Mechanisms of Antigen Presentation:
Role of non classical MHC class II molecule H2-O/HLA-DO and Tetraspanin molecule CD82

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I N D I A

Mechanisms of Antigen Presentation:
Role of non classical MHC class II molecule H2-O/HLA-DO and Tetraspanin molecule CD82

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Dedicated

to

my parents

Sri Kunapuli Sobhana Chalapathi Rao

and

Smt Kunapuli Subba Lakshmi

Aim of the PhD thesis

For presentation of antigen by MHC class II molecules to T cells, antigen is processed and loaded onto MHC II molecules in endosomal compartments of antigen presenting cells. There, MHC II molecules are complexed with the accessory molecules DM and DO. These complexes are also associated with tetraspanins, such as CD82, to form large supramolecular structures. It is well-known that DM serves to help loading of peptides onto MHC II molecules, but the function of DO is not yet clear. Likewise, very little is known about the biological function of CD82.

It was the aim of this thesis to elucidate the role of DO and CD82 molecules in antigen presentation. For this study, genetically modified mice were available expressing different levels of DO, namely DO knock-out mice and transgenic mice over expressing DO. Since DO is mainly expressed in B cells, BCR transgenic mice were included, which internalize antigen via the BCR, resulting in efficient antigen presentation by B cells. In addition, the question should be answered, if DO influences antigen presentation differentially in early versus late endosomal compartments.

For the analysis of CD82, respective knockout mice have been generated in the laboratory. Since nothing is known about the effects of deficient CD82 expression, first the phenotype of the CD82 knockout cells with regard to cell surface markers had to be extensively investigated. Next, the role of CD82 in proliferation of T and B lymphocytes and in antibody responses should be studied. Most importantly, these novel CD82 knockout mice allowed to investigate the long standing question whether or not CD82 would influence antigen presentation by MHC II molecule in the various endosomal loading compartments.

Summary

Presentation of antigen by MHC class II molecules to CD4 T cells depends on the loading of antigenic peptides onto MHC class II molecules in endosomal compartments of antigen-presenting cells. There, efficient loading of peptides requires the assistance of the accessory molecule HLA-DM. DM is found tightly associated with another molecule, HLA-DO in humans, or H2-O in mice, but the function of DO is not entirely clear. MHC class II, DM, and DO are further associated with tetraspanins such as CD82, but their role in antigen presentation, if any, is also not clear. In this thesis, the function of DO and CD82 in antigen presentation was studied.

1. DO in antigen presentation.

So far, the analysis of $H2O^{-/-}$ mice did not show a striking phenotype with regard to antigen presentation. Possible explanations are that the function of H2-O is allele-specific and does not affect presentation by A^b MHC class II molecules, the allele carried by $H2\text{-}O^{-/-}$ mice. Moreover, only few experimental systems are available for thorough testing of antigen presentation by th A^b allele. In this laboratory, transgenic mice were generated overexpressing H2-O, designated mDO mice, which express the A^k allele, and for which well-studied antigen presentation systems are available. The study presented here shows that DO downmodulates only presentation of epitopes that are loaded onto A^k molecules in early endocytic compartments, but not epitopes loaded in late endocytic compartments. Since DO is mainly expressed in B cells which predominantly take up antigen via their surface immunoglobulin (BCR, B cell receptor), we crossed the mDO with BCR transgenic mice. Again, DO downmodulated presentation of antigen only in the early endocytic compartment. These data suggest that DO may serve to skew antigen presentation to take place preferentially in late compartments.

2. CD82 in antigen presentation.

We have concentrated on the tetraspanin CD82, because CD82 was shown to be associated with MHC class II, DM, and DO in MHC class II loading compartments. In addition, CD82 is known interact with other transmembrane molecules such as TCR, CD4, CD8, etc.. CD82-deficient mice were generated in this laboratory for the present study. Expression of a large panel of surface markers was found to be unchanged in CD82-deficient mice. However, T cells from $CD82^{-/-}$ mice were hyper reactive to stimulation by anti-CD3 and anti-CD28 antibodies, suggesting that CD82 affects the TCR signalling pathway. When CD82-deficient dendritic cells were analyzed, decreased antigen presentation was found, but again only loading of epitopes on the early endocytic compartments was affected, but not loading in late compartments. When splenocytes were used, were most antigen-presenting cells are B cells, no differences between $CD82^{-/-}$ and wild-type cells were found with regard to antigen presentation. These data demonstrate for the first time a role for a tetraspanin in antigen presentation and suggest that CD82 enhances antigen presentation not only in a compartment-specific manner, but also in a cell type-specific fashion. The respective mechanisms need to be explored in the future. It is, however, striking to note that both DO and CD82 affect antigen presentation only in the early endosomal compartment. This may suggest a functional link between DO and CD82.

Zusammenfassung

T-Helferzellen erkennen Antigene, die von MHC Classe II Molekülen präsentiert werden. Die Beladung von MHC Klasse II mit Peptidfragmenten von Antigenen erfolgt in endosomalen Kompartimenten von antigenpräsentierenden Zellen, erfordert aber die Mitwirkung des akzessorischen Moleküls HLA-DM. DM wiederum ist relativ fest mit einem anderen Molekül assoziiert, HLA-DO im Menschen, bzw. H2-O in der Maus. Die genaue Funktion von H2-O ist jedoch noch unklar. MHC Klasse II, DM und DO sind ausserdem mit Teraspanin-Molekülen wie CD82 assoziiert, deren Rolle für die Antigenpräsentation noch nicht erforscht ist. In dieser Arbeit wurde die Funktion von DO und CD82 für Antigenpräsentation untersucht.

1. DO

Die Analyse von H2-$O^{-/-}$ Mäusen hat bisher keinen markanten Phänotyp in Bezug auf Antigenpräsentation ergeben. Eine mögliche Erklärung ist, dass H2-O allel-spezifisch wirkt und deshalb keinen Einfluss auf die Präsentation durch das A^b MHC Klasse II Molekül der H2-$O^{-/-}$ Mäuse hat. Eine weitere Schwierigkeit besteht darin, dass nur wenige experimentelle Systeme für eine umfassende Analyse der Antigenpräsentation durch das A^b-Allel verfügbar sind. In unserem Labor wurden sogenannte mDO-transgene Mäuse hergestellt, die H2-O überexprimieren und das A^k-Allel tragen, für welches eine Reihe von Antigenpräsentationssystemen verfügbar ist. Die Ergebnisse der Arbeit zeigen, dass DO die Präsentation von Antigenen negativ beeinflusst. Interessanterweise trifft das aber nur für Epitope zu, die in frühen endozytischen Kompartimenten auf A^k-Moleküle geladen werden, aber nicht für Epitope, die in späten endozytischen Kompartimenten geladen werden. Da DO vornehmlich in B-Lymphozyten exprimiert ist, die Antigen hauptsächlich über ihr Oberflächenimmunoglobulin (BCR) aufnehmen, haben wir die mDO-Mäuse mit BCR-transgenen Mäusen gekreuzt. Wiederum beeinflusste DO die Präsentation von Antigen nur im frühen endozytischen, aber nicht im späten Kompartiment. Diese Daten zeigen, dass DO Antigenpräsentation in späten Kompartimenten begünstigt.

2. CD82

Da MHC Klasse II, DM und DO in den MHC Klasse II-Beladungskompartimenten mit dem Tetraspanin CD82 assoziiert vorliegt, haben wir uns auf dieses Tetraspanin konzentriert. Zusätzlich interagiert CD82 mit anderen Transmembranmolekülen wie TCR, CD4, CD8, usw.. Für die Analyse von CD82 wurden in unserem Labor knockout-Mäuse hergestellt. Die Analyse ergab, dass die Expression einer Vielzahl von Oberflächenmarkern auf Lymphozyten in den $CD82^{-/-}$ Mäusen nicht verändert war. In funktionellen Untersuchungen wurden $CD82^{-/-}$ T-Zellen effizienter durch Antikörper gegen CD3 und CD28 stimuliert als $CD82^{+/+}$ T-Zellen. Diese Ergebnisse zeigen, dass CD82 die TCR-Signalkaskade beeinflusst. Dendritische Zellen von $CD82^{-/-}$ Mäusen zeigten eine verminderte Fähigkeit zur Antigenpräsentation, aber wie im Fall von DO war nur das Beladen mit Epitopen in frühen endozytischen Kompartimenten betroffen, aber nicht in späten endozytischen Kompartimenten. Im Gegensatz zu den dendritischen Zellen war Antigenpräsentation in B-Lymphozyten nicht durch die Abwesenheit von CD82 beeinflusst. Diese Daten zeigen zum ersten Mal, dass ein Tetraspanin die Präsentation von Antigen beeinflusst. CD82 unterstützt Antigenpräsentation nicht nur in einer kompartiment-spezifischen Weise, sondern auch in einer zelltyp-spezifischen Weise. Die zugrundeliegenden zellbiologischen Mechanismen müssen noch geklärt werden. Interessanterweise beeinflussen sowohl DO als auch CD82 Antigenpräsentation nur in den frühen endosomalen Kompartimenten. Dieses deutet auf eine funktionelle Beziehung zwischen DO und CD82 hin.

INTRODUCTION

1. Introduction

1.1 The Immune System: An overview

The immune system is a remarkably adaptive defense system that has evolved in vertebrates to protect them from invading pathogenic microorganisms. It is able to generate an enormous diversity of cells and molecules capable of specifically recognizing the large variety of foreign invaders. Functionally, an immune response can be divided into two related activities – *recognition* and *response*. Furthermore, the system is able to discriminate between foreign molecules and the body's own cells and proteins. Once a foreign organism has been recognized, the immune system enlists the participation of a variety of cells and molecules to mount an appropriate response, called an effector response, to eliminate or neutralize the foreign organism or substance. Later exposure to the same foreign material induces a *memory* response, characterized by a more rapid and heightened immune reaction that serves to eliminate the pathogen and prevent disease [1,2].

Components of Immunity

Immunity, the state of protection from infectious disease has both nonspecific and specific components. The nonspecific component, *innate immunity*, is a set of disease resistance mechanisms that are not specific to a particular pathogen. Phagocytic cells, such as macrophages, play an important role in many aspects of innate immunity. In contrast, the specific component, *adaptive immunity*, displays a high degree of specificity as well as the remarkable property of *memory*. The major cellular agents of adaptive immunity are lymphocytes.

Innate Immunity

Innate immunity can be seen to comprise four types of defensive barriers: anatomic (skin, mucous membranes), physiologic (temperature, low pH), Phagocytic ((macrophages, neutrophils, blood monocytes), and inflammatory (tissue damage and

infection induce leakage of vascular fluid, containing serum proteins with antibacterial activity).

Adaptive Immunity

Adaptive immunity is capable of recognizing and selectively eliminating specific foreign microorganisms and molecules. Unlike innate immune responses, adaptive immune responses are reactions to specific antigenic challenges and display four characteristic attributes: Antigen specificity, Diversity, Immunologic Memory, and Self/Nonself recognition.

Innate and adaptive immunity do not operate in total independence of each other. They cooperate in important ways to produce more effective immunity. The adaptive immune system produces signals and components that stimulate and increase the effectiveness of innate responses. More detailed explanation on this cross talk can be found in the following chapters.

1.1.1a Cells of the immune system

B cells

Its designation stems from its site of maturation, bursa of Fabricius in birds; bone marrow in a number of mammalian species, including humans and mice. B cells can be distinguished from other lymphocytes by their synthesis and display of membrane bound immunoglobulin (antibody) molecules, which serve as receptors for antigen (BCR). According to the clonal selection theory an individual B cell express membrane receptors that are specific for a distinct antigen. Each of the approximately $1.5x10^5$ antibody molecules on the membrane of a single B cell has an identical binding site for antigen. The diversity in the generation of pre determined antigen specific B cell receptors are possible because of the RAG 1 and RAG 2 gene products, which are essential for VDJ recombination. The potential number of antibodies of distinct sequences that the mammalian immune system is capable of generating is extremely high and exceeds $1x10^{10}$. Among the major other molecules expressed on the membrane of mature B cells are the following: IgM, IgD, B220 (CD45R), Class I&II

MHC, CD21 (CR2), CD35(CR1), Fcγ RII (CD32), CD80(B7-1), CD86(B7-2), and CD40.

T cells

T lymphocytes derive their name from their site of maturation in the thymus. T cells express T cell receptor (TCR), structurally distinct from immunoglobulin, most notably in the structure of its antigen binding site. The TCR does not recognize free antigen. Instead, the TCR recognizes only antigen that is bound to major histocompatibility complex (MHC) molecules. Fundamental differences between the humoral and cell mediated branches of the immune system is that the B cell is capable of binding soluble antigen, whereas the T cell is restricted to binding antigen displayed on self cells (antigen presenting cells). T cells that express the membrane glycoprotein molecule CD4 are restricted to recognizing antigen bound to class II MHC molecules, whereas T cells expressing CD8, a dimeric membrane glycoprotein, are restricted to recognition of antigen bound to class I MHC molecules. $CD4^+$ T cells generally function as T helper (T_H) cells and are class II restricted; $CD8^+$ T cells generally function as T cytotoxic (T_C) cells and are class I restricted. The ratio is approximately 2:1 in humans, but it may alter significantly in various disease conditions. T helper cell secretes various cytokines upon activation after the recognition of antigen presented by MHC II molecule. Changes in the pattern of cytokines produced by T helper cells can change the type of immune response that develops among other leukocytes. The T_H1 response produces a cytokine profile that supports inflammation and activates mainly certain T cells and macrophages, whereas the T_H2 response activates mainly B cells and immune responses that depend upon antibodies. The potential number of TCR of distinct specificity that the mammalian immune system is capable of generating is extremely high and exceeds $1x10^{12}$.Another subpopulation of T lymphocytes called regulatory T cells (T_{reg}), which suppress the auto reactive effector T cells. The mechanism of T_{reg} function is not completely understood.

Dendritic Cells (DCs)

The dendritic cell acquired its name because it is covered with maze of long membrane extensions that resembles the dendrites of nerve cells. Most dendritic cells process and present antigen to T_H cells. These cells can be classified by their location: Langerhans cells found in the epidermis and mucous membranes, Interstitial Dendritic cells, which populate most organs, Interdigitating dendritic cells present in T cell areas of secondary lymphoid tissue and the thymic medulla, Circulating dendritic cells include those in the blood, which consists of 0.1% of the blood leukocytes, and those in the lymph (known as veiled cells). The dendritic cells in different locations have different forms and functions. Despite their differences, all of these dendritic cells constitutively express high levels of both class I and class II MHC molecules and members of the co-stimulatory B7 family. DCs are the most important antigen presenting cells in the body. They will be discussed again in the section on antigen presentation.

Another type of dendritic cell, the follicular dendritic cell (FDC), appears to have a different origin and function from the antigen presenting dendritic cells. FDC do not express class II MHC and therefore do not function as antigen presenting cells for T_H cell activation. FDC express high levels of Fc receptors for antibody and complement receptors. Antigen antibody complexes on FDC can be retained for very long periods of time ranging from weeks to months or even longer. FDC play a very important role in germinal center reactions in the spleen [1,2].

Other Immune Cells:

In the innate immune system, there exist several additional cell types with important functions, such as NK cells, macrophages, mast cells, granulocytes and neutrophils. These cell types will not be described further here.

1.1.1b Organs of the Immune System

Thymus

The thymus is a flat, bilobed organ situated above the heart. Each lobe is organized into two compartments: the outer compartment or cortex is densely packed with immature T cells called thymocytes, whereas the inner compartment or medulla is sparsely populated with thymocytes. T cell precursors migrate from bone marrow to thymus for the selection process. Thymic stromal cells which express high levels of class I and class II MHC molecules, play a critical role in the selection process of thymocytes. Developing thymocytes that are unable to recognize self MHC molecules or that do have high affinity for self antigen plus self MHC (or self MHC alone) are eliminated by programmed cell death specifically termed as apoptosis. Thus, only those cells whose receptors recognize a self MHC molecule plus foreign antigen are allowed to mature. An estimated 95-99% of all thymocytes progeny undergo apoptosis within the thymus without ever maturing. Thymectomized mice show a dramatic decrease in circulating lymphocytes of the T cell lineage and an absence of cell mediated immunity. Aging is accompanied by a decline in thymic function. This may be one of the reasons for the development of autoimmune disorders in aged people [2].

Bone Marrow

The bone marrow is a central site for hematopoiesis as explained previously. B cells are produced in the bone marrow throughout life, but very few of those mature. There are several developmental stages during the development of B cells in bone marrow; pro-B cells, pre-B cells, immature B cells and mature B cells. It is estimated that in mice the bone marrow produces about $5x10^7$ B-cells/day but only $5x10^6$ (about 10%) actually are recruited into the recirculating B cell pool. The remaining 90% cells will undergo apoptosis without ever leaving the bone marrow. Like during thymic selection a selection process occurs within bone marrow eliminating B cells with self reactive antibody receptors [2].

Lymphatic System

When blood circulates under pressure, its fluid component seeps through the thin wall of the capillaries into the surrounding tissue, called lymph. Lymph flows from the spaces in connective tissue into a network of tiny open lymphatic capillaries and then into a series of progressively larger collecting vessels called lymphatic vessels. The largest lymphatic vessel, the thoracic duct, empties into the left subclavian vein near the heart. In this way, the lymphatic system captures fluid lost from the blood and returns it to the blood, thus ensuring steady state levels of fluid within the circulatory system. The heart does not pump the lymph through the lymphatic system; instead the flow of lymph is achieved as the lymph vessels are squeezed by movements of the body's muscles. A series of one way valves along the lymphatic vessels ensures that lymph flows only in one direction. When a foreign antigen gains entrance to the tissues, it is picked up by the lymphatic system (which drains all the tissues of the body) and is carried to various organized lymphoid tissues such as lymph nodes, which trap the foreign antigen. As lymph passes from the tissues to lymphatic vessels, it becomes progressively enriched in lymphocytes. Thus the lymphatic system also serves as a means of transporting lymphocytes and antigen from the connective tissues to organized lymphoid tissues where the lymphocytes may interact with the trapped antigen and undergo activation [2].

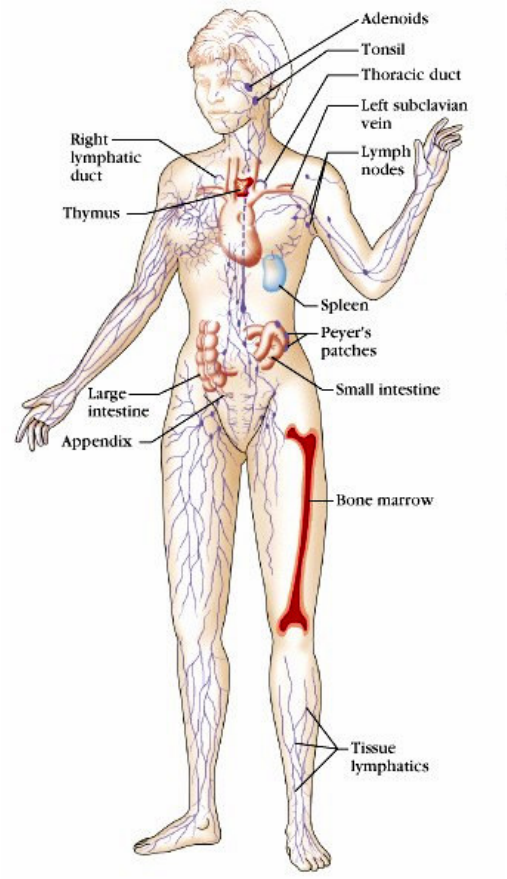


Fig.: 1a The Human Lymphatic System

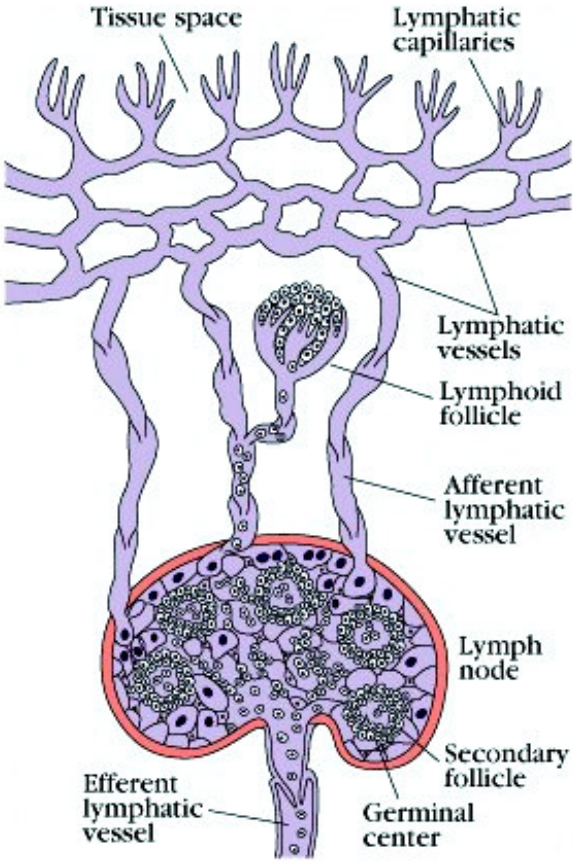


Fig.: 1b Lymphatic Vessels [2]

1.1.2 Secondary Lymphoid Organs

Various types of organized lymphoid tissues are located along the vessels of the lymphatic system. Lymph nodes and spleen are the most highly organized of the secondary lymphoid organs; they comprise not only lymphoid follicles, but additional distinct regions of T cell and B cell activity and they are surrounded by a fibrous capsule. Less organized lymphoid tissue, collectively called mucosal associated lymphoid tissue (MALT), is found in various body sites. MALT includes Peyer's patches, the tonsils and the appendix, as well as numerous lymphoid follicles within lamina propria of the intestine and in the mucous membranes lining the upper airways, bronchi, and genital tract. In a severe infection conditions, ectopic germinal centers can be formed any where in the body to make an interface between antigen presenting cell and T cell, in order to eliminate the pathogen from body rapidly [2].

Lymph Nodes

Morphologically, a lymph node can be divided into three roughly concentric regions: the cortex, the paracortex, and the medulla. The outermost layer, the cortex contains lymphocytes (mostly B cells), macrophages, and follicular dendritic cells arranged in primary follicles. After antigenic challenge, the primary follicles enlarge into secondary follicles, each containing a germinal center. Beneath the cortex is the paracortex, which is populated largely by T cells and also contains interdigitating dendritic cells thought to have migrated from tissues to the node. These interdigitating dendritic cells express high levels of class II MHC molecules, which are necessary for presenting antigen to T_H cells. The innermost layer of a lymph node, the medulla is more sparsely populated with lymphoid lineage cells; of those present, many are plasma cells actively secreting antibody molecules. Afferent lymphatic vessels pierce the capsule of a lymph node at numerous sites and empty lymph into the subcapsular sinus and lymph comes out of the node through efferent lymphatic vessel.

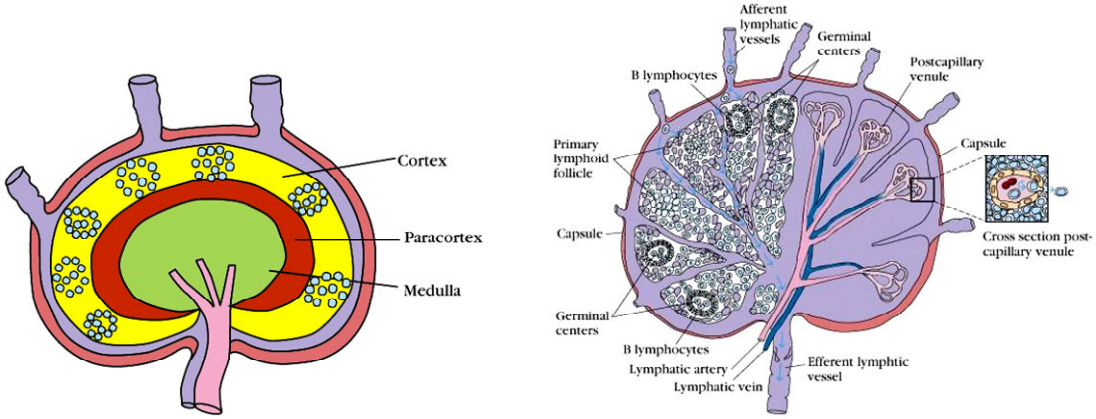


Fig.: 2 Structure of the Lymph Node [2].

Spleen

The spleen is a large, ovoid secondary lymphoid organ situated high in the left abdominal cavity. While lymph nodes are specialized for trapping antigen from local tissues, the spleen specializes in filtering blood and trapping blood borne antigens; thus, it can respond to systemic infections. Unlike lymph nodes, the spleen is not supplied by lymphatic vessels. Instead, blood borne antigens and lymphocytes are carried into the spleen through the splenic artery.

More recirculating lymphocytes pass daily through the spleen than through all the lymph nodes combined. The spleen is surrounded by a capsule that extends a number of projections (trabeculae) into the interior to form a compartmentalized structure. The compartments are of two types, the red pulp and white pulp, which are separated by a diffuse marginal zone. The splenic red pulp consists of a network of sinusoids populated by macrophages and numerous red blood cells, it is the site where old and defective red blood cells are destroyed and removed. Many of the macrophages within the red pulp contain engulfed red blood cells. The splenic white pulp surrounds the branches of the splenic artery, forming a periarteriolar lymphoid sheath (PALS) populated mainly by T cells. The marginal zone located peripheral to the PALS, is rich in B cells organized into primary lymphoid follicles. The effect of splenectomy on the immune response depends on the age at which the spleen is removed. In children, splenectomy often leads to an increased incidence of bacterial sepsis, whereas in adults it has less adverse effects, although it leads to some increase in blood borne bacterial infections (bacteremia) [2].

1.2 Antigen Presentation

Recognition of foreign antigen by a T cell requires that peptides derived from the antigen be displayed within the cleft of an MHC molecule on the membrane of a cell. The formation of these peptide-MHC (pMHC) complexes requires that a protein antigen to be degraded into peptides by a sequence of events called antigen processing. The degraded peptides then associate with MHC molecules within the cell interior, and the pMHC complexes are transported to the membrane, where they are displayed (antigen presentation). Class I MHC molecules bind peptides derived from endogenous antigens that have been processed within the cytoplasm of the cell. Class II MHC molecules bind peptides derived from exogenous antigens that are internalized by phagocytosis or endocytosis and processed within the endocytic pathway.

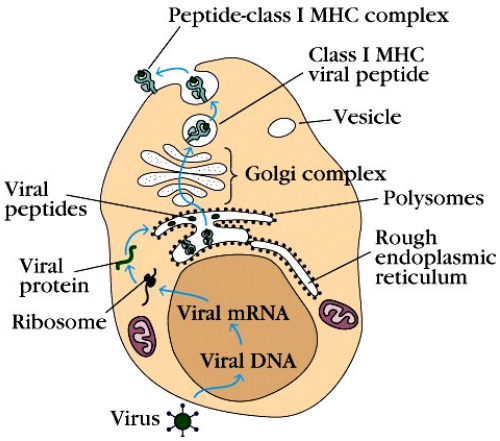
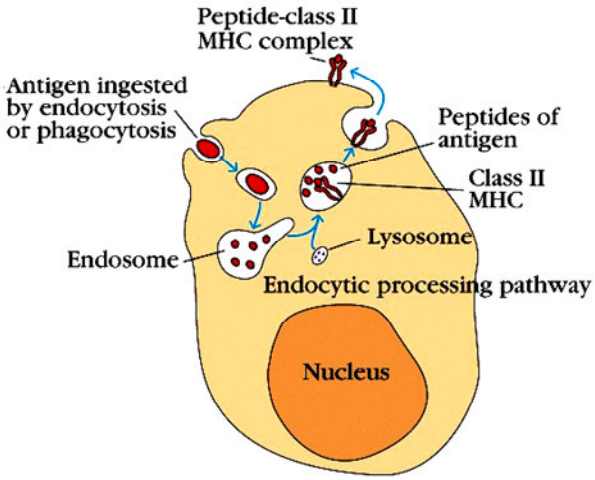


Fig.: 3a Cytosolic Pathway



3b Endocytic Pathway [2]

1.2.1 Major Histocompatibility Complex

The molecular center of the immune system consists of major histocompatibility complex (MHC) molecules: the cell surface receptors that govern, by interaction with membrane expressed molecules on crucial cells of the innate and adaptive immune systems. Work carried out in the 1940s by Peter Gorer and George Snell established that antigens encoded by the genes in the group designated II took part in the rejection of transplanted tumors and other tissue. Snell called these genes "histocompatibility genes"; their current designation as histocompatibility-2 (H-2) genes was in reference to Gorer's group II blood group antigens. Gorer died before his contributions were recognized fully, Snell was awarded the Nobel Prize in 1980 for this work. The MHC is a set of linked genes, located on chromosome 6 of the human, chromosome 17 of the mouse. The MHC molecules are not static surface receptors that merely bind another set of receptors to indicate their presence and number. They are structurally and conformationally dynamic: they modulate their structure by incorporating peptides derived from ingested foreign molecules or dysregulated proteins expressed in the same cell. Members of the family of MHC encoded molecules interact extensively with a number of other molecules, during both their biosynthesis and intracellular trafficking. These interactions regulate the quality and rate of their appearance in specialized intracellular compartments as well as their arrival at the cell surface. The MHC is referred to as the HLA complex in humans and as the H-2 complex in mice. Although

the arrangement of genes is somewhat different, in both cases the MHC genes are organized into regions encoding three classes of molecules:

- Class I MHC genes encode glycoproteins expressed on the surface of nearly all nucleated cells; the major function of the class I gene products is presentation of peptide antigens to T_C cells.

- Class II MHC genes encode glycoproteins expressed primarily on antigen presenting cells (macrophages, dendritic cells, and B cells), where they present processed antigenic peptides to T_H cells.

- Class III MHC genes generally encode various secreted proteins that have immune functions, including components of the complement system and molecules involved in inflammation.

Structure of Class II MHC Molecules

Class II MHC molecule contain two different polypeptide chains, a 33kDa alpha chain and a 28kDa beta chain, which associated by noncovalent interactions. Class II MHC molecules are membrane bound glycoproteins that contain external domains, a transmembrane segment, and a cytoplasmic anchor segment. Each chain in a class II molecule contains two external domains: α1 and α2 domains in one chain and β1 and β2 domains in the other. The membrane proximal α2 & β2 domains bear sequence homology to the immunoglobulin fold structure (for this reason, class II MHC molecules also are classified in the immunoglobulin superfamily). The membrane distal portion of a class II MHC molecule is composed of the α1 & β1 domains and forms the antigen binding cleft for processed antigen. The two chains of the class II MHC molecules are encoded by the: I-A and I-E regions in mice and by the DP, DQ and DR regions in humans. MHC genes are highly polymorphic, meaning that many alternative forms of the gene or alleles exist at each locus. MHC class I molecules consist of a α-chain containing these domains: α1, α2, and α3 and the associated β_2 microglobulin [1,2,7].

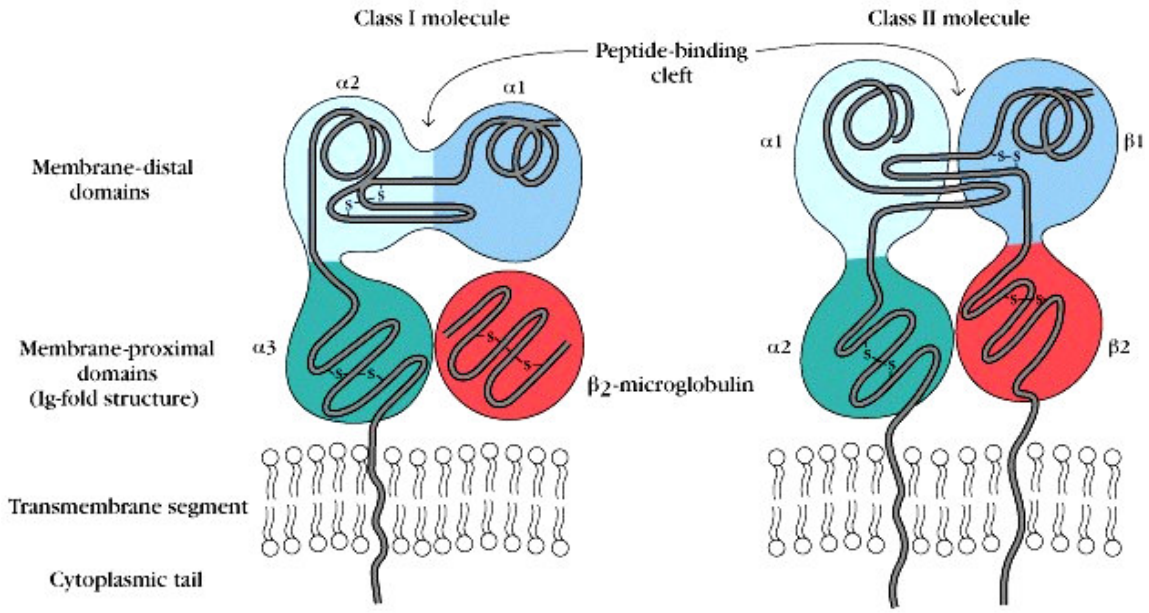


Fig.: 4 Schematic diagrams of Class I and Class II MHC molecules [2]

Peptide binding by MHC Molecules

Several hundred different allelic variants of class I and II MHC molecules have been identified in humans. Any one individual, however, expresses only a small number of these molecules- up to 6 different class I molecules and up to 12 different class II molecules. Yet this limited number of MHC molecules must be able to present an enormous array of different antigenic peptides to T cells, permitting the immune system to respond specifically to a wide variety of antigenic challenges. Thus peptide binding by class I and II molecules does not exhibit the fine specificity characteristic of antibodies and T cell receptors. Instead, a given MHC molecule can bind numerous different peptides, and some peptides can bind to several different MHC molecules. The peptide binding groove in class I molecule is blocked at both ends, whereas the groove is open in class II molecules. As a result of this difference, class I molecules bind peptides that typically contain 8-10 amino acid residues, while the open groove of class II molecules can accommodate slightly longer peptides of 13-18 amino acid residues. Another difference is that class I binding requires that the peptide contain certain amino acid residues, called anchor residues, near the N and C terminal ends; there is no such requirement for class II peptide binding. The K_D value for the peptide MHC molecule complex is approximately 10^{-6}; the rate of association is slow, but the rate of dissociation is even slower. This means, the peptide MHC molecule association

is very stable under physiologic conditions, thus most of the MHC molecules expressed on the membrane of a cell will be associated with a peptide of self or nonself origin.

MHC and Immune Responsiveness

Early studies by B. Benacerraf in which guinea pigs were immunized with simple synthetic antigens first showed that the ability of an animal to mount an immune response, as measured by the production of serum antibodies, is determined by its MHC haplotype [3]. Later experiments by H. McDevitt, G.J. Hämmerling and M. Sela used congenic and recombinant congenic mouse strains to map the control of the so called immune response genes to the class II MHC. By analyzing the products of these Ir or immune response genes, molecules were discovered by McDevitt and Hämmerling that we call today MHC class II molecules. We now know that the dependence of immune responsiveness on the class II MHC reflects the central role of class II MHC molecules in presenting antigen to T_H cells [4-6].

1.2.2 MHC Restriction of T cells

Both $CD4^+$ and $CD8^+$ T cells can recognize antigen only when it is presented with a self MHC molecule on the membrane of antigen presenting cell. This attribute, called self-MHC restriction. The self MHC restriction of $CD8^+$ T cells was first demonstrated by R. Zinkernagel and P. Doherty in 1974. In their experiments, mice were immunized with lymphocytic choriomeningitis (LCM) virus; several days later, the animals' spleen cells, which included T_C cells killed only syngeneic virus infected target cells. Later studies with congenic and recombinant congenic strains showed that the T_C cell and the virus infected target cell must share class I molecules encoded by the same haplotype. Thus, antigen recognition by $CD8^+$ T_C cells is class I MHC restricted. In 1996, they were awarded Nobel Prize for their contribution to the understanding of cell mediated immunity [1,2,11].

A. Rosenthal and E. Shevach showed that antigen specific proliferation of T_H cells occurred only in response to antigen presented by macrophages of the same MHC

haplotype[10, 11]. Together with McDevitt studies these experiments confirmed that the antigen recognition by $CD4^+$ T_H cell is class II MHC restricted [2,10].

1.3 Antigen Processing and Presentation Pathways

Early evidence for the necessity of antigen processing was obtained from the experiments conducted by K. Ziegler and E.R. Unanuae [12]. They contradicted the prevailing dogma that antigen recognition by B and T cells was basically similar. These researchers observed that T_H cell activation by bacterial protein antigens was prevented by treating the antigen presenting cells with paraformaldehyde prior to antigen exposure. However, if the antigen presenting cells were allowed to ingest the antigen and were fixed with paraformaldehyde 1-3 hr later, T_H cell activation still occurred. During the interval of 1-3 hr, the antigen presenting cells had processed the antigen and had displayed it on the membrane in a form able to activate T cells.

Three cell types are classified as professional antigen presenting cells; dendritic cells, macrophages and B lymphocytes. These cells differ in their mechanisms of antigen uptake, in whether they constitutively express class II MHC molecules, and in their co-stimulatory activity:

- Dendritic cells are the most effective of the antigen presenting cells. Because they constitutively express a high level of class II MHC molecules and co-stimulatory activity, they can activate naïve T_H cells.

- Macrophages must be activated by phagocytosis of microorganisms before they express class II MHC molecules or the co-stimulatory B7 molecules.

- B cells constitutively express class II MHC molecule but must be activated before they express the co-stimulatory B7 molecules.

Several other cell types (Thymic epithelial cells, vascular endothelial cells, Fibroblasts, Glial cells, Pancreatic beta cells, Thyroid epithelial cells), classified as non professional

antigen presenting cells, can be induced to express class II MHC molecules. Many of these cells function in antigen presentation only for short periods of time during a sustained inflammatory response.

Evidence for Two Processing and Presentation Pathways

Intracellular (endogenous) and extracellular (exogenous) antigens present different challenges to the immune system. Extracellular antigens are eliminated by secreted antibody, whereas intracellular antigens are most effectively eliminated by cytotoxic T lymphocytes (CTLs). To mediate these responses, the immune system uses two different antigen presenting pathways: endogenous antigens are processed in the cytosolic pathway and presented on the membrane by class I MHC molecules; exogenous antigens are processed in the endocytic pathway and presented on the membrane by class II MHC molecules [13,-15].

1.3.1 The Cytosolic Pathway

The pathway by which endogenous antigens are degraded for presentation with class I MHC molecules utilizes pathways involved in the normal turnover of intracellular proteins. In eukaryotic cells protein levels are carefully regulated. Every protein is subject to continuous turnover and is degraded at a rate that is generally expressed in terms of its half life. Some proteins have very short half life (for example transcription factors); denatured, misfolded or abnormal proteins also are degraded rapidly. Intracellular proteins are degraded into short peptides by a cytosolic proteolytic system present in all cells. Proteins targeted for proteolysis often tagged with ubiquitin, a small protein. Ubiquitin-protein conjugates can be degraded by a multifunctional protease complex called a proteasome. Each proteasome is a large (26S), cylindrical particle consisting of four rings of protein subunits with a central channel of 10-50 Å. A proteasome can cleave peptide bonds between 2 or 3 different amino acid combinations in an ATP dependent process. Degradation of ubiquitin-protein complexes is thought to occur within the central hollow of the proteasome, thereby preventing lysis of other proteins within the cytoplasm. The immune system appears to modify the proteasome by the addition of two subunits to the proteasome: LMP2 and LMP7 [2]. These

subunits are encoded within the MHC gene cluster and are induced by increased levels of IFN-γ.

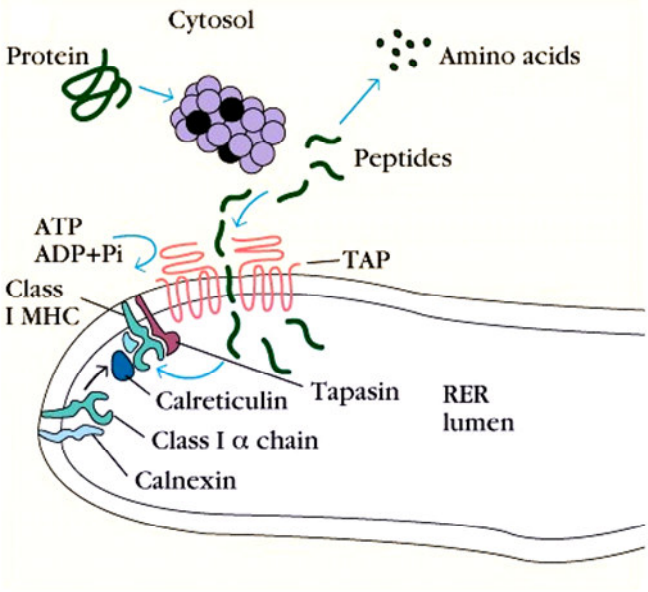


Fig.: 5 Generation of antigenic peptide class I MHC complexes in the Cytosolic Pathway [2]

Peptide Transport from the Cytosol to the ER

The transporter protein, designated TAP (for transporters associated with antigen processing) is a membrane spanning heterodimer consisting of two proteins: TAP1 and TAP2. In addition to their multiple transmembrane segments, the TAP1 and TAP2 proteins each have one hydrophobic domain, which is thought to project through the membrane into the lumen of the ER, and on ATP binding domain, which projects into the cytosol. Both TAP1 and TAP2 belong to the family of ATP binding cassette proteins, these proteins mediate ATP dependent transport of peptides. Peptides generated in the cytosol by the proteasome are translocated by TAP into the ER. TAP has the highest affinity for peptides containing 8-13 amino acids, which is the optimal peptide length for class I MHC binding. The calnexin associated class I MHC α chain binds to β_2 microglobulin, dissociates from calnexin and binds to calreticulin and to tapasin, which is associated with the transmembrane TAP protein (ERp57 may be involved at this step). This class I MHC complex then captures an antigenic peptide, which allows dissociation of the MHC-peptide complex from the chaperones. Finally, the peptide loaded class I MHC molecule is transported from the ER through the Golgi

complex to the plasma membrane. Studies from the tapasin knockout (KO) mice, ERp57 knockout mice and TAP knockout mice shows that reduced class I MHC expression on the cell surface. The extent of reduction of class I MHC with TAP KO mice is severe than tapasin KO and ERp57 KO. It suggests that loading complex members for class I MHC are very important not only for optimal peptide binding but also for the stability of the class I MHC [16-19].

1.3.2 Exogenous Pathway

Antigen presenting cells can internalize antigen by phagocytosis, endocytosis or both. Macrophages internalize antigen by both processes, whereas most other APCs are not phagocytic or poorly phagocytic and therefore internalize exogenous antigen only by endocytosis (either receptor mediated endocytosis or pinocytosis). B cells, for example, internalize antigen very effectively by receptor mediated endocytosis using specific membrane antibody as the receptor.

Peptide Generation in Endocytic Vesicles

Once an antigen is internalized, it is degraded into peptides within compartments of the endocytic processing pathway. The endocytic pathway appears to involve increasingly acidic compartments: early endosomes (pH 6-6.5); late endolysosomes (pH 4.5-5). Internalized antigen moves from early to late endosomes, encountering hydrolytic enzymes and a lower pH. Lysosomes contain a unique collection of more than 40 acid-dependent hydrolases, including proteases, nucleases, glycosidases, lipases, phospholipases and phsphatases. Within the compartments of the endocytic pathway, antigen is degraded into oligopepties of about 13-18 amino acid residues, which bind to class II MHC molecules [19].

Transport of Class II MHC Molecules to Endocytic Vesicles

Since antigen presenting cells express both class I and class II MHC molecules, some mechanism must exist to prevent class II MHC molecules from binding to the same set of antigenic peptides as the class I molecules. When a class II MHC molecule is synthesized within the ER, it associates with another protein called the invariant (Ii)

chain. Three pairs of the class II αβ chains associate with a preassembled invariant chain trimer. This trimeric protein interacts with the peptide-binding cleft of the class II molecule, preventing endogenously derived peptides and peptides from binding to the cleft while the class II molecule is within the ER. In addition to its role in preventing peptide or protein binding to class II MHC molecules [62], the invariant chain is also involved in the folding of the class IIα and β chains, their exit from the ER, and the subsequent routing of class II molecules to the endosomal compartments. The invariant chain contains sorting signals in its cytoplasmic tail that directs the transport of the class II MHC complex from the trans-Golgi network to the endocytic compartments [8].

Assembly of Peptides with Class II MHC Molecules

Class II MHC-invariant chain complexes are transported from ER through Golgi complex and trans-Golgi network to endosomes. As the proteolytic activity increases in endosomes the invariant chain is gradually degraded. However, a short fragment of the invariant chain termed CLIP (for Class II associated invariant chain peptide) remains bound to the class II molecule. CLIP physically occupies the peptide binding groove of the class II MHC molecule, presumably preventing and premature binding of antigenic peptide. A nonclassical class II MHC molecule, called HLA-DM (H2-M in mouse) ("DM"), plays a role in the removal of CLIP and in the subsequent loading of class II molecules with antigenic peptides.

DM – Catalyst, Chaperone and Peptide Editor

H2-M markedly enhances the release of CLIP, thereby accelerating the loading of class II molecules with cognate peptide. DM mediated peptide exchange follows the rules of Michaelis-Menten kinetics derived from enzyme catalysis, which suggests that DM acts like a catalyst. As indicated by both biochemical analysis and the crystal structure, DM cannot bind antigenic peptides, and is therefore unlikely to act as a peptide transfer molecule. Catalysis occurs at substoichiometric amounts compatible with a DM:HLA-DR ratio of about 1:5 in loading compartments. In agreement with the notion that peptide loading occurs mainly in acidic endo-lysosomal compartments, DM mediated

peptide exchange is most efficient at the low pH of these organelles (pH 4.5-5). After CLIP release, DM stabilizes the intermediate empty MHC class II molecules, which otherwise would denature and aggregate at low pH in the absence of a stabilizing peptide in their binding groove. This property is regarded as a chaperone function of DM. Under steady state conditions, about 20-25% of DR in loading compartments exists in empty and highly peptide receptive DM-DR complexes that enable the cell to respond rapidly to an antigenic challenge. Importantly, DM not only exchanges CLIP for cognate peptide but also exchanges low stability peptides (with a high off rate) for peptides that bind class II molecules more stably (peptides with a low off rate). Thus, DM acts as a peptide editor, and thereby favors the formation of stable MHC class II peptide complexes so that $CD4^+$ T cells have more time to screen antigen presenting cells for their protein content [20-25].

Conformational alterations of DR molecules imposed by DM

Class II molecules undergo conformational changes during maturation and loading. Some of these changes appear to be controlled by DM. For example, DR3-CLIP molecules do not react with the DR3 specific 16.23 antibody, but association with DM results in an empty DR3-DM intermediate, which has switched to a 16.23^+ conformation that is conserved after loading with cognate peptide. In the absence of DM, CLIP can also be exchanged for peptide through an allele specific self release mechanism of CLIP, but in this situation the resulting DR3 peptide complex remains 16.23^-. Notably, T cells can discriminate between the 16.23^+ and 16.23^- isoforms of DR3-peptide complexes. Thus conformational changes imposed by DM seem to be relevant to T cell recognition [31,32].

Peptide editing by DM in recycling compartments and at the cell surface

DM is known to exert its function mainly in late endosomal compartments, but it has been shown that the small amount of DM (about 10% of total DM) that can be found at the surface of B cells and immature dendritic cells also has a role. Surface DM

enhances the binding of exogenously added high stability peptide and decreases binding of low stability peptide. Interestingly, DM has been shown to counteract the presentation to T cells of myelin basic protein – an autoantigen implicated in multiple sclerosis that is loaded onto class II molecules at the cell surface and in the recycling compartment. Thus, DM acts also as a peptide editor at the cell surface and in recycling compartments. The implication of these findings is that disturbances of DM expression or activity may influence initiation and progression of autoimmune diseases [34].

Quality control model for DM

The available data are compatible with the following quality control model. The peptide binding groove of MHC class II molecules is in equilibrium between two principal conformational states: an open and a more closed state.

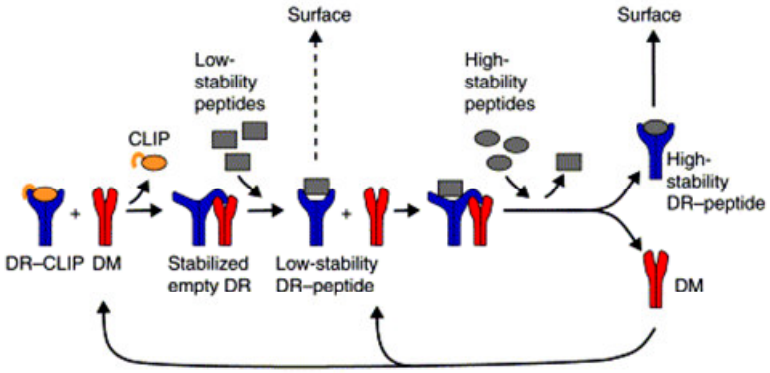


Fig.: 6 Functions of DM as Catalyst, Chaperone and Peptide Editor [31]

The preferential binding of DM to class II molecules that are empty or occupied with low stability peptides suggests that DM has a higher affinity for a more open conformation. Thus, binding of DM may shift the equilibrium towards a more open binding groove, probably breading conserved hydrogen bonds between the peptide backbone and MHC class II side chains, and thereby enabling the peptide, or CLIP to dissociate. Stably binding peptides would be able to force the groove into the closed state with concomitant dissociation of DM, allowing DM to engage another class II – CLIP complex [36-37]. If there is an abundance of low stability peptides, then these can bind but may be exchanged for a high stability peptide. Glycin and proline residues

at peptide anchor and non anchor positions have been identified as residues that decrease MHC class II complex stability and increase susceptibility to DM mediated release. This quality control model is consistent both with the kinetic identification of class II intermediates that are highly receptive to peptides and with empty DM-DR complexes observed in loading compartments. This model also explains the preferential, but not exclusive, loading with stably binding peptides that occurs in the presence of DM. Mutagenesis studies have mapped the interaction site with DM to the lateral face of DR3 molecules.

The function of DM is modulated by DO (HLA-DO in humans, H2-O in mouse), another non classical MHC molecule. In addition to DO it may be influenced by tetraspanin molecules such as CD82 and CD63, which appear to form large complexes with class II and DM molecules [31,33].

HLA-DO/H2-O: a modulator of DM

HLA-DO/H2-O molecules are MHC class II like heterodimers encoded in the MHC. Like DM, they display limited polymorphism and do not bind peptides. DO is expressed mainly in B cells and some thymic epithelial cells, but its low expression has also been reported in dendritic cells. In B cells, DO is stably complexed with DM during intracellular transport and in endo/lysosomal compartments, and recycles between MHC loading compartments and the cell surface. In fact DO require association with DM to leave the ER. It has been reported that germinal center B cells express reduced levels of DO, thereby DM to DO ratio substantially increases, which is a favorable situation for the germinal center B cells to take up the antigens displayed on the surface of follicular dendritic cells for the presentation to a T_H cell to get the help for the formation of memory B cells and plasma cells. The strong association and co-localization of DO with DM in loading compartments suggests that DO may have a role in modulating the functions of DM [31].

Influence of DO on MHC class II antigen Presentation

The function of DO has been a matter of debate. Over expression of DO in various cell lines has led to accumulation of DR-CLIP complexes at the cell surface in some studies; however, another study reports a decrease in surface DR-CLIP complexes. By contrast, no change in the numbers of class II CLIP complexes was found in engineered mice or murine B cell lines expressing different levels of DO. These conflicting results may reflect experimental artifacts in transfectants expressing non physiological amounts of DO, or differences between species, cell types or class II alleles. Cell transfectants, or transgenic or knockout mice expressing different levels of DO consistently show that DO down modulates presentation of several antigens to T cells in the context of different MHC class II alleles. Interestingly, this down modulatory activity of DO is observed only when the antigen is taken up via fluid phase endocytosis. In the case of antigen up take via the B cell receptor (BCR), presentation of some (but not all) antigens or epitopes is enhanced by DO.

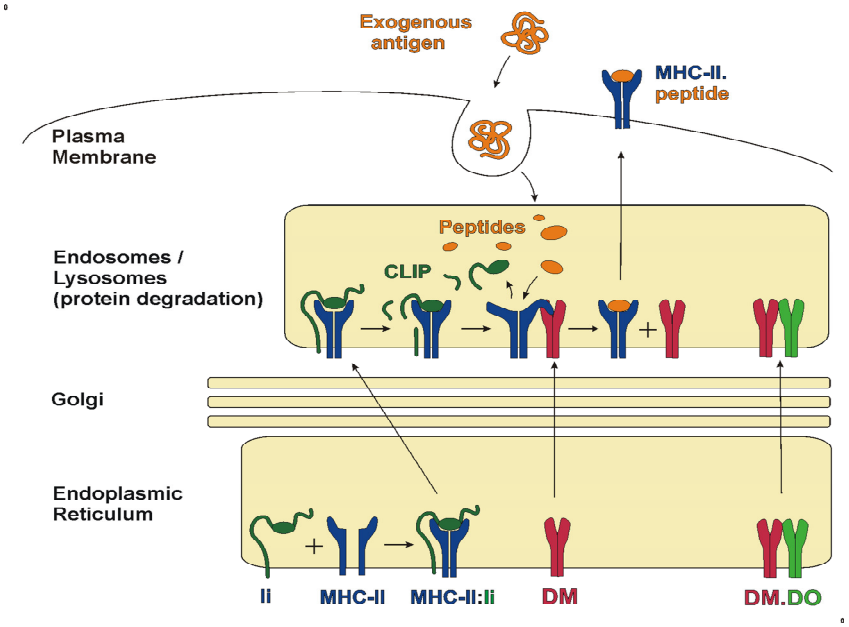


Fig.: 7 Role of H2-O/HLA-DO in antigen presentation

BCRs are likely to transport antigen into late compartments; by contrast, during fluid phase uptake the antigen can be processed and loaded in all the compartments along the endocytic pathway. Thus, DO seem to enhance loading in late compartments, but may decrease loading in early endosomes. This conclusion is supported by experiments with purified proteins, indicating that DO favors peptide loading at pH4.5-5.0, and

appears to reduce loading at pH6.0-6.5. But loading was not decreased by DO at pH6 in another study. The nature of the DO preparation or the peptide used might account for the differences. In comparison to DM alone, DM-DO complexes seem to bind more tightly to DR and are more efficient stabilizers of empty DR molecules at acidic pH. This explains the enhancement of peptide loading by DO at low pH, but not the inhibitory property of DO at high pH. Any model on the physiological role of DO must explain why DO is predominantly expressed in professional antigen presenting cells, B cells and DCs but not in macrophages [31,38-40].

1.4 Biology of Tetraspanin Proteins

The cell membrane contains many different cell surface proteins, some in motion, and some anchored to the cytoskeleton. Which execute different tasks (signaling, adhesion, etc.). Considerable evidence emerging out during the recent times suggests that the membrane molecules are arranged in rafts or microdomains. Here tetraspanins seem to be important for such organization. Their ability to interact with many other signaling molecules and participate in activation, adhesion, and cell differentiation could all relate to role as molecular facilitators that bring together large molecular complexes and allow them, through stabilization, to function more efficiently. The tetraspanin superfamily was first recognized in 1990, when comparison of the sequences of the newly cloned CD37, CD81 (TAPA-1), and sm23 genes with the tumor associated gene CD63 revealed sequence homology and a conserved predicted structure. The family has now grown to about 32 members in mammals, 35 in flies and 20 in worms. Individual tetraspanin proteins are often expressed at 30,000-100,000 copies per cell, and several different tetraspanin proteins are present in almost all cell types and species analysed so far. Whereas some tetraspanins are widely expressed on almost all cell types (for example CD81) or on most epithelial, endothelial and fibroblastic cells (for example CD151), others are more restricted (eg. CD137 and CD53 for lymphoid cells) [77].

1.4.1 Characteristics of Tetraspanins

Tetraspanins have four transmembrane domains (TM1-TM4), forming a small and a large extracellular loop (SEL and LEL), with short intracellular amino and carboxy tails and also several conserved amino acids, including an absolutely conserved CCG motif and two other cysteine residues that contribute to two crucial disulphide bonds within the LEL. General structural features of tetraspanin proteins are highlighted in Fig.:8. The tetraspanin proteins are often inaccessible to cell surface labeling reagents because of their diminutive stature, protruding only 4-5nm above the membrane.

Of the around 200-350 amino acids that are found in tetraspanins, 13-31 amino acid reside in the short extracellular loop (SEL), for which structural information is not yet available. The large extracellular loop (LEL, 69-132 amino acids) is subdivided into a constant region, containing three α-helices (A, B and E), and a variable region, which contains nearly all of the known tetraspanin protein-protein interaction sites. Most of the tetraspanins are modified by N-glycosylation; such as variably glycosylated or acylated, such as CD9. The glycosylation patterns between different tetraspanins vary widely, however some, like CD81 are not glycosylated. CD9 contains a glycosylation site in SEL, whereas most other glycosylated tetraspanins contain sites in LEL. The first, third and fourth transmembrane domains often contain polar residues of unknown function, and almost all tetraspanins are modified by the post-translational addition of palmitate to membrane proximal cysteine residues. Palmitoylation has a crucial influence in organizing tetraspanins and their neighbors into tetraspanin enriched microdomains (TEMs). Several features of TEMs imply that these are a new type of signaling platform, distinct from lipid rafts. Several tetraspanins such as CD151 have been physically linked to integrins, where they modulate integrin-dependent cell adhesion activities [77].

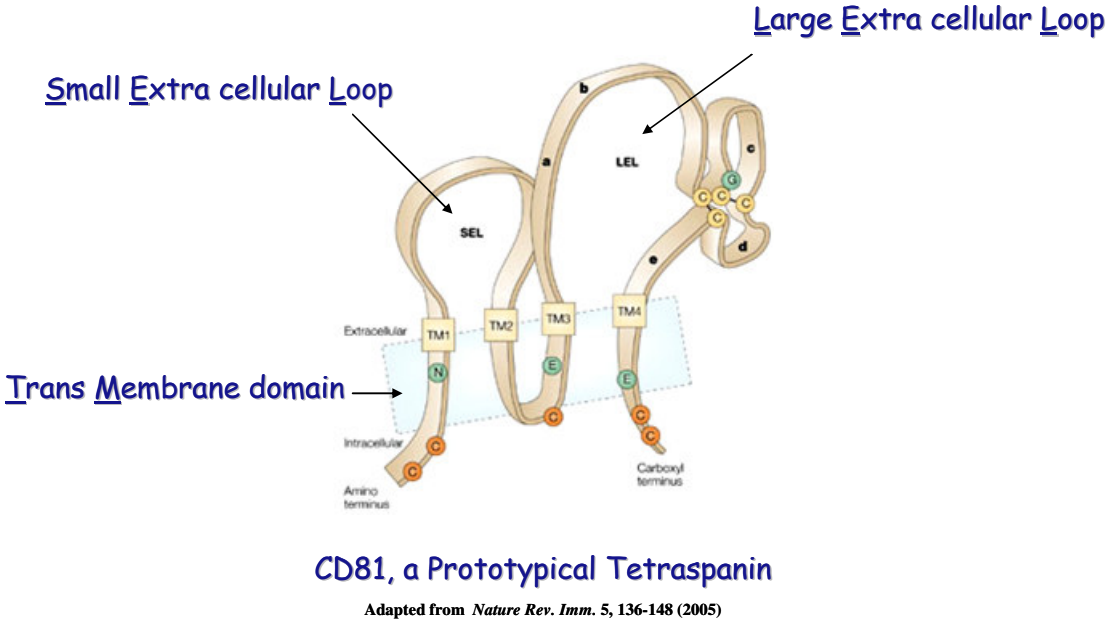


Fig.:8 Schematic structure of Tetraspanin molecule

1.4.2 Genetic insights into Tetraspanin Functions

Human Tetraspanins

Mutations in four different human tetraspanins result in notable phenotypes. Several individual mutations in the TALLA-1/A15 tetraspanin, each within the LEL loop, lead to a new form of X-linked mental retardation. However, little is known so far about the disease mechanism. For mutations of the peripherin/RDS tetraspanin, though, the disease mechanism is clearer. Any of over 70 different mutations in this tetraspanin disrupt the parallel layered structure of the outer segments of photoreceptor cells, which results in retinal dystrophies. Mutations in the tetraspanin ROM1 (a rod outer segment protein and partner for peripherin/RDS), can also synergize with a specific peripherin/RDS point mutation to yield digenic retinal disease (whereby two genes interact to produce the disease phenotype). Recently a disease causing mutation in human tetraspanin CD151 has been described. In a rare mutation, CD151 is truncated and lacks its integrin binding domain. Patients bearing this mutation have end stage hereditary nephritis and a type of skin blistering disease known as pretibial epidermolysis bullosa. Notably, mutations of human integrin $\alpha_6\beta_4$ and the mouse integrin α_3 subunit also cause kidney disruption and/or skin blistering diseases. Human patients with mutations also have sensorineural deafness (irreversible hearing loss involving nerve damage) and β-thalassaemia minor. It is possible that the observed patients might also have mutations in genes other than CD151. However, the most straightforward interpretation is that CD151 regulates basement membrane integrity in the inner ear and in bone marrow as well as in the skin and kidney [77].

Mouse Tetraspanins

In mice, the genes for seven different tetraspanins (CD9, CD81, CD37, Tssc6, CD151, Peripherin/RDS and ROM) have been deleted. Mice that lack the tetraspanins peripherin/RDS or ROM1 have severe or mild retinal degeneration, respectively, which is consistent with the results seen in humans. By contrast, mice lacking CD151 are surprisingly different from humans that are deficient in CD151. CD151KO mice were viable and fertile, their skin and kidney development was apparently normal, and there were no obvious alterations in the functions of their associated laminin binding

integrins in vivo. Possibly, truncated human CD151 is more harmful than a deleted CD151 in mice because there may be opportunity for compensation by other tetraspanins in the complete absence of CD151. Also, it has not yet reported whether humans with mutant CD151 have blood clotting defects such as those seen in CD151KO mice [65]. CD151KO keratinocytes have significantly dimished cell surface levels of the tetraspanin CD9. It is not yet known whether cell surface levels of other CD151 associated tetraspanins (such as CD81, CD82, CD63 and CD53) are also affected by the absence of CD151. Deletion of CD151 cause hyperproliferation of T cells in vivo, but did cause hyperproliferation of T cell receptor stimulated T cells in vitro. Deletion of the tetraspanins CD81, CD37 or Tssc6 also caused strikingly similar T cell hyperproliferation in vitro [63-65]. Hyperproliferation might arise from excessive activity of the tyrosine kinase LCK, thereby upregulating phosphorylation of important T cell signaling molecules. Knockout of CD9 did not affect mouse humoral immune responses or T cell proliferation [66]. However, oocyte fertilization was impaired in CD9KO mice, owing to a deficiency in sperm egg fusion. CD9 null mice also show defective myelination in the peripheral nervous system [66].

1.4.3 Tetraspanins and Malignancy

Some tetraspanins have been viewed as useful markers for the characterization of cancer cells. CD9 was initially described on the surface of cells of B-lineage acute lymphoblastic leukaemia. It is expressed on 90% of B lineage acute leukaemias, and on 50% of acute myeloid leukaemias and B lineage chronic lymphoid leukaemias. The tetraspanin TALLA-1 is expressed in acute neuroblastomas and T lymphoid leukaemias. CD63, the first tetraspanin to be cloned is strongly expressed at early stages of melanoma formation and is downregulated at advanced stages. CD82 expression is reduced during the tumoural progression of prostate, lung, pancreas and colorectal cancers. Recently it has been shown that CD82 specifically interacts with an endothelial cell surface protein, Duffy antigen receptor for Chemokines (DARC, also known as gp-Fy and encoded by *DARC*) by Yeast two hybrid screening. This interaction leads to inhibition of tumor cell proliferation and induction of senescence by modulating the expression of TBX2 and p21 [80]. Similarly, the level of CD9 was

lower in cell lines derived from metastasis of colon carcinoma as compared with cell lines derived from the primary tumour. Experimentally it was shown that transfection of CD9 or CD63 into melanoma cells induced a reduction of the metastatic potential of these cells. This phenomenon was also observed following the transfection of CD82 into prostate cancer cells [74].

There have been many reports showing a critical role for integrins in tumour development, invasion or metastasis. Therefore, a direct or indirect effect of tetraspanins on integrin function might lead to alterations of adhesion or migration properties of the malignant cells that could modify their metastatic potential. In cancer of the oesophagus, in which prognosis is linked to the expression of CD82, no mutations have been observed in the gene encoding CD82; thus downregulation is not a result of mutation. It has been suggested that CD82/KAI-1 expression is downregulated by the p53 tumour suppressor gene but this was not confirmed by other reports. Despite the vast amount of work devoted to the relationship of tetraspanin expression with the prognosis of tumours, tetraspanins are not used as markers in routine practice for assessing the prognosis of cancer in patients [74].

1.4.4 Tetraspanins in Viral infections

There is increasing evidence for the involvement of tetraspanins in infections by various viruses. The most extensively documented involvement of a tetraspanin in viral infection is CD81 in HCV infection. CD81 was the first protein ligand identified for HCV, specifically for E2 protein. The tetraspanin CD82 was identified as an antigen recognized by several antibodies found to inhibit HTLV-1 mediated syncytium formation. Coimmunopresipitation experiments show that HTLV-1 Env glycoproteins interact with highly glycosylated forms of CD82. It has been shown that HIV-1 infection is inhibited by antiCD63 antibodies, but not by antibodies to CD9 or CD81 [78]. Feline immuno-deficiency virus (FIV) infection also appears to involve tetraspanin CD9. Anti CD9 antibody has been shown to inhibit the FIV infection. These studies suggests that tetraspanins as the targets of antibodies which inhibit the infectivity of a range of viruses. Much intense studies needs to be done in this line, the

major obstacle is the availability of suitable reagents for detection and stimulating antibodies for a range of tetraspanin molecules both for human and as well as for mouse.

1.4.5 A role for Tetraspanins in Antigen Presentation!

It has been shown that the tetraspanin CD82 is a resident of MHC class II compartment, where it associates with HLA-DR, -DM and –DO molecules [61,79]. Similarly, CD63 also has been shown to associate with these molecules and both CD82 and CD63 associate with each other. The ability of CD82 and CD63 form complexes with class II MHC, DM, and DO suggests that the tetraspanin proteins may modulate presentation, but so far no functional evidence has been reported. Therefore, it was an aim of this thesis to study whether or not the tetraspanin CD82 would have an influence of antigen presentation.

MATERIALS & METHODS

2. Materials and Methods

2.1: Materials:

2.1.1: Mice

C57BL/6

H2-O-/-

mDO Tg

anti HEL BCR Tg

CD82-/-

CD53-/-

CBA/J

OTI TCR Tg

OTII TCR Tg

2.1.2: Chemicals and Reagents

Dimethyl Sulfoxide (DMSO)	Sigma
HEPES	Sigma
Ethylene diamine tetraacetic acid (EDTA)	Sigma
Potassium chloride	Sigma
Sodium citrate	Sigma
Glycine	Roth
Tris	Roth
Magnesium chloride	Roth
Sodium chloride	Roth

Sodium azide	Sigma
Bovine serum albumin (BSA)	Sigma
Agarose	Applichem
Polyacrylamide	Applichem
Sodiumdodecyl sulfate (SDS)	Roth
TEMED	Sigma
Ethydium bromide	Roth
Bromophenol blue	Roth
Acetone	Roth
Methanol	Roth
Ethanol	Roth
Chloroform	Roth
Isopropanol	Roth
Formaldehyde	Roth
Cell strainers	Hartenstein
Trypan blue	Sigma
Proteinase K	SIGMA-Aldrich
Complete Protease inhibitor	Roche
Gene Ruler™ 100bp Ladder	MBI Fermentas
Cycloheximide	Sigma-Aldrich
ECL chemiluminescence kit	Pierce
Scintillation fluid (Betaplate Scint)	Wallac
Nitrocellulose membrane	Amersham/ Bioscience
Whatman filter paper	Hartenstein
Whatman membrane for NTOC	Herolab
96 well cell culture plates	Cellstar
96 well ELISA plates	Nunc
6 & 24 well cell culture plates	Cellstar
12 well cell culture plates	TPP, Switzerland
6 mm Petri dish	Cellstar

T-25, T-75 & T-250 tissue culture flasks	Greiner
FACS tubes	BD
NBT, BCIP, anti Dig antibody	Roche

Peptides:

HEL peptide (34-45): FESNFNTQATNR

HEL peptide (46-61): NTDGSTDYGILQINSR

OVA peptide S8L: SIINFEKL

OVA peptide K17G: KISQAVHAAHAEINEAG

2.1.3: Antibodies

for proliferation assay :

αCD3ε (145.2C11)	BD Pharmingen
αCD28 (37.51)	BD Pharmingen

for ELISA:

IL-2 ELISA

Capture antibody JES6-1A12:	Anti-mouse IL-2 mAb
Detection antibody JES6-5H4:	Biotinylated anti-mouse IL-2 mAb
Avidin-horseradish peroxidase conjugate	Dianova
Recombinant mouse IL-2	R&D
Streptavidine-Eu+	PE

Cytometer setup beads	BD Biosciences
PE positive control detector	
FITC positive control detector	
Wash buffer	
Assay diluent	

for FACS staining:

All antibodies were purchased from BD Pharmingen unless otherwise mentioned.

Antibody	Clone
anti-CD4 PE	(GK1.5)
anti-CD4 FITC	(GK1.5)
anti-CD4 bio	(GK1.5)
anti-CD8 PE	(53-6.7)
anti-CD8 FITC	(53-6.7)
anti-CD3 FITC	(145.2C11)
anti-CD25 PE	(PC61)
anti-TCRβ bio	(H57-597)
anti-CD44 FITC	(IM7)
anti-CD5 bio	(53-7.3)
anti-CD69 bio	(H1.2F3)
anti-CD62L bio	(Mel 14)
anti-NK1.1 PE	(PK136)
anti-LFA1	(HI111)
anti-CD49 bio	(9F10)
anti-CD18 bio	(6.7)
anti-Fc receptor	(24G2)
anti-Rat IgG2aκ	(R35-95)

2.1.4: Media and Buffers

for cell culture, proliferation assay and FACS staining:

PBS (Phosphate buffered saline):

8 g	NaCl
0.2 g	KCl
1.44 g	Na_2HPO_4
0.24 g	KH_2PO_4

Dissolve in 800 ml dH_2O, adjust pH to 7.4 with HCl

Volume adjust to 1 L, autoclave and store at RT

FACS-Buffer:

PBS + 0.1% BSA + 0.01% Azide

MACS buffer:

EDTA	-	2mM
FCS	-	0.5%
Human ply Ig	-	1% in dPBS

RPMI$^+$ 1640 Medium:	*Invitrogen, Karlsruhe*
+L-Glutamin (0.07%)	GIBCO
+Na-Pyruvat (1 mM)	GIBCO
+β-Mercaptoethanol (0.05 mM)	GIBCO
+MEM (non essential amino acids) (1%)	GIBCO
+ Penicillin (100 U/ml),	Grunthal
+ Streptomycin (100 U/ml)	FatolGmbH
+ FCS (Foetal calf serum) (1-10%)	GIBCO
(heat inactivated at 56°C for 45 min)	

DC medium

400 ml commercial RPMI, 50ml heat inactivated (56^{o}C, 45min) filtered FCS, 50ml filtered F1/16 s/n as a source for GM-CSF, 1x glutamine, 1x P/S, mercaptoethanol.

for genomic DNA isolation and agarose gel electrophoresis:

Tail Lysis Buffer:

50 mM Tris pH 8.0

100 mM EDTA

100 mM NaCl

1% SDS

TBE Buffer (1x):

10.8 g Tris

5.5 g Boric acid

0.37 g EDTA

add 1 L H_2O

DNA Loading Buffer:

0.25% Bromophenol blue

0.25% Xylene Cyanol FF

30% Glycerol in water

store at 4°C

1% Agarose Gel: (150 ml)

1.5 g agarose

150 ml 1x TBE buffer

boil

add 4 µl Ethidium bromide (10 mg/ml)

for ELISA

Coating buffer (0.1 M Sodium Carbonate, pH 9.5)

8.40 g $NaHCO_3$

3.56 g Na_2CO_3

Volume adjusted to 1L with dH_2O

Wash buffer

PBS with 0.05% Tween-20

Substrate buffer

0.1M KH_2PO_4, pH ~ 6

Substrate solution

OPD (1mg/ml in substrate buffer)

Hydrogen peroxide (H_2O_2)

Stop solution

2 N H_2SO_4

Enhancement solution (from PE)

for Southern blotting

Wash buffer: 0.5x SSC; 0.1% SDS

Dig buffer I : 100mM Tris/HCl (pH 7.5),150mM NaCl

Dig buffer II : 2% Boehringer blocking reagent with Dig buffer I

Dig buffer III : 100mM Tris/HCl, 100mM NaCl, 50mM $MgCl_2$, pH 9.5

SSC : 175g NaCl+88.2g tri sodium citrate, pH 7

Staining solution: Dig buffer 3 (10ml)+NBT (45ul)+BCIP (35ul) per blot.

for cell counting

Trypan Blue Solution:

0.4% (w/v) Trypan Blue in PBS

Dilute 1:10 in PBS for live cell counting

ACK Lysis buffer:

0.15M NH4Cl	-	8.2 g
10mM KHCO3	-	1 g
0.1 mM Na2EDTA	-	37.2 mg

Add 800 ml of DDH2O and adjust pH to 7.2-7.4 with 1N HCl and finally makeup the volume to 1000ml. Sterilize by filtration with 0.2um membrane filter and store at 4°C.

SDS-PAGE

Composition of SDS-Poly-acryl amide gels

Resolving Gel:

	Component volumes (ml) per 12 ml gel mix	
	8%	12%
H_2O	5.52 ml	3.96 ml
30% acrylamide mix	3.24 ml	4.8 ml
1.5 M Tris (pH 8.8)	3.0 ml	3.0 ml
10% SDS	0.12 ml	0.12 ml
10% ammonium persulfate	0.12 ml	0.12 ml
TEMED	6 µl	4.8 µl

Stacking Gel:

	Component volumes (ml) per 3 ml gel mix
H_2O	2.1 ml
30% acrylamide mix	0.5 ml
1.0 M Tris (pH 6.8)	0.38 ml
10% SDS	0.03 ml
10% ammonium persulfate	0.03 ml
TEMED	3 µl

Protein Loading Buffer (1X SDS Gel Loading Buffer):

50 mM Tris.Cl (pH 6.8)

100 mM Dithiothreitol

2% SDS

0.1% Bromophenol Blue

10% Glycerol

Store at -20°C

Gel Running Buffer: (10X)

144.13 g Glycine

30.3 g Tris

100 ml 10% SDS

volume adjusted to 1L with dH_2O

store at RT

Protein Transfer Buffer: (10X)

145 g Glycine

29 g Tris

volume adjusted to 1L with dH_2O

store at RT

dilute 10X transfer buffer: methanol: dH_2O in 1:2:7 ratio before use

Ponceau S Solution:

2 g Ponceau S

30 g Trichloroacetic acid

30 g Sulfosalicylic acid

dH_2O to 100 ml

store at RT

Dilute 1:10 in dH_2O for working solution

Blocking Solution:

5% (w/v) nonfat dried milk in PBS/0.1% Tween

2.1.5: *Radioactive Material*

^{3}[H]-Thymidin	ICN Biomedicals

2.2 Instruments and Accessories

Agarose gel reader	Mitsubishi
Microscope	Leica
Multichannel pipette	CAPP
Heat block	Hartenstein
pH meter	Hanna Instruments
Harvester “MACH 3”	Tomtec
β-Counter “Micro Beta Trilux”	Wallac
Centrifuge “Megafuge 1,0R” and “Biofuge 13”	Heraeus Instruments
FACScan	Beckton Dickinson
FACSCalibur	Beckton Dickinson
FACSSorter vantage SE	Beckton Dickinson
VICTOR	PE
Ultraspec 2000, UV/Visible Spectrophotometer	Pharmacia Biotech
CO_2 incubator	Heraeus Instruments
Dynal Magnet	Dynal A.S.

2.3: Methods

2.3.1: Isolation and cultivation of bone marrow derived dendritic cells (bmDC) from mouse:

For the isolation of bone marrow derived dendritic cells from mouse the following protocol was used:

Day 0

- Femur and tibiae were removed from a freshly killed mouse, cleaned using paper tissues, and transferred into sterile ice-cold DPBS.

- The bones were dipped into in ice-cold 70 % ethanol for ~30-60sec for sterilization purpose.

- The bones were transferred into sterile ice-cold DPBS.

- Both ends of each bone were cut with surgical scissors and the bone marrow flushed out with ice-cold DPBS (by using a syringe with 0.45 mm ∅ needle) into a 15 ml Falcon centrifuge tube.

- The cells were centrifuged the cells at 1500 rpm for 10min at 4^{o}C.

- The cells were re-suspended thoroughly but gently with 1ml DC medium to eliminate clumps. 9ml DC medium was added to make up the final volume to 10ml.

- Avoiding the clumps in the bottom of the tube, 2 x 10^{6} viable cells were transferred to 100 mm Petri dishes (bacteriological grade, not culture grade, otherwise cells will adhere to surface).

- 10ml of DC medium was added per dish.

- Cells were incubated at 37^{o}C in a cell culture incubator.

Day 3

- 10 ml of warm DC medium was added to each plate slowly (through the edge of the dish).

Day 6

- 10ml of cells were collected carefully from the edge of the dish and centrifuged at 1500 rpm, for 5 min. Samples from different dishes of the same animal can be pooled.

- The cell pellet were re-suspended with the same volume of pre-warmed fresh DC medium and 10ml were transferred back to each dish.

Day 8

- The procedure described for day 6 was repeated.

Day 9 maturation of DCs

- 1µg/ml LPS was added to each plate; TNF-α at a final concentration of 500 U ml^{-1} can also be used instead of LPS to maturate the bmDCs (DC can be easily activated by pippeting, handling, etc. It is highly recommended to keep the DCs always on ice).

- Plates were incubated for 24h at 37°C.

Day 10 Bone marrow derived dendritic cells are ready to be used.

- Non-adherent cells (mostly dendritic cells) were collected by washing gently the bottom of the dishes. Adherent cells are mostly macrophages.

- Centrifuge the cells at 1500 rpm for 10min.

- Re-suspend the cells and adjust to the desired concentration.

2.3.2: Estimation of total IgG and IgM levels in mouse sera:

- Falcon 96 well ELISA plates (#353912) were coated with Goat anti mouse IgG+IgM (H+L) (Cat No.: 115-005-068, from Jackson Immuno Research) or TNP(34)BSA/TNP(3)BSA/NP(36)BSA/NP(2)BSA at the concentration of 1.8ug/ml in carbonate buffer, pH:9.6.

- The plates were incubated at 37°C for 1hr. 30 min. or at 4°C for over night

- The plates were washed with PBS-T, 3 times.

- Plates were blocked with Gelatin blocking solution for 1 hr. at 37°C or over night at 4°C. The plates were then covered with plastic foil to prevent evaporation of the liquid.

- Coated plates can be stored for min 2 months at 4°C.

- Sera samples were diluted with PBS-T for the measurement of Ig (starting dilution from 1:50 in 1→3 steps further dilution with PBS-T).

- Purified mouse IgG (Y3) can be used as positive control at the following dilutions; 320 ng/ml in 1→ 2 dilution steps until 5 ng/ml and PBST alone can be used as negative control.

- Standard mouse IgM (Pharmingen, # 550963) starting from 125ng/ml in 1 → 2 dilution steps until 1.95 ng/ml and PBST alone can be used as negative control.

- 100ul of diluted test sera samples or standard IgG or IgM samples per well were added.

- The plates were incubated for 1 hr at 37^{o}C.

- The plates were washed for 5 times with PBS-T.

- 100ul of secondary antibody solution was added per well to detect either IgG or IgM. For IgG detection, peroxidase conjugated secondary antibody anti mouse IgG-HRP was used at 1:2000 dilution in PBS-T; for IgM detection anti mouse IgM-HRP was added at 1:1000 dilution.

- The plates were incubated for 1 hr at 37^{o}C.

- The plates were washed with PBS-T for 3 times.

- 1mg/ml OPD substrate solution was prepared freshly with substrate buffer. Hydrogen peroxide (H_2O_2) was added at the concentration of 1ul/ml of substrate buffer.

- 100ul of substrate solution per well was added, waited until the colour change from colour less solution to olive green. This usually takes around 3-10min depending on the amount of antibody in the test sample. To avoid the influence of temperature on the colour development step may be incubate at 37^{o}C. At elevated temperatures like 37^{o}C the reaction of colour development is faster than at room temperature. Colour development may start in 2-5 min.

- The colour development reaction was stopped by adding 25ul of 2N H_2SO_4 per well. The colour will change from green to red after the addition of a strong acid.

- The optical density was measured at 490nm with VICTOR 96 well plate reader.

2.3.3: B cell/T cell/DC Isolation by using MACS:

- Spleens or LN were isolated from mice.

- The spleen was placed into a 6 cm petri dish with ice cold dPBS containing collagenase (1mg/ml) and DNase (100units/ml).

- Collagenase and DNase buffer was injected into both ends of the spleen with a 1ml syringe.

- The spleens were incubated for 5 min on ice.

- Small fragments of spleen were made with forceps.

- Cells were incubated for 20 min at 37°C.

- Cells that were attached to the dish during the incubation time were collected with a cell scrapper. The final volume was adjusted to 15 ml with ice cold MACS buffer.

- The cells were passing through the 40um Nylon filter into a 50 ml Falcon tube in order to remove clumps.

- The cells were centrifuged at 1500 rpm, 10 min at 4°C.

- The cell pellet was re-suspended in 1500ul ice cold MACS buffer with 5% Human poly IgG (to block the Fc receptors on the cell surface). Incubated for 5 min on ice.

- The cells were centrifuged at 1500 rpm, 10 min at 4°C.

- Re-suspend the cells in 200ul of MACS buffer.

- 50ul of Biotin-Antibody cocktail was added for $50X10^6$ cells (Approx. per spleen).

- Mix well but gently.

- Incubate for 10 min at 4°C.
- 150ul of MACS buffer and 100ul of Anti-Biotin Micro beads was added.
- Mix well and incubate for 15 min at 4°C.
- 10 ml ice cold MACS buffer was added and spin at 1500 rpm, 10 min at 4°C.
- The supernatant was removed carefully without disturbing the cell pellet.
- Re-suspend cell pellet in 500ul of MACS buffer.

Magnetic separation with LS column:

- The LS MACS column was equilibrated with 3 ml of ice cold MACS buffer.
- The cell suspension was transferred, into the column without air bubbles.
- The column was washed 3 times with 3ml cold MACS buffer per each step.
- The flow through consisting of B cells was saved.
- *Elution of the cells (T cells, DCs, Macrophages) retained in the column:* The column was removed from the magnet and placed into a 15ml Falcon tube. 5 ml of ice cold MACS buffer was added and pushed with the piston to elute the cells from the column.
- 10ml ice cold MACS buffer was added to the cells.
- The cells were filtered through 0.45um Nylon filter.
- The cells were counted with trypan blue staining.
- The cells were centrifuged at 1500 rpm, 4°C for 10 min.
- The cells were re-suspended in the volume required for the experiment.

Important steps: Incubation time and temperature with MACS bead are very important and critical. The amount of the beads and the volume of the reaction are also very important.

Important points to be noted:

- Around 60% of the cells in spleen are B cells
- One can obtain 10-25 million CD19+ cells from one spleen depending on the age of the mice.
- One may obtain 2-3 million cells CD11c+ (DCs) cells from one spleen.

MACS beads:		Cat #		
mouse antiB220-beads	-	130-049-501	-	Miltenyi Biotec GmbH
mouse antiCD19-beads	-	130-052-201	-	Miltenyi Biotec GmbH

Collagenase-4 - C-5138 from Sigma
DNase Type-4 - D5025 from Sigma

2.3.4 Hapten Inhibition Assay:

- Sera dilutions were prepared (1: 25 in PBST) to be tested for hapten inhibition assay.
- Hapten (TNP-L-Lys) dilutions were prepared (40mM) from the stock (250mM) with PBS-T. Serially diluted the hapten solution with PBS-T in 1→2 steps.
- Transfer 50ul/well of hapten solution into TNP(3)BSA coated plate.
- Transfer 50ul/well of sera dilution into TNP(3)BSA plate containing hapten solution (50ul).
- In the end sera dilution would be 1:50 and hapten concentration would be 20mM.
- Plates were incubated for 1 hr at 37^{o}C.
- Plates were washed with PBST for 3 times.
- Dispense 100ul of GAM-POX-IgG Fc specific secondary antibody at the dilution of 1:2000.
- Plates were incubated for 1 hr at 37^{o}C.
- Plates were washed with PBST for 3 times.
- OPD substrate was used to develop the colour reaction and measured the optical density at 490nm as explained previously.

2.3.5 Lysis of RBC cells with ACK lysis buffer:

- Cells were re-suspended (use 2 ml ACK buffer per one spleen) in ACK lysis buffer and incubated on ice for 5-10 min.

- Centrifuge the cells at 4000 rpm for 5 min. Cell pellet should look in white colour.

- Filter through nylon filter.

2.3.6 FITC-OVA uptake assay:

- BMDCs were re-suspended at the concentration of $2x10^6$ cells/ml in complete RPMI BMDC.

- Transfer 50,000 BMDCs per well by transferring 50ul of cell suspension per well into a Falcon U-bottom 96 well plate.

- Transfer 50ul of FITC-OVA (concentration 2mg/ml in completer RPMI) per well.

- Plate was incubated at 37°C cell culture incubator for the indicated time points: 0.5hr, 1.5, 2.5, 3.5, 4.5, 5.5, 6.5 (time in hrs) and over night.

- For each time point harvest the cells and block with HuPly Ig and stain with CD11c-APC and measure the fluorescence intensity by FACS.

- Non FITC-OVA pulsed bmDC serve as negative control.

2.3.7 Alum precipitation of antigen:

- Antigen (TNP(12)OVA/NP(12)OVA) and 10% Alum in PBS were mixed in 1:1 ratio in an eppendorf tube.

- One fifth quantity of the 6.25% Ammonia solution was added to the antigen-alum solution.

- Tube was incubated for 1hr at 4°C on ice.

- Centrifuge the reaction mixture at 10000 rpm for 10 min in a cold centrifuge.

- Remove the supernatant without disturbing the pale yellow pellet.

- Resuspend the pellet in sterile PBS for immunization.

2.3.8 Southern Blotting:

Day I Isolation of genomic DNA from mouse tail piece:

- Add 10ul of protinaseK to 700ul of tail buffer which consists of a mouse tail piece in an eppendorf tube.
- Tube was incubated for o/n at 56^{o}C.
- Tube was centrifuged at 13000 rpm for 5 min.
- Removed the supernatant without disturbing the pellet. (NOTE: Genomic DNA attaches to mouse skin fur. While removing the supernatant it is very important to remove the supernatant completely).
- Transfer the supernatant containing mouse genomic DNA into a new eppi.
- 500ul of iso propanol was added to the genomic DNA solution.
- Mix gently but thoroughly. You should see a white thread like precipitated genomic DNA.
- Take a glass capillary and role the genomic DNA at one end of the glass capillary.
- Genomic DNA was dipped in 70% ethanol first and then into 100% ethanol.
- Place the glass capillary consists of genomic DNA into a fresh eppi.
- 200-300ul of double distilled water was transferred to dissolve the genomic DNA. Tubes were placed at 68^{o}C for 15 min with shaking.
- Prepared mouse genomic DNA was stored at 4^{o}C.

Day 2 Restriction digestion of genomic DNA:

- Mix 20.5ul of genomic DNA, 3ul of 10x restriction buffer, 1.5ul of restriction enzyme (RE).
- Incubate for o/n at 37^{o}C

Day 3 Agarose gel electrophoresis and membrane transfer setup:

- 1% agarose gel was prepared as per the standard protocols.
- 25ul of genomic DNA digest solution was mixed with 5ul of 6xDNA loading dye.
- Dig labelled marker was loaded in one lane for MW reference.
- Run the gel at 90V until the dye front runs for ¾ of the gel.
- Transfer the gel into denaturation buffer and shake for 20min on a shaker.
- Wash the gel with water.
- Place the gel into neutralization buffer and shake for 2x15 min.
- In the meantime soak the nitrocellulose membrane in 20xSSC.
- Arrange the transfer setup without air bubbles as shown in M-1.
- Let the transfer run for o/n or weekend.

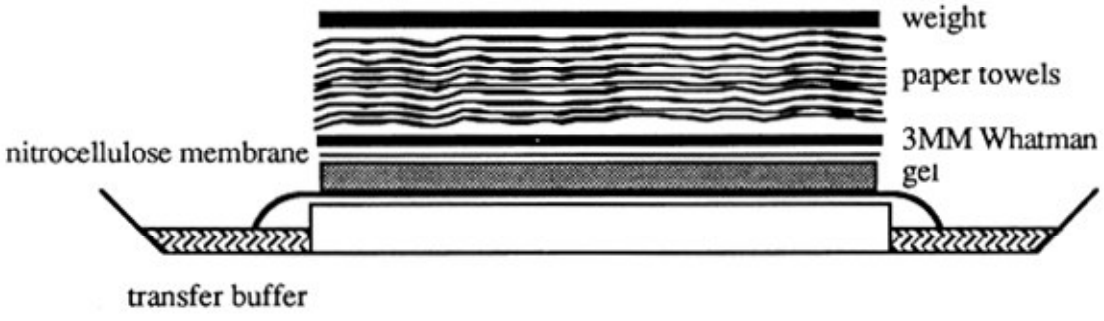


Fig. M-1: Transfer setup for Southern blotting.

Day 4 Hybridization with probe:

- Membrane was removed from transfer setup and let it dry by placing at 80^{o}C for 2hrs.
- Pre warm the prehybridization buffer to 65^{o}C.
- Membrane was placed in prehybridization buffer for 1 hr.
- Probe solution was boiled for 10 min.
- Remove the pre hybridization buffer and add 100ml of pre boiled probe solution.

Day 5 Development of membrane:

- Membrane was washed 3x10min at 65^{o}C with pre warm wash buffer. From this step onwards work at RT.
- Membrane was placed for 1 min in Dig buffer 1
- Membrane was placed for 30min in Dig buffer 2
- Membrane was placed shortly for Dig buffer 1
- Membrane was placed for 30min in antiDig antibody solution (1:5000).
- Membrane was washed for 2x15min with 100ml of Dig buffer 1.
- Membrane was washed for 2 min in Dig buffer 3.
- Membrane was incubated in the dark without shaking in BCIP-NBT staining solution until you see the bands (5min to o/n).
- Stop the colour development of bands just by washing the membrane with distilled water and dry the membrane between filter papers.

2.3.9 Mass spectrometry:

Spleen cells were lysed in 1% Triton X-100 and were precipitated with Y3P conjugated Sepharose beads. Peptides were eluted with 0.1% trifluoro acitic acid and were analysed on a Reflex III mass spectrometer (Bruker). Peptides were identified with multidimensional protein identification technology, which is based on two dimensional liquid chromatographic fractionation followed by mass spectrometric sequencing. Lyophilized peptides were resuspended in 5% acetonitrile, 0.5% acetic acid, 0012% heptafluoro butyric acid and 5% formic acid. Peptides were fractionated on a fused-silica micro capillary column (100um inner diameter) packed with C18 reverse phase material CD18-ACE 3 um (Pronto SIL 120-3-C18 ACE-EPS; Bischoff Chromatography) follwed by cation exchange material (Partishere SCX; 5um particle size; Whatman). A fully automated eight step gradient separation was done on an Agilent Technologies). The high performance liquid chromatography column was directly coupled to a Finnigan LCQ ion trap mass spectrometer (Finnigan) equipped with a nano-LC electrospray ionization source. Mass spectrometry in the MS/MS mode was done according to the manufacturer's protocol. Peptides were identified by using the SEQUEST and MASCOT algorithms against the Swiss-Prot database. Matched sequences were processed.

2.3.10 Antigen Presentation Assay and IL-2 estimation

- Purified spleen cells/DC from mouse or BMDCs (APC) was prepared following the standard protocols explained in the above sections.

- 50ul of antigen presenting cells ($5x10^4$/well) were seeded into a round bottom 96 well plate. 50ul of T cell hybridoma cells/TCR transgenic T cells ($5x10^4$/well) were added to the antigen presenting cells. In the end the ratio of antigen presenting cell to T cell is 1:1.

- 100ul of serially diluted 2x concentrations of protein Ags, hen egg lysozyme (HEL), or ovalbumin (OVA) or peptide controls were added to the APC+T cell mixture.

- The culture was maintained for 24-36 hrs at 37°C in a 5% CO_2 cell culture incubator.

- After incubation 100µl of the supernatant were used for the measurement of IL-2 by a europium-based fluorescence immunoassay by using purified anti-IL-2 mAb (clone JES6-1A12) and biotinylated anti-IL-2 mAb (JES6-5H4) (BD Pharmingen).

Estimation of mouse IL-2 levels by ELISA:

- Nunc black flat bottom 96 well plates were coated with IL-2 capture antibody (clone JES6-1A12) at concentration of 1.8ug/ml in carbonate buffer, pH:9.6.

- The plates were incubated at 37°C for 1hr. 30 min. or at 4°C for over night.

- The plates were washed with PBS-T, 3 times.

- The plates were blocked with Gelatin blocking solution (200µl/well) for 1 hr. at 37°C or over night at 4°C. The plates were wrapped with plastic foil to prevent evaporation of the liquid.

- These plates can be stored for a minimum of 3 months at 4°C without much loss of bioactivity.

- To detect the levels of IL-2 in a cell culture supernatant isolated from an antigen presentation assay, 100ul of antigen presentation assay cell supernatant were transferred into wells of anti-mIL-2 capture antibody coated plate.

- Purified mIL-2 standards were prepared by diluting with PBST, starting from 40ng/ml in 1→ 4 steps dilution for 10 titration steps. PBST alone in the 12^{th} well would serve as a background negative control for the IL-2 detection.

- The plates were incubated for 3 hrs at room temperature or for over night at 4^{o}C.

- The plates were washed with PBS-T for 5 times.

- 100ul/well of 0.25ug/ml anti mIL-2-bio antibody in PBST was added.

- The plates were incubated for 2 hrs at RT.

- The plates were washed with PBS-T for 3 times.
- 100ul/well Streptavidin-Europium in assay buffer in 1:1000 dilutions was added.
- The plates were incubated for 45 min at RT (Note: Be sure to incubate only 45 minutes ONLY. Longer incubation will increase the background).
- The plates were washed with PBS-T for 3 times.
- 150ul of enhancement solution was added per well and the plates were incubated for minimum 30min at RT. Alternatively the plates can be left at $4^{o}C$ for over night.
- The fluorescence was measured with VICTOR 96 well plate reader.
- Results were reported as fluorescence counts per second.

2.3.11 T and B cell Proliferation Assay:

T cell Proliferation Assay:

- U-bottom 96 well plates from Falcon were coated with 5µg/ml αCD3 mAb + 5µg/ml αCD28 mAb in PBS (100µl/well) overnight at $4^{\circ}C$. Several blank wells without antibody coating served as non stimulated control.
- The wells were washed 2 times with sterile PBS with a multi channel pipette.
- $5x10^4$ spleen cells were seeded per well in 200µl of complete RPMI medium.
- The plates were incubated for the indicated time points at $37^{\circ}C$ in a 5% CO_2 incubator for 24-96 hrs.
- The plates were covered with plastic foil to prevent evaporation.
- Radio labelled thymidine was added at a concentration of 1µCi/well in complete RMPI.

- 18 hrs after thymidine addition, cells were harvested and thymidine incorporation measured as CPM (counts per minute) by using a beta scintillation counter.

- Instead of αCD3 mAb+αCD28 mAb system we have also stimulated with soluble ConA (5µg/ml) in complete RPMI medium for the indicated time points.

B cell Proliferation Assay:

- $5x10^4$ spleen cells/well were seeded into a 96 well U-bottom Falcon plate in 200µl of complete RPMI medium. LPS was supplemented for stimulation of B cells at a concentration of 10µg/ml.

- The plates were incubated for indicated time points (24-96 hrs) at 37°C in a 5% CO_2 incubator.

- The plates were covered with plastic foil to prevent evaporation of the medium.

- Radio labelled thymidine was added at the concentration of 1µCi/well.

- 18 hrs after thymidine addition, cells were harvested to measure thymidine incorporation in a beta scintillation counter.

RESULTS

3.1 Results Part I: H2-O Project

As outlined in the introduction H2-O (HLA-DO in human) (DO) is a non classical MHC class II molecule mainly expressed in B cells and thymic epithelial cells. In previous studies DO have been reported to be absent from dendritic cells, but very recently it has been found to be expressed also in dendritic cells both in human and mouse [47-48]. DO is a dimeric protein consisting of two subunits α and β. H2-Oα knockout mice were generated in the laboratory of Dr. Lars Karlsson [54] and kindly provided for our analysis. H2-O transgenic mice which express 2-3 folds higher levels of H2-O were developed in our laboratory by Dr. Elena Armandola by injecting H2-O α and β cDNA controlled by the K^b MHC class I promoter into the pronucleus of fertilized eggs from (CBA/J x C57BL/6) F1 mice [56]. Since these mice were not on a B6 background I have back crossed with C57BL/6 for 10 times for the present study. These H2-O transgenic mice were also crossed with BCR transgenic mice for hen egg lysozyme (antiHEL BCR Tg mice, I-A^k haplotype obtained from Dr.Lee Leserman) in order to bring the BCR transgene into the DO transgenic background. The rationale for generating these mice was to investigate the role DO in antigen presentation in a situation where the antigen is internalized into B cells via the BCR, in comparison to antigen up take via the fluid phase uptake. H2-O-/- mice are of the H-2^b haplotype because they have been generated with ES cells from the H-2^b strain 129. Since the H2-O gene is located within the MHC gene cluster one can not obtain H-2^k mice deficient for H2-O by breeding of H2-O-/- (H-2^b) mice with H-2^k haplotype mice, as the latter would then contribute their own H2-O genes which are linked to the H-2^k genes.

3.1.1: FACS analysis of H2-O-/- and mDO Tg mice

A thorough analysis of H2-O -/- was not yet been presented. Therefore we have performed an extensive analysis of H2-O-/- mice and mDO Tg mice for the surface marker expression profiles of cells isolated from spleen, bone marrow, lymph node, and thymus.

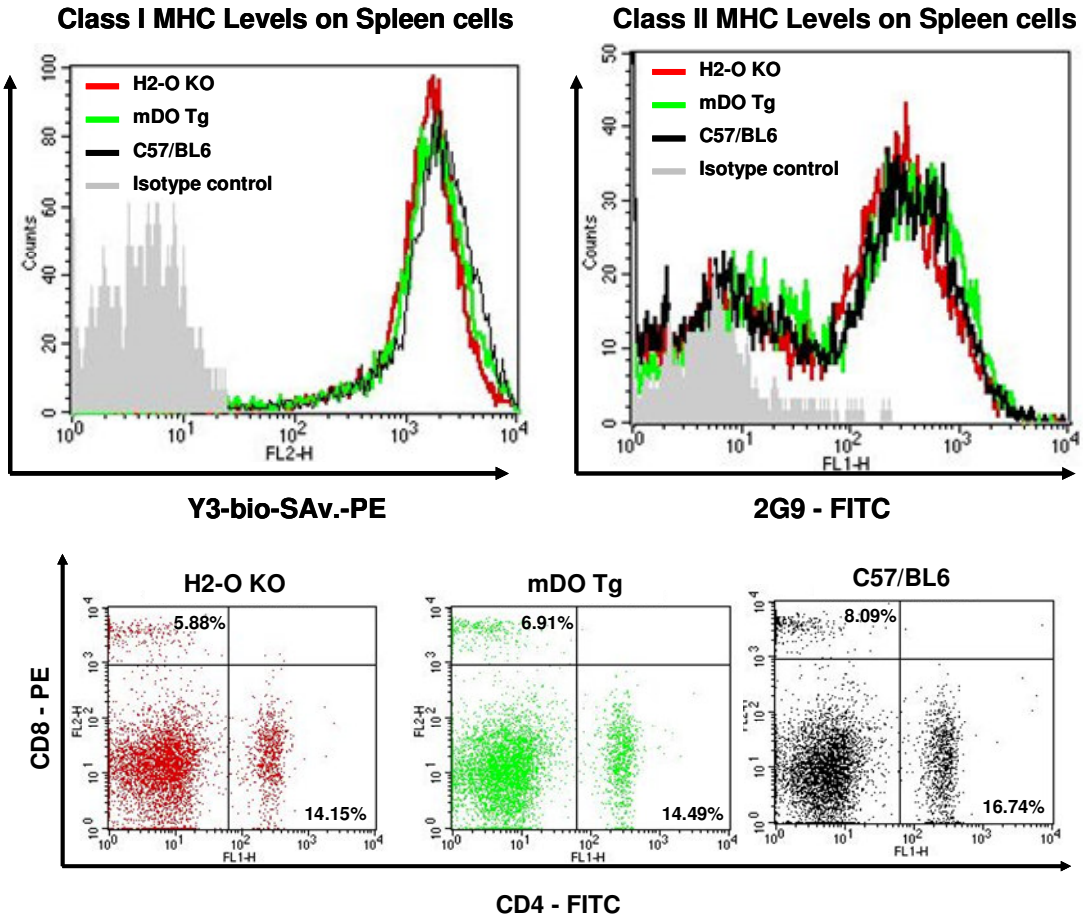


Fig.: RI-1 Expression profile of MHC class I and class II (top), CD4, CD8 (bottom) on spleen cells isolated from H2-O-/-, mDO Tg, and B6 mice.

As shown in fig. RI-1 no significant difference was observed in the expression profile of MHC class I and II molecules and ratios of CD4 to CD8 positive splenic T cells from H2-O -/-, mDO Tg and B6 mice.

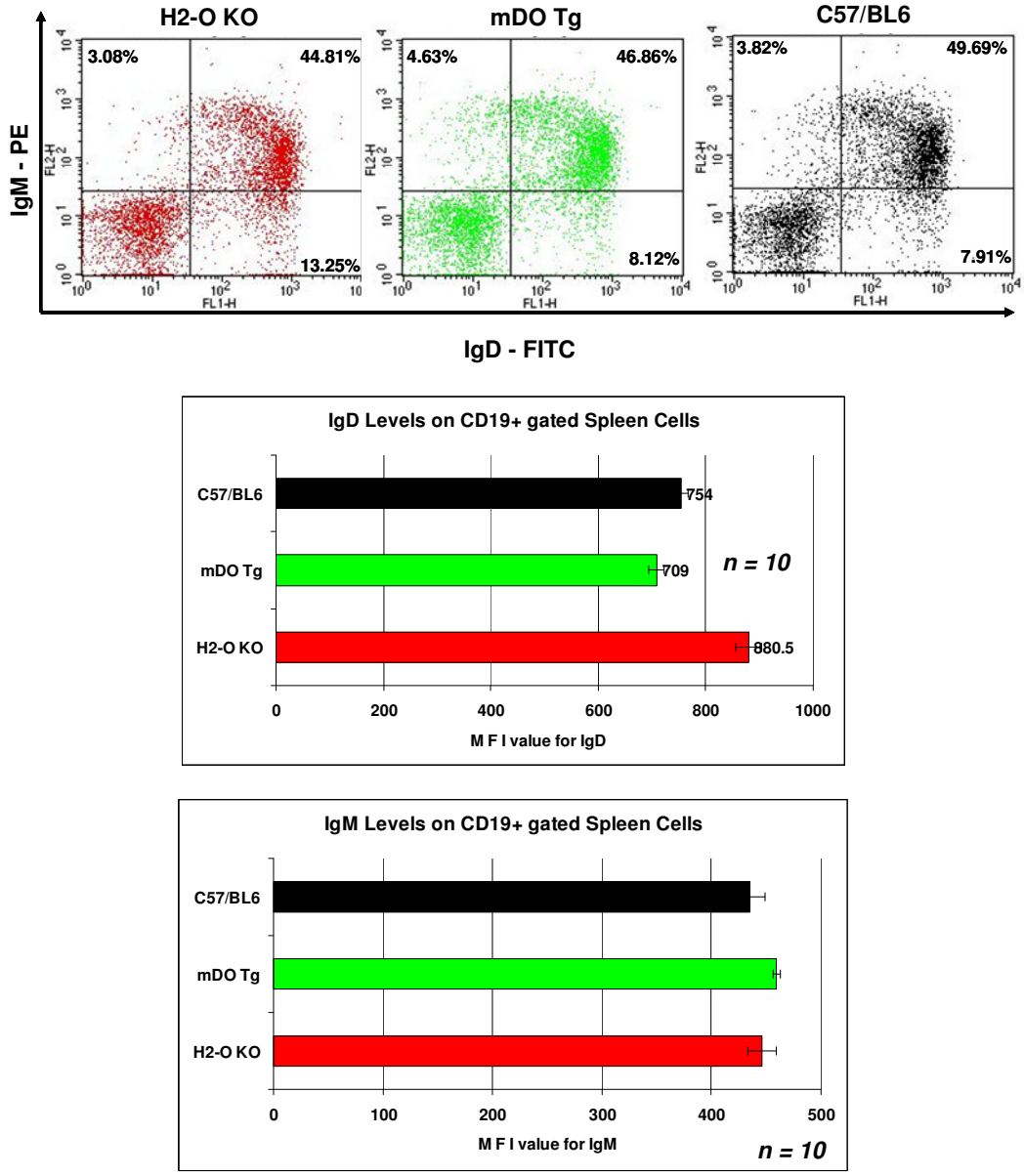


Fig.: RI-2 Expression profiles of BCR proteins IgD and IgM on spleen cells (top) and mean fluorescence intensity of IgD (middle) and IgM (bottom) on CD19 positive gated cells from H2-O-/- (red), mDO Tg (green), and B6 (black) mice.

As shown in fig.: RI-2, an about 1.5 fold increase in the number of IgD single positive B cells was observed in spleens from H2-O-/- mice (~13%) when compared with controls (~8%). This increase was observed in all 10 mice tested. We then analysed the level of IgD expression on CD19+ gated B cells isolated from spleen. Surprisingly, we found around 20% higher expression of IgD on H2-O-/- splenic B cells.

In contrast, IgM expression was unaltered in H2-O-/- mice when compared with controls. We next examined the early B lineage cells in the bone marrow to see whether is there any defect in the production of B cells in bone marrow of H2-O-/- mice. Early B cells are characterized by co-expression of the markers B220 and early B lineage marker.

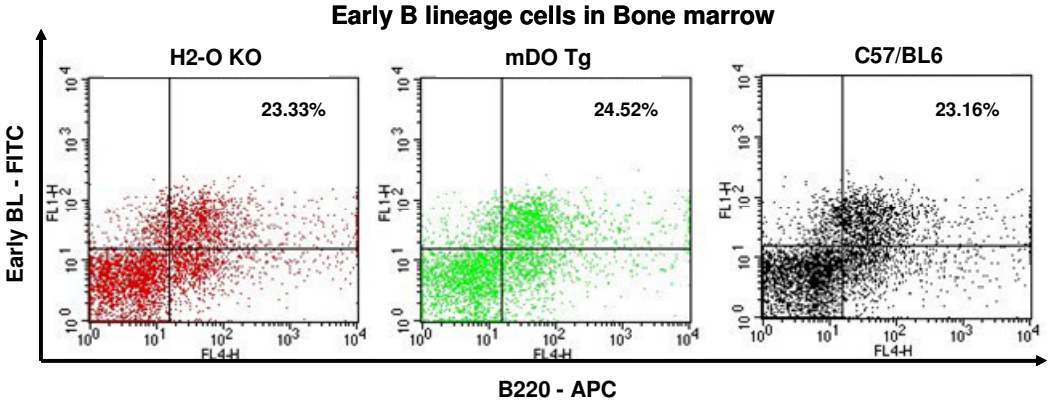


Fig.: RI-3 Expression profile of early B lineage cells in the bone marrow of H2-O-/-, mDO Tg, and B6 mice.

As shown in Fig.: RI-3 there was no difference observed in early B lineage cells production in H2-O-/- mice when compared with mDO Tg and B6 mice. These data suggest that B cell production in the bone marrow is intact in these mice. Instead, it seems that the matured B cell compartment in the periphery is altered in H2-O-/- mice.

3.1.2 Humoral Responses

3.1.2.1 Normal Levels of Immunoglobulins in mDO Tg mice

We next investigated the normal serum levels of immunoglobulins in mDO Tg mice and H2-O-/- mice.

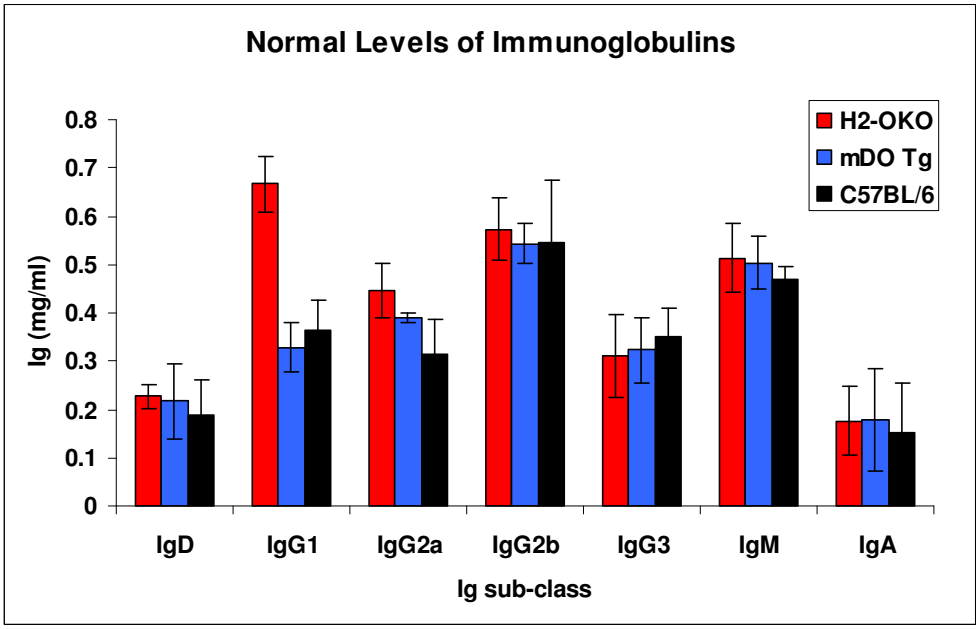


Fig.: RI-4 Normal levels of immunoglobulins in H2-O-/-, mDO Tg, and B6 mice sera samples estimated by ELISA.

As shown in the fig.: RI-4 there are elevated IgG1 levels in H2-O-/- mice when compared with the control mice. This data is in agreement with published data (x). In contrast, there was no difference with regard to the other subclasses of immunoglobulins including IgD for which elevated levels of expression have been observed on B cells (Fig.: RI-2). Likewise in the mDO Tg mice we have not found any significant differences in titers of the normal levels of immunoglobulins when compared with B6 mice.

3.1.2.2 T dependent antibody responses to antigen

We next investigated the H2-O-/-, and mDO Tg mice for antibody responses to the T dependent antigen. We choose 8-10 female mice of age 8-12 weeks from each genotype and immunized intraperitoneally with two different test antigens TNP(11)OVA and NP(12)OVA antigen with alum as an adjuvant. In principle, in response to the immunization with TNP(11)OVA we may expect a minimum of three different types of antibodies; 1) against the hapten TNP, 2) against various ovalbumin protein epitopes, and 3) for the junction part of TNP-ovalbumin. In order to measure only TNP specific antibodies and to eliminate the ovalbumin specific antibody from our ELISA measurements, we choose TNP-BSA. Since the ELISA plates were coated with TNP-BSA, only the hapten TNP specific antibodies can bind to the ELISA plates.

In order to see if DO influenced the affinity of the TNP antibodies we have coated the ELISA plates with TNP-BSA conjugate that carry a low or high density of TNP namely TNP(3)BSA and TNP(34)BSA. Low affinity antibody should not bind to the TNP low (TNP(3)BSA) coated plate or bind less efficiently, whereas both high and low affinity antibodies specific for TNP would bind to TNP high (TNP(34)BSA) plate. The reason why only high but not less affinity antibody binds to TNP low coated plates is caused by the fact that on a TNP low plate the TNP epitopes are spread so far apart that the antibody would be able to bind with one arm or binding site only. This results in monovalent binding for which high affinity is crucial. In contrast on a TNP high plate which is densely covered by the TNP epitope, antibodies can bind with both arms, resulting in increased avidity, so that also low affinity antibodies can bind.

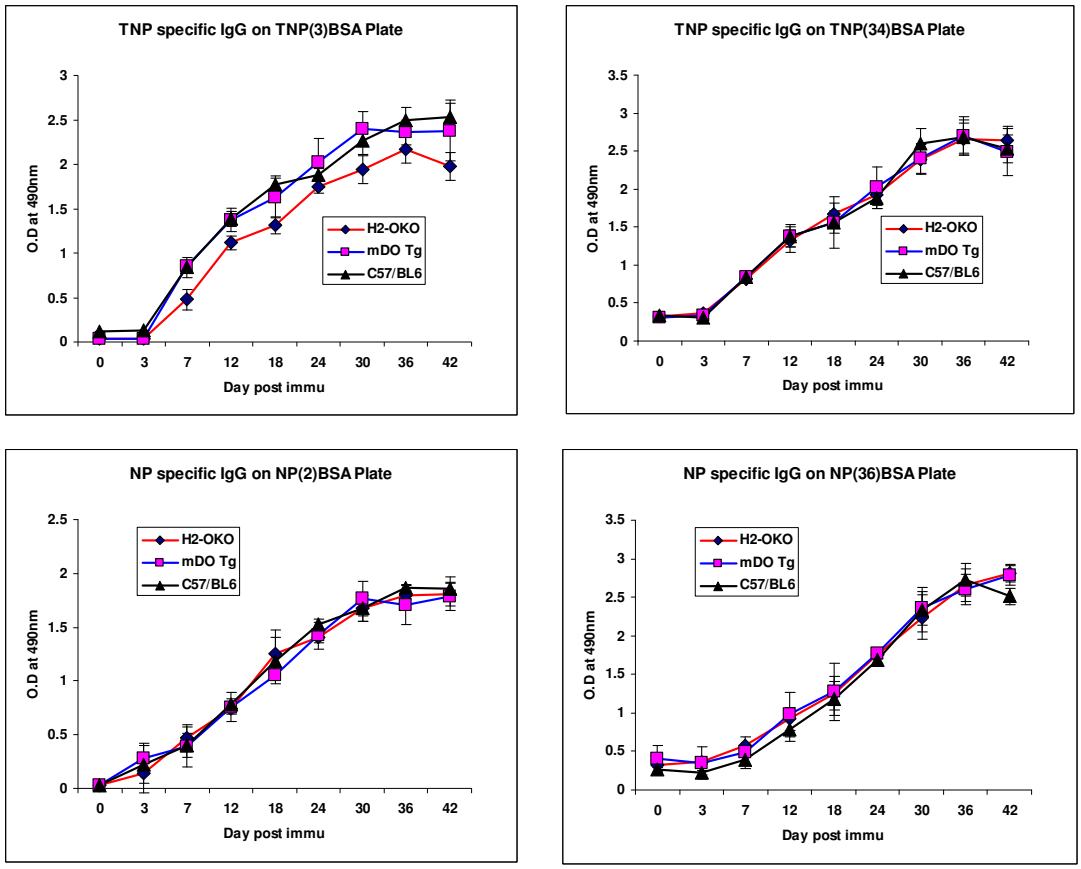


Fig.: RI-5 TNP specific IgG levels in sera samples isolated from H2-O-/-, mDOTg and b6 mice. IgG levels were measured by ELISA at the mentioned days of post immunization with TNP(11)OVA (top) and NP(12)OVA (bottom) in Alum as an adjuvant. Levels of TNP specific IgG on TNP(3)BSA coated plate (top left) and TNP(34)BSA coated plate (top right); NP(2)BSA coated plate (bottom left) and NP(36)BSA coated plate (bottom right). Eight to 10 female mice of age 8-12 weeks from each genotype were used in this study. Data shown is representative of minimum three experiments performed.

The results presented in fig.: RI-5 show a slight reduction in TNP specific antibody binding was observed with H2-O-/- mice on TNP low plates but not on TNP high plate. Suggesting that anti TNP antibodies of H2-O-/- mice are of slightly lower affinity. In contrast, no differences were seen for NP specific IgG levels among H2-O-/-, mDO Tg, and B6 mice. This data suggests that DO may influence the affinity of the antibody generated against the T dependent antigen TNP-OVA but not for NP-OVA. A possible explanation for the different results is that NP antibodies may be generally of higher affinity than TNP antibodies.

3.1.2.3 Determination of affinity of antibody raised in H2-O-/- mice by Hapten inhibition assay

We next investigated with a different method the affinity of the antibodies against TNP(11)OVA from H2-O-/- mice. As we have shown in fig.:RI-5 there was a slight reduction in the TNP specific IgG binding on TNP low plate suggesting that the affinity of antibodies secreted by H2-O-/- mice was lower when compared with control mice. To find out the affinity of the antibody we performed inhibition assays with the hapten TNP-L-Lys. It is known that the antibody class switching from IgM to IgG starts after 2 week of immunization, and that the affinity of antibodies increases with time. Therefore, we tested sera isolated from mice days 18, 30, 34, and 42 after immunization.

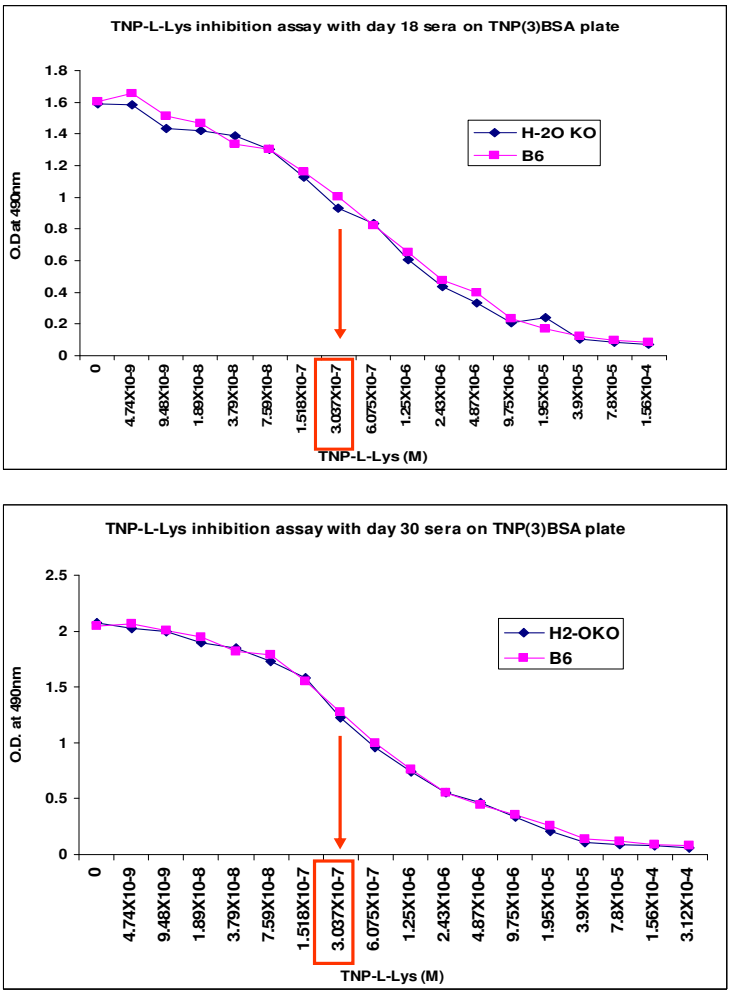


Fig.: RI-6a Affinity of antibody raised in H2-O-/- mice with day 18 (top) and 30 (bottom) sera samples. Sera dilutions were normalized by taking 1:50 for H2-O-/- and 1:80 for B6 with day 18 sample and with day 30 by taking 1:100 for H2-O-/- and 1:200 for B6.

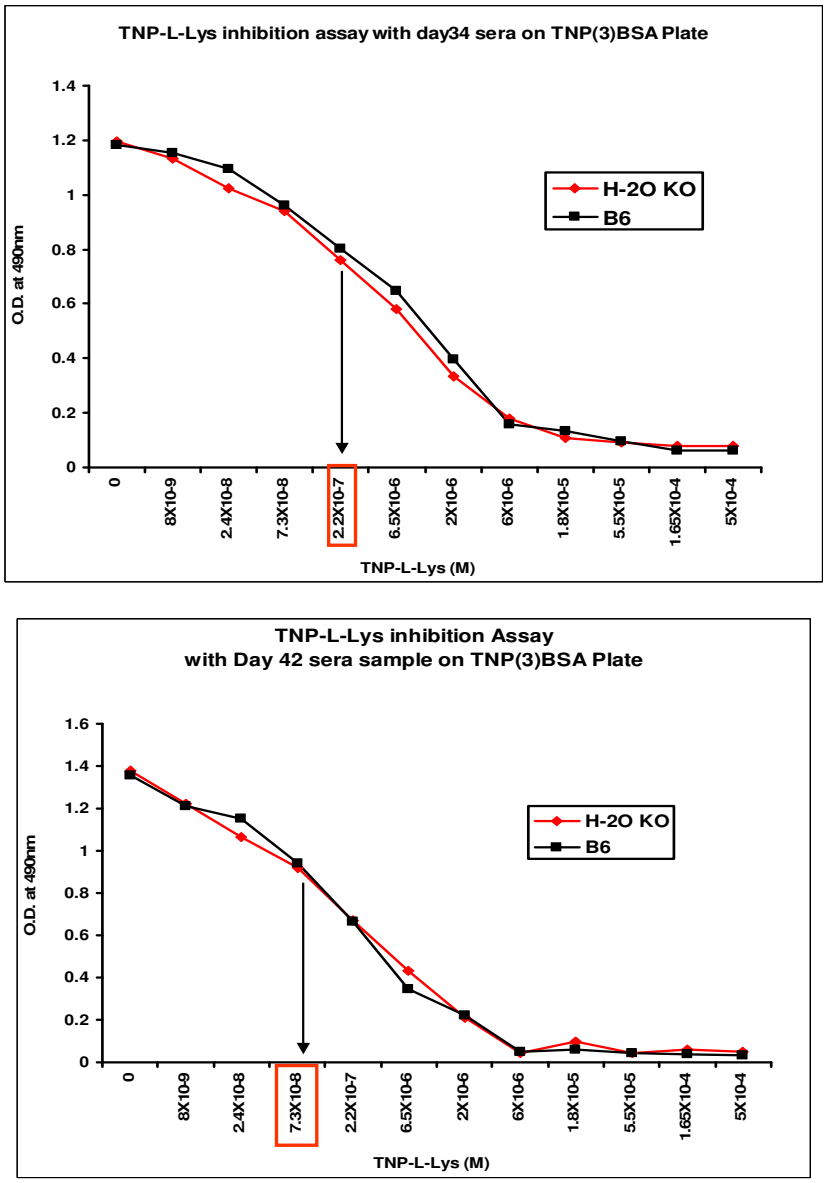


Fig.: RI-6b Affinity of antibody raised in H2-O-/- mice. with day 34 (top) and 42 (bottom) sera samples. Sera dilutions were normalized by taking 1:100 for H2-O-/- and 1:200 for B6 with day 34 sample and with day 42 by taking 1:200 for H2-O-/- and 1:400 for B6.

As shown in fig.:RI-6a and b, sera dilutions were normalized between H2-O-/- and B6 mice as indicated in the figure legend. This normalization is necessary so that both inhibition curves started with the same OD value. As presented in fig.:RI-6a and b we have not found any differences in the affinity of the antibodies raised in H2-O-/- mice as compared to B6 mice. The approximate affinity of the antibodies increased from $3x10^{-7}$M to $7.3x10^{-8}$M from day 18 to day 42 post immunization. It is possible that the

small differences seen on the TNP low plate in Fig.: RI-5, top left, reflect small differences in the antibody titer only but not in affinity.

3.1.2.4 T cell independent antibody responses

We next investigated the T independent antibody responses by injecting the T independent antigen TNP(77)Ficoll. B cells responding to multimeric antigens through multiple interactions and, do not need T cell help. Therefore, only IgM antibodies are produced, because Ig class switching does not occur.

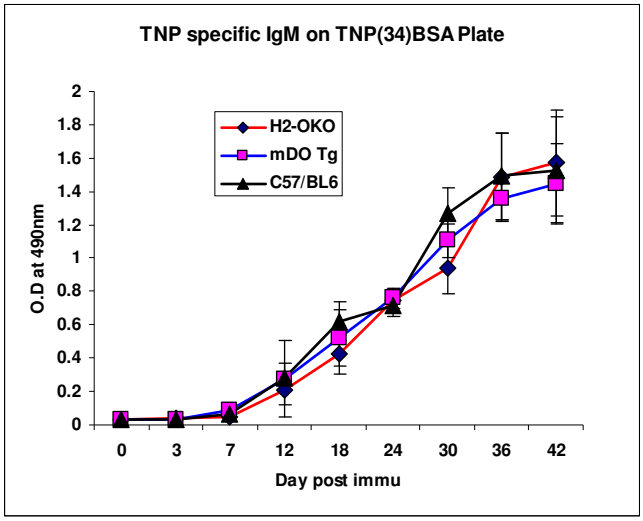


Fig.: RI-6 T independent antigen responses. Antigen TNP-Ficoll specific IgM titers were measured by ELISA on TNP(34)BSA coated plate. Sera isolated from days post immunization as show on *x* axis. Five female mice were used for each group, aged 8-12 weeks old.

No differences were observed in the T independent antigen TNP(77)Ficoll specific IgM titers between H2-O-/-, mDO Tg and wild type control mice (Fig.: RI-6). The titers were relatively low when compared with T dependent antigen.

3.1.3 Antigen Presentation Assays

3.1.3.1 DO inhibits antigen presentation in the early endocytic compartments but not in the late endocytic compartments

Previous studies on antigen presentation by H2-O-/- B cells following fluid phase uptake of antigen yielded at most only very minor effects [54-55,57]. In contrast, H2-O overexpression and anti-sense RNA knockdown studies in mouse B cell lines clearly showed that DO down modulates antigen presentation [56]. Likewise, transfection of H2-O into a mouse sarcoma cell line showed decreased antigen presentation as compared to control transfectants. These results collectively support the notion that H2-O can inhibit antigen presentation, likely via interference with the function of H2-M. To further clarify the role of H2-O in the class II MHC mediated antigen presentation pathway, we crossed our mDO Tg mice with anti HEL BCR Tg mice which express a high affinity BCR (IgM and IgD) specific for the antigen HEL. The resulting mDOxBCR Tg mice allowed us to study the effect of DO on antigen presentation when the antigenic cargo is delivered into the cell via a high affinity BCR. Because the mDO transgene is not genetically linked to the MHC locus this experimental system also allow to study the impact of DO on the A^k MHC allele. So far previous studies on DO were mainly performed with A^b and A^E MHC class II antigens [54-57].

In an antigen presentation assay antigen presenting cells take up the antigen, process it in specialized cellular compartments and then present the processed epitopes via MHC molecules. Upon recognition of these peptide MHC complexes T cells will be activated and secrete IL-2. This secreted IL-2 can be measured by a sandwich ELISA. Thus, the amount of IL-2 produced is a measure of the extent of the antigen presentation by an antigen presenting cell.

Two model antigens were used i.e., hen egg lysozyme (HEL) and ovalbumin (OVA). Both the antigens are well studied. Fig.:RI-11 shows the location of major epitopes in hen egg lysozyme.

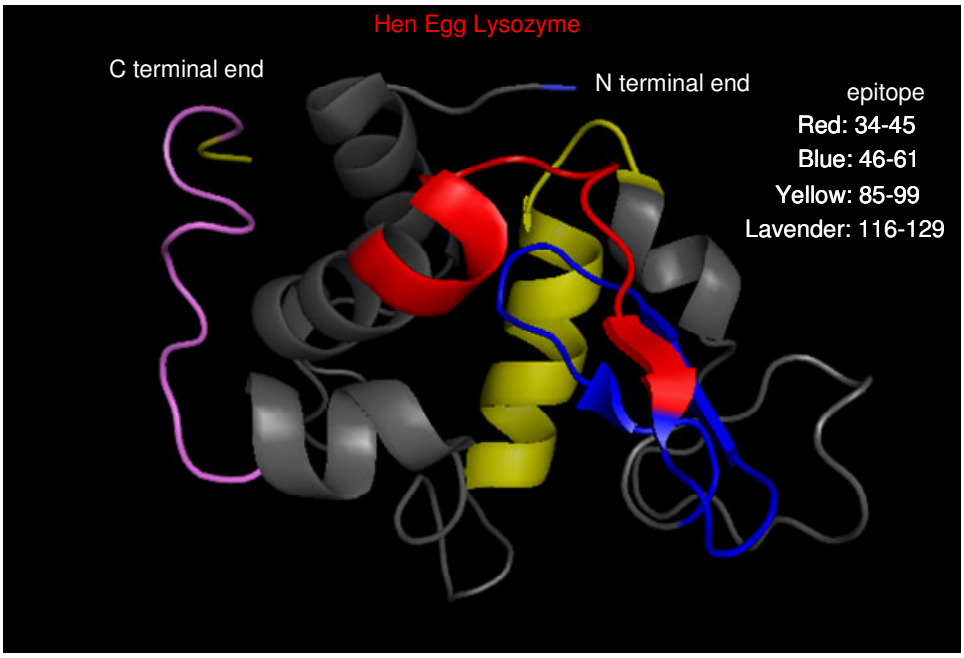


Fig.: RI-11 Solution structure of HEL (PDB: 1GXV). Different epitopes marked with different colors with *Py*MOL software. The immunodominent epitope of the HEL shown in red colour (HEL:46-61).

Previous studies have shown that the HEL epitope 33-47 is preferentially processed and loaded into I-A^k molecules in an early endocytic compartment. In contrast, epitopes 46-61 and 116-129 are preferentially processed and presented in late endocytic compartments. These conclusions were based on the observation that chloroquine which neutralizes the acidic late endocytic compartment, impairs presentation of the epitope 46-61 but has no or little effect on the neutral early endocytic compartments [41]. The T cell hybridoma 3B11 recognizes the HEL epitope region 34-45 in an I-A^k restricted manner. Whereas, the T cell hybridoma 3A9 recognizes HEL epitope 46-61 (immuno dominant epitope in an I-A^k restricted manner. For antigen presentation mice were expressing both, mDO and BCR, only mDO or BCR, or wild type mice.

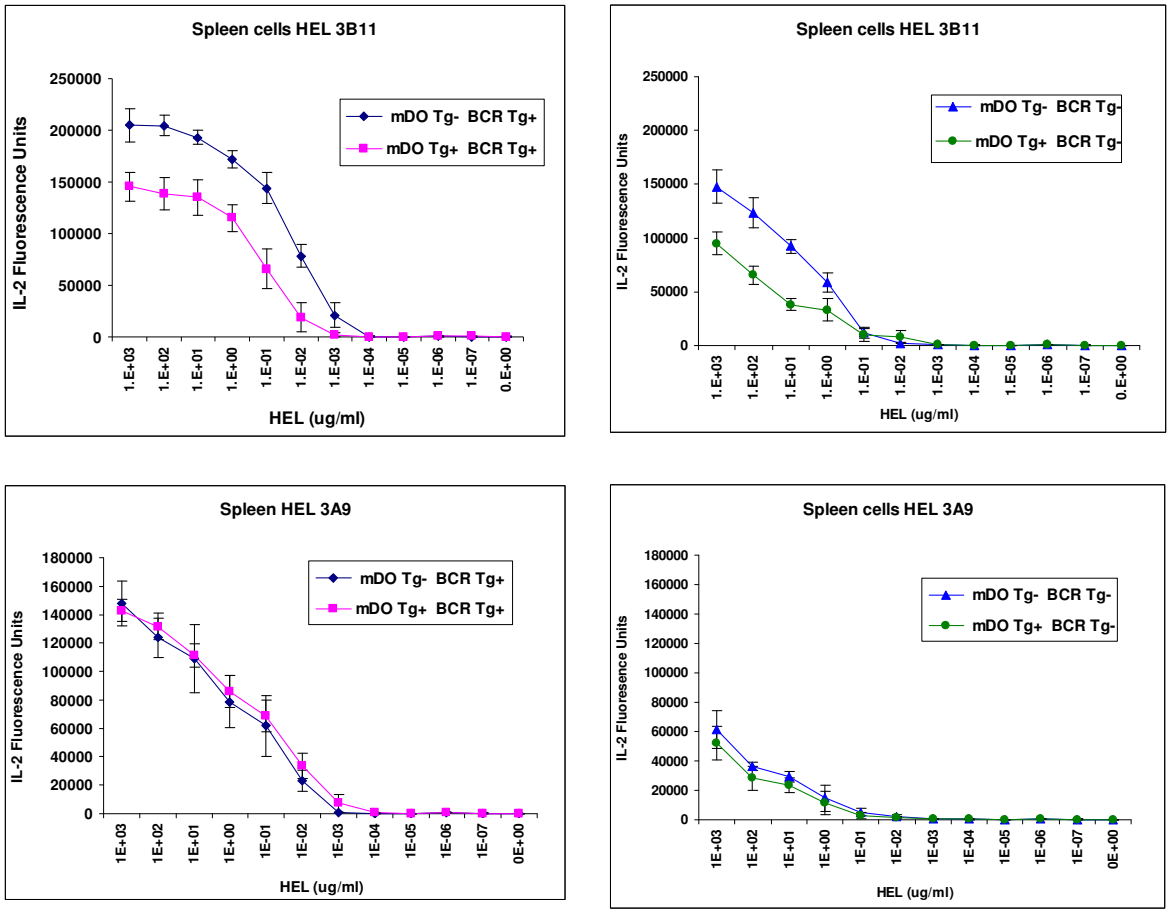


Fig.: RI-12 DO suppress antigen presentation in the early endocytic compartments but not in the late endocytic compartments. Antigen presentation was performed with 3B11 (top) and 3A9 (bottom) hybridomas with spleen cells isolated from corresponding genotypes as presented in the figure legend. HEL used as antigen. This experiment is a representative of minimum three experiments.

As shown in Fig.: RI-12, mDO Tg- BCR Tg+ spleen cells present the HEL epitope 34-45 better to T hybridoma 3B11 than mDO Tg+ BCR Tg+ (upper left panel). Likewise when HEL antigen was taken up by the fluid phase instead of the BCR, again the over expression of DO was found to down modulate presentation of epitope 34-45 to the T hybridoma 3B11. Thus, irrespective of the presence or absence of BCR Tg; mDO Tg+ spleen cells show impaired antigen presentation when tested for presentation of the epitope 34-45 with the 3B11 T cell hybridoma. When presentation of the epitope 46-61 was analysed with the T hybridoma 3A9, again much higher presentation was observed after uptake of antigen by the BCR as compared to fluid phase uptake (lower panels). However, over expression of mDO did not affect antigen presentation, irrespective of uptake via the BCR or fluid phase with 3A9 T cell hybridoma. When the spleen cells

from the different mouse strains were incubated with the cognate peptide HEL 34-45 or HEL 46-61 no difference in peptide presentation was observed (Fig.:RI-13) demonstrating that DO indeed affects antigen presentation in MHC II loading compartments.

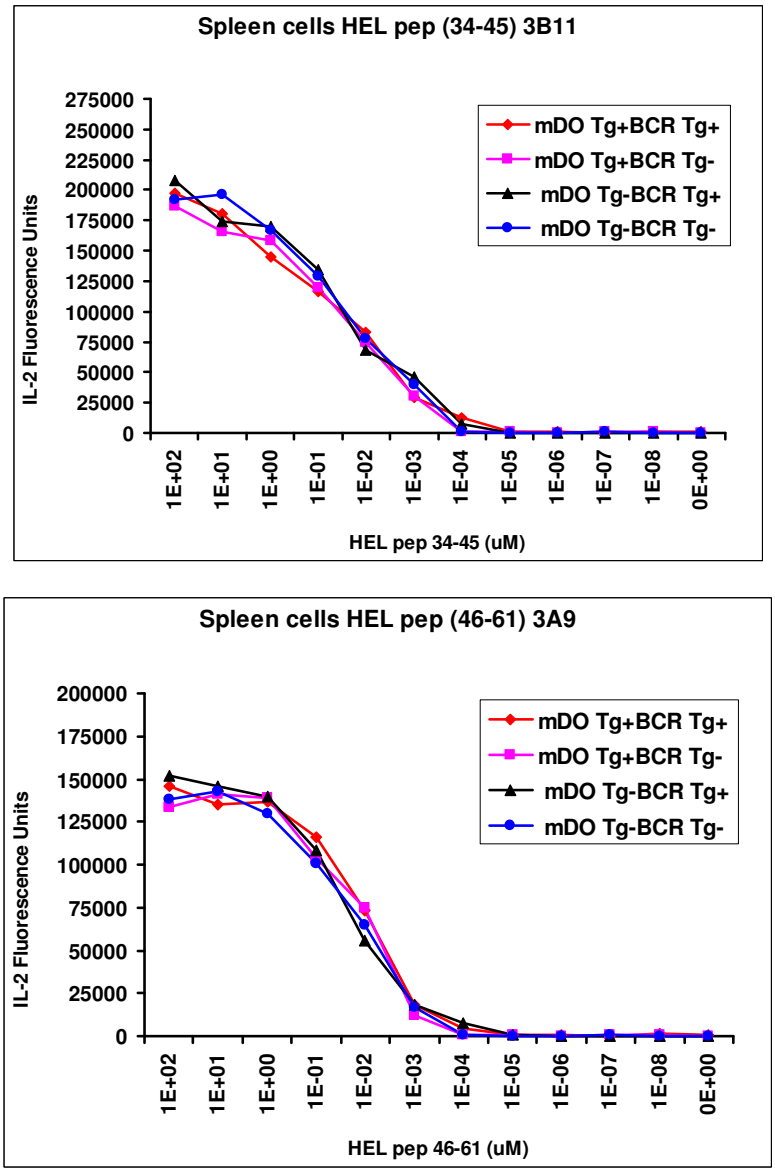


Fig.: RI-13 Antigen presentation was performed with two different HEL specific I-A^k restricted hybridomas 3B11 (HEL:34-45) (top) and 3A9 (HEL:46-61) (bottom) with peptide controls.

These observations suggest that DO favors antigens to be processed in late but not in early endocytic compartments. This makes sense in physiological context because primarily B cells need to respond to the antigens taken via their BCR but not via fluid

phase. In this way DO keeps control over antigen presentation machinery in the early compartments in the B cell.

3.1.3.2 High levels of DO inhibits antigen presentation in bmDC

As explained earlier expression of DO was recently found in dendritic cells (DC) both in mouse and human [40]. Therefore, we extended our studies to antigen presentation by bone marrow derived DC (bmDC) isolated from mDO Tg (A^k) mice and CBA (A^k) wild type mice in order to see if the elevated levels (2-3 fold) of DO suppressed antigen presentation in bmDC.

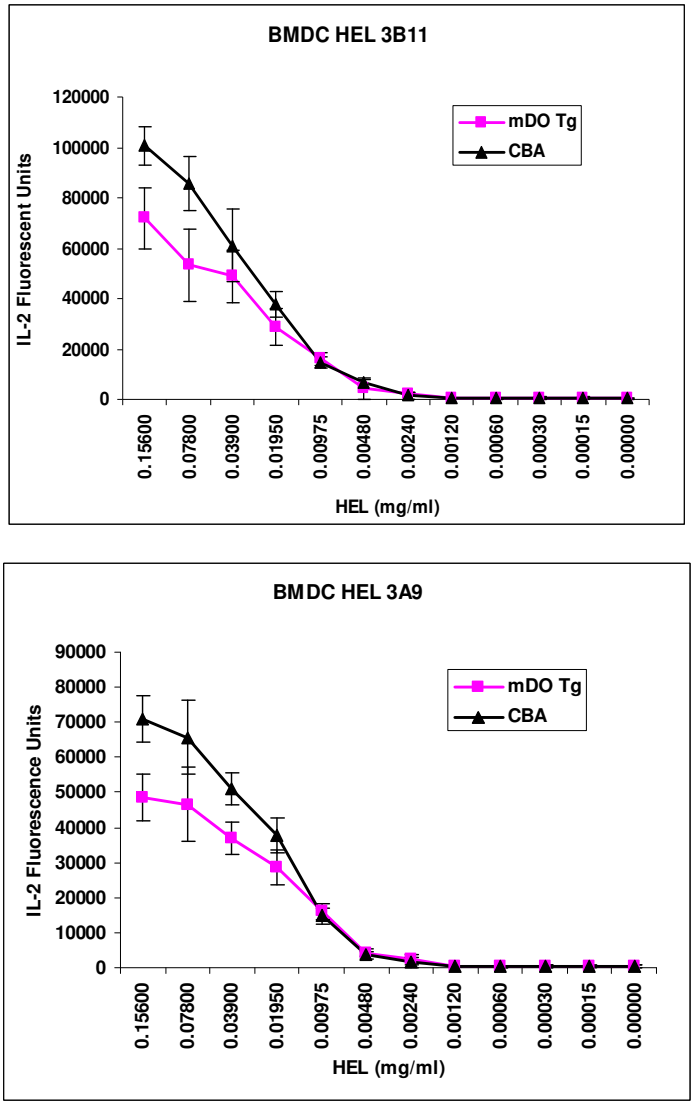


Fig.: RI-14 Elevated levels of DO suppress antigen presentation in bmDC. Antigen presentation was performed with 3B11 (top) and 3A9 (bottom) hybridomas with bmDC isolated from corresponding genotypes as presented in the figure legend. HEL used as antigen. This experiment is a representative of minimum three experiments.

As shown in fig.:RI-14 the increased levels of DO decrease antigen presentation in bmDC for both HEL epitopes 34-45 and 46-61 when compared to wild type controls. The impairment in antigen presentation is not as drastic as seen with BCR Tg+ mDO Tg+ spleen cells in Fig.: RI-12. The compartments for the generation of HEL epitopes 34-45 and 46-61 are not known for DC and need to be determined. When the bmDCs were incubated with the cognate peptide HEL 34-45 or HEL 46-61 no difference in peptide presentation was observed (Fig.:RI-15).

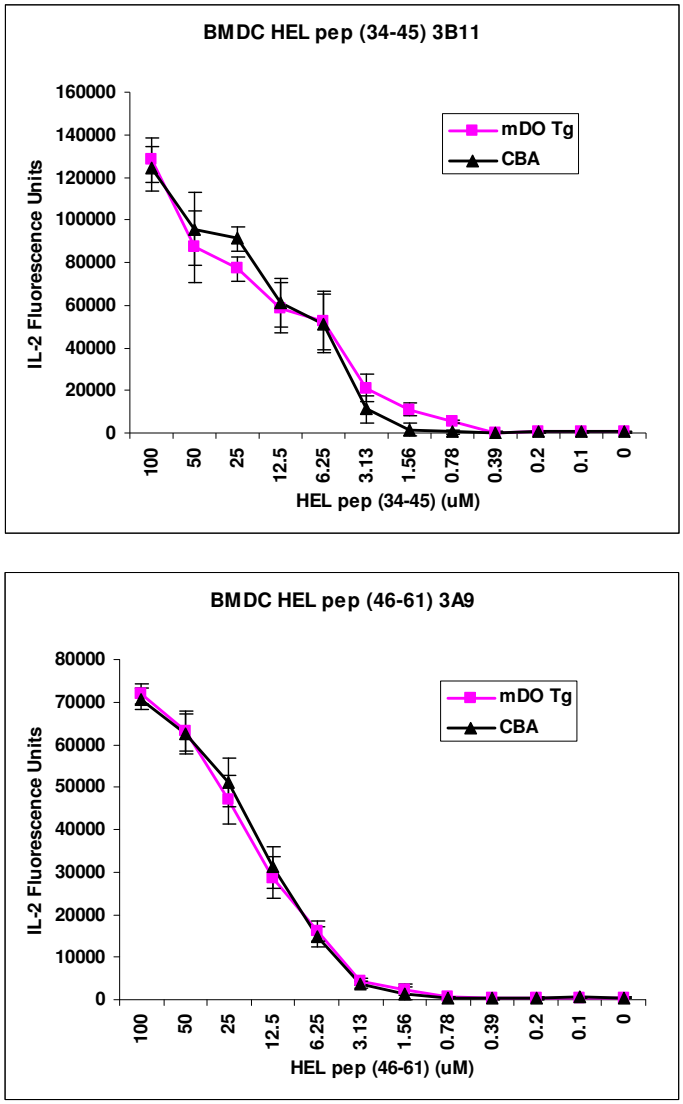


Fig.: RI-15 Antigen presentation was performed with two different HEL specific I-A^k restricted hybridomas 3B11 (HEL:34-45) (top) and 3A9 (HEL:46-61) (bottom) with peptide controls.

To further confirm that mDO would indeed impair antigen presentation and not expression of surface molecules that may be important for T cell recognition, such as MHC II or costimulatory molecules CD40 and CD80, we next investigated the surface marker expression profile of bmDC isolated from mDO Tg (A^k) and CBA mice.

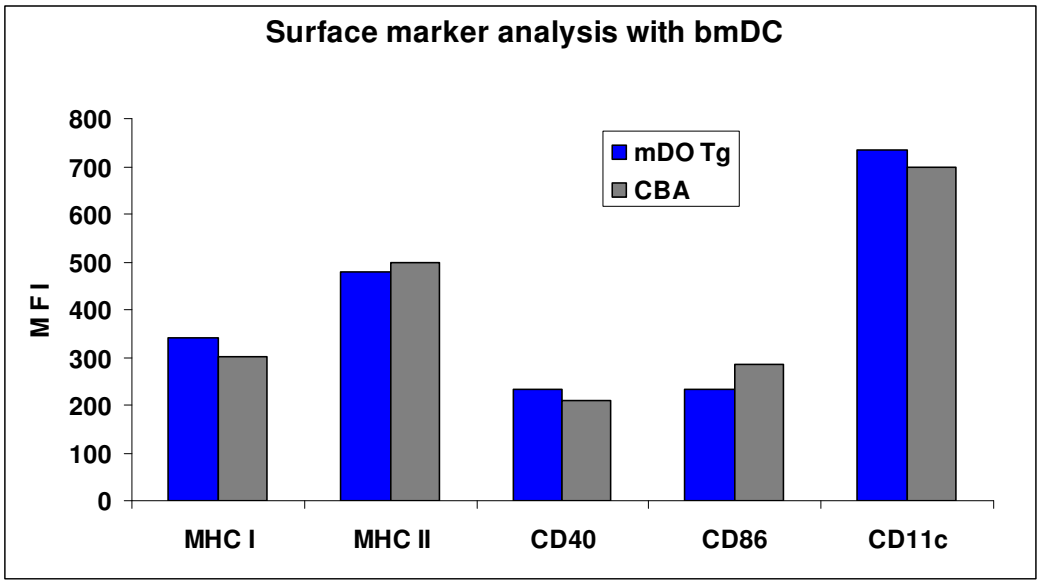


Fig.: RI-16 Surface marker expression profile of bmDC isolated from mDO Tg and CBA.

As shown in fig.: RI-16 there is no difference in the expression profile surface markers on bmDC isolated from mDO Tg and CBA. Together, it can be concluded that mDO down modulates antigen presentation in A^k DC.

3.1.3.3 Antigen presentation in bmDC from H2-O-/- mice

We next investigated the efficiency of antigen presentation with H2-O-/- bmDC (A^b) in comparison with mDO Tg (A^b) and B6 controls. Since H2-O-/- is of A^b haplotype we have used ovalbumin as antigen and OT I and OT II TCR transgenic T cells as responders. OTI TCR transgenic T cells recognize the OVA peptide S8L presented by the K^b MHC class I molecule, whereas OTII T cells recognize the OVA peptide K17G (OVA:323-339) presented by the A^b MHC class II molecule.

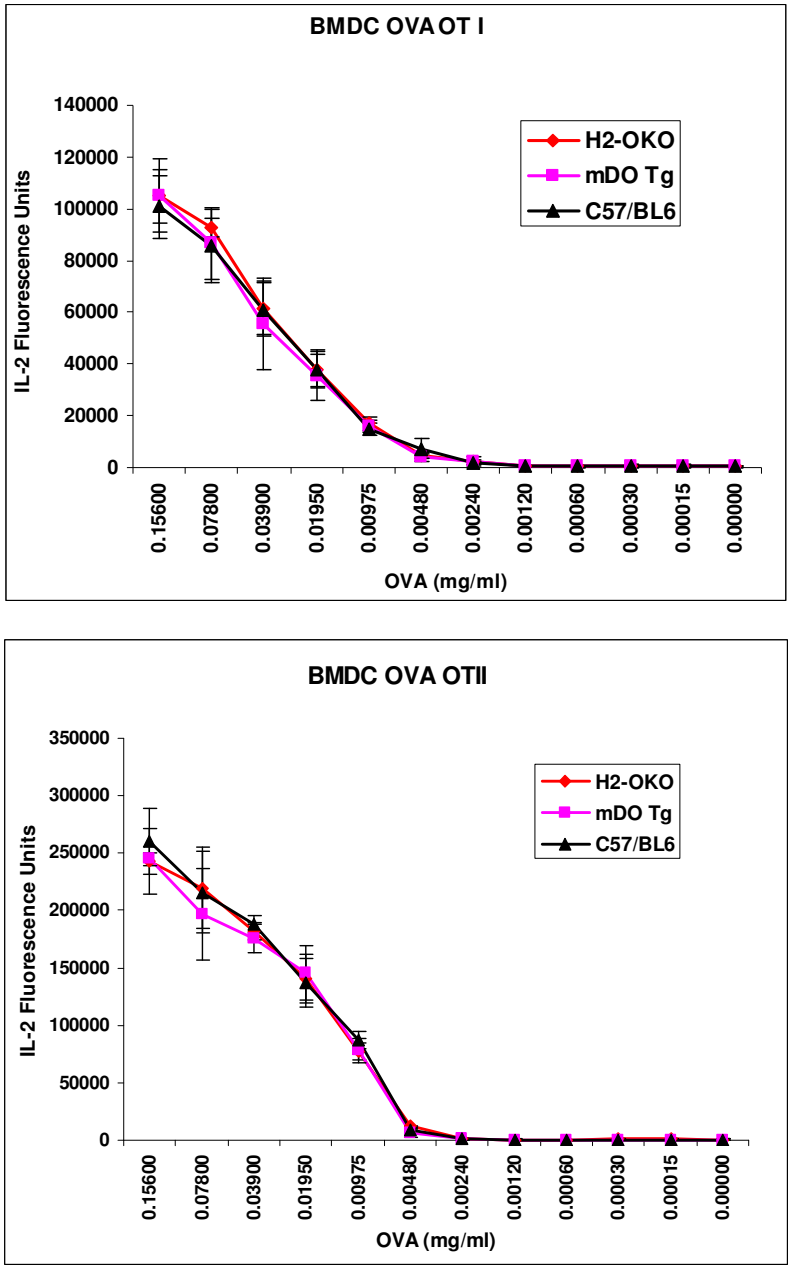


Fig.: RI-17 H2-O-/-(H2-K^b) or mDO Tg (H2-K^b) bmDC does not show any alteration in the capacity of antigen presentation when compared with B6 control. OTI (top) and OTII (bottom) TCR transgenic T cells were used as responders and ovalbumin used as antigen. This experiment has been reproduced several times.

As shown in fig.: RI-17 no differences were observed in antigen presentation by H2-O-/- or mDO Tg bmDC of the H-2^b haplotype when compared with B6 controls. This suggests that DO may not be crucial for antigen presentation in A^b bmDC. There are several potential explanations. For example, it is possible that the effect of DO is MHC

allele specific. Alternatively, DO may not affect the loading of the OVA peptide K17G on to A^b molecules, which is reminiscent of the observation that DO did not influence loading of the HEL 46-61 epitope onto A^k molecules (Fig.: RI-12). The result also show that the presentation of the S8L epitope by the MHC class I molecules was not affected. This, however, was to be expected because DO is not involved in the MHC I presentation pathway.

When the surface marker profiles of bmDC from H2-O-/-(H2-K^b), mDO Tg (H2-K^b), and B6 were investigated no striking differences were observed (Fig.: RI-18), which is in agreement with the analysis of A^k bmDC (see Fig.: RI-16).

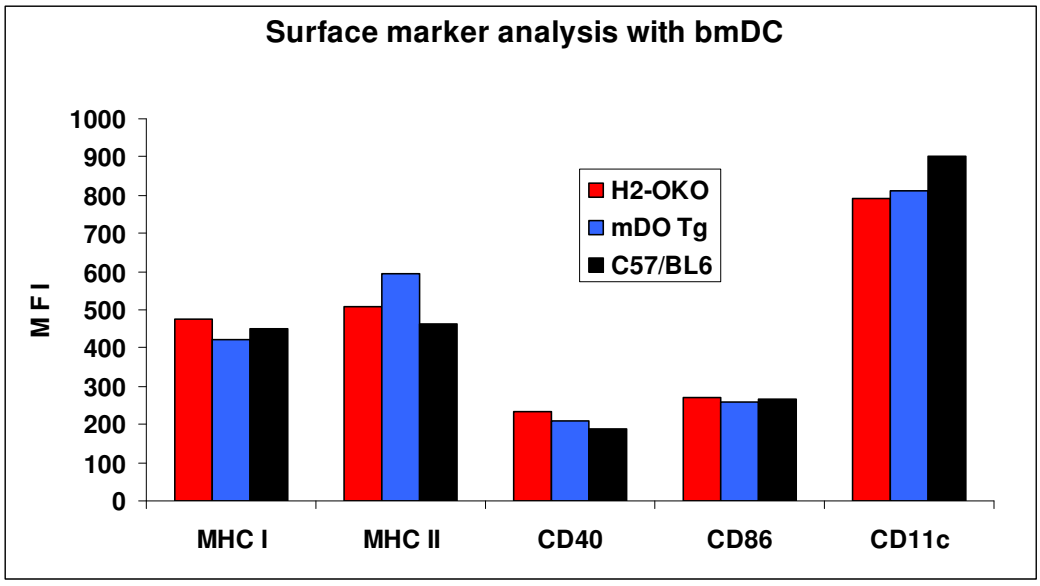


Fig.: RI-18 Surface marker expression profile of H2-O-/-(H2-K^b) or mDO Tg (H2-K^b), and B6 bmDC.

3.2: Results Part II – CD82 Project

3.2.1: Generation of CD82KO mice

As outlined in the introduction, tetraspanin proteins form a network with other tetraspanin family members and various membrane proteins, including MHC class I and MHC class II molecules. In order to see if the presence of tetraspanins in these supramolecular complexes influences antigen presentation by MHC molecules, Dr. Satoshi Tanaka from this laboratory has generated knockout mice for the tetraspanin molecules CD82 and CD53. The mouse CD82 gene is located on chromosome number 2 and contains 9 exons. The CD82 protein consists of 266 amino acid residues and the molecular weight of the unmodified protein is ~29,628 Da. The post translationally modified CD82 protein shows a molecular weight ranging from ~40,000 to 50,000 Da.

The targeting vector was constructed by replacing exons 4 and 5 by the neomycin resistance gene (see Fig.RII-1a) which was flanked with *flp* site. *Lox*P sites were introduced to flank the regions exon 4 and exon 5. The *flp* site allows us to delete the neomycin resistant gene, if the CD82 *floxed/floxed* mice are crossed with *flp* deleter mice. The l*ox*P site allows deletion of the CD82 exons 4-5 if the CD82 *floxed/floxed* mice are crossed with *Cre* deleter mice resulting in CD82 deletion in all cells in total mouse body. If desired, CD82 can also be deleted in specific tissues when mice are used for crossing that express *Cre* recombinase selectively in those tissues. In addition to the CD82 knockout mice, Dr. Satoshi Tanaka has also generated for the tetraspanin CD53.

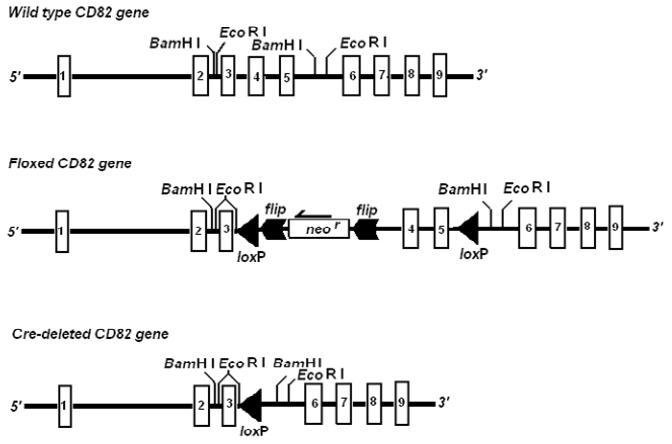


Fig.: RII-1a: ***CD82 gene inactivation*. Restriction maps are shown for the wild-type CD82 gene locus (top), targeting vector (middle) and recombinant gene locus (bottom). Exons are indicated as white boxes and un translated region was shown as dark line. *neo*^{r}; neomycin resistance gene, *lox*P; lox P site, & flip; flip site. *Bam* HI and *Eco* RI; restriction sites.**

As shown below in Fig.: RII-1b, there are 8 exons in mouse CD53 gene. The *lox*P sites were introduced downstream to exon 1 and up stream to exon 6. Thereby, exons 2 through 5 can be deleted upon crossing CD53 *floxed/floxed* mouse with a *Cre* deleter mouse. An inverted neomycin resistant gene was introduced down stream to exon 1 and *lox*P site.

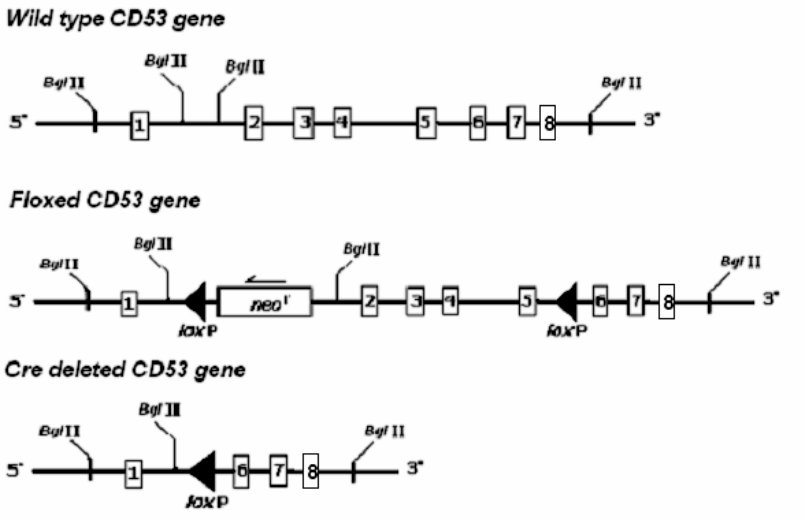


Fig.: RII-1b: ***CD53 gene inactivation*. Restriction maps are shown for the wild-type CD53 gene locus (top), targeting vector (middle) and recombinant gene locus (bottom). Exons are indicated as white boxes and un translated region was shown as dark line. *neo*^{r}; neomycin resistance gene, *lox*P; lox P site, & flip; flip site. *Bgl*II; restriction site.**

Deletion of the CD82 gene in homozygous/heterozygous mutants was identified by RT-PCR (not shown) and Southern blotting, which was also used to monitor breeding of the CD82-/- mice. As shown in Fig.: RII-2, a 6 kb band was released upon CD82 knockout mouse tail DNA digestion with *Bam*H I as determined by agarose gel electrophoresis, whereas a wild type mouse tail DNA released a 8 kb band.

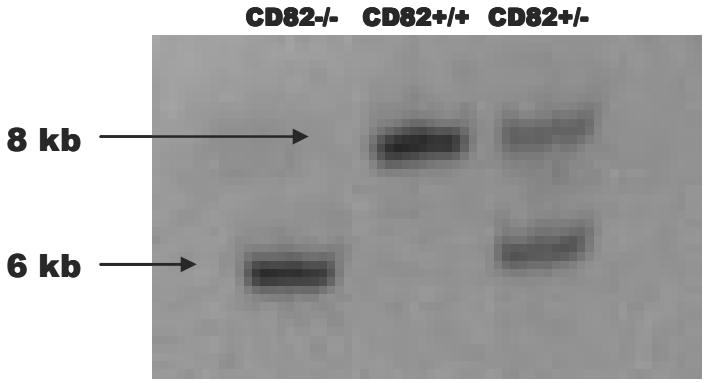


Fig.: RII-2 ***Southern Blotting analysis*** **with genomic DNA isolated from mouse tail digested with *Bam*HI for CD82 KO (CD82-/-) (left), heterozygous (CD82+/-) (right) mutants in comparison with WT (CD82+/+) (middle).**

Deletion of the CD53 gene in homozygous/heterozygous mutants was also determined by Southern blotting and RT-PCR (data not shown). The CD53-/- mouse have not yet been backcrossed to C57BL/6 or CBA. Therefore in this thesis I focused the analysis mainly on CD82-/- mice. A small portion of data that is generated with non backcrossed CD53-/- mice and CD82-/-xCD53-/- double knockout mice will be shown in the later sections.

The resulting CD82-/- and CD53-/- mice were born at the expected Mendelian frequency and raised normally under specific pathogen free (SPF) conditions at the animal facilities of the German Cancer Research Center. No obvious behavioral and physical abnormalities were detected. We also crossed CD82-/- mice with CD53-/- mice to generate double knockout mice for CD82 and CD53. The absence of both CD82 and CD53 proteins did not affected the embryonic development or litter size.

3.2.2 FACS profiles of various cell surface markers in CD82-/- mice

Expression levels of various cell surface markers on immune cells isolated from CD82-/- and wild type control mice that were backcrossed 5 times to C57BL/6 mice were measured by FACS. As mentioned in the previous section, tetraspanin proteins associate with many different cell membrane proteins including CD4, CD8, MHC class I and MHC class II etc. Therefore it is possible that lack of CD82 protein may modulate the expression levels of various cell surface proteins. With this objective I have tested various cell surface markers on cells isolated from spleen (Fig.:RII-3a and 3b), thymus, bone marrow and lymph nodes (data not shown).

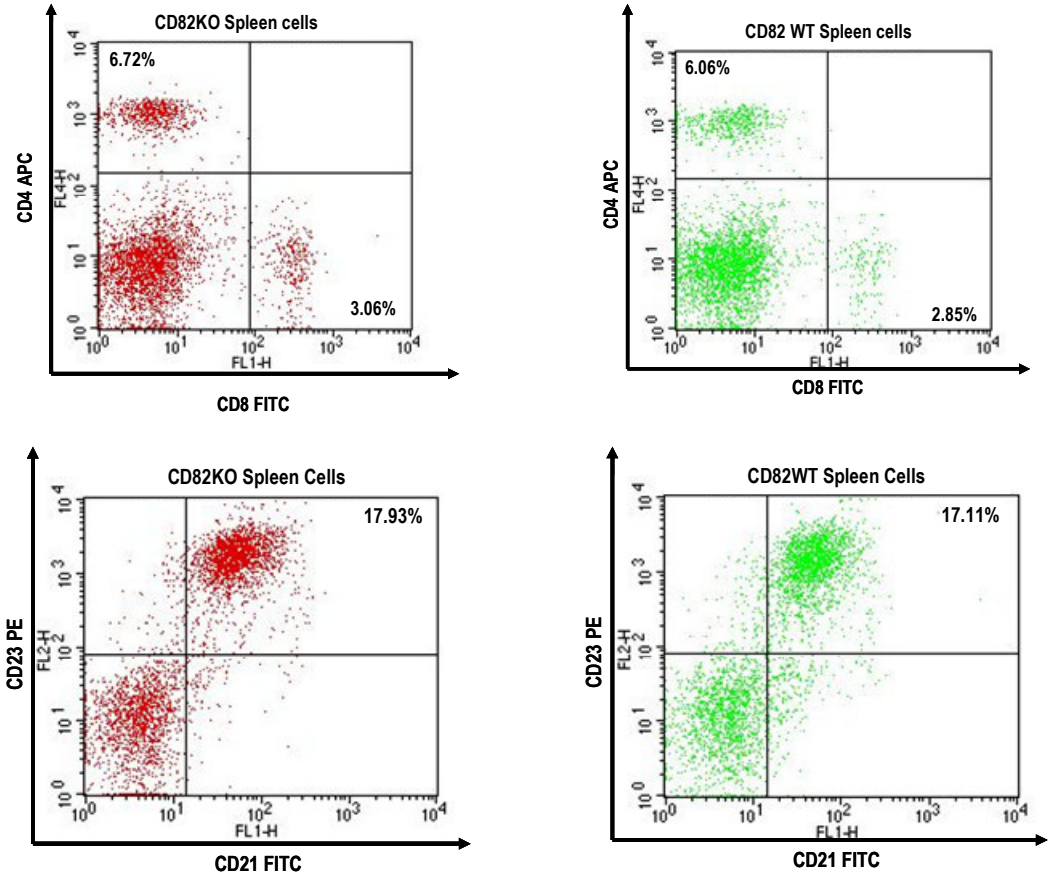


Fig.: RII-3a Expression levels of CD4, CD8 on CD82-/- (top left), CD82+/+ (top right) spleen cells. Percentage of mature B cells (CD21 and CD23 double positive) in CD82-/- (lower left) and CD82+/+ (lower right) in spleen. Total percentage of cells indicated in the respective quadrant.

Fig.:3a shows that CD82 deletion did not affect the percentage of CD4 T cells, CD8 T cells and B cells in the spleen, or the expression levels of CD4, CD8, CD21 and CD23 surface markers on these cells. Thus, T and B cell development appears to be normal.

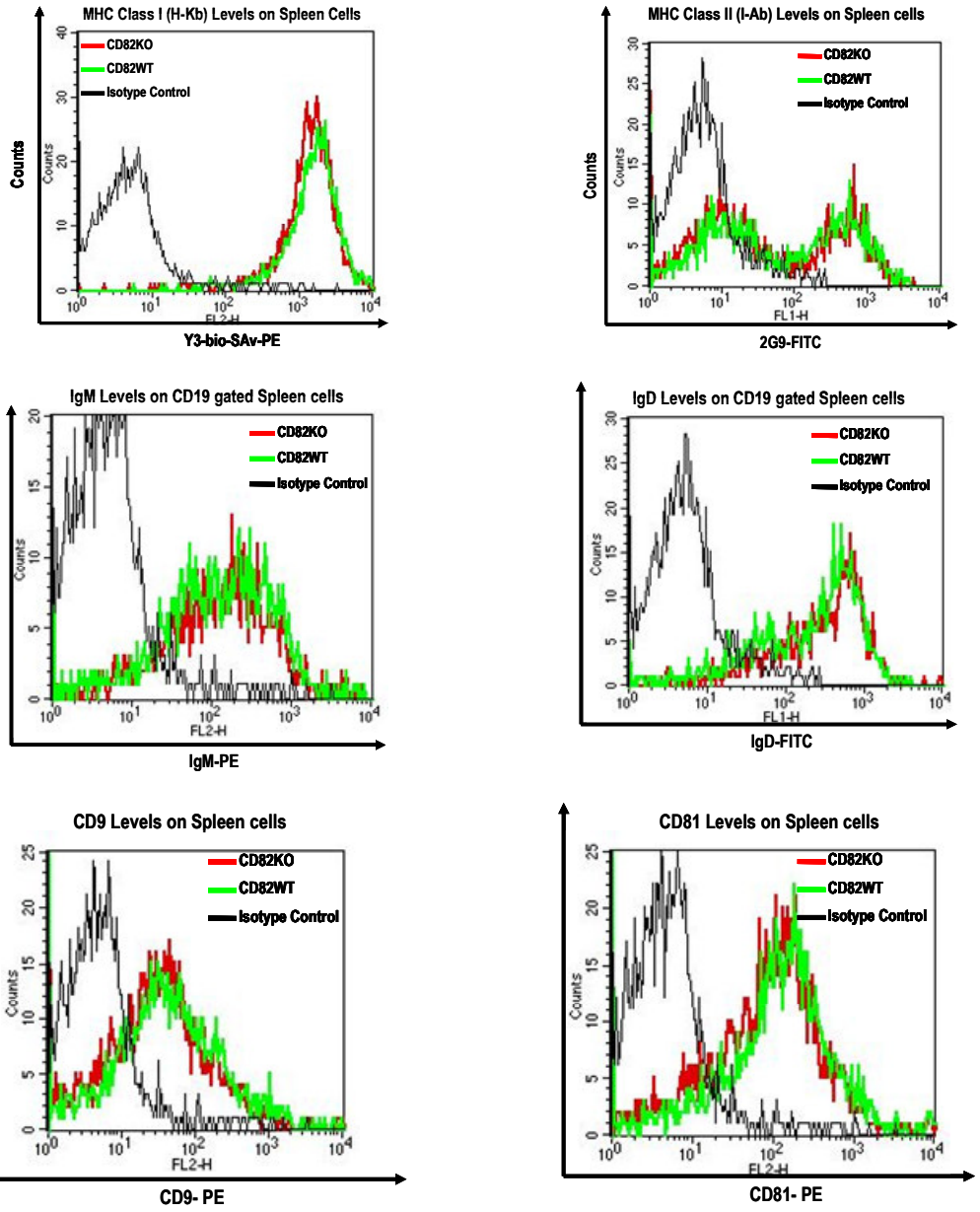


Fig.: RII-3b Expression levels of class I MHC (top left), class II MHC (top right), IgM (middle left), IgD (middle right), CD9 (lower left), and CD81 (lower right) on spleen cells isolated from CD82-/- (red), CD82+/+ (green); isotype control antibody staining was shown in gray colour.

Fig.RII-3b shows that there was no difference with regard to expression of MHC class I and class II molecules, surface IgM and IgD on B cells. The expression levels of other tetraspanin proteins CD9 and CD81 were also intact. In other lymphoid organs such as lymph node, bone marrow and thymus also no abnormalities were detected (not shown).

3.2.3 Humoral Responses

3.2.3.1 Normal levels of Immunoglobulins in CD82KO mice

The B cell receptor (BCR) is also known to associate with tetraspanins. To see whether normal endogenous levels of immunoglobulins are affected by the absence of the CD82 protein, we have measured the endogenous levels of different sub classes of immunoglobulin titers by ELISA in CD82-/- mice. The littermate CD82+/+ mice were used as controls. For this study we choose female mice of age 8-12 weeks old.

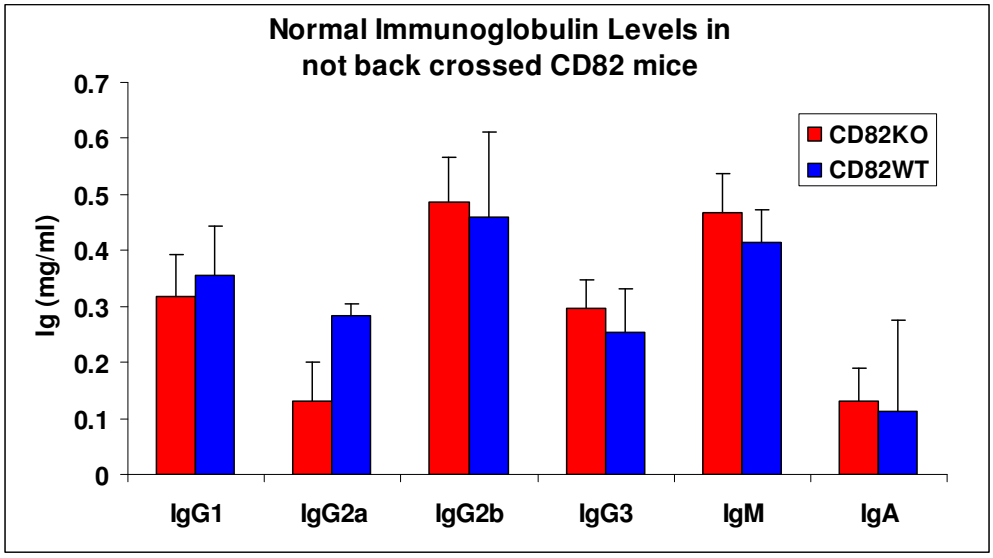


Fig.: RII-4 **Normal levels of immunoglobulins IgG1, IgG2a, IgG2b, IgM, and IgA in not back crossed CD82-/- mice and wild type litter mate control CD82+/+ mice as measured by ELISA. Sera isolated and tested from 5 female mice of age 8-12 weeks from each genotype that were not back crossed at all with B6 mice.**

It can be seen from Fig.: RII-4 that CD82-/- mice showed around 2 folds reduced IgG2a levels when compared with to their littermate control mice. No differences were observed for the other immunoglobulin classes. After backcrossing CD82-/- mice with C57BL/6 mice for 5 times we have again tested the normal levels of immunoglobulins to see if the results were reproducible in mice with a more homogenous genetic background.

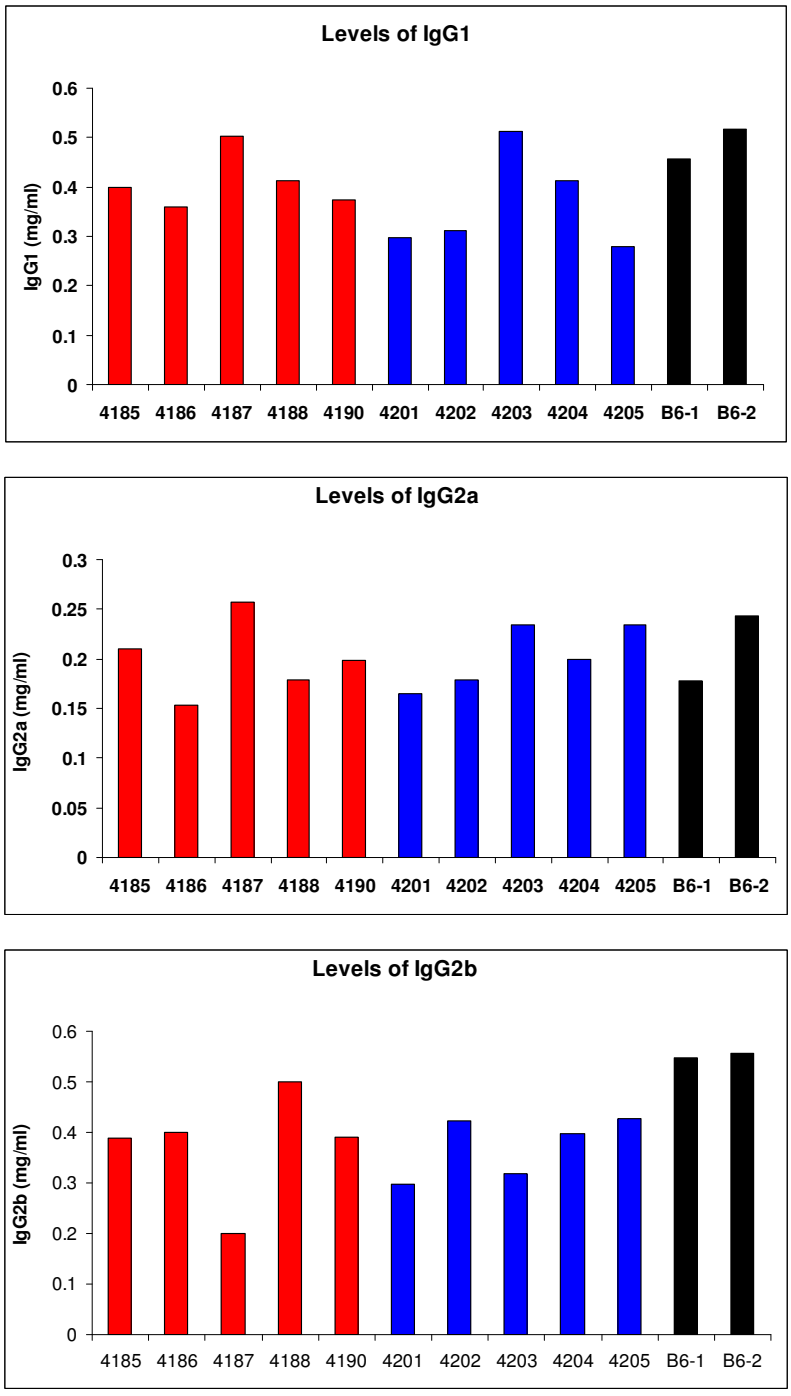


Fig.: RII-5 Normal levels of immunoglobulins IgG1(top), IgG2a (middle) and IgG2b (bottom) in 5 times backcrossed CD82-/-(red bars), littermate control CD82+/+ (blue bars) and C57BL/6 (black bars) mice. Sera isolated and tested from 5 female mice of age 8-12 weeks from each genotype that were backcrossed with C57BL/6 mice for 5 times. Data shown is representative of minimum three experiments performed.

Fig RII-5 ***continued in the next page*****.**

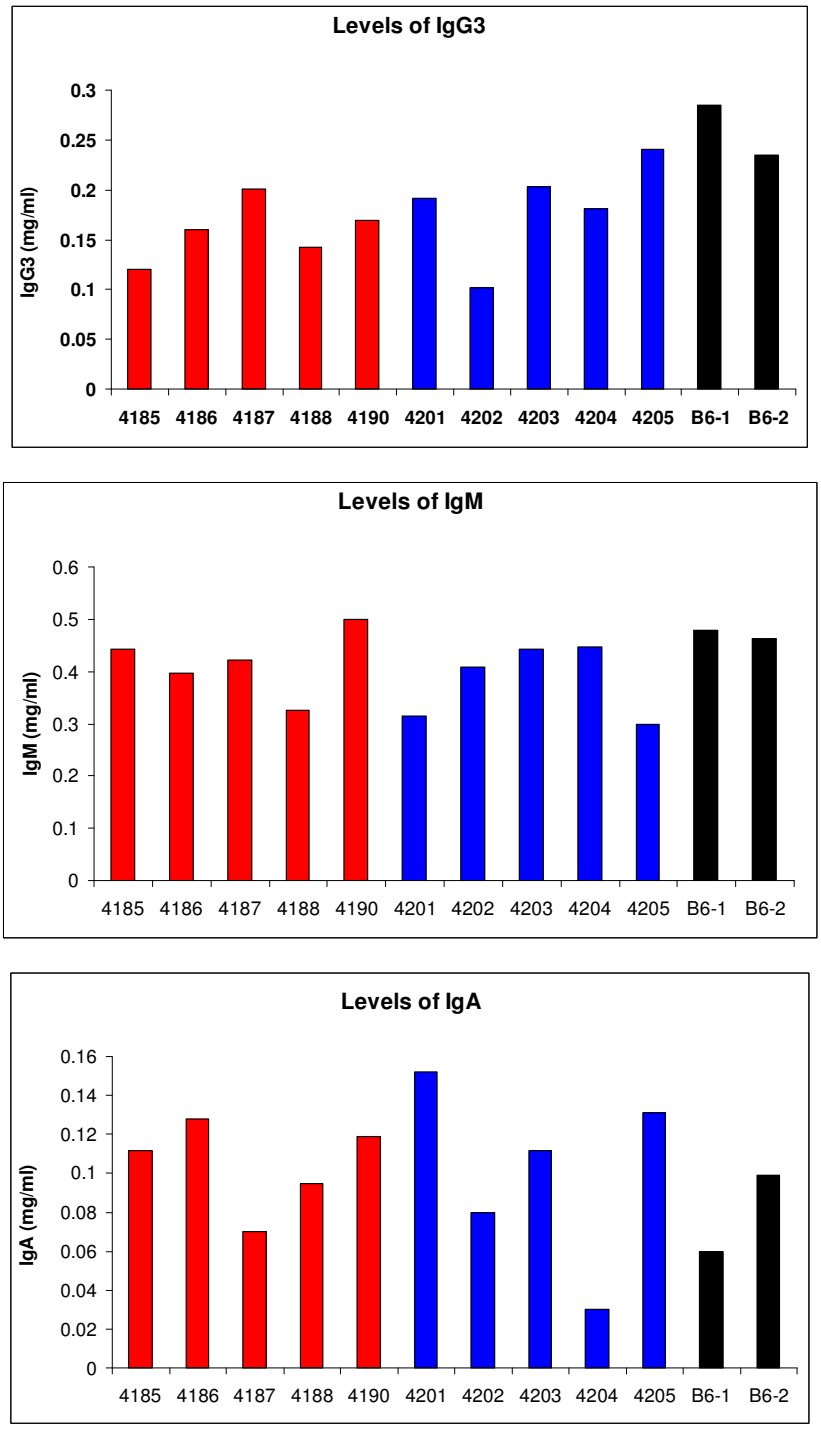


Fig.: RII-5 continuation: Normal levels of immunoglobulins IgG3(top), IgM (middle) and IgA (bottom) in 5 times backcrossed CD82-/-(red bars), littermate control CD82+/+ (blue bars) and C57BL/6 (black bars) mice. Sera isolated and tested from 5 female mice of age 8-12 weeks from each genotype that were back crossed with C57BL/6 mice for 5 times. Data shown is representative of minimum three experiments performed.

As shown in Fig. RII-5 that there is some variation with regard to absolute levels of immunoglobulins in individual mice, but we could not detect significant differences between CD82-/- and CD82+/+ mice and C57BL/6 mice. The IgG2a levels were also comparable in the 5 times back crossed mice. Most likely the differences in IgG2a levels shows in Fig.RII-4 can be attributed to the differences in the genetic back ground.

3.2.3.2 T cell dependent antibody responses to antigen

Next we investigated in CD82-/- mice the antibody responses to T dependent antigen TNP(11)OVA. We choose 8-10 female mice of age 8-12 weeks old from each genotype i.e., CD82-/- and CD82+/+ mice and immunized them intraperitoneally with TNP(11)OVA antigen with alum as an adjuvant.

As explained in previous section (3.1.2.2) in principle, in response to the immunization with TNP(11)OVA we may expect a minimum three different types of antibodies; 1) against the hapten TNP, 2) against various ovalbumin protein epitopes, and 3) for the junction part of TNP-ovalbumin. In order to measure only TNP specific antibodies and to eliminate the ovalbumin specific antibody from our ELISA measurements, we choose TNP-BSA. Since the ELISA plates were coated with TNP-BSA, only the hapten TNP specific antibodies can bind to the ELISA plates.

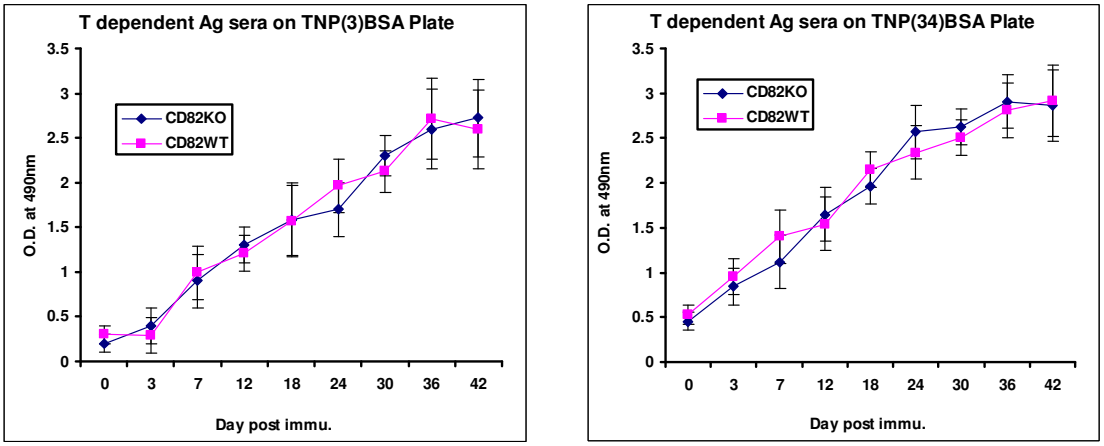


Fig.: RII-6 TNP specific IgG levels in sera samples isolated from CD82-/- and wild type mice. IgG levels were measured by ELISA at the mentioned days of post immunization with TNP(11)OVA in Alum as an adjuvant. Levels of TNP specific IgG on TNP(3)BSA coated plate (left) and TNP(34)BSA coated plate (right). Eight to 10 female mice of age 8-12 weeks from each genotype that were not back crossed at all with B6 mice were used in this study. Data shown is representative of minimum three experiments performed.

As seen in Fig.: RII-6, no significant differences in TNP specific IgG antibody titers were observed between CD82-/- and wild type control mice. Furthermore, there is no detectable difference observed between TNP high and low plates (Fig.: RII-6 left). We conclude that lack of CD82 protein does not influence the affinity of the antibody generated against the T dependent antigen. These data also mean that class switching from IgM to IgG in response to T dependent antigens is intact in the absence of CD82. Since there were no differences in titers of the antigen TNP specific antibody between CD82-/- mice when compared with wild type control mice, we can also conclude that the B cell priming is intact in the in the absence of CD82.

This experiment was performed with not back crossed CD82-/- and wild type mice and it needs to be repeated with backcrossed mice. Respective experiments are underway.

3.2.3.3 T cell independent antibody responses

We next investigated the T independent antibody responses by injecting the T independent antigen TNP(77)Ficoll. B cells respond to the multimeric T independent antigens through multiple interactions and, does not need T cell help. Therefore, only IgM antibodies are produced, and Ig class switching does not occur.

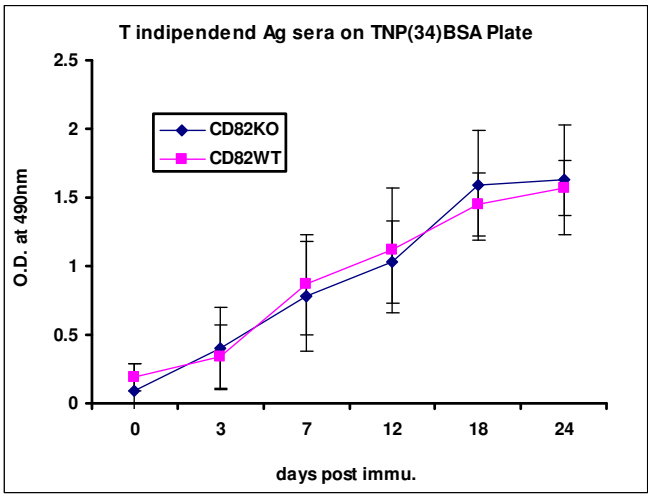


Fig.: RII-7 T independent antigen responses. Antigen TNP-Ficoll specific IgM titers were measured by ELISA on TNP(34)BSA coated plate. Sera isolated from days post immunization show on *x* axis.

No differences were observed in the T independent antigen TNP(77)Ficoll specific IgM titers between CD82-/- and wild type control mice (Fig.: RII-7). The titers were relatively low when compared with T dependent antigen because IgM is usually of low affinity. For this experiment 5 female mice were used for each group, aged 8-12 weeks old. The mice used for this experiment were not back crossed to C57BL/6 mice. Currently we are testing with the same antigen CD82-/- and wild type mice that were backcrossed to C57BL/6 for 5 times.

In conclusion, CD82 has no influence on BCR triggering and subsequent antibody production.

3.2.4 T and B cell Proliferation

3.2.4.1 CD82-/- T cells are hyper proliferative

We next investigated the proliferative responses of T cells isolated from spleens of CD82-/- and wild type mice upon various stimuli like αCD3+αCD28 and Con A. As mentioned previously it has been shown that CD82 associate with cell surface receptors CD4 and CD8 along with other membrane proteins. It has been also shown that human

CD82 delivers a co stimulatory signal in T cells [69]. The anti CD3 antibody was used to stimulate the T cells via the TCR, whereas the anti CD28 antibody causes a co-stimulatory signal. Thus, this frequently used procedure reflects the natural stimulation of T cell during immune responses.

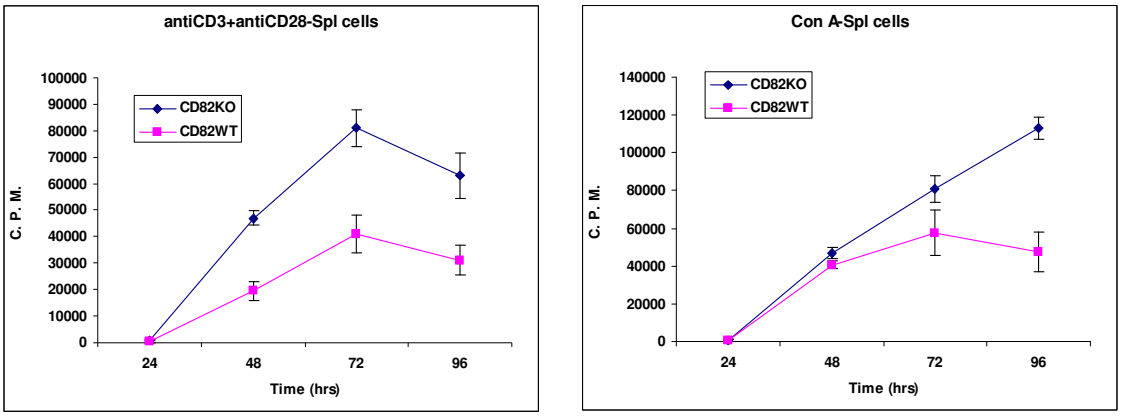


Fig.:8 CD82-/- T cells are hyper proliferative upon αCD3+αCD28 (left) and Con A (right). Data shown is representative of three individual experiments conducted with 5 times back crossed CD82-/-mice with C57BL/6. Similar results were obtained in all three experiments.

Interestingly, CD82-/- (H-2^b) splenocytes showed significantly higher proliferation upon stimulation with αCD3+αCD28 (5ug/ml each, soluble form). With the mitogen Con A, which stimulate also through TCR components, also a higher stimulation was found for CD82-/- T cells. This result may suggests that the phosphorylation of key T cell signaling molecules, such as the TCR ζ chain and ZAP 70 is influenced by the CD82 protein. The data shown in Fig.: RII-8 is a representative of three individual experiments conducted with 5 times back crossed CD82-/-mice with C57BL/6. Similar results were obtained in all three experiments.

3.2.4.2 CD82-/- B cells proliferate normally

We next examined the proliferative capacity of spleen cells following stimulation with LPS. The LPS acts mainly via the TLR4 membrane protein. The role of tetraspanin proteins in the context of TLR signaling is not known.

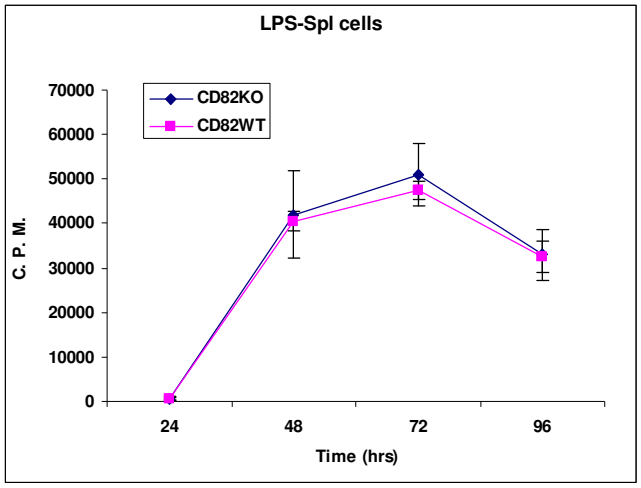


Fig.: RII-9 CD82-/- B cells proliferate normally upon LPS stimulation. Data shown is representative of three individual experiments conducted with 5 times back crossed CD82-/-mice with C57BL/6. Similar results were obtained in all three experiments.

No significant differences were observed in the proliferative capacity as shown in Fig.: RII-9 between CD82-/- and wild type mice spleen cells upon LPS stimulation. It seems that CD82 is not an essential protein in B cell proliferative responses. The data shown in Fig.: RII-9 is a representative of minimum three individual experiments conducted with 5 times backcrossed CD82-/- mice with C57BL/6. Similar results were obtained in all three experiments.

3.2.5 Antigen Presentation Assays

3.2.5.1 CD82-/-(H-2^k) dendritic cells show impaired antigen presentation

The tetraspanin molecules CD82, CD63, CD9 have been shown to interact with antigen presentation machinery components namely the MHC molecules and non classical MHC molecules HLA-DM and HLA-DO. Dendritic cells (DC) are the most powerful antigen presenting cells in the immune system. To see whether or not the tetraspanin molecules CD82 and CD53 would influence antigen presentation we use bone marrow derived dendritic cells (bmDC) from CD82-/- and wild type mice that were back crossed with CBA (H-2^k) mice for 5 times (from now on designated CD82-/- (H-2^k)).

Since these mice have the H-2^k MHC haplotype; we were able to utilize a panel of T cell hybridomas that are specific for hen egg lysozyme (HEL) epitopes in an I-A^k restricted manner.

As explained in previous section former studies have shown that the epitope 33-47 is preferentially processed and loaded into I-A^k molecules in an early endocytic compartment. In contrast, epitopes 46-61 and 116-129 are preferentially processed and presented in late endocytic compartments.

These conclusions were based on the observation that chloroquine which neutralizes the acidic late endocytic compartment, impairs presentation of the epitope 46-61 and 116-129 but has no or little effect on the neutral early endocytic compartments [41].

T cell hybridoma 3B11 recognizes the HEL epitope region 33-47 in an I-A^k restricted manner. The T cell hybridoma 3A9 recognizes HEL epitope 46-61 (immuno dominant epitope), and 2F9 recognize HEL: 116-129 in an I-A^k restricted manner. These later two hybridomas have been shown to be chloroquine sensitive. PA4.8G4 recognizes the HEL epitope 85-99, but its sensitivity to chloroquine is not yet known.

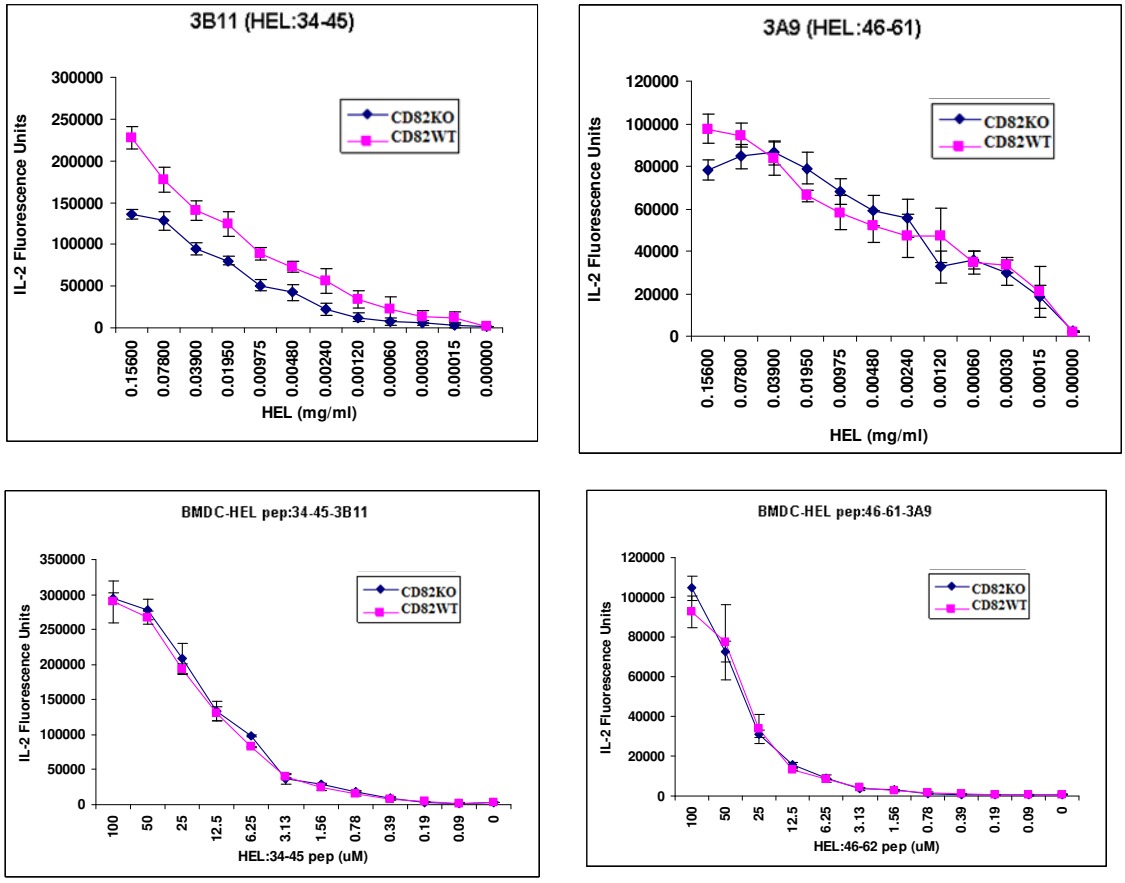


Fig.: RII-11 Antigen presentation is impaired in CD82-/- bmDC (H-2^k). Antigen presentation was performed with two different HEL specific I-A^k restricted hybridomas 3B11 (HEL:34-45) (top left) and 3A9 (HEL:46-61) (top right). Peptide controls were shown for 3B11 (lower left) and 3A9 (lower right).

As shown in Fig.: RII-11, CD82-/- bmDC present HEL ~2 folds less efficient to the T cell hybridoma 3B11 than wild type control cells. In contrast, no significant differences were observed with T cell hybridoma 3A9. When the bmDC were incubated with the cognate peptide HEL 34-45 no difference in peptide presentation was observed (Fig.:RII-11, lower left).

These results suggest that CD82 support antigen presentation in the recycling compartment (where recycling MHC mainly capture the processed peptides) but not in late endo-lysosomal compartment (where *de novo* synthesized MHC capture processed peptides). In order to verify the differences seen in CD82-/- and wild type dendritic cells (Fig.: RII-11) we generously received additional I-A^k restricted T cell hybridomas specific for HEL:33-47 epitopic region, E907, A6B3, and T cell hybridomas PA4.14A8; PA1.2F9 (HEL:116-129) and PA4.8G4 (HEL:85-99) from Dr.Jose Moreno and Dr.Dario Vignali.

It can be seen in Fig.:RII-12 (in the next page); that presentation of HEL was lower in CD82-/- bmDC for all these additional T cell hybridomas which recognize the HEL antigen 33-45 namely E907, A6.B3, and PA4.14A8. In contrast no differences were observed for the HEL epitope 116-129 and HEL: 85-99 specific hybridomas PA1.2F9 and PA4.8G4 respectively. Together, these observations suggest that there is impairment in the antigen processing and presentation mechanisms in the absence of the CD82 protein in a compartmental specific manner.

The data shown in Fig.: RII-11 and 12 is a representative of minimum three experiments and all the three experiments.

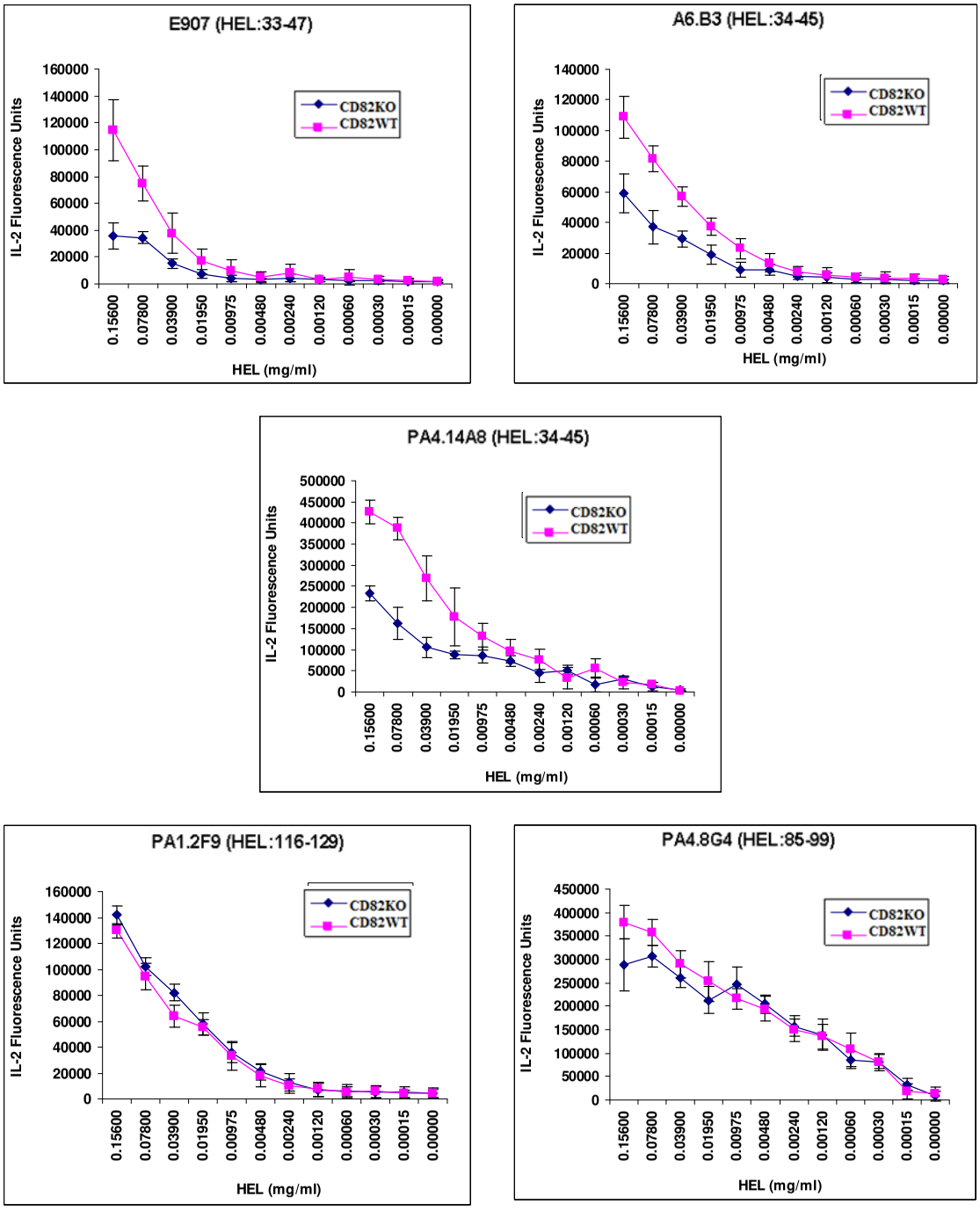


Fig.: RII-12 Antigen presentation is impaired in CD82-/- bmDC (H-2^k). Antigen presentation was performed with different HEL specific I-A^k restricted hybridomas E907 (HEL:33-47) (top left), A6.B3 (HEL:33-45) (top right), PA4.14A8 (HEL:34-45) (middle), PA1.2F9 (116-129) (lower left), and PA4.8G4 (HEL:85-99) (lower right).

In fig.:RII-3 only B and T cells had been analysed for surface markers but not DC. Because of the differences found in antigen presentation with bmDC, it was of interest to compare the expression levels of CD11c, MHC class I and class II and costimulatory molecules CD40 and CD86 on bmDC from CD82-/- and WT. However, as shown in Fig.:RII-13 no significant differences was observed in the expression levels of above mentioned surface molecules in CD82-/- bmDC when compared with wild type. Thus, CD82 does not modulate the surface levels of MHC II and costimulatory molecules, which might have explained the differences see in the antigen presentation studies.

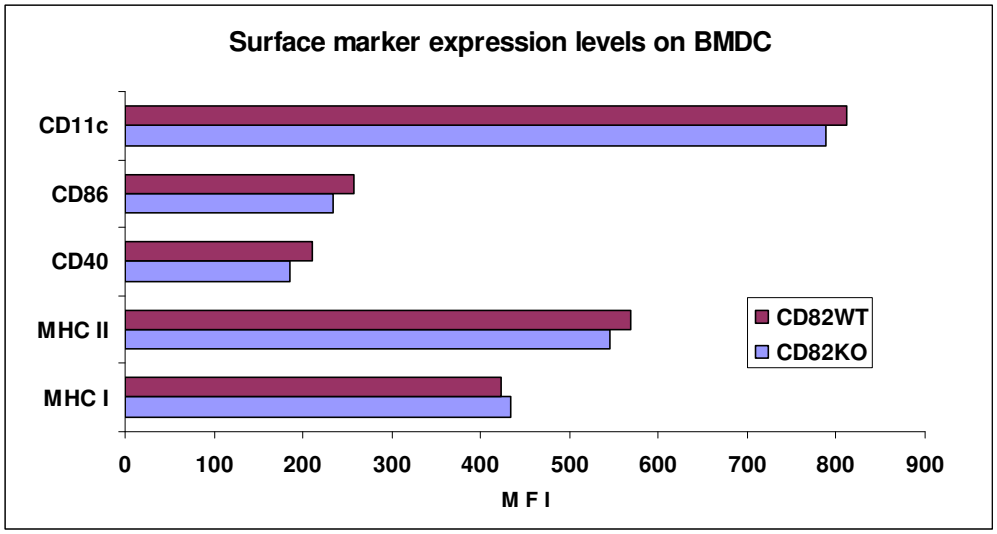
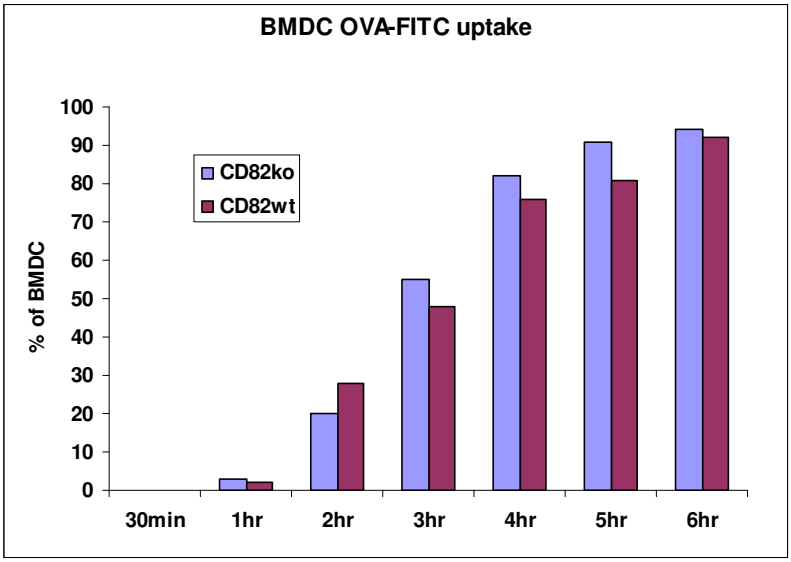


Fig.: RII-13 Surface marker expression levels on bmDC from CD82-/- and wt mice as measured by FACS. Mean fluorescence intensity values were plotted for CD11c, CD86, CD40, MHC II, MHC I.

3.2.5.2 FITC-OVA uptake assay with bmDC

An alternative explanation for the above shown effect of CD82 on antigen presentation is that CD82 may influence uptake of antigen into the antigen presenting cell bmDC. To test this question we performed a FITC-OVA uptake assay. A FITC conjugated ovalbumin was offered to bmDC in vitro for the different time points (next page, Fig.: RII-14) and then the percentage of cells positive for FITC-OVA was measured by FACS.



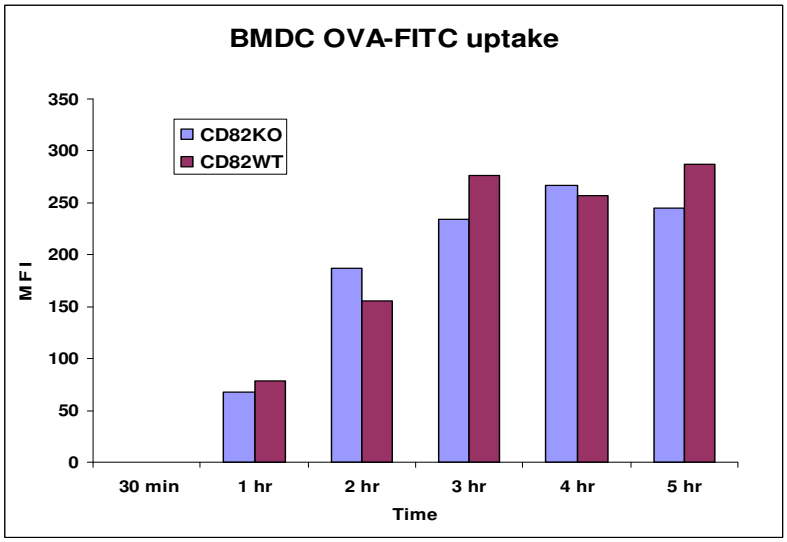


Fig.: RII-14 FITC-OVA uptake by CD82-/- bmDC and wild type bmDC. Percentage of bmDC positive for FITC-OVA measured (top) over the time period of 6 hrs and MFI values plotted (lower) for the same.

As shown in Fig.: RII-14 after 4 hrs most DC had taken up FITC-OVA. There was no difference between CD82-/- and wt DC with regard to kinetics of amount of antigen uptake. In the future it needs to be determined whether or not there is a difference in the uptake into early endocytic compartments.

3.2.5.3 CD82-/-(H-2^k) splenocytes do not show impairment in antigen presentation

We next performed antigen presentation assays with splenocytes isolated from CD82-/-(H-2^k) and WT mice. Around 50-60% of the cells in spleen are B cells, whereas DC represents only 1-3%.

Therefore in this system B cells are the major antigen presenting cells. The antigen used was HEL and the T cell hybridomas were the same as the ones used for the previous experiments with bmDC.

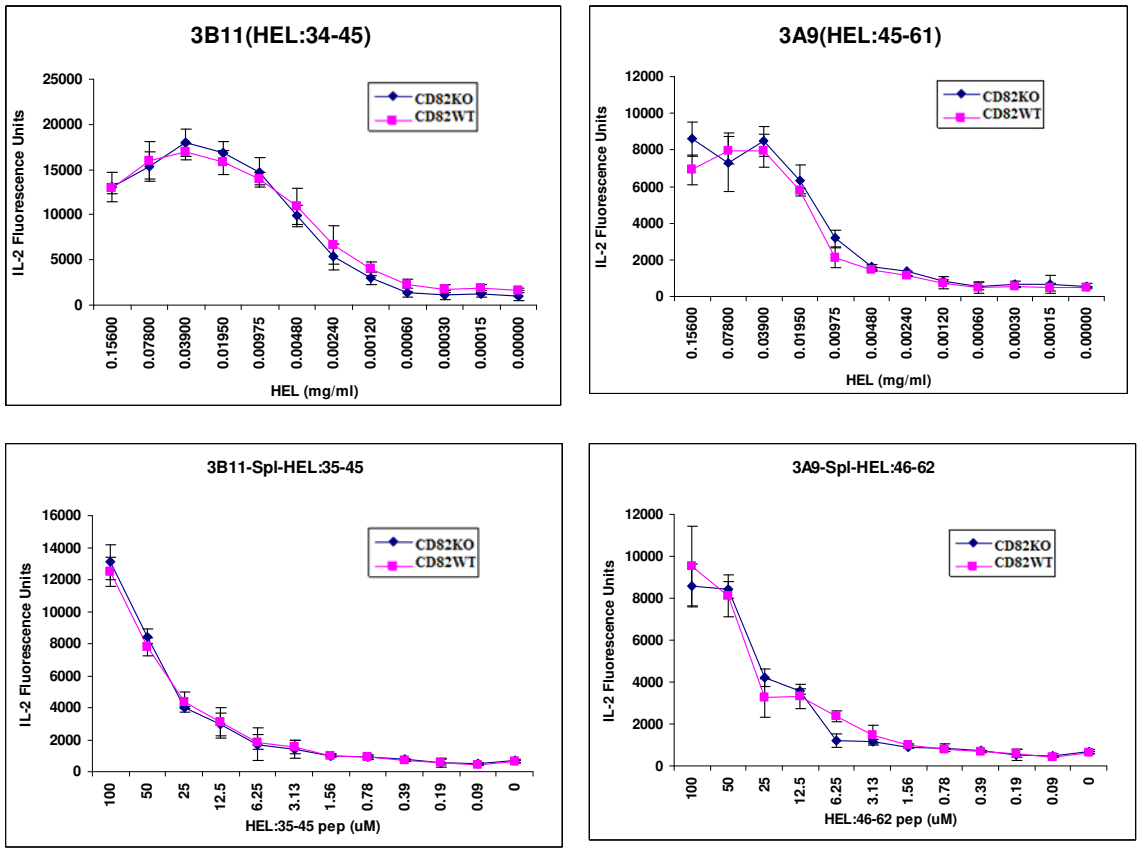


Fig.: RII-15a CD82-/- spleen cells show intact antigen presentation. Antigen presentation was performed with 3B11 (HEL:34-45) (top left), 3A9 (HEL:46-61) (top right), peptide controls for 3B11 (bottom left) and 3A9 (bottom right).

Fig.: RII-15b continued in the next page.

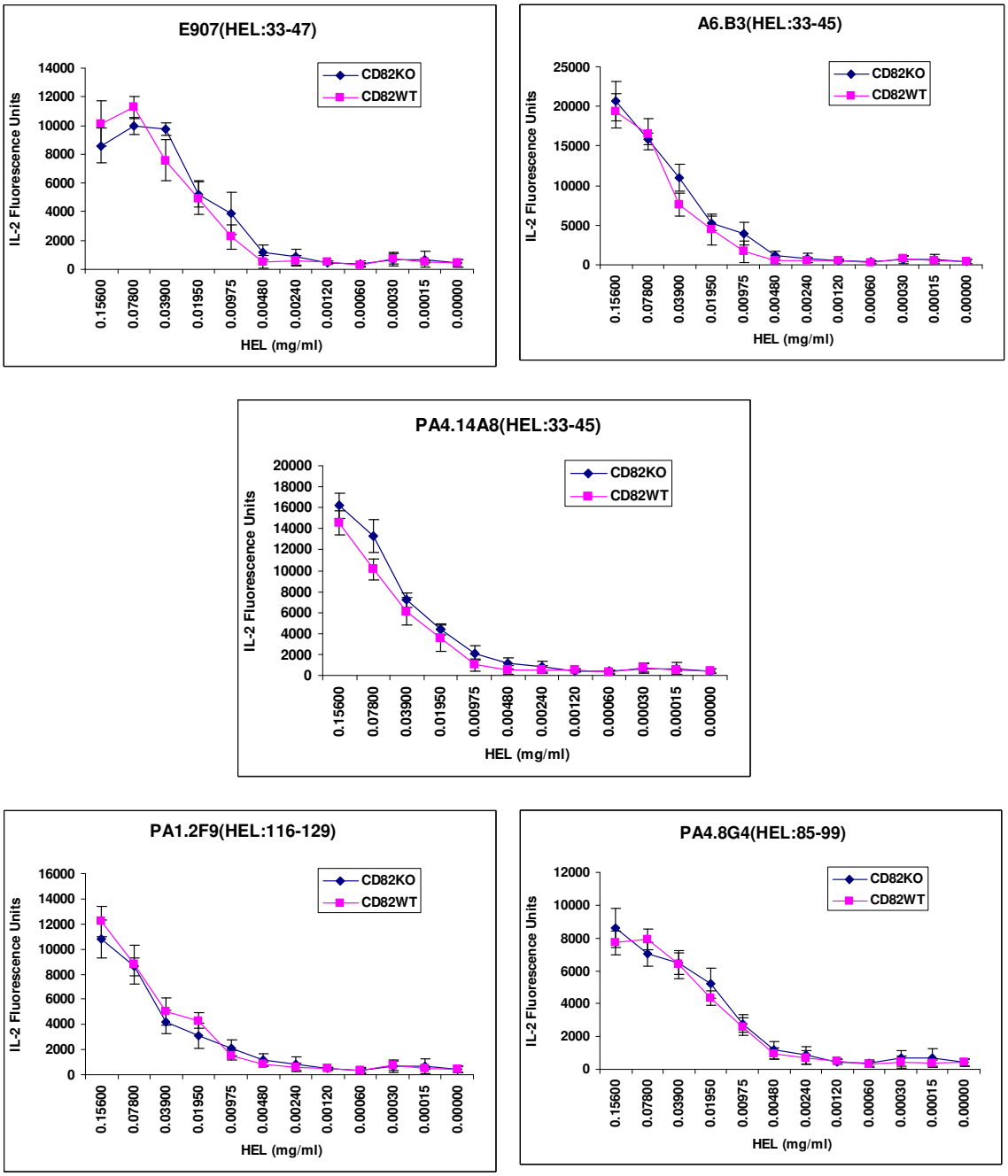


Fig.: RII-15b CD82-/- spleen cells show intact antigen presentation. Antigen presentation was performed with E907 (HEL:34-45) (top left), A6.B3 (HEL:34-45) (top right), PA4.14A8 (HEL:33-45) (middle), PA1.2F9 (HEL:116-129), and PA4.8G4 (HEL:85-99) (bottom right).

To our surprise we did not find an impairment of antigen presentation with CD82-/- (H-2^k) spleen cells. Thus, the tetraspanin molecule CD82 seems to affect antigen presentation in a cell type specific manner, namely in DC but not in B cells.

3.2.5.4 Antigen presentation by CD82-/- bmDC from C57BL/6 mice

As mentioned we have also back crossed the CD82-/- mice with C57BL/6 mice (H-2^b) which enabled us to investigate H-2^b restricted antigen presentation. We next examined the efficiency of antigen presentation with bmDCs isolated from CD82-/- (H-2^b) mice and wild type control mice. We have used the ovalbumin as antigen and OT I and OT II TCR transgenic T cells as responders. OTI TCR transgenic T cells recognize the OVA peptide S8L presented by the K^b MHC class I molecule, whereas OTII T cells recognize the OVA peptide K17G (OVA:323-339) presented by the A^b MHC class II molecule.

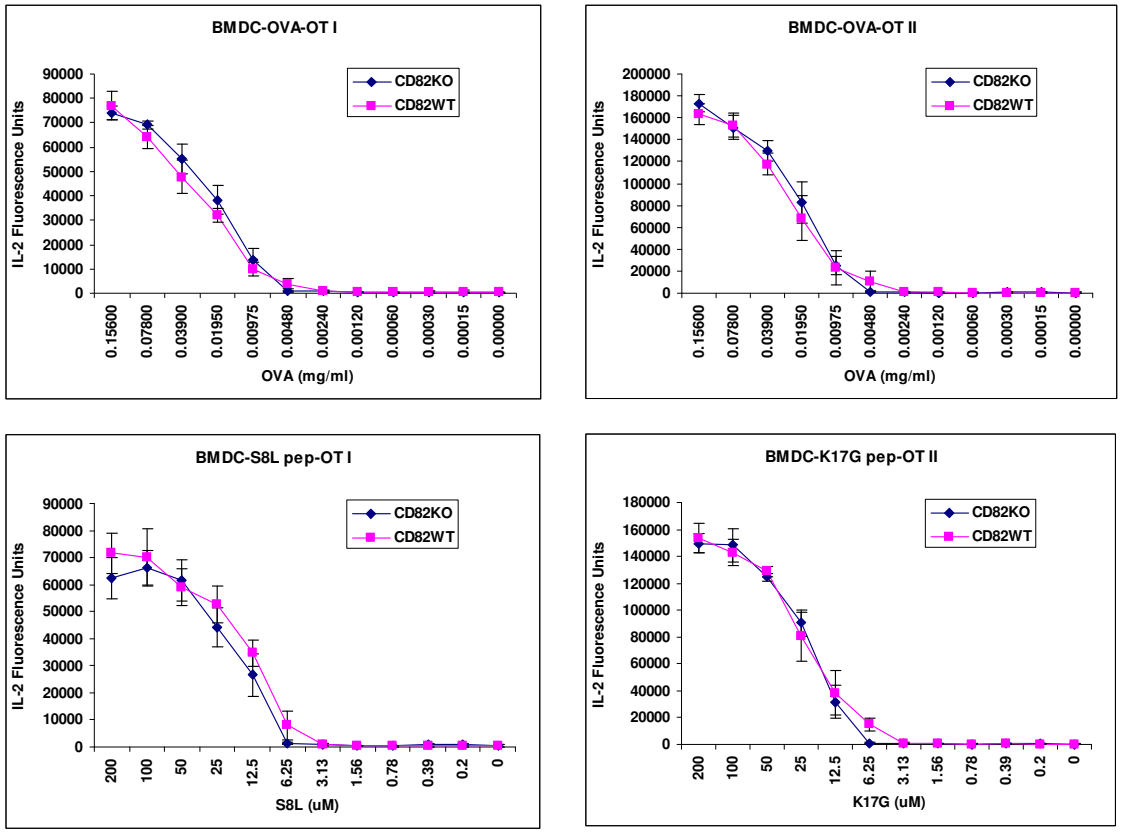


Fig.: RII-16 CD82-/- (H-2^k) bmDC show no impairment in antigen presentation when compared with wild type bmDC. bmDC with ovalbumin antigen+OTI (top left), with ovalbumin+OTII (top right), with S8Lpep+OTI (bottom left), and with K17G+OTII (bottom right). The data presented is a representative of three independent experiments performed with 5 times back crossed CD82-/- (H-2^b) mice.

As shown in Fig.: RII-16, we did not observe impairment of antigen presentation with CD82-/- (H-2^b) bmDCs when compared with WT controls. This data is not correlating with the previous observation with CD82-/- (H-2^k) bmDC data (Fig.RII-11 and 12). Possible explanations are that CD82 may influence antigen presentation in MHC

haplotype specific manner, or that presentation of the K17G epitope does not occur in a early endocytic compartment. With regard to MHC class I mediated presentation of the S8L epitope; there was also no improvement to be seen.

3.2.5.5 Antigen presentation by CD82-/-XCD53-/- double knockout bmDC

As mentioned earlier we have also generated knockout mice for CD53 and crossed the CD82-/- with the CD53-/- mice in order to generate a double knockout for CD82-/- and CD53-/-. I have performed a preliminary antigen presentation experiment with double knockout bmDC by taking ovalbumin as antigen and OTI and OTII as responder T cells. These mice have the H-2^b haplotype and are not back crossed to CBA (H-2^k) so that we could not use the panel of A^k restricted T hybridomas.

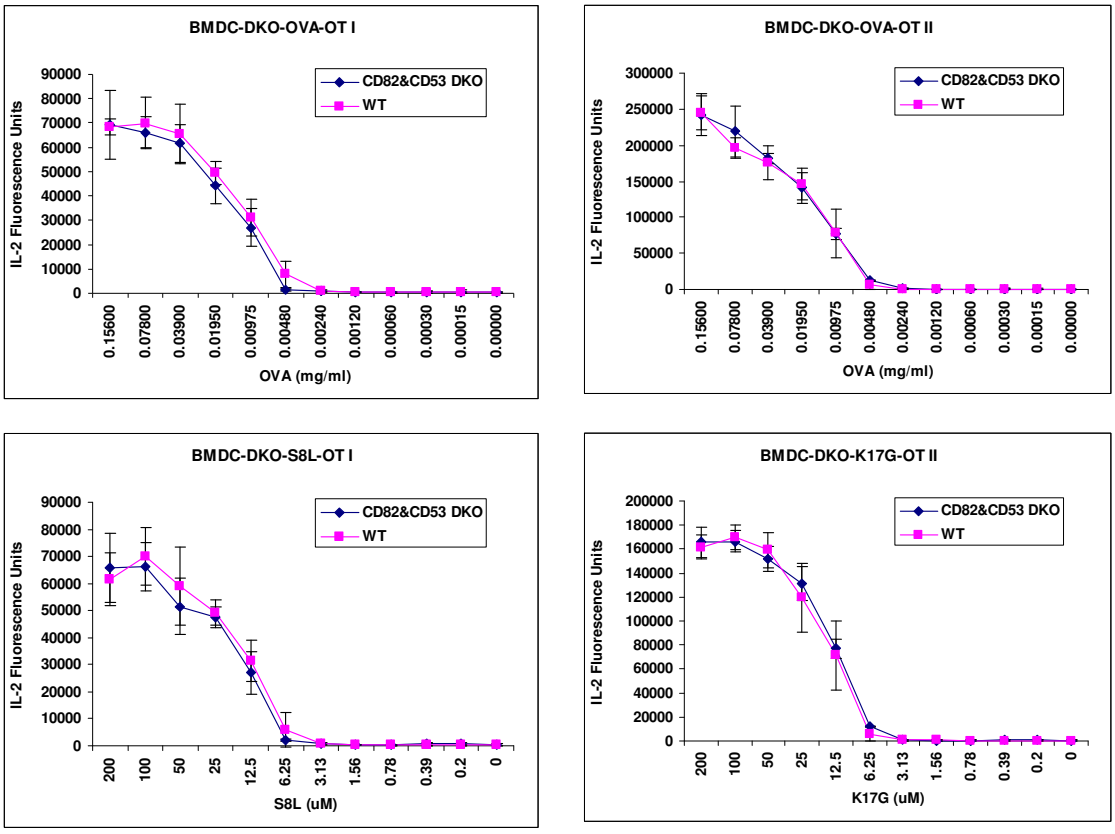


Fig.:17 Antigen presentation experiment with bmDC isolated from CD82-/-xCD53-/- double knockout mice. DKO bmDC+OVA+OTI (top left), DKO bmDC+OVA+OTII (top right), peptide controls with S8L (lower left), and K17G (lower right). The data presented is a representative of two independent experiments performed.

As shown in Fig.:17, no differences were observed in the levels of antigen presentation between bmDC isolated from CD82-/-xCD53-/- mice when compared with wild type control mice. This experiment has been reproduced two times. These results show that absence of two tetraspanin molecules, CD82 and CD53, does not influence the antigen presentation in bmDCs. There were also no differences in the surface expression of MHC class I and MHC class II on spleen cells from CD53-/- mice and double knockout mice (Fig.:RII-18).

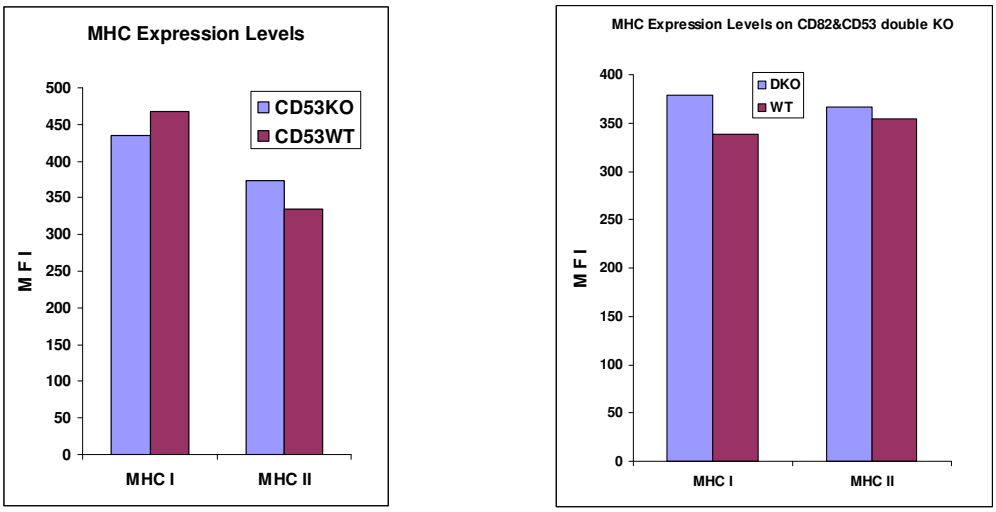


Fig.: RII-18 MHC class I and class II expression levels on spleen cells with CD53-/- and CD53+/+ (left); and with double knockout for CD82 and CD53 and wild type control mice (right).

3.2.5.6 Self peptide repertoires of CD82-/- spleen cells

To determine whether endogenous peptides were presented differentially in the absence of CD82 protein in a collaboration with Mr. Sebastian Spindeldreher and Dr. Harald Kropshofer, Hofmann LaRoche, Basel; peptides were we eluted peptides from affinity purified MHC class II molecules from CD82-/- and CD82+/+ spleen cells. Sequence analysis by a combination of two dimensional liquid chromatography and nano electrospray ionization tandem mass spectrometry was performed. The peptides were we analyzed peptides with the computer algorithms SEQUEST and MASCOT available on the Swiss-Port database (http://www.expasy.org/sprot/sprot-top.html).

Table: RII-1

Name of the protein	Sequence	CD82KO	CD82WT
Lymphocyte specific protein 1	REPDPEDAVGGSGEAEEHL	5	0
Proteasome subunit β type 3	FGPYYTEPVIAGLDPK	4	3
14-3-3 protein γ (PKC inhibitor)	KNVTELNEPLSNEERNL	5	2
IL-4 induced protein 1	GALLLNAPVVSITQGR	7	4
Complement C3	QQLAFKQPSSAYAAFN	5	6
H-2 class II A-S beta chain	RHNYEGVETHTSL	1	5
V-CAM 1	APKETTIWVSPSPILE	8	4
gamma 1 Actin	LRVAPEEHPVL	8	0
beta 1 hemoglobin	NDGLNHLDSLKGTF	4	0
hemoglobin alpha chain	SFPTTKTYFPHF	13	3
IgG 2A c region	TELNYKNTEPVLDSD	12	5

Table: RII-1: Self peptide repertoires of CD82-/- spleen cells. Peptides were eluted from affinity purified MHC class II molecules from CD82-/- and CD82+/+ spleen cells. Sequence analysis by a combination of two dimensional liquid chromatography and nano electrospray ionization tandem mass spectrometry. Peptides were analyzed with the computer algorithms SEQUEST and MASCOT on the Swiss-Port database. Numbers on the right hand side indicate the incidents of the same peptide identified in respective genotype.

As shown in Table: RII-1 some of the peptides (shown in yellow colour) were present larger amounts in CD82-/- mice, whereas, others were presented more in wild type mice (shown in green colour). List of all peptides sequenced is shown in appendix A-I. This observation suggests that CD82 influences the peptide repertoire presented by spleen cells. However, these are very preliminary data as not backcrossed mice were used for this study. This needs to be further investigated with backcrossed mice. It would be interesting to perform this study with bmDC because we have now found the interesting differences in antigen presentation with CD82-/- mice.

DISCUSSION

4.1 Discussion Part I – H2-O Project

Published studies to date have yielded inconclusive results concerning the function of DO in the class II antigen processing pathway. In vitro biochemical assays with purified molecules have mostly shown that the DM/DO complex is inactive in terms of its ability to catalyze peptide loading of class II molecules [21] suggesting that DO interferes with DM function. In contrast, functional antigen presentation assays with cell lines expressing different levels of DO have shown that DO can promote, inhibit, or have no effect on MHC class II antigen presentation. These differences may be due to different experimental systems and/or the antigens examined [53-59]. In vivo studies using H2-O sufficient and deficient mice have also failed to define a specific role for DO [53]. These failure and inconclusive results are is puzzling in the light of the fact that a major fraction of DM is always found to be tightly associated with DO. Since DM is crucial for peptide loading onto MHC II molecules, one would expect to easily see an effect of DO, but this is not the case.

For better to understanding of the biological role of DO our laboratory has generated a transgenic mDO mouse as a novel tool for the analysis of DO function. This mouse expresses 2-3 folds higher levels of H-2O since mDO is controlled by the K^b promoter, all MHC class I positive cells express the H2-O transgene. Previous studies have utilized H2-O-/- mice and therefore assessed a role for DO in presentation by A^b MHC class II molecules. The mDO ($H\text{-}2^k$) mice enabled us to extend the analysis to another MHC II allele, namely A^k. Since DO is strongly expressed in B cells, and because B cells usually take up antigen via their BCR we crossed the mDO Tg ($H\text{-}2^k$) mice with anti-HEL BCR Tg ($H\text{-}2^k$) mice. With these crossed mice we wanted to study if DO influenced in antigen presentation when the antigenic cargo delivered into endo-lysosomal compartments via a high affinity BCR. The HyHEL10 antibody used for the generation of anti-HEL BCR Tg mice [60] has an affinity of about 10^{-9}M.

4.1.1 H2-O-/- mice show higher percentage of IgD+ B cells

First we have analysed thoroughly the expression profile of various surface markers on cells from spleen, bone marrow, lymph node (data not shown) and thymus (data not shown). For most surface antigens, the expression was normal on H2-O-/- cells, but for IgD we have made the surprising observation that the percentage of IgD single positive cells in H2-O-/- spleen is increased consistently about 1.5 fold whereas IgM positive cells are unaltered. Moreover, H2-O-/- B cells displayed around 20% higher IgD levels than wild type controls (Fig.: RI-2). A misbalance in IgD expression may be explained by is a defect in the early B lineage committed bone marrow cells in H2-O-/- mice. However, when bone marrow precursors were analysed no defect in the early B lineage committed compartment was observed (Fig.: RI-2). Other groups have reported that during the germinal center reactions IgD is strongly reduced together with DO on B cells [50]. Together with our findings these observations may suggests that these DO and IgD control each other's expression but the link between IgD and DO expression is not yet understood and needs to be investigated in the future.

As mentioned the other cell surface markers namely MHC class I and class II, CD4, CD8, CD19, CD21, CD23, CD11c, CD5, CD40, IgM, TCR alpha, CD25, and B220 were not changed in H2-O-/-, or mDO Tg mice (Fig.:RI-1 and data not shown).

4.1.2: Antibody responses and affinity measurements

Since B cells in H2-O-/- mice display higher levels of surface IgD we expected that serum IgD levels may also be increased in these mice. Therefore we have estimated by ELISA the normal levels of immunoglobulins in H2-O-/-, mDO Tg and B6 mice, but no significant increase in IgD levels was seen in sera from H2-O-/- mice as compared to controls (fig.:FI-4). The other Ig subclasses were also not altered in H2-O-/-, mDO Tg mice except for IgG1 in H2-O-/- mice. Elevated IgG1 levels observed in H2-O-/- mice in agreement with published data [54].

Next we immunized H2-O-/-, mDO Tg and wild type mice with T dependent and T independent antigens together with the adjuvant alum. We have selected two T dependent antigens for immunization i.e., TNP(11)OVA and NP(12)OVA. TNP(3)BSA and TNP(34)BSA conjugates were used to compare the relative affinity of the anti TNP sera. When the anti TNP sera were analysed there was no difference in antibody titers. However, we observed a decreased affinity in the TNP sera from H2-O-/- mice. The decrease was small but reproducible in several experiments. For the antigen NP-OVA no differences with regard to affinity was found. (Fig.: RI-5 bottom). A possible explanation is that NP antibodies are so called germ line antibodies which may already have a fairly high affinity.

When we analyzed sera obtained by immunization with the T independent antigen TNP – Ficoll, no significant differences were observed in the IgM titers between H2-O-/- , mDO Tg and wild type control mice (Fig.: RI-6).

When we attempted to confirm the different affinities of the anti TNP sera from H2-O-/- mice by another method, namely inhibition by the hapten TNP-L-Lys, as shown in Fig.: RI-7 an increase in affinity was observed with time after immunization from $3x10^{-7}$M on day 18 to $7.3x10^{-8}$M on day 42. However, with the hapten inhibition method no difference in the affinity of TNP antibodies from H2-O-/- and wild type mice could be found. The reasons for the discrepancy are not clear, but we have to keep in mind that the differences observed with the TNP low/high technique were very small to start with.

4.1.3: DO inhibits antigen presentation in early but not late endocytic compartments

Comparative analysis of antigen presentation by H2-O-/- and wild type B cells following fluid phase uptake of antigen showed that most antigens were presented equally well [54-55]. In contrast H2-O over expression and anti sense RNA knockdown studies in mouse B cell lines have shown that DO downmodulates antigen presentation [56].

Using the mDOxBCR mice we have studied the antigen intake via a high affinity BCR into the B cell that express different levels of DO. As expected, uptake of HEL antigen via the BCR led to more efficient presentation of antigen than fluid phase uptake (Fig.: RI-12). DO was found to downmodulates presentation, irrespective of the pathway of antigen uptake (via BCR or fluid phase). However, only presentation of those epitopes was downmodulated that are loaded onto A^k molecules in early endocytic compartments, whereas presentation of epitopes in late compartments were not affected. Why should DO downmodulate antigen presentation only in early but not late compartments? The following potential explanation can be provided. A BCR binding a antigen which has low affinity is likely to loose this antigen following internalization already in the early endocytic compartment. Furthermore, BCRs for antigens are expected to be of low affinity because high affinity B cells with self reactivity will be deleted. Therefore, these antigens may be preferentially delivered to early compartments by the low affinity BCR. Since the immune system is trying to avoid the presentation of auto antibodies, it makes sense that there is a mechanism preventing presentation in the early compartments [31]. This is one of the reasons why we have studied the affinity of anti TNP antibodies. Our results were, unfortunately inconclusive and therefore neither support nor disprove this hypothesis. Sera against more antigens need to be tested. In addition, antigen presentation following uptake by BCRs with high and low affinity should be compared.

4.1.4 High levels of DO inhibits antigen presentation in bmDC

We extended our studies to bmDC isolated from mDO Tg (A^k) mice and CBA (A^k) to see whether or not elevated levels of DO would also suppress antigen presentation in DC. Indeed, high levels of DO suppress antigen presentation in bmDC, but in contrast to B cells suppression was seen for both HEL epitopes 34-45 and 45-61. It needs to be tested whether these epitopes are generated in the early or late compartments in dendritic cells. When the bmDCs were incubated with the cognate peptide HEL 34-45 or HEL 46-61 no difference in peptide presentation was observed (Fig.:RI-15). Surface markers on bmDC were not changed, indicating that suppression of antigen

presentation in DC is indeed caused by DO and not by indirect changes in the interaction between DC and T cells.

We also investigated the efficiency of antigen presentation with H2-O-/- (A^b) bmDC using taking ovalbumin as an antigen and OTI and OTII as responder T cells. However, there was no influence on antigen presentation was observed. One explanation is that DO may be modulate antigen presentation in an MHC II allele specific manner. Another possible reason could be DO does not affect the ovalbumin derived epitope. The use of OTI TCR cells shows that DO does not affect cross presentation or MHC class I mediated presentation. In this experimental system the ovalbumin epitope S8L is generated in the cytosol via cross presentation and presented by MHC class I molecule at the cell surface. Since for cross presentation antigen is first taken up by the endocytic pathway and then fed into the MHC I pathway by a not fully understood mechanism, it is possible that DO would interfere with cross presentation but that was not found in our study.

How does a two fold increase in DO affect antigen presentation? It has been shown that around 60-70% of DM is associated with DO. The remaining 30-40% of DM appears to be free and may be the fraction that supports antigen presentation. An increase in DO would decrease the fraction of free DM. However, we have to keep in mind that the stoichiometry of DM:DO association is not clear, whether it is a 1:1 association or whether one DM molecule is occupied by several DO molecules

It is possible that more than one DO molecule binds to a DM molecule and thereby interferes with its activity. The observation, however, that an about two fold increase of DO expression substantially inhibits antigen presentation both in spleen cells and bmDC, suggests the existence of a fraction of free DM molecules, which can be bound by additional DO molecules because DO binds very tightly to DM and dissociation has not been found to occur under physiological conditions, it is questionable, whether DO associates in vivo and occupies the next free DM molecule. Whatever the stoichiometry, at first sight it does not seem to make sense that a substantial fraction of

DM is continuously occupied and blocked. However, if one considers that inhibition of DM by DO functions only at around neutral pH in the early endocytic compartments, then this makes sense, because as in chapter 4.1.3 the immune system may want to inhibit peptide loading only in early but not in late compartments. When considering function and stoichiometry of DO:DM complexes, it should be kept in mind that in loading compartments MHC class II, DM and DO are embedded in super complexes, containing various tetraspanin molecules such as CD63, CD81, and CD82, which may influence the activity of DM:DO complex as will be discussed in the following section.

4.2: Discussion Part II: CD82 Project

The involvement of tetraspanin proteins in various cellular processes is well recognized, but poorly understood. In this study we have concentrated on the tetraspanin CD82. It has been shown that CD82 interacts with several transmembrane molecules including TCR, BCR, CD4, CD8, MHC class II, MHC class I and non classical MHC proteins DO and DM [61]. The interaction of CD82 with MHC class II, MHC class I and non classical MHC proteins DO and DM is of particular interest because it occurs in endocytic compartments where antigen processing and loading of peptides into MHC molecules is known to take place. We hypothesized that CD82 may play a role in antigen presentation. Therefore, we have generated knockout mice for CD82. There are 9 exons in CD82 gene; from which we have deleted exons 4 and 5 (Fig.:RII-1a). The truncated CD82 mRNA does not seem to be stable enough to express the truncated protein (unpublished observations S Tanaka). The CD82 *floxed/floxed* mice were crossed with *Cre* deleter mice in order to obtain the CD82-/- mice. Deletion of CD82 gene in all tissues is not lethal. The mice survived normally and gave birth at expected Mendelian ratio. This suggests that CD82 protein is not critical for developmental processes. Moreover, no apparent behavioral, physical or anatomical abnormalities were found with CD82-/- mice and their life span seemed to be normal.

Dr.S.Tanaka from our laboratory has also generated CD53 knockout. There are 8 exons in the mouse CD53 gene from which exons 2 to exon 5 were deleted (Fig.:RII-1b) by crossing the CD53 *floxed/floxed* mice with *Cre* deleter mice. Like with CD82, deletion of CD53 gene in mouse whole body is not lethal and CD53-/- mice breed normally with no apparent behavioral, physical or anatomical abnormalities. We have also intercrossed the CD82-/- with CD53-/- mice to obtain double knockout mice. These CD82-/- CD53-/- mice also bred normally. This suggests that the lack of both tetraspanin molecules CD82 and CD53 does not affect mouse embryonic development.

4.2.1: Surface marker expression pattern in CD82-/- mice is not changed

The expression profile of different cell surface markers like class I MHC, class II MHC, CD3, CD4, CD8, TCR, CD11c, B220, CD43, NK1.1, DX5, IgM, IgD, CD21, CD23, CD19, CD25, CD44 and CD40 on various immuno-competent cells isolated from bone marrow, spleen, thymus, and lymph nodes are normal as no significant changes were observed in the absence of CD82 when compared with wild type cells (Fig.: RII-3a&b, and data not shown). The expression levels of other members of the tetraspanin family CD9, CD81 are also intact in the absence of CD82 protein (Fig.: RII-3b). Taken together, no T and B cell developmental defects both in terms of number and surface expression of various cell surface markers are observed CD82 deficient mice. Con A stimulated and LPS stimulated spleen cells show no significant differences in the expression profile of various T and B cell activation markers (data not shown).

It has been shown that mice deficient for the tetraspanin protein CD81 have reduced levels of CD19, although expression of other surface markers in the B cell compartment was similar to that of WT mice. In CD82-/- mice there is no reduction of CD19. We have also investigated various cell adhesion molecules, for example ICAM1, LFA1, LFA2, VCAM on the surface of B, T, and DC surface and found no significant differences observed in the absence of CD82 protein (Fig.: RII-3a and data not shown). Taken together the absence of the CD82 protein does not affect the expression of the many cell surface markers tested here.

4.2.2: Antibody responses in CD82-/- mice

CD82 associates also with the BCR. To determine whether CD82 contributes to the regulation of the humoral immune response, the basal antibody levels of the immunoglobulin subclasses IgM, IgG1, IgG2a, IgG2b, IgG3, IgA were measured. No differences were found between CD82-/- and CD82+/+ mice (Fig.: RII-5). Next we immunized CD82-/- and wild type mice with T dependent and T independent antigens together with adjuvant alum. When we analyzed the sera samples isolated post

immunization for the antigen specific immunoglobulins IgM (in case of T independent antigen TNP-Ficoll) and total IgG (in case of the T dependent antigen TNP(11)OVA) no significant differences were observed between CD82-/- and wild type control mice (Fig.: RII-6 and 7).

This observation suggests that the priming of B cells for the formation of short lived plasma cells and production of antigen specific IgM in response to the T independent antigen TNP-Ficoll is independent of CD82 protein. The hapten specific IgG response in case of T dependent antigen TNP(11)OVA indicates that in the absence of CD82 protein B cells can undergo T cell dependent class switching and produce normal levels of IgG. Moreover, we have not observed any significant differences in the affinity of anti TNP antibodies raised in the absence of CD82 as compared to wild type controls (Fig.:RII-6). Together these observations suggest that the tetraspanin protein CD82 is not essential for the B cell priming and production of antibody against antigen, both in terms of titer and affinity. However, we have not tested suboptimal immunization protocols yet. The data presented in Fig.: RII-6 and 7 were performed with mice that have not yet backcrossed. However we do not expect that major differences with regard to antibody production will appear in backcrossed mice because there were no differences in the endogenous levels of Ig in 5 times backcrossed mice.

Other tetraspanin knockout mice have been studied with respect to humoral immune responses. For example, CD81-/- mice show partial impairment of responses to TNP-KLH [64]. This is probably explained by the fact that CD81 strongly associates with CD19 which is required for B cell proliferation. CD37-/- mice show also an impairment of antibody production, but only in suboptimal immunization procedures [63]. In the case of CD9-/- mice there is no impairment in humoral immune responses [76]. These observations suggest that B cell priming is influenced by some (CD81, CD37) but not other (CD82 and CD9) tetraspanin molecules.

4.2.3: CD82 influences proliferation of T cells but not B cells

In agreement with the lack of influence of CD82 on antibody responses we have not observed an impairment in the proliferation of CD82-/- B cells after stimulation with the mitogen LPS which acts through the TLR4 receptor on B cells. In contrast, when we analyzed the proliferative responses of T cells to Con A or antiCD3+CD28 we observed significantly enhanced proliferation in CD82-/- T cells (Fig.: RII-8). These observations suggest that CD82 may be involved in T cell signaling events. Upon TCR stimulation, the tyrosine kinase *Lck* plays a critical role in phosphorylation of key T cell signaling molecules, including the TCR ζ chain and ZAP 70. The natural ligand for CD82 was identified recently as the Duffy antigen receptor for chemokines (DARC) [80]. DARC is expressed in human erythrocytes and on endothelial cells lining post capillary venules in kidney and spleen. It has been shown that direct interaction of DARC with CD82 leads to inhibition of tumor cell proliferation and induction of senescence by modulating the expression of TBX2 and p21 [80]. This suggests that CD82 ligation with its natural ligand DARC induces anti-proliferative signals and is in line with our finding that the absence of CD82 in T cells enhances proliferation.

T cells lacking the tetraspanins CD37, CD81 and Tssc6 are also hyperproliferative to TCR stimulation [63-66, 68]. It has been shown that CD82 and CD81 are involved in the formation of the immunological synapse at the site of TCR engagement [69]. Thereby, these tetraspanin molecules may regulate T cell proliferation possibly by direct association with cell surface receptors that are essential for inducing proliferative signals or by controlling the formation of rafts responsible for proliferative events.

4.2.4: Role of CD82 protein in antigen presentation

Since CD82 associates with MHC II in loading compartments, we hypothesized that it may modulate antigen presentation. Indeed, the studies presented here show for the first time that the absence of a tetraspanin molecule, namely CD82, impairs antigen presentation. We consider this is to be the major finding with the CD82-/- mice. Strikingly, the defect in antigen presentation was observed only in bmDC but not in spleen cells (Fig.:RII-11&12 and Fig.: RII-15) and thus appears to be cell type specific.

Furthermore, presentation was found to be only affected in the recycling compartment but not in late endo-lysosomal compartments as will be discussed below.

I used two model antigens in the antigen presentation assays i.e., hen egg lysozyme (HEL) and ovalbumin (OVA). Both the antigens have been well studied in various laboratories. Upon uptake into endocytic compartments antigens are usually digested into various peptides or epitopes that can bind to MHC II molecules. It is known that some epitopes are preferentially generated and loaded into MHC II molecules in acidic late endocytic compartments, whereas for other epitopes this occurs in the early endocytic recycling compartments, probably depending on protease activities present in these compartments. Fig.:RII-10 shows the location of the major epitopes in hen egg lysozyme. Previous studies have shown that the epitope 33-47 is preferentially processed and loaded into I-A^k molecule in an early endocytic compartment. In contrast epitopes 46-61 and 116-129 are preferentially processed and presented in the late endocytic compartments. These conclusions were based on the observation that chloroquine, which neutralizes the acidic late endocytic compartment, impairs presentation of the epitope 46-61 and 116-129, whereas it has no or little effect on the presentation of epitopes in the neutral early endocytic compartment such as epitope 33-47. Thus, the use of T cell hybridomas specific for different epitopes of HEL presented by the A^k molecule allowed us to determine if presentation occurs predominantly in early or late endocytic compartments. In addition to T cell hybridomas specific for the above mentioned epitopes, we have also used the T cell hybridoma PA4.8G4 recognizing HEL epitope 85-99, but we do not yet know its sensitivity to chloroquine.

As shown in Fig.:RII-12; a 2-4 fold reduction in antigen presentation was observed in CD82-/- bmDC when we tested for the HEL epitope 33-47 with the specific T cell hybridomas E907, A6.B3, and PA4.14A8. No significant differences were observed for presentation of HEL epitopes 46-61, 116-129 and 85-99. The presentation of the synthetic 34-45 HEL peptides by bmDC was not affected by the absence of CD82 (Fig.: RII-11 and 12). Together these results strongly suggest that CD82 specifically supports antigen presentation in early but not in late endocytic compartments. Importantly,

when splenocytes were used, where the main antigen presenting cells are the B cells, no effect of CD82 were found (Fig.:RII-15). These results indicate that CD82 exerts its biological function in cell type specific manner. The mechanism is unclear. We considered the possibility that CD82 may influence the uptake of antigen into endocytic compartments. In a preliminary experiment we examined the rate of antigen internalization with CD82-/- bmDC versus wild type bmDC. As shown in Fig.: RII-14 the overall rates of internalization of FITC-OVA by CD82-/- bmDC and wild type bmDC are similar. It will be necessary to follow the uptake of antigen into early versus late compartments, which I have not yet performed because of time limitations.

The results discussed above show clearly that CD82 affects antigen presentation by MHC class II molecules. To investigate whether or not CD82 would also influence antigen presentation by MHC class I molecules, we made use of the TCR transgenic OT-I mice which recognize the ovalbumin-derived peptide SIINFEKL presented by the MHC class I molecule K^b. No difference in the presentation of SIINFEKL was observed between CD82-deficient and wild-type bmDC. This result is not completely unexpected, as in the MHC class I antigen presentation pathway the peptides are generated in the cytosol and then transported by TAP into the ER where they are loaded on MHC class I molecules with the help of a loading complex. CD82 is not part of the class I loading complex, which, therefore, may explain why CD82 has no influence on MHC class I loading. However, it has been reported that in addition to this major TAP-dependent pathway MHC class I may also be occasionally loaded with peptides in the recycling endosomal compartment by exchange of peptides. Since CD82 is found associated with MHC class I in endosomal compartments, it is possible that it may also influence peptide exchange by class I molecules in these compartments. This question will have to be addressed in the future.

For ovalbumin, there exists another TCR transgenic mouse, designated OT-II, which recognizes the OVA peptide 323-339 presented by the MHC class II molecule A^b. When bmDC were incubated with OVA, no difference was found between $CD82^{-/-}$ and wild-type cells with regard to presentation of the OVA epitope 323-339 to OTII T cells.

It is not yet known, if this epitope is presented in early or late endosomal compartments. An alternative explanation would be that the effect of CD82 on antigen presentation is specific for certain MHC alleles such as A^k. Such allele specificity has been observed for the accessory molecule HLA-DM which was found to support strongly antigen presentation by some MHC class II alleles, but not by others [81].

As mentioned above we have also generated CD82-/- X CD53-/- double knockout mice in order to see if deletion of two tetraspanins would have a more drastic effect on antigen presentation. When bmDC from CD82-/- X CD53-/- mice, which are of the H-2^b haplotype and are not yet backcrossed, were tested with OTI and OTII cells for MHC class I and class II mediated presentation of OVA respectively. Again no difference was found between knockout and wild type mice. Thus, these results are similar to those obtained OVA presenting CD82-/- APC. Backcrossing of CD53-/- mice to CBA (H-2^k) is planned and will allow us to use the large panel of A^k restricted hybridomas.

4.2.5: Influence of CD82 on peptide profiles.

Early after generation of the CD82 knockout mice in our laboratory, a collaboration with Sebastian Spindeldreher and Dr. Harald Kropshofer, Hofmann LaRoche, Basel, was initiated, aiming at the question whether the absence of CD82 would modify the overall repertoire of peptides presented by MHC class II molecules. S. Spindeldreher and H. Kropshofer have developed a sensitive technique by which they could extract peptides from one spleen of mice and sequence a large number of peptides by nano electrospray ionization tandem mass spectrometry. This procedure is laborious and time-consuming. Therefore, the results obtained so far are preliminary, as only a small number of mice could be tested. Nevertheless, these results indicate that there is a difference in the peptide repertoire of $CD82^{-/-}$ versus wild-type splenocytes (Table: RII-1) (see the peptides listed in appendix A-I). It seems that from various proteins more peptides are generated in $CD82^{-/-}$ cells, whereas from other proteins more abundant peptides are generated in wild-type mice. These findings suggest that CD82 affects the overall peptide repertoire presented by MHC class II molecules. However, we have to

keep in mind that the mice used for these studies were not yet back-crossed to B6. Moreover, there appear to be variations between individual mice because the above mentioned changes are not found in all mice tested so far. A more detailed study would require the analysis of a much larger number of mice preferably back-crossed mice. Since in spleens MHC class II molecules are mainly derived from B cells, and because DC but not B cells were found to differ in their capacity to generate and present certain epitopes, it would certainly be of interest to include DC, for comparison of the peptide repertoires of $CD82^{-/-}$ and wt DC.

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Appendix A-I List of Peptide Sequences

Peptides sequences from CD82 knockout spleen cells

SwissProt	Protein	Sequence	Epitope
Q9JJ00	Phospholipid scramblase 1	YPPPYPPAAFQGPSDHAAYPIP	15 - 36
		YPPPYPPAAFQGPSDHAAYPIPQ	15 - 37
Q7TMK0	Hypothetisches Protein	SPKLWIYYTSNLAPGVP[1]	38 - 54
		SPKLWIYYTSNLAPGVPA	38 - 55
Q8K352	SH3 Protein exprimiert in Lymphozyten	FKFIYVDVLPEEAVGPV	223 - 239
		KFIYVDVLPEEAVGPV[1]	224 - 239
P05367	Serum Amyloid A-2 Protein	GPGGVWAAEKISDARE	65 - 80
		GPGGVWAAEKISDARES	65 - 81
Q8VEB4	Lysosomale Phospholipase A2	MYQMYGGPVVLVAHSM	183 - 198
		YQMYGGPVVLVAHSM	184 - 198
O35649	Cyclophilin C-assoziiertes Protein	TTSSYSNPTIGYENR	498 - 512
		TSSYSNPTIGYENR	499 - 512
P18527	Ig schwere Kette V Region	FSSYAMSWVRQTPE	28 - 41
		FSSYAMSWVRQTPEK	28 - 42
Q61147	Ceruloplasmin (Ferroxidase)	DVDKEFYLFPTVFDENES	567 - 584
		VDKEFYLFPTVFDENE[1]	568 - 583
P17439	Glucosylceramidase	RPISLFASPWTSPTW	188 - 202
		RPISLFASPWTSPTWLK	188 - 204
P47753	*F-Actin capping Protein* α-1	WKFTITPPSAQVVG	176 - 189
		WKFTITPPSAQVVGVL[1]	176 - 191
Q61233	L-Plastin (LCP-1)	AVDLGKNQAKFSL	462 - 474
		AVDLGKNQAKFSLVG	462 - 476
Q8R2G5	*Slit-like 2 Protein*	ALAAVLLAVLAAAGAAYCVRRA[1]	581 - 602
		VLLAVLAAAGAAYCVRRA	585 - 602
P29699	Alpha-2-HS-Glykoprotein (Fetuin-A)	LPADPPASVVVGPVVVPRGLS	271 - 291
		DPPASVVVGPVVVPRGL[1]	274 - 290

Peptides sequences from CD82 knockout spleen cells

SwissProt	Protein	Sequence	Epitope
Q91X72	Hemopexin	ISARWKNPITSVDAA[1]	85 - 99
		ISARWKNPITSVDAAF	85 - 100
		SARWKNPITSVDAA[1]	86 - 99
Q8C815	Integrin-α 2b	LFLQPKGPQALSTPT	74 - 88
		LFLQPKGPQALSTPTL	74 - 89
		FLQPKGPQALSTPT	75 - 88
Q91XQ6	Lysosomal-assoziiertes Transmembranprotein 4β	LPPYDDATAVPSTAKEPP	203 - 220
		LPPYDDATAVPSTAKEPPP[1]	203 - 221
		LPPYDDATAVPSTAKEPPPP[1]	203 - 222
P05371	Clusterin (SGP-2)	KDNPKFMDTVAEKALQE	423 - 439
		KDNPKFMDTVAEKALQEY	423 - 440
		DNPKFMDTVAEKALQE[1]	424 - 439
Q9DC42	CD97	LPKGPFTYTSPSNTEL	246 - 261
		LPKGPFTYTSPSNTELS	246 - 262
		LPKGPFTYTSPSNTELSL[1]	246 - 263
P08101	FcγRII	LPEEVGEYRQPSGGSVPV	256 - 273
		LPEEVGEYRQPSGGSVPVSPG[1]	256 - 276
		EVGEYRQPSGGSVPVSPG	259 - 276
Q61190	Interleukin-10 Rezeptor β-Kette	LHLRFSAPQIENEP	127 - 140
		LHLRFSAPQIENEPE[1]	127 - 141
		LHLRFSAPQIENEPET	127 - 142
		HLRFSAPQIENEPE[1]	128 - 141
P08071	Lactotransferrin	RPYLNWNGPPASLEE	150 - 164
		RPYLNWNGPPASLEEA	150 - 165
		RPYLNWNGPPASLEEAV	150 - 166
		RPYLNWNGPPASLEEAVSKFFSKS	150 - 173
Q8BND5	Quiescin Q6	VQANRYSEAHPQEPA[1]	418 - 432
		VQANRYSEAHPQEPAD	418 - 433
		VQANRYSEAHPQEPADG	418 - 434
		QANRYSEAHPQEPADG	419 - 434
		ANRYSEAHPQEPAD	420 - 433
P10854	Histon H2B	VRLLLPGEL	98 - 106
		VRLLLPGELAKHAVSEGTKAVTKYT	98 - 122
		VRLLLPGELAKHAVSEGTKAVTKYTSS[1]	98 - 124

Peptides sequences from CD82 knockout spleen cells

SwissProt	Protein	Sequence	Epitope
		VRLLLPGELAKHAVSEGTKAVTKYTSSK[1]	98 - 125
		LLLPGELAKHAVSEGTKAVTKYTSS	100 - 124
P01872	Ig μ-Kette C Region, sezernierte Form	GGKYLATSQVLLSPKS	62 - 77
		GGKYLATSQVLLSPK	62 - 76
		GKNKDLHVPIPAVAE	94 - 108
		KNKDLHVPIPAVAE	95 - 108
		VPPRDGFSGPAPRKSK	117 - 132
		VPPRDGFSGPAPRKSKLI	117 - 134
P52760	*Heat-responsive protein 12*	IYKTYFQGSLPARAAYQ[1]	118 - 134
		YKTYFQGSLPARA	119 - 131
		YKTYFQGSLPARAA	119 - 132
		YKTYFQGSLPARAAY	119 - 133
		YKTYFQGSLPARAAYQ[1]	119 - 134
		KTYFQGSLPARAAYQ	120 - 134
P13020	Gelsolin	GWDDNYWSVDPLDRALAE	759 - 776
		WDDNYWSVDPLDRAL[1]	760 - 774
		WDDNYWSVDPLDRALA	760 - 775
		WDDNYWSVDPLDRALAE[1]	760 - 776
		WDDNYWSVDPLDRALAELAA	760 - 779
		WSVDPLDRAL[1]	765 - 774
		WSVDPLDRALAE[1]	765 - 776
		WSVDPLDRALAEL[1]	765 - 777
		WSVDPLDRALAELAA[1]	765 - 779
Q9CY54	Hämoglobin (*11 days embryo cDNA product, beta adult major chain, full insert sequence*)	GHHLGKDFTPAAQAAFQK	115 - 132
		GHHLGKDFTPAAQAAFQKVV	115 - 134
		HHLGKDFTPAAQAAFQK	116 - 132
		HLGKDFTPAAQAAFQ	117 - 131
		LGKDFTPAAQAAFQ	118 - 131
		LGKDFTPAAQAAFQK[1]	118 - 132
		GKDFTPAAQAAFQ	119 - 131
		GKDFTPAAQAAFQK[1]	119 - 132
		GKDFTPAAQAAFQKV	119 - 133
		FTPAAQAAFQKVVAGVAAALAHKYH[1]	122 - 146
		FQKVVAGVAAALAHKYH[1]	130 - 146
		AALAHKYH[1]	139 - 146
P04441	Invariante Kette (Ii) (CLIP)	SAKPVSQMRMATPLLMRPM[1]	83 - 101
		SAKPVSQMRMATPLLMRPMSM	83 - 103
		AKPVSQMRMATPLLMRP[1]	84 - 100
		AKPVSQMRMATPLLMRPM[1]	84 - 101

Peptides sequences from CD82 knockout spleen cells

SwissProt	Protein	Sequence	Epitope
		AKPVSQMRMATPLLMRPMS	84 - 102
		AKPVSQMRMATPLLMRPMSM	84 - 103
		KPVSQMRMATPLLMR	85 - 99
		KPVSQMRMATPLLMRP[1]	85 - 100
		KPVSQMRMATPLLMRPM[1]	85 - 101
		KPVSQMRMATPLLMRPMS	85 - 102
		KPVSQMRMATPLLMRPMSM	85 - 103
		VSQMRMATPLLMRP[1]	87 - 100
		VSQMRMATPLLMRPM[1]	87 - 101

Peptides sequences from CD82 wild type spleen cells

SwissProt	Protein	Sequence	Epitope
P01872	Ig μ-Kette C Region, sezernierte Form	NPNVNVFVPPRDGFSGPA NPNVNVFVPPRDGFSGPAP[1]	111 - 128 111 - 129
P07758	α-1-Antitrypsin	VHKAVLTIDETGTEAAA VHKAVLTIDETGTEAAAA	352 - 368 352 - 369
P13020	Gelsolin	SNDAFVLKTPSAAYLWVG NDAFVLKTPSAAYLWVG[1]	588 - 605 589 - 605
Q99PS6	Histidine-reiches Glycoprotein	NFPSFSLPNCNRSLQPE[1] FPSFSLPNCNRSLQPE	476 - 492 477 - 492
Q61318	Apolipoprotein B	NNYALFLSPRAQQASWQ[1] NYALFLSPRAQQASWQ	333 - 349 334 - 349
P48758	Carbonylreduktase [NADPH] 1	WVRTDMAGPKATKSPEE VRTDMAGPKATKSPEE	229 - 245 230 - 245
Q04519	Sphingomyelin Phosphodiesterase	ARETYGLPDAMPASWH[1] ARETYGLPDAMPASWHN	537 - 552 237 - 253
Q8K5E1	Gehirn cDNA, Klon MNCb-5546	FPPEFYEHAKALWEDE FPPEFYEHAKALWEDEG[1]	85 - 100 85 - 101
P17182	α-Enolase	KSFVQNYPVVSIEDPF[1] KSFVQNYPVVSIEDPFD[1]	280 - 295 280 - 296
Q925B1	*Lung seven transmembran receptor 2 [Gpr108]*	GEQKLFISPGLLPEAP[1] GEQKLFISPGLLPEAPT	134 - 149 134 - 150
P23953	Leber Carboxylesterase	GDIFFGIPAVLLSRS GDIFFGIPAVLLSRSL	409 - 423 409 - 424
P43432	Interleukin-12 β-Kette (IL-12B)	YPDSWSTPHSYFSLK YPDSWSTPHSYFSLKF	255 - 269 255 - 270
P09581	*Macrophage colony stimulating factor I receptor* (CSF-1-R)	VKDGYQMAQPVFAPK[1] KDGYQMAQPVFAPK	867 - 881 868 - 881

Peptides sequences from CD82 wild type spleen cells

SwissProt	Protein	Sequence	Epitope
Q61207	Sulfatiertes Glykoprotein 1 (Prosaposin)	QNMETAARCNAVDH[1]	537 - 550
		NMETAARCNAVDH	538 - 550
P09671	Superoxid Dismutase [Mn], mitochondrial	ATEEKYHEALAKGDVT	64 - 79
		EEKYHEALAKGDVT	66 - 79
P19226	60 kDa Hitzeschock Protein, mitochondrial (Hsp60)	IEQSWGSPKVTKDG	64 - 77
		IEQSWGSPKVTKDGVT	64 - 79
Q06770	Corticosteroid-bindendes Globulin (Transcortin)	FTLKYNRPFIFLAFDK	367 - 382
		FTLKYNRPFIFLAFDKY[1]	367 - 383
P35329	B-Zell Rezeptor CD22	AKLDVHYAPKAVTTVIQ	426 - 442
		LDVHYAPKAVTTVIQ	428 - 442
Q8BGB5	*Hypothetical LIM domain/ cytochrome c family heme-binding site containing protein*	WAHKEVDSGTK	116 - 126
		WAHKEVDSGTKTA	116 - 128
Q8BMK2	Ähnlich zu *FH1/FH2 domains-containing protein*	TRELWDSPEPASAPR	484 - 498
		TRELWDSPEPASAPRTPQ	484 - 501
Q80Y13	Malate Dehydrogenase, cytoplasmatisch	VPDDLLYSFPVVIKNK	280 - 295
		DLLYSFPVVIKNK	283 - 295
Q00623	Apolipoprotein A-I	DEPQSQWDKVKDF	25 - 37
		DEPQSQWDKVKDFANVY	25 - 41
P21981	Protein-Glutamin γ-Glutamyltransferase	DDREDITHTYKYPEGSPEE	434 - 452
		DITHTYKYPEGSPEER	438 - 453
P11835	Integrin β-2 (LFA-1 beta-subunit) (CD18)	RSNEFDYPSVGQLA[1]	296 - 309

Peptides sequences from CD82 wild type spleen cells

SwissProt	Protein	Sequence	Epitope
		RSNEFDYPSVGQLAH	296 - 310
Q8VCI0	RIKEN cDNA 1100001H23 Gen	SDPTGVHCATAYWSPESK[1]	36 - 53
		DPTGVHCATAYWSPESK	37 - 53
Q9CXK3	α-Herzactin	LKYPIEHGIITNWDDMEKIWHHTFYNE	69 - 95
		DDMEKIWHHTFYNEL	82 - 96
Q6PEM2	Pzp Protein	VDKDSFYCSPFTISGSPL	79 - 96
		VDKDSFYCSPFTISGSPLP[1]	79 - 97
		DKDSFYCSPFTISGSPLP	80 - 97
Q91W60	Inter-α-Trypsin Inhibitor	SLKYNFVTPLTHM*VVTKP	579 - 596
		SLKYNFVTPLTHM*VVTKPE	579 - 597
		LKYNFVTPLTHM*VVTKP	580 - 596
Q921A6	Ig leichte Kette, variable Region	GSGRDYSFSISNLEPED	199 - 215
		SGRDYSFSISNLEPE	200 - 214
		SGRDYSFSISNLEPED[1]	200 - 215
P29621	Serine Proteinase Inhibitor A3C	YKKLALKNPDTNIVFSPL	59 - 76
		KKLALKNPDTNIVFSPL	60 - 76
		KLALKNPDTNIVFSPL	61 - 76
P28665	Murinoglobulin 1	NKVDLRFSTSQSLPASQT	558 - 575
		KVDLRFSTSQSLPASQT	559 - 575
		VDLRFSTSQSLPASQT[1]	560 - 575
P06800	CD45	FSNFFSGPIVVHCSA[1]	689 - 703
		FSNFFSGPIVVHCSAG	689 - 704
		SNFFSGPIVVHCSA[1]	690 - 703
P51150	Rab-7	VLVFDVTAPNTFKTLD	85 - 100
		LVFDVTAPNTFKTLD	86 - 100
		LVFDVTAPNTFKTLDS	86 - 101
P29699	α-2-HS-Glykoprotein (Fetuin-A)	HDLRHAFSPVASVESA[1]	298 - 313
		HDLRHAFSPVASVESAS[1]	298 - 314
		DLRHAFSPVASVESA	299 - 313
Q02013	Aquaporin-CHIP	LAIDYTGCGINPAR	181 - 194
		LAIDYTGCGINPARS	181 - 195
		AIDYTGCGINPAR	182 - 194

Peptides sequences from CD82 wild type spleen cells

SwissProt	Protein	Sequence	Epitope
P61982	14-3-3 Protein γ(Protein Kinase C Inhibitor Protein-1) (KCIP-1)	KNVTELNEPLSNEER	27 - 41
		KNVTELNEPLSNEERN	27 - 42
		KNVTELNEPLSNEERNL[1]	27 - 43
Q9JLJ0	TBX1 Protein (LPS-induzierter TNF-α Faktor)	GPDGKGMNPPSYYTQPVPVPN	50 - 70
		GPDGKGMNPPSYYTQPVPVPNAN	50 - 72
		GPDGKGMNPPSYYTQPVPVPNANA[1]	50 - 73
Q80YR5	*Scaffold attachment factor B*	TSQELVRAPTAALSPEPQ	374 - 391
		SQELVRAPTAALSPEPQ[1]	375 - 391
		ELVRAPTAALSPEPQ	377 - 391
P05206	cAMP-abhängige Protein Kinase, katalytische β Untereinheit	GTPEYLAPEIILSKG[1]	200 - 214
		GTPEYLAPEIILSKGYN	200 - 216
		TPEYLAPEIILSKG	201 - 214
P55258	Rab-8A (Onkogen c-mel)	GIKFMETSAKANINVEN	144 - 160
		IKFMETSAKANINVE	145 - 159
		KFMETSAKANINVE	146 - 159
P11152	Lipoprotein Lipase	LDPAGPNFEYAEAPSRLSPDD	182 - 202
		LDPAGPNFEYAEAPSRLSPDDA	182 - 203
		AGPNFEYAEAPSRLSPDD	185 - 202
Q922L5	*Snd1-pending Protein*	IWRDYVPPTANLDQKD	300 - 315
		RDYVPPTANLDQK[1]	302 - 314
		RDYVPPTANLDQKD	302 - 315
O88342	*WD-repeat protein 1*	DEGKLLEAKGPVTDVAYSHDG	481 - 501
		GKLLEAKGPVTDVAYSHD	483 - 500
		KLLEAKGPVTDVAYSHD[1]	484 - 500
P14438	H-2 Klasse II Histokompatibilitätsantigen, A^b α-Kette	STPATNEAPQATVFPK	77 - 92
		TPATNEAPQATVFPK[1]	78 - 92
		TPATNEAPQATVFPKSPV	78 - 95
Q8BTS0	DEAD	IARQNFTEPTAIQAQG	109 - 124
		IARQNFTEPTAIQAQGWPV[1]	109 - 127
		RQNFTEPTAIQAQG	111 - 124
Q61592	Gas6	GSVHTYVGGLPEVSVIS	613 - 629

Peptides sequences from CD82 wild type spleen cells

SwissProt	Protein	Sequence	Epitope
		GSVHTYVGGLPEVSVISAPV[1]	613 - 632
		SVHTYVGGLPEVSVIS	614 - 629
Q62351	Transferrin Rezeptor 1 (TfR1)	VEYHFLSPYVSPR	684 - 696
		VEYHFLSPYVSPRESP	684 - 699
		VEYHFLSPYVSPRESPF	684 - 700
P01865 oder P01868	Ig γ2a oder γ1 Kette C Region	TWNSGSLSSGVHTF[1]	40 - 53
		TWNSGSLSSGVHTFPAVL	40 - 57
		TWNSGSLSSGVHTFPAVLQSDL	40 - 61
P29391	Ferritin leichte Kette 1 (Ferritin L Untereinheit 1)	APQGSLGEYLFERLTLKHD	164 - 182
		GSLGEYLFERLTLKHD	167 - 182
		FERLTLKHD[1]	174 - 182
Q9EPH1	Vermeintliches α-1B-Glykoprotein	LVREGFKTPFAVASTRS	452 - 468
		VREGFKTPFAVASTR	453 - 467
		VREGFKTPFAVASTRS	453 - 468
P97821	Dipeptidyl-Peptidase I (Cathepsin C / Cathepsin J)	DPFNPFELTNHAVL	394 - 407
		DPFNPFELTNHAVLL	394 - 408
		DPFNPFELTNHAVLLVGYGRD	394 - 414
		IAVAAIPIPKL	452 - 462
P16301	Phosphatidylcholin-Sterol Acyltransferase	YDHNFPYKDPVAALYEDG	351 - 368
		DHNFPYKDPVAALYED[1]	352 - 367
		DHNFPYKDPVAALYEDG[1]	352 - 368
		HNFPYKDPVAALYED	353 - 367
P08109	Hitzeschock Protein 71 kDa (Hsc70)	DAGTIAGLNVLRIINEPTAAAIAYG	160 - 184
		LNVLRIINEPTAAAIAYG[1]	167 - 184
		NVLRIINEPTAAAIAYG[1]	168 - 184
		VLRIINEPTAAAIAYG	169 - 184
Q9JME8	Hypothetisches Protein	ADKAAASGPASASAPA	14 - 29
		ADKADKAAASGPASASAPA[1]	14 - 32
		ADKADKAAASGPASASAPAA	14 - 33
		DKADKAAASGPASASAP	15 - 31
		DKADKAAASGPASASAPA	15 - 32
Q8CFG0	Extrazelluläre Sulfatase Sulf-2	GGLPDYSAPNPIKVTH	591 - 606
		GGLPDYSAPNPIKVTHR	591 - 607
		GLPDYSAPNPIK	592 - 603
		GLPDYSAPNPIKVT	592 - 605

Peptides sequences from CD82 wild type spleen cells

SwissProt	Protein	Sequence	Epitope
		GLPDYSAPNPIKVTH[1]	592 - 606
P01027	Komplement C3	SDKEGHKYVTVVANFGET	99 - 116
		DKEGHKYVTVVANFGET	100 - 116
		EGHKYVTVVANFGET[1]	102 - 116
		DAGLAFKTSQGLQTEQR	640 - 656
		AGLAFKTSQGLQTEQ	641 - 655
		AGLAFKTSQGLQTEQR	641 - 656
P06332	CD4 (T4 / Leu-3)	AEKDSFFQPWISF	244 - 256
		AEKDSFFQPWISFSIK[1]	244 - 259
		AEKDSFFQPWISFSIKN[1]	244 - 260
		AEKDSFFQPWISFSIKNK	244 - 261
		EKDSFFQPWISF	245 - 256
		EKDSFFQPWISFSIK[1]	245 - 259
P19973	Lymphocyten-spezifisches Protein 1 (LSP1)	KSSELDEDEGFGDW	76 - 89
		REPDPEDAVGGSGEA	141 - 155
		REPDPEDAVGGSGEAEE	141 - 157
		REPDPEDAVGGSGEAEEHL[1]	141 - 159
		REPDPEDAVGGSGEAEEHLIR	141 - 161
		AVGGSGEAEEHLIRH	148 - 162
		PSMAVASTKTL[1]	247 - 257
P01866	Ig γ2b Kette, C Region	WPSQTVTCSVAHPA	75 - 88
		WPSQTVTCSVAHPAS[1]	75 - 89
		WPSQTVTCSVAHPASS	75 - 90
		WPSQTVTCSVAHPASST[1]	75 - 91
		DDPDVQISWFVNNVEVHTAQTQ	158 - 179
		ISWFVNNVEVHTAQ	164 - 177
		ISWFVNNVEVHTAQT	164 - 178
		ISWFVNNVEVHTAQTQ[1]	164 - 179
		ISWFVNNVEVHTAQTQT	164 - 180
Q91V86	*11 days embryo cDNA, RIKEN full-length enriched library, clone*	VHLTDAEKAAVSGLW	1 - 15
		VHLTDAEKAAVSGLWGKVNADEVGGEALGRL[1]	1 - 31
		VHLTDAEKAAVSGLWGKVNADEVGGEALGRLL[1]	1 - 32
		LTDAEKAAVSGLWGKVNADEVGGEALGRL	3 - 31
		LTDAEKAAVSGLWGKVNADEVGGEALGRLL	3 - 32
		TDAEKAAVSGLWGKVNADEVGGEALGRL	4 - 31
		DAEKAAVSGLWGKVNADEVGGEALGRLL	5 - 32
		VSGLWGKVNADEVGGEALGRLL	11 - 32

Peptides sequences from CD82 wild type spleen cells

SwissProt	Protein	Sequence	Epitope
		WGKVNADEVGGEALGRL	15 - 31
		WGKVNADEVGGEALGRLL	15 - 32
		GKVNADEVGGEALGRLL	16 - 32
		KVNADEVGGEALGRL[1]	17 - 31
		KVNADEVGGEALGRLL	17 - 32
P60710	Actin, cytoplasmatisch (β-Actin)	LVVDNGSGMCKAGFAGDDAPR	8 - 28
		LVVDNGSGMCKAGFAGDDAPRAVF[1]	8 - 31
		VVDNGSGMCKAGFAGDDAPR[1]	9 - 28
		VVDNGSGMCKAGFAGDDAPRA	9 - 29
		VVDNGSGMCKAGFAGDDAPRAVF[1]	9 - 31
		VDNGSGMCKAGFAGDDAPRAVF	10 - 31
		CKAGFAGDDAPRAVF	17 - 31
		KAGFAGDDAPR[1]	18 - 28
		KAGFAGDDAPRA[1]	18 - 29
		KAGFAGDDAPRAV	18 - 30
		KAGFAGDDAPRAVFPS	18 - 33
		KAGFAGDDAPRAVFPSIVG	18 - 36
		KAGFAGDDAPRAVF[1]	18 - 31
		FAGDDAPRAVF[1]	21 - 31
		FAGDDAPRAVFPS[1]	21 - 33
		SGGTTMYPGIADRMQKE[1]	300 - 316
		SGGTTMYPGIADRMQKEIT	300 - 318
		YPGIADRMQKEIT	306 - 318

Abbreviations

Ab	antibody
Ig	immunoglobulin
Ag	antigen
APC	antigen presenting cell
BSA	bovine serum albumin
CD	cluster of differentiation
cRPMI-1640	complete RPMI-1640 medium
CTL	cytotoxic T-lymphocyte
DC	dendritic cell
BM	bone marrow
Thy	thymus
bmDC	bone marrow dendritic cell
HEL	hen egg lysozyme
OVA	ovalbumin
pep	peptide
spl	spleen
mDO	mouse DO (H2-O)
ELISA	enzyme linked immunosorbent assay
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FSC	forward scatter
IFN-γ	interferon gamma
IL	interleukin
ko	knockout
LFA-1	leukocyte function antigen-1
LEF-1	lymphocyte enhancer factor-1
MHC	major histocompatibility complex
NK cell	natural killer cell
o/n	over-night

PBS	phosphate buffered saline
PI	propidium iodide
RAG1	recombinase activating gene-1
RT	room temperature
TCR	T-cell receptor
Tg	transgenic
Th1	T helper type 1
Th2	T helper type 2
TNF-α	tumor necrosis factor α
WT	wild type
ZAP70	zeta associated protein 70