UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Role of the Endoplasmic Reticulum in Major Histocompatibility Class II Restricted Antigen presentation and Immune Response

A Dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

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by

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The Dissertation of Matthew Clay Wheeler is approved, and it is acceptable in quality and form for publication on microfilm:

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DEDICATION

For all of my friends and family, who may have wondered why, but were supportive nonetheless, and especially my fiancée Anna for not having to wonder why at all.

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ABSTRACT OF THE DISSERTATION

The Role of the Endoplasmic Reticulum in Major Histocompatibility Class II Restricted Antigen Presentation and Immune Response

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The generation of adaptive immune responses requires presentation of major histocompatibility (MHC) class II restricted immunogenic peptides to CD4 T cells by antigen presenting cells (APCs). These peptides are derived from the processing of protein antigens in acidic endosomal compartments. Classically, the generation of such peptides has been thought to have occurred through an exogenous pathway that involves internalization of antigens from the extracellular milieu. Recently, it has been demonstrated that peptides can also be generated from endogenously synthesized and retained antigen. While research in this field is burgeoning the physiological consequences of the processing of intracellular antigen are unknown.

This dissertation examines the *in vivo* consequences and *in vitro* pathways relevant to the processing and presentation of antigen that is endogenously synthesized and retained in different intracellular compartments by B lymphocytes. I show that peptides derived from the processing of intracellular antigen are able to prime a CD4 T cell response irrespective of intracellular targeting. Furthermore, I show that T cells primed by peptides derived from antigen retained in the endoplasmic reticulum (ER) induce higher secretion of IFN-γ and TNFα in responding cells. This increase is associated with upregulation of the ER stress responsive genes GADD34, Grp78 and CHOP, and down regulation of the costimulatory molecule OX40L in B lymphocytes. An OX40L reduced phenotype was recapitulated in CpG activated B lymphocytes by treatment with the ER stress inducing compounds tunicamycin and thapsigargin.

I also show that presentation of ER retained antigen is linked to the ER associated degradation (ERAD) pathway. Presentation of the model antigen Eα from an ER targeted IgH requires mannose processing, cytosolic translocation and degradation by the resident cytosolic protease tripeptidyl peptidase II. Additionally, ER stress inducing compounds are able to initiate presentation of a secretory IgH that traffics through the ER but is not normally presented.

Ultimately, the research presented here has far reaching implications for immune responses generated under states of cellular stress, such as viral

infection oncogenic transformation and anti-idiotype responses at the levels of antigen processing and immune priming. These results reveal important considerations for vaccine design, as ER stress may serve an important adjuvant effect in the induction of cellular immunity.

Chapter 1

Introduction

Classical MHC class II presentation pathway

CD4 T cells are regulators of the adaptive immune response, and provide help for the priming and memory functions of CD8 T cells (Janssen et al., 2003; Keene and Forman, 1982), the production of antibodies by B cells (Mitchison, 1971), and the priming of other CD4 T cells (Gerloni et al., 2000). CD4 T cells also help control self-reactivity in the periphery by regulating activated T cells (Sakaguchi et al., 1995). *In vivo*, CD4 T cell responses are primed after cognate interactions with peptides bound to MHC Class II molecules displayed on the surface of antigen presenting cells (APC) (signal 1), in tandem with proper co-stimulation (signal 2) (Bluestone et al., 1999).

It is generally accepted that antigen presenting cells (APCs) generate peptide:MHC class II complexes for CD4 T cell activation from exogenous antigen (Cresswell, 1994). Exogenous antigen is internalized through scavenger receptors, delivered to the endocytic pathway, where it is progressively unfolded, degraded by resident proteases and bound to MHC

class II molecules in MHC class II compartments (MIIC). MIIC are specialized vesicles present in the late endocytic pathway that are enriched in MHC class II and other accessory molecules necessary for peptide processing and loading. This being written, there is no clear consensus as to the exact make-up or morphology of MIIC, and this is further complicated by the fact that MIIC behave differently depending on the APC. Therefore, for the sake of simplicity and practicality, it is important to note that MHC class II is present at nearly every stop along the endocytic highway, from the early endosomes to terminal lysosomes, and that loading could ostensibly occur anywhere along the way given the proper proteolytic conditions.

MHC class II molecules reach these assorted vesicles by first assembling, as an α-ß heterodimer, in the endoplasmic reticulum (ER), with the chaperone invariant chain (Ii) (Cresswell, 1994). The most notable consequence of this interaction is that a portion of the mature Ii molecule, known as the Class II associated invariant chain peptide (CLIP), occupies the peptide-binding groove of the newly formed heterodimer. From the ER, the Class II-Ii complex is either delivered through the Golgi network to multivesicular bodies (MVB) in the endocytic pathway, or to the cell surface where a targeting motif in the cytoplasmic tail of the Ii directs rapid internalization and delivery to the endocytic pathway. Once delivered to the endocytic pathway, the Ii is degraded by resident proteases leaving the CLIP bound in the peptide binding groove (Cresswell, 1994). The CLIP is then

removed by interactions with the DM molecule and replaced by a peptide of sufficient binding affinity, The DM molecule (HLA-DM in humans; H-2M in mice) is a non-classical MHC molecule that lacks a traditional peptide binding groove and serves to stabilize empty MHC class II molecules as the CLIP-peptide exchange occurs (Sherman et al., 1995). By analogy this is the molecular equivalent of the scene in *Raiders of the Lost Ark* where Indiana Jones (DM) approaches a pedestal (MHC class II) and must quickly replace the golden idol (CLIP) with a bag of sand (foreign peptide) in order to avoid setting off a trap. In B cells, the activity of DM is further modulated by the DO molecule (HLA-DO in humans; H2-O in the mouse), ensuring that liberation of CLIP occurs only under the highly acidic conditions present in late endosomal compartments and MIIC (Alfonso et al., 2003). After binding of peptides in these late endocytic compartments peptide:MHC complexes are delivered to the cell surface where they are recognized by CD4 T cells bearing a T cell receptor (TCR) of the appropriate affinity.

Collectively, this process is known, and will be referred to in this dissertation, as the exogenous pathway of MHC Class II presentation. A schematic of this process is shown in Figure1. While this is a basic overview of the process there are two subtle points worth mentioning, as they may help the reader of this dissertation understand the results presented.

First, since MHC class II molecules are “open ended” they can bind peptides of varying lengths, including full-length proteins that exist in a

reduced or unfolded state. Thus, it is possible for the core immunogenic peptide determinant to bind the MHC class II molecule before proteases have fully processed the entire antigen. This process has been dubbed MHC guided processing (Sercarz and Maverakis, 2003). This is in contrast to processing and loading of MHC class I molecules, which have "closed" ends and require full antigenic processing before a final peptide of 8-10 amino acids can be bound.

Second, since the Ii chain itself requires protease processing to be liberated from the MHC class II molecule, immunogenic determinants tend to bind newly synthesized MHC only during the later stages of the endocytic process. However, it has been demonstrated that binding can also occur earlier in the endocytic pathway in recycling endosomes (sometimes referred to as CIIV) (Pathak and Blum, 2000; Pathak et al., 2001; Sinnathamby and Eisenlohr, 2003; Sinnathamby et al., 2004). This most likely occurs through a mechanism by which an MHC class II molecule already containing a processed peptide is internalized from the cell and is displaced by a peptide of higher affinity.

Presentation of intracellular viral antigens by MHC class II molecules

Do to the distribution of MHC class II and accessory molecules in the endocytic pathway it has long been a tacit assumption that the only relevant presentation of antigen via MHC class II was of immunogenic epitopes derived

from exogenous antigen. This notion, however, is quickly becoming passé as recent investigations and older studies revisited are examined. It now appears as if the MHC class II expressing APC will use any means necessary to present antigen derived from pathologically relevant sources such as viruses, intracellular bacteria and self antigen. Moreover, in contrast to presentation of peptides via the exogenous pathway, there appears to be at least three main pathways by which endogenous antigen is presented via MHC class II, indicating that there is, in fact, more than one way to skin the immunological cat.

The first studies indicating that MHC class II restricted peptides could be generated from endogenously synthesized antigen were used human viral systems. Since CD4 T cells are necessary for antiviral responses it seems unlikely that evolution would have left something as important as this to the mere chance of extracellular virus encountering scavenger receptor, especially during the early stages of viral infection where the presence of mature virions in the extracellular milieu may be quite low. This is supported by the fact that many viruses avoid internalization into the endocytic pathway, and the certain death of lysosomal destruction. The most famous example is that of the influenza virus, that utilizes a pH sensitive conformational change in its hemagglutinin (HA) molecule to escape the endocytic pathway (Skehel and Wiley, 2000).

The presence of an endogenous pathway for MHC class II presentation was first alluded to when it was found that UV inactivated PR8 influenza virus could block presentation of an immunodominant peptide derived from the HA molecule (amino acids 107-119, S3) and another derived from the neuraminidase molecule (amino acids 79-93, NA79) (Eisenlohr and Hackett, 1989). Presentation was also blocked by the protein synthesis inhibitor cyclohexamide indicating that recognition of these two epitopes by T cells required *de novo* synthesis of protein and was not a result of passive uptake, processing and presentation through an exogenous pathway. Later research showed that presentation of an epitope of the influenza matrix protein to an MHC class II restricted T cell line was blocked by brefeldin A (BFA) but not by chloroquine (Nuchtern et al., 1990). This observation also supported the presence of an endogenous pathway since most antigens that are presented via an exogenous pathway are blocked by chloroquine, a compound that prevents endosomal acidification. Recently, Tewari et al. found that presentation of the S3 and NA79 peptides were generated in the cytosol by the proteasome and were dependent on functional TAP (Tewari et al., 2005). It was also found that presentation was blocked by primaquine, a compound that inhibits the formation of recycling endosomes. Interestingly, in this study, when T cells from influenza infected mice were restimulated with infected antigen presenting cells treated with the proteasome inhibitor epoxomicin there was a reduction in the class II restricted response by 40%. These

studies elaborate a pathway by which endogenously synthesized antigen can be processed in the cytosol by the proteasome and translocated into the ER by the TAP molecule. From here it appears peptides make their way to recycling endosomes for binding to MHC class II molecules. How this latter process occurs is still unknown.

While it appears that MHC class II restricted presentation of endogenous antigens, in some cases, utilizes a pathway reminiscent of the MHC class I processing pathway, this is by no means the entire story. Malnati et al. discovered that presentation of a cytosol localized version of hemagglutinin, in contrast to previous reports was not dependent on functional TAP (Malnati et al., 1992). It is notable that a vaccinia vector, as opposed to natural influenza infection, in this case supplied the hemagglutinin, and thus, the differences in vector and route of infection may account for the discrepancy seen. Further evidence to suggest divergent pathways for presentation of endogenous antigen was revealed when it was found that the stability of a cytosolic antigen influenced whether it could be presented by MHC class II molecules (Gueguen and Long, 1996). Gueguen and Long modified the influenza matrix protein in order to alter its half-life in the cytosol, doing this they found that a more stable protein increased presentation of MHC class II restricted peptides. How long lived cytosolic proteins were ultimately processed was a mystery until the discovery that a cytosol localized neomycin resistance gene was presented by autophagy (Nimmerjahn et al.,

2003). Autophagy is a process by which a membranous vacuole known as an autophagosome non-specifically envelops portions of the cytosol and eventually fuses with endosomal compartments (Seglen and Bohley, 1992). It has been shown that many long-lived cytosolic proteins are degraded in this way (Levine and Klionsky, 2004). This pathway provides an attractive mechanism that allows cytosolic antigen to be delivered to MIIC. More significance was added to these results when it was found that a nuclear localized antigen from Epstein-Barr virus (EBV) was dependent upon autophagy for presentation (Paludan et al., 2005). Finally, using the more systemic approach of mass spectroscopy analysis of all MHC class II associated peptides, Dengjel et al. found that inducing autophagy by serum starvation increased the presence of peptides derived from intracellular antigen associated with MHC class II, in this case by 56% (Dengjel et al., 2005).

A relative laggard to the presentation party is the notion of chaperone mediated autophagy (CMA) as a method to deliver cytosolic proteins to the endosomal system. CMA differs from the aforementioned autophagy in that it requires direct-protein-protein interactions. In CMA the chaperone molecule heat shock cognate protein 70 (hsc70) binds to a KFERQ related sequence in the substrate protein, delivers it to the lysosomal compartment and, with the help of the lysosome associated membrane protein-2a (lamp2a) protein, imports it into the lysosome (Majeski and Dice, 2004). Importantly, CMA

requires proteins to be unfolded and does not require vesicular transport, as does regular autophagy. Zhou et al. found that presentation of cytosolic glutamate decarboxylase (GAD) protein by a B lymphoma cell line was dependent on hsc70 expression, and increased by over expression of LAMP-2a, but not LAMP2b (Zhou et al., 2005). CMA was also shown to be the dominant pathway for presentation of a mutant κ light chain that wasn't processed by the proteasome, indicating that proteins processed in the cytosol can also be dependent upon CMA for their presentation.

Ultimately, peptides from viral pathogens, presented through an endogenous route, may play a significant role in the immunity to these pathogens. To date presentation via endogenous pathways have been elucidated for influenza, measles, EBV and vaccinia virus (Jacobson et al., 1989; Jaraquemada et al., 1990; Nuchtern et al., 1990; Paludan et al., 2005). Most likely these viruses represent the tip of the iceberg concerning presentation of viral antigen via endogenous pathways begging the question of what is the importance that presentation of intracellular antigen has on the nascent immune response in virus infected organisms.

The work done in human systems on presentation of intracellular antigen has been important for two main reasons. First, these investigations provide significance for this phenomenon beyond the realm of biological curiosity. Second, they have delineated three main mechanisms by which presentation occurs bulk autophagy, chaperone mediated autophagy and a

pathway dependant on both TAP and the proteasome. A schematic of these three pathways is shown in figure 2.

Investigations into the MHC class II restricted presentation of intracellular antigen: Insights gained from model antigen systems

While much knowledge has been gained from human systems, work with the model mouse antigens hen egg white lysozyme (HEL), ovalbumin (OVA), and others has revealed much that is not apparent from viral systems.

Weiss and Bogen first demonstrated that T cell epitopes were generated for presentation from antigen targeted to the endoplasmic reticulum (ER) (Weiss and Bogen, 1991). Presentation of a peptide derived from an ER targeted λ chain was presented nearly as efficiently as the same peptide generated from exogenous antigen. Presentation was not influenced by the folding state of the molecule as an ER targeted λ that was mutated to abolish disulfide bonding was equally presented.

HEL, which was first used to study presentation of antigen through an exogenous pathway, has been modified by several groups to achieve intracellular targeting from an expression plasmid. When naïve mice are immunized with HEL 90% of the T cell response is directed against four immunodominant epitopes from amino acids 18-33, 31-47, 48-63 and 115-125 (Peterson et al., 1999). Interestingly, levels of presentation do not match the make up of the responding T cell pool. Peptide 48-63 comprises 98% of the

HEL derived peptides after pulsing cell with exogenous HEL, while peptide 18-33 comprises just 0.4% of this pool (Velazquez et al., 2001). Yet, each epitope makes up approximately 30% of the responding T cell repertoire (DiPaolo and Unanue, 2002). Since HEL generates multiple distinct epitopes, HEL that has been targeted to intracellular compartments can help illuminate the process by which epitopes are selected for MHC class II. Adorini et al. discovered that targeting endogenously synthesized HEL to the ER resulted in presentation of a similar but not identical subset of epitopes (Adorini et al., 1993). For example, peptides generated from ER localized HEL could stimulate a T cell hybridoma specific for amino acids 116-129, but not amino acids 112-124. On the other hand a secretory form of HEL generated peptides that could stimulate both T cell hybridomas. These results agree with those obtained using the OVA model antigen system where presentation of peptides derived from ER retained antigen was more inefficient then presentation of peptides generated from antigen targeted to the endocytic pathway (Brooks et al., 1991; Fernandes et al., 2000). Therefore, it appears that processing of ER retained antigen occurs in distinct compartments from exogenous or secretory antigens, and yields a distinct set of epitopes available to T cells. This, in fact could influence T cell responses by stimulating T cells that discriminate based on flanking residues generated by differential protealysis.

The site of processing for ER retained antigen is currently unknown. Peptides derived from ER retained HEL do not bind MHC class II nor was antigen degraded in the ER (Bonifaz et al., 1999). It is worth noting, however, that both HEL and OVA are normally extracellular antigens, designed by evolution to exist in harsh environments, and have been co-opted for studying presentation of endogenous antigen. The nature of these antigens may be such that they are not amenable to presentation through pathways that normally process intracellular antigen.

Information on processing and presentation of endogenous antigen from *in vivo* mouse models is relatively scarce with a few notable studies. *Legionella pneumophila* is an intracellular bacterium that escapes destruction in phagolysosomal compartments by diverting the compartments to form ER like organelles. Neild et al. discovered that presentation of MHC class II restricted antigens by macrophages from proteins synthesized after generation of the ER-like organelle are necessary for the CD4 T cell response to this organism (Neild and Roy, 2003). It has also been found that mice immunized with tumor cells that have been modified to express MHC class II, present intracellular antigen (Baskar et al., 1995). In this system, however, expression of the Ii chain and DM inhibited presentation of intracellular peptides. This phenomenon may be specific to this system, since the great majority of previous studies have used B lymphoma cell lines which naturally express all

components of the MHC class II presentation pathway, it is not yet know if priming occurs by APCs.

Concluding remarks

Overall, presentation of endogenously synthesized intracellular antigen has been shown in both mouse and human, using a variety of pathogens and model antigen systems. Presentation of peptides from antigen targeted to the cytosol proceeds by at least three different pathways that depend on either autophagy, chaperone mediated autophagy or TAP and the proteasome. Currently, It is not yet known how ER retained antigen is presented via MHC class II, although peptide binding in the ER is not likely given current data. Furthermore, most studies have focused on systems *in vitro*, therefore, it is not entirely certain how the *in vivo* immune response is influenced by the presentation of endogenous antigen. It is the goal of the work presented in this dissertation to elucidate cellular pathways that contribute to presentation of intracellular antigen, and to explore how this influences the CD4 T cell immune response generated.

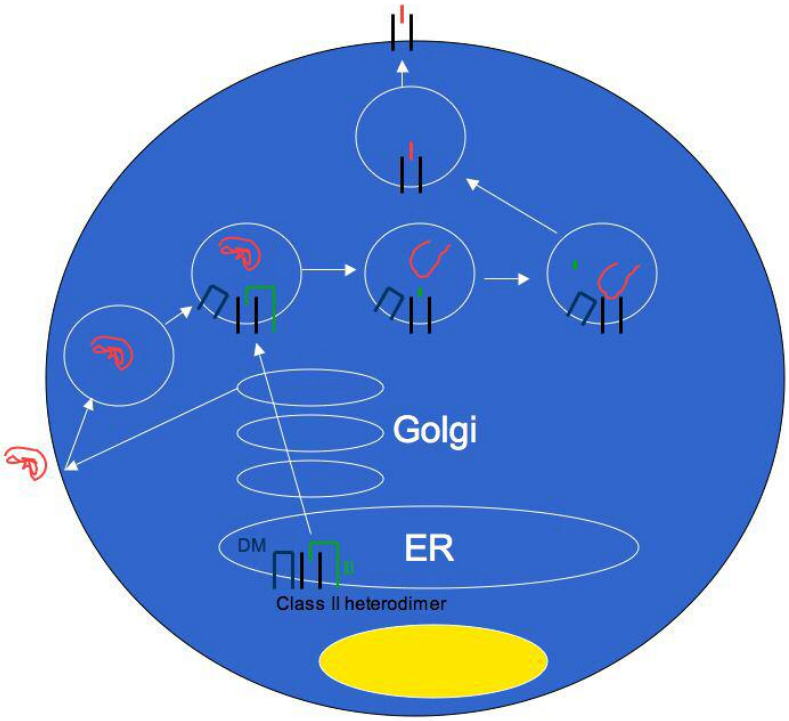

Figure 1.1 The classical MHC class II presentation pathway. Exogenous antigen is internalized into endocytic compartments and progressively degraded. Concomitantly, MHC class II molecules are synthesized, assemble in the ER and are transported to the endocytic pathway. Here the Ii is degraded and the CLIP is displaced, with the help of DM, by antigen of suitable affinity.

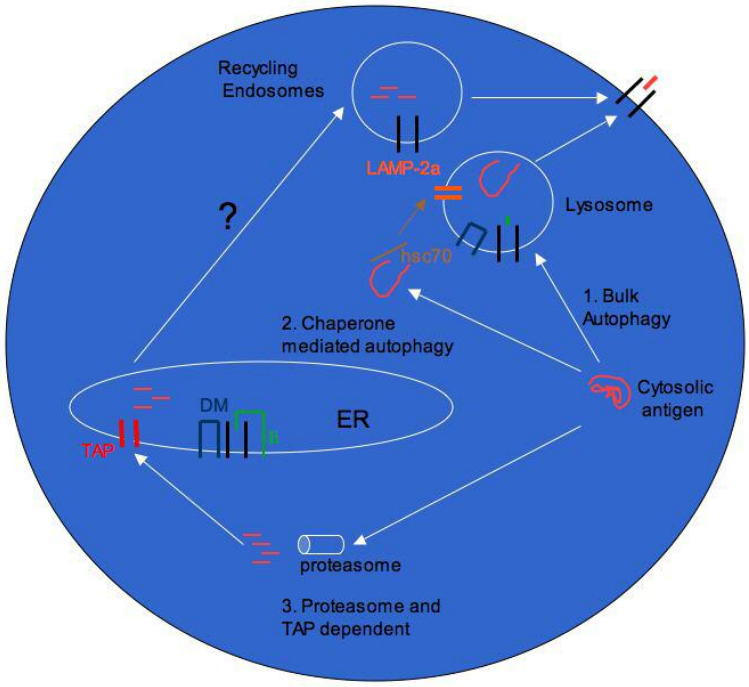

Figure 1.2 Presentation of cytosolic antigen through endogenous pathways. Resident cytosolic antigens are presented in three main ways 1) nonspecifically through bulk autophagy, 2) via specific protein-protein interactions by CMA or 3) by a TAP and proteasome dependent pathway.

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Chapter 2

Intracellular targeting of antigen in a model APC based DNA vaccine

Introduction

There is general enthusiasm for therapeutic DNA vaccines that use plasmids to express immunogenic antigens that result in T cell priming (Liu and Huang, 2002; Liu et al., 2004). Plasmid DNA vaccines require plasmid internalization, neo-synthesis of plasmid encoded antigens, processing and presentation of immunogenic peptides to T cells, and ultimately an adjuvant effect that results in APC activation and T cell priming. It has been thought that CpG motifs present in the backbone of plasmid vaccine vectors supplied that necessary adjuvant effect, by stimulating toll-like receptor 9 (TLR9) (Hemmi et al., 2000; Krieg et al., 1995; Sato et al., 1996; Ulevitch, 2003). Recently, however it has been shown that plasmid vaccines are capable of efficiently priming an immune response in both $TLR9^{+/+}$ and $TLR9^{-/-}$ mice (Cortez-Gozalez et al., 2006; Spies et al., 2003). Further studies cast more doubt on the role of CpG as an adjuvant for plasmid DNA vaccines, as a cytosolic DNA sensing pathway, that is non sequence specific, has recently been revealed (Ishii et al., 2006; Stetson and Medzhitov, 2006). Signaling

through this pathway is independent of TLR9 activation, and has been shown to result in robust expression of the type I interferons IFN-α/β. These results illustrate the need for basic inquiries into the optimization of plasmid DNA vaccine vectors that focus on improving and delineating pathways of peptide presentation and immune activation.

Previously my lab has reported on a new approach to program adaptive T cell responses termed transgenic lymphocyte immunization (TLI) (Zanetti et al., 2004) that is based on the use of antigen presenting B lymphocytes rendered transgenic with plasmid DNA through a process of spontaneous lymphocyte transgenesis (SLT) (Filaci et al., 2004). We have previously shown that only IgD+/IgM+ B cells are rendered functionally transgenic. The immunogenic plasmid codes for the heavy (H) chain of a secretory immunoglobulin (IgH) molecule composed of a rearranged murine variable (V) region and human γ1 constant region. Antigen specificity is imparted by inserting DNA sequences coding for immunogenic T cell epitopes into the complementarity-determining regions (CDRs) of the V region (Zanetti, 1992). We have shown that a single i.v. injection of transgenic B lymphocytes leads to trafficking to the spleen, and primes both CD4 and CD8 T cell responses against peptides expressed by the IgH transgene (Gerloni et al., 2004). The generation of a CD4 response requires MHC class II expression on the injected lymphocytes, and occurs in the absence of functional dendritic cells (DC), indicating that priming involves direct presentation to T cells (Castiglioni

et al., 2003). Therefore, this system constitutes an excellent model to examine the effects of endogenously synthesized antigen on CD4 T cell priming.

There are two model antigens that are used in the experiments shown in this chapter and throughout this dissertation. The NVDP model antigen is a peptide derived from the circumsporozite protein of *P. falciparum.* This peptide has the sequence NANPNVDPNANP and is immunogenic in C57Bl/6 mice (Gerloni et al., 1998). The second model antigen used is the Eα peptide, it is derived from amino acids 56-73 of the alpha chain of the E^b molecule (ASFEAQGALANIAVDKA). This peptide is presented by the A^b molecule, and is immunogenic in C57Bl/6 mice which do not express the E^b locus (Rudensky et al., 1991). Furthermore, this model antigen has the advantage of a monoclonal antibody, Y-Ae, that recognizes the peptide specifically in the context of the A^b molecule (Murphy et al., 1992).

In the results presented in this section I show that antigen targeted to the ER, cytosol or MIIC can prime an MHC class II restricted CD4 T cell response. Furthermore, I show that T cells primed by antigen that is retained in the ER of antigen presenting B cell secrete higher levels of IFN-γ and TNFα compared to T cells primed by cytosolic or secretory antigen.

Results

Targeting of hIgG to different intracellular compartments

In order to determine if the ability of endogenously synthesized antigen to prime a T cell response varies as a result of the intracellular localization of antigen, I altered a model secretory IgH to be either retained in the ER, targeted to the cytosol or to the MIIC. To achieve ER targeting I engineered a secretory IgH to express a C-terminal KDEL motif. The KDEL amino acid motif is present on many ER resident proteins and has been shown to induce ER retention in a normal secretory protein (Persic et al., 1997). To achieve cytosolic targeting I removed the secretory leader sequence from a WT IgH. Finally, to direct MIIC targeting by a normal IgH, I fused the transmembrane and cytosolic domain of the beta chain of the DM protein to the C terminus of the WT IgH. The β chain of the DM molecule contains a tyrosine based sorting motif that is sufficient to direct the DM protein to the endocytic pathway (Copier et al., 1996; Potter et al., 1999). A similar strategy using the LAMP-1 protein has previously been employed to direct protein targeting to the endocytic pathway (Fernandes et al., 2000). A schematic representation of the expressed IgH products is shown in figure 1 where $V_H\gamma_1$ denotes the wild type secretory IgH, $V_H\gamma_1$KDEL denotes ER targeting, Δ$L11V_H\gamma_1$ cytosolic targeting and, $V_H\gamma_1$DM targeting to the MIIC.

To ensure that the engineered $V_H\gamma_1$KDEL, Δ$L11V_H\gamma_1$ and $V_H\gamma_1$DM IgH molecules were targeted to the intended intracellular compartment, and not secreted, I generated stable transfectoma using J558L cells. J558L is a murine plasmacytoma that expresses a λ light chain in the absence of an

endogenous heavy chain. Thus, upon transfection with IgH transgenes J558L cells begin to secrete complete H_2L_2 (IgG) molecules, which can be detected by capture ELISA for the transgenic H chain. We found no transgenic IgG in $V_H\gamma_1$KDEL, ΔL11$V_H\gamma_1$ or $V_H\gamma_1$DM transfectoma supernatants as shown in Figure 2A. This was despite the fact that $V_H\gamma_1$KDEL, ΔL11$V_H\gamma_1$ and $V_H\gamma_1$DM transfectoma expressed the transgene by RT-PCR (Fig. 1b). By ELISA γ_1 protein was present in soluble intracellular fractions in the case of $V_H\gamma_1$KDEL and ΔL11$V_H\gamma_1$; and in insoluble membrane fractions in the case of $V_H\gamma_1$DM (data not shown).

Next, I used deconvolution microscopy to further verify that each transgenic product was reaching the proper intracellular compartment. To this end, stably transfected J558L cells were stained for both the ER marker calnexin and the human γ_1 portion of the IgH transgene (Figure 3). Three of the engineered IgH, the $V_H\gamma_1$, $V_H\gamma_1$KDEL and $V_H\gamma_1$DM proteins, showed a predominantly vesicular staining that co-localized with calnexin. This is expected for the $V_H\gamma_1$ since the secretory construct must transit through the ER in order to be paired with a light chain and secreted, and for the $V_H\gamma_1$DM since the DM molecule normally transits through the ER before endocytic targeting. The ΔL11 $V_H\gamma_1$ protein showed no co-localization with calnexin, and a predominantly puntiform staining, shown previously to indicate cytosolic localization of an IgH in cell lines expressing high levels of this protein (Persic et al., 1997).

To further ascertain the localization of the $V_H\gamma_1$DM protein in J558L, transfectoma cells were stained for LAMP-1 or MHC class II (not shown). No co localization was found with either marker. In order to determine if the reason for this was due to the acid labiality of antibody molecules, J558L cells were incubated with the endosomal acidification inhibitor chloroquine. Again, no colocalization was found with LAMP-1 or MHC class II (not shown). In fact no punctate staining, indicative of protein presence in small membrane bound organelles, was seen at all. This indicates that while the $V_H\gamma_1$DM is transcribed, produced, partitions into membrane fractions and is not secreted the final location may not in fact be lysosomal, or that the protein is highly unstable in endocytic compartments. The results from this section are summarized in Table 1.

T cell responses generated from mice immunized with targeting constructs coding for the NVDP peptide in the CDR2

In order to assess T cell priming by peptides generated from antigen targeted to different intracellular compartments, I employed a technique known as somatic transgene immunization (STI) to immunize naïve C57Bl/6. The main difference between TLI and STI is that the immunogenic plasmid, which does not differ between the two techniques, is injected directly intra-spleen instead of being used to render B lymphocytes transgenic *ex vivo.* Mice were immunized intra-spleen with 100μg of each plasmid coding for a targeted IgH

and the NVDP peptide expressed in the CDR2. Fourteen, days after immunization splenocytes from immunized mice were harvested and restimulated with either the cognate NVDP peptide, or the non T cell stimulating NANP peptide, and proliferation was measured by [^{3}H] thymidine incorporation. Data is expressed as a stimulation index (SI), which is the ratio of cpm obtained with NVDP divided by cpm obtained with NANP peptide. An SI of greater then three, denoting specific proliferation three times higher then background, is indicative of a positive response. When mice were immunized with the secretory $NV^2NA^3\gamma_1$ construct 6/6 mice responded with an average SI of 22.2±0.97 (Figure 4) in agreement with previous data from this lab. Disappointingly, 0/9 mice responded to immunization with $NV^2NA^3\gamma_1$KDEL with an SI of 1.3±0.2, and 0/12 to immunization with ΔL11$NV^2NA^3\gamma_1$ with an SI of 0.93±0.23 (Figure 4). Thus, IgH targeting to either the ER or cytosol of B cells does not result in priming when a model immunogenic peptide is inserted into the CDR2.

Mice immunized with the $NV^2NA^3\gamma_1$DM construct responded well with 8/8 mice responding with an average SI of 37.07±5.6 (Figure 4). This is significantly higher then mice immunized with the $NV^2NA^3\gamma_1$ plasmid ($p<0.05$). When more mice were immunized and responses were measured by limiting dilution assay, however, there was no significant difference in precursor frequency (Figure 5). Thus, even thought the $NV^2NA^3\gamma_1$DM construct primes a

more vigorous response this is not due to an increase in precursor cell frequency.

B lymphoma cells present CDR 3 associated peptides from cytosolic and ER retained IgH, but not from CDR2 associated antigen

Previous reports have shown that endogenously synthesized antigen is presented via MHC Class II in B lymphoma cell lines. Therefore, I investigated presentation of the $E\alpha_{56-73}$ peptide by the murine B lymphoma cell line B6-2. B6-2 is an, I-$A^{b/d}$ positive B lymphoma cell line that does not express the $E\alpha_{56-73}/A^{b}$ complex naturally (figure 6a). The Eα peptide as a model antigen allowed the use of the Y-Ae and the T cell hybridoma 1H3.1 to assess peptide presentation directly *in vitro.*

In order to further elaborate upon previous data I built separate constructs inserting the Eα peptide into the CDR2 or CDR3 of IgH that targeted the ER or cytosol, and produced stable transfectoma of B6-2 cells that expressed these IgH constructs. Not surprisingly, B6-2 cells that were transfected with ER or cytosol targeted IgH did not present the Eα peptide expressed in the CDR2 of the variable region despite expression at the mRNA level (Figure 6A and data not shown). In contrast when this same peptide was present in the CDR3 it was efficiently presented by B6-2 from IgH that targeted both the cytosol and the ER, as evidenced by Y-Ae binding and 1H3.1 reactivity (Figure 6 A& B). Interestingly, the Eα peptide was not presented

when inserted into either the CDR2 or CDR3 of the secretory IgH (not shown). Which may reflect that B6-2 cells do not easily present exogenous antigen (not shown) Thus, it appears that results concerning T cell priming from the previous section can be reconciled with data showing that ER or cytosolically targeted IgH do not present CDR2 associated peptides. The results from this section are summarized in table 1.

In vivo priming of CD4 T cells by intracellular antigen

Next, I investigated whether endogenously synthesized and processed antigen is able to prime a CD4 T cell response *in vivo*. I used transgenic B lymphocytes as APCs to immunize naïve C57Bl/6 mice, using TLI as a model immunization system. I injected mice with $5x10^3$ transgenic B lymphocytes via the tail vein per our published protocol (Gerloni et al., 2004). Fourteen days after immunization I harvested spleens, restimulated whole splenocytes with the $E\alpha_{56\text{-}73}$ peptide and monitored T cell proliferation by ^{3}H-thymidine incorporation. As shown in Figure 7 a proliferative response was readily detected after immunization with transgenic B lymphocytes, irrespective of the intracellular targeting by transgenic IgH. Splenocytes from C57Bl/6 mice immunized with B lymphocytes transgenic for $NV^2NA^3\gamma_1$, a plasmid coding for an IgH transgene that lacks the $E\alpha_{56\text{-}73}$ peptide, were used as a negative control. These showed no response in 4 mice, and responded with a mean normalized cpm±SEM of 31±31 (normalized cpm determined by subtracting

cpm with media from cpm with E$\alpha_{56\text{-}73}$ peptide). Overall, a CD4 T cell response was observed in 7 out of 10 mice immunized with B lymphocytes transgenic for E$\alpha^3\gamma_1$ with a mean normalized cpm of $6.5x10^4 \pm 1.0x10^4$. In contrast to experiments conducted with NVDP peptide in the CDR2, 11 out of 11 mice immunized with B lymphocytes transgenic for E$\alpha^3\gamma_1$KDEL responded with a mean normalized cpm of $7.3x10^4 \pm 8.6x10^3$. The same was seen for cytosolic antigen where 12 out of 12 mice immunized with B lymphocytes transgenic for ΔL11E$\alpha^3\gamma_1$ responded with mean normalized cpm of $7.0x10^4 \pm 9.2x10^3$. In contrast to results obtained using NVDP peptide, the DM targeting construct displayed no proliferative advantage when the Eα peptide was inserted into the CDR3 ($7.9x10^4 \pm 1.0x10^4$). There was no significant difference in mean normalized cpm between any of the four groups immunized with Eα containing plasmids. This indicates that T cell epitopes inserted into the CDR3 of an IgH molecule generate immunogenic peptides for MHC Class II presentation by an endogenous pathway, and are able to prime a T cell response in naïve mice irrespective of whether antigen is retained in the ER or in the cytosol.

In order to verify that the cells proliferating were in fact CD4 T cells I separated $CD4^+$ and $CD8^+$ T cells from the spleens of immunized mice by negative selection and stimulated each population separately with Eα peptide. The two T cell populations (CD4 and CD8) were > 90% pure by FACS analysis (data not shown). I then stimulated CD4 or CD8 T cells with

irradiated LPS blasts made from naïve syngeneic mice pulsed with the $E\alpha_{56\text{-}73}$ peptide. As shown in Figure 8 proliferation occurred only in the population enriched for $CD4^+$ T cells stimulated with LPS blast pulsed with the $E\alpha_{56\text{-}73}$ peptide. This confirms that CD4 T lymphocytes are in fact being primed *in vivo* by the $E\alpha_{56\text{-}73}$ peptide presented by transgenic B lymphocytes.

Priming occurs without the help of host APC

To determine if T cells primed *in vivo* were a consequence of direct antigen presentation by transgenic B lymphocytes, I immunized wild-type C57Bl/6 mice with transgenic B lymphocytes generated using MHC Class II deficient mice. I reasoned that even though secretion of the IgH protein had been abrogated by intracellular targeting, and I had *in vitro* evidence for presentation of the $E\alpha_{56\text{-}73}$ peptide generated from processing of the endogenously synthesized IgH protein, T cell priming *in vivo* could still be due to a "bystander" effect mediated by host APCs. This could result from (a) the shedding of peptides by the injected transgenic B lymphocytes, (b) low level secretion of antigen or (c) generation of apoptotic bodies of transgenic B lymphocytes, yielding secondary uptake by host APCs. Upon immunization with MHC Class II deficient B lymphocytes transgenic for either $E\alpha^3\gamma_1$KDEL or ΔL11$E\alpha^3\gamma_1$, C57Bl/6 mice did not produce a measurable T cell proliferative response (Figure 9). In contrast, mice immunized with MHC Class II competent transgenic B lymphocytes responded well. This was depite the fact

that MHC class II negative B lymphocytes were able to be transfected and transcribed antigen (not shown). This experiment demonstrates that MHC Class II expression on transgenic B lymphocytes used for immunization is necessary for the generation of immunity *in vivo*. Therefore, one can exclude the possibility that the previous T cell responses could be attributed to priming by recipient host dendritic cells, macrophages or B lymphocytes. This result is in agreement with similar experiments in *rel* B (-/-) bone marrow chimeric mice (Gerloni et al., 2004) whose dendritic cells are unable to present antigen (Castiglioni et al., 2003).

Th1 bias following priming by antigen retained in the ER

In the above experiments I saw no difference in the T cell response among mice immunized with B lymphocytes transgenic for the various plasmids, indicating that differential intracellular targeting does not affect the ability of endogenously synthesized antigen to prime CD4 T cells. That did not exclude, however, the possibility that the responding T cells may possess a different Th phenotype with respect to cytokine secretion.

To test the possibility that primed CD4 T cells could be distinguished by their cytokine profile I analyzed the cytokine content of supernatants of T cell cultures 48 hours after *in vitro* restimulation with the $E\alpha_{56\text{-}73}$ peptide. Cytokines (IL-2, IFN-γ, TNFα, IL-4) were measured by cytometric bead array assay. In comparison to priming with B lymphocytes transgenic for $E\alpha^{3}\gamma_{1}$ or

ΔL11 E$\alpha^3\gamma_1$, priming with B lymphocytes transgenic for Eα^3KDELγ_1 induced significantly higher secretion of the Th1 cytokines IFN-γ and TNFα by responding T cells (Figure 10, upper panels). However, there was no significant increase in IL-2 level between the three groups (Figure 10, lower left panel). Secretion of the Th2 cytokine IL-4 was similarly comparable in all three groups (Figure 10, lower right). The results of this section with background levels obtained with media are also captured in table 2.

Overall, priming with B lymphocytes transgenic for E$\alpha^3\gamma_1$KDEL resulted in higher secretion of Th1 cytokines IFN-γ and TNFα. This could be a result of retention of antigen in the ER since E$\alpha^3\gamma_1$ transgenic B lymphocyte priming does not result in such a response. This possibility is examined in detail in the next chapter.

Discussion

The results presented in this chapter help to delineate rules by which the *in vivo* processing and presentation of intracellular antigen can occur. When antigen is inserted into the CDR2 of an IgH molecule *in vivo* priming only occurs when antigen is targeted to the secretory pathway or the endocytic system. The observation that DM targeting induced a more intense immune response then secretory antigen is not surprising. In theory, the DM molecule would target the MIIC or late endosomes and increase the overall density of antigen in MHC class II loading compartments. This may be a result of more

efficient targeting of the MIIC due to the DM molecules normal association with MHC class II. While I could never formally demonstrate MIIC targeting circumstantial evidence seems to indicate that the DM targeting motif, at least, put the IgH in that vicinity. The relative instability of IgG molecules in acidic conditions, such as those present in endocytic compartments may have doomed the experiments to definitively localize the DM molecule. Previous studies that have tried to achieve endocytic targeting of antigen using either the LAMP-1 or the transferrin receptor molecule were conducted with proteins that are more stable, like OVA, thus allowing for easier detection in endocytic compartments. DM targeting conferred advantage when a peptide was inserted into the CDR2, but not, however, when inserted into the CDR3 indicating that the enhancement effect seen with DM targeting is dependent on the context of the immunogenic peptide in the targeted protein. Thus, practical use of DM targeting, to enhance immune response, may be limited and require evaluation on an individual basis

Experiments performed in vitro indicated that the Eα peptides inserted into the CDR2 of an IgH targeted to the ER or cytosol is not effectively processed or presented, explaining the lack of priming by CDR2 associated NVDP *in vivo*. These results indicate strongly that at least part of the processing pathway involved in presentation lies outside of endocytic compartments, since antigen targeting the MIIC effectively primes an immune response. In contrast, when the Eα peptide was inserted into the CDR3

presentation was readily observed after IgH targeting to the ER or cytosol. The reason for this discrepancy is not clear, but it does indicate that the selection of epitopes for presentation by MHC class II from intracellular antigens is more stringent then for exogenous antigens. Results that relate specifically to the processing and presentation pathway used by ER and cytosolic antigen will be discussed in more detail in chapter 4.

One of the major points of the work presented in this chapter is that peptides derived from processing of endogenously synthesized antigen are able to prime CD4 T cell responses in naïve mice irrespective of whether antigen is retained in the cytosol or in the ER. This point is predicated on whether the antigen can be effectively presented to begin with, since only antigens expressed in the CDR3 loop of an IgH were able to be presented *in in vitro* and prime an immune response *in vivo*. I assert that CD4 T cell priming *in vivo* was a result of antigen presentation by B lymphocytes that process antigen synthesized endogenously, in the absence of any secondary uptake of exogenous antigen. This is supported by the fact that transgenic B lymphocytes were prepared *ex vivo* before *i.v.* injection, no secondary uptake of antigen by non-transfected B lymphocytes could be demonstrated *in vitro*, and no immunity could be induced using transgenic B lymphocytes lacking MHC Class II. To my knowledge this is the first formal demonstration of Class II restricted priming of CD4 T cells, by primary professional APC *in vivo* where the conditions of intracellular localization of antigen, its processing and

presentation, were predetermined *ex vivo*. Although, it is known that many microbial pathogens are able to induce both CD4 and CD8 T cell responses *in vivo*, the precise mechanism for CD4 T cell priming remain obscure. For instance, during initial stages of infection by viruses or intracellular bacteria APCs generate peptides for MHC Class II presentation by processing antigens taken up from the extra-cellular milieu via scavenger receptors, or through phagocytosis or macropinocytosis. Thus, during viral or bacterial infection APCs are able to process and present exogenous and endogenously synthesized antigen. In the system described here, we show that antigen processed through an exogenous pathway can be dispensable for T cell priming.

A second consideration is that MHC Class II-restricted CD4 T cell responses primed by APCs in which antigen is endogenously synthesized induce T cells that undergo differential secretion of cytokines dependent on the intracellular targeting of antigen. Significantly higher levels of the Th1 cytokines (IFN-γ and TNFα) were induced in responding T cells by immunization with B lymphocytes transgenic for antigen retained in the ER. This observation has intriguing basic immunological and practical consequences.

From a basic immunological standpoint this result is interesting because it is not immediately clear why antigen retained in the ER might induce a CD4 T cell response with a Th1 bias when compared to cytosolic or

secretory antigen. *In vitro,* there is not an increase in presentation when compared to cytosolic antigen, ruling out increased presentation as a culprit. One possibility would be that an over accumulation of proteins in the ER triggers an ER stress response resulting in a heightened inflammatory environment, leading to more efficient CD4 T cell priming. This process may mirror events occurring during natural infection by viruses which in many instances induce a stress response in the ER. Herpes simplex virus, hepatitis B and C viruses, hantavirus as well as a neurovirulent murine virus, $FrCas^E$, can all induce some level of ER associated stress after infection (Cheng et al., 2005; Ciccaglione et al., 2005; Dimcheff et al., 2004; Li et al., 2005). Accordingly many viruses have developed strategies to evade the most immediate and threatening consequence of ER stress, cessation of protein production. For example HSV dephosphorylates EIF2α so that infected cells continue to translate viral RNA's (Boyce et al., 2005). While viruses can block some of the aspects of ER stress that are detrimental to their survival, it is not clear whether they can block effects that lead to inflammation and heightened immunogenicity.

From a practical stand point it appears that ER targeting of antigen in a DNA vaccine may provide an important adjuvant effect useful for therapeutic purposes. A Th1 bias in response to antigen retained in the ER of APCs may be beneficial for immune response to many pathogens. Th1 responses are necessary for a protective immune response to intracellular bacteria such as

M. tuberculosis and *L. monocytogenes,* responses that depend on IFN-γ (Kaufmann and van Embden, 1993). Similarly, the establishment of stable T cell responses that make susceptible mice resistant to *Leishmania major* require the induction of Th1 responses (Bretscher et al., 1992). Analogous considerations have been made for the human immunodeficiency virus (HIV) where an early loss of Th1 cytokines due to the rapid elimination of HIV-specific $CD4^+$ Th1 cell population is commonly observed and likely linked to the progression of AIDS (Shearer and Clerici, 1998). Finally, Th1 cytokines have been shown to suppress hepatocellular HBV gene expression in HBV transgenic mice by a noncytolytic process (Guidotti et al., 1994).

Enhanced Th1 responses are also considered of benefit in anti- tumor responses. The beneficial effect can be due to any of the following mechanisms: (a) sustained activation and maintenance of CD8 T lymphocytes (Janssen et al., 2003; Sun and Bevan, 2003), (b) upregulation of the MHC molecules on tumor cells of non hemopoietic origin hence increasing their elimination (Dighe et al., 1994), (c) direct cytotoxicity of tumor cells (Fransen et al., 1986; Williamson et al., 1983),and (d) induction of inhibitors of angiogenesis by tumor cells (Coughlin et al., 1998; Qin and Blankenstein, 2000). Thus, simple expedients to increase the production of IFN-γ and TNFα by vaccination with engineered APCs could be beneficial against for therapeutic responses against, latent viral infections, intracellular pathogens and tumor cells.

Figure 2.1 Schematic of targeting constructs. All IgH contain a mouse variable region (blue) and a human constant region (red). The secretory leader sequence was deleted for cytosolic targeting. A KDEL sequence (yellow) was added to achieve ER targeting. The Transmembrane and cytosolic portion of the β chain of the DM protein (green) was added to achieve MIIC targeting.

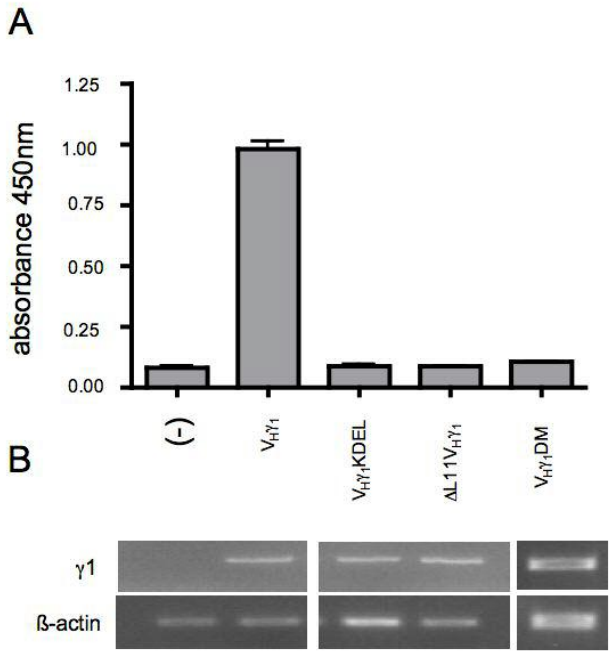

Figure 2.2 IgH targeted to intracellular compartments are not secreted. (A) Sandwich ELISA for the γ1 portion of each IgH performed on supernatants of IgH plasmid transfectoma. (-) denotes pSV2Neo (backbone vector) transfected cells (B) RT-PCR performed for human γ1 mRNA on J558L IgH plasmid transfectoma.

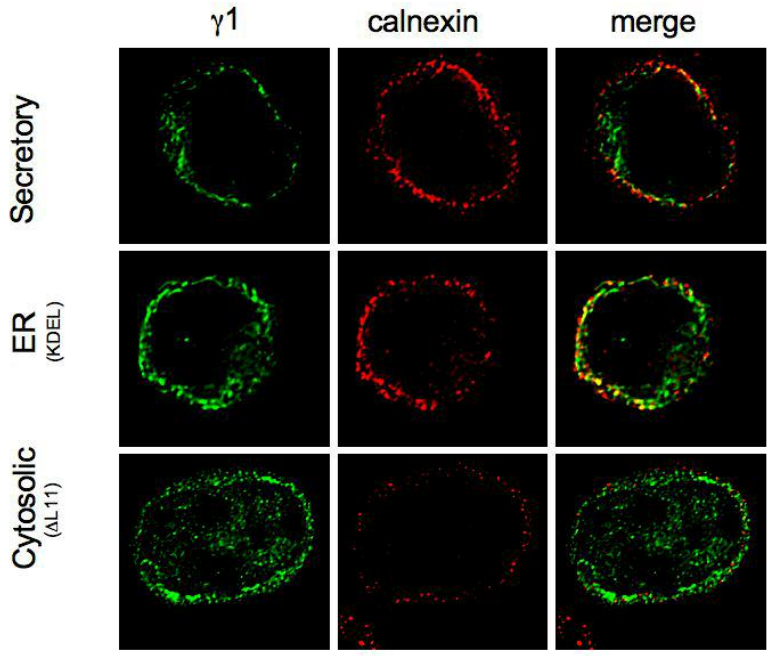

Figure 2.3 Localization of Intracellular targeting vectors with the ER marker calnexin. J558L transfectoma were stained for targeted IgH using a FITC conjugated goat anti-human γ1 antibody (green). Calnexin was detected using a goat anti-calnexin antibody and a donkey anti goat Cy3 labeled secondary (red). Colocalization is indicated in yellow. Cells were spun onto slides and analyzed by a fluorescence microscope at 100x and images were deconvolved using SoftWorx.

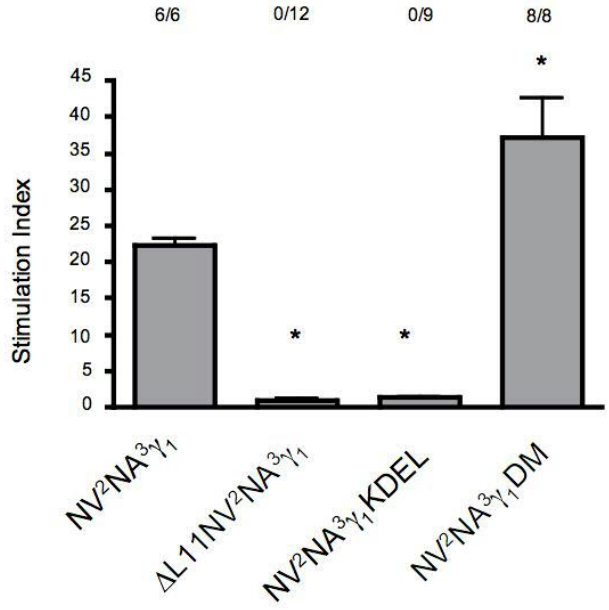

Figure 2.4. Immune response to IgH targeting plasmids with the NVDP peptide inserted in the CDR2. Naïve C57Bl/6 mice were immunized intra-spleen with 100μg of the indicated plasmid. After 14 days mice were sacrificed and restimulated with 50 ug/mL of NVDP peptide and proliferation was measured by ^{3}H-thymidine incorporation. Results are indicated as a stimulation index (cpm with peptide/cpm with media). Mice with a stimulation index significantly different from mice immunized with $NV^{2}NA^{3}\gamma_{1}$ ($p < 0.05$) are indicated with an *.

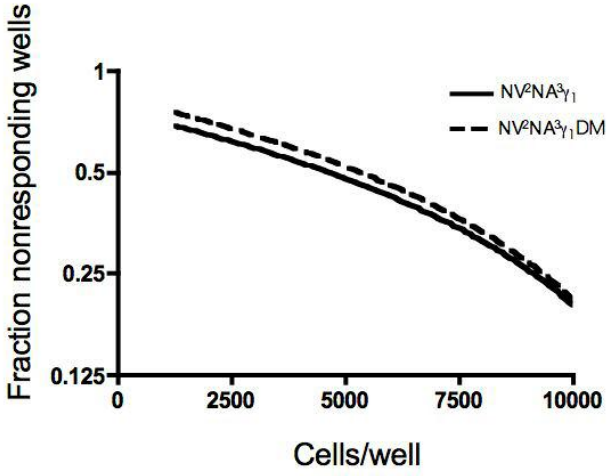

Figure 2.5 Limiting dilution analysis of mice immunized with $NV^2NA^3\gamma_1$ and $NV^2NA^3\gamma_1$DM. Naïve C57Bl/6 mice were immunized with 100µg of indicated plasmid by STI (n=3). Shown is the average fraction of responding wells. The two linear regressions are not significantly different

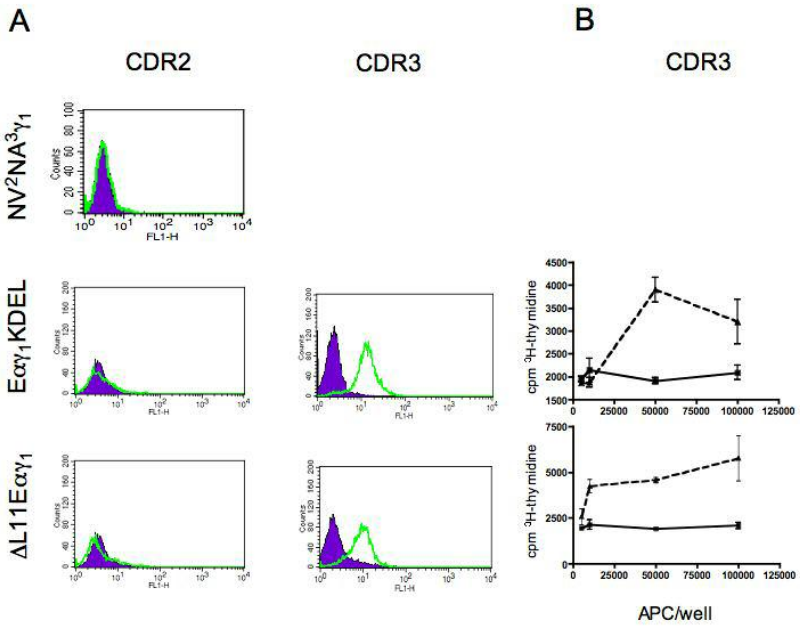

Figure 2.6 Presentation of Eα peptide by B6-2 cells. Transfectoma of B6-2 cells were made with the indicated constructs with the Eα peptide in either CDR2 or CDR3. (A) Cells were stained with FITC conjugated Y-Ae antibody (green line) and compared to unstained cells (purple fill). (B) T cell hybridoma 1H3.1 was incubated with the indicated number of $NV^2NA^3\gamma_1$ tansfectoma (solid line) or with $Ea^3\gamma_1$KDEL of ΔL11E$\alpha^3\gamma_1$ cells (dotted lines) for 24 hours and supernatants were used to stimulate CTLL-2 cells. Proliferation was measured by ^{3}H-thymidine incorporation

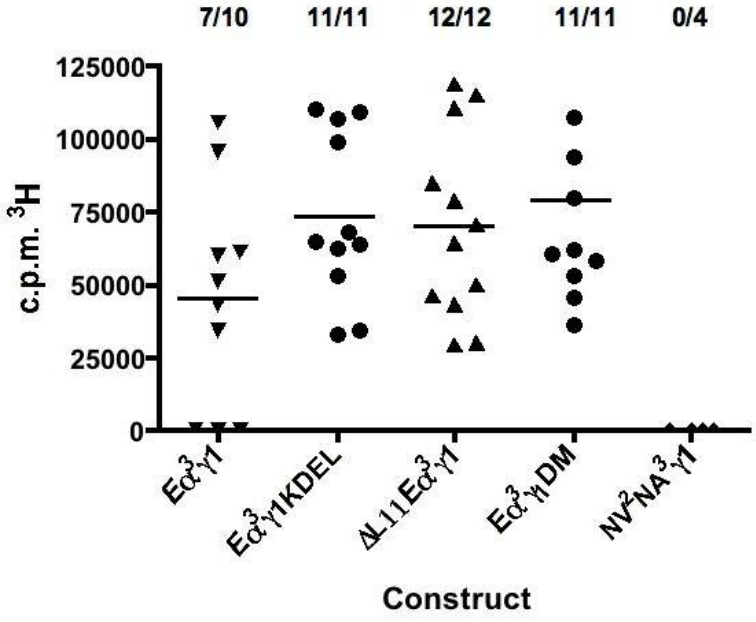

Figure 2.7 Immune response to IgH targeting plasmids with the Eα peptide inserted in the CDR3. Naïve C57Bl/6 mice were immunized by TLI with $5x10^3$ B lymphocytes transgenic for the indicated construct. After 14 days splenocytes were harvested and restimulated with 50ug/mL Eα peptide. Results are shown as normalized proliferation (cpm with peptide-cpm with media

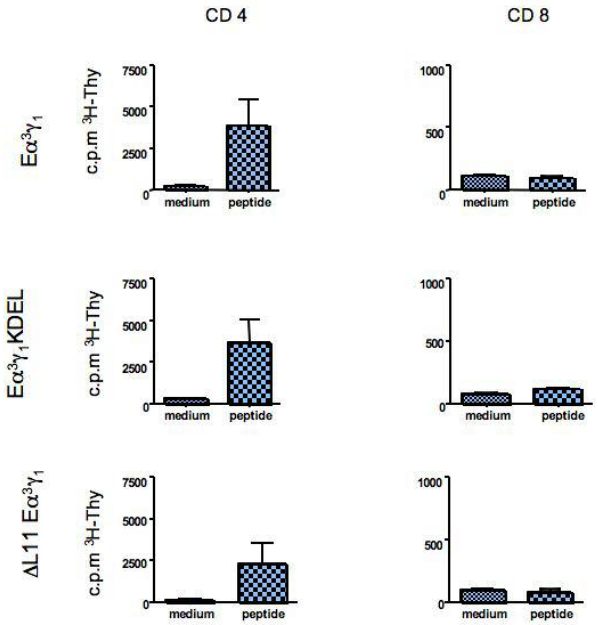

Figure 2.8. CD4 T cells respond to priming with intracellular antigen. Naïve C57Bl/6 mice were immunized by TLI with B lymphocytes transgenic for the specified construct (n=2). At 14 days mice were sacrifieced and spleens were pooled. CD4 or CD8 T cells were purified by negative selection and restimulated with LPS blasts prepared from naïve C57Bl/6 pulsed with Eα peptide or with media.

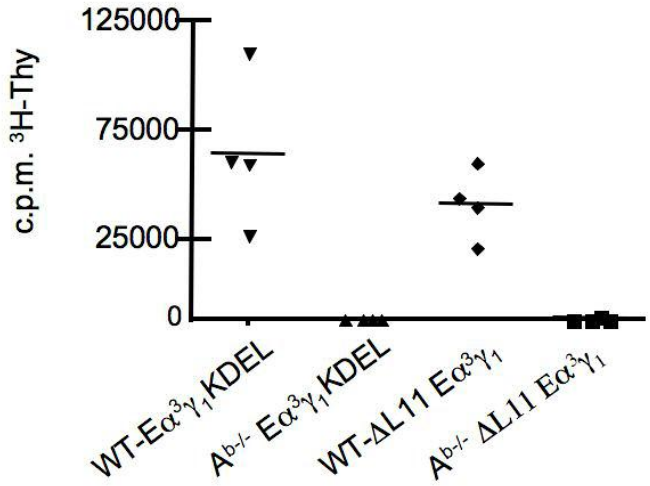

Figure 2.9 Transgenic B lymphocytes require MHC class II expression to prime a T cell response *in vivo*. Naïve C57Bl/6 mice were immunized with $5x10^{3}$ B cells, transgenic for the indicated construct, from either WT or A^{b}-/- mice. After 14 days splenocytes were harvested and restimulated with 50 μg/mL of Eα peptide and normalized proliferation was recorded.

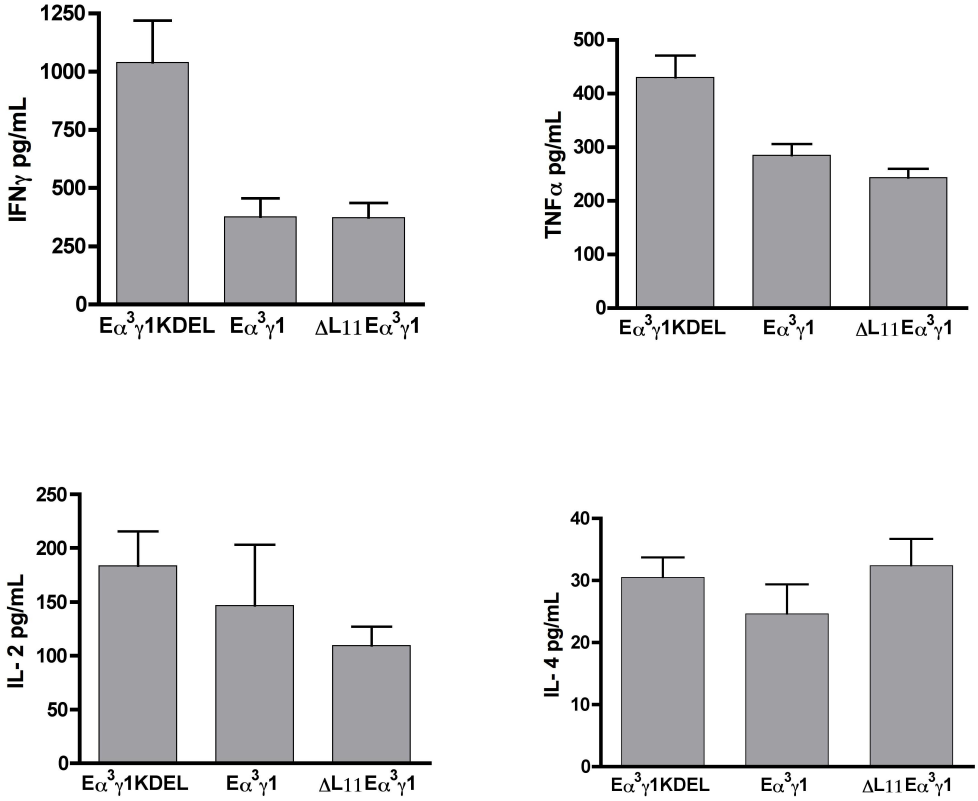

Figure 2.10 T cells primed with ER retained antigen secrete more IFN-γ and TNFα then CD4 T cells primed with secretory or cytosolic antigen. Naïve C57Bl/6 mice were immunized with $5x10^3$ B lymphocytes transgenic for the indicated construct. After 14 days splenocytes were harvested and stimulated with 50 μg/mL Eα peptide, supernatants were collected at 48 hours and analyzed by cytometric bead array.

Table 2.1 Summerization of *in vitro* targeting and antigen presentation experiments. N/D = Not Done

	Secreted by J558L cells	Colocalized with calnexin in J558L	B6-2 Presentation by Y-Ae	B6-2 Presentation by T-cell hybridoma
$NV^2NA^3\gamma_1$	Yes	Yes	No	No
$E\alpha^3\gamma_1$	Yes	Yes	No	No
$E\alpha^3\gamma_1$KDEL	No	Yes	Yes	Yes
$E\alpha^2\gamma_1$KDEL	N/D	N/D	No	N/D
ΔL11$E\alpha^3\gamma_1$	No	No	Yes	Yes
ΔL11$E\alpha^2\gamma_1$	N/D	N/D	No	N/D
$E\alpha^3\gamma_1$DM	No	Yes	Yes	Yes

Table 2.2. Cytokine levels from responding T cells. Levels are shown in pg/mL ± SEM, and levels that differ significantly from T cells primed with secretory antigen ($p<0.05$) are indicated in **bold**.

	IFN-γ peptide	IFN-γ medium	TNFα peptide	TNFα medium	IL2 peptide	IL2 medium	IL-4 peptide	IL-4 medium
$E\alpha^3\gamma_1$ (n=6)	376.2± 194.9	12.1± 16.6	285.0± 20.90	11.0± 10.2	146.5± 56.95	16.7± 15.1	24.62± 4.718	3.94± 1.7
$E\alpha^3\gamma_1$KDEL (n=7)	**1039.0± 179.5**	11.4± 16.2	**430.3± 40.72**	8.8± 5.3	183.5 ±32.17	21.0± 13.9	30.48± 3.185	5.4± 1.9
ΔL11$E\alpha^3\gamma_1$ (n=8)	372.2± 63.53	5.9± 7.7	242.8± 16.42	8.3± 4.6	109.5± 17.59	21.3± 10.8	32.41± 4.238	7.1± 2.1

Parts of chapter 2 have been submitted to The Proceedings of the National Academy of Sciences, USA under the title, “ER Retention of Antigen by B Lymphocytes Influences Costimulation and CD4 T Cell Priming.” Matthew C. Wheeler, Mara Gerloni, Marta Rizzi and Maurizio Zanetti. I was the main researcher on this paper and the other authors helped direct and design the research.

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Chapter 3

ER stress effects on APC function

Introduction

The ER is the initial destination for many proteins that are secreted or destined for other intra-cellular compartments. As such, the ER plays a pivotal role in the early events of protein production such as: disulphide bond formation, core glycosylation and proper folding. Due to this, the ER is sensitive to perturbations in the normal equilibrium of protein folding and secretion. The process by which the cell re-establishes proper folding/secretion equilibrium is tied to global changes in transcription, translation and activation of proteins that induce chaperone expression, ER biogenesis, protection from and, ultimately, initiation of apoptosis and expression of pro-inflammatory mediators. Overall, this process is known as the ER stress/ unfolded protein response (UPR) pathway.

Three main ER resident sentinel proteins initiate ER stress, PERK, Ire1 and ATF6 (Schroder and Kaufman, 2005; Schroder and Kaufman, 2006). The activity of these three proteins is mainly controlled by

association with the ER resident folding chaperone BiP (GRP78). BiP normally sequesters these proteins in the ER by binding to their N-terminal domains, but when the demand for BiP is high, due to the presence of unfolded proteins, they are released from their association with BiP and initiate ER stress signaling in three very different ways.

PERK (PKR like ER kinase) is a kinase that phosphorylates eukaryotic initiation factor 2α (EIF2α) and the bZIP transcription factor NRF2. The result of this is translational attenuation and upregulation of ER stress responsive genes. There are two different Ire1 genes, Ire1α and Ire1β. Ire1α is constitutively expressed, where as the β form is only expressed in the gut. Ire1 is a trans membrane kinase that auto-phosphorylates and uses an RNAse like domain to induce splicing of the XBP-1 mRNA (hac1 in yeast), spliced XBP-1 then becomes active in transcription of ER stress responsive genes. ATF6, like Ire1, comes in two flavors α and β, it is also a trans membrane molecule that is released by intra membrane protealysis. Upon release ATF6 translocates to the nucleus and by way of a bZIP motif directly induces expression of ER responsive genes. The fact that there are at least three distinct types of ER stress sensing proteins allows for the possibility of more nuanced response to various ER stress inducing conditions, as has been noted recently by Durose et al (Durose et al., 2006). This may be due to functionally distinct promoter elements that induce ER stress responsive genes, or differential

activation of ER stress sentinels. A schematic detailing a few of the ER stress activated gene transcripts is shown in figure 1.

The effect of ER stress on general inflammation has been documented in several studies. Activation of Ire1 proteins in response to stress results in activation of the TRAF2 protein, which in turn leads to activation of the pro-inflammatory proteins JNK and NF-κB (Xu et al., 2005). ER stress has been shown to increase secretion of the inducible inflammatory mediator COX-2 as a result of NF-κB induction (Hung et al., 2004), and promoteote leukocyte adhesion to aortic smooth muscle cells *in vitro*, and colonic epithelium *in vivo* (Majors et al., 2003). *In vivo*, Ire1β deficiency in mice results in chronic ER stress and increased susceptibility to colitis, most likely due the gut specific expression of this Ire variant (Bertolotti et al., 2001). A study by Zhang et al. has shown that ER stress induces cleavage, and nuclear translocation of an N-terminal fragment of the ER membrane CREBH protein that results in the expression of serum amyloid P-component (SAP) and C-reactive proteins (CRP) (Zhang et al., 2006). Interestingly, pro-inflammatory cytokines such as IL-6 and TNFα increased CREBH expression, but did not result in cleavage and, thus, activation. This study also found that ATF6 alone was able to induce expression of SAP and CRP proteins indicating that ER stress can have both direct and indirect effects on systemic inflammatory signals.

ER stress effects on adaptive immunity have not been well documented beyond a necessity for proper antibody folding and secretion. ER stress is a requisite checkpoint for antibody secretion and plasma cell differentiation (Gass et al., 2002; Iwakoshi et al., 2003; Reimold et al., 2001). This is not entirely surprising since plasma cells are highly specialized for the secretion of massive amounts of antibodies, and, thus, require a robust secretory pathway. In fact, an ER stress checkpoint is probably necessary to eliminate B cells producing IgG that cannot be efficiently folded. No studies to date, however, have focused on the role that ER stress might play in T cell priming by an antigen presenting cell. In this chapter I present results that indicate that ER stress may provide an adjuvant effect for the development of Th1 immunity, and show that ER stress concomitant with activation can alter surface expression of costimulatory molecules.

Results

Increase in Th1 response is not due to plasmid effects

To rule out that the effects seen in the previous chapter were not due to intrinsic properties of the plasmids used (e.g., immunostimulatory DNA motifs), I isolated B lymphocytes from naïve mice by negative selection, incubated $1x10^6$ with 25 µg/mL of either $E\alpha^3\gamma_1$KDEL or $E\alpha^3\gamma_1$ plasmid, and recorded proliferation at 48 hours. Since B cell proliferation is a classic

response to B cell mitogens like CpG (Krieg et al., 1995) or endotoxin I reasoned that this would rule out contribution by these factors to the results of the previous experiments. I found that when compared to LPS or CpG ODN, plasmid DNA induced the same proliferation as control ODN (Figure 2A), confirming that plasmid DNA is not mitogenic for B lymphocytes (Cortez-Gozalez et al., 2006; Spies et al., 2003). In contrast, B lymphocytes incubated with $E\alpha^3\gamma_1$KDEL or $E\alpha^3\gamma_1$ plasmid DNA did up regulate the costimulatory molecule CD86 (Figure2B), another marker of B cell activation. More importantly, both experiments show that the two plasmids do not differ in their ability stimulate B lymphocytes through immunostimulatory DNA motifs during the initial stages of B cell activation.

ER retention of antigen by B lymphocytes promotes ER stress and down modulates OX40L expression *in vivo*

I reasoned that ER retention of antigen might lead to ER stress in transgenic B lymphocytes, and that this may influence antigen presenting function. Therefore, I examined whether primary B lymphocytes rendered transgenic with $E\alpha^3\gamma_1$KDEL or $E\alpha^3\gamma_1$ plasmid DNA showed differences in their expression of ER stress responsive genes. When cells undergo ER stress they upregulate the expression of a number of stress responsive genes including GADD34 (MyD116) (Novoa et al., 2001), Grp78 (BiP) (Lee, 1992) and CHOP (GADD 153) (Chen et al., 1992; Wang et al., 1996). Here,

in order to only analyze transgenic B lymphocytes, we engineered plasmids that contained the selectable surface marker K^k, to aid identification and isolation of transgenic B lymphocytes after transgenesis or *in vivo* injection (A schematic is shown if Figure 3). When I analyzed B lymphocytes transgenic for either $E\alpha^3\gamma_1$KDEL-K^k or $E\alpha^3\gamma_1$-K^k at 24 hours I found no upregulation of the ER stress markers GADD34, CHOP and Grp78 (Figure 4a). At 48 hours post transfection I saw repeatable upregulation of ER stress marker genes in $E\alpha^3\gamma_1$KDEL-K^k transgenic B lymphocytes, but not in lymphocytes transgenic for $E\alpha^3\gamma_1$-K^k (Figure 4b). Purified B lymphocytes treated with tunicamycin, a compound that induces ER stress by preventing N-linked glycosylation (Dorner et al., 1990), served as a positive control and resulted in robust expression of all three markers at 24 hours. These results indicate that transgenic B lymphocytes that retain antigen in the ER accumulate enough protein to trigger an ER stress response.

To elucidate factors that could influence the antigen presenting function of B lymphocytes transgenic for $E\alpha^3\gamma_1$KDEL-K^k or $E\alpha^3\gamma_1$-K^k, C57Bl/6 mice were injected i.v. with $1x10^6$ B lymphocytes transgenic for each plasmid. Transgenic cells were then re-isolated from the spleen of injected mice based on expression of the K^k molecule after 3 days *in vivo*, and were compared to B lymphocytes transgenic for a plasmid that expressed only K^k (pMACS K^k). We chose a 3 day time point based on previous data indicating that T cells reach the height of clonal expansion 3 days after

encounter with antigen presenting B lymphocytes (Garside et al., 1998; Gerloni et al., 2000). Twenty four hours after transgenesis we saw no difference in the upregulation of I-A^b or of the costimulatory molecules CD86, CD40 and OX40L between B lymphocytes transgenic for $E\alpha^3\gamma_1$KDEL-K^k or $E\alpha^3\gamma_1$-K^k (Figure 5a-d). Initial experiments showed little movement in CD80 so analyses using this molecule were excluded from this dissertation. Both $E\alpha^3\gamma_1$KDEL-K^k and $E\alpha^3\gamma_1$-K^k transgenic lymphocytes showed upregulation of I-A^b, CD86 and CD40 when compared to non-transfected B cells isolated from the spleens of naïve mice, indicating that some activation had already occurred before injection. However, after 3 days *in vivo*, K^k positive B lymphocytes re-isolated from the spleens of mice injected with B lymphocytes transgenic for either $E\alpha^3\gamma_1$KDEL-K^k or $E\alpha^3\gamma_1$-K^k showed marked upregulation of I-A^b, CD86 and CD40 when compared to mice injected with B lymphocytes transgenic for pMACS K^k only (Figure 5e-g). When I examined these lymphocytes for expression of OX40L, however we found that B lymphocytes transgenic for $E\alpha^3\gamma_1$KDEL-K^k displayed a 3-fold reduction in OX40L expression relative to lymphocytes transgenic for $E\alpha^3\gamma_1$-K^k (Figure 5h). Therefore, transgenic B lymphocytes that express ER retained antigen show a defect in upregulation of the costimulatory molecule OX40L.

ER stress leads to defect in OX40L upregulation in B lymphocytes

I next tried to verify that a state of ER stress could account for down modulation of OX40L in B lymphocytes transgenic for $E\alpha^3\gamma_1$KDEL-K^k. To this end, B lymphocytes were isolated from the spleens of C57Bl/6 mice, then treated with CpG ODN and ER stress inducing compounds, and monitored for their expression of costimulatory molecules. In these experiments I used two compounds that induce ER stress by different mechanisms, tunicamycin and thapsigargin. Notably, thapsigargin inhibits calcium homeostasis in the ER, and does not block N-linked glycosylation like tunicamycin (Thastrup et al., 1990). After 24 hours, CpG treatment caused robust upregulation of cell surface I-A^b, CD86 and CD40 (Figure 6a-c). The inclusion of tunicamycin or thapsigargin, however, did little to impact I-A^b expression (figure 6a). CD86 surface expression was reduced by tunicamycin treatment only (figure 6b), while CD40 only by treatment with thapsigargin (figure 6c). CpG did not upregulate OX40L at this time point (figure 6d). By 40 hours we found that neither compound inhibited I-A^b expression (figure 6e). In fact, tunicamycin appears to even increase I-A^b expression. CD86 expression was inhibited only by tunicamycin (figure 6f), and CD40 (figure 6g) expression was similar after either treatment. However, in comparison to CpG treatment alone, both compounds blocked the cell surface expression of OX40L (figure 6h). These experiments serve as proof of principle that ER stress can lead to down modulation of OX40L expression in B lymphocytes.

Discussion

In the experiments presented in this chapter I show that primary B lymphocytes transfected with an IgH encoding a KDEL motif induces up regulation of ER stress markers. I further show that these same cells have reduced cell surface expression of OX40L after 3 days *in vivo*. Moreover, two different ER stress inducing compounds, tunicamycin and thapsigargin are able to block up regulation of OX40L on CpG stimulated B cells.

The fact that up regulation of ER stress markers is seen only in B lymphocytes transfected with ER retained IgH, and not secretory IgH, is significant since CD4 T cells primed by B lymphocytes presenting ER retained antigen exhibited significant increase in IFN-γ and TNFα release as compared to T cells primed by secretory antigen. How ER stress causes this increase is unclear since ER stress can induce secretion of general inflammatory cytokines, like COX-2 and TNFα (Hung et al., 2004), but not necessarily Th1 patterning cytokines. Costimulation, however, has a great effect on the ensuing immune response and the fact that OX40L appears to be specifically influenced by ER stress signals is potentially interesting.

OX40L is a member of the tumor necrosis factor receptor family (Watts, 2005). It reaches its peak of expression 2-3 days after APC activation and engages its cognate receptor OX40 that is expressed on

activated T cells (Croft, 2003). OX40-OX40L interactions have been shown to stabilize expression of the anti apoptotic molecules Bcl-2 and Bcl-XL (Rogers et al., 2001). In line with results in this chapter it has been implicated in the function of CD4 T cells by determining initial clonal expansion and memory (Gramaglia et al., ; Salek-Ardakani and Croft, 2006). It has been found to positively influence Th2 responses in mouse models (Linton et al., 2003; Salek-Ardakani et al., 2003), and engagement on B cells drives proliferation and cytokine secretion. Recently studies by Lund et al. have shown that the interaction between Th cells and B cells is important in instructing B cell commitment to type 1, IFN-γ secreting and type 2, IL-4 secreting (Harris et al., 2005a; Harris et al., 2005b). It is possible that the absence of OX40L signaling interferes with signaling necessary for type 2 commitment, therefore, allowing a greater niche for type 1 B cell development.

While it is normally portrayed as an APC specific costimulatory molecule, it is also expressed by activated T cells, epithelial cells, and at sites of inflammation associated with atherosclerotic lesions, and could be responsible for T cell adhesion to these sites (Watts, 2005), and the stabilization of recall responses (Mestas et al., 2005). Given this, it is likely that OX40L participates in immunity beyond initial priming and that ER stress effects on APC and non-APCs could impact T cell priming and subsequent peripheral immune responses.

In conclusion, ER stress, which has previously not been known to influence T cell priming, can play a role in the induction of CD4 T cell mediated immunity. The down stream effects of ER stress, as they relate to T cell priming, provides new fertile ground for explorations into how the cellular environment can influence immunity. The significance of these findings is supported by the fact that viral or bacterial infection has been associated with ER stress.

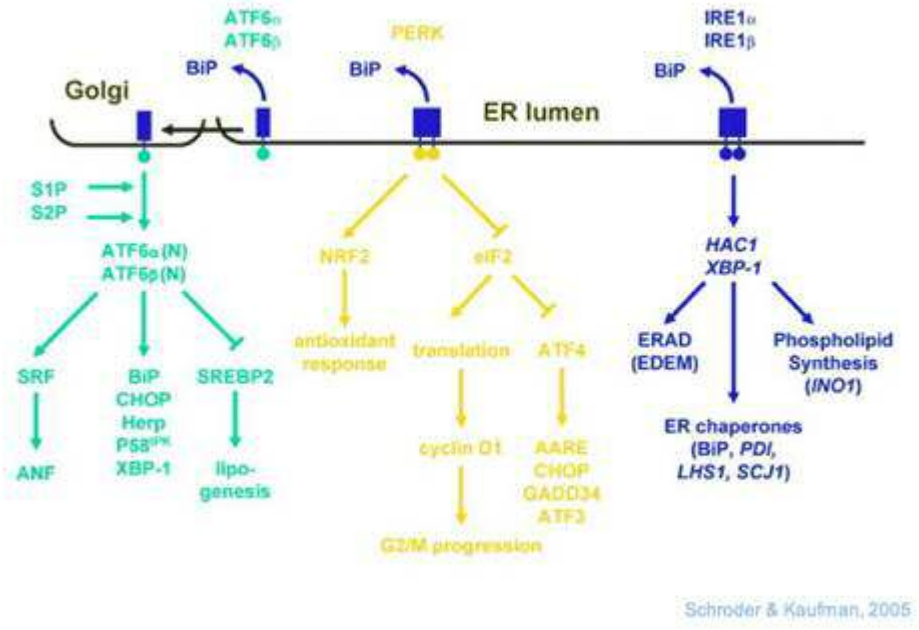

Figure 3.1 Schematic of the ER stress pathway. ER stress institutes a multifaceted transcriptional response. The three main ER sensors ATF6, PERK and IRE1 each initiate transcription of both distinct and overlapping genes

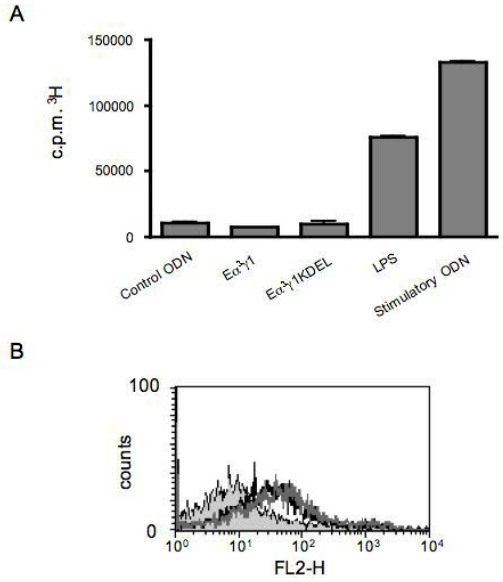

Figure 3.2 Differences in plasmid DNA composition do not account for different immune responses *in vivo*. (A) Splenic B cells were isolated by negative selection and incubated with non-CpG ODN (black bar), 25 µg/mL $E\alpha^3\gamma_1$(grey bar), 25 µg/mL $E\alpha 3\gamma_1$KDEL (open bar), 2.5 ug/mL LPS (checkered bar), or 0.1 µM CpG ODN 1826 (cross-hatched bar) for 24 hours. Proliferation was determined by ^{3}H-thymidine incorporation. Values represent means ± SD of triplicate wells. (B) Splenic B cells were isolated by negative selection and incubated with 25 µg/mL $\gamma 1E\alpha^3$ (grey line), 25 µg/mL $\gamma 1E\alpha^3$KDEL (black line) for 24 hours and then analyzed for CD86 by FACS. Untreated cells (grey fill) served as a control.

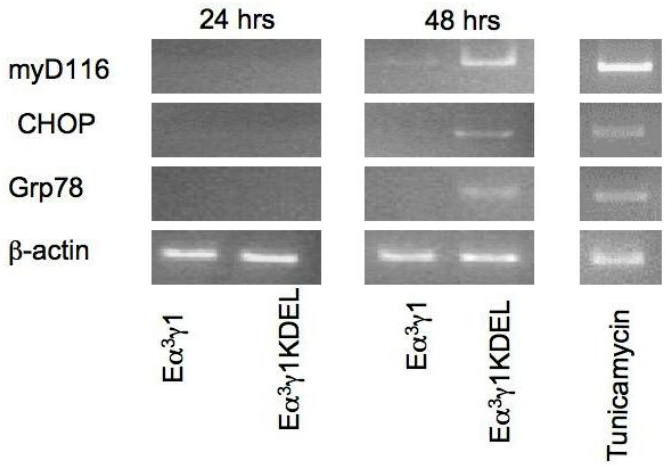

Figure 3.3 Primary B lymphocytes that express ER retained antigen upregulate transcription of ER stress responsive genes. B lymphocytes were rendered transgenic for either $E\alpha^3\gamma_1$-K^k or $E\alpha^3\gamma_1$KDEL-K^k and K^k positive cells were isolated at 24 and 48 hours. Total RNA was extracted and RT-PCR was performed for ER stress induced genes GADD34, Grp78 and CHOP. RNA from splenic B lymphocytes isolated by negative selection, and treated with 5ug/mL of tunicamycin for 24 hours served as a positive control.

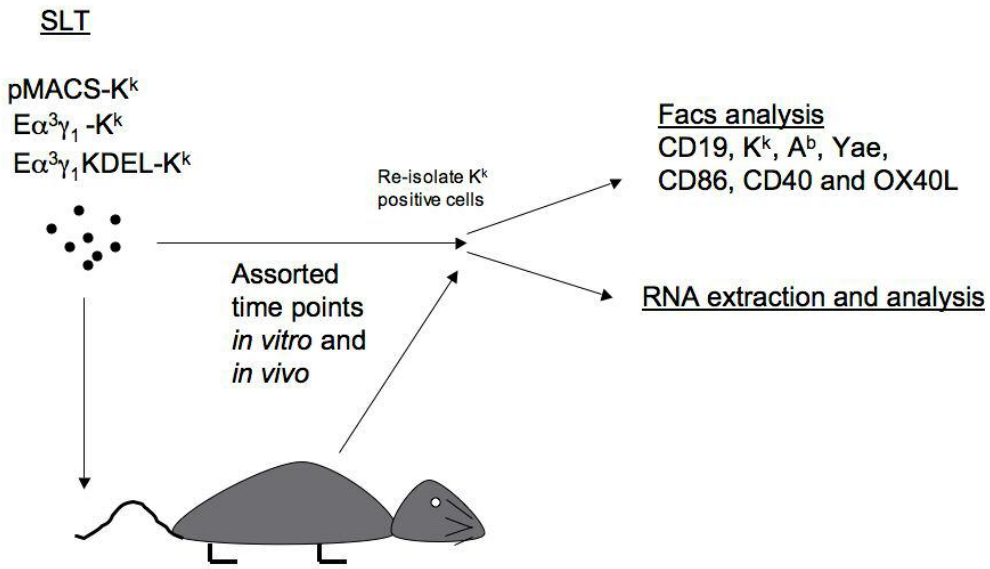

Figure 3.4 Schematic of re-isolation experiments.

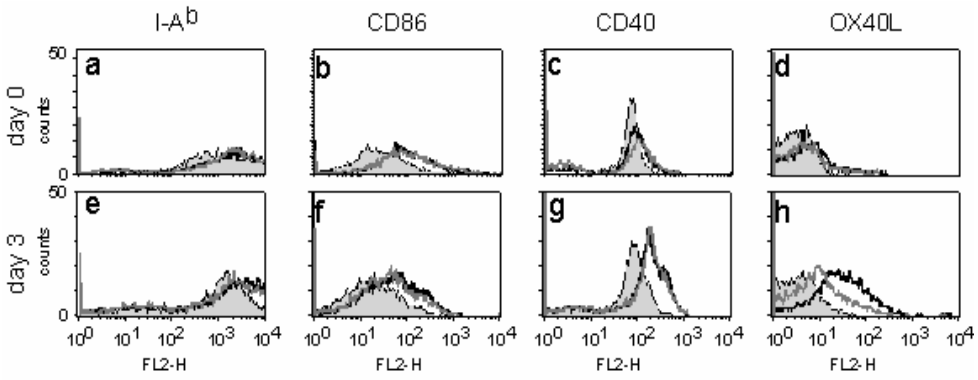

Figure 3.5 Primary B lymphocytes that express ER retained antigen show diminished OX40L expression *in vivo*. B lymphocytes were subjected to spontaneous transgenesis with either E$\alpha^3\gamma_1$-K^k (black line) or E$\alpha^3\gamma_1$KDEL-K^k (grey line). K^k positive lymphocytes were isolated 24 hours later, and analyzed for expression of I-A^b (a,e), CD86 (b,f), CD40 (c,g) or OX40L (d,h) at day 0 (top row) and day 3 (bottom row) after i.v. injection.

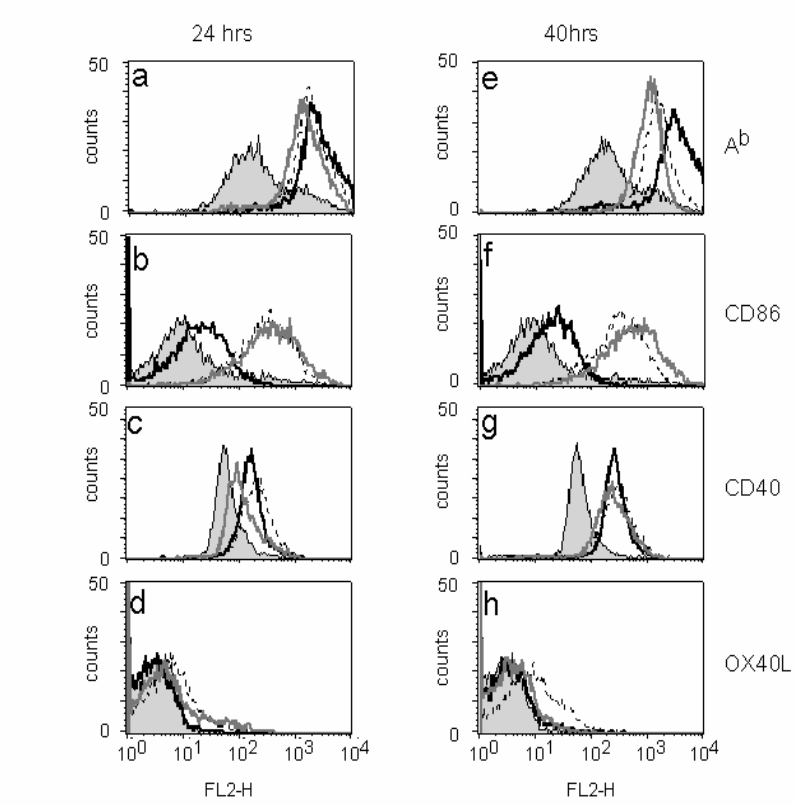

Figure 3.6 ER stress inducing compounds prevent OX40L upregulation in primary B lymphocytes. Primary splenic B lymphocytes were isolated by negative selection and treated with either 0.1 μM CpG ODN 1826 alone (dotted line), CpG ODN 1826 plus 5μg/mL tunicamycin (black line) or with CpG ODN 1826 plus 300 nM thapsigargin (grey line). Untreated cells (grey fill) served as a negative control. Expression of I-A^b (a,e), CD86 (b,f), CD40 (c,g), and OX40L (d,h) were measured at 24 (left column) and 40 hour (right column) time points by FACS.

Parts of chapter 3 have been submitted to The Proceedings of the National Academy of Sciences, USA under the title, "ER Retention of Antigen by B Lymphocytes Influences Costimulation and CD4 T Cell Priming." Matthew C. Wheeler, Mara Gerloni, Marta Rizzi and Maurizio Zanetti. I was the main researcher on this paper and the other authors helped direct and design the research.

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Chapter 4

Processing pathways for presentation of ER retained antigen

Introduction

While processing pathways have been defined for the presentation of cytosolic antigen, the same cannot be said for ER retained antigen. Previous work has disputed the ability of peptides to bind MHC class II in the ER. Further considerations also cast doubt on the ER as a main compartment for MHC class II restricted antigen processing, most notably the presence of the Ii chain, which prevents peptide binding, and the lack of proteolytic activity in this organelle. Previous work has indicated that antigen expressed in the cytosol can be processed in a number of ways, and, thus, may serve as a common staging area for the processing of intracellular antigens.

ER associated degradation

In the previous chapter I showed that primary B lymphocytes transfected with ER retained IgH up regulate expression of ER stress markers. One consequence of ER stress is the augmentation of ER

associated degradation (ERAD), a process that results in the cytosolic translocation and degradation, of misfolded proteins (Meusser et al., 2005). Degradation occurs by cytosolic proteases, usually the ubiquitin-proteasome pathway, but also through other resident cytosolic proteases (Fayadat et al., 2000; Mancini et al., 2003; Romisch, 2005; Schmitz and Herzog, 2004; Tsuda et al., 2006). One of these is the tripeptidyl peptidase II (TTPII), that, like the proteasome, has been implicated in the processing of MHC class I restricted antigens (Geier et al., 1999; Seifert et al., 2003). However, its role in processing antigen has never been explored.

The main purpose of ERAD is to provide a quality control checkpoint that ensures that all proteins entering the ER interact with appropriate chaperones, and are folded properly before transport to the golgi complex and more subtle protein manipulations occur. Enzymes that process the core oligosaccharide added to asparagines, present on most secretory proteins, control the ERAD pathway (Helenius and Aebi, 2004). This oligosaccharide consists of three glucoses, nine mannoses and two N-acetylglucosamines ($Glc_3Mann_9GlcNAc_2$, Figure 1). The two terminal glucose residues are progressively cleaved by glucosidase I & II. This allows glycoproteins to interact with the chaperone calnexin (cnx) or calreticulin (crt). After proteins are bound to cnx/crt and allowed to properly fold the final glucose is cleaved releasing it from cnx/crt. Proteins that are still misfolded are recognized by UDP glucose:glycoprotein glucosytransferase, reglucosyltated and re-enter

the cnx/crt cycle . Proteins that are unable to fold properly are, in time, trimmed by α-mannosidase I, extracted from the cnx/crt cycle by the ER degradation enhancing α-mannosidase like protein (EDEM) (Molinari et al., 2003) and ultimately translocated to the cytosol through the Sec61 channel protein. The exception is that some glycoproteins such as immunoglobulins bind to BiP upon entry into the ER, and therefore do not require glucosidase processing (Gala and Morrison, 2004; Molinari and Helenius, 2000). However, they are still processed by α-mannosidase I (Fagioli and Sitia, 2001). In this chapter I will detail experiments that examine the role of ER stress and ERAD in the MHC class II processing and presentation of ER retained antigen.

Results

Inhibition of a cytosolic protease reduces presentation of an ER restricted antigen

In order to further elucidate pathways that lead to presentation of an ER retained antigen, I used the B6-2 transfectoma generated in chapter two as an *in vitro* system of Eα peptide presentation. I incubated B6-2 cells transfected with $E\alpha^3\gamma_1$KDEL and ΔL11$E\alpha^3\gamma_1$ with compounds previously shown to inhibit processes that have been implicated in MHC class II presentation, and looked for reduction in staining with the Y-Ae antibody, indicative of Eα peptide presentation.

Lactacystin or MG-132, both proteasome inhibitors, had no impact on presentation of the Eα peptide (Figure 2 and data not shown) by either cell line, indicating that the proteasome is not responsible for presentation of the Eα peptide from ER or cytosolically targeted IgH. Inhibition of bulk autophagy by 3-methyladenine (3MA) or wortmanin (Figure 2 and data not shown) had no effect on presentation in either cell line, ruling out bulk autophagy as a main route for the presentation of Eα peptide from cytosolic and ER targeted IgH. This is further supported by the fact that inhibitors of lysosomal processing chloroquine and leupeptin had paradoxical effects with leupeptin but not chloroquine inhibiting Eα presentation (Figure2). Overall, this implies that lysosomal processing is not necessary for Eα presentation, since leupeptin is not a highly specific protease inhibitor, if lysosomal processing were occurring then chloroquine would also have a negative effect on display of the Eα peptide. Most likely leupeptin is non specifically inhibiting a non-lysosomal protease that is involved in the processing of IgH. Interestingly, the TTPII inhibitor AAF-CMK (Figure 2) strongly inhibited presentation of the Eα peptide in both cell lines. This implies that in this system cytosolic and ER retained antigen share a common pathway that requires both antigens access the cytosol. Of course this could be the result of general toxicity of AAF-CMK to antigen presentation, but this is made unlikely since AAF-CMK does not reduce overall levels of A^b (not shown) and is thought to be a specific inhibitor of the TTPII (Geier et al., 1999).

In order to discern whether the TTPII was acting on ER and cytosolically targeted IgH in the cytosol, and not in some other cellular compartment, I incubated both E$\alpha^3\gamma_1$KDEL and the ΔL11E$\alpha^3\gamma_1$ transfected cells with AAF-CMK or leupeptin, and used deconvolution microscopy to localize undegraded antigen in the cell. Cells were incubated with AAF-CMK or leupeptin for 16 hours and then fixed, permeabilized and stained for both the γ1 portion of each IgH and the ER marker calnexin (Figure 3). With out AAF-CMK γ1 staining in E$\alpha^3\gamma_1$KDEL transfected B6-2 cells was largely vesicular and associated closely with calnexin positive vesicles (Figure 3A). After AAF-CMK treatment for 16 hours γ1 staining showed less association with calnexin positive vesicles and a more diffuse staining (Figure 3B) indicative of cytosolic localization that is seen with ΔL11E$\alpha^3\gamma_1$ transfected B6-2 cells (Figure3D). These results were similar for leupeptin treatment (Figure 3C), indicating that leupeptin may have some inhibitory activity against the TTPII. Therefore, it appears that the TTPII is acting upon both the E$\alpha^3\gamma_1$KDEL and the ΔL11E$\alpha^3\gamma_1$ IgH in the cytosol.

Presentation of ER retained IgH is partially dependent on Mannose trimming

Since it appeared that ER retained IgH was being delivered to the cytosol for processing I used inhibitors for Glucosidase I, Mannosidase I and Mannosidase II to probe the contribution of these enzymes to Eα peptide

processing. In these experiments B6-2 transfected with ΔL11E$\alpha^3\gamma_1$ serves as a negative control since IgH in these cells should not be processed by enzymes in the ERAD pathway due to cytosolic localization. Australine, an inhibitor of the glucosidase I enzyme had no effect on Eα presentation from cytosolic or ER retained IgH (Figure 4). This is to be expected for the E$\alpha^3\gamma_1$KDEL IgH since it associates with the chaperone BiP and not calnexin. Kifunensine on the other hand, a specific inhibitor of mannosidase I, had a small, but repeatable effect on presentation of the Eα peptide only from E$\alpha^3\gamma_1$KDEL IgH (Figure 4). This decrease in Eα peptide presentation agrees with data from Fagioli et al. showing that orphan IgM heavy chain and J chain degradation is impaired by kifunensine and not australine, but this impairment is not absolute, as kifunensine treatment merely slows degradation (Fagioli and Sitia, 2001). The fact that the decrease is small may reflect that other unknown pathways may also be responsible for IgH degradation. Also, this decrease may be partially masked by slight A^b upregulation in Kifunensine treated cells (not shown). Swainsonine, a mannosidase II inhibitor, failed to inhibit presentation of the Eα peptide, as neither the E$\alpha^3\gamma_1$KDEL nor the ΔL11E$\alpha^3\gamma_1$ IgH is expected to be processed by this enzyme which has little impact on the ERAD pathway (Figure 4).

ER stress plays a role in the presentation of peptides from ER retained antigen

In order to probe the requirement for ERAD mediated processing for Eα peptide presentation I used a previously published siRNA target sequence to knockdown expression of the EDEM protein (Molinari et al., 2003). Using the BLOCK-iT siRNA vector system, I made a construct that allowed expression of siRNA from a plasmid that conferred blasticidin resistance. This allowed the generation of B6-2 cell lines that expressed either $E\alpha^3\gamma_1$KDEL or ΔL11$E\alpha^3\gamma_1$ IgH, and siRNA against EDEM, or non-specific siRNA to serve as a negative control. The results of this experiment yielded unexpected results, as siEDEM and $E\alpha^3\gamma_1$KDEL transfected B6-2 cells showed a 70% upregulation of EDEM expression by real time PCR and a down modulation of Eα peptide display (Figure 5). Even more puzzling is the fact that siEDEM and ΔL11$E\alpha^3\gamma_1$ transfected cells actually saw a 45% decrease in EDEM expression by real time PCR, with no impact on Eα peptide display (Figure 5). Results published since then have shed light on this experiment as the discovery of another EDEM variant, EDEM2, has been discovered (Olivari et al., 2005). This variant is highly similar to EDEM and expression is induced by ER stress. Looking back at the siRNA primers used, indicates that the regions targeted are dissimilar between EDEM1 and EDEM2, where as the sequences targeted by the real time PCR primers are conserved between the two proteins. Thus, siRNA against the constitutively expressed EDEM1 would not have been active against the ER stress inducible EDEM2, but the real time primers used would have detected both mRNAs. This is consistent with the data shown

since the expression of the ER stress induced version of EDEM would be exacerbated in E$\alpha^3\gamma_1$KDEL transfected B6-2 due to the KDEL ER retention motif.

Since EDEM2 upregulation is controlled by XBP-1 splicing (Olivari et al., 2005) which is a hallmark of ER stress that in turn increases ERAD. I next sought to determine how ER stress influenced presentation of an ER retained antigen using the ER stress inducing compounds tunicamycin and thapsigargin. Consistent with previous results, tunicamycin and thapsigargin both reduced expression of Eα peptide presentation in E$\alpha^3\gamma_1$KDEL transfected B6-2 (Figure 6). Unexpectedly, however, both compounds also reduced Eα peptide display by ΔL11E$\alpha^3\gamma_1$ transfected cells. This was despite upregulation of A^b by both compounds. One reason for this could be the result of an inhibitory effect caused by accelerated ERAD that would subsequently flood the cytosol with antigens and compete with IgH for processing machinery and MHC class II binding. This would account for ER stress mediated inhibition of a cytosolic IgH as well.

To determine if ER stress inhibited Eα presentation due to general toxicity for MHC class II mediated presentation, or to competition as a result of accelerated ERAD, I used E$\alpha^3\gamma_1$ transfected B6-2 to determine if ER stress was able to initiate presentation of Eα peptide from IgH that did not normally present Eα peptide. In chapter two I indicated that B6-2 does not present peptides from E$\alpha^3\gamma_1$ transfected cells even though the IgH was expressed at a

high level and trafficked through the ER. In this cell line treatment with tunicamycin would mimic ER retention, lead to cytosolic translocation, processing by cytosolic proteases and presentation of Eα peptide. Tunicamycin treatment of $E\alpha^3\gamma_1$ transfected B6-2 led to presentation of Eα peptide when compared to tunicamycin treated $NV^2NA^3\gamma_1$ (Figure 7). The fact that presentation was not as robust as $E\alpha^3\gamma$1KDEL transfected cells is not surprising, since treatment with tunicamycin is a heavy handed approach that would result in ERAD mediated degradation of most ER retained proteins. $E\alpha^3\gamma$1KDEL transfected cells, on the other hand, would more specifically degrade only IgH resulting in less competition, by other protein, for ERAD machinery and MHC class II. Thus, it appears that ER stress has a paradoxical effect on presentation of ER retained antigen. ER stress can augment ERAD, thus creating more substrates for antigen processing and an increase in presentation of peptides derived from ER retained antigens, but, by virtue of this, any one particular antigen may be lost in the flood leading to reduced presentation due to competition with other antigens.

Presentation of ER retained IgH is not sensitive to TAP inhibition

At this point I decided to examine the role of TAP in presentation of peptides derived from the ER. In order to inhibit TAP function I used a FLAG tagged version of the MK3 protein from murine γ herpes virus 68. This protein ubiquitinates MHC class I molecules directing their destruction. Loss of MHC

class I leads to subsequent down regulation of the TAP1 subunit (Boname et al., 2004; Boname et al., 2005; Boname and Stevenson, 2001). Upon transfection with an expression vector for FLAG-MK3 B6-2 cells that expressed E$\alpha^3\gamma_1$KDEL showed expression of the FLAG tagged protein by FACS and deconvolution microscopy (Figure 8A and data not shown), down regulation of TAP1 expression (Figure 8B) and no alteration of A^b levels when compared to vector transfected E$\alpha^3\gamma_1$KDEL cells (Figure 8C). Despite the fact that the FLAG-MK3 protein was able to inhibit TAP1 expression there was no inhibition of Eα peptide presentation (Figure 8D). Thus, TAP does not play a role in presentation of peptides from ER retained antigen.

Discussion

In this chapter I show that ER retained antigen is processed in the cytosol by the TTPII. This is an interesting result for two reasons: first, that the TTPII is implicated in the processing of an MHC class II restricted antigen, and second, that ER retained antigen is translocated to the cytosol for processing. I also show that this translocation requires glycoprocessing by a member of the ERAD pathway, in this case α-mannosidase I. Lastly, I show that acceleration of ERAD by ER stress induction can initiate presentation of peptides not normally presented, and can also have a detrimental effect on any single peptide due to a competitive effect.

That ER stress/ERAD can control presentation of ER retained antigens has important implications for the immune response against viruses and the immuno surveillance of oncogenesis. This pathway may be utilized by host cells to aide presentation of viral peptides during infection. Likewise, viruses may count upon a “bulk ER stress effect” in order to reduce presentation of immunodominant epitopes, due to a flood of self-antigens, and hide in plain site. Since many tumor cells undergo ER stress during oncogenic transformation the presentation of unique peptides coupled with an ER stress induced adjuvant effect may be beneficial to cancer immune surveillance.

ERAD also creates a link between protein quality control and antigen presentation. The fact that the model antigen used here is an antigenized antibody is particularly germane to antigen presentation for anti-idiotype responses. The presence of the Eα peptide in the CDR3 serves as a pseudo idiotype peptide. Turnover of Ig via ERAD could create a steady stream of peptides to initiate ant-idiotype responses.

As can be seen, the ERAD pathway is of great utility when it comes to presentation of ER retained antigen. The fact that ER stress positively regulates ERAD allows for tantalizing possibilities linking antigen presentation with inflammatory signals and the induction of anti-viral, anti-tumor and regulatory responses.

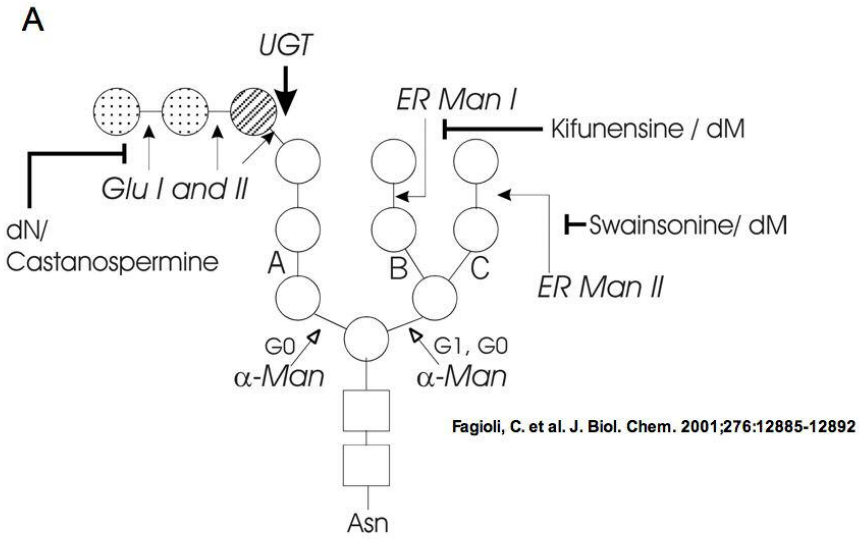

Figure 4.1 Structure of $Glc_3Mann_9GlcNAc_2$ glycans and processing sites. Glu I & II trimming are necessary for cnx/crt entry. Mann I processing is necessary for extraction from cnx/crt cycle.

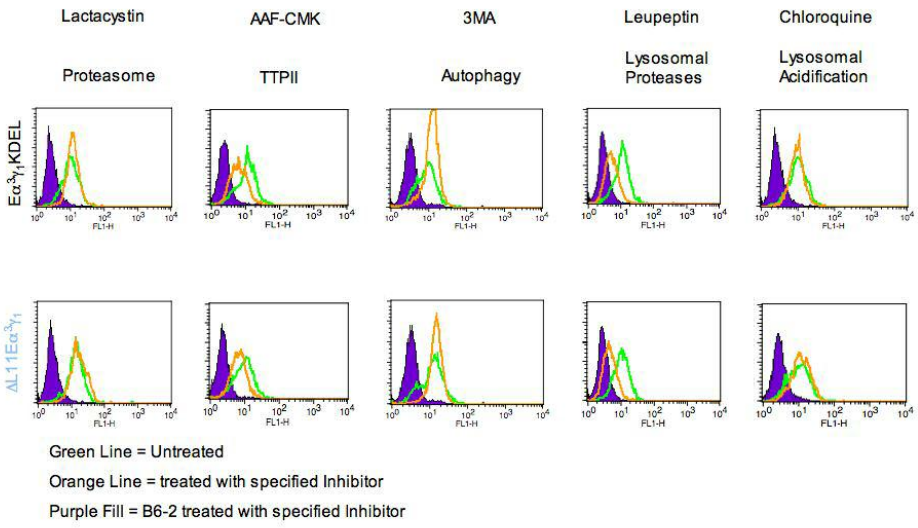

Figure 4.2 Sensitivity of Eα presentation to protease inhibitors. B6-2 cells transfected with the indicated constructs were incubated for 24 hours with the specified inhibitor. Concentrations used were Lactacystin (10 μM), AAF-CMK (50μM), 3MA (7.5 μM), leupeptin (250/μg/mL) and chloroquine (50μM) (orange line), and compared to untreated cells (green lines) or treated B6-2 (purple fills) . Cells were then analyzed by FACs using the Y-Ae antibody.

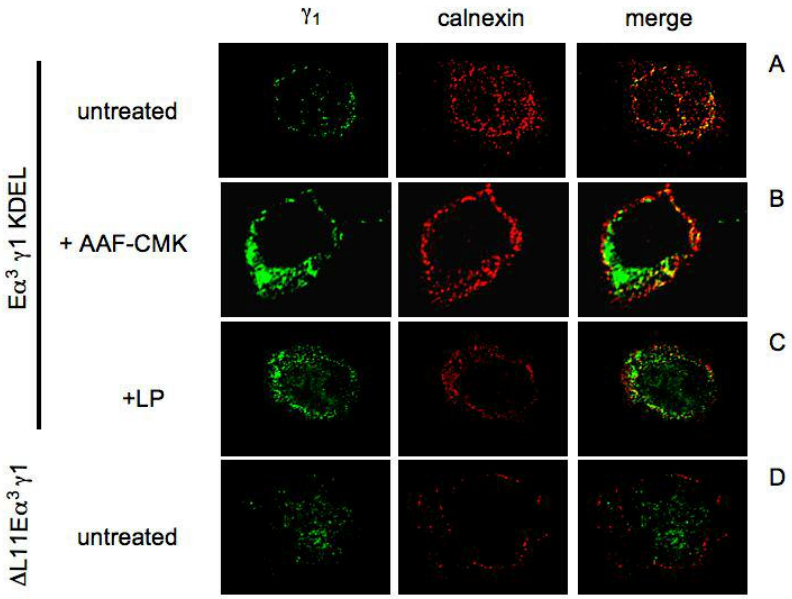

Figure 4.3 Treatment with AAF-CMK or leupeptin induces cytosolic retention of unprocessed IgH. B6-2 transfected with the indicated construct were left untreated (A & D) or treated with AAF-CMK (B) or leupeptin (C). Cells were fixed, permeabilized, and stained then imaged at 100x magnification by epi-fluorescence . Images were deconvolved using Softworx software. Yellow indicates colocalization.

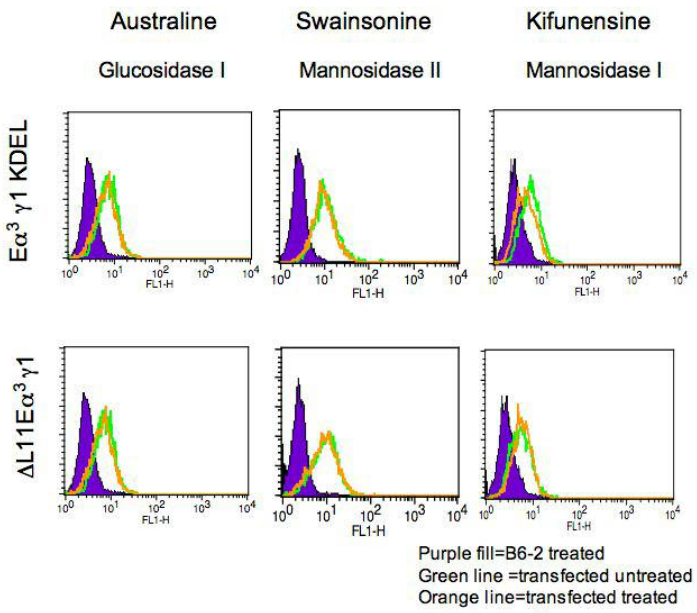

Figure 4.4 Inhibition of mannosidase processing reduces Eα peptide display. B6-2 cells transfected with the indicated construct were treated with 100 μM of australine, swainsonine or kifunensine for 24 hours (orange lines) and compared to untreated cells (green lines) or B6-2 cells (purple fills). Cells were then washed and stained with the Y-Ae antibody and analyzed by FACS.

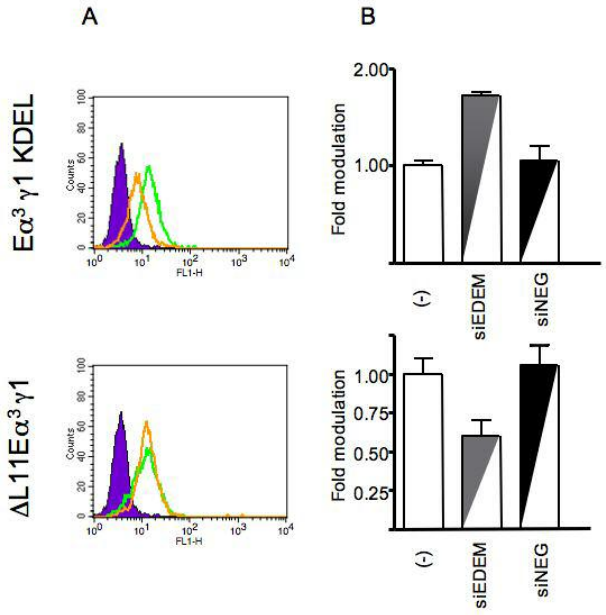

Figure 4.5 Knockdown of EDEM1 in B6-2 transfected with ER or cytosol targeted antigen has varying effects. (A) B6-2 transfected with siEDEM (orange lines) and a negative control plasmid (green lines) were compared to untransfected B6-2 (purple fills) and analyzed for Eα peptide presentation by Y-Ae antibody. (B) Real time PCR for total EDEM mRNAs. Samples were normalized to β-actin, and analyzed by the comparative CT method.

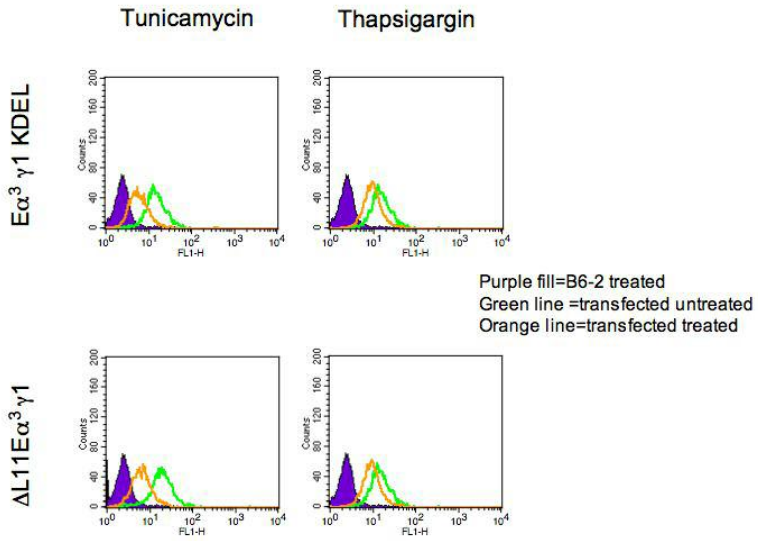

Figure 4.6 ER stress reduces Eα peptide presentation from intracellular IgH. B6-2 transfected with the indicated construct were analyzed by Y-Ae staining after treatment with 5 μg/mL tunicamycin or 300nM thapsigargin (orange line) for 24 hours. Staining was compared to transfected untreated cells (green line) and vector control treated cells (purple fill)

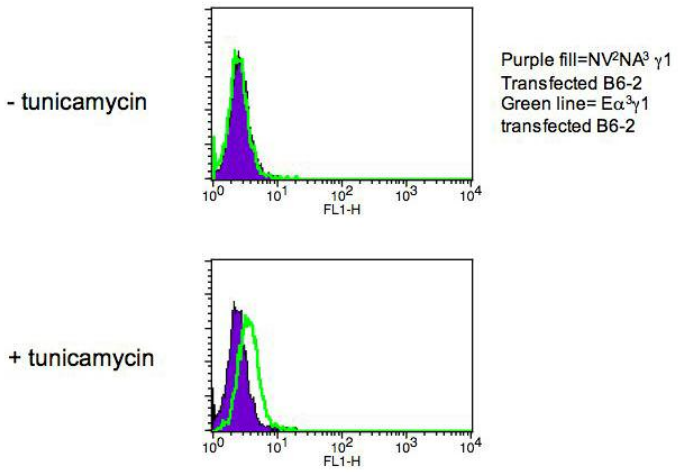

Figure 4.7 Tunicamycin promotes presentation of secretory antigen. Eα$^{3}\gamma_{1}$ transfected (green line) or NV^{2}NA$^{3}\gamma_{1}$ transfected (purple fill) B6-2 cells were not treated (upper panel) or treated with 5 μg/mL tunicamycin (lower panel) for 24 hours and analyzed for Eα peptide presentation by Y-Ae antibody.

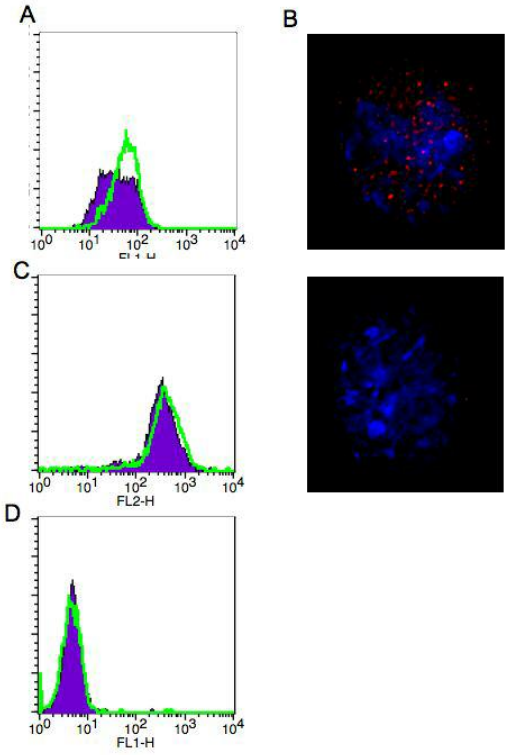

Figure 4.8 Presentation of Eα peptide from ER retained IgH is not dependent on TAP function. $E\alpha^3\gamma_1$KDEL transfected B6-2 cells were stably transfected with plasmids expressing FLAG-MK3 (green line) or vector alone (purple fill) were analyzed for expression of FLAG-MK3 (A), TAP (B, upper panel vector control, lower panel FLAG-MK3 transfected, blue is nuclear staining and red anti-TAP1), A^b (C) and Eα peptide (D).

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Chapter 5

Conclusions

Immunogenic peptides derived from ER and cytosol targeted antigen are presented by B cells in vitro, and prime a CD4 T cell response in vivo

In experiments detailed in this dissertation I show that that peptides derived from endogenously synthesized antigen that is targeted to the ER or cytosol is able to prime an immune response in vivo. This observation is not trivial given that most previous studies have focused on the presentation of intracellular antigen in vitro by B lymphoma cells using model antigens, in most cases, HEL and OVA (Adorini et al., 1993; Bonifaz et al., 1999; Brooks et al., 1991; Fernandes et al., 2000).

While antigen presentation is requirement for T cell priming, in vitro experiments are unable to reveal the full extent of the interplay between costimulatory molecules present on, and cytokines secreted by, APCs and T cells. This is the Achilles heel of the usual tool for examining antigen presentation in vitro, the T cell hybridoma. T cell hybridomas while able to properly reflect MHC class II:TCR interactions have a relaxed requirement for costimulation. This is not at all a moot point since proper intracellular trafficking of MHC class II:peptide complexes is necessary for entry into lipid

rafts and the immunological synapse (Eren et al., 2006; Meyer zum Bueschenfelde et al., 2004; Poloso et al., 2004). Furthermore, it is not certain how peptide loading through endogenous routes influences the antigen presenting capability of APCs.

Previously, it has been shown that MHC class II transduced tumor cell lines are able to present peptides in vivo (Baskar et al., 1995; Qi et al., 2000). However, this system utilizes viral transduction to express MHC class II in the absence of the peptide loading chaperones Ii and DM, and recent evidence indicates that there may, in fact, be some APC involvement in priming (Dolan et al., 2006). In contrast, the experiments here use primary B lymphocytes that express both of these molecules. Most importantly, experiments conducted in vivo show that primary B lymphocytes do upregulate known costimulatory molecules CD86 and CD40, as well as total A^b, indicating that the requirement for costimulation is also met.

MIIC targeting

The optimization of DNA vaccine delivery vectors is a current goal in the field of vaccine development (Barouch, 2006; Muthumani et al., 2002). One way to enhance the efficacy of both therapeutic and prophylactic vaccines is to enhance presentation of both immunodominant and subdominant epitopes. This can be achieved by increasing the efficiency of antigen processing, and the overall display of these peptides by MHC class II

molecules. Targeting MHC class II loading compartments is a logical way to either increase peptide density, or allow subdominant epitopes to be more efficiently processed. The results presented in Chapter 2 show that the cytosolic and transmembrane targeting signal from the β chain of the DM molecule, fused to the C terminus of an IgH, can significantly increase immune responses to the NVDP model peptide when inserted into the CDR2. In contrast, there was no advantage conferred by DM targeting when the Eα peptide was inserted into the CDR3 of an IgH. The reasons for this are not immediately clear, but *in vitro* data suggests that the CDR2 is not as amenable to antigen processing as is the CDR3. This is supported by the fact that presentation of peptides derived from ER and cytosol retained antigen are dependent upon CDR context. Thus, inserting peptides into the CDR3 may already predispose an antigen for maximal processing regardless of ER, cytosolic or MIIC targeting. CDR2 antigens, however, may need to be processed in a discreet compartment from that targeted by cytosolic or ER retained antigen, a compartment accessed by DM targeting. This possibility is not unlikely given that ER and cytosol targeted antigens are likely processed in similar compartments. Therefore, DM targeting may not increase the immune response to all potential T cell epitopes. Instead, it may allow the MHC class II restricted presentation of epitopes not normally, or only minimally displayed during the course of natural virus infection or oncogenic transformation. This naturally leads to tantalizing applications for vaccine development for cancer

and latent viral infections where responses to sub-dominant epitopes are sought after (Newman et al., 2002).

ER retention of antigen by B lymphocytes induces ER stress and enhances IFN-γ and TNFα secretion in primed T cells

The cytokines IFN-γ and TNFα play important roles as pro-inflammatory cytokines during an adaptive immune response. Generally, IFN-γ secretion is associated with Th1 patterning, and TNFα with inflammatory effects. Here, I show that primary B cells transgenic for a plasmid that expresses an ER retained IgH primes CD4 T cells that exhibit greater secretion of these two cytokines when compared to T cells primed by B lymphocytes expressing a secretory or cytosolic form of IgH.

This does not appear to be due to differences in innate activation of B cells by the different plasmids, as the two plasmids, secretory or with ER retention signal, did not differ in their ability to stimulate B cell proliferation or CD86 up regulation. Both of these are hallmarks of TLR activation and would detect subtle differences in CpG content of the plasmid or endotoxin content of the preparation. In fact, while both plasmids readily induced CD86 upregulation, neither was effective at inducing proliferation a phenomenon that has been previously documented in B lymphocytes (Cortez-Gozalez et al., 2006; Spies et al., 2003). This may result due to inhibitory sequences in the plasmid DNA that interfere with TLR activation (Lenert et al., 2003; Stunz et

al., 2002), or may denote that initial activation is through an alternate pathway. This is supported by the recent publication of two studies that implicate a hypothetical cytosolic receptor that recognizes DNA in a non-sequence specific way (Ishii et al., 2006; Stetson and Medzhitov, 2006). The activation of this pathway does not induce MAPK signaling but does result in secretion of the type I interferons IFN α/β, and upregulation of costimulatory molecules on APCs. The extent to which this pathway is utilized when mice are immunized by transgenic B lymphocytes is not yet known, but is currently being explored.

The fact that ER retention appears to determine greater IFN-γ and TNFα secretion leads to the possibility that cellular alterations due to an ER stress response are the main culprit. In support of this B lymphocytes transgenic for ER retained IgH exhibit increased expression of the ER stress markers GADD34, Grp78 and CHOP at 48 hours after transfection. That these results are not seen at 24 hours may be a result of the temporal dynamics of IgH accumulation, to a certain ER stress inducing threshold, and induction of ER stress responsive genes which peak 12 to 24 hours after a stress inducing stimulus. ER stress is known to influence many aspects of cellular function such as apoptosis/cell survival, translation, ER biogenesis and chaperone expression (Schroder and Kaufman, 2005; Xu et al., 2005).

The interactions between the ER stress signaling pathway and events that are necessary for T cell priming have not been previously explored. Therefore, I decided to examine the expression of costimulatory molecules,

that have previously been implicated in CD4 T cell priming, by B lymphocytes transgenic for ER retained IgH. After three days *in vivo*, B lymphocytes transgenic for secretory or ER retained IgH showed equally high expression of the costimulatory molecules CD86, CD40 and A^b. The expression of OX40L, however, was three-fold lower in cells transfected with ER retained IgH. I further tested whether ER stress could block upregulation of OX40L, by using primary B lymphocytes activated by CpG and treated with the ER stress inducing compounds tunicamycin and thapsigargin. Both compounds were able to block upregulation of OX40L but not CD86, CD40 or A^b showing that blockade of OX40L upregulation *in vivo* was due to ER stress.

OX40L is a member of the tumor necrosis factor receptor family (Watts, 2005). It reaches its peak of expression 2-3 days after APC activation and engages its cognate receptor OX40 that is expressed on activated T cells (Croft, 2003). OX40-OX40L interactions have been shown to stabilize expression of the anti apoptotic molecules Bcl-2 and Bcl-XL (Rogers et al., 2001). Supporting results in this chapter it has been implicated in CD4 T function by determining initial clonal expansion and memory (Gramaglia et al., 2000; Salek-Ardakani and Croft, 2006). It has, also, been found to positively influence Th2 responses in mouse models (Linton et al., 2003; Salek-Ardakani et al., 2003), and engagement on B cells drives proliferation and polarizing cytokine secretion (Stuber et al., 1995). Recently, studies by Lund et al. have shown that the interaction between Th cells and B cells is important in

instructing B cell commitment to a type 1, IFN-γ secreting, or type 2, IL-4 secreting phenotype (Harris et al., 2005a; Harris et al., 2005b). It is possible that the absence of OX40L signaling interferes with signaling necessary for type 2 commitment, therefore, allowing a greater niche for type 1 B cell development. This idea is supported by work showing that B cell signaling through OX40L helps to promote IL-4 release in naïve T cells (Flynn et al., 1998). These considerations do not take into account pro-inflammatory cytokines that may be secreted by the antigen presenting transgenic B lymphocyte, which may play a role in Th patterning. This possibility is currently under exploration.

ERAD provides a pathway for presentation of ER retained antigen by MHC class II molecules

One of the methods by which a stressed cell restores the normal folding and secretion equilibrium is by accelerating the degradation of ER retained proteins (Meusser et al., 2005; Romisch, 2005). This process is known as ERAD, and it has been previously implicated in cross presentation of MHC class I restricted OVA peptides (Imai et al., 2005). However, it has not been examined with regards to MHC class II restricted presentation of ER retained antigen. In this dissertation I show that peptides derived from ER retained IgH are presented through a pathway that relies on the movement of antigen from the ER to the cytosol, and that once in the cytosol IgH is processed by the

tripeptidyl peptidase II. This is indicated by the fact that AAF-CMK is able to inhibit presentation of peptides from ER retained antigen, and that cells treated with this inhibitor also show cytosolic accumulation of unprocessed IgH. This is notable since the TTPII is a cytosolic protease, and is the first indication that a cytosolic protease other than the proteasome is involved in processing of antigen for presentation of MHC class II peptides.

Leupeptin also had similar effects on presentation and may be a result of a notorious lack of protease specificity. It appears to be working at the cytosolic level due to the accumulation of undegraded IgH in the cytosol of treated cells. Whether leupeptin can also inhibit the TTPII is unknown, but its effects may stem from action on another protease that is involved in the cytosolic degradation of IgH.

Proteins are marked for ERAD mediated degradation by glyco-processing enzymes. Here, presentation of ER retained antigen is dependent on the action of mannosidase I, as evidenced by kifunensine mediated inhibition of antigen presentation. Mannosidase I is a slow acting ER restricted enzyme responsible for removing a terminal mannose residue from N-linked core glycans marking them for degradation through ERAD. This is not entirely unexpected since it has been shown by others that unassembled Ig-μ and J chains require mannose trimming for degradation (Fagioli and Sitia, 2001). J chains probably most reflect the glycosylation profile of IgH since they have only one N-linked glycosylation site, and bind to BiP instead of

calnexin/calreticulin. The effect of kifunensine, seen here, was modest, it should be noted that, while it is a potent mannosidase I inhibitor, kifunensine merely reduces ERAD and does not eliminate it entirely. This may have to do with the amount of N linked glycans present on IgH as the degradation of μ chains, which have five N-linked glycans, are more sensitive to kifunensine treatment then J chains. Therefore, while it appears as though mannose trimming creates substrates for ERAD and subsequent MHC class II restricted presentation, there may be other mechanisms capable of driving substrate translocation from ER to cytosol for MHC class II processing.

ER stress effects presentation of ER retained antigen by MHC class II molecules

ER stress signaling increases ERAD, by upregulating ERAD components (Schroder and Kaufman, 2005; Schroder and Kaufman, 2006). Therefore I developed the hypothesis that ER stress can facilitate presentation of antigens that traffic through the ER. Supporting this hypothesis tunicamycin treatment leads to presentation of an MHC class II restricted peptide from a secretory form of IgH that is not presented under normal conditions. This is ostensibly caused by ER retention and subsequent ERAD mediated degradation of IgH, thus, creating antigenic substrates for cytosolic processing. The fact that tunicamycin or thapsigargin do not enhance but inhibit presentation of ER and cytosol restricted peptides is initially

disconcerting. However, tunicamycin and thapsigargin both induce massive amounts of ER stress. Tunicamycin, due to inhibition of all N-linked glycosylation (Nishimura et al., 1987), would create ERAD substrates out of every newly synthesized glycoprotein leading to bulk accumulation, and ERAD mediated degradation of protein thereby competing with ER retained IgH for processing and presentation. Since the site for processing of ER retained and cytosolic antigen appears to be the same this would inhibit presentation of antigen from both compartments. This is supported by the fact that thapsigargin has a more modest effect on presentation as it would have less of an effect on folding in the ER while still accelerating ERAD, causing more cytosolic translocation then normal, but not nearly as much as tunicamycin. Plus, there is evidence suggesting that thapsigargin and tunicamycin induce different ER stress expression profiles (Durose et al., 2006), providing an explanation for why the effect of thapsigargin on antigen presentation is less pronounced.

Implications for the Immune response and vaccine design

Overall the results detailed in this dissertation reveal important aspects for the functioning of an APC under conditions of cellular stress. Viral infection (Cheng et al., 2005; Ciccaglione et al., 2005; Dimcheff et al., 2004; Li et al., 2005; Tardif et al., 2002) and oncogenic transformation (Bi et al., 2005; Nakamura et al., 2006) are two instances in which ER stress is induced *in*

vivo. Processes linked to ER stress may be important for the immune responses against both occurrences.

The influence on OX40L may be a key factor contributing to viral latency. The influence on OX40L may not just extend to APCs, but to other cell types as well. Activated T cells and endothelial cells are two examples of non APCs that express OX40L (Watts, 2005), and both are important sites of viral infection. In these cells OX40L serves as an adhesion molecule (Imura et al., 1996), thus, contributing to viral latency through evasion.

The ER stress influence on antigen presentation may help to augment immune surveillance by initiating presentation of ER derived peptides by cells that are undergoing oncogenic transformation. Since, peptides from ER resident proteins are not normally presented, T cells against them have probably escaped thymic deletion and peripheral tolerization and may therefore be activated given the proper inflammatory environment.

The use of an ERAD pathway for presentation, and an ER stress mediated adjuvant effect may have important implications in the development of anti-idiotype responses. Anti-idiotype vaccines have been posited as a potentially useful way to immunize against tumor antigens(Hsu et al., 1997; Stevenson et al., 1995). These two events may, also, underscore the effect of natural regulatory circuits that are important, *in vivo*, for anti-idiotype and anti-isotype responses.

The use of ER retention to promote ER stress may find more general practical use when trying to optimize a plasmid based DNA vaccine. The inclusion of ER retention motifs in proteins to be used in potential vaccines could lead to greater IFN-γ and TNFα release by primed CD4 T cells. This may be practical for use against infection by mycobacterium, intracellular bacteria and virus, as IFN-γ has been shown to be critical for these responses (Bretscher et al., 1992; Guidotti et al., 1994; Kaufmann and van Embden, 1993; Shearer and Clerici, 1998). This may also be important for therapeutic vaccines against cancer, as IFN-γ is beneficial for T cell reactions against tumor cells (Yu et al., 1996; Zitvogel et al., 1996).

While not examined in this dissertation the effect of ER stress on CD8 T cell priming may also be beneficial as IFN-γ is necessary for enhanced CD8 T cell function (Croft et al., 1994). However, high levels of inflammation during a primary response do not necessarily translate to improvements in memory response, as previously shown in a mouse model of LCMV infection (Badovinac et al., 2005). Moreover, CD4 T cells aide crucial aspects of CD8 T cell memory recall and maintenance (Janssen et al., 2005; Janssen et al., 2003), and alterations in OX40L may effect CD4 T cell memory responses more than primary responses. Thus, the role of ER stress in CD8 T cell primary and memory responses also needs to be examined.

Concluding remarks

Overall the role of the ER in the immune response against MHC class II restricted antigens may be more involved then previously thought. The ER plays an active role in the generation of MHC class II restricted peptides through the use of the ERAD pathway. This not only effects presentation of ER restricted antigen but also cytosolic antigen, as the point of processing for both appears to be the cytosol.

The involvement of the ER in the immune response also extends beyond mechanistic considerations for antigen presentation. The ER stress response can also have dramatic consequences for adaptive immunity by effecting costimulation and altering the ensuing T cell response. The results presented in this dissertation likely represent the tip of a very large iceberg, as ER stress effects on immunity and inflammation need to be examined in more diverse cell types and model systems of *in vivo* immunity.

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Appendix

Methods

Plasmids

Plasmids encoding the secretory IgH genes were made as previously described (Xiong et al., 1997). For cytosolic targeting the leader sequence was removed using a Quick Change XL site directed mutagenesis kit from Stratagene (La Jolla, CA). For ER targeting a Cla I and a Not I site was added 3' of the γ1 coding region using a Quick Change XL site directed mutagenesis kit from Stratagene. A pair of complementary oligonucleotides coding for SEKDEL with a 3 alanine spacer were inserted between these restriction sites. Heterologous peptides Eα (ASFEAQGALANIAVDKA), NV (NANPNVDPNANP) and NA (NANPNANPNANP) were inserted into the variable regions as previously described. Plasmids containing DMβ cDNA were obtained from Lars Karlsson (Johnson & Johnson, San Diego.). Plasmid pEGFP was purchased from Clontech (Mountainview, CA). pMACS K^k plasmid was purchased from Miltenyi Biotech (Auburn, CA). A plasmid encoding the FLAG-MK3 was a kind gift of Dr. Phillip Stvenson, and

transferred into pcDNA3.1-Hygro (Invitrogen, Carlsbad, CA). The siEDEM plasmid was made using the BLOCK-iT Pol II miR RNAi expression vector kit (Invitrogen), and primers with the target sequence of AACGTGAACATGTTCAGTGGG as previously described (Molinari et al., 2003). All plasmid sequences were confirmed by sequencing at the UCSD Cancer Center Core Sequencing Facility. Plasmids were prepared using Wizard Plus Maxi-prep Kit from Promega (Madison, WI).

Mice

C57Bl/6 Mice were purchased from Harlan (Indianapolis, IN). MHC class II deficient mice on a C57Bl/6 background were purchased from Taconic (Germantown, NY). All mice were housed at the UCSD Animal Facility and handled in accordance with University of California-San Diego Animal Subjects Program Guidelines.

Cell lines

J558L cells were originally obtained from Dr. S. Morrison (University of California-Los Angeles). B6-2 cells were obtained from the NIH. T cell Hybridoma 1H3.1 and B cell Hybridoma Y-Ae were obtained from Alexander Rudensky (University of Washington, Seattle). CTLL-2 cells were obtained form ATCC (Manassas, VA).

Peptides, CpG and Antibodies

Eα peptide 56-73 (ASFEAQGALANIAVDKA) was synthesized at the Ohio State University Peptide Synthesis Facility. All protease inhibitors and

Tunicamycin and thapsigargin were purchased from Calbiochem (San Diego, CA). CpG ODN 1826 was kindly provided by Dr. P. Lenert (University of Iowa). Goat anti-calnexin antibody was purchased from Santa Cruz Biotech (Santa Cruz, CA). Donkey anti-goat-Cy3 and donkey anti-human γ1 FITC antibodies were purchased from Jackson Immuno Research (West Grove, PA). Goat anti-human IgG antibodies labeled with horse radish peroxidase was purchased from Sigma (St. Louis, MO). PE conjugated antibodies anti-mouse I-A^b, CD19, CD86, CD40, OX40L CD4 and CD8, and a bitotinylated anti-mouse K^k were purchased from Pharmingen (San Diego, CA). YA-e antibody was purified from hybridoma supernatent and FITC labeled in house.

Spontaneous Lymphocyte transgenesis and *in vivo* immunization

Spontaneous lymphocyte transgenesis was performed as previously described with the following modifications (Filaci et al., 2004). Briefly, for immunization, $4x10^6$ splenocytes were incubated with 5 μg of pMACS Kk, 10μg of IgH plasmid and 10μg of pEGFP-N1 in PBS with out calcium and magnesium for 1 hour and then cultured overnight in RPMI with 10% FBS. The reaction was scaled up to get enough cells for immunization. Cells were isolated the next day using a Macselect kit (Miltenyi Biotech), and analyzed by FACS for transfection efficiency. Mice were then immunized with $5x10^3$ EGFP positive cells i.v.

For experiments with IgH-K^k plasmids cells were transfected in the same way, except that only 10 μg of IgH plasmid was used. Cells were then

isolated using an Easysep biotin selection kit from Stem Cell Technologies Inc., (Vancouver, BC, Canada) coupled with a Biotin conjugated anti K^k and analyzed at the indicated time points.

Generation of stable transfectoma

J558L and B6-2 cells ($10x10^6$) were transfected by electroporation with 50 μg of linearized plasmid DNA in ice cold PBS without Ca^{2+} and $Mg^{2+}$and shocked twice at 300 mV. Cells were incubated on ice for 15 min and then allowed to recover in RPMI containing 10% Fetal Bovine Serum (FBS) for 48 hours. Cells were then grown under the appropriate antibiotic for 14 days 1mg/mL for G418 selection, 250 μg/mL for hygromycin selection and 5ug/mL for blasticidin selection.

Secretion Assay

J558L hybridoma were seeded at $1x10^6$ cells per well in a 12 well plate in 2mL of media. Supernatents were harvested 24 hours later and used in a capture ELISA to detect the γ1 portion of each IgH at a dilution of 1:10. Plates were read using a Molecular Devices plate reader at a wavelength of 450 nm.

Microscopy

J558L transfectoma were fixed and permeabilized using the CytoFix/CytoPerm kit from Pharmingen. Cells were then incubated with goat anti-calnexin primary antibody for 1 hour at RT. Cells were then incubated with donkey anti-goat-Cy3 and donkey anti-human-FITC antibodies. Cells were washed and spun onto slides using a cytospin centrifuge. Stained cells were imaged at the

UCSD Cancer Center Digital Imaging Shared Resource using a Photometrics CCD mounted on a Nikon TE-220 inverted epifluorescence microscope at 100x magnification. Images were deconvoluted using Soft Worx (Applied Precision, Inc.; Issaquah, WA)

RT-PCR

Total RNA was extracted from cells using an Rneasy kit from Qiagen Inc. (Valencia, CA). cDNA was synthesized using a poly(T) primer from 50 ng of total RNA using Sensiscript reverse transcriptase (Qiagen) for primary cells, and 300ng of total RNA using Omniscript reverse transcriptase for cell lines. Primers used for PCR analysis had the following sequence: human γ1; tcaaggactacttccccgaacc and tactccttgccattcagccagccagtcc, GADD34; gagaagagggagtggctgagc and agcattccgacaagggtgacc, CHOP; ccctgcctttcaccttgg and ccgctcgttctcctgctc, Grp78; ctgggtacaatttgatctgacgg and gcacctggtggctttcccagcattc, and β-actin tgggccgccctaggcacc and cggttggccttagggttcag. PCR was carried out for 25 cycles using platinum *Pfx* polymerase from Invitrogen at an annealing temp of 62 degrees.

Real time PCR

Real time PCR was performed at the Center for Aids Research San Diego real time PCR core facility. Reactions were normalized against β-actin, primers with the sequence AAGCCCTCTGGAACTTGCG and AACCAATGGCCTCTCTGG were used for Syber green detection. Results were analyzed using comparative CT analysis.

Y-Ae staining flow cytometry

Cells were incubated in FACS buffer (PBS+0.5%BSA+0.05% Sodium Azide) with 2% normal mouse sera for 15 min, and then incubated with 100 ng/mL of FITC labeled Y-Ae antibody. Primary B lymphocytes were stained in FACS buffer using commercial antibodies at 200 ng/mL Cells were then analyzed by flow cytometry using a Becton Dickinson (Palo Alto, CA) FACScalibur and CellQuest software .

Hybridoma assay

Transfected B6-2 cells were seeded in triplicate at the indicated concentrations in 96 well plates and incubated with $1x10^5$ 1H3.1 T cell hybridomas for 24 hours. Supernatants were then harvested and incubated for 24 hours with $5x10^3$ CTLL-2 cells. 1μCi of [^{3}H]-labeled thymidine was then added and cells were incubated for an additional 24 hours. Cells were then harvested onto filter mats and read on a ß-counter (Wallac,Turku, Finland).

Cell separation

CD4+ and CD8+ T cells were isolated from the spleens of immunized mice by negative selection using CD4 and CD8 isolation kits from Stem Cell Technologies Inc. B lymphocytes were isolated from spleens of naïve C57/Bl6 mice by negative selection using a B cell isolation kit from Stem Cell Technologies Inc. Cells populations were greater than 90% pure as determined by flow cytometry with appropriate antibodies. K^k positive cells

were isolated using a biotinylated anti-K^k antibody, and a biotin selection kit from Stem Cell Technologies.

T cell proliferation

$4x10^5$ splenocytes were incubated, in triplicate, in a round bottom 96 well plate with either medium or 50 μg/mL of Eα peptide. At 72 hours 1μCu of [^{3}H]-labeled thymidine was added to each well, and cells were harvested onto filter mats and read on a Wallac ß counter 16 hours later. Results obtained by stimulation with peptide are expressed as c.p.m. from which c.p.m. of cultures in medium only were subtracted. Tests were performed in triplicate.

Cytokine analysis

Supernatants of 48 hour triplicate cultures were pooled and assayed with a mouse Th1/Th2 cytometric bead array (Pharmingen). Data were acquired using a BD FACScalibur, and analyzed with BD CBA software (Becton Dickinson). Statistical analysis was performed using Prism software from GraphPad (San Diego, CA), and significance was determined using a two-tailed unpaired *t*-test.

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