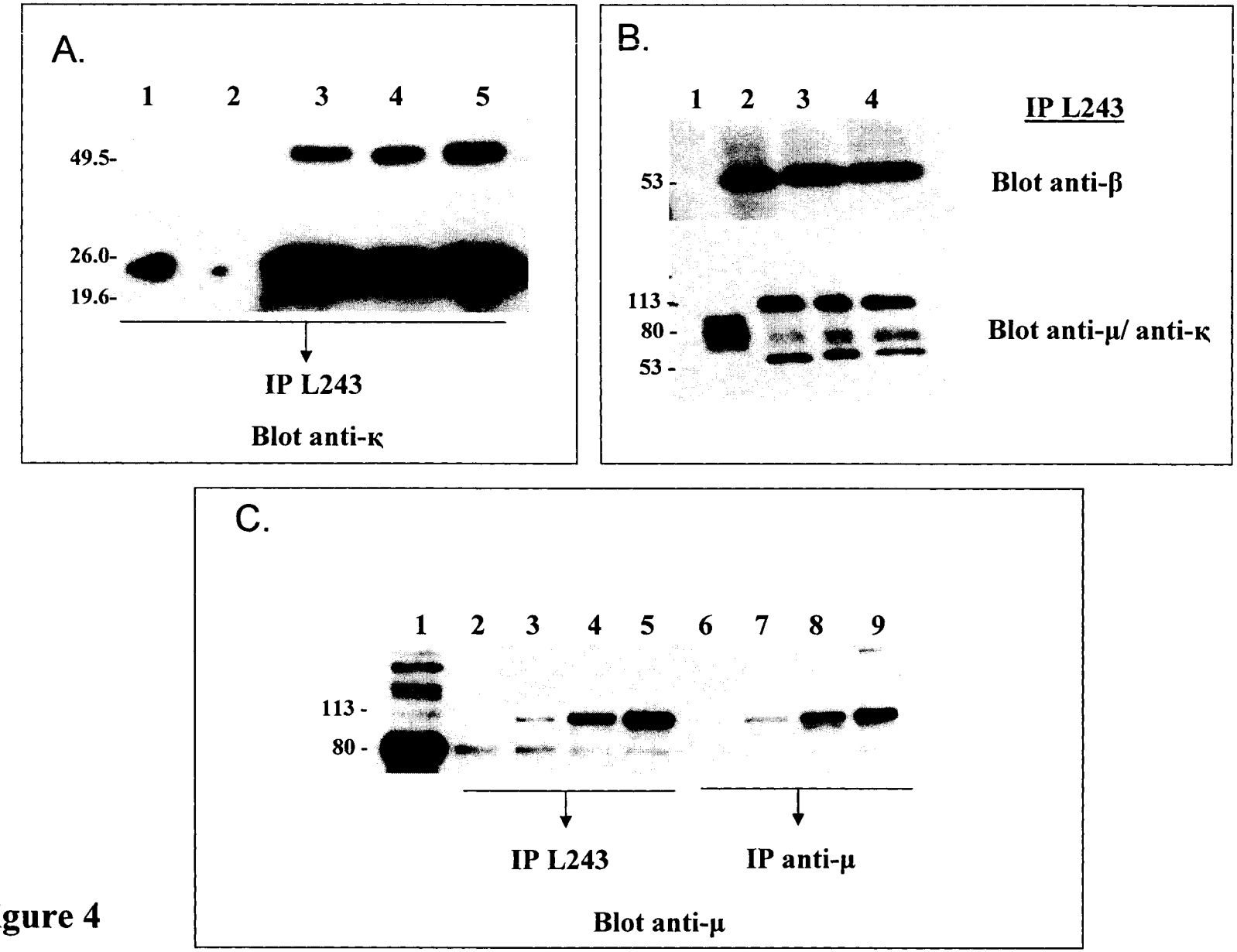

A.
1 2 3 4 5
49.5-
26.0-
19.6-
IP L243
Blot anti-κ
B.
1 2 3 4
IP L243
53 -
Blot anti-β
113 -
80 -
53 -
Blot anti-μ/ anti-κ
C.
1 2 3 4 5 6 7 8 9
113 -
80 -
IP L243
IP anti-μ
Blot anti-μ

Figure 4

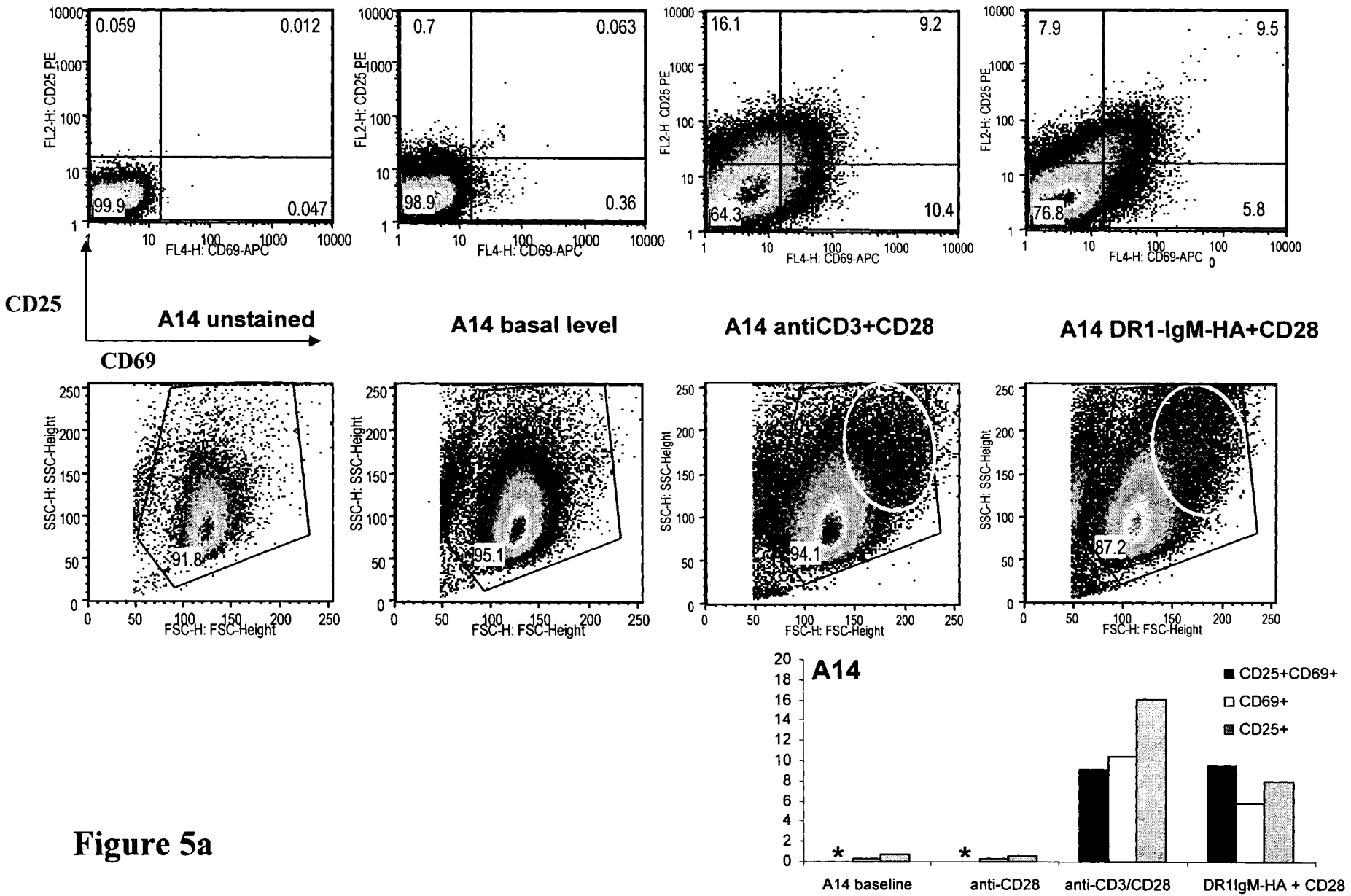

0.059
0.012
99.9
0.047
0.7
0.063
98.9
0.36
16.1
9.2
64.3
10.4
7.9
9.5
76.8
5.8
FL2-H: CD25 PE
FL4-H: CD69-APC
CD25
CD69
A14 unstained
A14 basal level
A14 antiCD3+CD28
A14 DR1-IgM-HA+CD28
SSC-H: SSC-Height
FSC-H: FSC-Height
91.8
95.1
94.1
87.2
A14
CD25+CD69+
CD69+
CD25+
A14 baseline
anti-CD28
anti-CD3/CD28
DR1IgM-HA + CD28

Figure 5a

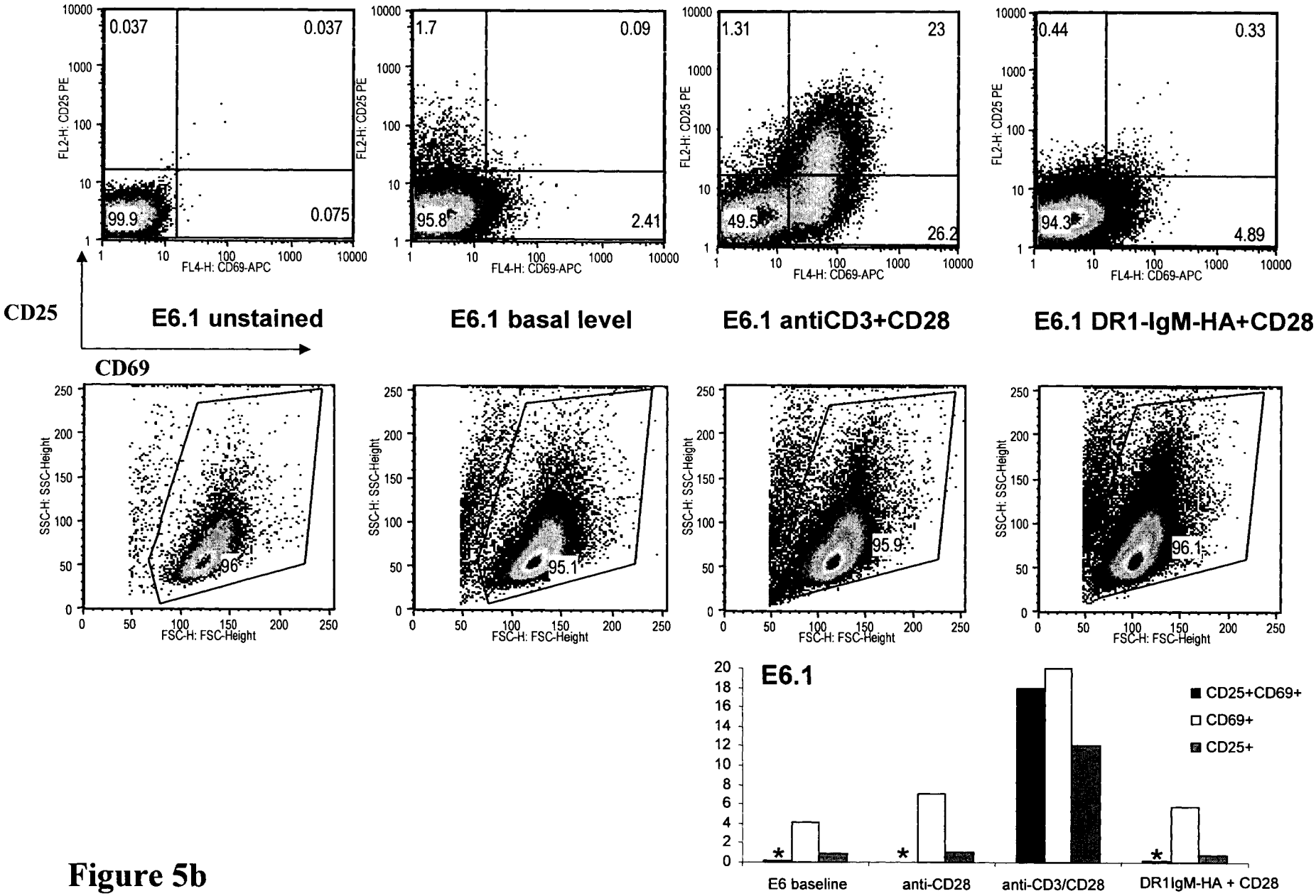

CD25
CD69
E6.1 unstained
E6.1 basal level
E6.1 antiCD3+CD28
E6.1 DR1-IgM-HA+CD28
0.037
0.037
99.9
0.075
1.7
0.09
95.8
2.41
1.31
23
49.5
26.2
0.44
0.33
94.3
4.89
FL2-H: CD25 PE
FL4-H: CD69-APC
SSC-H: SSC-Height
FSC-H: FSC-Height
96
95.1
95.9
96.1
E6.1
CD25+CD69+
CD69+
CD25+
E6 baseline
anti-CD28
anti-CD3/CD28
DR1IgM-HA + CD28

Figure 5b

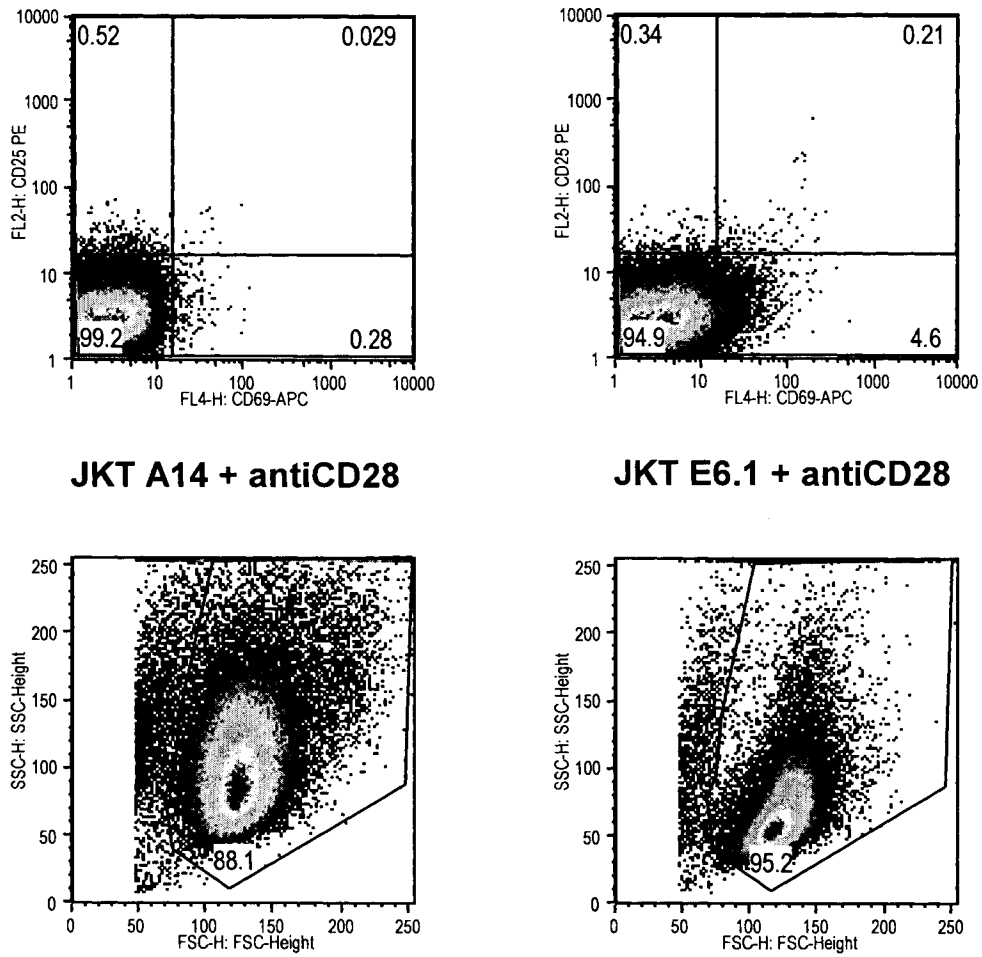

0.52
0.029
99.2
0.28
FL2-H: CD25 PE
FL4-H: CD69-APC
JKT A14 + antiCD28
0.34
0.21
94.9
4.6
JKT E6.1 + antiCD28
88.1
95.2
SSC-H: SSC-Height
FSC-H: FSC-Height
CD25
CD69

Figure 5c

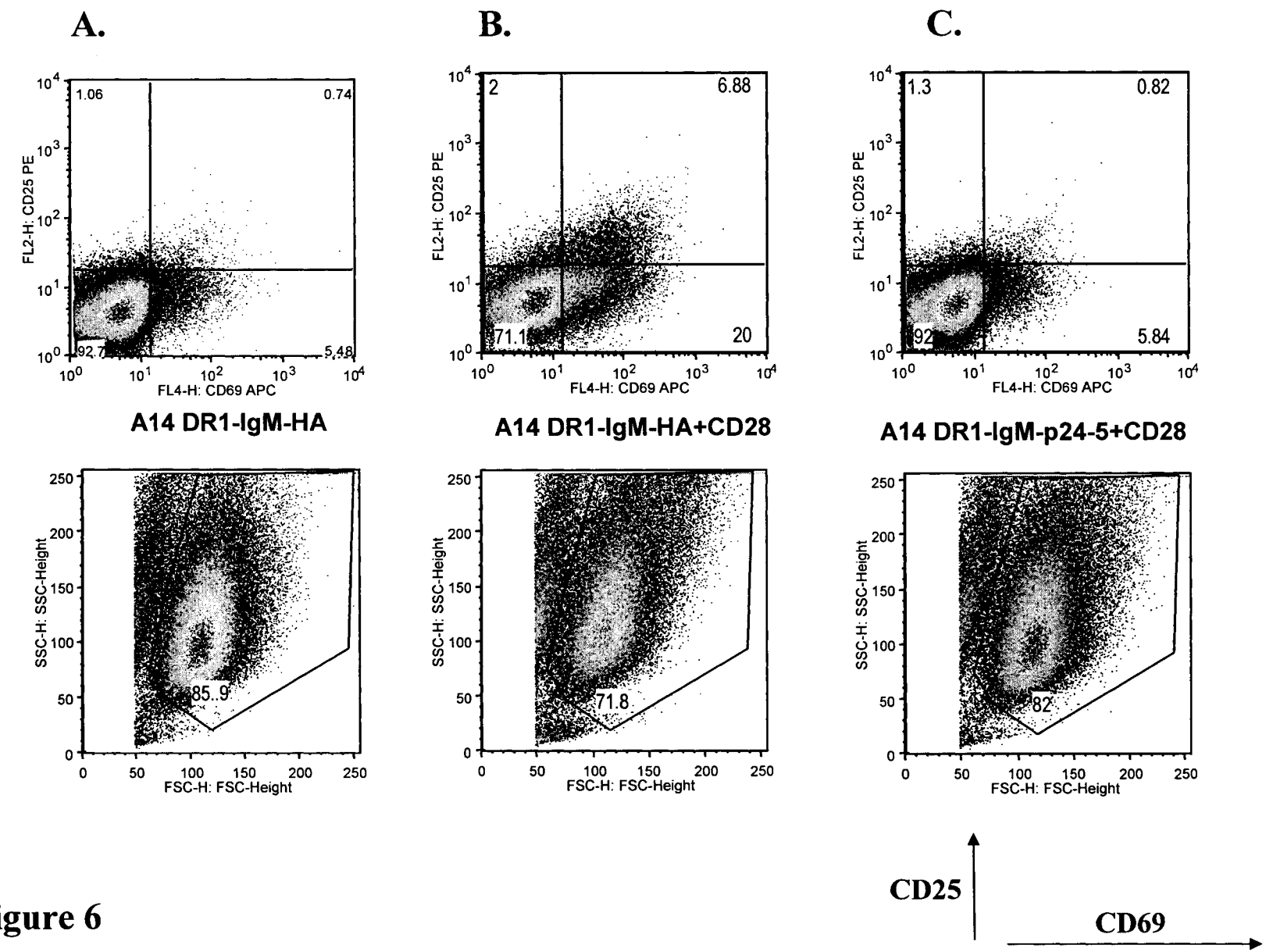

A.
B.
C.
1.06
0.74
92.7
5.48
2
6.88
71.1
20
1.3
0.82
92
5.84
FL2-H: CD25 PE
FL4-H: CD69 APC
A14 DR1-IgM-HA
A14 DR1-IgM-HA+CD28
A14 DR1-IgM-p24-5+CD28
SSC-H: SSC-Height
FSC-H: FSC-Height
85..9
71.8
82
CD25
CD69

Figure 6

4 Original contribution to scientific knowledge

The results presented in this thesis contribute originally to scientific knowledge in several aspects:

The mucosal immunizations carried out in the humanized mouse model in Chapter 2 set forth a unique model for pre-clinical evaluation of new generation T cell vaccines. We showed that mucosal administration of viral vector based vaccines results in antigen-specific, systemic primary T cell responses. Moreover, we demonstrated that the presentation of epitopes from the HIV-1 subtype A Gag immunogen was restricted by the human MHC transgene. Previously identified epitopes in HIV-1 patients were found to be immunogenic in these mice. Therefore, we propose this model as a platform for mapping and identification of novel T-cell epitopes restricted by human MHC Class I and Class II molecules as well as the delineation of functional signatures following mucosal immunization using viral vector based mucosal T cell vaccines.

The reagents developed in Chapter 3 are pioneering in the attempt to generate conformationally active multimers of human MHC Class II DR1 molecules covalently linked to antigenic epitopes. We showed that these molecules assemble indeed into higher valence multimers in an insect expression system without the need of further *in vitro* multimerization steps. We further tested the production of these molecules in mammalian systems. We demonstrated that these reagents were capable of the *in vitro* activation of Jurkat T cells in an antigen-specific manner. The development of such reagents for the detection and *ex-vivo* expansion of low-avidity T cell receptor (TCR) bearing T lymphocytes has critical implications in the context of such conditions as autoimmune diseases.

5 Discussion

5.1 Current status of HIV vaccine research

The UNAIDS '2006 Report on the Global AIDS Epidemic' stated that in 2005 nearly 39 million people worldwide were living with HIV, including 4.1 million new cases, and that an estimated 2.8 million lost their lives to the disease that year (*www.unaids.org*).

Since it was first identified over 20 years ago, the HIV/AIDS epidemic has undoubtedly exceeded all anticipations of severity of global demographic, social, and economic impact [78]. In addition, the inefficacy and inaccessibility of retroviral treatment regimens in areas worst hit by this expanding epidemic, highlight the imperative need of tangible progress toward a vaccine. In this respect, the 'XVI International AIDS Conference' 2006, joining over 24,000 stakeholders of the field in Toronto, Canada, rightfully adopted the slogan '*Time to Deliver*'.

Until recently, approaches to vaccine development for a range of different infectious diseases have been mainly empirical. Identification and delineation of the intricate immune correlates of protection during the natural course of an infection or following vaccine administration are becoming vital to the design and development of new generation vaccines against emerging as well as existing diseases, such as AIDS [4,42,124, 125].

The transmission of HIV-1 occurs primarily at mucosal surfaces. The mucosa is also possibly involved in the initial selection of the genotypic and phenotypic minor variants found during acute phases of infection [79, 80]. To prevent dissemination of the virus to draining lymph nodes and systemic lymphoid tissue, and thwart the establishment of local and systemic reservoirs, generation of protective humoral and cell-mediated immune responses at mucosal sites of initial viral encounter must be targeted through the rational design of mucosal vaccines. Vaccines intended to prevent mucosal transmission of HIV should ideally be able to induce multiple immune effectors in the host including antibodies and cell-mediated immune responses both at mucosal and systemic sites [80,81].

The pathogenesis of HIV infection is complex and multipronged, with genetic susceptibility, host responses, and viral factors all playing important roles in disease prognosis. The development of small animal models addressing some of these variables, including genetic background, is paramount in assessing the impact of different therapeutic, immune-intervention, and vaccine strategies.

5.2 Rationale for mucosal delivery

Recent understanding of the mechanisms involved in mucosal HIV transmission and the role of immune modulators, such as integrins and chemokines, in mucosal trafficking can aid the tailoring of new strategies to enhance AIDS vaccine efficacy [80,82]. The stimulation of a mucosal response can be achieved through the administration of immunogens at mucosal inductive sites, where specialized, organized lymphoepithelial follicular infrastructures exist. Subsequently, the common mucosa-associated system regulating and coordinating immune responses at mucosal surfaces helps in the generation of responses at distal mucosal effector sites [83]. This system, called the mucosa-associated lymphoid tissue, is based on primed T and B lymphocytes that leave the original site of antigen presentation through the lymphatics and blood, and selectively home to lymphoid tissue at distant sites in gastrointestinal, respiratory, genitourinary, and other mucosa-associated regions. Furthermore, it has been observed that immunization by mucosal delivery of a host of live-attenuated or recombinant viral vectors expressing the antigen of interest, results in significant humoral and cellular responses both mucosally and systemically [84, 85].

5.3 Development of the transgenic CHAD model

Over the years, transgenic animal models have contributed greatly to the progress made toward understanding the pathogenesis and immune mechanisms involved in many diseases. The Major Histocompatibility

Complex (MHC), a set of polymorphic genes expressed on the surface of host cells, is involved in antigen presentation to immune cells. The Class I HLA-A*0201 and Class II HLA-DRB1*0101 alleles cover nearly 40% and 10% of the human population, with some groups having frequencies as high as 56% and 31%, respectively (source: *www.allelefrequencies.net*).

Moreover, it has been previously demonstrated that in the generation of HLA transgenic models, the substitution of the CD8-binding α3 domain of A2.1 with its murine counterpart leads to the effective generation of murine CTL responses to HLA-A2.1 restricted epitopes previously identified in humans [86, 87]. Similarly, the substitution of α2 / β2 CD4 biding domains of DR with a murine counterpart enhanced interaction with murine CD4, resulting in more potent responses [88, 89,90].

Taken the above into account, and in conjunction with Dr. D.B. Weiner's laboratory at the University of Pennsylvania, we generated a humanized chimeric A2DR (CHAD) transgenic mouse model. These mice concurrently express MHC Class I A*0201 and Class II DR*0101 molecules. Over the past 3 years, we undertook careful derivation and selection of progeny to establish a colony of animals which are high expressors of the chimeric transgenes.

Our study in Chapter 2 exploits this model to study HLA-A2-restricted cytotoxic T lymphocyte (CTL) and HLA-DR1-restricted CD4 helper responses following immunization with viral vectors expressing Gag. We observed that epitopes in Gag that serve as the dominant HLA-A2.1 or DR1-restricted determinant in the equivalent human response were also dominant in these mice. The use of synthetic peptide matrices covering the complete Gag sequence can further reveal similarities between the murine and human response repertoire and be crucial in the identification of novel immunodominant epitopes following immunization.

When considering T cell responses by different species, our results confirm earlier results that the α1 and α2 domains of HLA-A2.1 and the α1 and β1 domains of HLA-DR1 play a dominant role in determining the ensuing CD8

and CD4 repertoire, despite the chimeric murine regions of the molecules. Our results therefore support previous studies which have used HLA transgenic system for the delineation of HLA-Class I and Class II -restricted responses, providing a rational animal model for the design and assessment of new generation vaccines. [91,92,93].

Interestingly, a recent study describes that the use of the HLA-A2/K^b transgenic mice allowed the definition of an HLA-A*0201 binding peptide epitope that would have been rejected on the basis of predicted major histocompatibility complex binding affinity. This peptide was shown to bind to HLA-A*0201 but not murine D^b or K^b molecules on the cell surface. Furthermore this peptide was shown to be immunogenic *in vitro* to human T cells from HLA-A2 healthy donors [94].

Altogether, this model emerges as a versatile small animal system (in lieu of non-human primates or human subjects) for the study of the correlates of protection and immune mechanisms involved in a multiplicity of vaccines in development and corresponding preclinical strategies. This transgenic mouse model also constitutes a unique system and platform in validation of the functionality of immune monitoring tools in preclinical stages, including novel multimeric tools, which following proof-of-concept, can be extrapolated to non-human primate or human samples.

Still, species differences in structure and function of the immune system should be remembered and vigilance must be practiced when designing strategies and regimens, and extrapolating data to humans. Successful approaches in mice and even primates have often disappointingly failed when tested in humans. Results should always be considered with great care before applying to human disease especially if new concepts for therapy are concerned. Thus, animal models of HIV immunizations are relevant for primate or human trials, as long as the limitations of this approach are kept in mind.

5.4 Use of viral-vector vaccine delivery systems

Live recombinant vesicular stomatitis viruses (rVSV) expressing foreign viral proteins have been used successfully in animal models, in immunizations against a variety of viral pathogens such as HIV, Influenza virus, Measles virus, Papillomavirus, Rabies virus, etc [95]. rVSV vaccines administered mucosally induce potent systemic humoral and cellular immune responses, thus making this vector a promising candidate in the design of HIV prime/boost immunization strategies. In addition, attenuated VSV-vectors have a major advantage of low-level seropositivity in the human population. This makes VSV-based vaccines candidates for heterologous prime-boost protocols [96, 97, 98]. Similarly, recombinant adenovirus based vaccines have been used recently in a variety of prime-boost regimens and have yielded promising data [84,41]. Adenoviral vectors are limited however by pre-existing seropositivity in the population [99].

We based our choice of vaccine vector and delivery regimen on preliminary data obtained from collaborating two CANVAC research laboratories and on results from the litterature. As described in Chapter 2, we adapted a mucosal vaccination protocol involving two viral vectors, namely rVSV and Adenovirus serotype 5, expressing HIV Gag from Subtype A. These vectors are also endowed with inherent adjuvant properties, thus dispensing the need for mucosal adjuvant co-delivery. The efficacy of this strategy, as well as the generation and dissection of the ensuing cellular immune response was investigated.

Our main interest lies in the identification and characterization of HIV-specific T cell subsets both in the CD4 and CD8 compartments following immunization, and the generation of long-term memory. To this end, thorough and multiparameter immune monitoring tools set forth in our laboratory and protocols carefully adopted to murine samples are employed. These allow the meticulous assessment of the phenotype, functional signature, and epitope

specificity of $CD4^+$ and $CD8^+$ T lymphocytes at different time points following immunization.

Proliferation assays, class I and class II tetramer staining, ICS, and a panel of phenotypic markers are currently used through multiparameter flow cytometry to characterize the phenotype, receptor specificity, and functionality of the generated T cell subsets both at the acute and memory phases following vaccine administration. Phenotypic subsets of CD4+ T cell compartment are not fully elucidated in the mouse. In this report, we showed that i.n. route of immunization using two different viral vector based vaccines resulted in the generation of significant systemic antigen-specific CD4+ T-cell responses in the primary phase of the immune response. Our objective is to dissect the exact nature of this response.

On the other hand, even though in the current study we did not investigate specific mucosal immune responses following vaccine administration, previous studies have shown that i.n. immunization with an adenoviral vector expressing HIV-1 Gag resulted in enhanced IgA responses in body secretions [84]. Further experiments for longitudinal analysis of these responses are discussed below, in Section 5.7.

5.5 Development of multimeric MHC Class II molecules

Immunotherapies for human immune-mediated diseases are proliferating rapidly. With these changes comes the need to monitor patients for immune responses to therapy based on early surrogate markers for clinical responses. The development of fast and sensitive bioassays is central towards this end.

As reviewed in Chapter 1, over the past decade, novel state-of-the-art technologies for detecting cellular immunity have been developed. These tools allow the detection of T cells based on the antigen-specificity of their TCR. Concurrently, many of the new immunotherapeutic approaches and vaccine strategies that are currently being developed - in infectious disease, autoimmune disorder, or tumor models – are highly targeted and disease

specific. Thus careful assessment of the quantitative and qualitative nature of antigen-specific T cell response is critical in the validation of the efficacy of such treatments and immunizations, especially in clinical studies.

In this context, optimization of multimeric reagents which characterize specific cellular immune responses is an ongoing process and of utmost value as a mean of detecting and tracking the fate of antigen-specific T lymphocytes as surrogate markers of clinical trials. For long, many attempts to ligate the T cell receptor to protein substrates failed. This led to the assumption that the affinity of the T cell receptor was too low.

Following the unravelling of the molecular structure of MHCs and antigen presentation of peptides to TCR, Mark Davis and John Altmann pioneered the development of oligomeric MHC reagents through the realization that by increasing the avidity of MHC molecules one could ligate the corresponding TCR [67]. Since, MHC Class I and Class II Tetramers have been developed and tested in identification of antigen specific T cell responses in a variety of disease models and their impact in the field of immunology has been significant in elucidating many specificities of the immune response.

Our objective is to develop a novel, multimeric, versatile tool and ultimately assess its functionality within a specific HIV- immunization framework. Staining intensity being a direct function of T- cell receptor affinity, low affinity TCR's might not be recognized by existing tools such as tetramers. These low-affinity interactions, which are involved in processes such as autoimmunity, may often be missed. The elaboration of methods to efficiently activate and expand antigen specific CD4+ T cells *ex vivo* is crucial for the development of effective T cell based immunotherapies.

On the other hand, it is established that the avidity of the TCR–MHC–peptide interaction depends not only on the affinity of each TCR for its MHC–peptide ligand, but also on the density and clustering of TCR on the T cell surface. It is important to state that the higher binding affinity of the MHC-multimers is caused by the increased avidity, which is the sum of the individual affinities of

the multiple MHC and TCR interactions. Moreover, together with factors such as the density of the MHC on the surface of the antigen-presenting cells, the concentration of peptide, and the type of costimulatory ligands available, TCR signaling leads to variable outcomes influenced by the strength of the interaction.

Recent studies have compared activation profiles among T cells stimulated with dimers (bivalent class II–peptide complexes), trimers, or tetramers. These studies indicate that multimerization of TCRs occurs early after interaction with MHC oligomers, followed by rapid T cell activation. Fluorescent staining with the multimers was enhanced by this activation, suggesting that clustering of receptors and/or endocytosis occurs, which augments the fluorescent tetramer signal detection. There is thus a relationship between avidity, activation, and tetramer binding. It appears that, while high-avidity T cells are readily detected using MHC tetramer fluorescence technology, detection of low-avidity T cells may require simultaneous measurement of activation markers or methods to facilitate enhanced tetramer staining by improving TCR clustering and/or internalization [72,100,101]. Researchers have exploited this association between tetramer binding and T cell activation, since even in the absence of antigen-presenting cells, soluble tetramers of sufficient avidity can activate T cell clones, as determined by both surface activation markers and cytokine secretion [70, 72,101].

On the other hand, while low-affinity TCRs were shown to only bind MHC class II-peptide complexes at 37°C, high-affinity TCR stained at 4°C [102]. Fluorescently labeled liposomes added to $CD4^+$ T cells co-localized with markers for endocytotic compartments, indicating that these T cells have the potential to internalize multimers.

In the present report, and in order to increase the overall avidity of soluble MHC Class II reagents, we used the murine immunoglobulin M (IgM) as a molecular scaffold to multimerize HLA-DR1 molecules. As demonstrated in Chapter 3, this system allows the identification (through stimulation) of

antigen-specific T cells, offering a new tool to study and manipulate the intensity and quality of the T cell responses [117].

It is noteworthy that the hinge region in the immunoglobulin scaffold provides a flexible access for T-cell binding in contrast to the conventional rigid Streptavidin moieties used in monomer oligomerization in the generation of class I and class II tetramers. Similarly, IgM takes a characteristic 'staple' conformation upon binding to antigen in physiologic conditions, and this structural tendency would ultimately assist the concurrent ligation of multiple TCRs.

Approaches based on enhancing the clustering and/or internalization of TCR–tetramer complexes are desirable and additional molecular tools which facilitate TCR multimerization on the cell surface and increase the overall avidity of interaction achieved in large complexes may lead to new avenues in the better characterization of low-affinity T lymphocytes and the isolation of those populations.

5.6 Rationale for insect expression

S2 *Drosophila* cells have conserved a homologue (heat shock cognate protein: hsc72) of the immunoglobulin binding chaperone protein (BiP). Hsc72 was shown to chaperone the secretion of mature and conformationally active IgG, with a production efficiency of 1μg/ml (by ELISA) [103]. In addition, our laboratory and others have used insect expression systems for the production of MHC Class II monomers [69].

We based our hypothesis on the above observations, and undertook expression of IgM based multimers in the Drosophila expression system. Since, we have introduced several modifications to support and optimize production of a dodecameric molecule that measures over 1400 kDa.

5.7 Future Directions and Conclusion

5.7.1 Prime-boost immunization strategies and immune monitoring.

Studies are currently underway in mice, which include the study of T cell repertoire diversity following different combinations of prime/boost strategies using the vectors characterized in primary responses in Chapter 2, namely VSV-AV3 and Adenovirus serotype 5 expressing Gag A from HIV-1. Furthermore, we have adapted our multiparameter functional assays to murine samples, and functional signatures following immunization will be explored using a combination of proliferation and intracellular staining assays.

In addition, we have established the protocol and necessary logistics and ethical requirements to be able to perform vaccinia challenge in immunized HLA A2DR mice to assess ensuing protection. Other studies that are planned include the longitudinal study of memory generation (including adoptive transfer of antigen-specific cells into naïve transgenics) and the delineation of different murine CD4 memory subsets, an area that so far has not been fully ventured.

This mouse model is also being exploited as a model to validate immune monitoring tools, such as HLA-DR1 tetramers presenting different epitopes from HIV. Given the fact that these mice responded to common immunodominant epitopes indentified in human subjects, we are in the process of establishing immortalized clones from splenocytes of Gag immunized groups, for quality control of our in house MHC Class II tetramer production. These clones will also be useful in the validation of the novel multimeric reagents developed in Chapter 3, namely the p24-5 DR1*IgM dodecamer. The efficacy of these reagents will be tested *versus* conventional MHC Class II tetramers.

5.7.2 Large-scale production and validation of multivalent MHC*IgM

As mentioned above, the HLA-A2DR mouse model will be of valuable use in the validation and optimization of the multivalent MHC reagent developed in Chapter 3.

Since its development and production in insect Drosophila system, we have generated an array of constructs incorporating both variants of the murine immunoglobulin, that is in the presence or absence of the variable domains of the heavy and light chains of IgM. We have further tested these constructs in several systems, including a *Baculovirus* expression system, and eukaryotic 293T and 293E expression systems, using vectors such as pcDNA3 (Figure S8 and S9 in Appendix) and SRalpha (Figure S6-S10 in Appendix).

A visual summary of these approaches is presented in the subsequent Appendix section of this thesis.

Briefly, in collaboration with BD Biosciences, Pharmingen, we have cloned the respective chimeric chains into the Baculovirus *2p10* expression vector, which similarly to the Drosophila system, incorporates both chains within a single vector. We have successfully up-scaled production of the chimeric protein in the BD in-house high-throughput Baculovirus system (Figure S5 in Appendix). On the other hand, in the mammalian expression system we have adopted an approach of co-transfection using dual vectors, each expressing either the chimeric DRα-IgMμ or pep-DRβ-IgMκ chains (Fig. S6-S10 in Appendix).

The reagents developed through the different systems above are yet to be evaluated for their valency, conformation, stability, and function. The biochemical complexity of this biomolecule requires fine control along each step of production and purification. Furthermore we are in the process of testing, standardizing, and optimizing direct staining protocols of IgM-MHC class II oligomers on T cell lines and primary cells. In collaboration with BD Biosciences we are also considering approaches for direct coupling of this

multivalent molecule to fluorochromes to allow performance of single step direct visualization assays.

In conclusion, the MHC multimeric tools developed in the framework of this project will permit the identification and characterization of antigen-specific CD4 T cells bearing low affinity TCR against peptide/MHC complexes, the characterization of peptide epitopes derived from cDNA library targeted by major histocompatibility complex (MHC)-restricted CD4 T cells of unknown specificity, and the *ex-vivo* tailoring of the quality and of T cell responses.

Once established, we would test these novel multimers in the context of acute antiviral immune responses and adapt conditions for use in the analysis of mouse or human samples.

6 Reference List

1. Kawai, T. & Akira, S. Pathogen recognition with Toll-like receptors. *Curr. Opin. Immunol.* **17**, 338-344 (2005).

2. Levashina, E.A., Moita, L.F., Blandin, S., et al. Conserved role of a complement-like protein in phagocytosis revealed by dsRNA knockout in cultured cells of the mosquito, Anopheles gambiae. *Cell* **104**, 709-718 (2001).

3. Christophides, G.K., Vlachou, D., & Kafatos, F.C. Comparative and functional genomics of the innate immune system in the malaria vector Anopheles gambiae. *Immunol. Rev.* **198**, 127-148 (2004).

4. Pantaleo, G. & Koup, R.A. Correlates of immune protection in HIV-1 infection: what we know, what we don't know, what we should know. *Nat Med* **10**, 806-810 (2004).

5. Kraehenbuhl, J.P. & Neutra, M.R. Epithelial M Cells: Differentiation and function. *Annu. Rev. Cell Dev. Biol.* **16**, 301-332 (2000).

6. Nonaka, M. & Yoshizaki, F. Primitive complement system of invertebrates. *Immunol. Rev.* **198**, 203-215 (2004).

7. Lemaitre, B., Nicolas, E., Michaut, L., Reichhart, J.M., & Hoffmann, J.A. The Dorsoventral regulatory gene cassette spatzle/Toll/cactus controls the potent antifungal response in Drosophila adults. *Cell* **86**, 973-983 (1996).

8. Medzhitov, R., Preston-Hurlburt, P., & Janeway, C.A., Jr. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature* **388**, 394-397 (1997).

9. O'Neill, L.A. Therapeutic targeting of Toll-like receptors for inflammatory and infectious diseases. *Current Opinion in Pharmacology* **3**, 396-403 (2003).

10. Pantaleo, G. & Harari, A. Functional signatures in antiviral T-cell immunity for monitoring virus-associated diseases. *Nat Rev Immunol* **6**, 417-423 (2006).

11. Smith, P.L., Lombardi, G., & Foster, G.R. Type I interferons and the innate immune response--more than just antiviral cytokines. *Mol. Immunol.* **42**, 869-877 (2005).

12. Flajnik, M.F. & Kasahara, M. Comparative genomics of the MHC: glimpses into the evolution of the adaptive immune system. *Immunity* **15**, 351-362 (2001).

13. Mayer, W.E., Uinuk-ool, T., Tichy, H., Gartland, L.A., Klein, J., & Cooper, M.D. Isolation and characterization of lymphocyte-like cells from a lamprey. *PNAS* **99**, 14350-14355 (2002).

14. Uinuk-ool, T., Mayer, W.E., Sato, A., Dongak, R., Cooper, M.D., & Klein, J. Lamprey lymphocyte-like cells express homologs of genes involved in immunologically relevant activities of mammalian lymphocytes. *PNAS* **99**, 14356-14361 (2002).

15. Abi-Rached, L., Gilles, A., Shiina, T., Pontarotti, P., & Inoko, H. Evidence of en bloc duplication in vertebrate genomes. *Nat Genet* **31**, 100-105 (2002).

16. Chaplin, D.D. 1. Overview of the human immune response. *Journal of Allergy and Clinical Immunology* **117**, S430-S435 (2006).

17. Klein, J. & Sato, A. The HLA System- Second of two parts. *The New England Journal of Medicine* **343**, 782-786 (2000).

18. Klein, J. & Sato, A. The HLA System- First of two parts. *The New England Journal of Medicine* **343**, 702-709 (2000).

19. Kumanovics, A., Takada, T., & Lindahl, K.F. Genomic organization of the mammalian MHC. *Annual Review of Immunology* **21**, 629 (2003).

20. Fischer Lindahl, K. On naming H2 haplotypes: functional significance of MHC class Ib alleles. *Immunogenetics* **46**, 53-62 (1997).

21. Pieters, J. MHC class II-restricted antigen processing and presentation. *Adv. Immunol* **75:159-208.**, 159-208 (2000).

22. Alfonso, C. & Karlsson, L. Nonclassical MHC Class II molecules. *Annual Review of Immunology* **18**, 113-142 (2000).

23. Douek, D.C. & Altmann, D.M. HLA-DO is an intracellular class II molecule with distinctive thymic expression. *Int. Immunol* **9**, 355-364 (1997).

24. Engelhard, V.H. Structure of peptides associated with class I and class II MHC molecules. *Annu. Rev. Immunol* **12:181-207.**, 181-207 (1994).

25. Rudensky, A.Y., Preston-Hurlburt, P., Hong, S.C., Barlow, A., & Janeway, C.A., Jr. Sequence analysis of peptides bound to MHC class II molecules. *Nature* **353**, 622-627 (1991).

26. Villadangos, J.A. Presentation of antigens by MHC class II molecules: getting the most out of them. *Mol. Immunol.* **38**, 329-346 (2001).

27. Milner CM, C.RD. Genetic organization of the human MHC class III region. *Frontiers in bioscience* **6**, D914 (2001).

28. Hathaway, L.J. & Kraehenbuhl, P. The role of M cells in mucosal immunity. *Cellular and Molecular Life Sciences (CMLS)* **57**, 323-332 (2000).

29. Kraehenbuhl, J.P. & Neutra, M.R. Molecular and cellular basis of immune protection of mucosal surfaces. *Physiol. Rev.* **72**, 853-879 (1992).

30. Kiyono H. & Fukuyama S. NALT- versus Peyer's-Patch - mediated mucosal immunity. *Nature Rev. Immunol* **4**, 699-710 (2004).

31. Garside, P., Millington O., & Smith K.M. The Anatomy of mucosal immune responses. *Ann NY Acad Sci* **1029**, 9-15 (2004).

32. Frankel, A.D. & Young, J.A. HIV-1: fifteen proteins and an RNA. *Annu. Rev. Biochem.* **67:1-25.**, 1-25 (1998).

33. Wahren, B., Ljungberg, K., Rollman, E., Levi, M., et al. HIV subtypes and recombination strains-strategies for induction of immune responses in man. *Vaccine* **20**, 1988-1993 (2002).

34. Wainberg, M.A. HIV-1 subtype distribution and the problem of drug resistance. *AIDS* **18** , S63-S68 (2004).

35. Kulkarni, P.S., Butera, S.T., & Duerr, A.C. Resistance to HIV-1 infection: lessons learned from studies of highly exposed persistently seronegative (HEPS) individuals. *AIDS Rev.* **5**, 87-103 (2003).

36. Younes, S.A., Yassine-Diab, B., Dumont, A.R., Boulassel, M.R., Grossman, Z., Routy, J.P., & Sekaly, R.P. HIV-1 viremia prevents the establishment of Interleukin 2-producing HIV-specific Memory CD4+ T Cells Endowed with Proliferative Capacity. *The Journal of Experimental Medicine* **198**, 1909-1922 (2003).

37. Harari, A., Dutoit, V., Cellerai, C., Bart, P.A., Du Pasquier, R.A., & Pantaleo, G. Functional signatures of protective antiviral T-cell immunity in human virus infections. *Immunol. Rev.* **211**, 236-254 (2006).

38. Burgers, W.A. & Williamson, C. The challenges of HIV vaccine development and testing. *Best Practice & Research Clinical Obstetrics & Gynaecology* **19**, 277-291 (2005).

39. Amara, R.R. & Robinson, H.L. A new generation of HIV vaccines. *Trends in Molecular Medicine* **8**, 489-495 (2002).

40. Pincus, S., Tartaglia, J., & Paoletti, E. Poxvirus-Based Vectors as Vaccine Candidates. *Biologicals* **23**, 159-164 (1995).

41. Santra, S., Seaman, M.S., Xu, L., Barouch, D.H., et al. Replication-Defective Adenovirus Serotype 5 Vectors Elicit Durable Cellular and Humoral Immune Responses in Nonhuman Primates. *The Journal of Virology* **79**, 6516-6522 (2005).

42. Dumont, A.R., Kalfayan, L.H., & Sekaly, R.P. Modulation of immune responses - strategies for optimising vaccines. *Expert Opinion on Biological Therapy* **4**, 627-630 (2004).

43. Woodland, D.L. Jump-starting the immune system: prime-boosting comes of age. *Trends in Immunology* **25**, 98-104 (2004).

44. Yuki Y. & Kiyono, H. New generation of mucosal adjuvants for the induction of protective immunity. *Rev. Med. Virol.* **13**, 293-310 (2003).

45. Holmgren, J. & Czerkinsky, C. Mucosal immunity and vaccines. *Nat Med* (2005).

46. Ryan, E.J., Daly L.M., & Mills K.H.G. Immunomodulators and delivery systems for vaccination by mucosal routes. *Trends in Biotechnology* **19**, 293-304 (2001).

47. Lefrancois, L. & Puddington, L. Intestinal and pulmonary mucosal T cells: Local Heroes Fight to Maintain the Status Quo. *Annual Review of Immunology* **24**, 681-704 (2006).

48. Mestas, J. & Hughes, C.C.W. Of Mice and Not Men: Differences between Mouse and Human Immunology. *The Journal of Immunology* **172**, 2731-2738 (2004).

49. Haley, P.J. Species differences in the structure and function of the immune system. *Toxicology* **188**, 49-71 (2003).

50. Gordon, J., Grafton, G., Wood, P.M., Larche, M., & Armitage, R.J. Modelling the human immune response: can mice be trusted? *Current Opinion in Pharmacology* **1**, 431-435 (2001).

51. Gregersen, J.W., Holmes, S., & Fugger, L. Humanized animal models for autoimmune diseases. *Tissue Antigens* **63**, 383-394 (2004).

52. Taneja, V. & David, C.S. HLA class II transgenic mice as models of human diseases. *Immunol. Rev.* **169**, 67-79 (1999).

53. Taneja, V. & David, C.S. HLA Transgenic Mice as Humanized Mouse Models of Disease and Immunity. *J. Clin. Invest.* **101**, 921-926 (1998).

54. Bercovici, N., Duffour, M.T., Agrawal, S., Salcedo, M., & Abastado, J.P. New Methods for Assessing T-Cell Responses. *Clinical and Vaccine Immunology* 7, 859-864 (2000).

55. Maecker, H.T. & Maino, V.C. T Cell Immunity to HIV: Defining Parameters of Protection. *Current HIV Research* **1**, 249-259 (2003).

56. He, X.S., Rehermann, B., Boisvert, J., Mumm, J., et al. Direct functional analysis of epitope-specific CD8+ T cells in peripheral blood. *Viral Immunol* **14**, 59-69 (2001).

57. Kalyuzhny, A.E. Chemistry and biology of the ELISPOT assay. *Methods Mol Biol.* **302**, 15-31 (2005).

58. Letsch, A. & Scheibenbogen, C. Quantification and characterization of specific T-cells by antigen-specific cytokine production using ELISPOT assay or intracellular cytokine staining. *Methods* **31**, 143-149 (2003).

59. Morgan, E., Varro, R., Sepulveda, H., Ember, J.A., et al. Cytometric bead array: a multiplexed assay platform with applications in various areas of biology. *Clinical Immunology* **110**, 252-266 (2004).

60. K.L.Barlow, J.Green, & J.P.Clewley. Viral genome characterisation by the heteroduplex mobility and heteroduplex tracking assays. *Reviews in Medical Virology* **10**, 321-335 (2000).

61. Soudeyns, H., Campi, G., Rizzardi, G.P., et al. Initiation of antiretroviral therapy during primary HIV-1 infection induces rapid stabilization of the T-cell receptor beta chain repertoire and reduces the level of T-cell oligoclonality. *Blood* **95**, 1743-1751 (2000).

62. Bercovici, N., Delon, J., Cambouris, C., Escriou, N., Debre, P., & Liblau, R.S. Chronic intravenous injections of antigen induce and maintain tolerance in T cell receptor-transgenic mice. *Eur. J. Immunol.* **29**, 345-354 (1999).

63. Ria F., van den Elzen P., Madakamutil L.T., Miller J.E., Maverakis E., & Sercarz E.E. Molecular Characterization of the T Cell Repertoire Using Immunoscope Analysis and its Possible Implementation in Clinical Practice. *Current Molecular Medicine* **1**, 297-304 (2001).

64. Hernandez-Fuentes, M.P., Warrens, A.N., & Lechler, R.I. Immunologic monitoring. *Immunol. Rev.* **196**, 247-264 (2003).

65. Dunbar, P.R. & Ogg, G.S. Oligomeric MHC molecules and their homologues: state of the art. *Journal of Immunological Methods* **268**, 3-7 (2002).

66. Klenerman, P., Cerundolo, V., & Dunbar,P.R. Tracking T cells with tetramers: new tales from new tools. *Nature Rev. Immunol* **2**, 263-272 (2002).

67. Altman, J.D., Moss, P.A.H., Goulder, P.J.R., Barouch, D.H., et al. Phenotypic analysis of antigen-specific T lymphocytes. *Science* **274**, 94-96 (1996).

68. Ogg,G.S. & McMichael,A.J. HLA-peptide tetrameric complexes. *Curr. Opin. Immunol* **10**, 393-396 (1998).

69. Cameron,T.O., Norris,P.J., Patel,A., Moulon,C., Rosenberg,E.S., Mellins,E.D., Wedderburn,L.R., & Stern,L.J. Labeling antigen-specific CD4+ T cells with class II MHC oligomers. *Journal of Immunological Methods* **268**, 51-69 (2002).

70. Cameron,T.O., Cochran,J.R., Yassine-Diab,B., Sekaly,R.P., & Stern,L.J. Cutting edge: detection of antigen-specific CD4+ T cells by HLA-DR1

oligomers is dependent on the T cell activation state. *J. Immunol.* **166**, 741-745 (2001).

71. Kalandadze,A., Galleno,M., Foncerrada,L., Strominger,J.L., & Wucherpfennig,K.W. Expression of Recombinant HLA-DR2 Molecules. Replacement of the hydrophobic transmembrane region by a leucine zipper dimerization motif allows the assembly and secretion of soluble DR alpha beta heterodimers. *J. Biol. Chem.* **271**, 20156-20162 (1996).

72. Kwok,W.W., Ptacek,N.A., Liu,A.W., & Buckner,J.H. Use of class II tetramers for identification of CD4+ T cells. *Journal of Immunological Methods* **268**, 71-81 (2002).

73. Nepom,G.T., Buckner,J.H., Novak,E.J., Reichstetter,S., Reijonen,H., Gebe,J., Wang,R., Swanson,E., & Kwok,W.W. HLA class II tetramers: tools for direct analysis of antigen-specific CD4+ T cells. *Arthritis Rheum.* **46**, 5-12 (2002).

74. Hugues,S., Malherbe,L., Filippi,C., & Glaichenhaus,N. Generation and use of alternative multimers of peptide/MHC complexes. *Journal of Immunological Methods* **268**, 83-92 (2002).

75. Fahmy,T.M., Bieler,J.G., & Schneck,J.P. Probing T cell membrane organization using dimeric MHC-Ig complexes. *Journal of Immunological Methods* **268**, 93-106 (2002).

76. Batard,P., Peterson,D.A., Devevre,E., Guillaume,P., Cerottini,J.C., Rimoldi,D., Speiser,D.E., Winther,L., & Romero,P. Dextramers: New generation of fluorescent MHC class I/peptide multimers for visualization of antigen-specific CD8+ T cells. *Journal of Immunological Methods* **310**, 136-148 (2006).

77. Giannoni,F., Barnett,J., Bi,K., Samodal,R., Lanza,P., Marchese,P., Billetta,R., Vita,R., Klein,M.R., Prakken,B., Kwok,W.W., Sercarz,E., Altman,A., & Albani,S. Clustering of T cell ligands on artificial APC membranes influences T Cell activation and protein kinase C {theta} translocation to the T cell plasma membrane. *The Journal of Immunology* **174**, 3204-3211 (2005).

78. Piot,P., Bartos,M., Ghys,P.D., Walker,N., & Schwartlander,B. The global impact of HIV/AIDS. *Nature* **410**, 968-973 (2001).

79. Smith P.D., Li L., & Meng G. Mucosal Events in the Pathogenesis of Human Immunodeficiency Virus Type 1 Infection . *The Journal of Infectious Diseases* **179**, S436-S440 (1999).

80. Belyakov,I.M. & Berzofsky,J.A. Immunobiology of Mucosal HIV Infection and the Basis for Development of a New Generation of Mucosal AIDS Vaccines. *Immunity* **20**, 247-253 (2004).

81. Belyakov,I.M., Hel,Z., Kelsall,B., Kuznetsov,V.A., Ahlers,J.D., Nacsa,J., Watkins,D.I., Allen,T.M., Sette,A., Altman,J., Woodward,R.,

Markham,P.D., Clements,J.D., Franchini,G., Strober,W., & Berzofsky,J.A. Mucosal AIDS vaccine reduces disease and viral load in gut reservoir and blood after mucosal infection of macaques. *Nat. Med.* **7**, 1320-1326 (2001).

82. Belyakov,I.M., Ahlers,J.D., Clements,J.D., Strober,W., & Berzofsky,J.A. Interplay of cytokines and adjuvants in the regulation of mucosal and systemic HIV-specific CTL. *J. Immunol.* **165**, 6454-6462 (2000).

83. Ogra,P.L., Faden,H., & Welliver,R.C. Vaccination Strategies for Mucosal Immune Responses. *Clin. Microbiol. Rev.* **14**, 430-445 (2001).

84. Lemiale,F., Kong,W.p., Akyurek,L.M., Ling,X., Huang,Y., Chakrabarti,B.K., Eckhaus,M., & Nabel,G.J. Enhanced Mucosal Immunoglobulin A Response of Intranasal Adenoviral Vector Human Immunodeficiency Virus Vaccine and Localization in the Central Nervous System. *The Journal of Virology* 77, 10078-10087 (2003).

85. Gherardi,M.M., Perez-Jimenez,E., Najera,J.L., & Esteban,M. Induction of HIV Immunity in the Genital Tract After Intranasal Delivery of a MVA Vector: Enhanced Immunogenicity After DNA Prime-Modified Vaccinia Virus Ankara Boost Immunization Schedule. *The Journal of Immunology* **172**, 6209-6220 (2004).

86. Vitiello,A., Marchesini,D., Furze,J., Sherman,L.A., & Chesnut,R.W. Analysis of the HLA-restricted influenza-specific cytotoxic T lymphocyte response in transgenic mice carrying a chimeric human-mouse class I major histocompatibility complex. *The Journal of Experimental Medicine* **173**, 1007-1015 (1991).

87. Irwin,M.J., Heath,W.R., & Sherman,L.A. Species-restricted interactions between CD8 and the alpha 3 domain of class I influence the magnitude of the xenogeneic response. *The Journal of Experimental Medicine* **170**, 1091-1101 (1989).

88. Woods, A., Chen, H.Y., Trumbauer, M.E., Sirotina,A., Cummings,R., & Zaller,D.M. Human major histocompatibility complex class II-restricted T cell responses in transgenic mice. *The Journal of Experimental Medicine* **180**, 173-181 (1994).

89. Yamamoto,K., Fukui,Y., Esaki,Y., Inamitsu,T., Sudo,T., Yamane,K., Kamikawaji,N., Kimura,A., & Sasazuki,T. Functional interaction between human histocompatibility leukocyte antigen (HLA) class II and mouse CD4 molecule in antigen recognition by T cells in HLA-DR and DQ transgenic mice. *The Journal of Experimental Medicine* **180**, 165-171 (1994).

90. Rosloniec,E.F., Brand,D.D., Myers,L.K., Whittington,K.B., Gumanovskaya,M., Zaller,D.M., Woods,A., Altmann,D.M., Stuart,J.M., & Kang,A.H. An HLA-DR1 Transgene Confers Susceptibility to

Collagen-induced Arthritis Elicited with Human Type II Collagen. *The Journal of Experimental Medicine* **185**, 1113-1122 (1997).

91. Firat H., Garcia-Pons F., Tourdot S., Pascolo S, Scardino A, Garcia Z, Michel ML, Jack R, Jung W, Kosmatopoulos K, Mateo L, Suhrbier A, Lemonnier F.A., & Langlade-Demoyen P. H-2 class I knockout, HLA-A2.1-transgenic mice: a versatile animal model for preclinical evaluation of antitumour immunotherapeutic strategies. *Eur. J. Immunol.* **29**, 3112-3121 (1999).

92. BenMohamed,L., Krishnan,R., Longmate,J., Auge,C., Low,L., Primus,J., & Diamond,D.J. Induction of CTL response by a minimal epitope vaccine in HLA A*0201/DR1 transgenic mice: dependence on HLA class II restricted TH response. *Human Immunology* **61** , 764-779 (2000).

93. Pajot A., Michel ML, Fazilleau,N., Pancre V., Auriault C., Ojcius,D.M., Lemonnier F.A., & Lone,Y.-C. A mouse model of human adaptive immune functions: HLA-A2.1-/HLA-DR1-transgenic H-2 class I-/class II-knockout mice. *Eur. J. Immunol.* **34**, 3060-3069 (2004).

94. McCarthy C., Youde S.J., & Man S. Definition of an HPV18/45 cross-reactive human T-cell epitope after DNA immunisation of HLA-A2/KB transgenic mice. *Int J Cancer* **118**, 2514-2521 (2006).

95. Lichty,B.D., Power,A.T., Stojdl,D.F., & Bell,J.C. Vesicular stomatitis virus: re-inventing the bullet. *Trends in Molecular Medicine* **10**, 210-216 (2004).

96. Ramsburg,E., Rose,N.F., Marx,P.A., Mefford,M., Nixon,D.F., Moretto,W.J., Montefiori,D., Earl,P., Moss,B., & Rose,J.K. Highly Effective Control of an AIDS Virus Challenge in Macaques by Using Vesicular Stomatitis Virus and Modified Vaccinia Virus Ankara Vaccine Vectors in a Single-Boost Protocol. *The Journal of Virology* **78**, 3930-3940 (2004).

97. Rose,N.F., Marx,P.A., Luckay,A., Nixon,D.F., Moretto,W.J., Donahoe,S.M., Montefiori,D., Roberts,A., Buonocore,L., & Rose,J.K. An Effective AIDS Vaccine Based on Live Attenuated Vesicular Stomatitis Virus Recombinants. *Cell* **106**, 539-549 (2001).

98. Haglund,K., Leiner,I., Kerksiek,K., Buonocore,L., Pamer,E., & Rose,J.K. Robust Recall and Long-Term Memory T-Cell Responses Induced by Prime-Boost Regimens with Heterologous Live Viral Vectors Expressing Human Immunodeficiency Virus Type 1 Gag and Env Proteins. *The Journal of Virology* **76**, 7506-7517 (2002).

99. Sumida,S.M., Truitt,D.M., Lemckert,A.A.C., Vogels,R., Custers,J.H.H.V., Addo,M.M., Lockman,S., Peter,T., Peyerl,F.W., Kishko,M.G., Jackson,S.S., Gorgone,D.A., Lifton,M.A., Essex,M., Walker,B.D., Goudsmit,J., Havenga,M.J.E., & Barouch,D.H. Neutralizing Antibodies to Adenovirus Serotype 5 Vaccine Vectors Are

Directed Primarily against the Adenovirus Hexon Protein. *The Journal of Immunology* **174**, 7179-7185 (2005).

100. Stone,J.D. & Stern,L.J. CD8 T Cells, Like CD4 T Cells, Are Triggered by Multivalent Engagement of TCRs by MHC-Peptide Ligands but Not by Monovalent Engagement. *The Journal of Immunology* **176**, 1498-1505 (2006).

101. Cochran,J.R., Cameron,T.O., & Stern,L.J. The relationship of MHC-peptide binding and T cell activation probed using chemically defined MHC class II oligomers. *Immunity.* **12**, 241-250 (2000).

102. Reichstetter,S., Ettinger,R.A., Liu,A.W., Gebe,J.A., Nepom,G.T., & Kwok,W.W. Distinct T Cell Interactions with HLA Class II Tetramers Characterize a Spectrum of TCR Affinities in the Human Antigen-Specific T Cell Response. *The Journal of Immunology* **165**, 6994-6998 (2000).

103. Kirkpatrick,R.B., Ganguly,S., Angelichio,M., Griego,S., Shatzman,A., Silverman,C., & Rosenberg,M. Heavy Chain Dimers as Well as Complete Antibodies Are Efficiently Formed and Secreted from Drosophila via a BiP-mediated Pathway. *J. Biol. Chem.* **270**, 19800-19805 (1995).

104. Chaplin D. D. Overview of the Immune System. *J Allergy Clin Immunol.* **111**(2 Suppl), S442-59 (2003).

105. Cheroutre H, Madakamutil L. Mucosal effector memory T cells: the other side of the coin. *Cell Mol Life Sci.* **62(23)**, 2853-66 (2005).

106. Gallo RC, Sarin PS, Gelmann EP, et al. Isolation of Human T- Cell Leukemia Virus in Acquired Immune Deficiency Syndrome (AIDS). *Science* **220**, 865-867 (1983).

107. Marx JL. Human T-Cell Leukemia Linked to AIDS. *Science* **220**,806-809 (1983).

108. Barre-Sinoussi F, Chermann JC, Rey F, et al. Isolation of a T-Lymphotrophic Retrovirus from a patient at Risk for Acquired Immune Deficiency Syndrome (AIDS). *Science* **220**, 868-871 (1983).

109. Dempsey PW, Vaidya SA, Cheng G. The art of war: Innate and adaptive immune responses. *Cell Mol Life Sci.* **60(12)**, 2604-21 (2003).

110. Belyakov IM, Berzofsky JA. Immunobiology of mucosal HIV infection and the basis for development of a new generation of mucosal AIDS vaccines. *Immunity* **20(3)**, 247-53 (2004).

111. Pajot A, Pancre V, Fazilleau N, Michel ML, et al. Comparison of HLA-DR1-restricted T cell response induced in HLA-DR1 transgenic mice deficient for murine MHC class II and HLA-DR1 transgenic mice

expressing endogenous murine MHC class II molecules. *Int Immunol.* **16(9)**, 1275-82 (2004).

112. Xu XN, Screaton GR. MHC/peptide tetramer-based studies of T cell function. *J Immunol Methods.* **268(1)**, 21-8 (2002).

113. Appay V, Rowland-Jones SL. The assessment of antigen-specific CD8+ T cells through the combination of MHC class I tetramer and intracellular staining. *J Immunol Methods.* **268(1)**, 9-19 (2002).

114. Lehner T, Bergmeier L, Wang Y, Tao L, Mitchell E. A rational basis for mucosal vaccination against HIV infection. *Immunol Rev.* **170**, 183-96 (1999).

115. Lehner T, Wang Y, Ping L, Bergmeier L, et al. The effect of route of immunization on mucosal immunity and protection. *J Infect Dis.* **179** Suppl 3, S489-92 (1999).

116. Medzhitov R. Toll-like receptors and innate immunity. *Nat Rev Immunol.* **1(2)**, 135-45 (2001).

117. Ge Q, Stone JD, Thompson MT, et al. Soluble peptide-MHC monomers cause activation of CD8+ T cells through transfer of the peptide to T cell MHC molecules. *Proc Natl Acad Sci U S A.* **99(21)**, 13729-34 (2002).

118. Trachtenberg E, Korber B, Sollars C, et al. Advantage of rare HLA supertype in HIV disease progression. *Nat Med.* **9(7)**, 928-35 (2003).

119. Choi,E.M., M.Palmowski, J.Chen, and V.Cerundolo. The use of chimeric A2Kb tetramers to monitor HLA A2 immune responses in HLA A2 transgenic mice. *Journal of Immunological Methods* **268,** 35-41 (2002).

120. Greten TF, Korangy F, Neumann G, Wedemeyer H, et al. Peptide-beta2-microglobulin-MHC fusion molecules bind antigen-specific T cells and can be used for multivalent MHC-Ig complexes. *J Immunol Methods.* **271(1-2)**, 125-35 (2002).

121. Yu YY, Netuschil N, Lybarger L, Connolly JM, Hansen TH. Cutting edge: single-chain trimers of MHC class I molecules form stable structures that potently stimulate antigen-specific T cells and B cells. *J Immunol.* **168(7)**, 3145-9 (2002).

122. Knabel M, Franz TJ, Schiemann M, Wulf A, et al. Reversible MHC multimer staining for functional isolation of T-cell populations and effective adoptive transfer. *Nat Med.* **8(6)**, 631-7 (2002).

123. Estcourt MJ, Letourneau S, McMichael AJ, Hanke T. Vaccine route, dose and type of delivery vector determine patterns of primary CD8+ T cell responses. *Eur J Immunol.* **35(9)**, 2532-40 (2005).

124. Harari A, Pantaleo G. Understanding what makes a good versus a bad vaccine. *Eur J Immunol.* **35(9)**, 2528-31 (2005).

125. Lambert PH, Liu M, Siegrist CA. Can successful vaccines teach us how to induce efficient protective immune responses? *Nat Med.* **11(4 Suppl)**, S54-62 (2005).

126. Levy JA. The importance of the innate immune system in controlling HIV infection and disease. *Trends Immunol.* 22(6), 312-6 (2001).

127. Bourinbaiar AS, Metadilogkul O, Jirathitikal V. Mucosal AIDS vaccines. *Viral Immunol.* **16(4)**, 427-45 (2003).

128. Cheroutre H. Starting at the beginning: new perspectives on the biology of mucosal T cells. *Annu Rev Immunol.* **22**, 217-46 (2004).

129. Cheroutre H, Madakamutil L. Acquired and natural memory T cells join forces at the mucosal front line. *Nat Rev Immunol.* **4(4)**, 290-300 (2004).

130. Strober W, Fuss IJ, Blumberg RS. The immunology of mucosal models of inflammation. *Annu Rev Immunol.* **20**, 495-549 (2002).

131. Greten TF, Schneck JP. Development and use of multimeric major histocompatibility complex molecules. *Clin Diagn Lab Immunol.* **9(2)**, 216-20 (2002).

132. Perfetto SP, Chattopadhyay PK, Roederer M.Seventeen-colour flow cytometry: unravelling the immune system. *Nat Rev Immunol.* **4(8)**, 648-55 (2004).

133. Tan GS, McKenna PM, Koser ML, et al. Strong cellular and humoral anti-HIV Env immune responses induced by a heterologous rhabdoviral prime-boost approach. *Virology.* **331(1)**, 82-93 (2005).

134. Epstein H, Hardy R, May JS, Johnson MH, Holmes N. Expression and function of HLA-A2.1 in transgenic mice. *Eur J Immunol.* **19(9)**, 1575-83 (1989).

135. Depil S, Angyalosi G, Morales O, Delacre M, et al. Peptide-binding assays and HLA II transgenic Abeta degrees mice are consistent and complementary tools for identifying HLA II-restricted peptides. *Vaccine.* **24(13)**, 2225-9 (2006).

136. Fournillier A, Dupeyrot P, Martin P, Parroche P, et al. Primary and memory T cell responses induced by hepatitis C virus multiepitope long peptides. *Vaccine.* **24(16)**, 3153-64 (2006).

137. Cockrell AS, Kafri T. HIV-1 vectors: fulfillment of expectations, further advancements, and still a way to go. *Curr HIV Res.* **1(4)**, 419-39 (2003).

138. van Maanen M, Sutton RE. Rodent models for HIV-1 infection and disease. *Curr HIV Res.* **1(1)**, 121-30 (2003).

APPENDIX

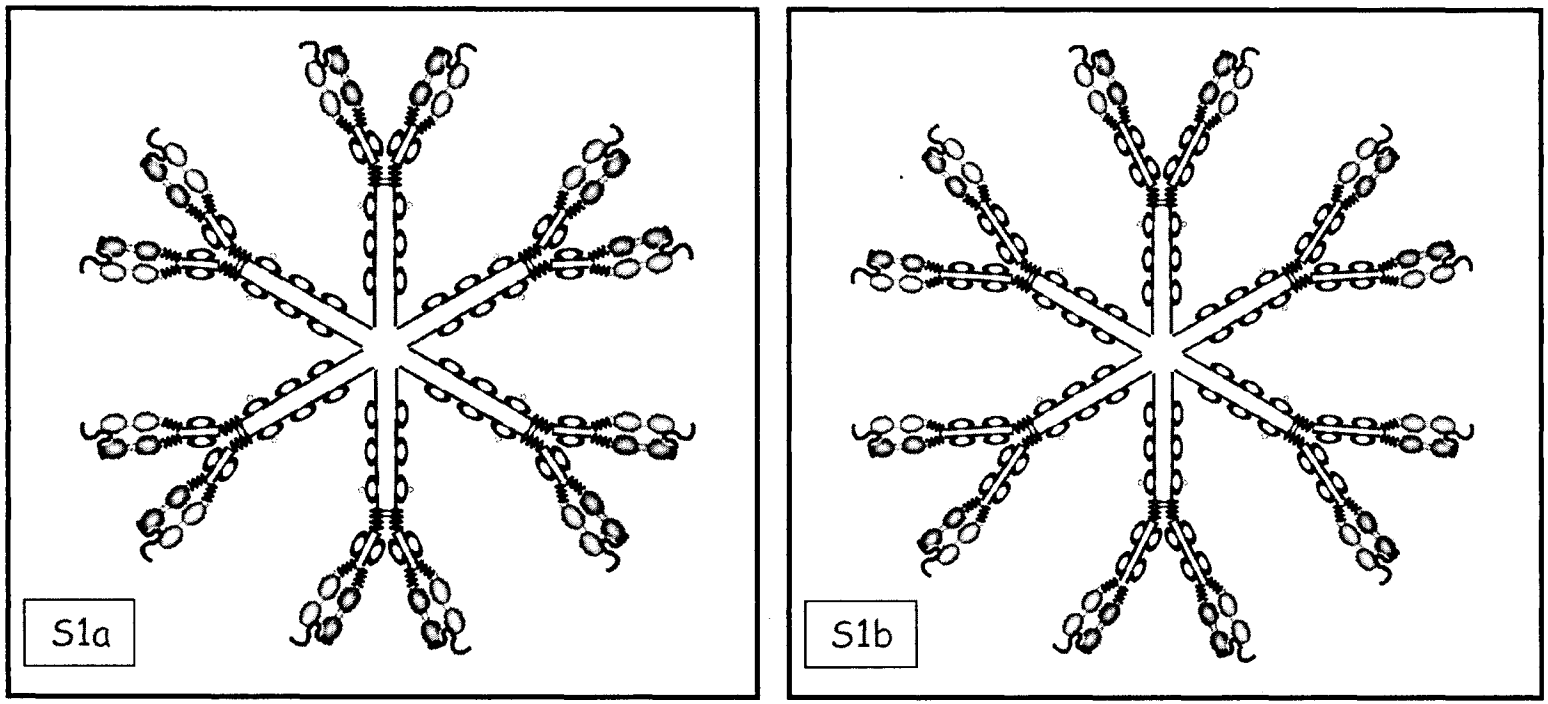
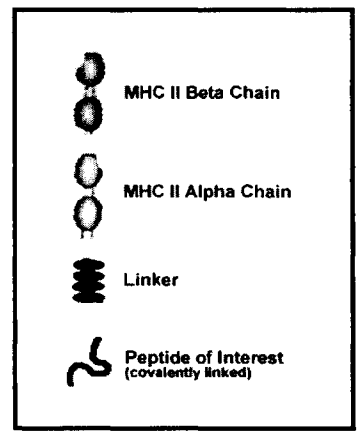


Figure S1. Schematic presentation of mIgM*MHCII dodecamer

S1a. Cartoon of MHCII*IgM constant construct (without variable domain)
S1b. Cartoon of MHCII*IgM construct including variable domain

S1c. Western Blot with monoclonal anti-IgM heavy chain



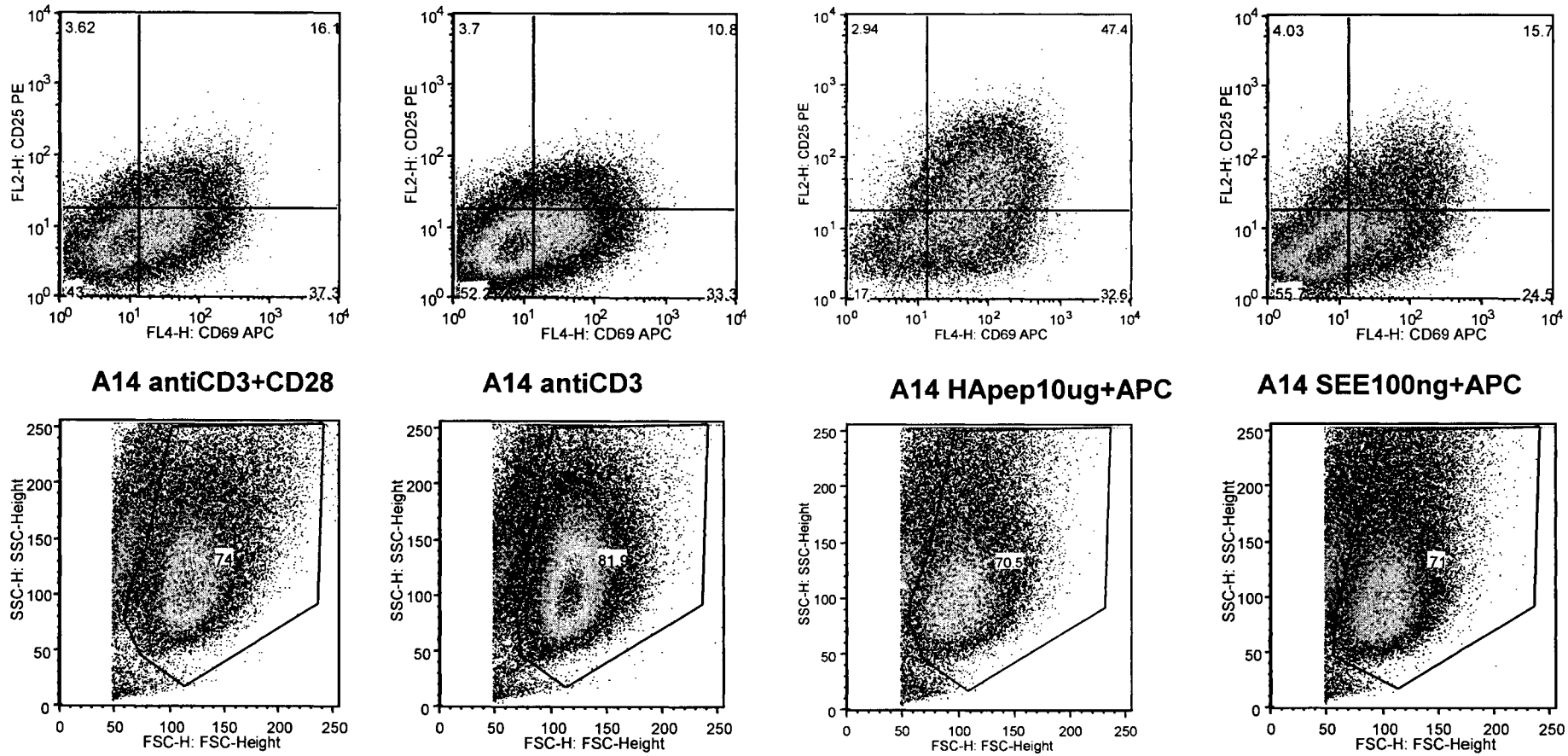


Figure S2. Stimulation Controls of Jurkat A14 T cells with HA peptide in presence of APCs

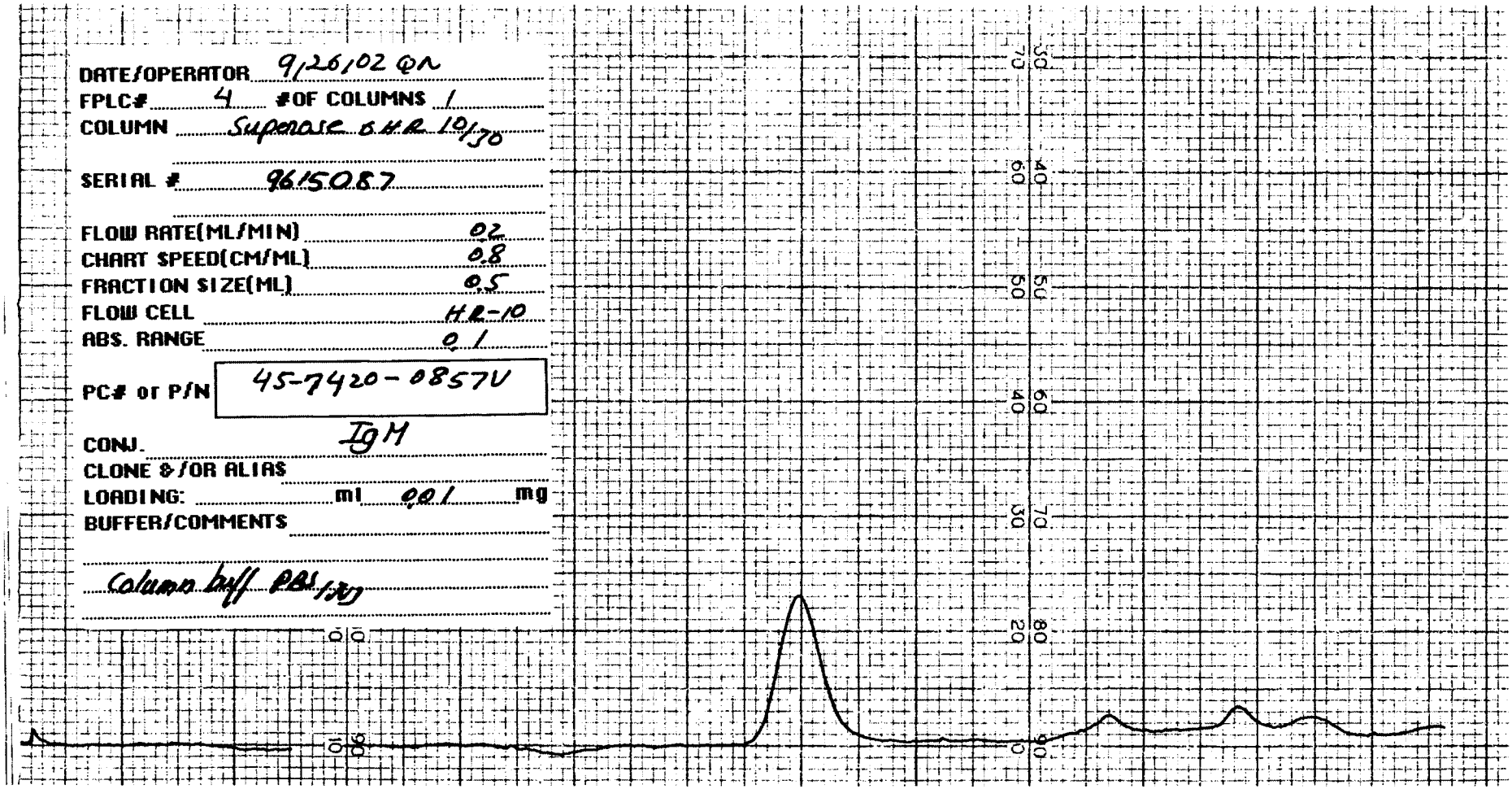


Figure S3. Gel Filtration Chromatography (FPLC Superose6) on control pentameric mouse IgM

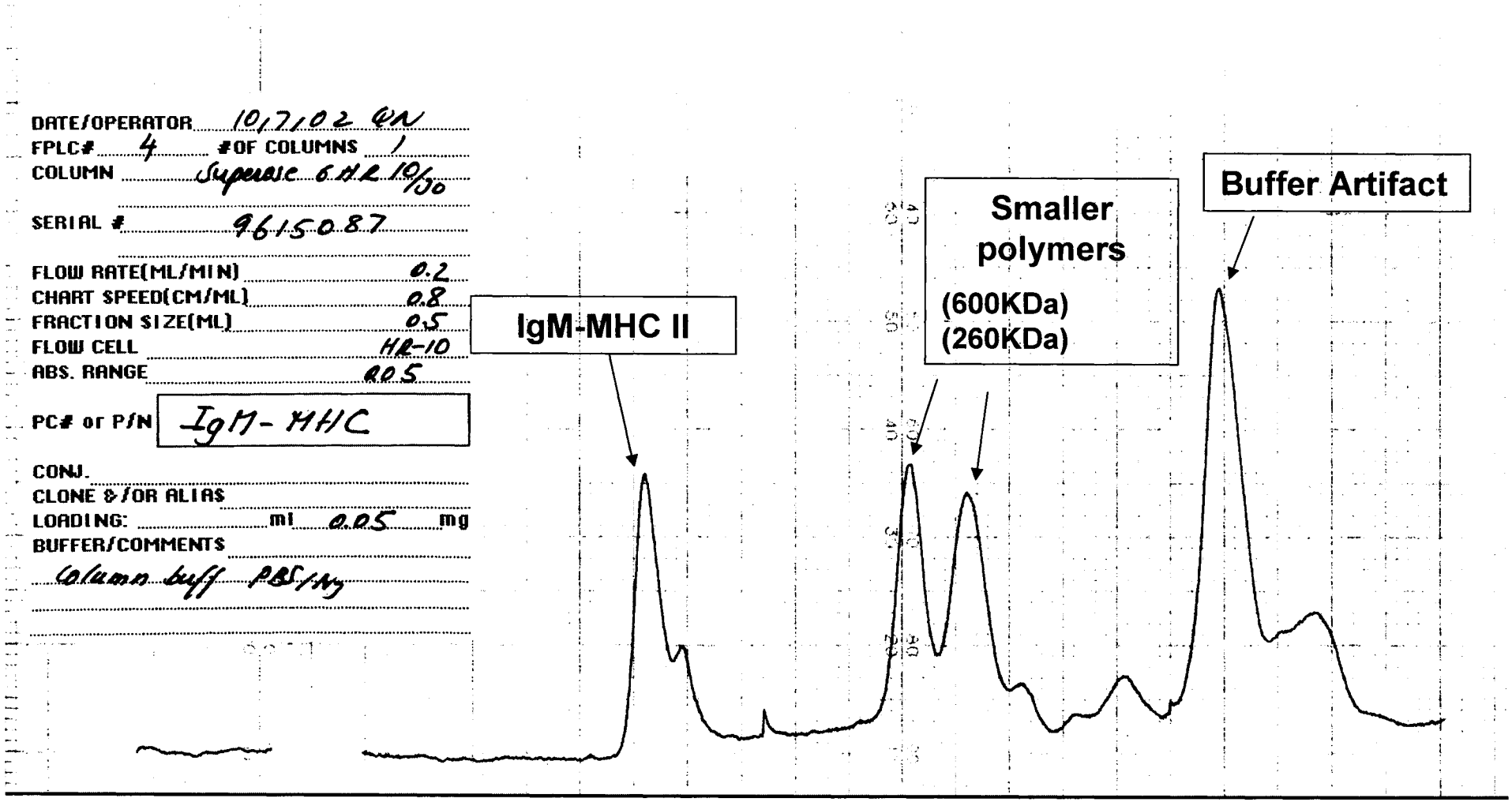
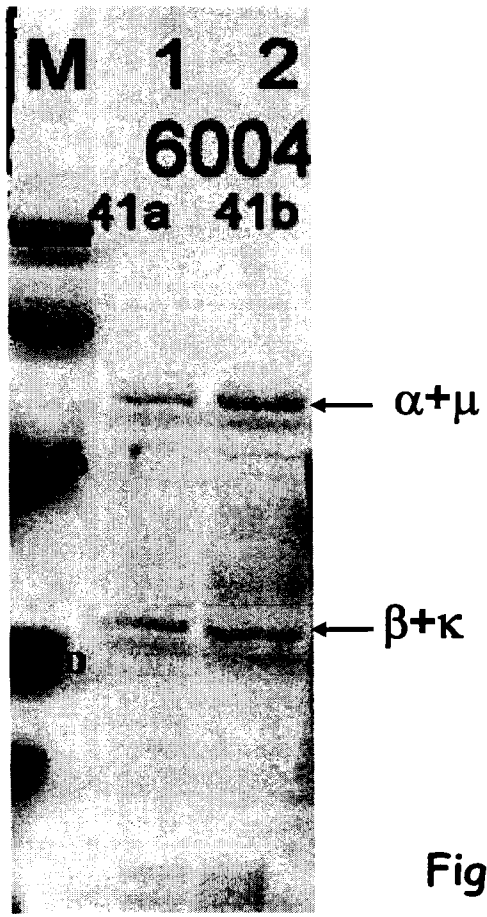


Figure S4. Gel Filtration Chromatography (FPLC Superose6) on mouse IgM-MHC II DR1 Chimera



- Expression using optimized in house Baculovirus shuttle vector established at BD/Pharmingen.
- Lane 1 and Lane 2: heavy and light chain fusion proteins of mIgM*MHCII constant construct (2 clones) as shown on SDS-PAGE.

Figure S5. Coomassie Blue staining of mIgM*DR1 expressed in *Baculovirus* expression system.

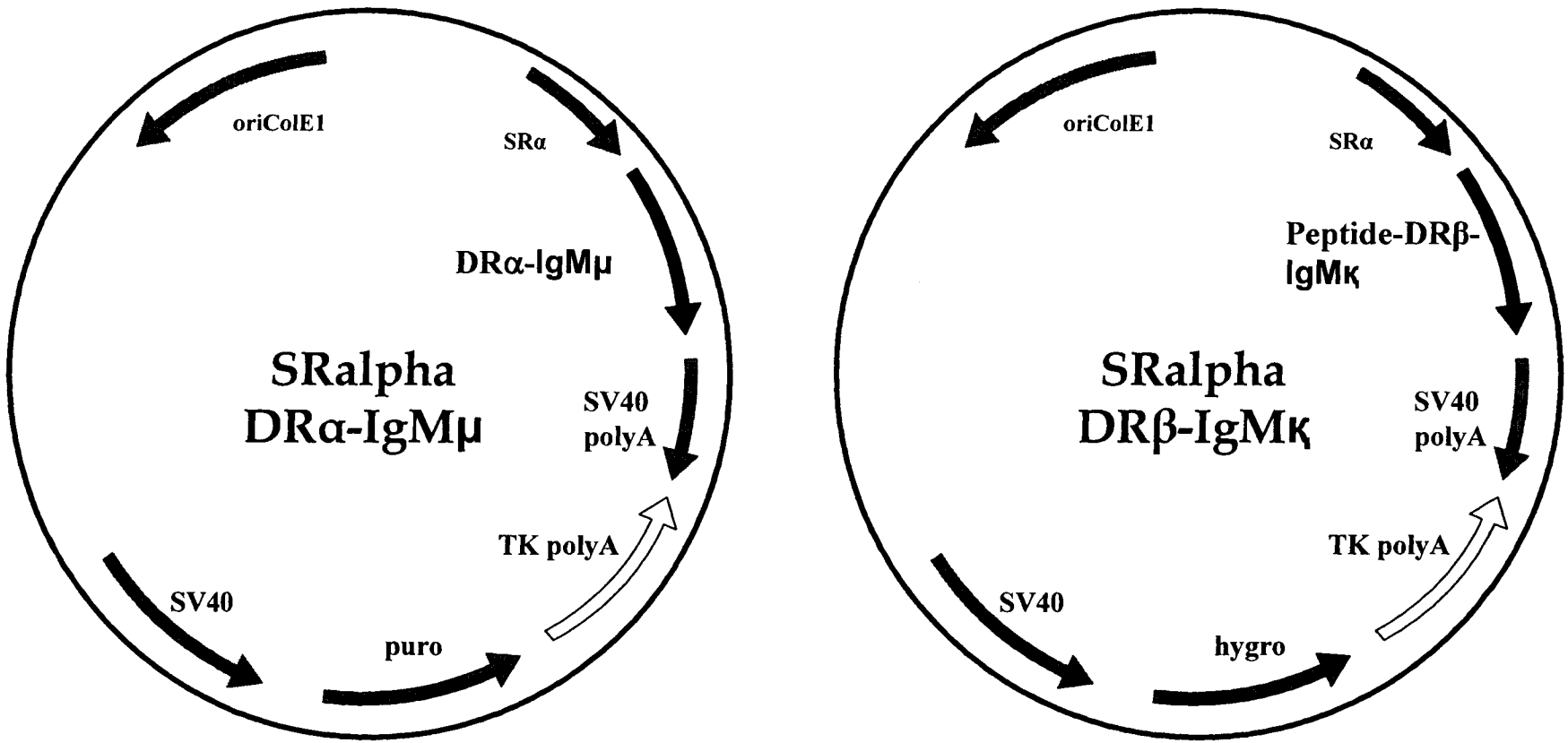


Figure S6. Clones of IgM*DR1 chimeric chains in SRalpha mammalian expression system.

The SRalpha promoter is composed of SV40 early promoter/enhancer & the R-U5' of the LTR of the HTLV-1.

Comments for pcDNA3
5446 nucleotides

CMV promoter: bases 209-863
T7 promoter: bases 864-882
Polylinker: bases 889-994
Sp6 promoter: bases 999-1016
BGH poly A: bases 1018-1249
SV40 promoter: bases 1790-2115
SV40 origin of replication: bases 1984-2069
Neomycin ORF: bases 2151-2945
SV40 poly A: bases 3000-3372
ColE1 origin: bases 3632-4305
Ampicillin ORF: bases 4450-5310

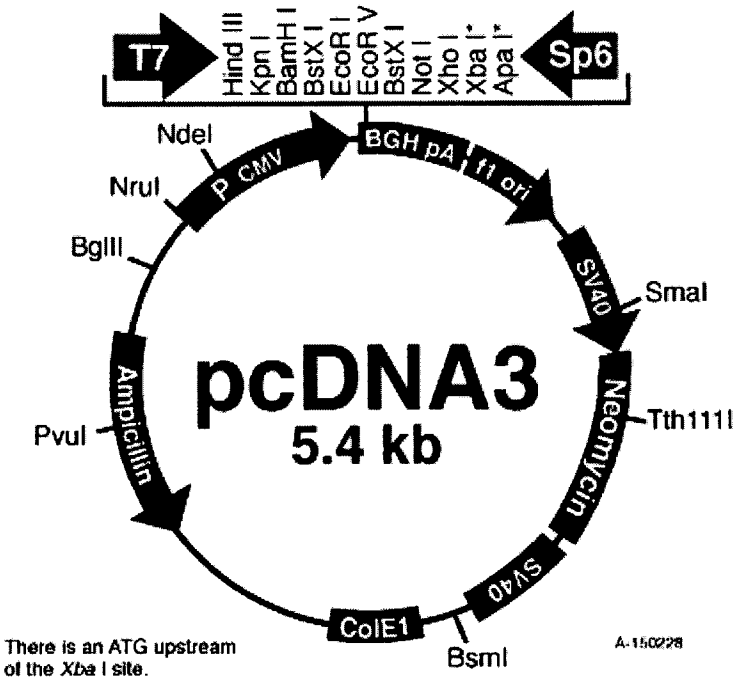


Figure S7. pcDNA3 mammalian expression system.

Adapted from:

Invitrogen™
life technologies

www.invitrogen.com

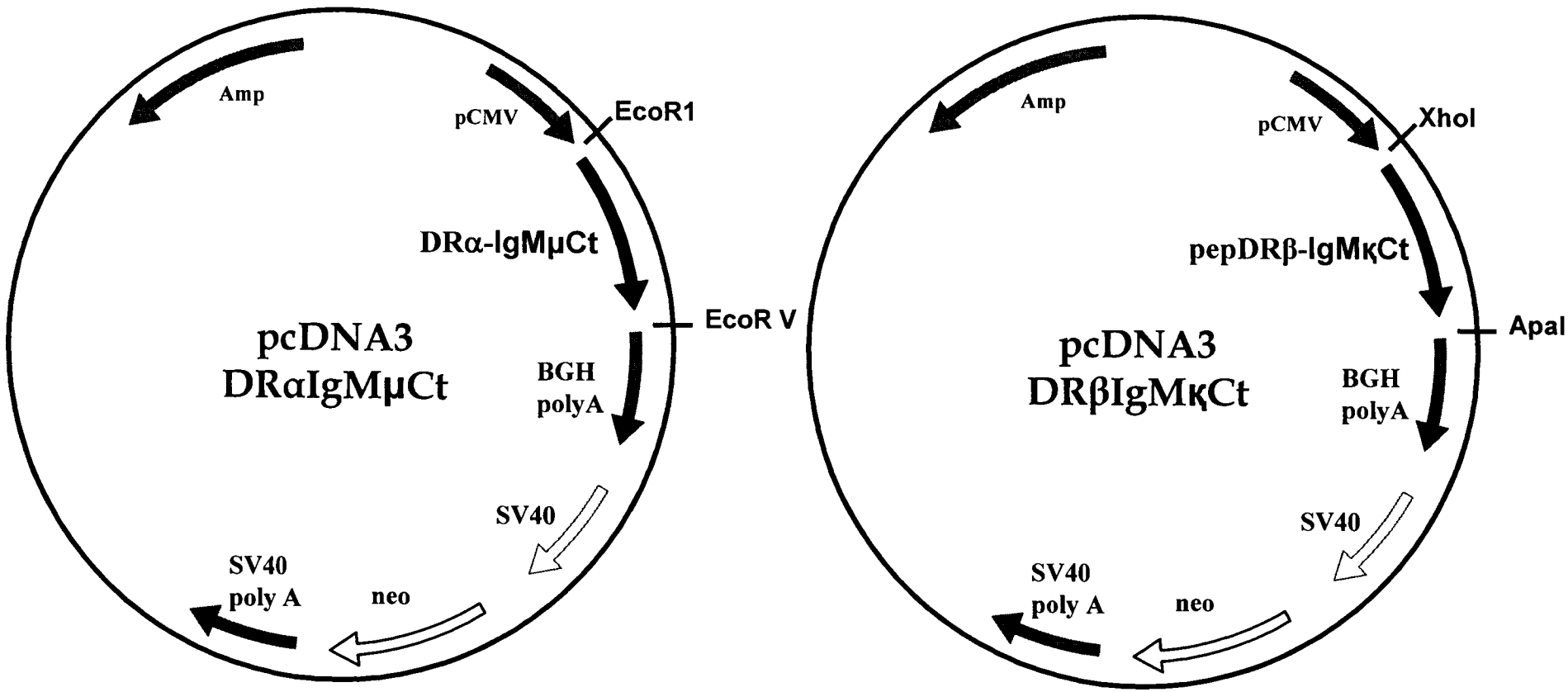


Figure S8. Clones of IgMCt*DR1 chimeric chains in pcDNA3 mammalian expression system.

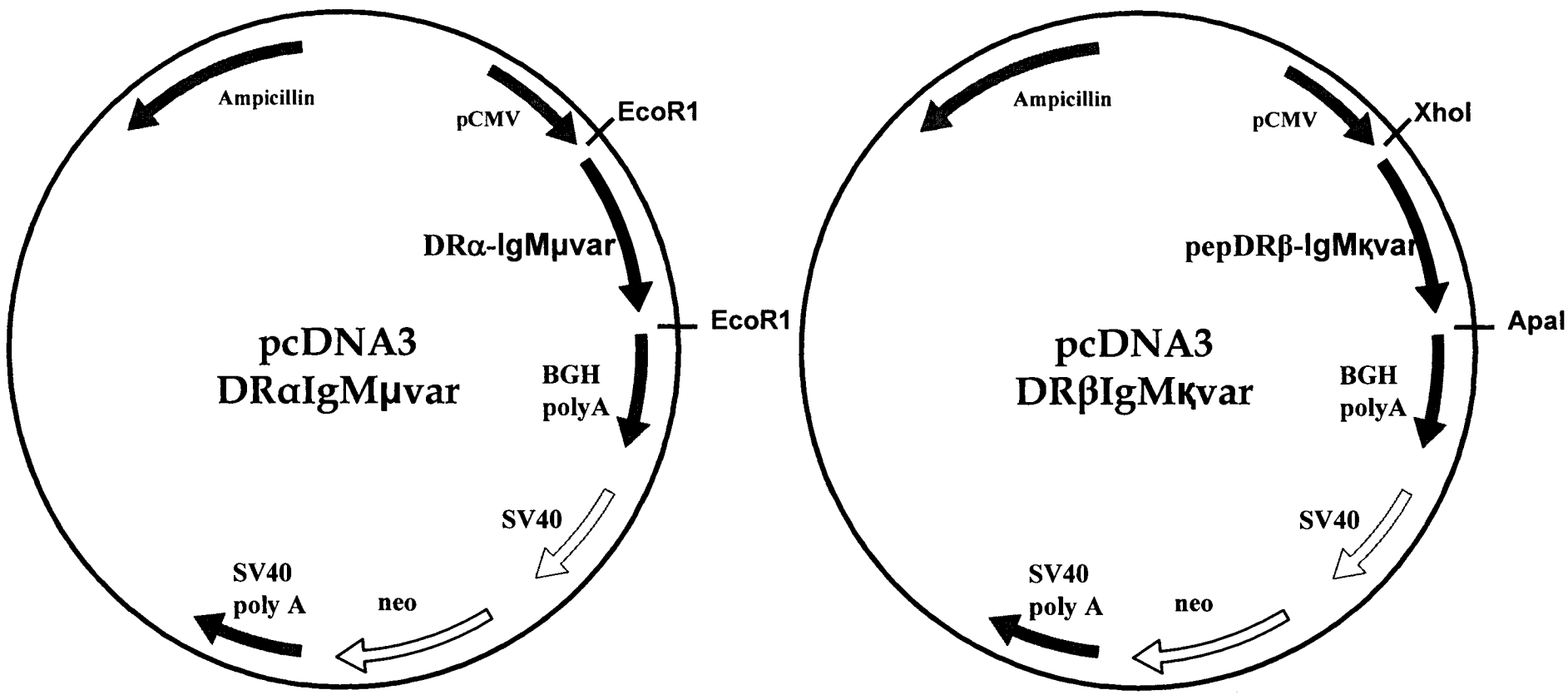


Figure S9. Clones of IgMvar*DR1 chimeric chains in pcDNA3 mammalian expression system.

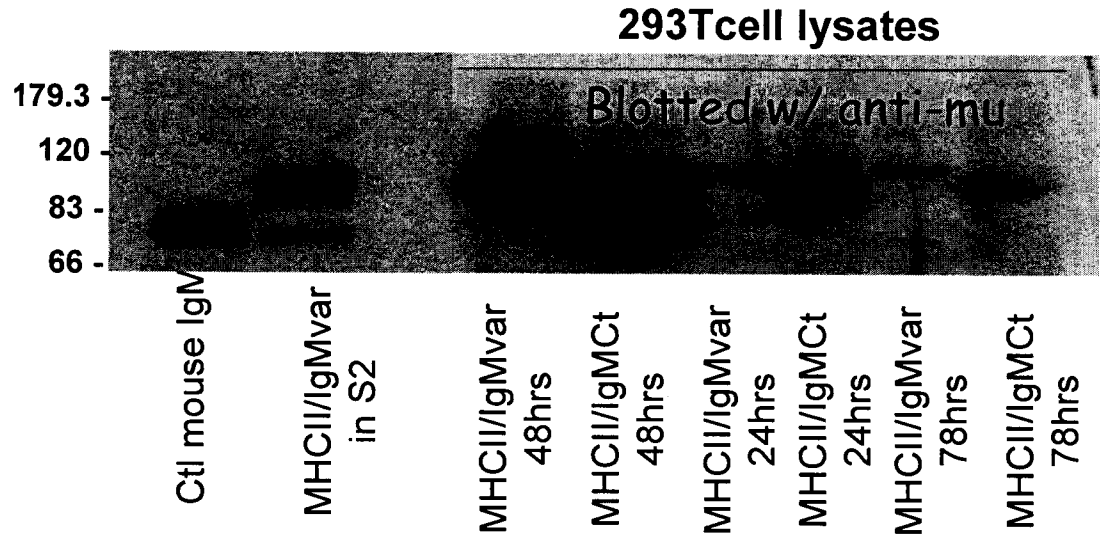
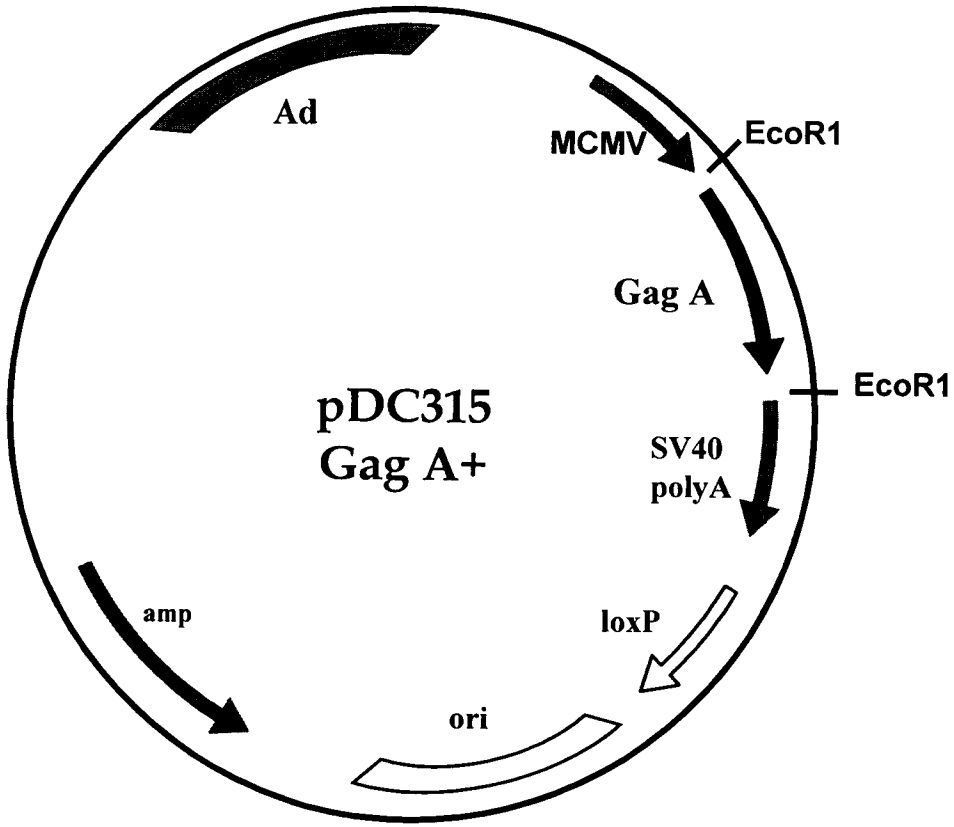
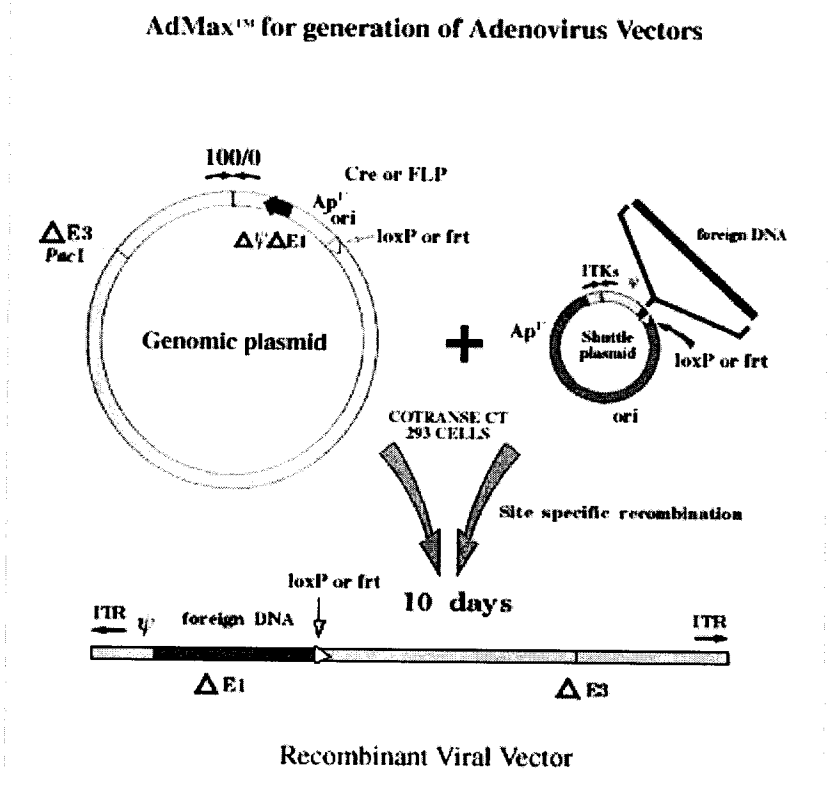


Figure S10. Western Blot using monoclonal anti-μ chain Ab on 293T cell lysates following 24hr, 48hr, and 78hr of transfection by SRalpha vectors of either constant or variable constructs.



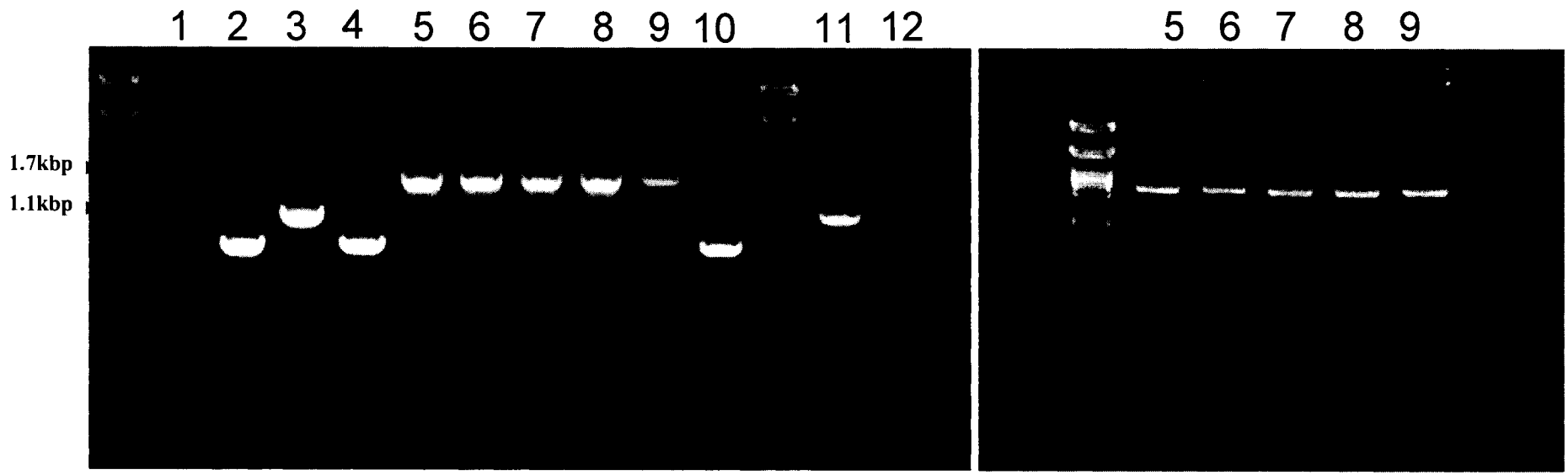


Ad Gag A shuttle vector

Adapted from:
Microbix AdMax™
http://www.microbix.com/products/040301.html

Figure S11. Adenovirus serotype 5 GagA viral vector generation.

PCR on 293 Cell Lysates: Amplification of HIV-1 Clade A gag gene



1. Uninfected 293
2. mOX40L GagB
3. m41BBL GagB
4. mCD70 GagB
5, 6, 7, 8, 9. Adeno Gag Clad A Plaques 1- 5
10. h41BBL Steap
11. Pos PCR
12. Neg PCR

Figure S12. PCR on 293 cell lysates following Ad5GagA transfection.

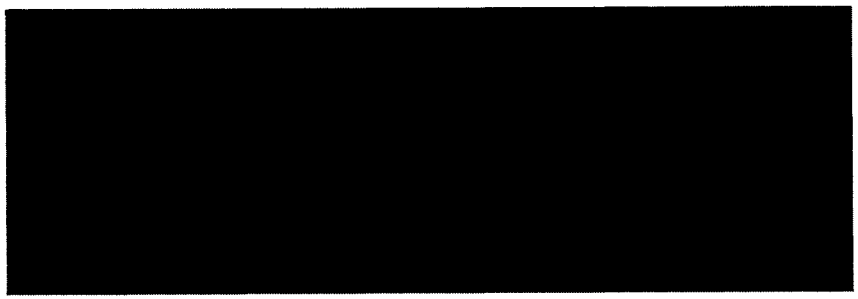
Amplification of HIV-1 Gag A gene

Figure S13. Western Blot on 293 cell lysates: adenoviral expression of HIV-1 clade A gag gene. (p24-antiserum)

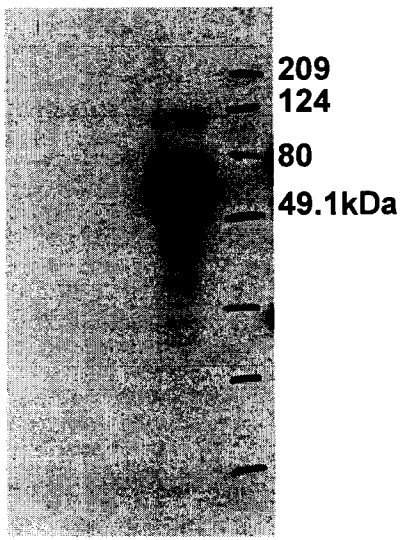
Lanes: 1, 2, 3, 4. Adeno Gag Clad A Plaques 1- 4

Lane 5: h41BBL + STEAP (Neg Ctrl)

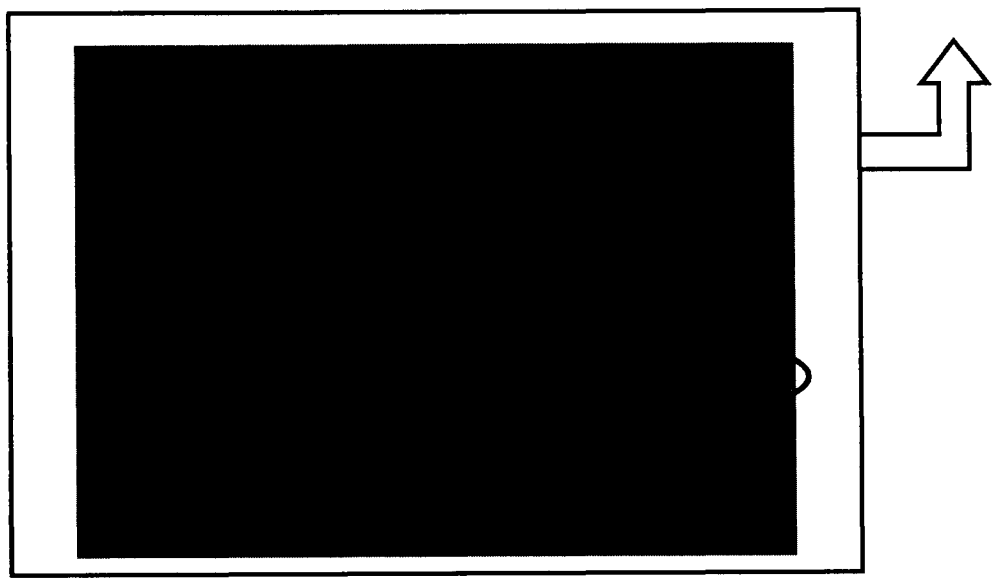
Lane 6: mCD70 GagB (Pos Ctrl)



Production



Plaque Screening



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AFTERWORD

Looking forward to getting this degree - once rightfully known as *Doctor of Philosophy* - and which, in our current day realities, has shrunk to a mere Ph.D., in my bewilderment, I allow myself to end this phase of my life with this afterword, inviting all modern-day scientists, doctors of philosophy, philosophers, thinking minds, and whoever might one day come across this manuscript, to take a moment to reflect upon the following quote from the 11th century Sufi poet Hakim Sanai:

"If, my love, I had to describe to you the way,
I would say clearly:
Turn to Life for the Truth
And not to empty truths for Life..."

From the 'Walled Garden of Truth' -
'Hadiqat ul-Haqiqat', Hakim Sanai.