Recombinant antibody-based vaccines:

A study on the role of asparagine residues as a part of or flanking T cell epitopes

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ABBREVIATONS

Aa: amino acid
Ab: antibody
AEP: asparaginyl endopeptidase
Ag: antigen
APC: antigen presenting cell
BCR: B-cell receptor
C: constant
CDR: complementarity-determing regions
CLIP: class II associated Ii peptide
CME: clathrin-mediated endocytosis
DC: dendritic cell
EBV: Epstein-Barr virus
ELISA: enzyme-linked immunosorbent assay
ER: endoplasmatic reticulum
Fab: fragment antigen binding
Fc: fragment crystalline
FcR: Fc receptor
Cγ3: IgG3 constant heavy chain
GILT:gamma-interferon-inducible lysosomal thiol reductase
H: heavy
HA: hemagglutinin
HEL: hen egg lysozyme
IFN-γ: interferon gamma
Ig: immunoglobulin
Ii: invariant chain
IL: interleukin
L: light
LN: lymph node
LPS: lipopolysaccharide
MBP: myelin basic protein
MHC: major histocompatibility complex
MIIC: MHC class II-loading compartment
MΦ: macrophage
N: asparagine
NK: natural killer cells
NPP: p-nitrophenyl phosphate
Tc: cytotoxic T cell
Th: helper T cell
TCR: T cell receptor
TGN: trans-Golgi network

TTCF: tetanus toxin C fragment
ON: over night
OriP: origin of replication
OVA: ovalbumin
PAMP: pathogen associated molecular patterns
PBST: PBS with 0.05% Tween
PCR: polymerase chain reaction
Pm: plasma membrane
PRR: pattern recognition receptor
PVDF: polyvinylidine fluoride
RE: restriction enzyme
RISC: RNA-induced silencing complex
RNAi: RNA interference
RT: room temperature
SDS-PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis
SN: supernatant
Strep-ALP: streptavidine alkaline phosphatise
V: variable heavy
Wt: wildtype

*The same form is used for both singular and plural

ABSTRACT

There is a fine balance between Ag presentation and by the destruction of Ag peptides. In a "Troybody" vaccine design it is of great importance that the introduced epitopes are properly excised from the Ab molecule. Specific proteases can affect vaccine processing, and the contribution of AEP on processing of the recombinant Ab molecules remains to be elucidated. The aa 89-105 sequence of $\lambda 2^{315}$ has been introduced in all loops in all constant domains of a human IgG3. Secretion was observed for all mutants except one, loop 2 in CH1. All recombinant Ab with aa 89-105 introduced in CH2 and CH3 induced T cell activation. Only one mutant with aa 89-105 introduced in CH1 induced T cell activation. We could not find an obvious reason for the fact that the peptide is presented from all positions in CH2 and CH3, and not from loop 1, 3, and 5 in CH1. Prediction of AEP cleavage sites within every mutated hIgG3 H chain was performed with NetAEP (http://theory.bio.uu.nl/kesmir/AEP), provided by Can Kesmir at Utrecht University, The Netherlands. This program revealed more restriction sites in the CH2 and CH3 domains than in the CH1 domain. Earlier studies had also shown that the OVA peptide neither was able be presented from LOOP 1 in CH1. We therefore decided to focus on AEP in LOOP 1 in CH1. Both the OVA and the $\lambda 2^{315}$ peptides contain N in its aa sequence. The deficiency of presentation could be a result of the lack of AEP restriction sites, inaccessibility for AEP restriction sites, or the fact that the epitopes are destructed as shown for the Myelin basic protein (MBP) epitope. A known model epitope, HA, does not contain N. We therefore decided to introduce HA in loop 1 in CH1. Additionally, recognition site for AEP was introduced C-terminally for all three epitopes. Both $\lambda 2^{315}$ epitopes was included in the study. We found the HA epitope to be presented from loop 1 in CH1, but none of the other two, $\lambda 2^{315}$ or OVA, to be. Presentation of HA was not enhanced by the proximity of the introduced AEP recognition site. This may be explained by AEP cleavage to occur in another position in the CH1 domain, and the $\lambda 2^{315}$ and OVA epitopes to be unable of presentation because they are destructed. Alternatively, the mechanism of AEP does not affect the observed results. To prepare comparing studies in a negative cell line, the mouse fibroblast cell line CA.36.2.1 was studied for expression of AEP. We found this cell line to express AEP. An alternative cell must therefore be found to in order to perform this study.

1 Introduction

1.1 General introduction

The immune system is a complex network of organs, cells and molecules that interact to eliminate foreign invaders, pathogens and altered self, i.e. cancer. Central to the immune system is its biologic ability to distinguish self from non-self and altered-self. It is of critical importance that the destructive effector mechanisms of the immune system are not attacking the host's own cells, witch can result in allergy and autoimmunity. The immune system consists of several parts, and is traditionally divided into the innate and the adaptive immune system.

The innate immune system constituting intact skin, mucosal linings, cytokines and certain white blood cells, provides a first line of defense. The cells in this arm of the immune system are comprised mainly of different phagocytic cells, such as macrophages (MΦ), dendritic cells (DC) and neutrophils. These cells ingest and kill the invading pathogens after recognition by germ-line encoded receptors. The innate immune system also prepares the adaptive immune system to recognize the pathogen through cytokine and chemokine production (reviewed in [1]).

In contrast to the limited number of germ line encoded receptors recognized by the cells in the innate immune system, the adaptive system utilizes a large repertoire of rearranged receptors. The adaptive immune system is characterized by specificity and takes days to develop. After clonal selection, lymphocytes can specifically eliminate pathogens. The adaptive immune system can be divided into a humoral part involving production of antibodies (Ab) by B cells and plasma cells and a cell mediated part which involves T cells. An important aspect in adaptive immunity is that the immune system learns to respond more rapidly and effectively to previously encountered pathogens, a property referred to as immunological memory. The immunological memory is the end result of a productive immune response, and the final goal of vaccination.

1.2 Immunoglobulins

Ab are large glycoproteins produced by B cells. They have the ability to bind and neutralize antigens (Ag) in plasma, or tag them for uptake and destruction by phagocytes. The Y-shaped Ab molecule can be found as the membrane bound B cell receptor (BCR) or as secreted form in body fluids. An Ab molecule consists of four polypeptide chains, two heavy (H) and two light (L) chains that are connected by disulfide bonds.

The Ab are also known as immunoglobulins (Ig) and are built from several constant (C) and variable (V) Ig domains. The H chain consists of four or five Ig domains; VH, CH1, CH2 and CH3 (and CH4), and the L chain consist of two Ig domains; VL and CL. The Ig molecule consists of a Fab fragment and a Fc portion. The Fab-fragments are connected to the Fc fragment via the hinge region which contributes flexibility to the molecule. The Fab fragment includes the Ag binding part of the molecule, and the Fc region determines the isotype. In mammalian species, the different Ig classes are IgM, IgG, IgD, IgA and IgE, providing Ab with different effector functions. Figure 1 is a ribbon presentation of a complete Ab and the Fab fragments are shown in more detail in Figure 2.

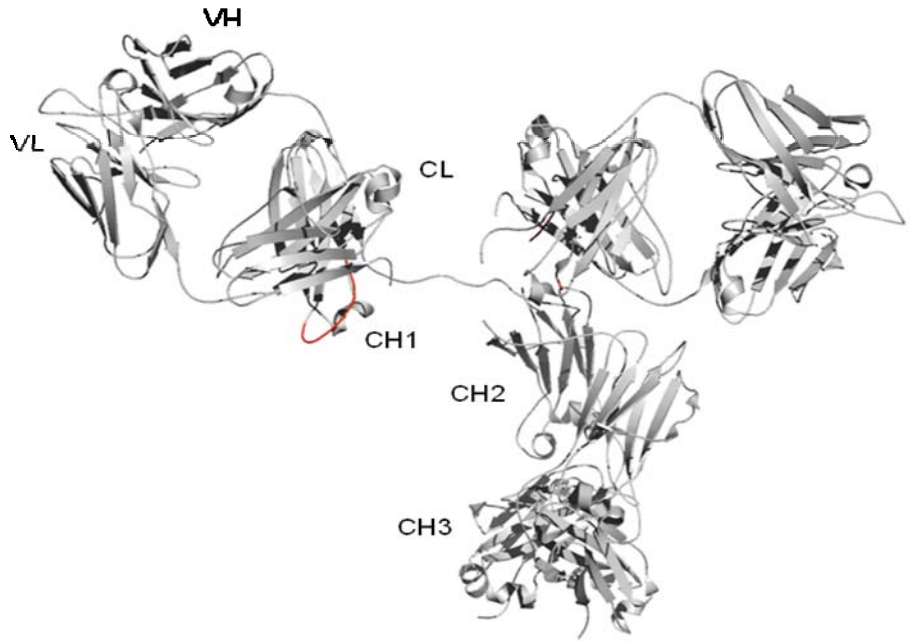


Figure 1: Ribbon presentation of a human IgG1 Ab crystal structure. *(PDB ID: 1HZH) [2]. The two Fab arms, each composed of VLCL/VHCH1, are connected to the Fc region (CH2CH3) via the hinge region. Loop1 in CH1 is indicated in red. The image was created by using PyMOL v0.98RC5 (DeLano Scientific LLC, CA, USA)*

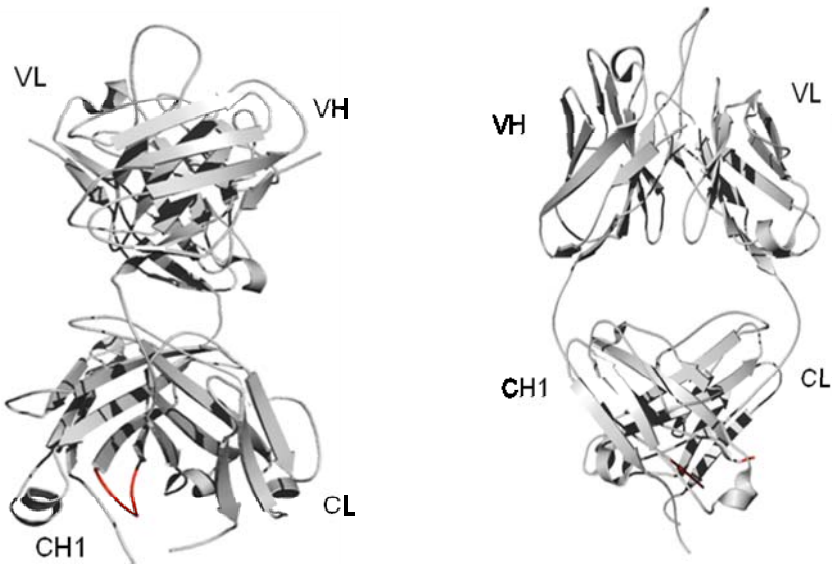
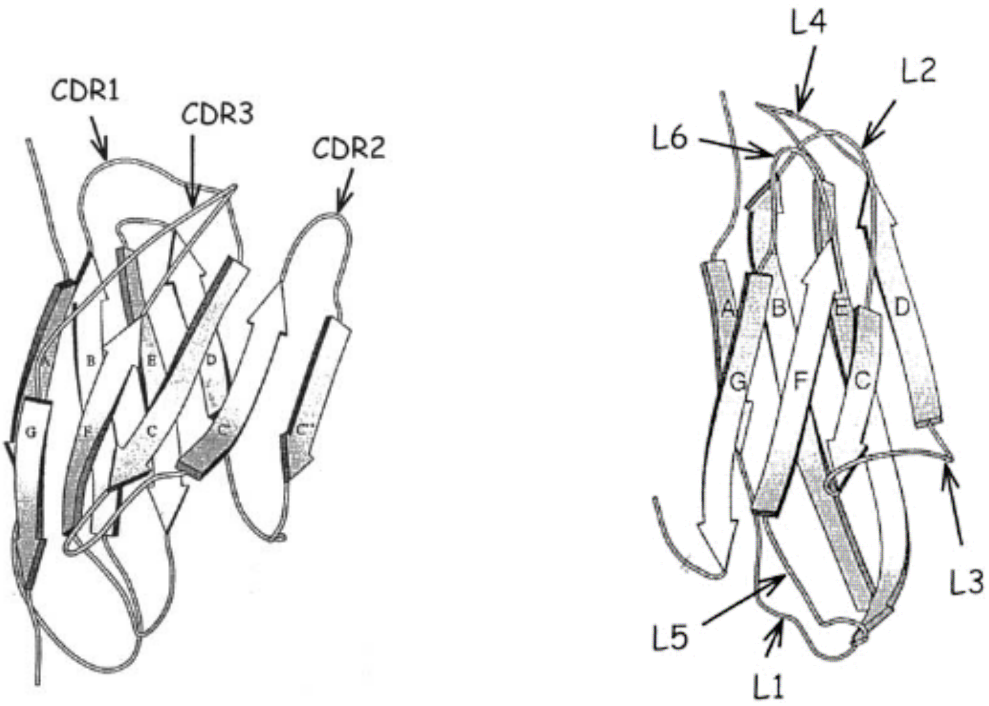


Figure 2: Ribbon presentation of the two Fab fragments, VLCL/VHCH1, from a human IgG1 Ab crystal structure. *(PDB ID: 1HZH) [2]. The images are rotated to show Loop1 in CH1which are indicated in red. The image was created by using PyMOL v0.98RC5 (DeLano Scintific LLC, CA, USA)*

1.2.1 The Immunoglobulin fold

Igs contain a characteristic structure called the Ig fold. The Ig fold consists of antiparallel β-strands that create a 2-layer sandwich of β-sheets linked together by a conserved disulfide bond. The Ig V and C domains are presented in Figure 3. The V domains are composed of nine β-strands and the C domain of seven β-strands. These β-strands are connected by loops, resulting in six loops in the C domain and eight in the V domain. The Ag specificity of the Ig-molecule is determined by the complementarity-determining region (CDR) composed of three N terminal loops in the V region, denoted CDR1, CDR2 and CDR3.



V domain C domain

Figure 3: The Ab V domain and C domain*. The framework regions form the β-sheets that provide the structural framework of the domain. CDRs in the V domain are indicated as CDR1, CDR2 and CDR3. Loops 1-6 in CH1 are indicated. Adapted from [3]*

1.3 Antigen presentation

1.3.1 The antigen presenting cell

APC are divided into the two categories, professional and non-professional. Whereas almost every cell has the ability to present Ag to $CD8^+$ T cells via major histocompatibility-molecules (MHC) class I molecules the term APC is considerably broad. Some more specialized cells express MHC class II as well as MHC class I molecules, and are often called professional APC. The professional APCs are further characterized as being efficient Ag internalizers, and able to give the co-stimulatory signal leading to T cell activation. DC and MΦ both differentiate from circulating bone-marrow precursors and complete their

differentiation upon leaving the blood stream and take residence in peripheral tissues. Both cell types take up Ag by phagocytosis, and upon maturation they up regulate the expression of MHC class II molecules and become efficient APC. MΦ utilizes innate immune receptors, pattern recognition receptors (PRR), to recognize conserved motifs on pathogens, collectively referred to as pathogen associated molecular patterns (PAMPs). Once activated, they express appropriate molecules and become efficient APC, reviewed in [4]. Activated DCs are especially potent $CD4^+$ T cell activators because they express co-stimulatory molecules and are able to stimulate naïve T cells. B cells are inefficient APC for most Ag, but can very efficiently present the Ag to which their Ab is directed. The BCR are able to recognize low-affinity and rare ligands, and activates signaling pathways to efficiently capture and process Ag. By crosslinking the BCR, internalization of Ag is rapidly accelerated [5]. B cells constitutively express high levels of MHC class II, and are for this reasons often referred to as a professional APC.

1.3.2 Major histocompatibility-molecules

MHC also known as human leukocyte-associated Ag are a cluster of cell surface glycoproteins. There are two classes of MHC molecules, MHC class I and II which display Ag to $CD8^+$ and $CD4^+$ T cells, respectively. MHC class I mainly present proteins that have been synthesized within the host cell while MHC class II presents exogenous proteins that have been digested and proteolytically processed. Class I MHC molecules are expressed on virtually all nucleated cells, whereas class II molecules, are normally confined to professional APC and thymic epithelial cells. MHC class I molecules are membrane bound heterodimers composed of an α-chain (~44 kD) and a β2- microglobulin (12 kD). The α-chain gene encodes three extracellular domains (α_1, α_2 and α_3), a trans-membrane domain and an intracellular domain that anchors the molecule to the membrane surface. α_1 and α_2 combine to form a groove, in which Ag peptides can bind. MHC class II molecules, like class I molecules, are composed of two polypeptide chains. Both chains are transmembrane and are designated α (~35 kD) and β (~27 kD). The α-chain has two extracellular domains (α_1 α_2) and so does the β-chain (β_1 β_2). The α_1- and β_1-chains combine to form a groove similar to the one found in MHC class I. This peptide binding groove is open ended and able to bind peptides larger than the length of the groove, typically 12-25 aa residues reviewed in [6]. In the following Ag processing and peptide loading on MHC class II molecules will mainly be described.

1.3.3 MHC class II pathway

In the MHC class II pathway endogenous Ag can be presented to $CD4^+$ T cells after endocytosis and processing by the APC. Invariant chain (Ii) is a non polymorphic type II transmembrane protein that binds MHC class II in ER, making MHC class II-Ii complexes. The MHC class II-Ii complexes are sorted to the endocytic pathway. Degradation of Ii and Ag begins in early endosome, and the Ii are exchanged with Ag peptides in the recycling compartments. The events are described in more detail in the following sections.

1.3.3.1 Endocytosis

Cells take up particles and solutes from the extracellular matrix in a process referred to as endocytosis. The endocytic pathway comprises the early endosomes, late endosomes and lysosomes. Internalized molecules travel to early endosomes, where they are sorted further. The internalized cargo can be recycled back to the plasma membrane (pm) or be sent to lysosomes for degradation. The membrane transport in this pathway has long been a subject of debate. The transport may be considered to take one of two forms which can be described in the maturation model or in the vesicular model. In the maturation model, the composition of early endosomes is believed to change until they become late endosomes and then lysosomes. Here, the lysosomally targeted ligands are delivered to early sorting endosomes which themselves mature into late endosomes. In the vesicular model, the early and late endosomes are stable subcellular compartments, connected by vesicular transport. The lysosomally targeted ligands pass through preexisting endosomes and are then selectively transported to long lived late endosomes in carrier vesicles [7]. The emerging pattern of endocytosis is increasingly complex, with individual routes relying on different components of the endocytic machinery. The current knowledge on the classical and new endocytic routes is reviewed in [8]

APC capture extracellular Ag via endocytosis. This occurs by multiple mechanisms that fall into two categories; phagocytosis or pinocytosis [9]. Phagosytosis in mammals are performed by professional phagocytes like MΦ, DC and granulocytes. Phagocytic receptors provide cells to recognize infectious Ag or apoptotic cells. When a small particle or pathogen

is coated with Ab, the Fc region of the Ab may bind Fc receptors (FcR) in APC pm and initiate phagocytosis [10]. Among the other receptors involved in phagocytosis are the scavenger receptors [11], complement receptors [12] and Toll like receptors [13].

Pinocytosis occurs by at least four different mechanisms: macropinocytosis, caveolin-mediated endocytosis, clathrin-and caveolin-independent endocytosis and clathrin-mediated endocytosis (CME) [9]. CME was previously referred to as "receptor-mediated" endocytosis, but it is now clear that most pinocytotic pathways actually involve specific receptor-ligand interactions. CME is constitutive in all mammalian cells and occurs at specialized sites where surface proteins concentrate into "coated pits" for internalization. The coated pits invaginate and pinch off to form clathrin coated vesicles. Ag captured by specific receptors provides an efficient uptake and B cells internalize Ag by such receptor mediated endocytosis. The BCR is able to recognize low-affinity and rare ligands, and activates signaling pathways to efficiently capture and process Ag. By crosslinking the BCR, internalization of Ag is rapidly accelerated [5].

1.3.3.2 Antigen degradation

Lysosomal degradation contributes to maintain intracellular homeostasis. Obstruction of the degradation process in human leads to severe diseases, generally called lysosomal storage diseases [14]. Furthermore, protein processing is a key feature in Ag presentation. After internalization into the acidic environment in endosomes and lysosomes, the proteins are processed to yield peptide substrates that can be loaded in the groove of MHC class II. Proteases are involved both in the processing of Ii and in the fragmentation of protein Ag.

Endocytosed Ag encounters a variety of proteolytic enzymes in the endosomal and lysosomal compartments. Endopeptidases, exopeptidases and γ-interferon induced lysosomal thiol reductase (GILT) [15], are among the enzymes involved in the processing pathway. The proteolytic enzyme GILT reduces the protein disulphide bonds optimally at acidic pH. Cathepsin S, L and B are the principal papain-like cystein proteases that have been described.

Endopeptidases are characterized by introducing a small number of nics that "unlock" the folded protein substrate. A cystein endopeptidase that has been described is the Asparaginyl Endopeptidase (AEP) [16]. AEP deficient mice were generated to study the physiological role of AEP in mammals *in vivo* [17]. The knockout mice showed enlarged lysosomes in an age-dependent manner, suggesting that matherials to be degraded are

accumulated within the lysosomal compartments. Normally, AEP is abundantly expressed in proximal tubule cells in the kidney cortex, and co localizes with the marker "lysosome associated membrane protein", LAMP- 2, on the apical side of the cells.

Activation of AEP is triggered by acidic pH and appears to be autocatalytic [18]. AEP is produced as an inactive zymogen that requires proteolytic cleavage to gain activity. The proteolytic cleavages are first a C terminal 110-residue propeptide and then an 8 residue propeptide. These cleavages occur at asparagine (N) 323 and aspartic acid 25 in the 433 aa sequence of AEP. The mature, active enzyme was produced following lipopolysaccharide (LPS) induced maturation of DC. The precursor and the mature forms of the enzyme were found at distinct locations along the endocytic pathway. Some compartments contain significant levels of the precursor form, whereas other, LAMP-1 positive compartments appear to contain the mature form.

In the processing of Ii, Cathepsin S has been shown to be crucial [19]. It has also been suggested that AEP plays an important role in initiating Ii processing [20]. A short peptide of about 3kDa is the final fragment of Ii that is present in the groove of MHC class II. This fragment of Ii, called class II associated Ii peptide (CLIP) and must be degraded to allow interaction of the MHC molecule with other peptides. Degradation of CLIP is mediated by the accessory protein HLA-DM, associated with HLA-DO in B-cells [21].

The reducing environment in endosomal compartments is contributed by the acidic milieu. The endosomal pH decreases along the endosomal pathway from early (pH 6,5-6,0) to late endosomes/lysosomes (pH 4,5-4,0). The acidic pH leads to degradation of proteins, and to the activation of most proteases. Regulation of lysosomal pH has been described as a target for pathogens. An example is *Helicobacter*, which by pH regulation hinders the presentation on MHC CLASS II [22]. Another key feature in protein degradation is the accessibility for the proteases in the protein. Peptides that are exposed are more susceptible to the processing enzymes than the ones situated inside the Ag core. Consequently, the tertiary protein structure greatly affects how the Ag are being processed.

It is commonly assumed that proteins are processed to relatively short fragments before they are captured by MHC II, in a "trim first, bind later" model. An alternative model, the "bind first, trim later" model, proposes a view were unfolded proteins or large Ag fragments are trimmed to final peptides after MHC class II binding (reviewed in [23]). Because partially processed Ag are likely to be more sensitive to destructive processing, rapid

engagement of MHC class II molecules would seem desirable. The open ended binding groove of MHC class II is ideally suited and perhaps designed for this process.

1.3.3.3 Loading on MHC class II and the role of Ii

Loading of MHC class II takes place in the late endosomal structures referred to as MHC class II containing compartments (MIIC) [24]. Ii binds MHC class II in ER, making MHC CLASS II-Ii complexes as described earlier. In the case of MHC class I, the initial events of Ag processing are segregated from the events of MHC peptide assembly in the ER membrane. In contrast, a single compartmental system hosts exogenous Ag processing and the loading of peptides onto MHC class II molecules. The association between MHC class II and Ii prevents peptide loading in the ER, and support the ER exit and correct sorting of MHC class II molecules to MIIC [25].

The correct travelling of MHC class II from the trans-Golgi network (TGN) to MIIC is mediated by dileucine-based motifs in the cytoplasmic tail of Ii [26]. The transport of MHC II –Ii complexes can occur from TGN directly to lysosomes, via the cell surface and endosomes, or via the early endosomes as described in Figure 4.

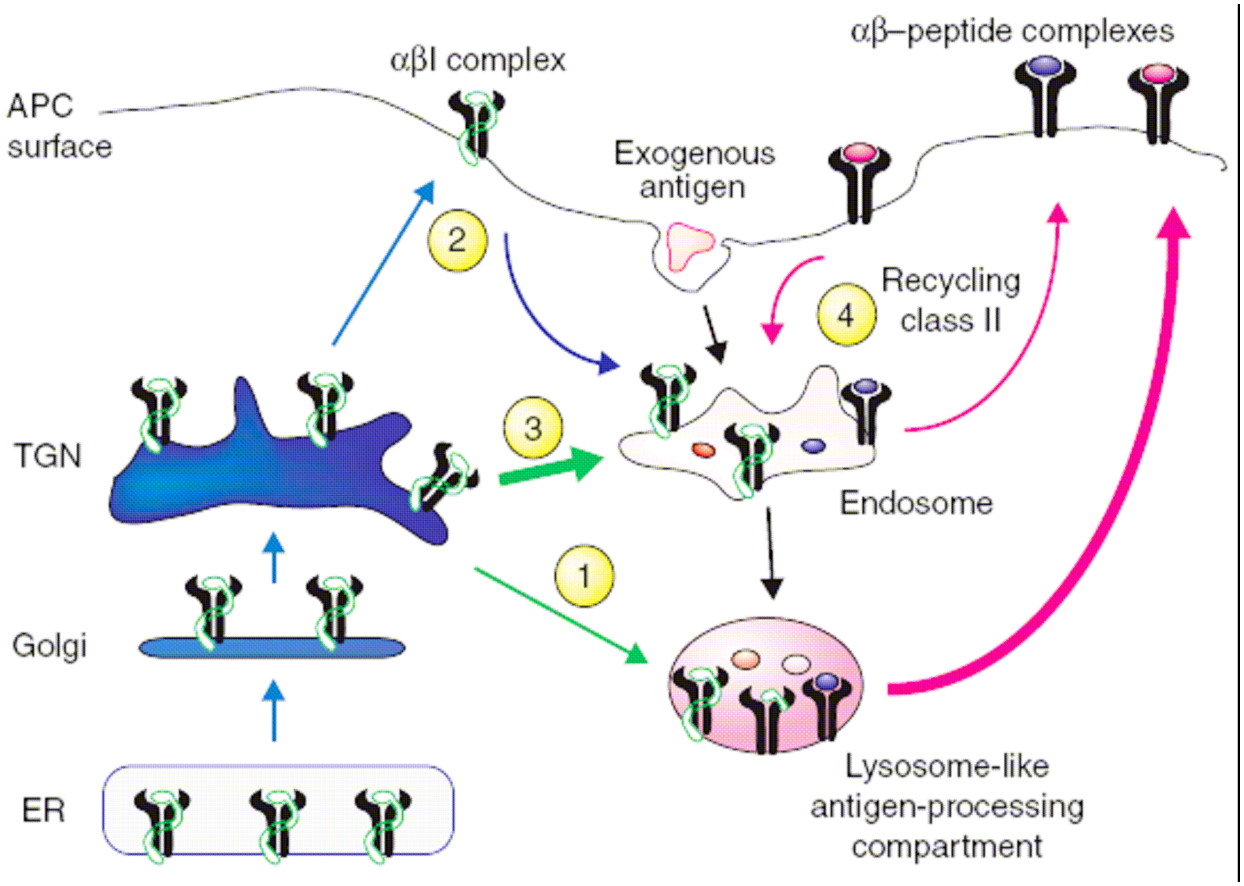


Figure 4: Transport of MHC class II molecules within APC*. MHC class II dimers (black pincers) associate with Ii (white snakes) in the ER. The MHC CLASS II-Ii complex are sorted to the endocytic pathway either directly from TGN to lysosomes (1), via the cell surface and endosomes (2) or via the early endosomes (3). Degradation of Ii and Ags begins in early endosomes. In the recycling compartments, peptides are exchanged with Ag peptides (4). Ag peptides and αβ–peptide complexes are subsequently transported to the cell surface (thick pink arrow). Pre-existing surface αβ–peptide complexes can also internalize and recycle through endosomes, where peptides can be exchanged (4). At the plasma membrane, the peptide and MHC-II complexes are presented for CD4+ T cells with complementary TCRs. The figure is adopted from [25]*

1.3.3.4 Cell surface expression of MHC class II-peptide complexes

When the groove of MHC class II has been loaded with peptide, the MHC class II-peptide complex is competent for transport to the pm, and presentation to T cells. Several mechanisms have been proposed for this transport. MHC class II^+ vesicles have been shown to directly migrate to and fuse with the pm [27], and B cells have been shown to transport and secrete the MHC class II-peptide complexes in the form of exosomes [28]. This pathway is still poorly understood.

1.3.3.5 TCR recognition

T cells are defined by expression of the heterodimeric αβ T cell surface receptor and specific co-receptor expression. The α- and β-chains are both transmembrane polypeptides with extracellular, Ig like, V domains (VαVβ) and C domains (CαCβ). The V domains of the TCRs include CDRs 1, 2, and 3. Each T cell bears TCRs with a single specificity. The huge receptor repertoire is enabled by the variation found within the CDRs, but opposed to Ab V domains, there is no affinity variation.

During maturation in thymus, T cells differentiate into discrete subpopulations with defined effector functions. Two major categories are defined by selective expression of the co-receptors CD4 or CD8. $CD4^+$ T lymphocytes are generally known as T helper cells (Th) and $CD8^+$ T cells are known as cytotoxic T cells (Tc). The TCRs recognize Ag in the context of self-MHC molecules with Ag peptide displayed in its groove. $CD4^+$ T cells recognize Ag presented on MHC class II molecules, whereas $CD8^+$ T cells recognize Ag in a MHC-class I context. CD8 and CD4 serve as co-receptors for MHC class I-peptide and MHC class II-peptide recognition, respectively [29]. The TCR interacts both with the presented peptide and with the flanking α-helixes of the MHC groove, a concept known as MHC restriction. The contact area between a T cell and an APC is a dynamic structure enriched in receptors and is called the immunological synapse (reviewed in [30]).

1.3.3.6 Post translational modification of antigenic peptides

Post translational modification affects Ag processing and T cell recognition. Among the posttranslational modifications are glyosylation, isoaspartylation, citrunillation, deamidation and phosphorylation (reviewed in [31]). These modifications provide heterogenicity to the protein Ag. T cell responses may be specific for peptides that have been modified, the modification may disturb the T cell recognition or it may perturb the processing events. Spontanous deamidation of N residues have been shown to diminish Ag presentation, by removing recognition sites for AEP [32]. In addition, downregulation of GILT in myeloma cells alters the presentation of disulfide containing peptides on MHC class II [33].

1.3.4 Peptide vaccination

Various vaccine strategies are used in the treatment of infection and cancer. The defined Ag can be delivered as gene-based vaccines, proteins or as peptides. Synthetic peptides have been an attractive approach to therapeutic vaccination, since the preparation of peptides is easy and cost-affordable, the autoimmune potential is minimal, and the peptides may be modified to increase their immunogenicity. By targeting T cells using vaccines consisting of synthetic peptides, the peptides can be directly loaded on MHC class II molecules *in vivo* if the required peptide is available at sufficient concentrations in the extracellular space, or the peptides can enter the cells either by pinocytosis or via the endocytic pathway [34, 35]. Peptides may also be cross presented on MHC class I molecules, reviewed in [36].

Challenges in the development of therapeutic peptide vaccines include lack of immunogenicity. Protein and peptide based vaccines are therefore usually given in combination with adjuvant to provide stimulation of APC. Further challenges are the selection of appropriate Ag and peptides, and the biodegradability of peptides.

1.3.5 Targeting of antigens to APC

The goal of preventive vaccination is to induce an Ab response capable of removing invading organisms before they have a chance to establish themselves. Thus, critical in vaccine development is the design of immunogens that give a strong and specific T lymphocyte response able to induce immunological memory. $CD4^+$ T helper cells play a pivotal role in orchestrating nearly all Ag-specific immune responses, as they secrete cytokines and give help to B cells and $CD8^+$ T cells. Consequently, strategies that modulate $CD4^+$ T cells are of great interest. APC have the ability to present Ag to $CD4^+$ T cells and several strategies have been used to target Ag to APC. A fusion protein with Ag peptide genetically coupled to the C-terminus of an Ab molecule targeted to DEC-205 on DC has been described [37]. In this Ag delivery system, the DCs were loaded with Ag in steady state. The Ag induced a T cell response, but the response was not sustained, and the T cells became unresponsive to systemic challenge with the Ag. Additional stimuli, such as coinjection with an anti-CD40 agonist Ab was required to induce T cell activation and immunity.

The focus of this thesis is the introduction of Ag peptides into Ab molecules that have specificity for surface molecules on APC. The term antigenized Ab was first used by Zanetti

et al in 1992 [38]. The use of recombinant Ab as vectors for delivery of Ag to APC has several advantages. The Ag are carried in stabile Ab molecules protected from degradation in serum. Intact Ab are divalent molecules possessing two bindings sites for the target of interest giving the Ab high functional affinity (avidity) for its target.

Genetic engineering made it possible to introduce T cell epitopes into Ab. Zanetti et al expressed the Ag peptides in the CDR regions of the Ab. One disadvantage of introducing foreign epitopes into the V region of an Ab is that the Ab loses its specificity and is dependent on APC entrance via the FcR or fluid phase endocytosis. By introducing Ag into the C domains of the Ab molecule, this problem can be circumvented. Ab molecules carrying Ag in the C domains have been constructed, and have been given the name Troybodies

1.3.6 Troyan Horse vaccine strategy

Like the Troyan horse carried soldiers into the city of Troy, Ab molecules are able to carry T cell epitopes into APC. By genetically engineering Ag peptides into Ab molecules, recombinant Ab carrying T cell epitopes can be constructed. The recombinant Ab are targeted to APC, by use of the specific V regions on the Ab molecule. After endocytosis and processing, the recombinant Ab can activate epitope specific $CD4^+$ T cells. This subset of T lymphocytes is involved in establishing an inflammatory environment and also serves as a source of help for B cells [39]. The Troybodies described in this thesis, contains T cell epitopes that replace the first loop (L1) connecting AB β-strands in the CH1 domain. This loop is indicated as red in Figure 2 and 3.

Initially, loops in the CH1 domain that corresponded to the CDR loops in the V-domain were exchanged with an epitope from the light chain of the M315 myeloma protein $\lambda 2^{315}$, aa 91-101. The three loops BC (L2), DE (L4) and FG (L6) in both human IgG3 [40] and mouse IgG2b [41] were exchanged with this epitope. Except from L2 in hIgG3, all Ab mutants were secreted, and activated epitope specific T cells. Secretion is an indication of proper folding. To investigate effect of targeting, the V-regions were replaced by V regions with IgD specificity. This had an *in vitro* effect up to 1000-fold compared to the NIP specific control Ab [42]. The *in vivo* targeting effect was further studied. Three commonly used model epitopes aa 110-120 of hemagglutinin, aa 323-330 of ovalbumin and aa 46-61 of hen egg lysosome were exchanged with loop 6 in CH1. After *in vivo* injection, the epitopes targeted to IgD on B cells was shown to activate specific T cells. Little or no activation could be detected

without targeting, even after the amount of Ag injected was increased 100-fold or more [43]. Corresponding results are also observed when epitopes are introduced in loops of CH2 and CH3 ("Loops in all three constant domains of an Ig heavy chain exchanged with a T cell epitope", Flobakk M, Rasmussen I B, Lunde E, Berntzen G, Michaelsen T E, Bogen B and Sandlie I, manuscript in preparation).

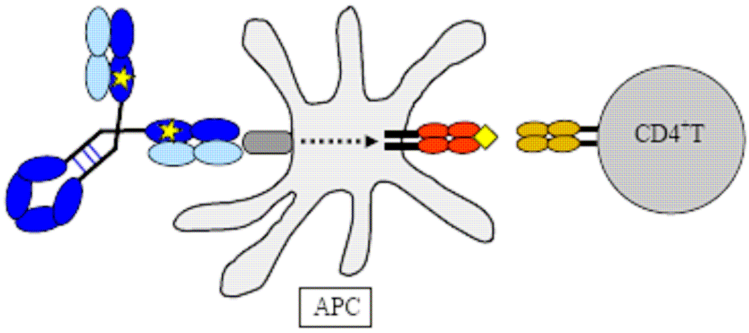


Figure 3: The Troybody strategy *A Troybody with T cell epitopes (stars) is internalized and processed by the APC, and peptides are presented on MHC CLASS II molecules to CD4+ T cells*

1.4 The role of AEP in antigen processing and presentation

AEP is also referred to as mammalian legumain, and is a processing enzyme with strict specificity for the carbonyl side of exposed N residues [16, 44]. The specificity observed for AEP is unusual among lysosomal enzymes, which are a group of enzymes often referred to as redundant and with broad substrate specificity. AEP belongs to the cystein peptidase family C13, and murine legumain shares 83% homology with the human protein [44]. Several alternatively spliced transcript variants have been described, but the biological validity of only two has been determined. These two encode the same isoform.

Watts et al studied the tetanus toxin Ag to analyze Ag processing in the class II MHC pathway. By *in vitro* lysosomal degradation of the 47kDa C-terminal domain of the tetanus toxin Ag (TTCF) in B cells, they found one enzyme to dominate the digestion pattern. The commonly used lysosomal protease inhibitors could not inhibit this enzyme. The processing products showed that in each case the cleavage had occurred after an N residue. This processing activity of TTCF was showed to be from the human form of legumain that was earlier described in plants [16]. Because the term legumain refers to plant entity, the

mammalian enzyme is referred to as "asparaginyl endopeptidase". TTCF contains 47 N residues, but cleavage was observed at only 3 of them. Obviously, N context and local secondary structure affects whether cleavage will actually occur. Further, in TTCF AEP cuts at two sites were pairs of N residues lies. Mutagenesis of the individual N target residues confirmed that optimal presentation of this Ag required their presence [45]. N-glycosylation of N was also shown to block AEP action *in vitro*. This finding suggests that N-glycosylation could eliminate sites of processing in mammalian proteins, allowing preferential processing of microbial Ag [16]. Further, by using TTCF protein as a test case, spontaneous N deamidation also inhibited AEP action [32].

T cell epitopes can be destroyed and thereby prevented from presentation on MHC class II. This process is referred to as negative processing. MBP is believed to be a major autoantigen in the pathogenesis of multiple sclerosis. The MBP (85-99) epitope contains a processing site for AEP in the peptide core. In a study with MBP in an Epstein-Barr virus (EBV) B-cell line, AEP was shown to destroy the MBP (85-99) epitope by cleaving at the N residue situated in position 94 [46]. This study showed an inverse relationship between the AEP activity in human APC and presentation of the MBP (85-99) epitope. Cell types that are likely to mediate negative selection were shown to express an active form of the AEP protease. This may lead to destruction of the MBP (85-99) epitope, and possibly eliminate autorective T cells in thymic selection [46].

There is a fine balance between Ag presentation and destruction of Ag peptides. In the case for TTCF processing, AEP is required for the generation of the immunodominant epitope. In contrast, AEP acts as a destructive protease on the MBP (85-99) epitope. In a "Troybody" vaccine it is of great importance that the introduced epitopes are properly excised from the Ab molecule. Specific proteases can affect vaccine processing, and the contribution of AEP on processing of the recombinant Ab molecules remains to be elucidated.

Amino acid 89-105 of $\lambda 2^{315}$ has been introduced in all loops in all constant domains of a human IgG3. Secretion was observed for all mutants except one, the loop 2 CH1 mutant. The recombinant Ab was mixed with APC and T cells with specificity for I-E^d and peptide (aa 91-101), and it was found that all recombinant Ab with aa 89-105 introduced in CH2 and CH3 induced T cell activation. Only one of the mutants with aa 89-105 introduced in CH1 induced T cell activation. This was surprising, because we had in earlier studies found that recombinant Ab with aa 91-101 introduced in loop 4 in CH1 induced T cell activation. We focused on loop 4 recombinant Ab, and compared two, one carrying aa 89-105 and one

carrying aa 91-101 in loop 4. It appeared that recombinant Ab carrying aa 91-101 induced T cell activation more effectively than a recombinant Ab with the aa 89-105 substitution. Studies on binding specificity to I-E^d showed that aa 89-105 contain two epitopes. The alternative loading frame is predicted to be more advantageous than aa 91-101. This loading frame is not detected in the activation studies because the T cell is specific for aa 91-101. It is reasonable however, to predict that the alternative loading reduce the loading of aa 91-101. Whether the presence of an "alternative loading frame" affects presentation differently in different domains is not known.

To further study why the peptide is efficiently presented from all positions in CH2 and CH3, and not from loop 1, 3, and 5 in CH1, prediction of AEP cleavage sites within every mutated hIgG3 H chain was performed with NetAEP (http://theory.bio.uu.nl/kesmir/AEP), provided by Can Kesmir at Utrecht University, The Netherlands. This program revealed more recognition sites in the CH2 and CH3 domain than in the CH1 domain. We therefore decided to focus on AEP. This is consistent with the "bind first, trim later" model, in which cutting by AEP is an early event in Ag processing.

The OVA peptide, aa 323-339 of ovalbumin, is a commonly used model epitope. Earlier studies had also shown that neither the OVA peptide were presented from loop 1 (L1) in CH1. Both the OVA and the $\lambda 2^{315}$ peptides contain N in their aa sequence. The lack of presentation could be a result of the lack of AEP recognition sites, inaccessibility for AEP recognition sites, or the fact that the epitopes are destructed as shown for the MBP (85-99) epitope. A known model epitope HA, which are aa 110-120 of hemagglutinin, does not contain N. We therefore decided to introduce HA in L1 in CH1. Additionally, recognition sites for AEP were introduced C-terminally of all three epitopes. Both $\lambda 2^{315}$ epitopes (91-101 as well as 89-105) were included in the study.

We found the HA epitope only to be presented from L1 in CH1. Presentation of HA was not enhanced by the proximity of the introduced AEP recognition site.

2. Aim of the project

This study was set up to study the effect of N residues on Ag presentation from CH1. The lack of presentation that was seen from CH1 might have several explanations. Among them are negative processing imposed by AEP on the epitopes, and the lack of recognition sites in the CH1 sequence. We wanted to investigate both. The two epitopes containing N in their aa sequence, $\lambda 2^{315}$ and OVA (323-339), were introduced, as well as one epitope, HA (aa 110-120 of hemagglutinin), that did not. Furthermore, recognition sites for AEP were introduced C-terminally of all. Two different sequences from $\lambda 2^{315}$ epitopes were included in the study (91-101 as well as 89-105). The effect of N residues in the sequence was investigated in presentation studies with the resulting 8 different recombinant Ab.

3. Materials and methods

3.1 Molecular cloning

3.1.1 Vectors

The pUC19γ3 vector contains a 2,8 kb fragment encoding human IgG3 (hIgG3) constant heavy chain (Cγ3) between the restriction sites for HindIII and BamHI. The expression vectors pLNOH2γ3 and pLNOκ [47] contain the gene fragments that encode Cγ3 and C kappa light (L) chain, respectively. Both C sequences are located downstream of a murine variable (V) region gene, and both vectors are designed to facilitate exchange of V and C region genes as cassettes [47]. V light (VL) and V heavy (VH) region genes were cloned from the Ig(5a)7.2 hybridoma that produces monoclonal Ab with specificity for the murine IgD[a] allotype. These V genes were subcloned into pLNOκ and pLNOH2γ3, respectively, to make the constructs pLNOκ$_{IgD}$ and pLNOH2$_{IgD}$ as previously described [42]. The expression vectors carry genes for the Epstein-Barr virus origin of replication (oriP) [48]. The vectors are further described in Figure 1.

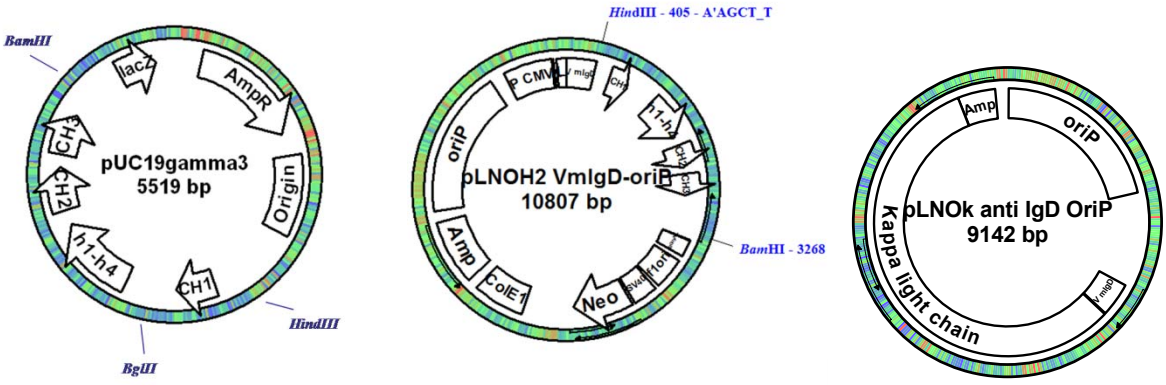


Figure 1: pUC19γ3 cloning vector, pLNOH2 and pLNOκ expression vectors *pUC19γ3 contains the 2,8 kb fragment encoding the Cγ3 of hIgG3 between HindIII-BamHI restriction sites. The expression vectors pLNOH2γ3 and pLNOκ contain the gene fragments that encode Cγ3 and C kappa L chain, respectively.*

3.1.2 T cell epitopes

Amino acid (aa) 110-120 of hemagglutinin (HA) from influenza PR8 virus [49], aa 323-339 of ovalbumin (OVA) [50, 51], and two peptides from MOPC315 plasmacytoma λ2 light (L) chain ($\lambda2^{315}$) aa 91-101 [52] and aa 89-105. The nucleotide and aa sequence of the T cell epitopes are described in Table 1.

Table 1 Nucleotide and aa sequence of T cell epitopes

T cell epitope	Nucleotide sequence	aa
HA	5'- tca ttc gaa ag ttc gaa ata ttc cca aag gaa -3'	5'- SFERFEIFPK -3'
OVA	5'- atc tct cag gct gtc cat gca gca cat gca gaa atc aat gaa gca ggc agg -3'	5'- ISQAVHAAHAEINEAGR -3'
$\lambda2^{315}$(91-101)	5'- gct cta tgg ttc aga aac cac ttt gtg ttc ggt -3'	5'- ALWFRNHFVFG -3'
$\lambda2^{315}$(89-105)	5'- ttc tgt gct cta tgg ttc aga aac cac ttt gtg ttc ggt gga ggt acc aaa -3'	5'- FAALWFRNHFVFGGGTK -3'

3.1.3 Mice and cell lines

293E is a genetic variant of the human embryonic cell line 293 [53]. 293E cells are obtained from ADCC. The 293E cell line expresses the EBV nuclear Ag 1 (EBNA1). Plasmids that contain OriP support increased protein expression in cells expressing EBNA1. CA.36.2.1 is an L cell line stably transfected with the genes encoding the I-E^d MHC II molecule [54]. The T cell hybridoma specific for the S1 determinant of HA PR8 virus [55] was a gift from Walter Gerhard (The Wistar Institute, Philadelphia, US). The HA epitope is recognized in context with I-E^d MHC CLASS II molecules [56]. The IL-2 dependent CTLL-2 cell line was purchased from ADCC. Spleen cells from Balb/c mice were used as APC. The $\lambda2^{315}$- specific TCR-transgenic mice on Balb/c background have been described [57]. The DO.11.10 TcR-transgenic mice with specificity for aa 323-339 of OVA were originally produced by Murphy

et al [58]. The TCRs recognize aa 91-101 and aa 89-105 $\lambda 2^{315}$ in complex with the MHC class II molecule I-E^d, and aa 323-339 OVA in complex with the MHC CLASS II molecule I-A^d.

All cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Bio-Whittaker) or RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (PAA), 2 mM L-glutamine (DMEM only), 25μg/ml streptomycin, and 25 U/ml penicillin (both from Bio-Whittaker) at 37°C in 5% CO_2. CTLL-2 cells were maintained in media that additionally included IL-2 (20U/ml).

3.1.4 Construction of "Troybodies"

HA (110-120), OVA (323-339), $\lambda 2^{315}$(91-101) and $\lambda 2^{315}$(89-105) peptides were expressed on Cγ3 by substituting the AB loop (L1) in the first constant domain (CH1). The loops in the domain were numbered consecutively following the linear aa sequence. Each C domain harbors six loops, and L1 is the first loop and pointing towards the C-terminal end of the molecule. The resulting recombinant Ab are denoted HA, OVA, L(91-101) and L(89-105), respectively. OVA (323-339) in L1CHI and $\lambda 2^{315}$(89-105) in L1CH1 were constructed previously in pUC19γ3 vector ("Loops in all three constant domains of an Ig heavy chain exchanged with a T-cell epitope", Flobakk M, Rasmussen I B, Lunde E, Berntzen G, Michaelsen T E, Bogen B and Sandlie I, manuscript in preparation).

HA (110-120) and $\lambda 2^{315}$(91-101) in L1CH1 were constructed as follows: pUC19γ3 served as template in the *in vitro* mutagenesis reactions. Mutagenesis was performed by PCR Quick change mutagenesis as described [59]. Reagents used for the mutagenesis were obtained from Stratagene. Synthetic oligonucleotides were purchased from MWG Biotech AG and shown in Table 2. The oligonucleotides encoding HA (110-120) and $\lambda 2^{315}$(91-101) epitopes included silent restriction sites for SspI and DraIII, respectively (underlined in Table 2). The PCR included 20 cycles with an annealing temperature of 55°C. Negative controls were template and forward primer only, template and reverse primer only, and template only. Dpn1 treatment (Stratagene) digests the methylated and hemimethylated parental DNA, whereas newly synthesized DNA is not affected. Mutants were analyzed with restriction cutting and further confirmed by sequencing (GATC, Germany).

Table 2 Oligonucleotide sequence for introduction of HA (110-120) and λ2^{315} (91-101)

Primer name	Primer sequence	Tm
HAL1CH1 Forward	5'- ccc atc ggt ctt ccc cct gtc att gca aag ctt cga aat att[a] ccc aag aaa cag cgg ccc tgg gct gc - 3'	78^{o}C
HAL1CH1 Reverse	5'- cag ccc agg gcc gct gtt tcc ttt ggg aat att tcg aac ctt tcg aat gac agg ggg aag acc gat ggg – 3'	78^{o}C
L(91-101)L1CH1 Forward	5' – ccc atc ggt ctt ccc cct ggc tct atg gtt cag aaa cca ctt tgt gtt cgg tac agc ggc cct ggg ctg – 3'	80^{o}C
L(91-101)L1CH1 Reverse	5' – gca gcc cag ggc cgc tgt acc gaa cac aaa gtg gtt tct gaa cca tag agc cag ggg gaa gac cga tgg g – 3'	80^{o}C

a) Silent restriction sites for SspI (in HA) and DraIII (in L(91-101)) are underlined

3.1.5 Construction of mutants with restriction sites for AEP

Processing sites for AEP were introduced C-terminally to HA (110-120), OVA (323-339), λ2^{315} (91-101) and λ2^{315}(89-105) expressed in L1 CH1 in γ3. The resulting Ab constructs are denoted HA-NN, OVA-NN, L(91-101)-NN and L(89-105)-NN, respectively.

The Ab containing restriction sites for AEP were constructed as follows: pUC19γ3 with genes encoding HA, OVA , L(91-101) and L(89-105) in L1CH1 served as template in the *in vitro* mutagenesis reactions that were performed as described above. Oligonucleotides were purchased from DNA Technology Denmark, and are described in Table 3. The oligonucleotides contain the sequence encoding NN (aac aac).

Test PCR was performed with Vent polymerase (NEB, Ipswich, MA, USA). The forward primer (Pre CH1 fwd) anneals upstream of the CH1-gene and the reverse primer (NN pos) anneals with introduced nucleotides, aac aac. The primers are described in Table 3. Positive clones were analyzed and confirmed by sequencing (GATC, Germany).

Table 3 Primer sequences for introduction of restriction sites for AEP, and primers used in test-PCR

Primer name	Primer sequence	Tm
HA-NN forw	5'- gaa ata ttc cca aag gaa **aac aac**[a] aca gcg gcc ctg gg-3'	68°C
HA-NN rev	5'- cc cag ggc cgc tgt **gtt gtt** ttc ctt tgg gaa tat ttc-3'	68°C
OVA-NN forw	5'- c aat gaa gca ggc agg **aac aac** aca gcg gcc ctg gg-3'	71°C
OVA-NN rev	5'- cc cag ggc cgc tgt **gtt gtt** cct gcc tgc ttc att g-3'	68°C
L(91-101)-NN forw	5'- ac cac ttt gtg ttc ggt **aac aac** aca gcg gcc ctg gg-3'	70°C
L(91-101)-NN rev	5'- cc cag ggc cgc tgt **gtt gtt** acc gaa cac aaa gtg gt-3'	70°C
L(89-105)-NN forw	5'- c ggt gga ggt acc aaa **aac aac** aca gcg gcc ctg gg-3'	71°C
L(89-105)-NN rev	5'- cc cag ggc cgc tgt **gtt gtt** ttt ggt acc tcc acc g-3'	71°C
Pre CH1 forw	5'-cgg ata aca att tca cac ag-3'	48°C
NN-pos	5'- cag ggc cgc tgt **gtt gtt**-3'	53°C

a) Restriction sites for AEP indicated as bold.

3.1.6 Subcloning

The mutant fragments corresponding to CH1 in Cγ3 were exchanged with the corresponding wt sequence using HindIII-BglII sites in pUC19γ3. The resulting mutant Cγ3 genes were sub-cloned as HindIII-BamHI fragments into the expression vector pLNOH2 $_{IgD}$. The plasmids were transformed into Ca^{2+} competent *E.coli* Top10^{F} cells. In the transformations *E.coli* Top10^{F} cells are incubated with plasmid DNA and then briefly heat shocked (42°C for 3 minutes). The heat shock causes DNA to enter the cell. Ampicillin resistant clones were picked and cultured in 1xLB medium. The Wizard® Plus SV Minipreps DNA Purification System (Promega) was used for DNA purification, according to the protocol supplied with the kit. Restriction enzyme (RE) digestion was performed under optimal conditions for each

enzyme or enzyme combination. All restriction enzymes, except Dpn1 (Stratagene), were provided by New England BiolAb (NEB) (Ipswich, MA, USA). DNA fragments and polymerase chain reaction (PCR) products were separated on 1% agarose gels. DNA was purified from the agarose gels using QIAquick Gel Extraction Kit (QIAGEN), according to the protocol included. Ligations were performed by use of T4 DNA Ligase (Roche)

3.2 Production, purification and analysis of the antibodies

3.2.1 Transient Transfection of 293E cells

hIgG3 is produced in transiently transfected 293E cells as described [48]. The L chain gene, encoded on pLNOκ$_{IgD}$, and each of the pLNOH2$_{IgD}$ variants, were transiently cotransfected in 293E cells. Briefly, the day before transfection 5 ml volumes 293E cells were plated at 2x10^6 cells/ml. 5μg of the pLNOH2$_{IgD}$ variants and 5μg of the pLNOκ vector were diluted in 0.5 ml of serum-free medium (OPTI-MEM® I, Gibco/BRL, Grand Island, NY), as were 20μl of LipofectAMINE^{TM} (LF2000) reagent (Invitrogen). After incubation for 5 min at room temperature (RT), diluted DNA was combined with diluted LF2000 reagent and incubated at RT for another 20 min. The DNA–LF2000 reagent complexes were each then added directly to the cell culture. Supernatant (SN) was harvested at day 3, 5, 7, 10, 12 and 14.

3.2.2 Enzyme-linked immunosorbent assay

The amounts of IgD specific Ab mutants secreted after transfection were measured in sandwich enzyme-linked immunosorbent assay (ELISA). Generally: 96 well microtiter plates were coated with a hIgG3 specific Ab and incubated ON at RT. Then, samples of 100μl diluted supernatants were added to each well and detected with a second biotinylated hIgG3 specific Ab. Streptavidine alkaline phosphatase (Strep-ALP) (1:3000) was added. Detection was done with the substrate for ALP, p-nitrophenyl phosphate (NPP) (Sigma-Aldrich) diluted in diethanolamine buffer to 1 mg/ml. The aborbance was measured in a Tecan Sunrice Remote Control at 405nm. A hIgG3 wild type (wt) preparation was used as standard in a three-fold dilution starting at 3μg/ml.

Ab used in ELISA were: S303, a polyclonal sheep α-human IgG Fab Ab [60], 132c8, a monoclonal mouse α-human IgG3 hinge Ab [61] and S298, a polyclonal sheep α-IgG Fc specific Ab kindly provided by T. E. Michaelsen, National Institute of Public Health, and IgD. Three different Ab combinations were used as coat and detecting agent: s303 (2µg/ml) and 132c8-bio (1:6000), s303 (2µg/ml) and s298-bio (1:8000), as well as mouse IgD (2µg/ml) and s298-bio (1:8000), respectively.

3.2.3 Ammonium sulphate precipitation of antibodies

Proteins in SN were precipitated by 1:1 addition of portions of a saturated ammonium sulphate solution. After precipitation, the tubes were incubated at RT for 20 min. The solutions were centrifuged using Sorvall centrifuge (OneMed, Norway) for 10 min at 17000g in 4°C. Pellets were dissolved in 2ml dH_2O. The precipitates were dialysed to PBS/Azide solution (0,02% Azide). Dialysis was performed in dialysis probes with a 12000 MW cut off (ChemiTek). Three dialysis shifts were included. The dialyzed solutions were centrifuged for 10 min at 4600 rpm in 4°C. This centrifugation step removes aggregates and particles, which precipitated during the dialysis process. The supernatants were stored at -80°C.

3.2.4 SDS-PAGE and Western blot analysis

75ng recombinant Ab was analysed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) on a Criterion XT Bis Tris 10% pre cast gel (BioRad, Hercules, CA, US). 4x XT sample buffer (Bio-Rad, Hercules, CA, USA) was added to the samples before they were preheated for 5 min at 95°C. Molecular weight marker covering the range from 6-175 kDa was preheated at the same temperature for 3 minutes. The samples were loaded on the gel and separated for 1 hour and 40 min at 140 V in 1x SDS/Tris-glycin electrophoresis buffer. Proteins were blotted onto a polyvinylidine fluoride (PVDF) membrane (Millipore, Madison, US) soaked in methanol for 1 minute and then washed in dH_2O for 1 minute before incubation in blotting buffer for 5 minutes. Blotting was performed using a semi-dry blotting apparatus (BioRad, Hercules, CA, USA) for 30 min at 20V. The membranes were blocked in 2% skim milk at 4°C over night (ON) or at RT for 2h. After three washes in PBS with 0.05% Tween 20 (PBST), the membrane was incubated for 2h at RT with HRP conjugated Ab. HRP activity on the membranes were visualized by SuperSignal

(BioRad) and exposed on a BIOMAX MR Film. Stripping buffer (BioRad) was used for 1h in RT.

Ab used in four Western blot analysis are: HRP conjugated polyclonal goat α-human IgG Ab (A0293) (Sigma), mouse Ab specific for human κ L chain (K13) [62] and subsequent detection with HRP-conjugated α-mouse IgG (A9044) (Sigma), biotinylated polyclonal sheep α-IgG Fc specific Ab (S298) (provided by T. E. Michaelsen, National Institute of Public Health) and subsequent detection with Strep-HRP and HRP conjugated Protein G (Calbiochem).

3.3 Antigen presentation and T cell activation

3.3.1 Antigen presentation of $\lambda 2^{315}$- and OVA-epitopes

3.3.1.1 Preparation of Balb/c spleen cells and lymph node cells

The APC was prepared by meshing Balb/c spleen tissue through a stainless steel mesh in ACT. ACT lyses the red blood cells. The cells were washed three times in RPMI medium by centrifugation for 7 min at 1200rpm in 4^{o}C. The splenocytes were irradiated at 2000cGy. Lymph node (LN) cells from transgenic (Tg) mice were prepared by crushing LN tissue through a stainless steel mesh. LN cells were washed in RPMI as described for spleen cells.

3.3.1.2 T-cell activation assays on antigen presentation of $\lambda 2^{315}$- and OVA-epitopes

The recombinant Ab that carry the OVA-epitope (OVA, OVA-NN) and the recombinant Ab that carry the $\lambda 2^{315}$ –eptiopes (L(91-101), L(91-101)-NN, L(89-105) and L(89-105)-NN) were tested as follows: Irradiated splenocytes from Balb/mice ($5x10^5$ cells per well) were incubated for 48 hours with LN cells ($1x10^5$ cells per well) and graded concentrations of Ag. The Ab were added as triplicates in flat-bottomed 96-well microtiter plates (Nunc, Denmak). All Ab mutants were diluted in 5-fold series. Activation of T-cells was assessed by pulsing the cultures with 1μCi [^{3}H]dThd. Culturing was continued for an additional 16-24h. The cells were then harvested onto filters using an Edvards pump. [^{3}H]dThd incorporation was counted using Top Count NXT scintillation counter (Packard, Meriden, CT).

Positive controls of OVA-specific T-cells were OVA protein (Sigma), a synthetic peptide corresponding to aa 323-339 of OVA (from B. Fleckenstein, University of Oslo) and a recombinant Ab expressing the OVA-epitope in loop 6 (L6) in CH1 [43]. A synthetic peptide according to aa 89-107 $\lambda 2^{315}$ and $\lambda 2^{315}$(91-101) in L6 in CH1 [40] was included as positive control in assays studying activation of $\lambda 2^{315}$-specific T-cells. OVA protein, OVA in L6 CH1 and $\lambda 2^{315}$(91-101) in L6 CH1 was added in five-fold dilutions and the synthetic peptides were added in ten-fold dilutions. Negative control was hIgG3wt with IgD specificity, and wells containing APC only, APC and T-cells only and T-cells only.

3.3.2 Antigen presentation of HA-epitopes

3.3.2.1 Limiting dilution of the HA-T-cell hybridoma

Hybridoma T-cells (gift from W.Gerhard, The Wistar Institute, Philadelphia, US) were cloned by limiting dilution. The hybridoma T-cells were seeded in four 96-well plates (Nunc, Denmark) at concentrations of 10, 1, 0.1 and 0.05 cells per well. Day 8 after seeding, the resulting colonies were analysed. Wells containing only one colony consisted of a clone of identical cells produced from a single progenitor. Ten different colonies were picked and grown in 24-well plates, before they were transferred to and grown in 5ml bottles.

3.3.2.2 T-cell assay and CTLL-2 assay studying activation of HA-specific T cells

APC was cultured with synthetic HA-peptide (aa110-120) and HA-specific T cells. The T cells were either a blend of HA-hybridoma cells or the ten HA-hybridoma clones. Synthetic HA-peptide was added as Ag in ten-fold dilutions starting at 10µg/ml. HA-hybridoma clone was used in T cell assays were recombinant Ab carrying the HA-epitope, HAL1CH1 and HA-NNL1CH1, were set as Ag. Ab mutants were diluted in 5-fold series. Positive controls included a synthetic peptide corresponding to aa 110-120 of HA and HA-epitope in loop 6 CH1 [43]. The synthetic peptide was added in ten-fold dilutions, and the recombinant Ab expressing the HA-epitope in L6 CH1 was added in five-fold dilutions. hIgGwt with IgD specificity was negative control.

CTLL-2 assays were performed as described: The Ag was added in triplicates to flat-bottomed 96-well microtiter plates (Nunc, Denmak). Irradiated splenocytes from Balb/mice

($5x10^5$ cells per well) were cultured for 20 hours with T cell hybridomas ($5x10^4$ cells per well) and graded concentrations of Ag. Triplicates of IL-2 standard was included in every plate in five-fold dilutions starting at 100U/ml. Activation of T cells was assessed by measuring production of IL-2 in the culture supernatant. This was done by [^{3}H]dThd incorporation by using IL-2 dependent CTLL-2 cells. In brief, diluted culture supernatants (1/3, 1/30 and 1/300) were incubated with CTLL-2 cells ($5x10^3$ per well) in 96-well plates for 24h. Prior to addition to the plates, CTLL-2 cells were washed four times in RPMI. The CTLL-2 cells were cultured with [3H]dThd for 12–14h, before harvesting. [3H]dThd addition and harvesting was performed as described above.

3.4 Comparing studies in Balb/c spleen cells and CA.36.2.1

3.4.1 Isolation of total RNA of CA.36.2.1 and spleen Balb/c mouse cells

Absolutely RNA miniprep kit (Stratagene) was used to isolate total RNA. The procedure followed the protocol included with the kit. In short, $5x10^6$ CA.36.2.1 and Balb/c spleen cells were harvested. The cells were lysed in lysis buffer and lysate was bound to a silica based fibre matrix by filtration. RNA was eluted in elution buffer, and the resulting total RNA was measured in an ND-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

3.4.2 cDNA synthesis of CA.36.2.1 and spleen Balb/c mouse cells

cDNA was synthesized from total RNA that was isolated from CA.36.2.1 and Balb/c spleen cells. Random Hexamers (500ng) (Promega), dNTP (5mM) (Amersham Biosciences) and dH_2O was added to the total RNA. After incubation at 65^{o}C for 5 min and at 4^{o}C for 2 min, Buffer (5x) (Invitrogen), DTT (0,1M) (Invitrogen) and rRNasin (40U/µl) (Promega) was added to the tube. The tube was further incubated at 25^{o}C for 2 min. SuperscriptII RNase (200U) (Invitrogen) was added and the tubes were incubated at 25^{o}C for 10 min, at 42^{o}C for 60 min and at 70^{o}C for 15 min. The cDNA was stored at -80^{o}C.

3.4.3 PCR-amplifications of the AEP gene

Expression of the gene encoding AEP was detected using PCR. The PCR was performed on cDNA from CA.36.2.1 and Balb/c spleen cells, with primers annealing to the gene encoding AEP. Synthetic oligonucleotides flanking the 1222 bp AEP gene were purchased from MWG Technologies. The primers: "AEP forward" and "AEP reverse" are described in Table 4. A touch down PCR was performed with annealing temperature from 72°C-60°C in 2°C steps and Phusion Polymerase (Finnzymes). These PCR products served as templates in a new nested PCR reaction.

To further amplify the gene encoding AEP, a nested PCR was designed. Synthetic oligonucleotides were purchased from DNA Technologies Denmark. The primers: "AEP nested fw" and "AEP nested Rv" are shown in Table 4. The primer pair amplifies a 1000 bp fragment of the AEP gene. Both the PCR product from touch down PCR and the total cDNA served as templates in the nested PCR. These PCR reactions had an annealing temperature at 63°C. The PCR reactions were performed with Phusion Polymerase (Finnzymes).

3.4.3.1 Verification of the AEP gene by restriction analysis

The AEP gene fragment that was amplified in the nested PCR contained a recognition site for HindIII. The fragment is 1000 bp and the restriction site is situated in position 663. The PCR product was cleaned by QIAquick®PCR Purification Kit (Qiagen), following the protocol included with the kit. The PCR product was further digested with HindIII (BioLAb). The restriction analysis was investigated on a 1% agarose gel.

Table 4 Primers for detection of the AEP gene

Primer name	Primer sequence	Tm
AEP forward	5' –atg acc tgg aga gtg gct gtg – 3'	58°C
AEP reverse	5' – gta gtg act aag aca cac ttt gtc cat g – 3'	57°C
AEP nested fw	5'- att acc gac acc agg cag ac-3'	60°C
AEP nested rv	5'- tgt gag cat ggt cct ctc tg- 3'	60°C

4 Results

4.1 Construction of mutant Ig H chains with model T cell epitopes in the CH1 domain

Amino acid 89-105 of $\lambda 2^{315}$ has earlier been introduced in all loops in all constant domains of a hIgG3. As all mutants harbouring epitope in CH2 and CH3 activated specific T cells, only one mutant with aa 89-105 introduced in CH1 induced T cell activation. Earlier studies has shown that the OVA peptide neither were able be presented from L1 in CH1. We wished to find an obvious reason for this. Prediction of AEP cleavage sites revealed more restriction sites in the CH2 and CH3 domain than in the CH1 domain. We focused on the proteolytic enzyme AEP with cleavage specificity for N, and how this affects presentation. Both the OVA and the $\lambda 2^{315}$ peptides contain N in its aa sequence. HA does not contain N and were introduced L1 in CH1. Additionally, recognition site for AEP was introduced C-terminally for all three epitopes.

To study the effect of N-residues as part of or flanking T cell epitopes, a series of mutants were made. All mutants were recombinant Ab with an aa replacement in L1 in CH1. The aa sequences all comprised commonly used model epitopes, either aa 110–120 of hemagglutinin, aa 323–339 of ovalbumin or aa 91-101 of myeloma protein of MOPC315 L chain, $\lambda 2^{315}$. Two sequences from $\lambda 2^{315}$ were included in the study, one consisting of aa 91-101 and one of aa 89-105.

Mutant H chain HA (110-120) and $\lambda 2^{315}$ (91-101) in L1 CH1 were constructed as follows: The nucleotides encoding the L1 in IgG3 CH1 gene were deleted and replaced by nucleotide sequences encoding the T cell epitope. Further recognition sites for the specific processing enzyme AEP were introduced. AEP has cleavage specificity for N [44]. Recognition sites were introduced in all four H chain mutants, as two N C-terminally to the T cell epitopes. A test PCR was designed to screen for positive clones. In clones with the nucleotides encoding the AEP recognition site, a gene segment will be amplified. The resulting eight different Ab constructs are shown in Figure 5. Each mutant H chain gene was co-transfected with a corresponding L chain gene into 293E cells. SN from transfected cells

were harvested at day 3, 5, 7, 12 and 14. The recombinant Ab are denoted HA, HA-NN, OVA, OVA-NN, L(91-101), L(91-101)-NN, L(89-105) and L(89-105)-NN.

Total protein was ammonium sulphate precipitated and dialyzed. Secretion of hIgG3 mutants was measured by two different sandwich ELISAs using pairs of hIgG3 specific Ab. In each pair, one Ab was used as coat and the other, which was biotinylated, as detection reagent. The coat and detection Ab pairs were: s303 (specific for Fab) and 132c8-bio (specific for hinge), and mouse IgD (Ag) and S298-bio (specific for Fc). The ELISAs are named ELISA1 and ELISA2, respectively. We found that all mutants were secreted. The IgD specificity of the wt hIgG3 was retained in all mutants, as confirmed in an IgD specific ELISA using murine IgD as coat (Figure 6). The large variations found in concentration of IgG3wt in the two ELISAs seem unlikely, but have unknown reasons. The low secretion level for L(91-101) and L(89-105) is consistent with earlier observations. Mutants containing the NN sequence C-terminally to the epitopes were secreted in higher amounts than the corresponding mutants containing only the Ag peptides. This may be due to an addition of two polar aa in the relatively hydrophobic sequence of the C terminal end of the $\lambda 2^{315}$ epitopes. Both the V region and Fc part of the Ab were determined in ELISA 2. Concentrations determined from this ELISA were used in further calculations.

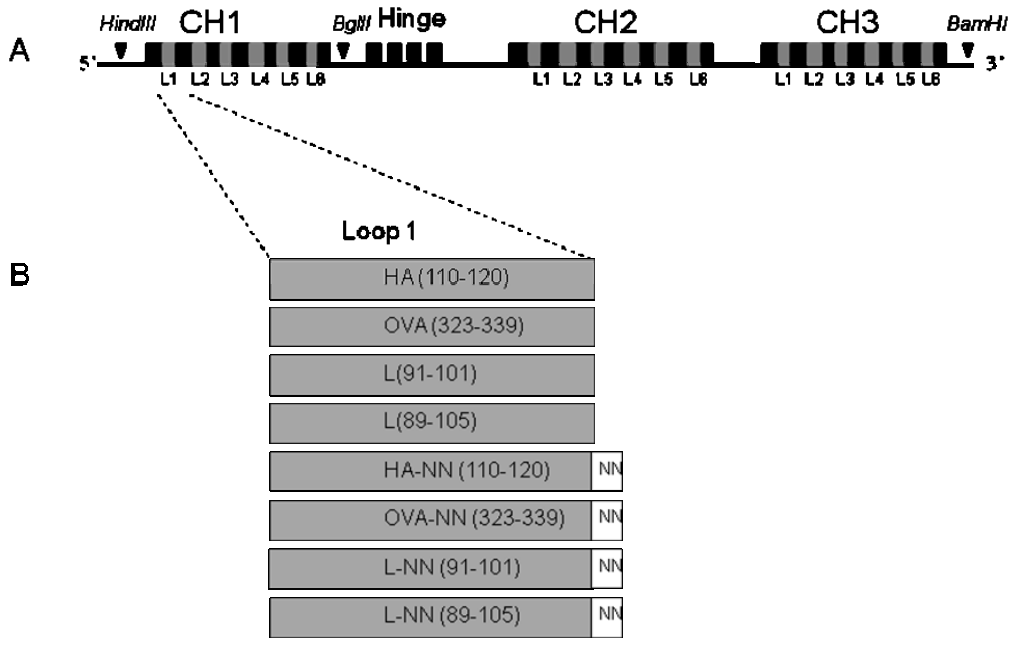


Figure 5: Overview of gene constructs. ***A:*** *Map of the Cγ3 gene. The boxes represent exons. Each CH encoding exon is shown with loop (grey) and framework (black) regions. Loops are numbered 1-6 from the 5'end. Restriction sites for HindIII, BglII and BamHI are indicated.* ***B:*** *Loop 1 in CH1of Cγ3 is substituted with the sequence listed. HA(110-120) 5'-SFERFEIPK-3', OVA (323-339) 5'-ISQAVHAAHAEINEAGR-3', $\lambda 2^{315}$ (91-101) 5'-ALWFRNHFVFG-3' and $\lambda 2^{315}$ (89-105) 5'-FAALWFRNHFVFGGGTK-3' Recognition sites for AEP are inserted C-terminally to the epitopes, as two asparagines (NN) (bottom four).*

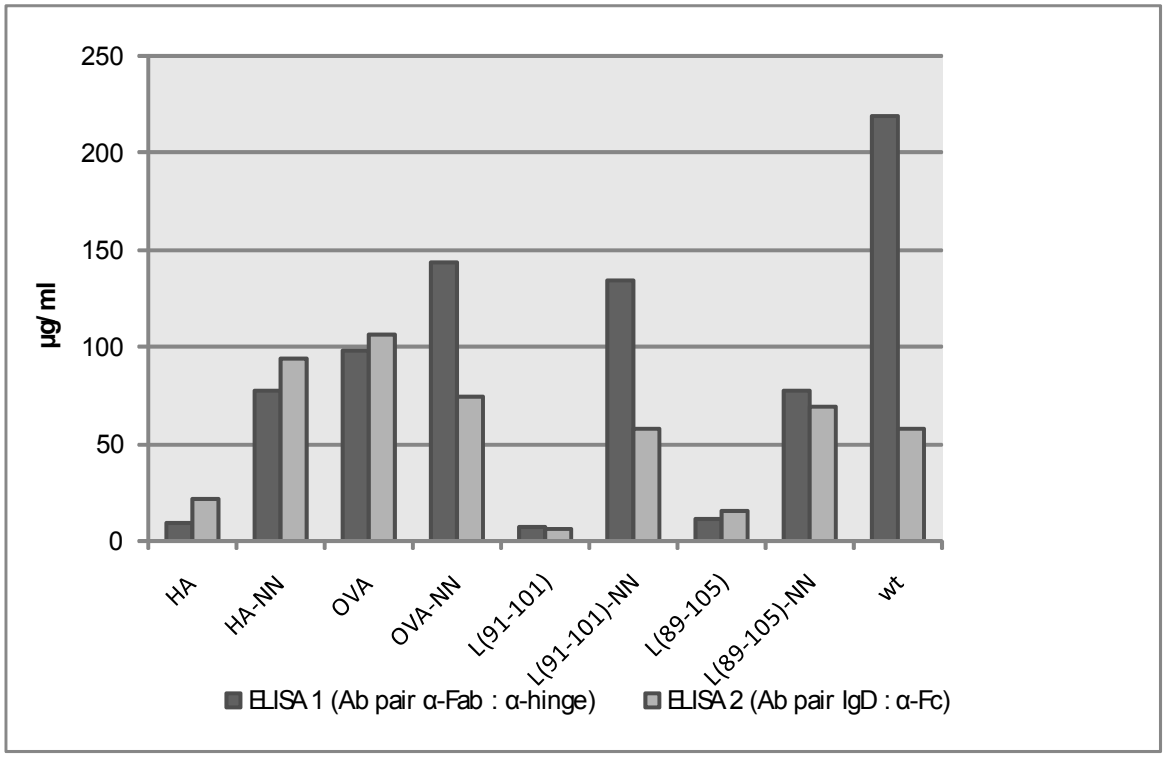


Figure 6: Concentration of recombinant IgG3s in supernatants after ammonium sulfate precipitation, determined in two different ELISAs. *pLNOH2$_{IgD}$ variants and pLNOκ were transiently transfected into 293E cells as described in Matherials and methods. Cells were plated as 2x10^6 cells /ml the day before transfection. Supernatants were harvested on day 3, 5, 7, 12 and 14. Concentrations of the total recombinant IgG3 after ammonium sulphate precipitation were determined in two different sandwich ELISAs. ELISA1: IgG3 was captured with a polyclonal sheep α-human IgG Fab Ab (S303) and detected with a monoclonal mouse α-human IgG3 hinge Ab (132c8). ELISA2: IgG3 was captured by mouse IgD and detected with sheep α-human IgGFc Ab (s298).*

4.2 Western blot analysis

The recombinant Ab samples were examined by SDS/PAGE and Western blot analysis. 75 ng samples were run on a Criterion XT Bis Tris 10% pre cast gel and blotted onto a PVDF membrane. The recombinant Ab were detected with specific Ab on the PVCF membrane. The Ab used for detection are: a polyconal α-human IgG Ab (A0293), a sheep α-human IgG Fc Ab (S298), a mouse α-human κ light chain Ab (K13) and Protein G. All demonstrated that the eight mutants were secreted as ~170 kDa proteins characteristic of complete disulfide bonded Ab consisiting of two H chains and two L chains, H_2L_2.

Figure 7 shows the Ab detected with the polyconal α-human IgG Ab. Proteins with a molecular weight at ~50 kD and ~25 kDa were present. There were large variations in signal among the different Ab. The ones denoted HA, L(91-101) and L(89-105) showed larger fractions of proteins at 50kDa and 25 kDa compared to the 170 kDa fraction than their 170 kDa counterparts. Additional bands were detected above the bands corresponding to H_2L_2. This was present for the Ab that showed the weakest of the H_2L_2 size, and is probably Ab aggregates.

In another Western blot the recombinant Ab were detected with a sheep α-human IgG Fc Ab. This is shown in Figure 8. Bands corresponding to the size of a complete H_2L_2 Ab appeared. Additionally, proteins which had migrated slightly further in the gel than H_2L_2 were detected, and for some of the mutants this bond was stronger to the H_2L_2 bond. Two bonds were observed for all Ab constructs by this detection might. This may be explained by glycosylation of the Ab, or the existents of Ab half molecules. In both cases, the corresponding bonds would have been detected with both polyconal α-human IgG Ab and the α-human κ light chain Ab. As this is not the case, we can not explain this fraction of proteins. The bands corresponding to proteins at ~50 kDa and ~25 kDa were not detected by this Ab. Single H chain and dimers of L chain have a molecular size at ~50 kDa and L chain monomers have a molecular size at ~25 kDa. Thus, the protein bands detected with the polyconal α-human IgG Ab were dimers of L chains and L chain monomers. If this fraction consisted of single H chain, it was expected to be detected with this Fc specific Ab.

The recombinant Ab were further detected with a mouse α-human κ light chain Ab. Bands were observed at sizes corresponding to the size of complete H_2L_2 molecules. Above the H_2L_2 bands, fainter bands were detected. This is most probably Ab aggregates. As expected, bonds corresponding to proteins at 25 kDa and 50 kDa were also detected by the α-kappa light chain. The picture is shown in Figure 9.

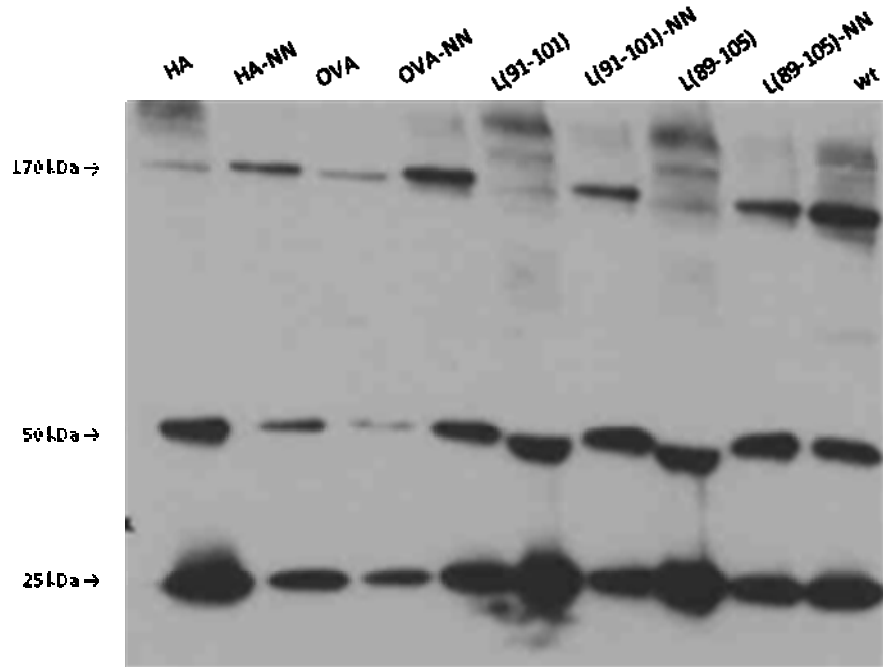


Figure 7: Recombinant IgG3s detected with a polyclonal goat α-human IgG Ab (A0293) by Western blot analysis *Samples of 75ng IgG3 were boiled at 95°C for 3 minutes and added to a non reducing 10% SDS-PAGE as described in Matherials and methods. After transfer to a PVDF membrane by semi dry blotting, IgG3 was detected with a polyclonal goat α-human IgG Ab (A0293). Molecular sizes at 170 kDa, 50 kDa and 25 kDa determined from protein standard are indicated.*

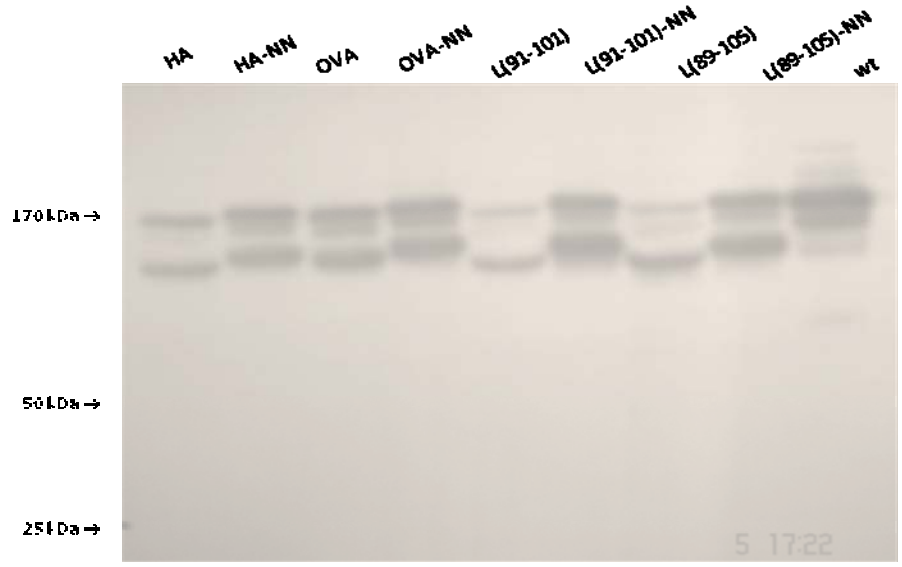


Figure 8: Recombinant IgG3s detected with sheep α-human IgG Fc Ab (S298) by Western blot analysis
Samples of 75ng IgG3 were boiled at 95°C for 3 minutes and added to a non reducing 10% SDS-PAGE as described in Matherials and methods. After transfer to a PVDF membrane by semi dry blotting, IgG3 was detected with a sheep α-human IgG Fc Ab (S298). Molecular sizes at 170 kDa, 50 kDa and 25 kDa determined from protein standard are indicated.

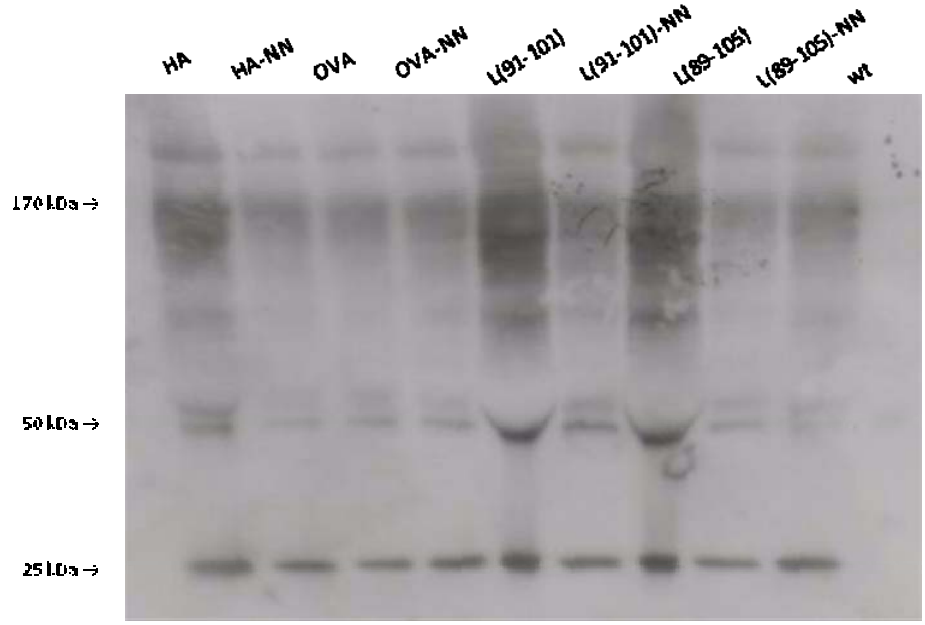


Figure 9: Recombinant IgG3s detected with a mouse α-human κ light chain Ab (K13) by Western blot analysis *Samples of 75ng IgG3 were boiled at 95°C for 3 minutes and added to a non reducing 10% SDS-PAGE as described in Matherials and methods. After transfer to a PVDF membrane by semi dry blotting, IgG3 were detected with a α-human kappa light chain Ab (K13). Molecular sizes at 170 kDa, 50 kDa and 25 kDa determined from protein standard are indicated.*

4.3 Antigen presentation and T cell activation

To investigate presentation of the introduced T cell epitopes in mutant hIgG3s, T cell proliferation assays were performed. As the recombinant Ab mutants were designed to target the introduced aa sequence to APC and thus induce increased, specific T cell activation, it is crucial that they are internalized so as to enter the Ag processing pathway, and that the specific epitopes are properly excised from the recombinant Ab carrier to bind MHC and transported to the cell surface. The mutants were used as Ag in a dose-response T cell activation assay. BALB/c spleen cells as APC, T-cells and recombinant Ab were combined. The APC were irradiated and thus, in this case, incorporation of radioactivity reflects T-cell proliferation upon Ag stimulation.

4.3.1 Antigen presentation of $\lambda 2^{315}$- and OVA-epitopes

OVA, L(91-101) and L(89-105) induced no detectable T cell responses (Figure 10 and Figure 11). OVA-NN, L(91-101)-NN, L(89-105)-NN were neither capable of activating the T cells, indicating that the introduced NN sequence did not contribute to Ag presentation. Positive controls are mutant Ab with (323-339) OVA or (91-101) $\lambda 2^{315}$ exchanged with L6 CH1, denoted OVAL6CH1 and L(91-101)L6CH1. Additionally, OVA protein, and a synthetic $\lambda 2^{315}$ peptide, aa 89-107, were included. IgG3 wt is negative control. As the graphs illustrate, only the positive controls activated the T cells. Negative controls consisting of T cells alone, APC alone or T cells and APC, showed no T cell activation (data not shown).

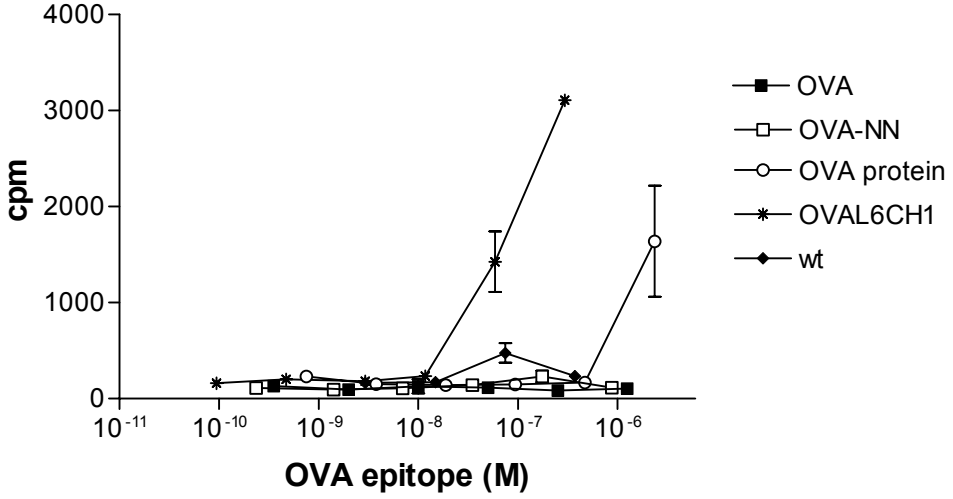


Figure 10: Activation of OVA specific T cells in response to presentation of the OVA-epitope from recombinant IgG3s or OVA protein. *LN cells from OVA specific TCR Tg mice were cultured together with irradiated Balb/c spleen cells and increasing amounts of recombinant Abs (OVA, OVA-NN and OVAL6CH1) or protein as described in Materials and methods. Tg LN cell proliferation was measured as [^{3}H]dThd incorporation.*

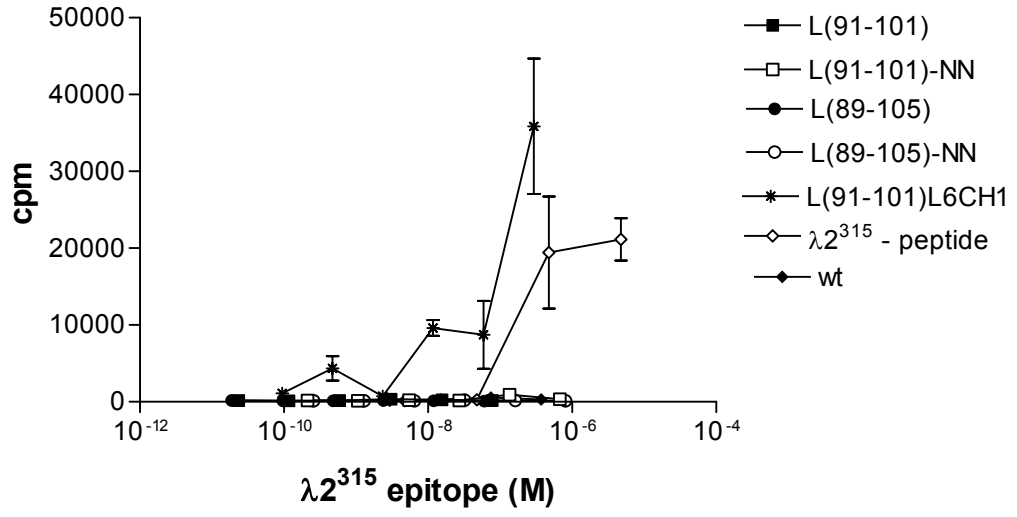


Figure 11: Activation of $\lambda 2^{315}$ specific T cells in response to presentation of the $\lambda 2^{315}$-epitopes from recombinant IgG3 or $\lambda 2^{315}$(89-107) peptide. *LN cells from OVA specific TCR Tg mice were cultured together with irradiated Balb/c spleen cells and increasing amounts of recombinant Abs or synthetic peptide as described in Materials and methods. Tg LN cell proliferation was measured as [^{3}H]dThd incorporation.*

4.3.2 Antigen presentation of HA-epitopes

4.3.2.1 Preparation of a HA-T cell hybridoma clone by limiting dilution

A HA-T cell hybridoma clone was cultured after limiting dilution. By limiting dilution it is possible to prepare a clone of cells derived from a single cell which is thus monoclonal in nature. Initially, before limiting dilution, hybridoma cells were pulsed with increasing amounts of synthetic peptide. Then a CTLL-2 assay was performed as described under "Matherials and Methods". In short; HA T cells were pulsed with HA-peptide. SN from the HA T cells was harvested. The SN was diluted and added to CTLL-2 cells. The CTLL-2 cells were then pulsed with [^{3}H]dThd, which incorporates in the activated CTLL-2 cells.

Activation was observed in the mixture of HA-hybridoma cells after stimulation of 1μg/ml peptide, as illustrated in Figure 12. Limiting dilution was then performed, and the reactivity of the ten clones were tested further. The HA-hybridoma clones were stimulated with increasing amount of HA-peptide. The activation of the individual clones is described in Figure 13A. Four out of ten clones showed reactivity to HA-peptide. The clones secreted

varying amounts of IL-2, determined from an IL-2 standard curve. The best one, named "Clone 4", secreted 36µg/ml IL-2, and was used in later experiments (Figure 13B).

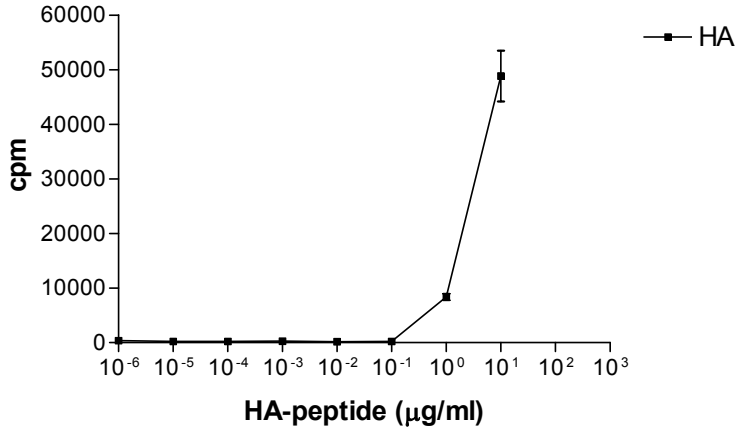
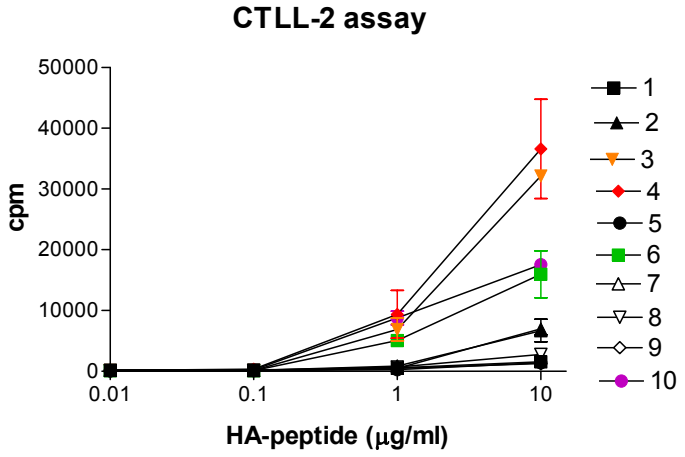


Figure 12: Activation of HA hybridoma T cells in response to presentation of the HA-peptide. *A mixture of HA hybridoma cells were cultured together with irradiated Balb/c spleen cells and titrated amounts of HA-peptide. Activation was measured as incorporation of [^{3}H]dThd into IL-2 dependent CTLL-2 cells.*

A



B

Clone	Amount IL2 in SN
3	29µg/ml
4	36 µg/ml
6	12 µg/ml
10	26 µg/ml

Figur 13: Proliferation of ten HA-specific T cell clones in response to presentation of the HA-peptide

A: *HA hybridoma cells (1-10) were cultured together with irradiated Balb/c spleen cells and increasing amounts of HA-peptide. Activation was measured as incorporation of [^{3}H]dThd into IL-2 dependent CTLL-2 cells.*

B: *The amount of IL-2 produced by four of the T-cell clones determined from an IL2 standard curve.*

4.3.2.2 CTLL-2 assays studying presentation of HA-epitopes

Recombinant Ab denoted HA and HA-NN were tested in the T cell activation assay. Measurement of the IL-2 concentration in the SN from HA T cells showed that these recombinant Ab induced a significant stimulation of the HA T cells. As described in Figure 14, [^{3}H]dThd was incorporated into the IL-2 dependent CTLL-2 cells. The Ab containing the introduced NN sequence C-terminally to the HA-epitope stimulated the HA hybridoma T cells in a similar manner as the Ab without there additional NN. Thus, both HA and the HA-NN are immunogens. Positive controls included in the experiments mutant were Ab with aa 110-120 HA exchanged with L6 in CH1, and a synthetic aa 110-120 HA-peptide. IgG3wt is a negative control. The negative controls consisting of T cells alone, APC alone or T cells and APC, showed no T cell activation (data not shown).

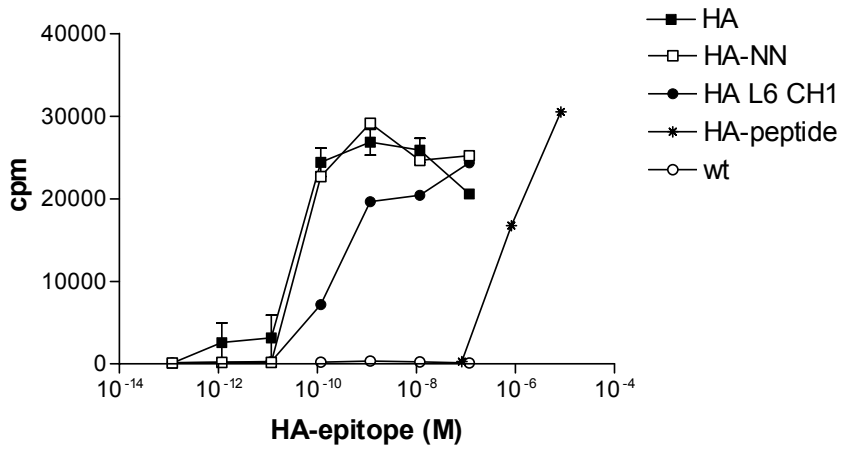


Figure 14: Activation of HA specific T cells in response to presentation of the HA-epitopes from recombinant antibodies or HA- peptide *HA specific hybridoma T cells were cultured together with irradiated Balb/c spleen cells and increasing amounts of recombinant IgG3 or synthetic peptide. Activation was measured as incorporation of [^{3}H]dThd into IL-2 dependent CTLL-2 cells.*

4.4 Detection of AEP in Balb/c spleen cells and CA.36.2.1

4.4.1 PCR-amplifications of the gene encoding AEP

To prepare comparing studies in a negative cell line, the mouse fibroblast cell line CA.36.2.1 was studied for expression of AEP. Total RNA isolation and cDNA synthesis was performed from Balb/c- and CA.36.2.1-cells. The The gene encoding AEP was amplified in a PCR reaction with primers flanking the 1378 bp gene. A nested PCR was performed on this PCR-product. A 998 bp fragment was amplified, using primers annealing the 1378 bp fragment. Primers used in this nested PCR were tested on both the PCR product and on cDNA from Balb/c spleen cells and CA.36.2.1. Figure 15 shows the PCR-product for both cell lines after PCR on cDNA. The PCR product from the nested PCR gave similar results (data not shown).

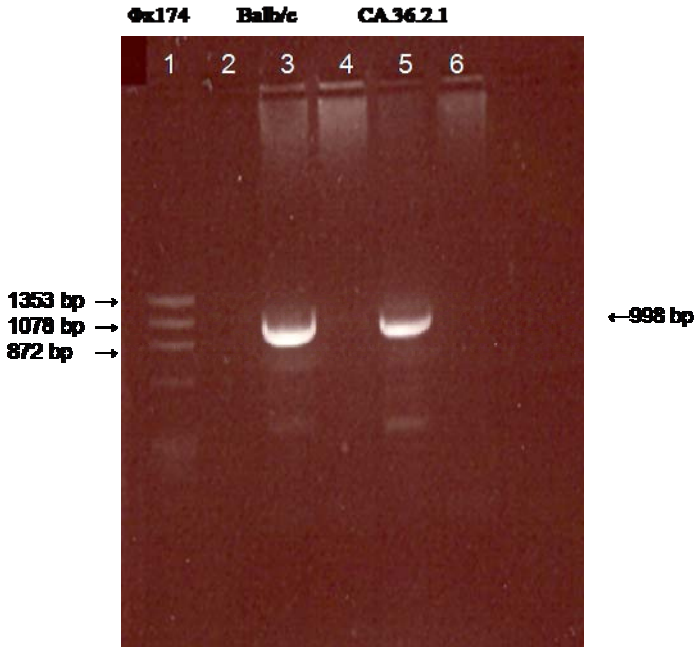


Figure 15: PCR-product from PCR on cDNA from Balb/c mouse and CA.36.2.1 with nested primers. *The fragments of the Φx174 molecular weight standard are indicated to the left, and the size of the PCR product to the right. The 998 bp fragment is detected in both cell lines, well 3 and 5. Non-template controls are included in wells 4 and 6.*

4.4.2 Verification of the AEP gene by restriction analysis

In order to verify the 998 bp PCR product after PCR on cDNA, restriction analysis was performed. The fragment included a restriction site for HindIII in position 663 in the 224-

1222 gene fragment of AEP. The PCR product from both cell lines was verified by this analysis. Two bonds were created, and had the expected sizes at 439 bp and 559 bp. Both bonds are localized between the 603 bp and 310 bp marker in the Φx174 ladder (Figure 16).

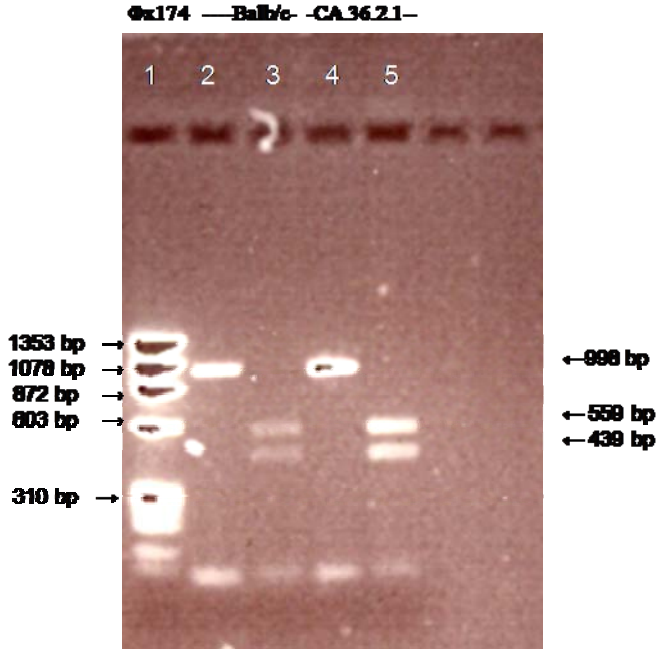


Figure 16: Restriction analysis of PCR product after PCR reaction on cDNA with nested primers. *The fragments of the Φx174 molecular weight standard are indicated to the left, and the size of the PCR fragments to the right. The PCR-product was digested with HindIII, creating a 559bp and a 439 bp fragment of the 998 bp AEP gene fragment, and loaded in well 3 and 5. Well 2 and 4 shows the 998bp PCR fragments from Balb/c and CA36.2.1 respectively.*

5 Discussion

As Ab are stable molecules and may be given unique specificities that allow targeting to APC, they are ideal vehicles for delivery of aa sequences that contain T-cell epitopes for vaccination purposes. It is essential that the Ab-epitope fusion protein is secreted from producing cells, and furthermore, processed by the APC after internalization such that the epitope is loaded on MHC and presented to specific T-cells. Here, we compared T-cell activation ability of 8 mutant rAb, to study the effect of N-residues as part of or flanking T cell epitopes. The T cell epitopes were introduced in L1 in CH1.

The substitution made in the CH1 domain was tolerated as the recombinant Ab were secreted as H_2L_2 molecules. This was not surprising, because the CH1 domain interacts with the CL domain through a hydrophobic interface that enhances the stability of both domains [63]. The finding was consistent with previous studies where OVA (323-339) and $\lambda 2^{315}$ (89-105) was exchanged with this loop ("Loops in all three constant domains of an Ig heavy chain exchanged with a T cell epitope", Flobakk M, Rasmussen I B, Lunde E, Berntzen G, Michaelsen T E, Bogen B and Sandlie I, manuscript in preparation). Total protein was ammonium sulphate precipitated. An alternative and more specific purification strategy could have been to purify the recombinant Ab by affinity chromatography on murine IgD. N is classified as a polar aa and the addition of two polar N in a position that is exposed on the surface on the Ab molecule showed a positive effect on the secretion level.

The efficiency of Ag presentation depends to a large extent on the concentration of peptide/MHC complexes on the APC surface. It is therefore of great importance that the same amount of recombinant Ab are added to APC in the presentation studies. ELISA made it possible to quantify the amount of recombinant Ab that actually binds murine IgD. The recombinant Ab contain manipulations in the Fab region, making this ELISA reliable for accurate quantifications. Ab detected in this ELISA retained both the specificity for IgD and effector function in the Fc part, and the concentrations determined here were used for further calculations.

In Western analysis the amount of Ab added in each well were standardized according to calculations made in ELISA. Even after standardization the mutations that were detected to be secreted in lowest amounts resulted in the weakest bonds on the membrane. This implies that ELISA underestimates the intern differences among the constructs.

We wished to study the effect of N-residues as part of or flanking T cell epitopes, and thus, a series of mutants were made. Most lysosomal proteases has rather broad specificity [64], while AEP is an Ag processing enzyme with strict cleavage specificity for N residues [16, 44]. Introduction of processing sites for AEP into Ag is complex because AEP has been described to cleave at only about 10% of the asparaginyl bonds in a sequence. The Cγ3 gene aa sequence contain 14 N residues [65]. The AEP cleavage server (http://theory.bio.uu.nl/kesmir/AEP/) predicts primarily two cleavage sites in the Fc region, one in CH2 and one in CH3. Thus, additional sites in CH1 might improve the activation potential of the CH1 mutants. The AEP cleavage server predicted the introduction of NN C-terminally as a preferred substrate for AEP in the given context for all mutants. L1 in CH1 is a water exposed loop, and by introduction of the two N, we expected the loop to become even more hydrephilic.

To assess presentation of the epitopes, T cell activation studies were performed. We found that recombinant Ab carrying OVA and $\lambda2^{315}$ epitopes were unable to activate specific T cells, and that introduction of recognition sites for AEP did not influenced the excision of the introduced epitopes. However, both mutant Ab carrying the HA epitope, induced T cell activation. We speculated that the $\lambda2^{315}$ and OVA sequences could be subject to destructive processing. This is in consistence with previous studies, were AEP has been described to be involved in negative processing of MBP [46].

According to the AEP cleavage server (http://theory.bio.uu.nl/kesmir/AEP/), the N situated in the OVA epitope is susceptible to cleavage, but the N situated in the $\lambda2^{315}$ epitope is not. Even so, the inability for the epitopes to be presented might be due to negative processing. The fact that HA, which does not contain N in its sequence, is presented supports this theory. Alternatively, the activation observed could be explained by this HA specific T cells to be more sensitive than the OVA and $\lambda2^{315}$ specific cells.

Earlier studies with recombinant Ab has described various T cell epitopes to be exchanged with loop 6 (L6) CH1 in hIgG3 [40, 43, 66-68]. In these studies, aa sequences containing N has been introduced. Both OVA (323-339), $\lambda2^{315}$ (91-101) and $\lambda2^{315}$ (89-105) activated specific T-cells when situated in this position. This indicates that the destructive effect imposed by AEP could not be seen for L6 in the same domain.

As no crystal structure of hIgG3 has been published, human IgG1 (hIgG1) has been crystallized and are shown in Figure 1. The two hIgG3 subclasses contain a high degree of sequence homology in the CH1 domains, and the crystal structure of hIgG1 could therefore

provide insight into the CH1 domain structure of hIgG3. As described in Figure 2 the structure of L1 in CH1 appears open and accessible.

It could be argued that the accessability would affect the excision of epitopes containing recognition sites for AEP C-terminally, resulting in Ag presentation from these recombinant Ab. This was not seen for the Ab containing OVA or $\lambda 2^{315}$. Further for the HA-mutants, the two N did not have any additional effect on the presentation of the HA epitope from L1. The T cell activation could be observed at an Ag concentration of 10^{-11} M for both HA and HA-NN. In fact, the Western blot analysis found the H_2L_2 fraction of HA-NN to be higher than the fraction of HA. Thus, more recombinant Ab was probably added to APC. This may indicate that HA is even more immunogenic than HA-NN. However, regarding AEP as an endopeptidase which cleaves at only 10% of the N residues situated in a sequence, the addition of N will not necessarily lead to cleavage at this position. Many N in hIgG3 molecules may never meet AEP, and thereby not be a substrate for the enzyme. AEP will act on the Ag at a specific time in the processing pathway, and its proteolytic effect surely depends on the overall structure and local secondary context in addition to the presence of N residues.

The T cell activation studies were performed with Balb/c splenocytes as APC. The gene encoding AEP were detected in the Balb/c splenocytes. In addition to be rich in B-lyphocytes, the spleen further contains DCs, natural killer (NK) cells and MΦ as reviewed in [69]. The recombinant Ab studied in this work were targeted to IgD^+ B cells. Detection of the gene encoding AEP in splenocytes can thereby not directly imply the existents of AEP in the B cell pool. However, AEP has earlier been identified in the endocytic compartment of B lymphoblastoid cells [16].

To prepare additional studies in an AEP negative cell line, the mouse fibroblast cell line CA.36.2.1 was studied for expression of AEP. The CA.36.2.1 cell line has earlier been used for Ag presentation studies. CA.36.2.1 is a mouse fibroblast cell line stably transfected with $I\text{-}E^d$. Both $\lambda 2^{315}$ and HA are presented in complex with this MHC CLASS II molecule, making presentation studies with these epitopes possible. In an earlier described Ag presentation study the $\lambda 2^{315}$ (91-101) were exchanged with loop 2, 4 and 6 in the CH1 domain of a hIgG3, constructing three different mutant H chains. The mutant H chains were transiently tranfected into CA.36.2.1 cells, and presentation of the epitopes for $CD4^+$ T cells was observed for all three mutants [40]. We found this cell line to express AEP. This made

this cell line inappropriate for the planned experiment, and an alternative cell must therefore be found to be able to perform this study.

The expression and function of AEP is currently being explored. AEP has been found to be present in thymic dendritic cells, were it is involved in the elimination of the immunodominant epitiope in MBP [46]. The degradation of this epitope might therefore contribute to the poor intratymic presentation of this epitope, which may enable MBV specific autoreactive T cells to escape negative selection. AEP has also been identified as an inhibitor of osteoclast formation and is associated with bone resorption [70]. Further, AEP is found to be expressed in solid tumors and in the endothelial cells of the tumor vasculature. Here it was demonstrated to affect cell migration, and was associated with enhanced tissue invasion and metastasis [71]. These properties lends AEP as a useful target for activating anti-tumor pro-drugs Much remains to be determined about this proteolytic enzyme, but its unique specificity and immense involvement in Ag processing may be a valuable tool in vaccine design.

6 Further perspectives

The results obtained in this study suggest that AEP is involved in Ag presentation of T cell epitopes in hIgG3. We believe that AEP affects the processing of the recombinant Ab, and that the action of this proteolytic enzyme explains why an epitope lacking recognition site for AEP can be presented to specific T cells and an epitope containing this restriction site can not. This effect is probably due to the processing events that AEP performs at N residues in the recombinant Ab.

One approach to study this further is to investigate the presentation of an additional T cell epitope without N in its aa sequence. Besides HA (110-120), aa 46-61 of hen egg lysosome (HEL) is a commonly used model epitope that does not contain any N. This epitope could be genetically engineered into L1CH1, and the immunogenity of the resulting recombinant Ab could be tested for Ag presentation in a T cell assay. HEL(46-61) has earlier been shown to activate specific T cells when it was exchanged with both L3 in CH1 [72], and when it was exchanged with L6 in CH1 [43].

Another approach is to perform comparative studies on Ag presentation with a cell line that is not expressing AEP. We expect that negative processing of Ag peptides will not happen in such cells. Alternatively, processing in general will be severely suppressed, and no presentation of any epitope detected. Cells used in Ag presentation studies must express MHC CLASS II. At least three different sources of APC could be used. An AEP knockout mouse is one source. Spleen cells from $AEP^{-/-}$ mice could be used in a T cell assay. This experiment would have the same design as described in Materials and methods. Recombinant Ab should be added in titrating amounts, and the proliferation of specific T cells would detect Ag presentation of the different epitopes. It might be favorable to find an alternative source of APC than knockout mice for both practical and economic reasons. In AEP knockout mice unknown events besides Ag processing might be affected, like Ii processing and processing of pro-protein precursors. These disruptions might have unwanted consequences on the Ag presentation.

The mouse fibroblast cell line CA.36.2.1 stably transfected with $I\text{-}E^{d}$ could be used in this Ag presentation studies. For the CA.36.2.1 cell line to be suitable, we needed to determine if the cell line express AEP. After detection with specific primers, CA.36.2.1 cells were shown to express AEP. To be able to compare the effect of Ag processing in a cell line

lacking AEP, the gene encoding could be deleted with RNA interference (RNAi). A third attempt to achieve suitable APCs could be by stably transfect I-E^d into a cell line not expressing AEP.

A reasonable solution would be to down regulate the amount of endogenous AEP in CA.36.2.1 cells by RNAi. RNAi is a method for knocking down target mRNA, leading to silencing of the gene product. The mechanism consists of two main steps. First, the dsRNA is recognised by Dicer, an enzyme of the RNaseIII family of endonucleases [73]. Dicer will cleave the dsRNA into smaller, double-stranded fragments, referred to as siRNAs. Second, the siRNAs are incorporated into the RNA-induced silencing complex (RISC) which unwinds the siRNA duplex. The single-stranded antisense strand then guides RISC to a complementary strand of mRNA which is then cleaved into smaller pieces. Chemically synthesized siRNA have the ability to bind RISC, and have been a powerful tool to inactivate gene expression. The mechanisms of RNAi are reviewed in [74]. The design of siRNAs involves choosing a target sequence of about 21 nucleotides long that fulfils certain requirements. [75]. Inactivation of AEP could be performed in CA.36.2.1 cells as described: The selection of siRNA could be determined from the target mRNA sequence, using a siRNA sequence selection web tool [76]. The siRNA sequences need to be checked for specificity using the Basic Logic Alignment Search Tool (BLAST) in the National Center for Biotechnology Information (NCBI) database, to insure specific knockdown of the gene in interest only, and minimize the off target effects. The selected siRNA can further be synthesized from a siRNA-licensed reagent supplier, Ambion, Dharmacon, Qiagen or Sigma Proligo [76]. Transient transfection of synthetic siRNA can further be done using Oligofectamine^{TM} reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's specifications. The changes in target protein level must be compared to an endogenously expressed control protein. This could be β-actin. Investigation of mRNA levels could be achieved by real-time reverse transcriptase-polymerase chain reaction (Real-time PCR). Real-time PCR could be performed using primers specific for AEP and for β-actin, as described [17]. Anti AEP has been described [17], and might be used in Western blot to detect the level of AEP in the cell lysate.

If we succeed in making CA36.2.1 cells not expressing AEP, Ag presentation can be studied. This could examine the presentation of the HA and $\lambda 2^{315}$ epitopes which are both presented on I-E^d. The mutant Cγ3 H chains expressing the epitopes in L1CH1 could be transiently transfected into CA.36.2.1 cells. A Th1 T cell clone 7A10B2 can be used for studying presentation of $\lambda 2^{315}$ the epitopes in these studies. The HA specific hybridoma cell

line could be used to detect presentation of the HA epitope. Activation of 7A.10.B2 cells could be performed by measuring an IFNγ secreted by the activated T cells, and presentation of the HA epitope could be measured by IL-2 dependent CTLL-2 cells as described previously.

We expect the HA epitope to be presented on I-E^d in the CA.36.2.1 cells. The recombinant Ab carrying HA serves as a positive control in the comparing experiment. If the proteolytic action of AEP is the reason why the $\lambda 2^{315}$ epitope can not be presented, we expect to see an presentation of these epitopes in CA.36.2.1 cells lacking AEP activity.

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