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Type B Presentation from HEL Protein in the Context of Inflammation

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ABSTRACT OF THE DISSERTATION

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Peptides from the model antigen hen egg white lysozyme (HEL) are capable of binding MHC II in multiple conformations. Resting antigen presenting cells (APC) load peptide in early endosomal compartments where multiple conformations of a given pMHC pair are allowed, generating both type A and B pMHC complexes. Protein traffics to the late endosomal compartment and is loaded onto nascent MHC II in the presence of the editor H2-DM where type A pMHC conformers are selectively formed, generating exclusively type A pMHC from protein. Previous work demonstrated that type B pMHC are also generated when HEL is administered *in vivo* along with certain inflammatory stimulants.

Using an *in vitro* system I established, I found that TLR ligands and type I interferons (IFN) act directly on dendritic cells (DC), allowing generation of type B pMHC from HEL. Both $CD8\alpha^+$ and $CD8\alpha^-$ DC present type B pMHC with TLR stimulation, but the relative effectiveness varied based on the ligand used. Using a type I IFN receptor (IFNAR1) blocking antibody and DC from mice deficient in the receptor, I

found that TLR-induced type I IFN production amplifies but is not required for TLR-induced type B presentation from HEL.

While I have not determined a sub-cellular mechanism for TLR-induced type B presentation, I have excluded mechanisms and made several observations. DC deficient in H2-DM are capable of TLR-induced type B presentation, indicating that regulation of H2-DM is not a critical mechanism. DC do not release meaningful levels of peptides allowing generation of type A or B pMHC. Additionally, generation of type B pMHC from HEL is a delayed event, requiring eighteen hours for appearance at the cell surface. While surface MHC II levels only modestly increase, there is a significant increase in total pMHC complexes after TLR or type I IFN stimulation.

I employed two-photon microcopy to observe type A and B T cells in intact lymph nodes. While there were no remarkable differences in T cell behavior between the two groups, I determined that DC present type A or both type A and B but never exclusively type B pMHC. Using mice that express HEL as a membrane-bound protein on APC (mHEL), I found that TLR stimulation increases type B presentation from membrane-bound protein. I found that while immunization of mHEL mice with CpG increases presentation of type B pMHC from pseudo-self HEL and primed anti-HEL CD4 T cells, no observable disease was detected.

These results identified the cells and signals required for inflammation-associated type B presentation from HEL and established tools for future investigation of the sub-cellular mechanism controlling these events.

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List of Abbreviations

Antigen Presenting Cell (APC)

Bone marrow-derived DC (BMDC)

Complete Freund's Adjuvant (CFA)

Dendritic cell(s) (DC)

Hen egg white lysozyme (HEL)

Interferon (IFN)

Incomplete Freund's Adjuvant (IFA)

Lipopolysaccharide (LPS)

Listeriolysin-o (LLO)

Major Histocompatability Complex Class II (MHC II)

Peptide-MHC Complex (pMHC)

Polyinosine-polycytidylic acid (Poly (I:C))

Pyrogen-Free Saline (PFS)

Toll-Like Receptor (TLR)

Quantitative Reverse Transcription PCR (qRT-PCR)

Chapter 1

Introduction

Antigen presenting cells (APC) play a critical role in the initiation of the adaptive immune response. APC acquire and process antigen, load peptides onto MHC II molecules, and present pMHC complexes to CD4 T cells. While the basic steps required for antigen presentation are understood clearly, there remain many fine details that require further examination. This dissertation explored the effects of inflammatory stimulants on a specific pathway of MHC II processing and presentation, the generation of unstable pMHC conformers termed type B that normally form exclusively from peptide but not protein antigen.

Initial Identification of Type A & B Reactivity

Eli Sercarz's group began studying T cell responses to multiple epitopes within individual proteins in the 1980's and 1990's (Sercarz, Lehmann et al. 1993). They found that T cells elicited in response to protein antigen had varying recall responses to epitopes from the protein. Some epitopes elicited strong T cell responses and were termed immunodominant. Other epitopes elicited weak responses and were termed subdominant. Finally, some epitopes yielded no T cell response and were termed cryptic. They argued that dominant epitopes were hearty peptides that survived processing and were thus highly expressed on the cell surface. Subdominant epitopes were mediocre at surviving processing and cryptic epitopes were destroyed and therefore not presented.

During the same time, the Unanue lab and other groups had begun to investigate naturally processed peptides presented on MHC II (Rudensky AYu, Preston-Hurlburt et

al. 1991; Chicz, Urban et al. 1992; Hunt, Michel et al. 1992; Nelson, Roof et al. 1992; Falk, Rötzschke et al. 1994). In these studies, peptides were eluted from surface MHC II molecules and the peptides were identified by Edman degradation and mass spectrometry. One of the proteins studied by our lab was the model antigen hen egg white lysozyme (HEL.) Surprisingly, it was found that one of the epitopes of HEL that was termed cryptic by the Sercarz group, 84-96 bound to I-E^k, actually represented a minimum of 7% of the total I-E^k complexes presented by an individual APC (Viner, Nelson et al. 1995). The issue was explored further to reconcile the fact that though this antigen was highly expressed on the cell surface, it yielded no T cell response following protein immunization. Mice were immunized with either HEL protein or 84-96 peptide. In addition to using recall responses in draining lymph nodes as the Sercarz group had, T cell hybridomas were made from the draining lymph nodes following immunization. When the T cell hybridomas were challenged with APC fed peptide or protein, nearly all of the T cells from peptide-immunized mice responded only to peptide but not protein. These peptide-reactive T cells were termed type B T cells in contrast to conventional type A T cells that can respond to protein or peptide antigen. The lab proposed that type A and B T cells recognized the same peptide-MHC II pair but that the complexes somehow differed based on the route of presentation.

It is important to note that not all cryptic epitopes are type B epitopes. There are certainly peptides that do not survive processing or have poor binding to MHC molecules.

Conformational Isomerism of HEL (48-61)-I-A^k

Following the original identification of type A and B reactivity, the Unanue lab shifted to studying the 48-61 epitope of HEL bound to I-A^k due to the biochemical and structural data available for the complex (Allen, Strydom et al. 1984; Nelson, Roof et al. 1992; Nelson, Viner et al. 1996; Fremont, Monnaie et al. 1998). Over the next decade, many observations were made that clarified the type B paradigm. The lab found that type A and B T cells shared a similar range of reactivity to their cognate antigen, eliminating the possibility that type B T cells were simply weakly reactive T cells (Viner, Nelson et al. 1996). Importantly, it was found that type A and B T cells respond to the same naturally processed, unmodified peptide-MHC II pair (Viner, Nelson et al. 1996). These data implied that something about the route of presentation of peptide versus protein dictated a conformational difference in the binding of the same pMHC pair.

Localization of pMHC Generation and the Role of H2-DM

Experiments using liposome-mediated targeting of peptide to different parts of the endosomal pathway revealed that type A and B conformers are generated in different compartments within the APC (Pu, Carrero et al. 2002; Pu, Lovitch et al. 2004). Liposomes are lipid bilayers with differing pH sensitivities, which allows the cargo to be unloaded at low or high pH (Yatvin, Kreutz et al. 1980; Connor, Yatvin et al. 1984; Harding, Collins et al. 1991). Using this technique, Pu and Lovitch found that when peptide was targeted to late endosomal compartments, only type A pMHC were generated (Pu, Lovitch et al. 2004). However, when peptide was targeted to early endosomal compartments, both type A and B pMHC formed. These data indicated that

some feature of the late compartment was refractory to generation of type B pMHC conformers. Interestingly, when peptide was targeted to the late compartments of APC deficient in H2-DM, type B pMHC were now able to form. This indicated that at least when a large pool of peptides is available for loading onto MHC II, H2-DM is capable of editing off the type B pMHC and allowing or promoting generation of type A conformers. The ability of H2-DM to select for type A conformers was definitively shown in experiments using plate-bound I-A^k. They found that when I-A^k-CLIP was loaded via peptide exchange, in the absence of H2-DM, both type A and B conformers formed. However, when I-A^k-CLIP was loaded in the presence of plate-bound H2-DM, only type A conformers were formed. This demonstrated that H2-DM acted not only as a peptide editor selecting for good binding peptides, as had previously been known (Sant, Chaves et al. 2005), but also as a conformational editor selecting for the most stable conformers of individual peptide-MHC pairs.

Biological Relevance of HEL-Reactive Type B T Cells

In an effort to understand the biological relevance of type B T cells, Dan Peterson in the lab used limiting dilution analysis to determine the frequency of type A and B T cells in response to peptide immunization. Briefly, limiting dilution analysis involves incubating cells from the draining lymph nodes of mice isolated one week after immunization at limiting numbers. The cells are expanded and tested for proliferation in response to peptide or protein antigen. Following peptide immunization of B10.BR mice, type B T cells comprised 30-40% of the T cell response to the 48-61 epitope of HEL, indicating that type B T cells comprise a significant component of the CD4 T cell

compartment. Peterson's findings became more interesting when he immunized two transgenic mouse lines expressing HEL. The first, ML-5, expresses HEL as a secreted protein under the metallothionein promoter (Goodnow, 1988.) In the ML-5 mice, type B T cells represented 80-90% of T cells elicited from peptide immunization. The second mouse line, mHEL, expresses HEL as a fusion protein tagged to the transmembrane domain of H-2L^{d} under control of the Eα promoter (Ho, 1994.) When the mHEL mice were immunized with peptide, the only T cells elicited were type B T cells. This indicated that the type B conformer was not adequately presented from either soluble protein or membrane-bound protein on APC, resulting in escape of these potentially self-reactive T cells from negative selection (Peterson 1999.)

Type B Presentation and Inflammation

The Unanue lab has had a longstanding interest in whether states of inflammation alter the scenario of type A and B pMHC presentation, allowing for generation of type B pMHC from protein antigen. Due to the high frequency of type B T cells in response to non-self peptide immunization, it would be favorable to engage this portion of the T cell population during an infection. Conversely, since type B T cells against self-antigens are capable of escaping negative selection, it could be detrimental if their epitopes could be generated from self-proteins in the periphery during inflammatory conditions.

In order to study the biological relevance of type B T cells, Scott Lovitch generated a transgenic mouse expressing the T cell receptor (TCR) of a type B T cell hybridoma specific for the dominant epitope of HEL, 48-62, bound to I-A^{k} (Lovitch, 2007.) This mouse line, termed MLA11.2, has been used alongside another TCR

transgenic line, 3A9, which is specific for the type A conformer of the same 48-62-I-A^k pair. Lovitch found that while MLA11.2 T cells responded the same as the type B hybridomas—proliferating *in vitro* in response to peptide-pulsed but not protein-pulsed APC and *in vivo* to peptide-immunized but not protein-immunized mice—they also proliferated *in vivo* when mice were immunized with HEL protein along with lipopolysaccharide (LPS,) listeriolysin O (LLO,) or complete Freund's adjuvant (CFA.) This indicated that type B epitopes could be presented from protein antigen during some states of inflammation. This dissertation sought to explore the cells and signals that are responsible for allowing type B pMHC to be generated from protein HEL in the presence of inflammatory signals.

Type B Reactivity with Other Antigens

It is worth mentioning, that type A and B reactivity has been observed in other models. The Unanue lab has found that type B T cells recognizing 37-53 of the MHC II $A\beta^k$ chain exist and escape negative selection in $A\beta^k$ -expressing mice (Lovitch, Walters et al. 2003). The 52-69 epitope of Eα on I-A^b is highly expressed on the cell surface of Eα-expressing mice (Barlow, He et al. 1998; Gyotoku, Fukui et al. 1998; Viret, He et al. 2001; Viret, He et al. 2003). Like our type B epitopes, the peptide-MHC II complex formed from exogenous Eα peptide is much less stable than the conformer formed from endogenous protein. Also, all type A T cells recognizing the endogenously processed Eα are deleted during negative selection, but type B T cells can be elicited with peptide immunization.

The immunodominant epitope of myelin basic protein (MBP) in the experimental allergic encephalomyelitis (EAE) model is an unusually short, unstable peptide (Fairchild, Wildgoose et al. 1993; Harrington, Paez et al. 1998). Structural data of the peptide-MHC complex interacting with an encephalitogenic TCR demonstrated that the peptide can bind in two conformations with the I-A^u molecule (He, Radu et al. 2002; Maynard, Petersson et al. 2005). Extensions of the peptide that result in a more stable complex do not lead to pathogenesis. Additionally, in mice transgenically expressing a human MHC II allele associated with multiple sclerosis, HLA-DRB5*0101, type B T cells against human MBP escape negative selection (Kawamura, McLaughlin et al. 2008).

The Unanue lab has also found that in NOD mice, peptide-reactive type B T cells comprise the majority of insulin-reactive T cells that escape negative selection (Mohan, Levisetti et al. 2010). However, with insulin (9-23) bound to I-A^{g7}, the difference between type A and B pMHC is register shift, not conformational isomerization (Mohan, Petzold et al. 2011). While the mechanism of why one register forms exclusively from peptide antigen while the other register forms from either peptide or protein antigen is still unclear, the general features of type B reactivity hold true—the type B pMHC are less stable than the type A, type B pMHC are more sensitive to editing by H2-DM, and T cells against the type B epitope are more able to escape negative selection when their cognate antigen is expressed in the thymus.

Effects of TLR Ligands on MHC II Pathway

Dendritic cells play a central role in the initiation of immune responses. Tissue-resident immature DC patrol the periphery, constantly taking in material. DC become activated upon encountering inflammatory compounds expressed by pathogens recognized via several pathways including Pattern Recognition Receptors (PRRs) and Toll-like receptors (TLR.) Resting DC are capable of processing and presenting antigen to T cells (Veeraswamy, Cella et al. 2003; Wilson, El-Sukkari et al. 2004) but many changes occur to DC upon encountering TLR ligands. DC maturation via TLR stimulation leads to increased migration of DC to draining lymph nodes, production of cytokines, and the increased surface expression of many co-stimulatory molecules and MHC I and II (Inaba, Witmer-Pack et al. 1994; MacPherson, Jenkins et al. 1995; Cella, Engering et al. 1997; Reis e Sousa, Hieny et al. 1997; Jakubzick, Bogunovic et al. 2008).

MHC II Expression, Localization, and Stability

TLR stimulation alters many components of the MHC II processing and presentation pathway. One of the initially described characteristics of DC maturation was the change in MHC II trafficking and surface expression. Exposure to TLR ligands induces a transient increase in MHC II synthesis followed by a near silencing of the locus and increased surface stabilization of MHC II (Cella, Engering et al. 1997). Immature DC recycle surface peptide-MHC II quickly and retain much of their MHC II within endosomes. Upon maturation, surface peptide-MHC recycling decreases significantly and most MHC II appear on the surface of the cell (Cella, Engering et al. 1997; Villadangos, Cardoso et al. 2001). MHC II surface expression is regulated by

polyubiquitination of lysine 225 in the tail of the β chain (Ohmura-Hoshino, 2006; van Niel, 2006; Shin, 2006) with ubiquitination leading to internalization. Ubiquitination of MHC II and its subsequent decreased surface retention has been shown to be mediated by MARCH-I (Matsuki, Ohmura-Hoshino et al. 2007; De Gassart, Camosseto et al. 2008; Thibodeau, Bourgeois-Daigneault et al. 2008). In conventional DC, expression of MARCH-I, and thus ubiquitination of MHC II, has been shown to increase with IL-10 treatment (Thibodeau, Bourgeois-Daigneault et al. 2008) and decrease with exposure to TLR ligands (Shin, Ebersold et al. 2006; van Niel, Wubbolts et al. 2006; De Gassart, Camosseto et al. 2008; Young, Wilson et al. 2008; Walseng, Furuta et al. 2010; Walseng, Furuta et al. 2010). Peptide-loaded MHC II complexes appear to be primarily targeted by MARCH-I (Walseng, Furuta et al. 2010). In addition to direct regulation of MARCH-I expression following TLR exposure, CD83, whose expression is increased during DC maturation (Zhou, Schwarting et al. 1992; Buelens, Verhasselt et al. 1997), has been shown to interfere with the ability of MARCH-I to down-regulate both MHC II and the co-stimulatory molecule CD86 (Tze, Horikawa et al. 2011). Thus, it appears that pMHC surface expression is regulated to be short-lived in resting DC and anti-inflammatory settings and long-lived in mature DC exposed to pro-inflammatory TLR signals.

Regulation of surface MHC II could be particularly important in understanding presentation of type B epitopes during inflammatory conditions. Since the type B conformer is less stable this surface stabilization could allow complexes to remain on the surface for greater amounts of time, increasing the chance for them to be seen by T cells.

Antigen uptake

In addition to changes in MHC II regulation, uptake of extracellular antigen and catabolism are altered in DC following exposure to TLR stimulation. Following exposure to TLR ligands phagocytosis is transiently increased and then decreases over time (West, Wallin et al. 2004). Decreased phagocytosis in response to TLR stimulation is regulated by expression of cdc42 (Garrett, Chen et al. 2000; West, Wallin et al. 2004). Despite this, DC are capable of taking up and processing antigen encountered after TLR exposure (Ruedl, Koebel et al. 2001; Young, Wilson et al. 2007). However, while there is debate regarding whether presentation of soluble antigen encountered after TLR stimulation is eliminated or simply reduced, it seems clear that processing and presentation of antigen acquired via receptor-mediated uptake remains intact (Ruedl, Koebel et al. 2001; Henri, Siret et al. 2007; Young, Wilson et al. 2007; Platt, Ma et al. 2010).

Catabolism

The catabolic rate of phagocytosed antigen is low in DC compared to macrophages (Delamarre, Couture et al. 2006). This appears to be due to lower content of proteases in lysosomes and differential regulation of the maturation rate of phagosomes in DC (Lennon-Duménil, Bakker et al. 2002; Delamarre, Couture et al. 2006). Catabolism is accelerated in DC upon exposure to TLR ligands, purportedly via increased recruitment of V1 sectors of the V-ATPase complex to lysosomal membranes (Trombetta, Ebersold et al. 2003). Transient increases in internalization and catabolism of antigen may favor increased generation of pMHC complexes, although this has not been directly shown.

H2-DM and H2-DO

The expression of H2-DM and H2-DO has also been shown to be affected by DC stimulation. IL-6 been shown to reduce mRNA levels and protein levels of H2-DM in bone marrow-derived DC (BMDC) which correlated with a decrease in SDS-stable MHC II dimmers (Kitamura, Kamon et al. 2005). In this system, reduced H2-DM and stable MHC II dimmers appeared to be a result of enhanced cathepsin activity. TLR ligands appear to have difference effects on H2-DM and H2-DO in DC. One report showed that *in vivo* administration of LPS has no effect on H2-DM protein levels but decreases H2-DO protein levels in splenic DC (Chen, Reed-Loisel et al. 2006) We have demonstrated that *in vitro* stimulation of DC with TLR ligands induces a transient increase in mRNA for H2-DM and H2-DO followed by a decrease in expression that leads to minimal changes in protein levels (Strong and Unanue 2011). More recently, it has been shown that *in vivo* TLR stimulation has minimal effects on H2-DM protein levels in DC, but decreased H2-DO levels primarily in $CD8\alpha^-$ DC (Porter, Yi et al. 2011). It is unclear whether these TLR induced changes in the ratio of H2-DM and H2-DO have biological effects on MHC II presentation.

Contribution of Type I IFN to TLR-Initiated Events

Stimulation of DC through TLR-3, 4, 7, and 9 have long been known to induce production of type I interferons (IFN) (Akira, Uematsu et al. 2006). More recently, using highly purified TLR 2 ligands, it has been recognized that stimulation through this receptor also leads to production of type I IFN (Barbalat, Lau et al. 2009; Dietrich, Lienenklaus et al. 2010). The contribution of type I IFN to TLR-initiated events has been

linked to many critical *in vivo* events including maturation of DC, DC migration to draining LN, antibody production, and bystander activation of NK and T cells (Van Uden, Tran et al. 2001; Hoshino, Kaisho et al. 2002; Hoebe, Janssen et al. 2003; Honda, Sakaguchi et al. 2003; Kamath, Sheasby et al. 2005; Longhi, Trumpfheller et al. 2009; Swanson, Wilson et al. 2010; Oh, Kurche et al. 2011). While the contribution of type I IFN to TLR-initiated events in the MHC II pathway has not been explored, type I IFN has been shown to be important for TLR-induced cross priming (Van Uden, Tran et al. 2001; Le Bon, Etchart et al. 2003; Durand, Wong et al. 2004; Oh, Kurche et al. 2011) and specifically for CpG-induced cross presentation of proteins like soluble OVA (Kuchtey, Chefalo et al. 2005).

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Chapter 2

Presentation of Type B Peptide–MHC Complexes from Hen Egg White Lysozyme by TLR Ligands and Type I IFNs[1]

Abstract

In APCs, presentation by MHC II molecules of the chemically dominant peptide from the protein hen egg white lysozyme (HEL) generates different conformational isomers of the peptide–MHC II complexes (pMHC). Type B pMHCs are formed in early endosomes from exogenous peptides in the absence of H2-DM, whereas in contrast, type A pMHC complexes are formed from HEL protein in late vesicles after editing by H2-DM. Thus, H2-DM edits off the more unstable pMHC complexes, which are not presented from HEL. In this study, we show that type B pMHC complexes were presented from HEL protein only after stimulation of dendritic cells (DC) with TLR ligands or type I IFN. Type I IFN contributed to most TLR ligand induced type B pMHC generation, as presentation decreased in DC lacking the receptor for type I IFNs (IFNAR1$^{-/-}$). In contrast, presentation of type A pMHC from HEL and from peptide was minimally affected by TLR ligands. The relative effectiveness of CD8α^+ DC or CD8α^- DC in presenting type B pMHC complexes varied depending on the TLR ligand used. Thus, TLR ligands and type I IFN alter the pathways of presentation by MHC II molecules of DC such that type B pMHCs are generated from protein Ag.

[1] Chapter 2 is used with permission, with minor adaptations, from the first publication that resulted from this thesis: Strong, B.S.I. and Unanue, E.R. (2011). Presentation of Type B Peptide-MHC Complexes from Hen Egg White Lysozyme by TLR Ligands and Type I IFNs Independent of H2-DM Regulation. J. Immunol. 187(5): 2193-201.

Introduction

The chemically dominant peptide from the model protein hen egg white lysozyme (HEL) binds to the MHC II molecule, I-Ak, in multiple conformations (Viner, Nelson et al. 1996; Pu, Carrero et al. 2002; Lovitch and Unanue 2005). Both the more stable peptide–MHC II (pMHC) complexes, termed type A, and the less stable conformers, type B, bind to the same peptide, DYGILQINS, and in the same register (Viner, Nelson et al. 1996; Pu, Carrero et al. 2002; Pu, Lovitch et al. 2004; Lovitch and Unanue 2005). The type B pMHC conformers result when APC interact with peptides in early endosomes but are removed by H2-DM in late endosomes (Pu, Lovitch et al. 2004). In contrast, the type A pMHCs are formed in late vesicles where H2-DM edits off the unstable complexes, allowing the persistence of only the type A (Pu, Lovitch et al. 2004). The type B-specific T cells to the HEL 52–60 are frequent (30–50% of CD4 T cells) and escape tolerance, for example, in transgenic mice expressing HEL by all APCs (Peterson, DiPaolo et al. 1999). Because T cells to type B pMHC complexes are found to autologous proteins, it is critical to understand how type B responses could be protective during infection or detrimental in the context of inflammation-associated autoimmunity (Peterson, DiPaolo et al. 1999; Lovitch, Walters et al. 2003; Mohan, Levisetti et al. 2010). We previously reported that type B pMHC were generated from HEL protein in vivo when administered along with inflammatory stimuli such as LPS, listeriolysin O, or CFA (Lovitch, Esparza et al. 2007). In this study, we explore the signals that regulate TLR-induced generation of type B pMHC in vitro.

Dendritic cells (DC) play a key role in initiating the adaptive immune pathway through the presentation of Ag on MHC II molecules to activate CD4 T cells. Although resting DC are able to process and present exogenous Ag on MHC II molecules, there are noticeable changes that occur following exposure to inflammatory stimulants (Cella, Engering et al. 1997; Askew, Chu et al. 2000; Inaba, Turley et al. 2000; Turley, Inaba et al. 2000; Veeraswamy, Cella et al. 2003; Durand, Wong et al. 2004; West, Wallin et al. 2004; Wilson, El-Sukkari et al. 2004; Blander and Medzhitov 2006). TLR signaling induces DC maturation involving the production of critical cytokines, increased migration to draining lymph nodes, and increased expression of MHC I, MHC II, and many costimulatory molecules (Inaba, Witmer-Pack et al. 1994; MacPherson, Jenkins et al. 1995; Reis e Sousa, Hieny et al. 1997; Jakubzick, Bogunovic et al. 2008). Although many details of how TLR signaling affects the MHC II pathway are still to be considered, it is clear that TLR engagement leads to a transient increase in endocytosis, a transient increase in MHC II transcription, and stabilization of surface pMHC (Cella, Engering et al. 1997; West, Wallin et al. 2004; Wilson, El-Sukkari et al. 2004; Shin, Ebersold et al. 2006; van Niel, Wubbolts et al. 2006).

Stimulation through TLR3, -4, -7, and -9 leads to production of type I IFN by DC (Akira, Uematsu et al. 2006). TLR2 also initiates type I IFN production (Barbalat, Lau et al. 2009; Dietrich, Lienenklaus et al. 2010). The contribution of type I IFN signaling to TLR-initiated responses has been explored primarily in vivo and shown to be important for events ranging from DC maturation to Ab production by B cells to bystander activation of T cells and NK cells (Van Uden, Tran et al. 2001; Hoshino, Kaisho et al.

2002; Hoebe, Janssen et al. 2003; Honda, Sakaguchi et al. 2003; Kamath, Sheasby et al. 2005; Longhi, Trumpfheller et al. 2009; Swanson, Wilson et al. 2010). In the MHC I pathway, type I IFN has been shown to be important for TLR-induced cross priming (Van Uden, Tran et al. 2001; Le Bon, Etchart et al. 2003; Durand, Wong et al. 2004) and specifically for CpG-induced cross presentation of proteins like soluble OVA (Kuchtey, Chefalo et al. 2005). However, the contribution of type I IFN to TLR-initiated events in the MHC II pathway has not been explored. In this study, we show that most TLR ligands and type I IFN-induced presentation of type B pMHC by DCs from HEL and that TLR-induced type I IFN contributed to induced presentation of type B pMHC.

Materials and Methods

Mice

Mice were maintained under specific pathogen-free conditions according to institutional animal care guidelines. B10.BR mice were either from a colony maintained in our facility or purchased from The Jackson Laboratory (Bar Harbor, ME). MyD88$^{-/-}$ mice and the type 1 IFNR gene knockout (IFNAR1$^{-/-}$) mice were backcrossed to the B10.BR background.

Stimulants

Stimulants used were Ultra-pure LPS-EB from Escherichia coli 0111:B4 (Invivogen, San Diego, CA), gardiquimod (Invivogen), flagellin (Invivogen), CpG-B: ODN 1826

(Integrated DNA Technologies, Coralville, IA), polyinosinic-polycytidylic acid [Poly (I:C)] (Sigma-Aldrich, St. Louis, MO), zymosan A from Saccharomyces cerevisiae (Sigma-Aldrich), resiquimod (a kind gift from Dr. Marco Colonna, Washington University), recombinant IFN-α and IFN-β (PBL InterferonSource, Piscataway, NJ), and IFN-γ (kind gift from Dr. Robert Schreiber, Washington University). TLR ligands used in this study are listed in Table I.

Flow Cytometry

For cell sorting, DC were stained with CD11c(N418; eBioscience, San Diego, CA; or BioLegend, San Diego, CA), CD8α (53-6.7; eBioscience), CD45RA (14.8; BD Pharmingen, San Diego, CA), Siglec H (440c; a kind gift from Dr. Marco Colonna), B220 (RA3-6B2; BD Pharmingen), and CD19 (1D3; BD Pharmingen). For analysis of DC, the following Abs were used: CD4 (RM4-5; eBioscience), CD40 (3/23; BD Pharmingen), CD80 (16-10A1; BD Pharmingen), CD86 (GL1; BD Pharmingen), I-Ak (40F; made in house), and H-2Kk (36-7-5; BD Pharmingen). Flow cytometry data were collected on a BD FACS LSR II or BD FACSCalibur (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR).

DC isolation and Flt3 ligand treatment

DC were isolated from spleens of mice injected with 10 μg Flt3 ligand i.p. for 3 consecutive days. On day 8, spleens were harvested and digested with 0.14 U/ml Liberase Blendzyme 3 or 1.67 U/ml Liberase TL (Roche Applied Science, Indianapolis, IN) to

make single-cell suspensions from which DC were isolated by CD11c magnetic beads (Miltenyi Biotec, Auburn, CA). Enriched DC were >95% pure as determined by flow cytometry. For sorting, DC isolated as above were stained for surface markers and re-suspended in phenol-free DMEM (Invitrogen, Carlsbad, CA) supplemented with 2% FCS and 2 mM EDTA. Cells were sorted on a BD FACSAria II (BD Biosciences). Conventional DC (cDC) were sorted as $CD11c^{high}CD45RA^{-}B220^{-}$ or $CD11c^{high}$Siglec H^{-} $B220^{-}$. cDC were further separated by CD8α expression.

Assays

For Ag presentation assays, 10^5 DC were incubated with HEL protein or the HEL (48–62) peptide (DGSTDYGILQINSRW) with or without stimulants for 18 h in 100 μl volume in V-bottom 96-well plates. Stimulant concentrations used were: 10 μg/ml zymosan A, 10 μg/ml Poly (I:C), 1 μg/ml LPS, 1 μg/ml gardiquimod, 6 μg/ml resiquimod, 1 μM ODN 1826 (CpG-B), 100 U/ml IFN-γ, 10 U/ml IFN-α, and 10 U/ml IFN-β. DC were washed three times with serum-free DMEM after incubation before adding 5 x 10^4 T cell hybridomas/well in a 200-μl volume; 24 h later, the release of IL-2 was measured in the culture fluid using CTLL as an indicator cell. Most experiments used two previously characterized T cell hybridomas: 11A10, which only reacts with type B pMHC, and 3A9, which recognizes type A pMHC. Sorted DC were pretreated with 1 μg/well anti-IFNAR1 mAb MAR1-5A3 [a kind gift of Dr. Robert Schreiber (Sheehan, Lai et al. 2006)], or control antihuman IFN-γ receptor-1 GIR.208 Ab for 1 h at 37°C. Without washing, HEL or peptide was added with or without stimulants to the wells. The

remaining assay followed the protocol described above. For examination of surface markers by flow cytometry, DC isolated as described above were incubated with or without stimulants for 18 h in 100 μl volume in 96-well V-bottom plates.

Quantitative real-time PCR

Sorted cDC were incubated for 2, 6, or 18 h untreated or stimulated with TLR ligands or IFN-β. Total RNA was isolated using an RNAqueous-Micro kit according to the manufacturer's instructions (Applied Biosystems, Carlsbad, CA). Equal amounts of RNA were used to generate cDNA using the High Capacity Reverse cDNA Transcription Kit according to the manufacturer's instructions (Applied Biosystems). Forty nanograms cDNA were used per reaction for quantitative real-time PCR (qRT-PCR) analysis. qRT-PCR was performed using a Fast SYBR Green kit and DDCT calculations on a StepOnePlus Instrument (Applied Biosystems). Primers used were the following: 18S forward (F), 5'-GTAACCCGTTGAACCCCATT-3'; 18S reverse (R), 5'-CCATCCAATCGGTAGTAGCG-3'; IFN-β F, 5'-ATAAGCAGCTCCAGCTCCAAG-3'; IFN-β R, 5'-GTCTCATTCCACCCAGTGCTG-3'; 18S was used as the qRT-PCR standard.

Results

TLR ligands induce type B presentation from HEL.

To assess the ability of TLR ligands (Table I) to affect type B pMHC presentation, a culture system was set up involving the culture of DC with HEL in the

presence of the stimulants for 18 hours after which the stimuli were removed, and the extent of presentation was assayed by adding the indicator T cell hybridomas. In agreement with the basic definition of type B presentation, unstimulated DC presented type B pMHC only when incubated with the HEL:48–61 peptide, but not HEL (Fig. 1A, 1C). However, all TLR ligands except flagellin (Fig. 2A) allowed for the presentation of type B pMHC conformers from HEL, referred to as induced type B presentation. Notably, TLR ligands had little effect on presentation from peptide (Fig. 1C, 1D), only affecting type B presentation from peptide at low levels (0.01 μM), and modestly augmented presentation to 3A9, a T cell that recognizes type A pMHC, only at low levels of HEL (0.01 μM) (Fig. 1B). IFN-γ did not induce type B presentation from HEL (Fig. 1A). The induced type B presentation by TLR ligands was reproducible, testing three different type B T cell hybridomas recognizing the HEL 48–61 epitope (data not shown.) Induced type B pMHC presentation by MyD88-deficient DC followed the expected pattern, with TLR7 and -9 ligands being completely eliminated, TLR3 ligands being minimally affected, and TLR4 ligands only partially reduced, reflecting the degree to which each TLR depends on MyD88 as a signaling adapter (Fig. 2) (Kawai and Akira 2010).

Role of DC subsets in induced presentation.

Whether a subset of DC was responsible for the presentation of type B epitopes from HEL was examined. Plasmacytoid DC comprised ~10% of the total DC population (identified as $CD11c^{int/low}$Siglec $H^{+}B220^{+}$), but were not essential for induced presentation as sorted cDC, identified as $CD11c^{hi}$ Siglec $H^{-}B220^{-}$ presented type B

pMHC from HEL with TLR stimulation (data not shown.) To examine the role of the cDC subsets, sorted $CD8\alpha^+$ and $CD8\alpha^-$ DC were incubated with HEL protein with or without CpG as in previous experiments. $CD8\alpha^+$ DC strongly presented type B pMHC from HEL upon CpG stimulation (Fig. 3B), but not in the absence of stimuli. $CD8\alpha^-$ DC, however, had some degree of presentation of type B pMHC in the absence of stimulation at high HEL doses: it increased upon CpG stimulation, but not as strongly as the $CD8\alpha^+$ DC (Fig. 3C). The ability of $CD8\alpha^+$ DC or $CD8\alpha^-$ DC to present type B pMHC from HEL slightly differed, depending on the TLR ligands, except for gardiquimod, a ligand of TLR7/8 (Fig. 3D). Gardiquimod induced a small level of presentation only by $CD8\alpha^-$ DC, which is not surprising as TLR7 is minimally expressed in $CD8\alpha^+$ DC (Edwards, Diebold et al. 2003). Of note, $CD8\alpha^-CD4\alpha^+$ DC have been shown to lack TLR3 expression but $CD8\alpha^-CD4^-$ DC express intermediate levels of TLR3 (Edwards, 2003.) Our $CD8\alpha^-$ DC population is a mixture of $CD4^+$ and $CD4^-$ so it is expected that these cells would express TLR3. Therefore it is not surprising that both DC subsets respond to Poly (I:C). Additionally, Poly (I:C) has been shown to signal through MDA5 in addition to TLR3 (Kato, 2006.) Since we do not have trif-/- mice on our genetic background, we do not know if Poly (I:C) is signaling through TLR3, MDA5, or a combination of the two pathways. There was no significant difference in presentation of HEL peptide by the DCs (Fig. 3E). Of note is that the degree of presentation of HEL at high doses by the unstimulated $CD8\alpha^-$ DC varied greatly from no response whatsoever to the small response found in Fig. 3B, reflecting most likely a degree of activation by environmental stimuli. In sum, these data indicate that both splenic DC subsets are capable of presenting

type B pMHC from protein HEL in response to TLR stimulation.

Role of type I IFN in induced type B presentation

In addition to TLR ligands, IFN-α and IFN-β induced type B pMHC presentation from HEL (Fig. 4A), but only affected type A presentation marginally at low levels of protein (Fig. 4B). Type I IFNs had minimal effects on presentation from peptide, as with TLR ligands, only affecting type B pMHC presentation at low peptide doses (0.01 μM) (Fig. 4C, 4D). qRT-PCR was used to assess expression of IFN-β by sorted cDC. Stimulation of cDC with CpG, Poly (I:C), LPS, gardiquimod, and zymosan A induced expression of IFN-β as indicated by increased mRNA levels after 2 h, decreasing by 6 h after stimulation (Fig. 4E). To test the role of TLR-induced type I IFN on presentation, MAR1-5A3 Ab was used to block signaling through the type I IFNα/β receptor (IFNAR1) (Sheehan, Lai et al. 2006). DC were pretreated with control or MAR1-5A3 Ab before adding HEL with or without stimulants. MAR1-5A3 completely blocked IFN-β–induced type B presentation by $CD8\alpha^+$ DC (99.7% at half-maximum, Fig. 5A). It reduced CpG-induced presentation by 57% (at half-maximum) by $CD8\alpha^+$ DC. Blocking the IFNAR1 receptor had little effect on presentation from peptide (data not shown) and followed a similar trend with $CD8\alpha^-$ DC (Fig. 5B).

Of note, MAR1-5A3 treatment reduced the level of type B presentation by unstimulated $CD8\alpha^-$ DC, indicating that cytokine production may explain why $CD8\alpha^-$ DC were able to present low levels of type B pMHC from protein in the absence of stimulation. To more critically address the role of TLR-stimulated type I IFN in induced presentation, DC from

IFNAR1-deficient mice were examined. Although IFN-β and IFN-α had no effect on presentation by IFNAR1-deficient DC (Fig. 5C, 5D, Fig. 6A), CpG-induced presentation was decreased by ~60% (at half-maximum) in IFNAR1-deficient DC compared with wild type DC (Fig. 5C, Fig. 6A). The role of TLR-induced type I IFN was explored with the remaining TLR ligands shown to induce type B pMHC presentation from protein HEL using IFNAR1-deficient DC. Poly (I:C)- and zymosan A-induced type B presentation were reduced by 25 and 36%, respectively, in IFNAR1-deficient DC (Fig. 5D, Fig. 6A). Gardiquimod-induced presentation was completely inhibited, and LPS-induced presentation was unaffected. These data show that type I IFN signaling contributed differently to each TLR pathway of induced type B pMHC presentation, augmented presentation through TLR3 and TLR9, was the sole mediator through TLR7, and had no effect on induction through TLR4. Of note, presentation from peptide and type A pMHC presentation from protein was unaltered in IFNAR1-deficient DC (Fig. 6B, 6C). Together, these results suggest that TLR and type I IFN signaling most likely initiate the same subcellular events and that TLR-induced type I IFN can amplify the signal, allowing for augmented type B pMHC presentation.

Role of type I IFN on expression of costimulatory molecules

Because costimulatory molecule expression is a component of DC maturation, the contribution of type I IFN to expression of costimulatory molecules was examined to see if it mirrored the contribution to induced type B pMHC presentation. DC from B10.BR wild type or IFNAR1$^{-/-}$ mice were analyzed for surface expression of MHC II and

costimulatory molecules after 18 h in culture with or without stimulation. Although MHC II levels changed little after stimulation, CD40, CD80, and CD86 were all significantly increased on B10.BR DC after exposure to TLR ligands and IFN-β (Fig. 7, Fig. 8). Costimulatory molecule expression after stimulation was lower on IFNAR1$^{-/-}$ DC than B10.BR DC for all stimulants tested, indicating that type I IFN signaling augmented upregulation of costimulatory molecule expression by all TLR ligands tested. As expected, increased surface expression of costimulatory molecules by IFN-β was completely dependent on type I IFN signaling, as IFNAR1$^{-/-}$ DC were unable to respond (Fig. 7F, 7G). Zymosan A-stimulated costimulatory molecule expression was minimally dependent on type I IFN signaling (Fig. 7A). Poly (I:C)- and CpG-stimulated costimulatory molecule expression was more significantly dependent on type I IFN signaling (Fig. 7B, 7E). Gardiquimod-stimulated costimulatory molecule expression was almost completely dependent on type I IFN signaling, mirroring the complete requirement for type I IFN signaling for induced type B pMHC presentation (Figs. 5D, 7D). The stimulation of costimulatory molecule expression by LPS was dependent partially on type I IFN signaling (Fig. 6C), which was not the case for induced type B presentation (Fig. 5D, Fig. 6A).

Discussion

The present report adds not only to the understanding of presentation of type B pMHC conformers of HEL, but also to the general effects of TLR ligand and type I IFN on the MHC II processing and presentation pathway. In the current study, exposure of DC to

TLR ligands and type I IFN changed HEL protein handling, allowing strong processing and presentation of type B pMHC complexes. Notably, in the absence of TLR or type I IFN stimulation, as reported before, DC did not present type B pMHC complexes from HEL unless at very high concentrations. Both major DC subsets participated in induced type B pMHC presentation of HEL, although the degree to which each participated varied depending on the inflammatory signal.

Type I IFN signaling was important for type B pMHC presentation initiated by CpG, Poly (I:C), gardiquimod, and zymosan A, but not for LPS-initiated events. Type I IFN has been argued to be important for both cross-priming of CD8 T cells and direct priming of CD8 T cells (Le Bon, Etchart et al. 2003; Ahonen, Doxsee et al. 2004; Durand, Wong et al. 2004) and for cross-presentation induced by CpG (Kuchtey, Chefalo et al. 2005). In this study, with MHC II, we found a strong effect not on presentation of the conventional type A epitopes, but rather on the type B pMHCs. Induction of CD40 by LPS, Poly (I:C), and CpG on GM-CSF bone marrow-derived DC has been shown to be at least partially dependent on type I IFN signaling (Hoshino, Kaisho et al. 2002; Hoebe, Janssen et al. 2003; Honda, Sakaguchi et al. 2003; Longhi, Trumpfheller et al. 2009). Similarly, the increase in CD40, CD80, and CD86 expression after stimulation with zymosan A, Poly (I:C), LPS, and CpG was partially dependent on type I IFN signaling. Gardiquimod-induced expression of these costimulatory molecules absolutely required type I IFN signaling. In our experiments, we used T cell hybridomas that do not require costimulation, allowing examination exclusively of changes in levels of pMHC. Previous studies showed effects of TLR ligands on the generation of class II pMHC

(Inaba, Turley et al. 2000; Turley, Inaba et al. 2000; Blander and Medzhitov 2006) as well as on the modulation of costimulatory molecules (Hoshino, Kaisho et al. 2002; Hoebe, Janssen et al. 2003; Honda, Sakaguchi et al. 2003; Longhi, Trumpfheller et al. 2009). These findings have varied in the kind of assays the APC used for examination and the amounts and forms of ligands, so a general consensus has not emerged on the key mechanisms of action. An issue thus is to separate processing and generation of pMHC from other components of the presentation pathway. We and others (Veeraswamy, Cella et al. 2003; Wilson, El-Sukkari et al. 2004) have shown subsequently, comparing results with T cell hybridomas and primary T cells, that both resting and TLR-activated DC were capable of processing and presenting Ag about equally.

We posit that presentation may be explained by changes in the dynamics of vesicular traffic, leading to a flow of peptides from late vesicles into endosomes lacking H2-DM. Such a mechanism of action is now the subject of ongoing examination. This could happen through changes in acidification of, or recruitment of proteases to, an early or recycling compartment. Interestingly, when bone marrow-derived DC were treated with CpG prior to Ag exposure, MHC II processing and presentation occurred in early and recycling compartments (Askew, Chu et al. 2000), a finding compatible with our hypothesis on how TLR ligands could promote presentation of type B pMHC of HEL.

A longstanding issue has been whether T cells recognizing type B epitopes from self-antigens could be involved in autoimmunity. This study indicates that TLR- and type I IFN-activated DC present type B pMHC from soluble HEL. It will be critical to determine whether type B pMHC from self-proteins are presented by TLR- and/or type I

IFN-activated DC, allowing priming of autoreactive T cells in vivo. We have previously shown that naturally arising insulin-reactive T cells against type B pMHC can transfer diabetes in the NOD model (Mohan, Levisetti et al. 2010).

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TLR	TLR2/6	TLR3	TLR4	TLR5	TLR7	TLR9
Agonist(s)	Zymosan A	Poly(I:C)	LPS	Flagellin	Gardiquimod Resiquimod	ODN 1826 (CpG-B)
	Also binds Dectin-1					

Table I. TLR Ligands used in this study.

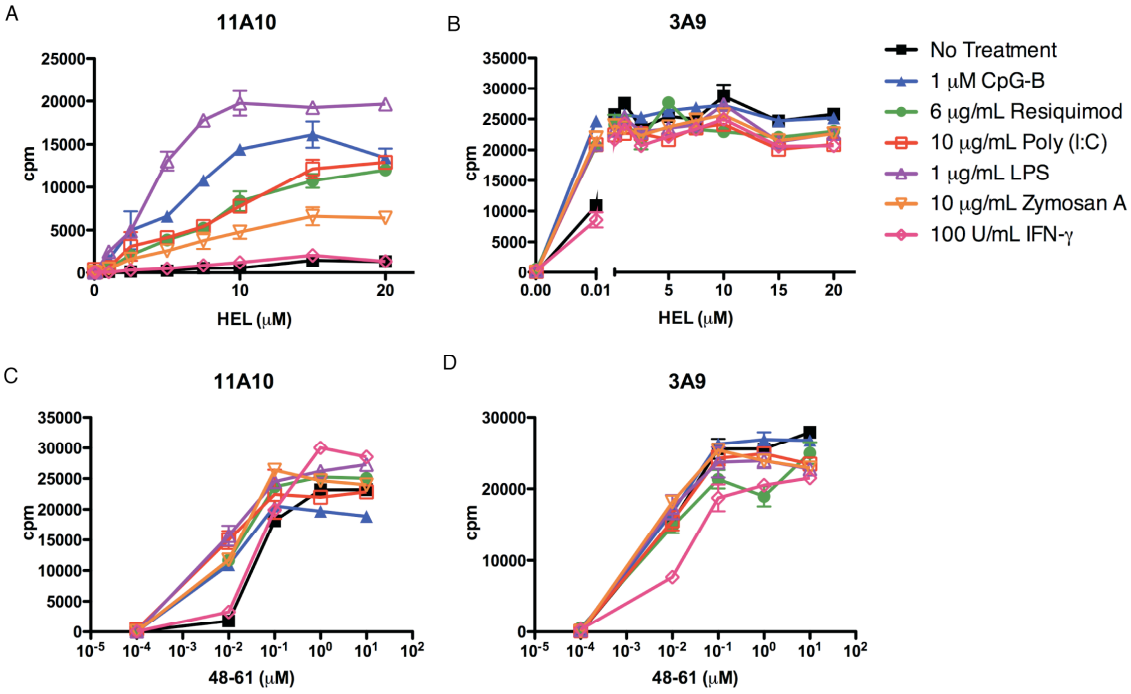


Figure 1. TLR Ligand-Induced Presentation of Type B pMHC from HEL. Presentation by DC incubated with HEL (A, B) or HEL:48-61 peptide (C, D) to type B 11A10 (A, C) or type A 3A9 (B, D) T cell hybridomas, in the presence or absence of TLR-ligands. Representative of at least four independent experiments. Error bars represent SD.

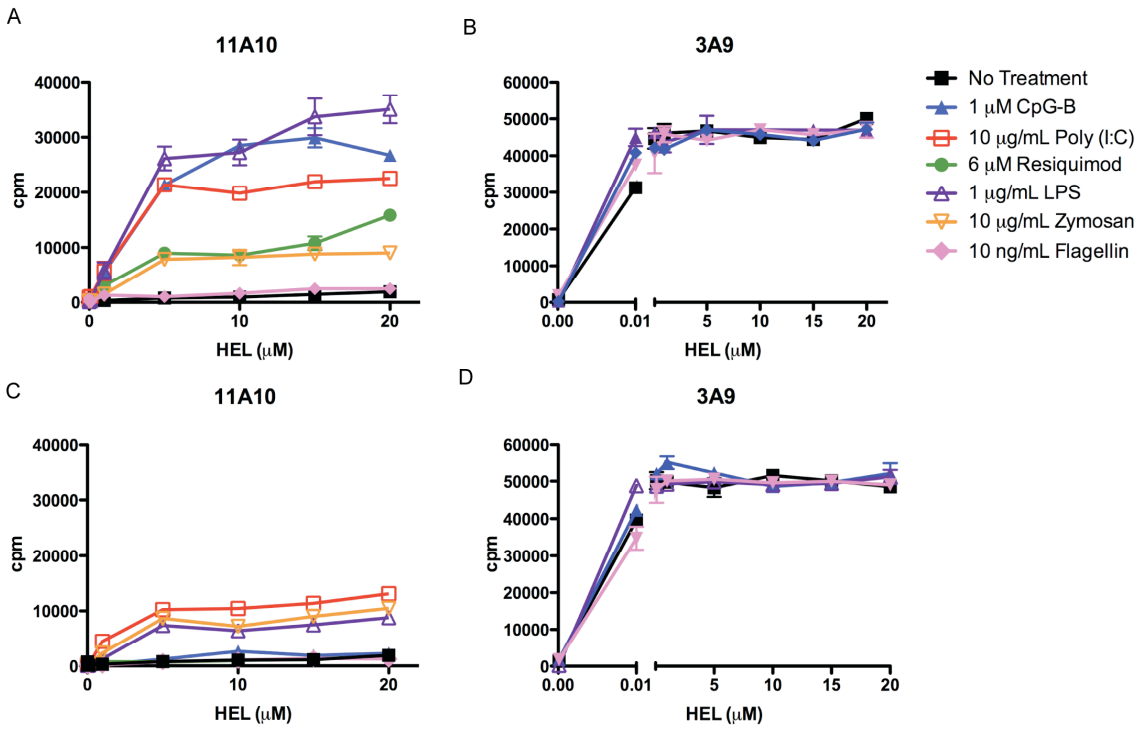


Figure 2. Dependence on MyD88$^{-/-}$ DC for TLR Ligand-Induced Presentation of Type B pMHC from HEL. Presentation to type B 11A10 (A, C) or type A 3A9 (B, D) T cell hybridomas by DC from wild-type (A, B) or MyD88$^{-/-}$ mice (C, D) incubated with HEL, with or without TLR ligands. Error bars represent SD. Representative of three independent experiments.

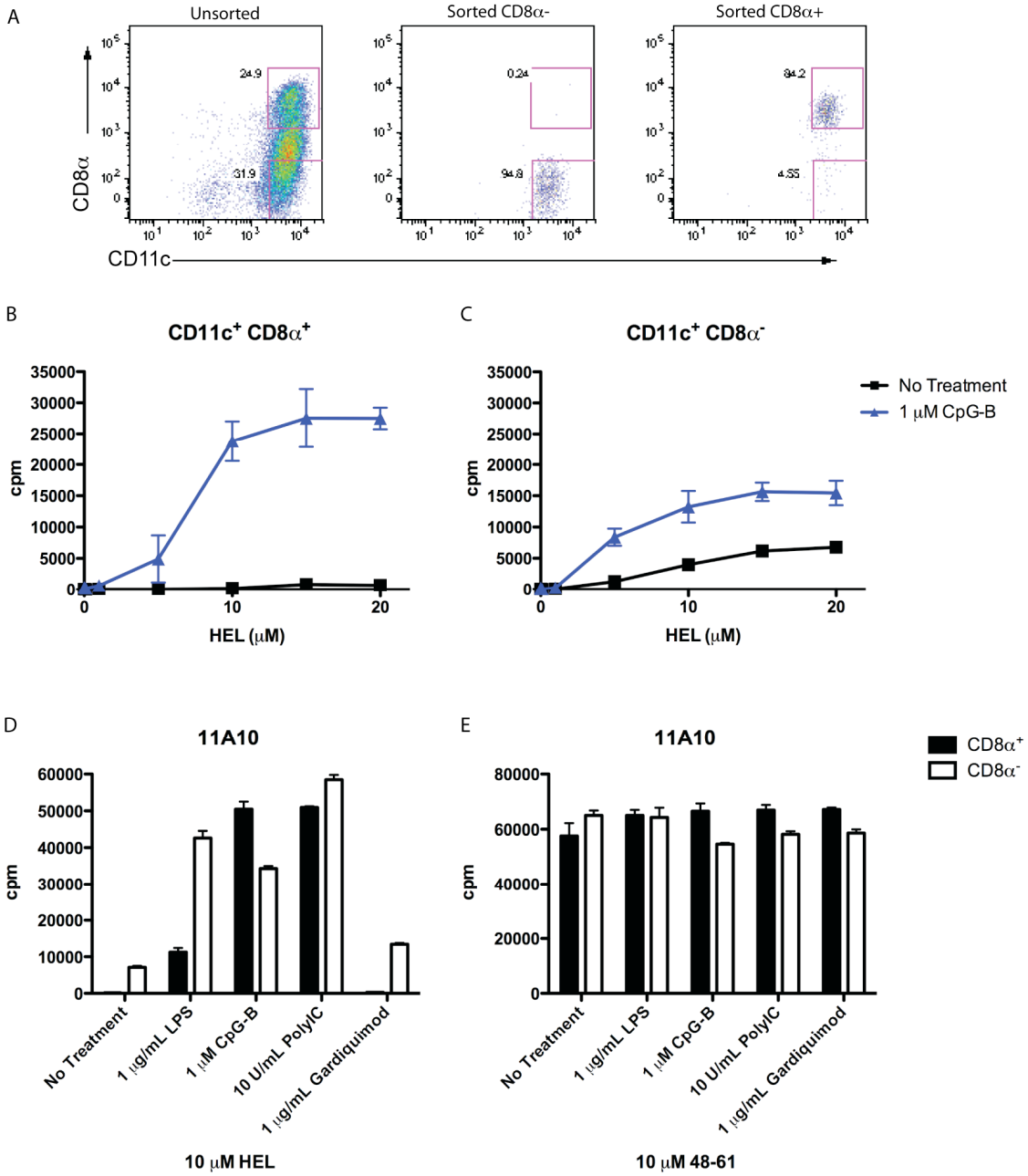


Figure 3. Presentation by Sorted CD8α$^+$ and CD8α$^-$ DC. (A) Representative flow cytometry assay on sorted cells showing CD11c and CD8α expression on unsorted and sorted DC populations. (B, C). Presentation to type B 11A10 T cell hybridoma by sorted CD8α+ (B) and CD8α- (C) DC incubated with HEL protein, with or without CpG. (D, E). Presentation to type B 11A10 T cell hybridoma by sorted DC incubated with HEL protein (D) or peptide (E) with or without TLR stimulants. Error bars represent SD. B and C representative of eleven independent experiments; D and E representative of four independent experiments.

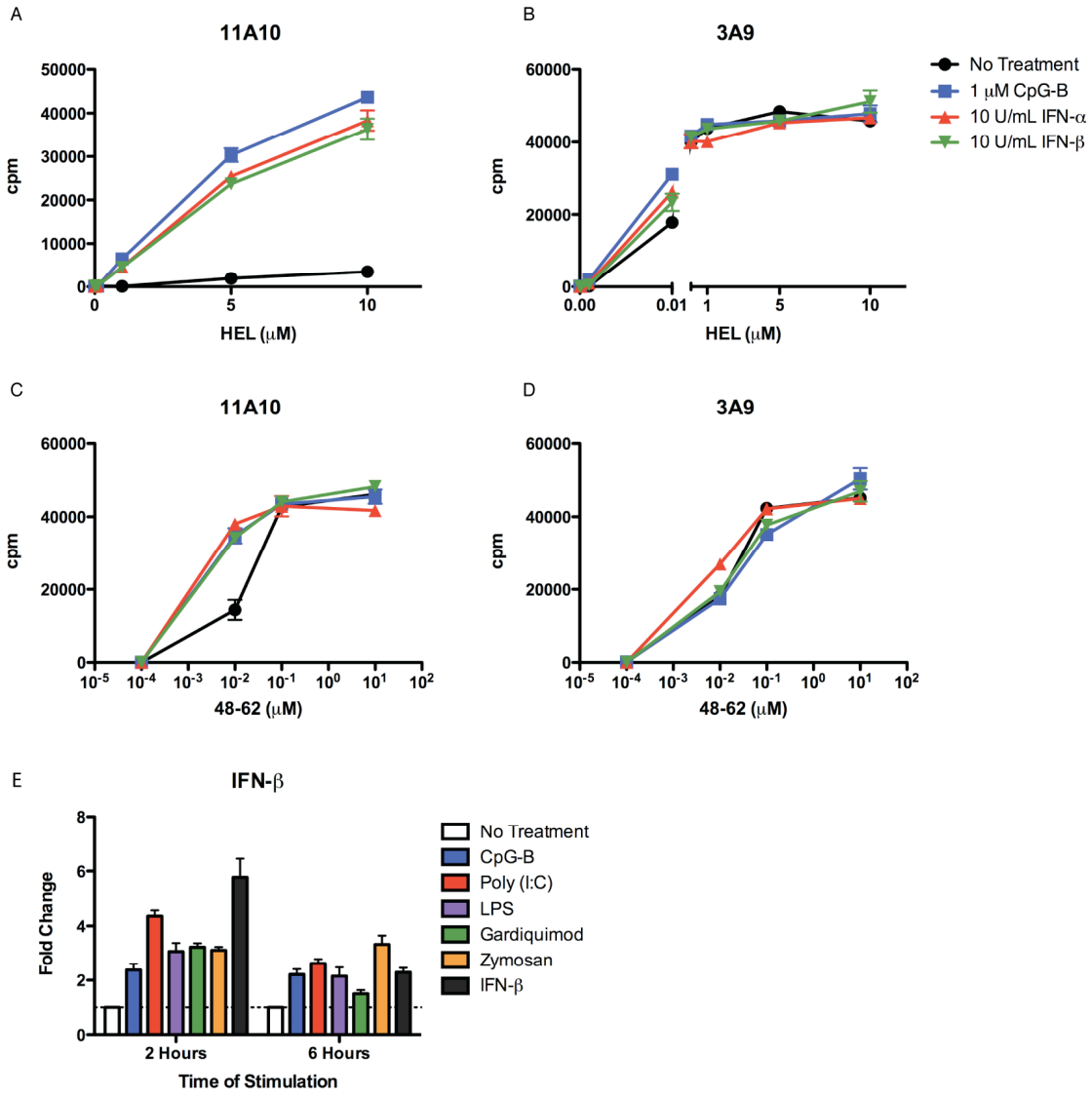


Figure 4. Type I IFN-Induced Presentation of Type B pMHC from HEL and Production of Type I IFN by TLR Stimulation. (A-D) Presentation by DC incubated with (A, B) HEL or (C, D) HEL peptide 48-62 with or without CpG or recombinant IFN-α or IFN-β to (A, C) type B 11A10 or (B, D) type A 3A9 T cell hybridomas. Representative of twelve independent experiments. (E) Sorted cDC were incubated with or without stimulants for 2 or 6 hours before RNA was isolated. qRT-PCR was performed to assess transcript levels of IFN-β. ΔΔCT calculations were performed using 18S as the standard.

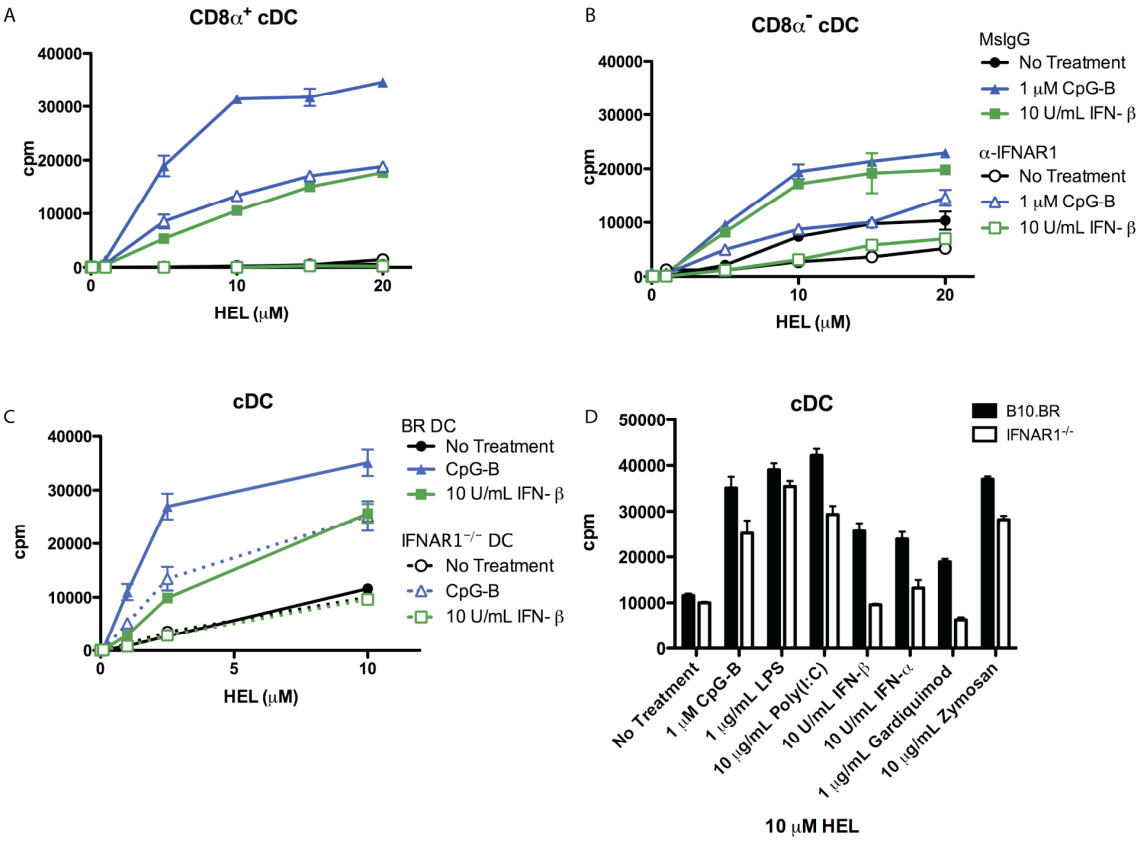
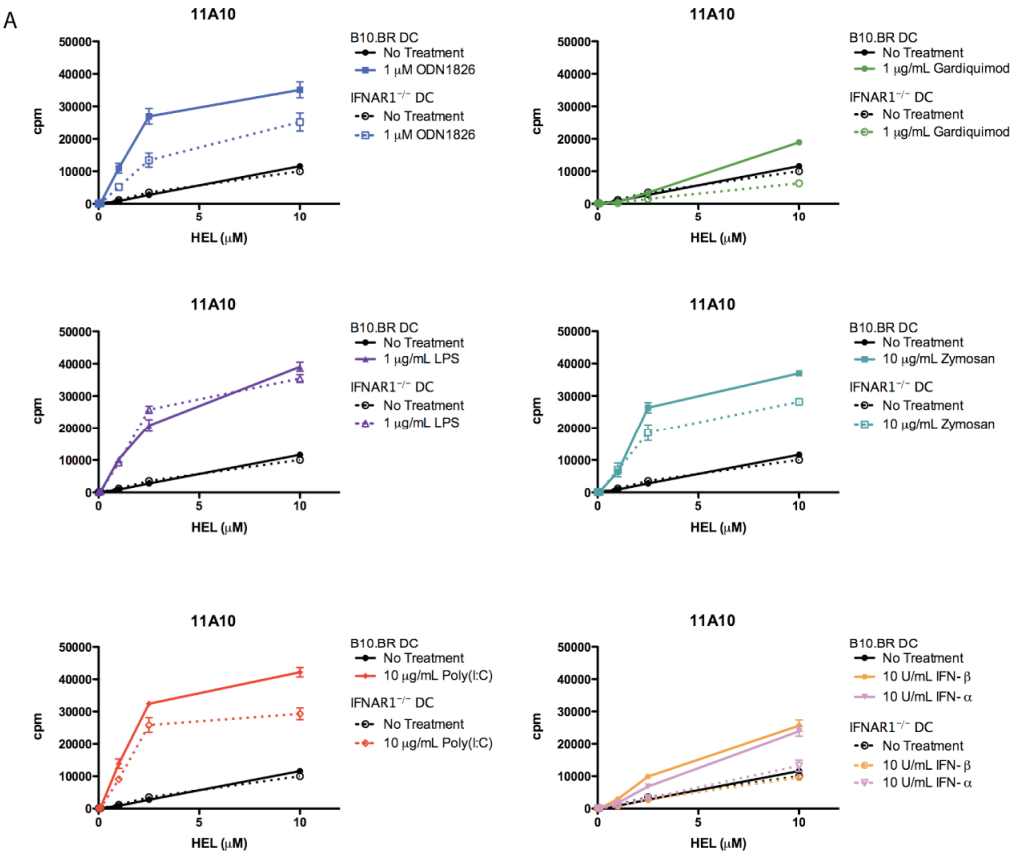
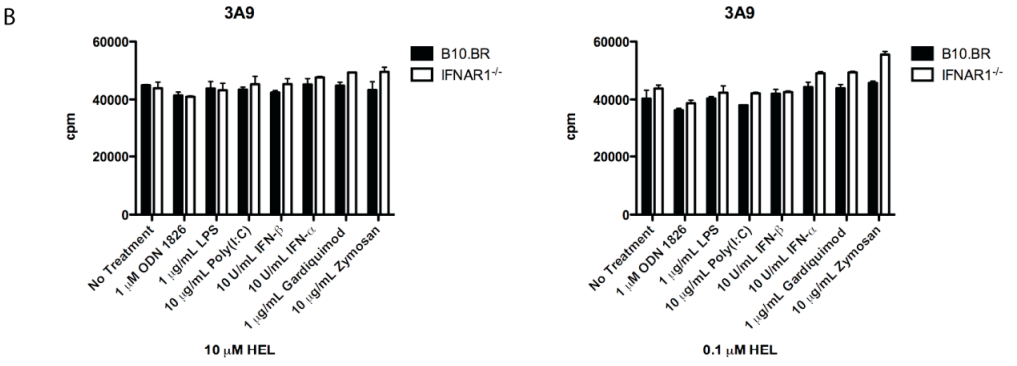


Figure 5. Role of Type I IFN Signaling in CpG-Induced pMHC Presentation from HEL. (A, B) Presentation to type B 11A10 T cell hybridoma by sorted $CD11c^{hi}CD8a^{+}$ (A) versus $CD11c^{hi}CD8a^{-}$ (B) cells pre-treated with MAR1-5A3 (anti-IFNAR1) or control mouse IgG before adding antigen and stimulants. (C) Presentation to type B 11A10 T cell hybridoma by sorted cDC from B10.BR or $IFNAR1^{-/-}$ mice incubated with HEL protein with or without stimulants. (D) Presentation to 11A10 by cDC treated as in B. Results shown for 10 μM HEL protein dose. Complete antigen titrations are shown in Figure 6. Error bars represent SD. All graphs are representative of two independent experiments.


A
11A10
B10.BR DC
No Treatment
1 μM ODN1826
IFNAR1−/− DC
No Treatment
1 μM ODN1826
cpm
HEL (μM)
11A10
B10.BR DC
No Treatment
1 μg/mL Gardiquimod
IFNAR1−/− DC
No Treatment
1 μg/mL Gardiquimod
11A10
B10.BR DC
No Treatment
1 μg/mL LPS
IFNAR1−/− DC
No Treatment
1 μg/mL LPS
11A10
B10.BR DC
No Treatment
10 μg/mL Zymosan
IFNAR1−/− DC
No Treatment
10 μg/mL Zymosan
11A10
B10.BR DC
No Treatment
10 μg/mL Poly(I:C)
IFNAR1−/− DC
No Treatment
10 μg/mL Poly(I:C)
11A10
B10.BR DC
No Treatment
10 U/mL IFN-β
10 U/mL IFN-α
IFNAR1−/− DC
No Treatment
10 U/mL IFN-β
10 U/mL IFN-α


B
3A9
B10.BR
IFNAR1-/-
cpm
No Treatment
1 μM ODN 1826
1 μg/mL LPS
10 μg/mL Poly(I:C)
10 U/mL IFN-β
10 U/mL IFN-α
1 μg/mL Gardiquimod
10 μg/mL Zymosan
10 μM HEL
3A9
0.1 μM HEL

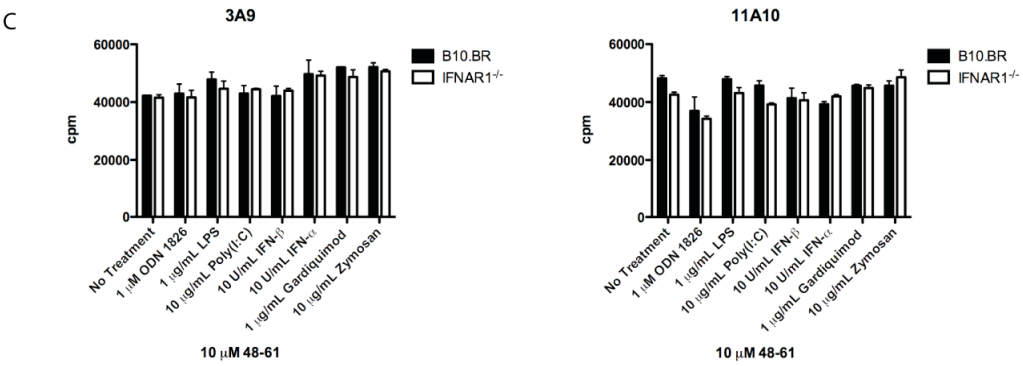

C
3A9
11A10
B10.BR
IFNAR1-/-
cpm
10 μM 48-61

Figure 6 (previous page.) Complete Results from Comparison of B10.BR and IFNAR1$^{-/-}$ DC. (A) Presentation to type B 11A10 T cell hybridoma by sorted cDC from wild type B10.BR or IFNAR1$^{-/-}$ mice incubated with HEL protein with or without stimulants. (B) Presentation to type A 3A9 T cell hybridoma by sorted cDC from B10.BR or IFNAR1$^{-/-}$ mice incubated with HEL protein with or without stimulants. Bar graphs for 10 and 0.1 μM HEL protein doses shown. (C) As in A and B except cDC incubated with 10 μM HEL peptide 48-61. Error Bars represent SD. Data are representative of two independent experiments.

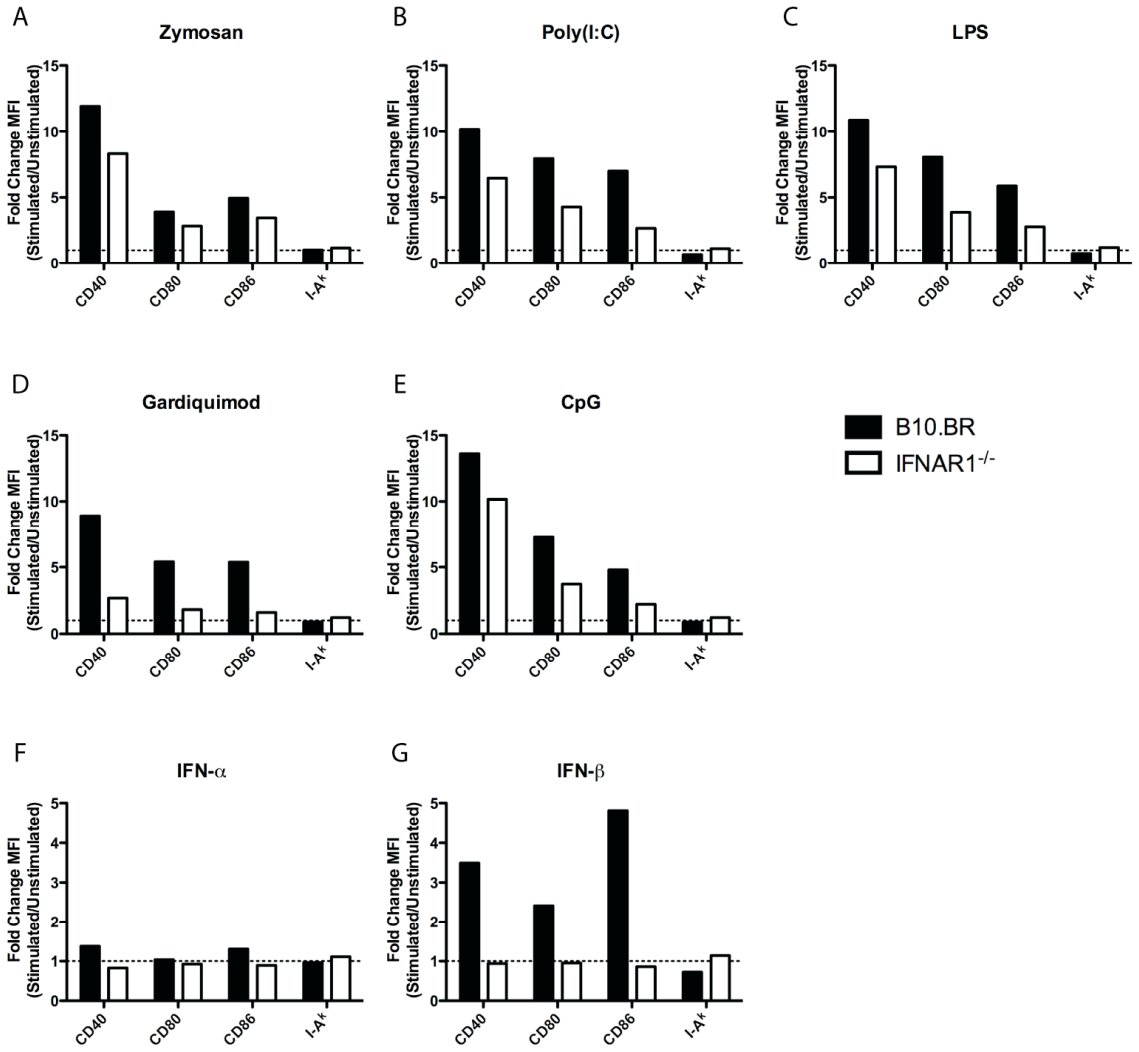


Figure 7. Role of Type I IFN Signaling in TLR-Induced Changes in Costimulatory Molecule Expression. DC from wild type B10.BR or IFNAR1$^{-/-}$ mice were incubated alone or with (A) Zymosan A, (B) Poly (I:C), (C) LPS, (D) Gardiquimod, (E) CpG, (F) IFN-α, or (G) IFN-β for 18 hours. DC were stained for CD40, CD80, CD86, or I-A^{k} and analyzed by flow cytometry. Data represented as fold change MFI of stimulated over un-stimulated DC. Data gated on live CD11c^{high} Siglec H^{-} cells. Data are representative of two independent experiments. Full histograms are shown in Figure 8.

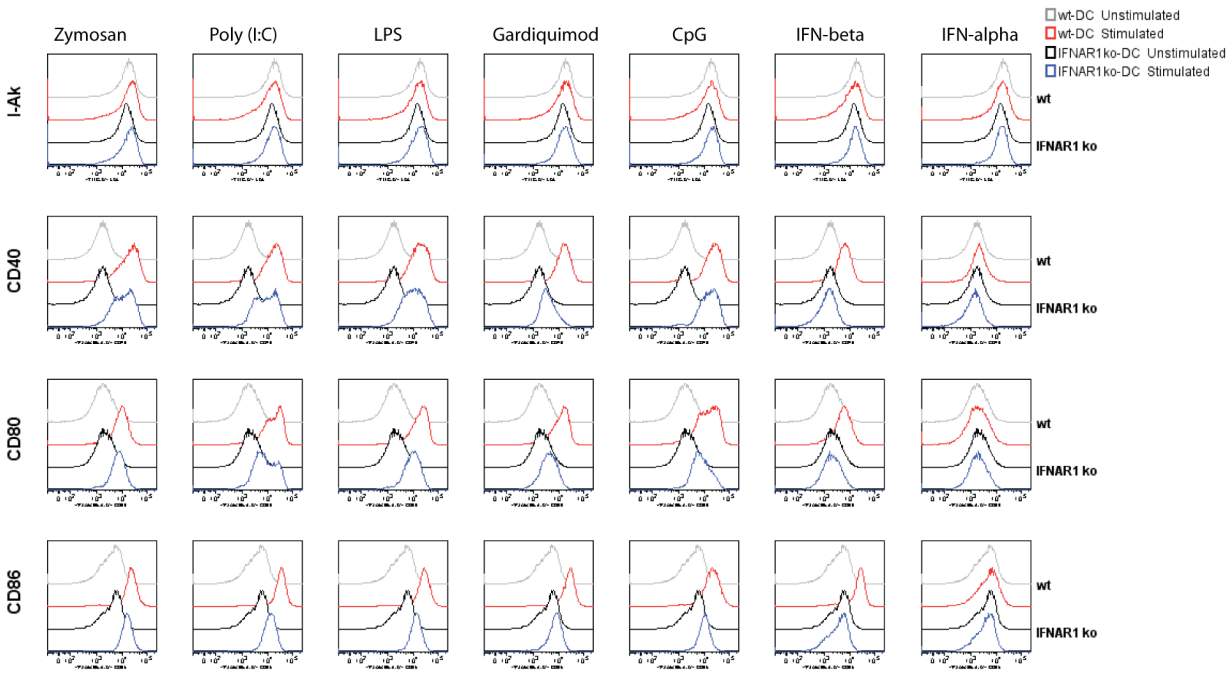


Figure 8. Histograms from Role of Type I IFN Signaling in TLR-Induced Changes in Costimulatory Molecule Expression. DC from wild type B10.BR or IFNAR1$^{-/-}$ mice were incubated alone or with stimulants for 18 hours. DC were stained for CD40, CD80, CD86, or I-A^{k} and analyzed by flow cytometry. Data gated on live CD11c^{high} SiglecH^{-} cells. Data are representative of two independent experiments.

Chapter 3

Sub-Cellular Events Involved in TLR-Induced Type B Presentation from HEL[2]

Abstract

We have shown that TLR ligands and type I IFN act directly on cDC, allowing for generation of type B pMHC complexes from HEL protein. While we still lack a specific understanding of the events initiated inside DC that allow for this change in presentation, we have learned several things about the process. We have found that generation of type B pMHC from HEL in the presence of TLR ligands is an extremely delayed event. We have also found that inducible type B presentation does not appear to be a result of modulation of H2-DM or the release of peptides. CpG stimulation has a modest effect on increasing the rate of HEL catabolism. CpG dramatically increases the total amount of HEL protein taken up by DC, however other TLR ligands and IFN-β have a more modest effect. All stimulants tested have minimal effects on surface MHC II levels but significantly increase the number of HEL 48-61-I-A^k complexes on the surface.

Introduction

Resting APC present only type A pMHC from HEL protein but both type A and B pMHC conformers from peptide antigen (Viner, Nelson et al. 1996; Pu, Carrero et al. 2002; Lovitch and Unanue 2005). Stimulation of DC with TLR ligands or type I IFN

[2] A portion of Chapter 3 is used with permission, with minor adaptations, from the first publication that resulted from this thesis: Strong, B.S.I. and Unanue, E.R. (2011). Presentation of Type B Peptide-MHC Complexes from Hen Egg White Lysozyme by TLR Ligands and Type I IFNs Independent of H2-DM Regulation. J. Immunol. 187(5): 2193-201

allows these APC to generate both type A and B pMHC conformers from HEL protein (Strong and Unanue 2011). Many changes occur to the MHC II pathway following TLR stimulation that could contribute to this change in presentation of type B conformers. MHC II synthesis is transiently increased following exposure to TLR ligands (Cella, Engering et al. 1997). Stimulation increases the half-life of pMHC complexes at the plasma membrane (Cella, Engering et al. 1997; Villadangos, Cardoso et al. 2001). Additionally, antigen uptake is increased following exposure to TLR ligands (West, Wallin et al. 2004). One report has indicated that exposure to CpG, a ligand of TLR9, changes the location of MHC II processing and presentation from late to early endosomes (Askew, Chu et al. 2000). Here we explore several potential sub-cellular events that could contribute to TLR-induced type B presentation from protein.

Materials and Methods

Mice

Mice were maintained under specific pathogen-free conditions according to institutional animal care guidelines. B10.BR mice were either from a colony maintained in our facility or purchased from The Jackson Laboratory (Bar Harbor, ME). H2-DMa–deficient mice were also described previously (Koonce, Wutz et al. 2003).

Stimulants

Stimulants used were Ultra-pure LPS-EB from Escherichia coli 0111:B4 (Invivogen, San Diego, CA), CpG-B: ODN 1826 (Integrated DNA Technologies, Coralville, IA),

polyinosinic-polycytidylic acid [poly (I:C)] (Sigma-Aldrich, St. Louis, MO), gardiquimod (Invivogen) IFN-α, and IFN-β (PBL Interferon Source, Piscataway, NJ).

Flow cytometry

For cell sorting, DC were stained with CD11c (N418; eBioscience, San Diego, CA; or BioLegend, San Diego, CA), CD8α (53-6.7; eBioscience), CD45RA (14.8; BD Pharmingen, San Diego, CA), Siglec H (440c; a kind gift from Dr. Marco Colonna), B220 (RA3-6B2; BD Pharmingen), and CD19 (1D3; BD Pharmingen). For analysis of DC, the following Abs were used: I-Ak (40F; made in house) and AW3.18 (made in house.) For intracellular staining, cells were fixed and permeabilized using a Cytofix/Cytoperm kit according to the manufacturer's instructions (BD Pharmingen). Abs for intracellular staining were H2-DM (2E5A; BD Pharmingen), goat anti-rat–Cy5 (Zymed, Carlsbad, CA), and H2-DO Alexa 647 (Mags.Ob1; a kind gift from Dr. Lisa Denzin, Memorial Sloan-Kettering Cancer Center). For examination of surface markers by flow cytometry, DC isolated as described below were incubated with or without stimulants for indicated times between one and 18 hours in 100 μl volume in 96-well V-bottom plates. Flow cytometry data were collected on a BD FACS LSR II or BD FACSCalibur (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR).

HEL Uptake

HEL was labeled with HiLyte Fluor 647 (AnaSpec, Fremont, CA) according to manufacturer's instruction. DC were incubated with labeled HEL in the presence or absence of stimulants at the same concentrations used for presentation assays indicated

below for 1-18 hours. After incubation, DC were washed extensively before staining for surface markers expression and analysis by flow cytometry.

DC isolation and Flt3 ligand treatment

DC were isolated from spleens of mice injected with 10 μg Flt3 ligand i.p. for 3 consecutive days. On day 8, spleens were harvested and digested with 0.14 U/ml Liberase Blendzyme 3 or 1.67 U/ml Liberase TL (Roche Applied Science, Indianapolis, IN) to make single-cell suspensions from which DC were isolated by CD11c magnetic beads (Miltenyi Biotec, Auburn, CA). Enriched DC were >95% pure as determined by flow cytometry. For sorting, DC isolated as above were stained for surface markers and re-suspended in phenol-free DMEM (Invitrogen, Carlsbad, CA) supplemented with 2% FBS and 2 mM EDTA. Cells were sorted on a BD FACSAria II (BD Biosciences). Conventional DC (cDC) were sorted as $CD11c^{high}CD45RA^{-}B220^{-}$ or $CD11c^{high}$Siglec H^{-} $B220^{-}$. cDC were further separated by CD8α expression. For analysis of H2-DM and H2-DO, cDC were further selected as $CD19^{-}$.

Isolation of other APC

Spleens were digested 1.67 using U/ml Liberase TL (Roche Applied Science, Indianapolis, IN) to make single-cell suspensions from which cells were isolated by CD11b or CD19 magnetic beads (Miltenyi Biotec, Auburn, CA) for isolation of splenic macrophages and B cells, respectively. Bone marrow-derived DC (BMDC) were isolated as previously described (Veeraswamy, Cella et al. 2003) and used on day six of culture.

Antigen Presentation Assays

For Ag presentation assays, 10^5 DC were incubated with HEL protein or the HEL (48–

62) peptide (DGSTDYGILQINSRW) with or without stimulants for 18 h in 100 μl volume in V-bottom 96-well plates. Stimulant concentrations used were: 10 μg/ml zymosan A, 10 μg/ml poly (I:C), 1 μg/ml LPS, 1 μM ODN 1826 (CpG-B), 100 U/ml IFN-γ, 10 U/ml IFN-α, and 10 U/ml IFN-β. DC were washed three times with serum-free DMEM after incubation before adding 5 x 10^4 T cell hybridomas/well in a 200-μl volume; 24 h later, the release of IL-2 was measured in the culture fluid using CTLL as an indicator cell. Most experiments used two previously characterized T cell hybridomas: 11A10, which only reacts with type B pMHC, and 3A9, which recognizes type A pMHC. For assays determining the timing of antigen presentation, DC were fixed at the times indicated in 1% paraformaldehyde for 5 min, incubated with 0.2 M DL-Lysine for 10 min, and washed extensively in media containing 5% FBS.

Peptide release assay

DC from CB.17 mice were incubated overnight with HEL with or without 1 μM CpG-B. Supernatants were collected and spun twice to remove cells and debris. Supernatants were serially diluted and added to paraformaldehyde-fixed DC from B10.BR mice. DC were fixed as indicated above. T cells were added as described above.

HEL catabolism

DC were incubated for 18 h with 1 μM CpG-B (ODN1826) or without stimulation. Equal numbers of DC were incubated with [125I]-HEL for 2 hours at room temperature with intermittent mixing. After the incubation, DC were washed and re-plated for indicated times. At each time point, TCA-soluble and -precipitable fractions were collected from the cells and the supernatant.

Quantitative real-time PCR

Sorted cDC were incubated for 2, 6, or 18 h untreated or stimulated with TLR ligands or IFN-β. Total RNA was isolated using an RNAqueous-Micro kit according to the manufacturer's instructions (Applied Biosystems, Carlsbad, CA). Equal amounts of RNA were used to generate cDNA using the High Capacity Reverse cDNA Transcription Kit according to the manufacturer's instructions (Applied Biosystems). Forty nanograms cDNA were used per reaction for quantitative real-time PCR (qRT-PCR) analysis. qRT-PCR was performed using a Fast SYBR Green kit and DDCT calculations on a StepOnePlus Instrument (Applied Biosystems). Primers used were the following: 18S forward (F), 5'-GTAACCCGTTGAACCCCATT-3'; 18S reverse (R), 5'-CCATCCAATCGGTAGTAGCG-3'; DMa F, 5'-TAGGGCTCTCGGAGACCTATG-3'; DMa R, 5'-AGTCGAAGGAGAAGAGTTCGT-3'; DOa F, 5'-CCGCAATGAGCTTCCTGAGTCCC-3'; DOa R, 5'-GTGGTCGGCCTTGATGGCCCTT-3'; H2-Aa F, 5'-TCAGTCGCAGACGGTGTTTAT-3'; and H2-Aa R, 5'-GGGGGCTGGAATCTCAGGT-3'. 18S was used as the qRT-PCR standard.

Results

The Role of H2-DM in TLR-Induced Type B Presentation from HEL

Using liposome-encapsulated antigen, Pu et al (Pu, Lovitch et al. 2004) found that when peptide was targeted to early endosomes, both type A and B conformers were presented but when peptide was targeted to late endosomes, only type A conformers were

presented. These data demonstrated that type B pMHC formed preferentially in early endosomes and suggested that late endosomal compartments were refractory to generation of the type B conformers. Strikingly, when peptide was targeted to late endosomes of H2-DM-deficient APC, there was generation of type B conformers. This showed that when a large pool of free peptide is available to load onto MHC II, H2-DM effectively eliminates type B conformers from forming in late endosomes. Based on these data, our first hypothesis regarding the mechanism of CpG-induced presentation of type B pMHC from protein was that CpG stimulation shifts presentation of HEL protein from late endosomes to early endosomes. There is a precedent for this concept, as CpG has been shown to shift presentation from late endosomes to early endosomes in GM-CSF BMDC (Askew, Chu et al. 2000).

Since H2-DM prevents type B pMHC from being generated (Pu, Lovitch et al. 2004), regulation of H2-DM was considered as an explanation for the effects of TLR ligands. The function of H2-DM can be modulated by H2-DO (Denzin, Sant'Angelo et al. 1997; van Ham, Tjin et al. 1997; Denzin, Fallas et al. 2005), so it was necessary to examine expression of both molecules.

To determine if expression of H2-DM or H2-DO was altered by TLR ligand stimulation, sorted cDC were incubated for 2, 6, or 18 hours with or without CpG-B, LPS, or IFN-β stimulation and H2-DM, H2-DO, and H2-Aα (MHC II) were examined by qRT-PCR. Their expression increased two hours after stimulation, dropping by six and eighteen hours (Figure 1A). Protein levels of H2-DM and H2-DO were examined by intracellular staining after eighteen hours of stimulation. CpG-B did not significantly

alter levels of H2-DM protein, but did modestly increase expression of H2-DO (Figure 1B). LPS has previously been shown to have a similar effect on splenic DC, having no detectable effect on H2-DM protein levels, however they observed a modest decrease in H2-DO protein levels (Chen, Reed-Loisel et al. 2006) More recently, a report showed that after *in vivo* administration of TLR ligands, H2-DO is selectively down-regulated at the protein level in CD8α^- spleen DC while H2-DM protein levels remain minimally changed(Porter, Yi et al. 2011).

To examine whether regulation of H2-DM contributed to induced type B pMHC presentation, cDC from B10.BR wild type or H2-DM-deficient mice were incubated with HEL with or without stimulants as previously described. Consistent with previous reports (Koonce, Wutz et al. 2003), presentation of the type A pMHC was decreased by about ten fold in the H2-DM-deficient cDC, an indication that H2-DM is important for the assembly of HEL pMHC complexes (Figure 1 D, F.) Importantly, H2-DM-deficient cDC presented type B pMHC from HEL protein in response to CpG and LPS, albeit at reduced levels compared to wild-type cDC (Figure 1 C, E.) These data indicate that while regulation of H2-DM may have some contribution to TLR and type I IFN-induced type B presentation from HEL, it is not the key mechanism controlling the event. It is important to note that H2-DM-deficient resting DC have been shown to be deficient for H2-DO protein (Chen, Reed-Loisel et al. 2006), meaning that at least resting DC are deficient for both proteins.

Timing of Generation of Type A and B pMHC from HEL

Initial experiments looking at the mechanism of type B presentation from HEL protein indicated that generation of the type B pMHC complexes was delayed with respect to the type A pMHC complexes. To determine how long DC must be exposed to HEL and CpG, DC were incubated with antigen with or without CpG for varying times between three and eighteen hours (Figure 2A.) After incubation, DC were washed and T cell hybridomas were added to the cultures. We found that HEL and CpG must be present for a minimum of twelve hours for type B pMHC to be generated (Figure 2B, D.) Type A pMHC, however, were generated at all tested times (Figure 2C, E.)

To assess when pMHC appeared at the cell surface, DC were incubated with a set amount of HEL (30 μM) with or without CpG for varying times between twelve and thirty-eight hours. After incubation, DC were fixed, serially diluted, and incubated with T cell hybridomas (Figure 2F.) We found that type B pMHC were not present at the cell surface until after a minimum of eighteen hours after addition of HEL and CpG (Figure 2G.) Type A pMHC, however, were generated at all tested times (Figure 2H.) While the significance of this delay is not understood, it suggests that either accumulation of type B pMHC requires significant time to reach detectable levels or that significant reprogramming of DC must occur before type B pMHC can be generated by DC from protein.

Timing of Addition of TLR Stimulation and Antigen

We observed that during certain time periods DC could be isolated from spleen and present type B pMHC from HEL protein in the absence of stimulation. Out of

concern that this might be due to environmental fluctuations, we established a colony of our primary mouse line, B10.BR, in a cleaner animal facility. DC from mice in the cleaner facility consistently behave as expected, only presenting type B pMHC from peptide antigen or from HEL protein in the presence of TLR ligands or type I IFN. To directly test whether prior exposure to TLR ligands primed DC to handle protein differently, DC were incubated for eighteen hours in the presence or absence of CpG stimulation. DC were washed and antigen was added with or without CpG for an additional eighteen hours after which cells were washed again before adding T cell hybridomas. CpG-activated DC were capable of presenting type B pMHC from HEL even when CpG was not present at the same time as antigen (Figure 3B) albeit at a lower level than resting DC treated with HEL and CpG at the same time (Figure 3A.) This indicated that prior activation of DC primed the cells to handle protein in a way that they could generate type B pMHC from protein antigen. Interestingly, CpG-activated DC also presented higher levels of type B pMHC from peptide antigen at low levels (0.01 μM) similar to the enhancing effect on presentation from peptide when CpG and peptide are given together (Figure 3C, D.) This suggests that activated DC, for example during infection, might be able to present type B pMHC from subsequently encountered protein.

Presentation by other types of APC

Early attempts to find an *in vitro* system to evaluate inflammation-associated type B presentation from HEL added antigen, stimulants, and T cell hybridomas at the same time for a total of eighteen hours. We now know that this did not allow sufficient time for generation of type B pMHC from HEL and T cell hybridoma production of IL-2. Based

on this knowledge, it became compelling to test whether APC other than splenic DC were capable of induced type B presentation when given sufficient time to generate the complexes. In order to evaluate this question, we chose to test three other APC populations—splenic macrophages, splenic B cells, and GM-CSF bone marrow-derived DC (BMDC)—for their ability to present type B pMHC from protein.

Splenic CD11b+ cells (a mixture of macrophages and DC) and B cells (CD19+) were isolated from spleens of B10.BR mice and were used in the same presentation protocol as the DC—overnight incubation with antigen with or without TLR ligands followed by an overnight incubation with T cell hybridomas. While CD11b+ and CD19+ splenocytes were capable of presenting type B pMHC from HEL protein in response to TLR stimulation, the magnitude of presentation was minimal compared to levels presented by splenic DC (Figure 4A-D.) The same low level of type B pMHC was also seen with GM-CSF BMDC. (Figure 4E, F) These data indicate that while many APC populations can present type B pMHC from HEL in response to TLR stimulation, these other APC are not as efficient in the process as splenic DC.

Effect of TLR Ligands on HEL Uptake and Processing Efficiency

One of the described effects of TLR ligand stimulation on antigen processing and presentation was the observation that TLR stimulation led to a transient increase in the uptake of antigen followed by significantly decreased uptake (West, Wallin et al. 2004). Based on these findings, we found it pertinent to test what effect CpG had on uptake and processing efficiency in our system.

To test the effect of TLR ligands on HEL uptake, DC were incubated with HiLyte Fluor-647-labeled HEL protein in the presence or absence of TLR ligands for times ranging from one to twenty-four hours. Cells were washed, stained for surface markers and analyzed by flow cytometry. We initially explored the effects of CpG-B on uptake of HEL protein by DC. We found that as early as after one hour of exposure, CpG-B-treatment dramatically increased the amount of HEL protein taken up by DC (Figure 5, Figure 6A.) However, while CpG-B and Zymosan remarkably increased the amount of HEL protein taken up by DC, the other TLR ligands only had a minimal effect (Figure 6A.) Notably, Poly (I:C) and LPS, both induce similar levels of type B pMHC from HEL as CpG, however they induce significantly lower levels of HEL uptake by DC. These data indicate that it is unlikely that increased uptake of protein is the primary mechanism of TLR-induced type B pMHC presentation.

To test the effect of CpG on the efficiency of HEL processing, DC were incubated with unlabeled HEL with or without CpG, as described above. Cells were stained with anti-I-A^k (40F) or anti-48-61-I-A^k (AW3.18.) The AW3.18 antibody specifically recognizes the chemically dominant epitope of HEL bound to I-A^k and is capable of blocking recognition by both type A and B T cells against the epitope, indicating that it recognizes both conformers (Dadaglio, Nelson et al. 1997). Staining was assessed by flow cytometry. TLR ligand and IFN-β stimulation resulted in increased levels of surface pMHC complexes from HEL protein (Figure 6B.) Interestingly, all stimulants tested also increased levels of complexes generated from peptide (Figure 6C.) The observation that TLR stimulation increased levels of pMHC complexes on the surface from both peptide

and HEL pulses suggests that this effect is independent of effects on antigen processing since the peptide does not require processing for presentation. TLR-stimulation minimally increased surface levels of MHC II, (Figure 6D) suggesting that increased epitope generation is not a result of dramatically increased surface MHC II but rather an effect on longevity of loaded complexes at the cell surface or increased loading of exogenous antigen onto MHC II molecules.

Effect of CpG on Peptide Release by DC

A possible mechanism of TLR-induced type B pMHC presentation is generation or release of peptides into the extracellular milieu. This could occur directly by DC expelling digested protein, release of proteases by DC leading to extracellular processing, (Santambrogio, Sato et al. 1999), extracellular processing of protein by serum proteases (Accapezzato, Nisini et al. 1998), or processing and release by bystander cells such as neutrophils (Potter and Harding 2001). To test peptide release, we incubated DC from CB.17 mice with a high concentration of HEL (60 μM) in the presence or absence of CpG. After overnight incubation, the supernatants were collected, centrifuged multiple times to remove intact cells or debris, serially diluted, and added to fixed DC from B10.BR mice or to fixed C3.F6, a B cell lymphoma line. T cell hybridomas were added to the cultures. Results with B10.BR DC and C3.F6 were the same. There was no meaningful level of peptide released from resting or CpG-activated DC leading to generation of either the type A or type B pMHC complexes (Figure 7.)

HEL Catabolism

We examined if CpG-B changed the rate of catabolism of I^{125}-HEL. DC were incubated for eighteen hours alone or with CpG-B, then incubated with I^{125}-HEL for two hours at room temperature followed by one, two, three, or four hour chases in media free of HEL. TCA soluble and precipitable fractions were collected from intracellular (Figure 8A) and supernatant (Figure 8B) fractions at each time point. CpG-B-activated DC showed slight acceleration of HEL catabolism compared to resting DC. This increased rate of catabolism could allow CpG-activated DC to generate more peptides for presentation but it is difficult to tie back into a mechanism for induced type B pMHC presentation.

Discussion

Although this study does not identify the way by which the type B pMHC epitopes were presented, some issues are pointing to possible mechanisms of action. The initial data indicate that H2- DM, a logical molecule to examine, may not be key in the effects seen with TLR ligands. Pointedly, H2-DM–deficient DC presented type B pMHC from HEL protein after TLR ligand exposure. Although TLR stimulation regulated expression of H2-DO, which controls H2-DM activity, modulation of H2-DM function was ultimately not the key mechanism of type B presentation, as shown with the H2-DM gene knockout DC.

We have ruled out the release of peptides from the treated APC, whereas the effects on catabolism by CpG were modest. The observation that TLR ligands and type I IFN increase the total levels of 48-61—I-A^k complexes at the surface from both peptide

and protein antigen suggests that there is increased presentation that is independent of effects on processing activity. Since total surface MHC II levels were minimally increased by stimulation, it appears unlikely that this increase in surface pMHC is due solely to increased MHC II expression. It is possible that the increased epitope expression could be due to increased surface stability of pMHC that has been previously reported (Walseng, Furuta et al. 2010).

Another possible mechanism of induced type B presentation is a change in the site of antigen processing and/or presentation to a compartment that is favorable towards generation of type B pMHC conformers. It is possible that factors other than H2-DM are refractory to type B pMHC formation in the late compartment. CpG-stimulation of GM-CSF BMDC has been previously shown to allow a brefeldin A-insensitive pathway of MHC II presentation, suggesting that this occurs in early endosomes on recycling MHC II molecules (Askew, Chu et al. 2000) an observation that would be consistent with our hypothesis. As will be discussed in detail in Chapter 6, we have been unable to experimentally prove or disprove whether TLR stimulation alters the site of HEL processing or presentation.

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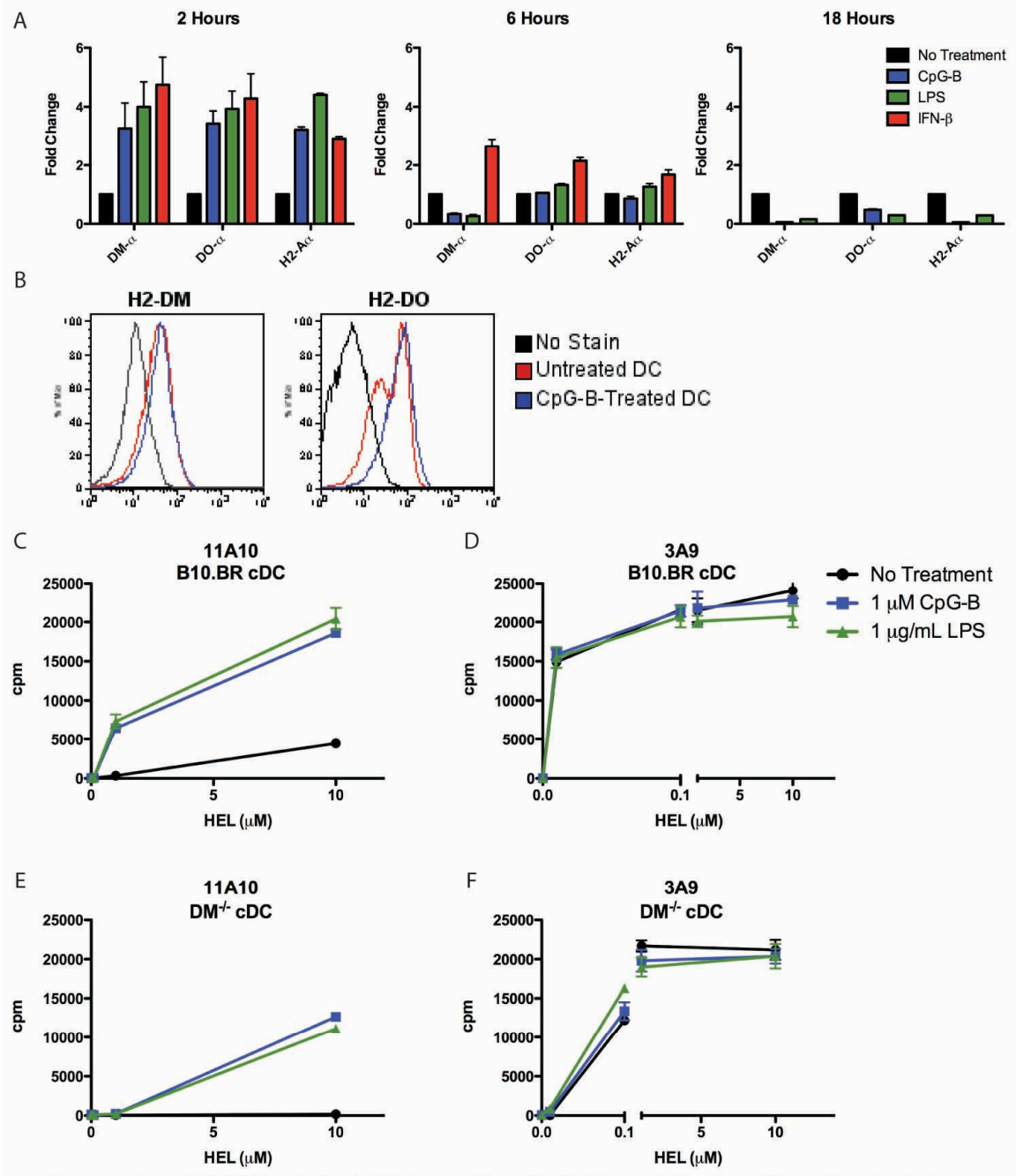

Figure 1. Role of H2-DM Regulation in TLR-Induced Type B pMHC Presentation from HEL. (A) qRT-PCR analysis of RNA isolated from sorted cDC ($CD11c^{high}SiglecH^{-}CD19^{-}$) were incubated with stimulants for 2, 6, or 18 hours. (B) Intracellular flow-cytometry analysis of H2-DM and H2-DO protein levels in sorted cDC after 18 hours of stimulation. (C-F) Presentation to (A, E) type B 11A10 or (B, D) type A 3A9 by sorted (C, D) wild-type B10.BR or (E, F) $H2\text{-}DM^{-/-}$ cDC incubated with HEL protein with or without stimulants.

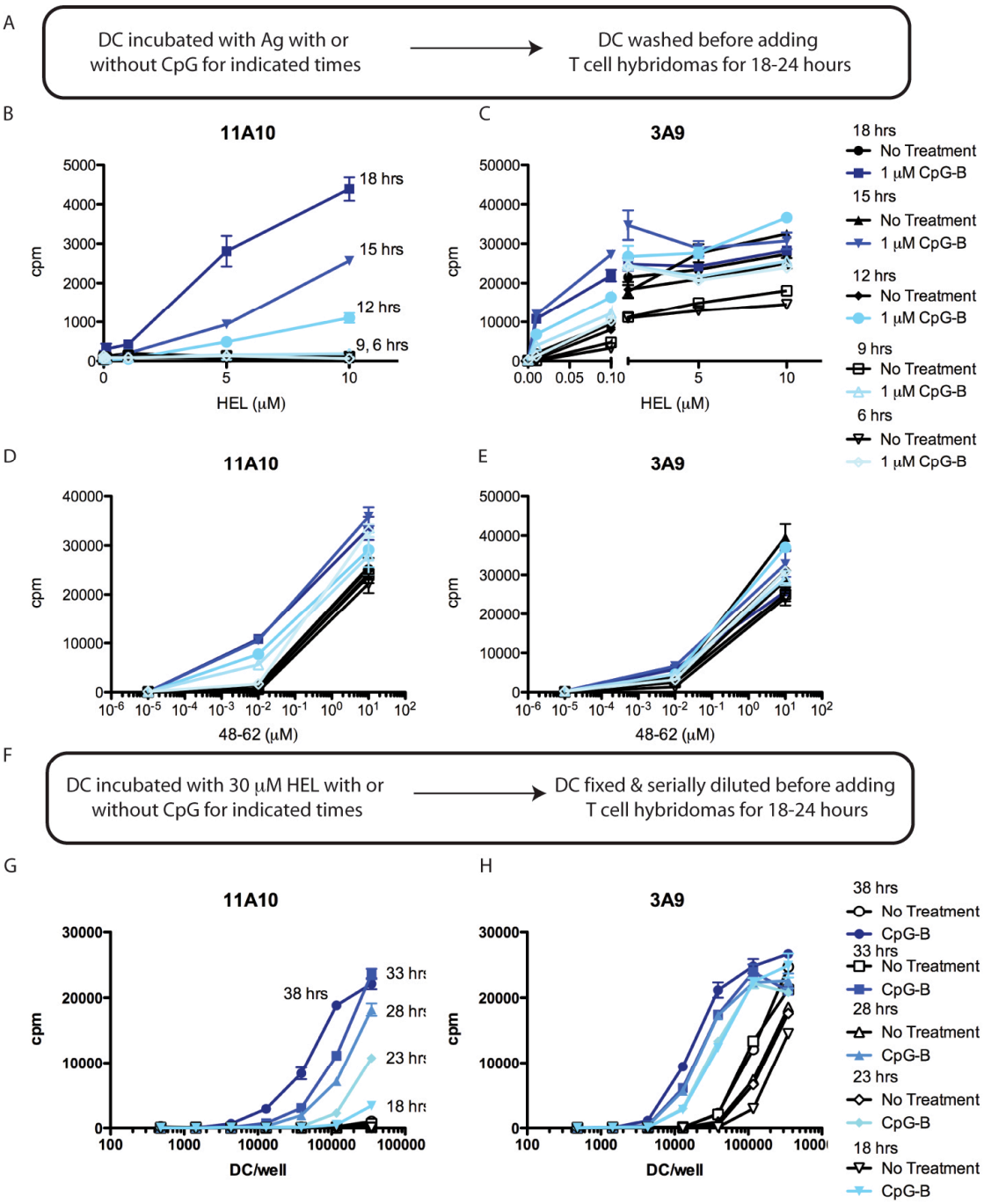

Figure 2. Timing of Generation of Type A and B pMHC from HEL. (A-E) DC incubated with antigen for the indicated times in the presence or absence of CpG-B, washed, and incubated with (B, D) type B 11A10 or (C, E) type A T cell hybridomas. (F-H) DC incubated with 30 μM HEL with or without CpG-B for the indicated times, fixed with PFA, serially diluted and incubated with (G) type B 11A10 or (H) type A 3A9 T cell hybridomas. Error bars represent SD.

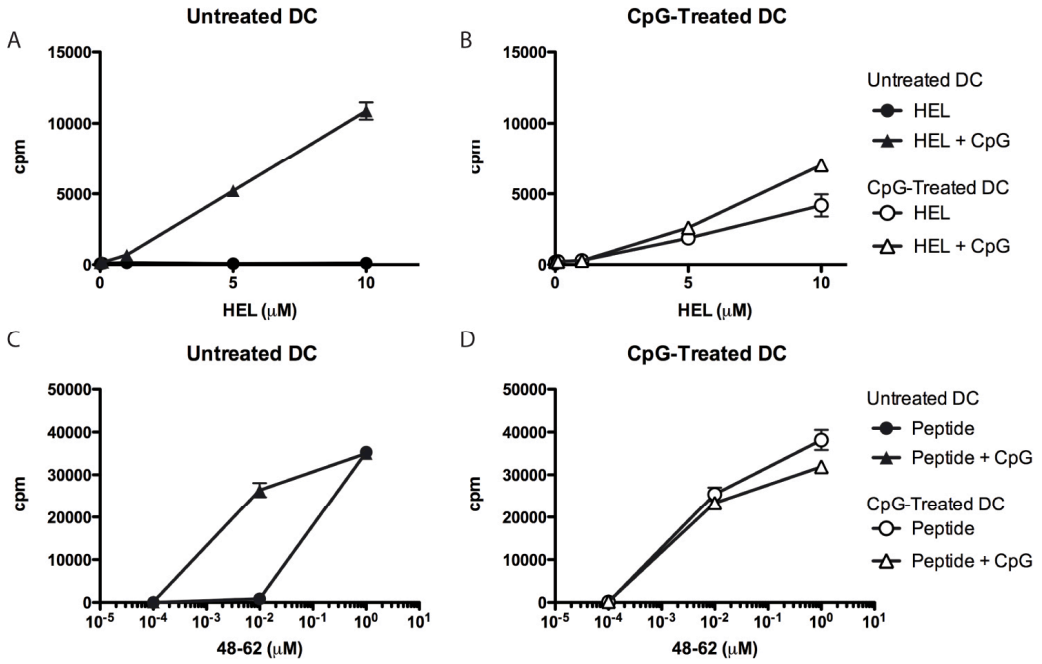

Figure 3. Pre-activation of DC with CpG Allows Type B Presentation from HEL. Presentation to type B T cell hybridoma 11A10 by DC incubated for eighteen hours with (A, C) media or (B, D) CpG-B before adding antigen with or without CpG for an additional eighteen hours. Representative of two independent experiments. Error bars represent SD.

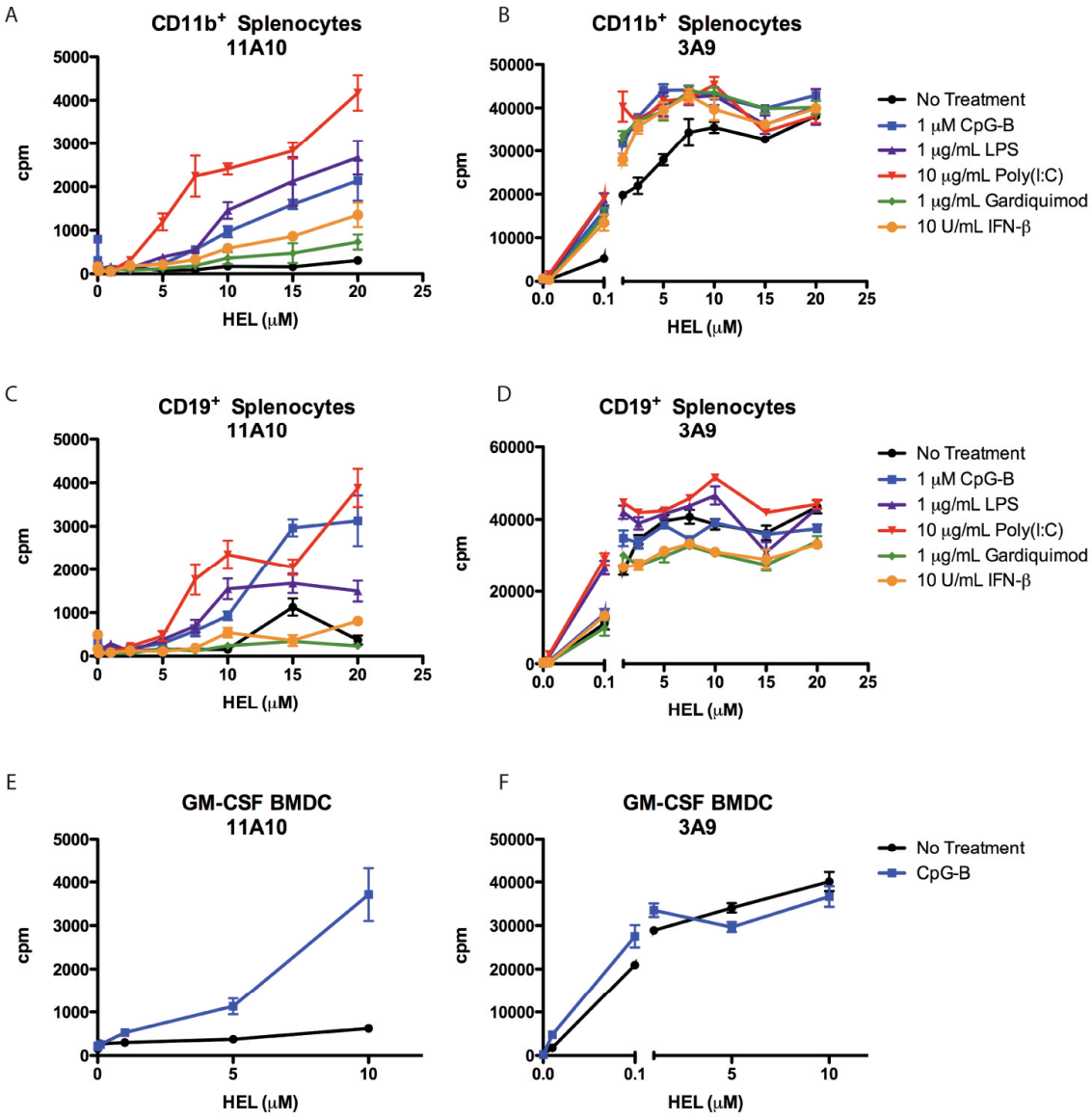

Figure 4. Presentation by CD11b+ Splenocytes, CD19+ Splenocytes, and GM-CSF BMDC. Presentation to (A, C, E) type B 11A10 or (B, D, F) type A T cell hybridomas by (A, B) CD11b+ splenocytes, (C, D) CD19+ splenocytes, or (E, F) GM-CSF BMDC. Error bars represent SD. Representative of two experiments.

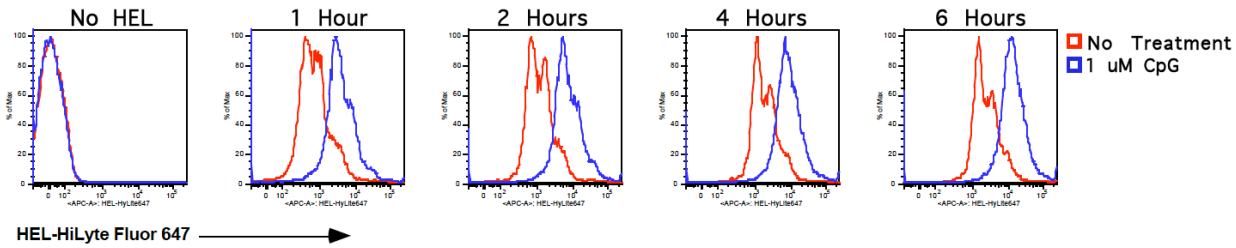

Figure 5. Effect of CpG-Stimulation on Uptake of HEL. Uptake of HEL-HiLyte Fluor 647 by DC incubated with 10 μM labeled protein with or without stimulants for 1-6 hours. Histograms represent events gated as $CD11c^{+}$. Representative of three independent experiments.

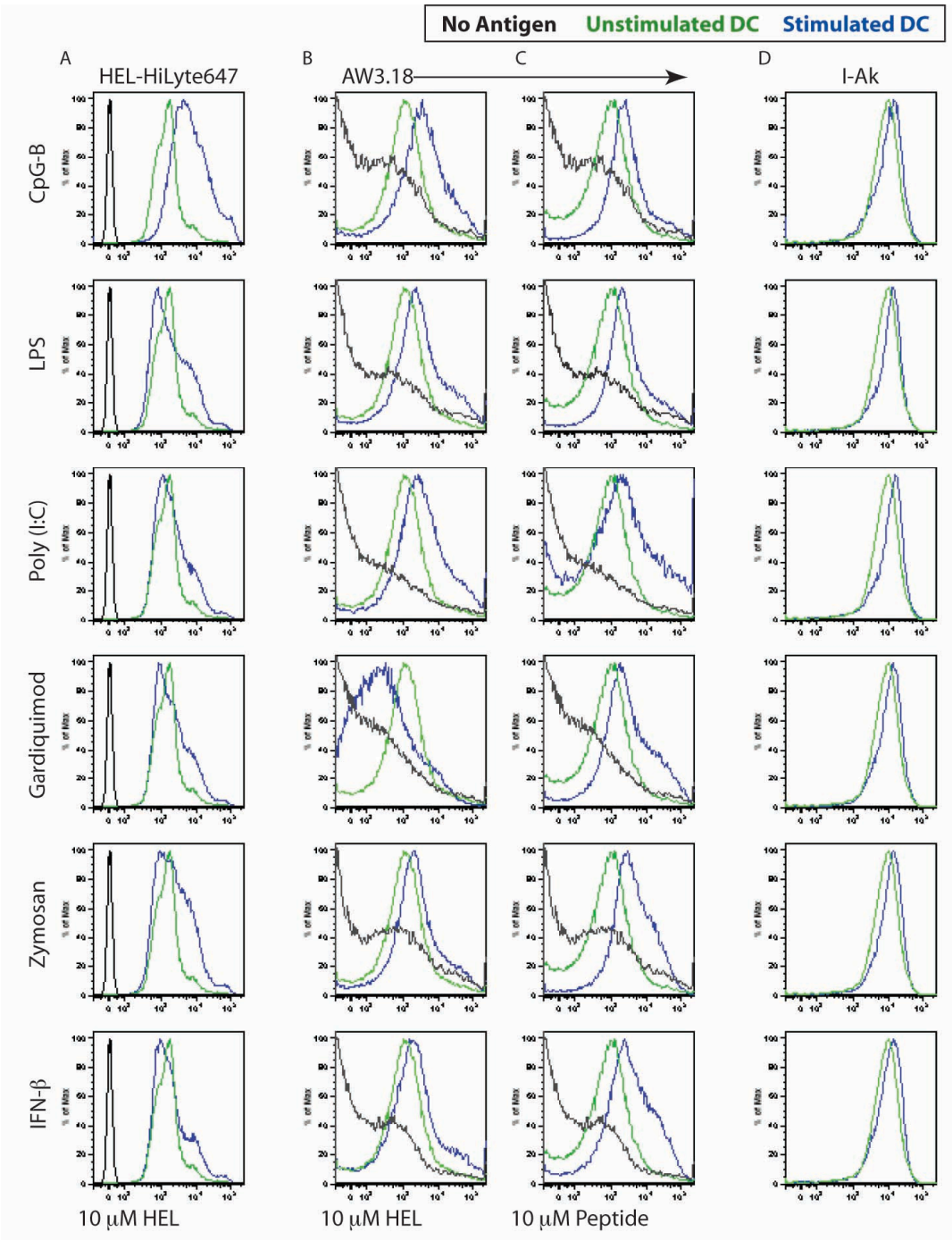

Figure 6. Effect of TLR Ligands and Type I IFN on HEL Uptake, Epitope Generation and Surface I-A^k Levels. (A) Uptake of HEL-HiLyte647 by DC incubated with 10 μM labeled protein with or without stimulants for 6 hours. (B, C) Surface staining for 48-62-I-A^k complex on DC after 24 hours of incubation with (B) 10 μM HEL or (C) 10 μM 48-62 peptide. (D) Surface I-A^k staining after 24 hours of incubation with or without stimulants. Representative of two experiments.

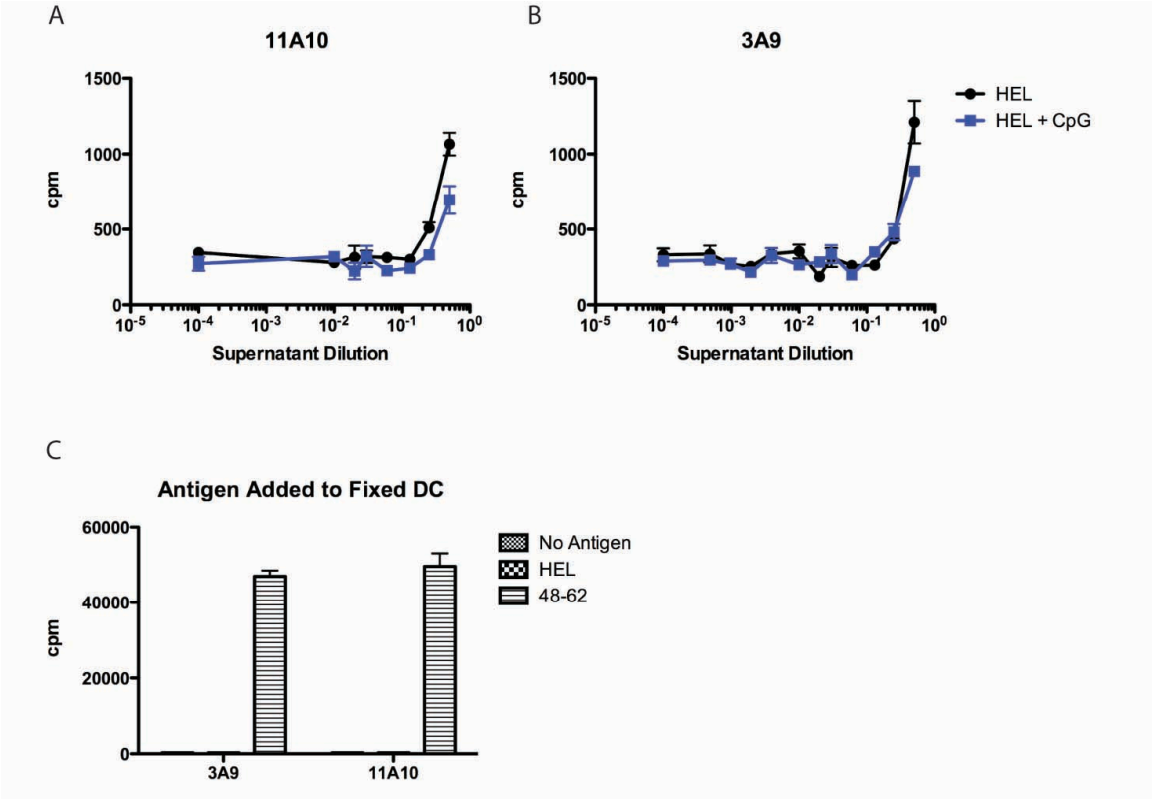

Figure 7. CpG Treatment of DC Does Not Induce Functional Peptide Release Leading to Antigen Presentation. (A, B) CB.17 DC were incubated overnight with 60 μM HEL with or without 1 μM CpG-B. Cell-free supernatants were serially diluted and added to fixed B10.BR DC and (A) 11A10 or (B) 3A9 T cell hybridomas were added to assess presentation. (C) 10 μM HEL or 48-62 peptide were added to fixed B10.BR DC with 11A10 or 3A9 T cell hybridomas.

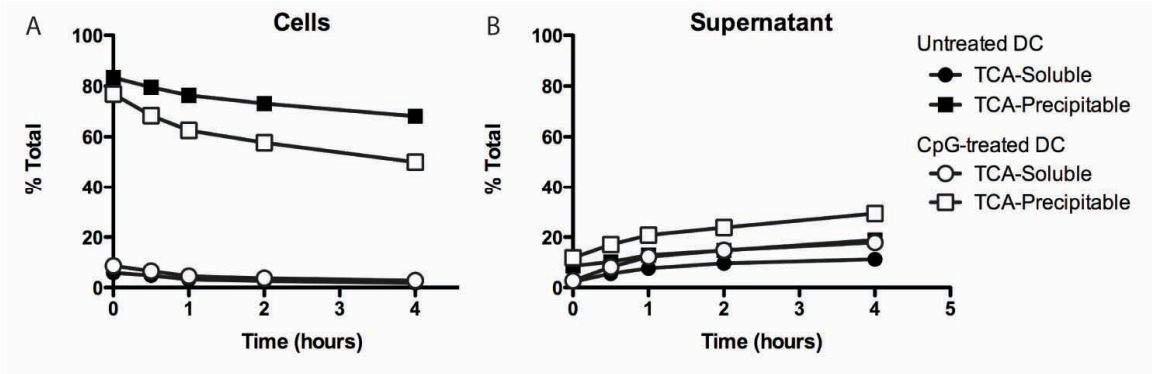

Figure 8. HEL Catabolism. (A, B) DC were incubated for 18 hours with or without CpG-B, washed, incubated with I^{125}-HEL for two hours at room temperature, washed extensively, then chased for indicated times at 37°C. TCA-Soluble and TCA-Precipitable fractions were collected from the (A) intracellular and (B) supernatant fractions. Data plotted as percentage of total cpm at each time point.

Chapter 4

Two-Photon Imaging of Type A and B T Cells-Type A and B pMHC are Presented by the Same DC

Abstract

Resting APC generate type B pMHC from peptide but not from protein antigen while generating type A pMHC from both peptide and protein. We have shown that certain types of stimulation—TLR ligands and type I IFN—allow APC to generate type B pMHC from protein HEL *in vivo* and *in vitro*. This study set to evaluate whether there are differences between activation of type A and B T cells *in vivo* in intact lymph nodes. We show that type A and B T cells become activated in similar regions of the lymph node, that type A and B pMHC appear to be presented by the same DC, and that some T cell cooperativity may exist with the presence of type A T cells being beneficial for activation of type B T cells.

Introduction

Studies employing two-photon microscopy have provided insight into *in vivo* dynamics of T cell behavior (Miller, Wei et al. 2002; Stoll, Delon et al. 2002; Bousso and Robey 2003; Hugues, Fetler et al. 2004; Mempel, Henrickson et al. 2004; Miller, Hejazi et al. 2004; Miller, Safrina et al. 2004; Celli, Garcia et al. 2005) There are some unresolved disputes regarding the specific order and significance of events when T cells encounter antigen-bearing dendritic cells, but it is generally accepted that T cells go through several phases characterized by changes in contact with DC and in movement dynamics. In the absence of antigen, T cells scan numerous DC (Bousso and Robey

2003; Miller, Hejazi et al. 2004). Upon antigen exposure, T cells begin to slow down, display restricted movement, and increase contact duration with antigen-bearing DC. Initially these contact times are relatively short, then increase to around 11 minutes compared to 3 minutes with non-antigen-bearing DC (Mempel, Henrickson et al. 2004; Miller, Safrina et al. 2004). During the next phase of interactions, beginning around 3 hours after initial contact, T cells increase contact time to around one hour. This phase lasts up to approximately 16 hours after initial contact. The next phase involves "dynamic swarming" where T cells retain relatively long contacts with DC, around 20 minutes, but will release and reinitiate contact with DC repetitively (Miller, Wei et al. 2002). During this phase, T cells become visually larger, indicating that they are entering a blast phase. Finally, after 24 hours, T cells resume short DC interactions and intermittent cell division (Miller, Safrina et al. 2004). We sought to use this technique to determine whether there were differences in the activation of type A and B T cells *in vivo* in intact tissue.

For all of the experiments described, T cells were analyzed in terms of their interaction with APC and their movement dynamics. T cell-DC interactions were analyzed by counting the average number of T cells clustering on individual DC, the duration of the T cell-DC contact, and whether T cells interacted with only one DC or had serial interactions with multiple DC. To study T cell behavior, changes in velocity and motility were observed. Motility was assessed by the motility coefficient that analyzes how far a cell moves over time. Naïve T cells have high motility coefficients, meaning that their movement is not biased to a specific region. Conversely, T cells

encountering antigen-bearing APC have lower motility coefficients, meaning that their movement is biased towards a restricted area.

To study activation of type A and B T cell *in vivo*, a model was established involving incubation of flt3L-expanded splenic DC with LPS with or without antigen (HEL peptide or protein) for one hour. Following incubation, DC were fluorescently labeled and transferred subcutaneously into the footpads of mice. DC were transferred approximately twenty-four hours before imaging, since it has been shown that activated DC require a minimum of eighteen hours to migrate to the draining lymph node (MartIn-Fontecha, Sebastiani et al. 2003). T cell populations were fluorescently labeled and transferred intravenously into the DC-immunized mice. For most experiments, T cells were transferred twelve hours after DC immunization. T cells arrive rapidly in the LN following intravenous transfer and are present before DC enter the lymph nodes. For these studies we used MLA11.2, a type B TCR transgenic, and 3A9, a type A TCR transgenic, which both recognize the chemically dominant epitope of HEL bound to I-A^k (Sakaguchi, Ermak et al. 1994; Lovitch, Esparza et al. 2007). The transgenic populations were transferred together or separately paired with bulk CD4 T cells isolated from naïve B10.BR mice.

It is important to note that the experiments described in this chapter were performed before all others described in this dissertation.

Materials and Methods

Mice

Mice were maintained under specific pathogen-free conditions according to institutional animal care guidelines. B10.BR mice were either from a colony maintained in our facility or purchased from The Jackson Laboratory (Bar Harbor, ME). The following strains—MLA11.2$\alpha^{-/-}$, 3A9$\alpha^{-/-}$, CD11c-EYFP—were from a colony maintained in our facility. The mouse lines listed as $\alpha^{-/-}$ were deficient for the TCR alpha chain. The CD11c-EYFP mice were provided by Mark Miller (Washington University in St Louis) and have been bred onto the B10.BR background. All mice were used between 6-10 weeks of age.

Isolation of Primary T Cells:

Spleen and peripheral lymph nodes (cervical, brachial, mediastinal, mesenteric, and popliteal) were isolated from TCR transgenic mice or B10.BR mice. The organs are diced and dispersed. After lysis of red blood cells with ACK lysis buffer, T cells were isolated using magnetic bead negative-selection (Miltenyi Biotec, Auburn, CA.)

DC isolation and Flt3 ligand treatment

DC were isolated from spleens of mice injected with 10 μg Flt3 ligand i.p. for 3 consecutive days. On day 8, spleens were harvested and digested with 0.14 U/ml Liberase Blendzyme 3 (Roche Applied Science, Indianapolis, IN) to make single-cell suspensions from which DC were isolated by CD11c magnetic beads (Miltenyi Biotec, Auburn, CA). Enriched DC were >95% pure as determined by flow cytometry.

Two-Photon Microscopy Experiments

DC isolated as indicated above were incubated with 1 μg/mL LPS (Sigma-Aldrich, St Louis, MO) with or without HEL protein or 48-62 peptide (1 or 10 μM antigen.) After incubation, DC were washed, labeled with 5 μM CFDA-SE (Invitrogen, Carlsbad, CA) for 25 min, 37°C in CO_2-independent media (Gibco, Carlsbad, CA). DC were washed and injected into the footpads or scruff of the neck of B10.BR mice 24 hours before imaging. T cells were isolated as described above, labeled with (10 μM) CMTPX, (10μM) CMTMR, or (40 μM) CMAC in CO_2-independent media (Gibco) for 35 min, 37°C. T cells were washed and injected intravenously into DC immunized mice, twelve hours before imaging. Draining lymph nodes were isolated, glued to plastic coverslips using Vetbond (3M, St. Paul, MN) and placed in a warmed flow chamber (37°C) perfused with warmed DMEM bubbled with 95% O_2 5% CO_2. Images were acquired on an Olympus BX51WI fluorescence microscope equipped with a 20x objective (Olympus) controlled with Image Warp (BitFlow, Woburn, MA). A chameleon Ti-Sapphire laser (Coherent, Santa Clara, CA) was used to excite the sample. Tissue volumes of 200 x 220 x 50 μm were captured at 27 second intervals by acquiring 21 sequential 2.5 μm Z-steps every 0.5 seconds. Recordings of an individual site lasted 30-40 minutes. Multidimensional rendering and three-dimensional cell tracking were performed using Imaris software (Bitplane AG, Zurich, Switzerland). Only tracks 3.5 minute and longer were included in analyses. All tracks were generated automatically using Imaris and manually checked for integrity.

Results

Cooperativity Between Type A and B T Cells

In preliminary experiments MLA11.2 and 3A9 T cells were transferred together into mice immunized with mock-loaded, peptide-loaded, or HEL-loaded LPS-treated DC, focusing on the second phase of DC-T cell encounter six to twelve hours after both cells reach the lymph node. During this time, activated T cells typically slow down and display restricted movement. In the presence of mock-loaded DC both T cell populations had high velocities and motility coefficients, a phenotype consistent with naïve T cell behavior (Figure 1.) Conversely, both T cell populations had lower velocity and motility coefficients, consistent with an activated phenotype, when in the presence of peptide-loaded or HEL-loaded DC (Figure 1.) It is important to note that the activation of MLA11.2 T cells in the presence of HEL-loaded LPS-treated DC *in vivo* was expected because CFSE-labeled MLA11.2 T cells proliferate in similar immunization conditions (Lovitch, Esparza et al. 2007).

As a control, the T cell populations were analyzed separately. To do this MLA11.2 or 3A9 T cells were transferred together or with a bulk naïve CD4 T cell population isolated from B10.BR mice. 3A9 behavior was consistent when transferred with MLA11.2, or with a bulk naïve population into mice immunized with all three conditions—mock or HEL-loaded DC (Figure 2.) MLA11.2 T cells were consistent when transferred with 3A9 or with a bulk naïve population when encountering mock-loaded (high velocity and motility.) However, when MLA11.2 was transferred in the absence of 3A9 into mice immunized with HEL-loaded DC, MLA11.2 T cells had high

velocities and motility coefficients, consistent with naïve T cell behavior (Figure 2.) This indicated that something about the presence of 3A9 increased the ability of MLA11.2 to "see" antigen or increased presentation of type B epitopes at this early time after immunization with HEL-loaded DC.

MLA11.2 and 3A9 T cells interacted differently with DC presenting antigen. 3A9 T cells formed large clusters of T cells around DC when transferred with MLA, alone, or with a bulk naïve population into mice immunized with peptide- or HEL-loaded DC (Figure 3.) MLA11.2 T cells, however, formed much smaller clusters when transferred with 3A9 into mice immunized with HEL-loaded DC. Since cluster size was dose-dependent for both T cell populations, it is possible that the smaller cluster size with MLA11.2 T cells indicates that even with peptide, the type B pMHC could be at a significantly lower density than the type A pMHC. For CD8 T cells, T cell-DC contact time and biased T cell motility have both been shown to be antigen dose-dependent (Henrickson, Mempel et al. 2008).

Localization of T Cell Activation Within the Lymph Node

We became interested in exploring whether presentation of type B epitopes is spatially restricted in the lymph node. The subcapsular sinus (SCS) region of the lymph node acts as an efficient physical filter for cells and particles entering the lymph node. Three groups have demonstrated that SCS macrophages are capable of retaining intact antigen and passing it off to B cells (Carrasco and Batista 2007; Junt, Moseman et al. 2007; Phan, Grigorova et al. 2007). Two of these studies showed that when antigen-specific B cells take antigen from SCS macrophages they retain the antigen, but antigen-

non-specific B cells transport the antigen to follicular DC (Carrasco and Batista 2007; Phan, Grigorova et al. 2007). Interestingly, it has also been shown that a similar phenomenon occurs in the spleen with antigen captured in the marginal zone being taken up by B cells and transported to follicular DC (Cinamon, Zachariah et al. 2008). We hypothesized that the subcapsular sinus could be a likely source for exogenous peptide and protein that could be released from dead and dying cells captured in the SCS.

To study this we began taking full section images before each experiment described above. To do this we took a large set of pictures beginning at the lymph node surface and ending as deep in the lymph node as could be imaged, usually around 100-250 μm. These images allowed us to observe where T cell activation occurred within the lymph nodes in all of our experiments.

Based on our data, there appeared to be no bias for type B presentation to a specific region of the lymph node. We observed T cell—DC interactions both close to the SCS and also deep in the cortex, however, the majority of T cells observed interacted with DC deep in the cortex. We found no biased localization of MLA11.2 or 3A9 T cell populations. The transferred DC that enter the cortex were usually confined to a single region of the lymph node and T cells often became concentrated in the same region of the LN. The DC population typically is distributed from the surface down deep into the cortex. However, the majority of the time the cells deep in the cortex are the most motile and interact the most with active T cells. It is important to note that this system observes DC that acquired antigen before transfer and migrated from the periphery to the LN. The situation may differ during an infection or following an immunization in the footpad.

Presentation of Type A and B pMHC by DC

When we began the two-photon microscopy experiments one question we wanted to address was whether type A and B peptide-MHC conformers were presented by the same or different DC. In our experiments we observed that MLA11.2 and 3A9 T cells often cluster on the same DC (Figure 4.) This indicated that the presentation of A and B epitopes are not mutually exclusive. Another interesting finding was that we never observed a DC that interacts exclusively with MLA11.2 T cells. All observed DC interacted with neither T cell, only with 3A9, or with both 3A9 and MLA11.2. Since the type A complex is more thermodynamically favorable it would be unlikely that a situation could exist where only type B conformers are presented. It is, however, an important concept that DC can present exclusively type A conformers or mixed A and B conformers but cannot exclusively present type B conformers (Figure 4.)

This is also interesting in the context of the data indicating that cooperation may exist between type A and B T cells. While the literature on T cell clonal competition and cooperativity presents numerous contradictions between experimental models, the one constant is that presentation of antigen by the same DC is essential to observe competition or cooperation between T cells (Kedl, Kappler et al. 2003).

In addition to analyzing whether type A and B pMHC were presented by the same or different DC populations, we also wanted to explore whether antigen could be transferred between DC, leading to generation of type B pMHC. To examine this issue, DC were incubated with LPS with or without HEL, labeled with Cell Tracker Red (CMTPX) and transferred into CD11c-EYFP mice. Technical restrictions allowed

observation of only one T cell population at a time. Because of this, the following T cell groups were transferred into the DC-immunized mice: [1] CFSE-labeled MLA11.2 alone, [2] CFSE-labeled MLA11.2 and unlabelled 3A9, [3] unlabeled MLA11.2 and CFSE-labeled 3A9, or [4] CFSE-labeled 3A9 alone. An important note is that even though the emission spectra of CFSE and EYFP are similar, the difference between endogenous DC and transferred T cells is easily distinguished by eye, as seen in Figure 5. The draining lymph nodes from these mice were isolated and imaged using two-photon microscopy.

When transferred along with 3A9 T cells, MLA11.2 T cells interacted with three DC populations. First, MLA11.2 interacted with transferred DC (primary antigen carrier.) Second, MLA11.2 interacted with endogenous DC that had red labeling apparently inside the DC, suggesting that the DC had engulfed transferred primary antigen-carrying DC. Third, MLA11.2 interacted with endogenous DC that, as far as could be told visually, had not engulfed transferred DC (Figure 5.) This indicated that antigen can be transferred and type B epitopes can be presented either when a primary antigen-bearing DC is phagocytosed and processed by a neighboring DC or by some other mechanism of antigen transfer. Additionally, we occasionally observed physical interactions between transferred and endogenous DC, suggesting a possible physical transfer of antigen or other molecules (Figure 6.)

Discussion

Observing type A and B T cells *in vivo* allowed us to determine that there are not remarkable differences between the two populations in terms of the site of their activation and the observation that they are activated by the same DC population. These findings

are relevant in the context of our observation that TLR ligands and type I IFN act directly on DC, allowing them to generate type B pMHC from HEL protein (see Chapters 2 and 3.) This indicates that not only do signals act directly on DC, but that TLR stimulation allows for DC to handle protein in a way that type B pMHC are generated, but not at the expense of generation of type A pMHC since the same DC are capable of generating both conformers.

The observations about potential T cell cooperativity between type A and B T cells and antigen transfer between DC are preliminary and require further experimentation to understand their significance. To truly establish that the presence of type A T cells is beneficial for activation of type B T cells, it would be ideal to test MLA11.2 T cells in the presence of another clonal T cell population that recognizes an irrelevant antigen to confirm that the effect is not due to simply the presence of another clonal population. We attempted to analyze this phenotype using an *in vitro* CFSE system where primary 3A9 and MLA11.2 T cells were incubated together or separately with DC in the presence or absence of antigen. While the system was not exhaustively tested, we did not see a significant difference in the proliferation of MLA11.2 T cells in the presence or absence of 3A9. It is possible that if earlier time points or markers of activation such as CD69 were observed, that there may be a discernable early enhancing effect of the presence of 3A9. What was more compelling about the *in vitro* CFSE assays was the discovery that CpG or LPS stimulation of splenic DC allowed for presentation of type B pMHC from HEL. It would also be possible to test this hypothesis by incubating primary 3A9 T cells or irrelevant transgenic T cells with DC incubated with or without

antigen, then remove the primary T cells and add type A or B T cell hybridomas to determine if the presence of 3A9 affects the level of pMHC presentation of the two conformers.

The observation that antigen was potentially transferred between DC, leading to type B presentation was from two experiments and would need to be repeated to substantiate the data. Additionally, to more convincingly show that transfer of antigen leads to generation of antigenic pMHC, a system would need to be set up where allogeneic (for example I-A^b) antigen-bearing DC were transferred into F1 (I-A^b x I-A^k) mice. In this scenario, the only way for MLA11.2 or 3A9 to become activated would be if the endogenous I-A^k-expressing DC acquired antigen from the transferred I-A^b-expressing DC. Measures of T cell activation could be assessed to determine if antigen transfer lead to meaningful levels of presentation.

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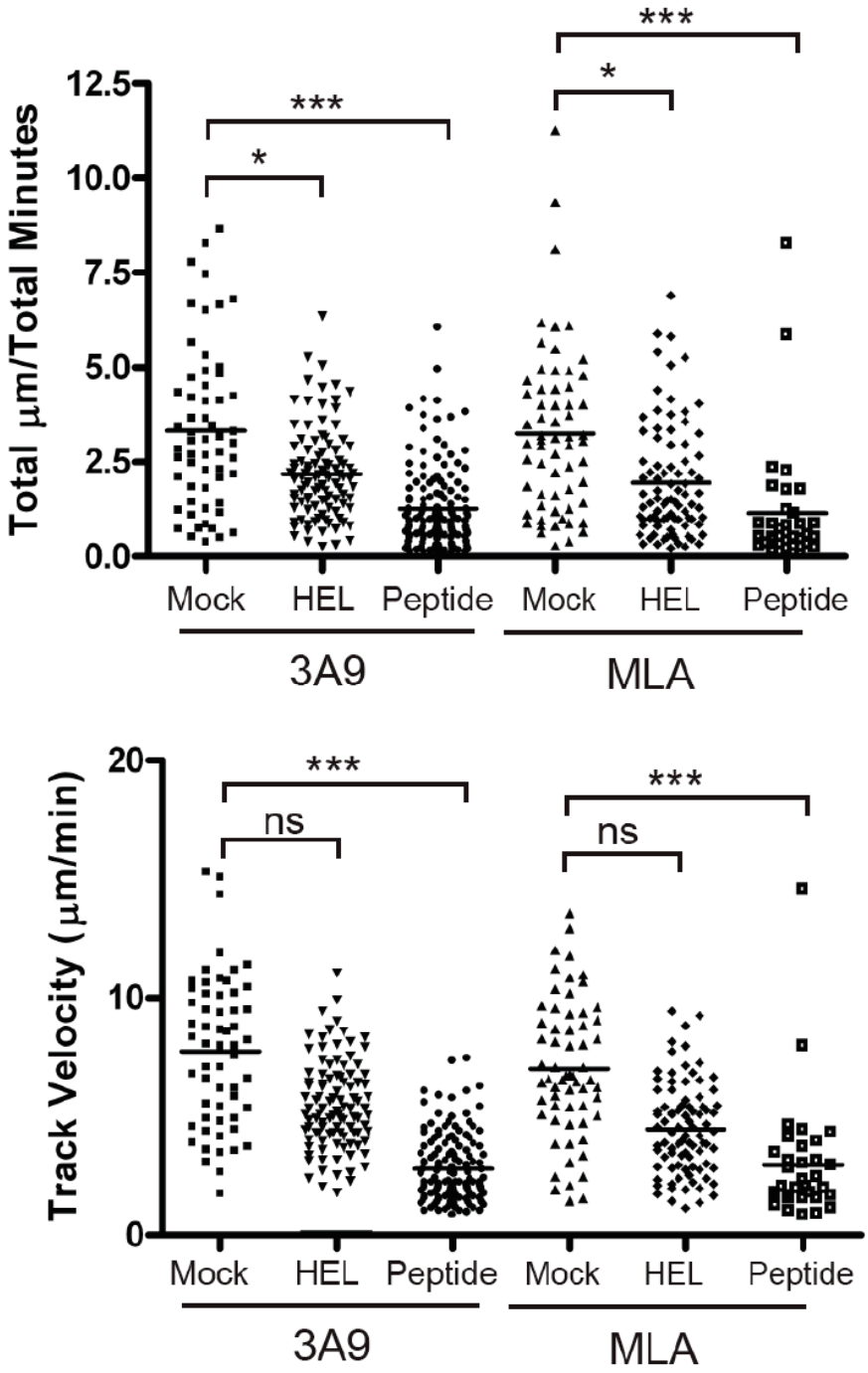

Figure 1. Velocity & Motility of MLA and 3A9 from co-transfer two-photon microscopy experiments. (A) Motility and (B) Velocity of transgenic type A 3A9 and type B MLA T cells transferred into mice immunized with mock, HEL, or Peptide-loaded DC. Statistics performed using Prism4 Mann-Whitney Test.

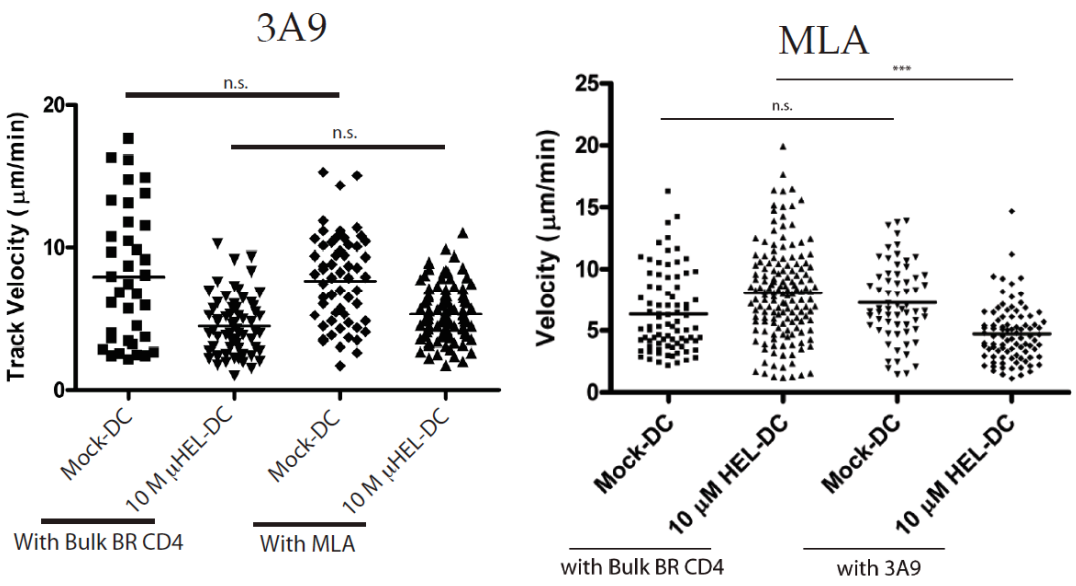

Figure 2. Velocity of 3A9 & MLA11.2 T Cells when transferred separately. Velocity of type A 3A9 and type B MLA transgenic T cells when transferred together or with equal numbers of bulk CD4 T cells from B10.BR mice into mice immunized with mock-loaded or HEL-loaded DC. Statistics performed using Prism4, Mann-Whitney.

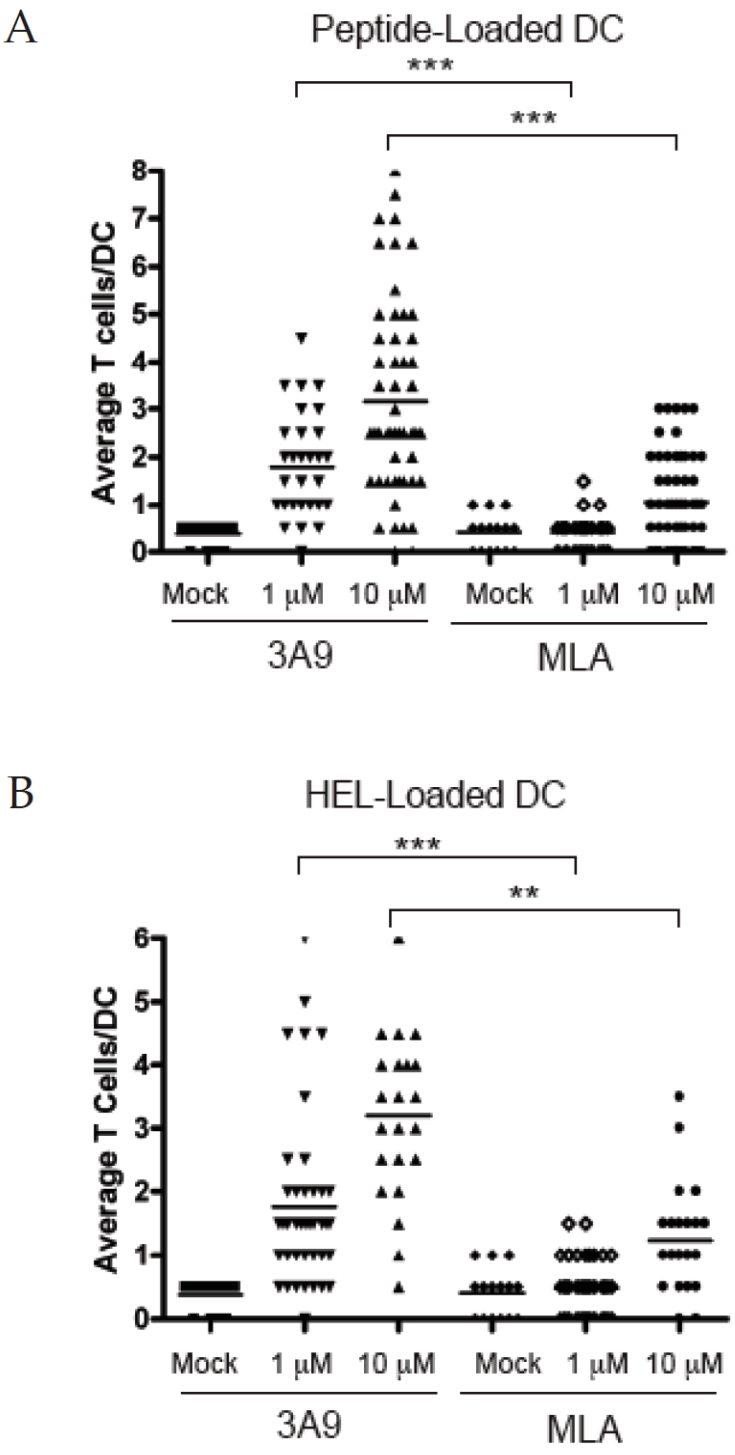

Figure 3. 3A9 T cells form larger clusters around DC than MLA T cells. Average number of T cells contacting individual DC over a thirty-minute time period in mice immunized with (A) peptide-pulsed or (B) HEL-pulsed DC. Statistics performed using Prism4, Mann-Whitney.

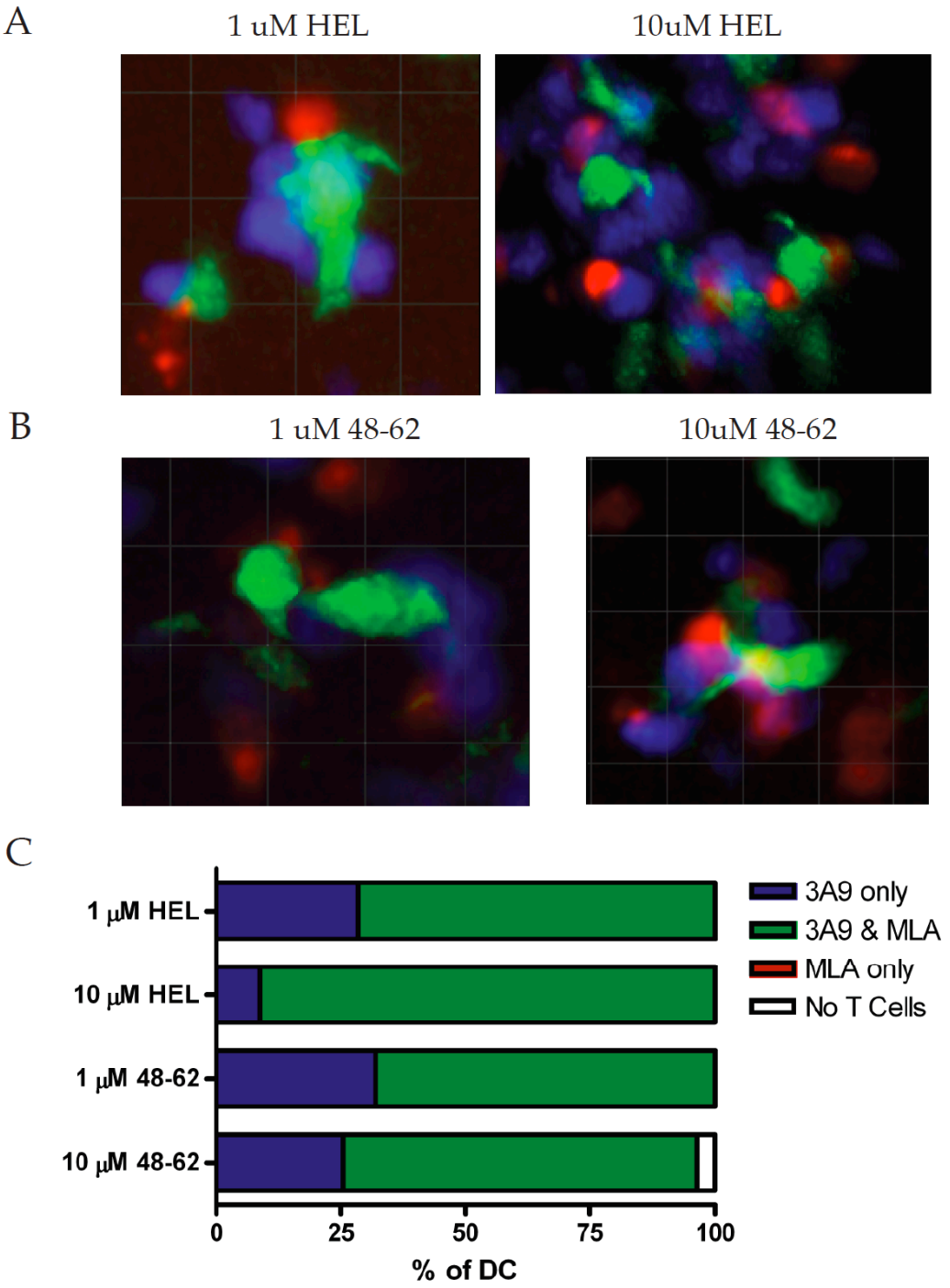

Figure 4. DC interact with 3A9 T cells, 3A9 and MLA T cells, or no T cells. (A, B) CMAC-labeled 3A9 (blue) and CMTPX-labeled MLA (red) T cells interact with CFSE-labeled DC (green) loaded *ex vivo* with (A) HEL or (B) 48-62 peptide. (C) Percentage of observed DC interacting with 3A9 only, both 3A9 and MLA, MLA only, or no T cells during a thirty-minute timeframe.

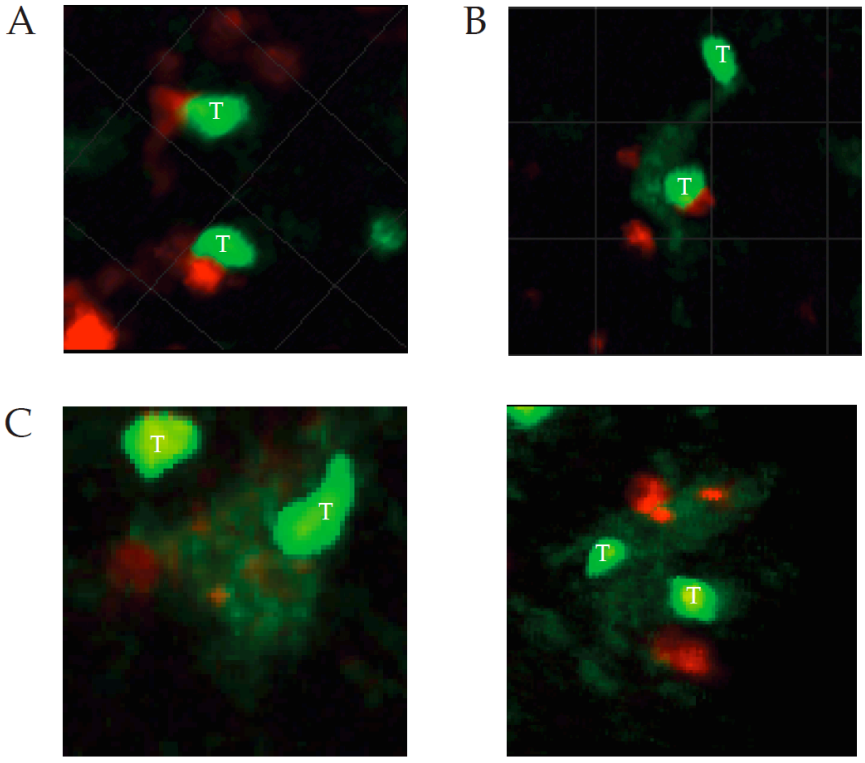

Figure 5. Antigen transfer between transferred and endogenous DC. CFSE (green)-labeled type B MLA T cells transferred with unlabelled type A 3A9 T cells into CD11c-EYFP mice immunized with CMTPX (red)-labeled HEL-loaded DC. MLA T cells labeled (T) in figures. MLA T cells interacting with (A) primary antigen-carrying DC, (B) endogenous DC, (C) and endogenous green DC that appear to contain fragments of transferred DC.

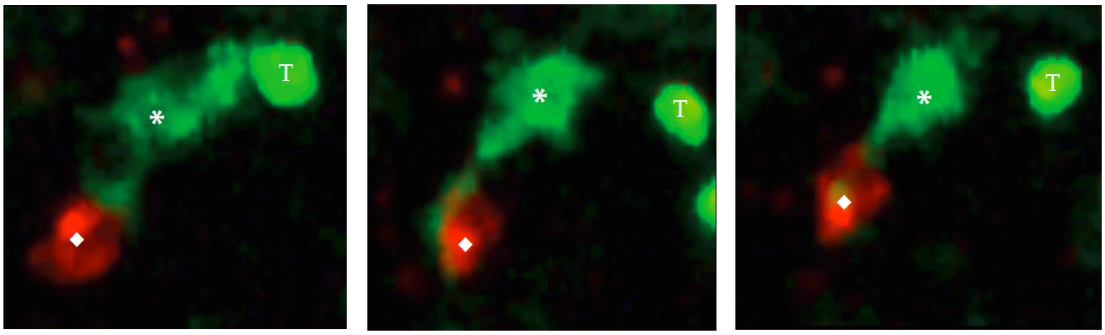

Figure 6. Physical interaction between transferred and endogenous DC. CFSE (green)-labeled type B MLA T cells transferred with unlabelled type A 3A9 T cells into CD11c-EYFP mice immunized with CMTPX (red)-labeled HEL-loaded DC. MLA T cells (T) Transferred DC is red. (◊) Endogenous DC is green (*.)

Chapter 5

CpG Stimulates Presentation of Type B pMHC Conformers From Self-Protein and Primes Autoreactive T Cells

Abstract

Conventional type A CD4+ T cells respond to their cognate antigen whether an APC encounters the antigen in peptide or protein form. In contrast, type B CD4+ T cells respond to APC given peptide but not to the same peptide processed from intact protein. Type B T cells against the model antigen hen egg white lysozyme (HEL) are prevalent, comprising 30-50% of HEL-reactive CD4 T cells after immunization, and escape negative selection in the thymus when HEL is expressed on all APC or in the periphery as a secreted serum protein. We have previously shown that type B pMHC, the epitope for type B T cells, can be presented *in vivo* from protein HEL during some states of inflammation and *in vitro* in response to TLR ligands and interferon-α/β. This raised the possibility that exposure to inflammatory agents may lead to presentation of type B pMHC from self protein *in vivo* and induce autoreactivity. Here we show that exposure to CpG, a ligand of TLR9, *in vitro* or *in vivo* induces type B pMHC presentation from "pseudo-self" membrane-bound HEL by DC and that immunization with CpG *in vivo* primes auto-reactive type B T cells but not auto-reactive B cells.

Introduction

Type B T cells were originally characterized as CD4 T cells that respond to their cognate antigen when given as peptide but not protein to an APC (Viner, Nelson et al.

1996; Pu, Carrero et al. 2002; Lovitch and Unanue 2005). In contrast, type A T cells respond to both peptide and protein antigen. It was later shown that type B T cells escape negative selection when their cognate antigen is expressed in the thymus both in transgenic systems with the model antigen hen egg white lysozyme (HEL) and in non-transgenic systems with the MHC class II E-α and A-β^k chains, insulin, and myelin basic protein (Barlow, He et al. 1998; Gyotoku, Fukui et al. 1998; Peterson, DiPaolo et al. 1999; Lovitch, Walters et al. 2003; Kawamura, McLaughlin et al. 2008; Mohan, Levisetti et al. 2010). In the HEL system, type B T cells recognize a conformational isomer of their cognate peptide-MHC II (pMHC) ligand that is distinct from the more stable type A pMHC conformer that forms from both peptide and protein. The type A pMHC complexes are selectively formed from protein due to the ability of H2-DM to selectively edit for these more stable complexes in the late endosomal compartment where protein is processed and presented (Pu, Lovitch et al. 2004). Peptide, however, is loaded in early or recycling compartments where H2-DM is not present or active, resulting in the generation of multiple conformations of a given peptide-MHC pair.

A longstanding issue has been whether potentially auto-reactive type B T cells that escape negative selection can be activated in the periphery during states of inflammation. Our group has shown that naturally arising insulin-reactive type B T cell clones are capable of transferring diabetes in NOD mice, indicating that type B T cells can be pathogenic in an autoimmune-prone system (Mohan, Levisetti et al. 2010). Additionally, type B myelin basic protein-reactive T cells escape negative selection in mice expressing human multiple sclerosis-susceptibility MHC II allele HLA-DRB5*0101

but do not cause disease unless activated and transferred into irradiated hosts (Kawamura, McLaughlin et al. 2008). While these studies indicated that type B T cells could be pathogenic in the proper context, they did not address whether inflammation induces presentation of type B pMHC from self-protein and primes naturally arising type B T cells. We have previously shown that type B pMHC are generated from HEL protein when co-administered with some inflammatory agents—LPS, listeriolysin O, or complete Freund's adjuvant—*in vivo* and also when protein is given with TLR ligands or type I interferons to DC directly *in vitro* (Lovitch, Esparza et al. 2007; Strong and Unanue 2011).

Here we show that exposure to CpG induces generation of type B pMHC from self-proteins *in viv*o and *in vitro* and primes auto-reactive T cells, but failed to elicit an anti-self HEL B cell response or detectable autoimmune disease.

Materials and Methods

Mice

Mice were maintained under specific pathogen free conditions at Washington University in St Louis according to institutional animal care guidelines. B10.BR mice were either from a colony maintained in our facility or purchased from Jackson laboratory. mHEL mice (expressing membrane-bound HEL under the MHC class II promoter) were previously described (Peterson, DiPaolo et al. 1999). MLA mice were previously described (Lovitch, Esparza et al. 2007) and were crossed to the mHEL mice. The resulting F1 generation was heterozygous for mHEL, TCR alpha, and MLA transgenes.

Stimulants

Stimulants used were ODN 1826 (CpG-B) (Integrated DNA Technologies, Coralville, IA) Poly(I:C) (Sigma-Aldrich, St. Louis, MO); Ultra-pure LPS-EB from *E*. coli 0111:B4 (Invivogen, San Diego, CA) and IFN-γ (kind gift from Dr. Robert Schreiber, Washington University in St. Louis.)

Immunizations

Mice were injected subcutaneously with Pyrogen-free saline (PFS) or 50 μg CpG-B in Incomplete Freund's Adjuvant (IFA) (Sigma-Aldrich, St. Louis, MO) per footpad as described in individual figure legends. For experiments with MLA x mHEL animals, mice were injected with PFS or 50 μg CpG-B in PFS in a 50 μL volume per footpad

Dendritic cell isolation and Flt3L treatment

DC were isolated from spleens of mice injected with 10 μg Flt3L intraperitoneally for three consecutive days. On day eight, spleens were harvested, digested with 0.14 U/mL Liberase Blendzyme 3 or 1.67 U/mL Liberase TL (Roche Applied Science, Indianapolis, IN) to make single cell suspensions from which DC were isolated by CD11c magnetic bead (Miltenyi Biotec Inc, Auburn, CA.) Enriched DC were ≥ 95% pure as determined by flow cytometry.

Isolation of Lymph Node APC

mHEL mice were immunized in the footpads as described above. 24 hours after immunization, draining popliteal lymph nodes were isolated, lymph nodes were digested with 1.67 U/mL Liberase TL (Roche Applied Science, Indianapolis, IN) to make single cell suspensions. Cell suspensions were fixed with 1% paraformaldehyde for 5 minutes,

incubated in 0.4 M DL-Lysine for 10 min, and washed extensively in media containing 5% FBS. Presentation assays were performed with T cell hybridomas as described below.

T cell presentation assays

For *in vitro* presentation assays DC were serially diluted and incubated with or without stimulants for 18 hours in 100 μL volume in v-bottom 96-well plates. Stimulant concentrations used were 10 μg/mL Poly (I:C), 1 μg/mL LPS, 1 μM CpG-B, 100 U/mL IFN-γ. DC were washed three times with serum-free DMEM after incubation before adding $5x10^4$ T cell hybridomas per well in a 200 μL volume; 24 hrs later the release of IL-2 was measured in the culture fluid using CTLL as an indicator cell. Two previously characterized T cell hybridomas were used, 11A10, which only reacts with type B-pMHC, and 3A9, which recognizes type A-pMHC.

ELISPOT Assays

Mice were immunized with Pyrogen-free saline or 50 μg CpG-B (ODN 1826) in incomplete Freund's adjuvant (IFA) (Sigma-Aldrich, St Louis, MO) per footpad in a 50 μL volume. Multiscreen–IP plates (Millipore, Billerica, MA) were coated overnight with 5 μg/well IL-2 or IFN-gamma capture antibody (BD Biosciences, San Diego, CA) for 24-48 hours at 4°C. T cell responses in the draining lymph nodes were detected 7 days post-immunization using whole lymph node suspension. Cells were recalled with 10 μM HEL or HEL peptides for 20 hours. Cytokine-producing cells were detected with 2 μg/well IL-2 or IFN-gamma detection antibody and 0.1 μL/well streptavidin-AKP (BD Pharmingen.) BCIP®/NBT (Sigma-Aldrich) was used to develop the plates. Spots were

detected and counted using an ImmunoSpot Analyzer and ImmunoSpot software (Cellular Technology Ltd, Shaker Heights, OH).

Anti-HEL Antibody Detection

Blood was collected weekly after immunization. ELISA plates were coated overnight at 4°C with 1 μg per well HEL protein in Carbonate Buffer. Serum was serially diluted in DMEM containing 5% Fetal Bovine Serum and incubated with HEL-coated plates for 1 hour, room temperature. Serum antibody was detected with donkey anti-mouse IgG-HRP and ABTS.

Results

TLR ligands induce presentation of endogenous "self" protein in vitro

The observation that TLR stimulation allowed for effective presentation of type B pMHC from exogenous protein raised the question of whether TLR exposure could cause presentation of type B pMHC from self-proteins and autoreactivity *in vivo*. mHEL mice express HEL under the MHC II promoter, allowing transgenic expression of membrane-bound HEL on all APC. In these mice all type A T cells recognizing HEL are deleted by negative selection, but most type B T cells escape into the periphery (Peterson, DiPaolo et al. 1999). When splenic mHEL DC were incubated with CpG or LPS there was a log-increase in type B presentation indicating that TLR ligand stimulation augmented type B presentation from membrane-bound protein in addition to soluble protein (Figure 1A, 1B.) Incubation of mHEL DC with Poly (I:C) resulted in an approximately ½-log increase in type B pMHC presentation (Figure 1C.) Consistent with the previously

described effect observed with soluble HEL, IFN-gamma had no effect on presentation of type B pMHC by mHEL DC (Figure 1D.) TLR ligands and IFN-gamma had minimal effects on presentation of type A pMHC by mHEL DC (Figure 1E, F.) The same trend of CpG increasing type B pMHC presentation by approximately a log-fold was also seen with lymph node (LN) DC from mHEL mice (data not shown.) As with splenic DC, CpG-B treatment minimally increased presentation of type A pMHC by LN DC (data not shown.)

Effect of CpG on Presentation of self-protein in vivo

To test whether CpG had the same effect *in vivo* that it had *in vitro* on presentation from membrane-bound HEL, mHEL mice were immunized in the footpads with saline/IFA or CpG/IFA emulsions. Twenty-four hours after immunization, draining lymph nodes were isolated, fixed with paraformaldehyde and incubated with T cell hybridomas. Lymph nodes cells from mice immunized with CpG/IFA presented approximately 1-log more type B pMHC than cells from mice immunized with saline/IFA, similar to the results of DC treated *in vitro* (Figure 2A.) Presentation of type A pMHC was minimally enhanced in mice immunized with CpG/IFA (Figure 2B)

CpG Breaks Tolerance by Priming Type B anti-HEL T Cells in vivo

Since TLR agonists induced an increase in presentation of type B pMHC by mHEL DC, we investigated whether TLR treatment of mHEL mice allowed for presentation of the epitopes and priming of type B T cells *in vivo*. To do this, mHEL mice were immunized with saline/IFA or CpG/IFA in the footpad. Seven days after immunization T cell responses in the draining lymph node were assessed by ELISPOT *ex*

vivo. Mice receiving CpG/IFA had peptide-specific T cell responses indicated by high numbers of IL-2 and IFN-gamma-producing cells in response to all three dominant epitopes of HEL compared to wells with no HEL (Figure 3A, 3B.) There were no significant cytokine-producing cells in response to HEL protein recall, confirming that the T cell response observed was against type B pMHC. There was no significant response from mice immunized with PFS/IFA. It is critical to note that the only antigen available to prime T cells in the system is expressed endogenously by the APC in the mouse. These data indicate that a single exposure to CpG/IFA is sufficient to allow for *in vivo* presentation of type B epitopes from endogenous membrane-bound protein, leading to priming of autoreactive type B T cells.

CpG is Insufficient to Break B Cell Tolerance against Self-HEL

To test whether B cell tolerance was also broken with administration of CpG, mHEL mice were immunized as described for the T cells assays and monitored for twenty-eight days. None of the immunized mHEL mice developed anti-HEL antibodies (Figure 3C, 4A-D) even after an additional intravenous challenge with CpG three weeks after immunization (data not shown), indicating that B cell tolerance was still intact in the mice. Animal weight and serum HEL-specific antibody levels were assessed weekly. Mice immunized with CpG twice (in saline or emulsion) had a marked weight gain one week after immunization (Figure 4E), consistent with previous reports indicating that CpG exposure causes extramedullary hematopoeisis and weight gain (Sparwasser, Hültner et al. 1999). Histology of lung, heart, liver, spleen, pancreas, and kidney at twenty-eight days post immunization indicated that there were no detectable differences

between the control saline/IFA immunized group and any experimental group (data not shown.)

CpG Primes TCR Transgenic Type B T Cells in Dual-Transgenic Mice

To confirm that administration of CpG primes type B T cells, mice transgenic for both mHEL and type B TCR MLA11.2 were tested. MLA11.2 recognizes the type B conformer of the chemically dominant 48-61 epitope of HEL bound to I-A^k. MLA11.2 T cells represent approximately 4% of peripheral CD4 T cells in these mice. This system allows the observation of the effect of CpG where HEL-reactive type B T cell frequency is increased relative to the naturally arising population. mHELxMLA11.2 mice were immunized with saline or CpG-B in saline and cytokine-producing cells were assessed by ELISPOT seven days post-immunization. CpG-injection dramatically increased the number of peptide-reactive IL-2 and IFN-gamma producing cells in mHELxMLA11.2 mice (Figure 5.) These results confirm that exposure to CpG primes type B T cells against self-protein.

Discussion

Our previous reports have indicated that LPS, CFA, and LLO were capable of allowing for type B pMHC presentation from protein HEL *in vivo* (Lovitch, Esparza et al. 2007) and that direct exposure of DC to inflammatory agents such as TLR ligands and type I IFN are sufficient to alter protein handling in a way that allows for processing and presentation of type B pMHC from intact protein antigen (Strong and Unanue 2011). We

now show that TLR ligands can induce type B pMHC presentation from membrane-bound "self-protein," priming naturally arising autoreactive T cells.

The observation that TLR ligands are capable of allowing presentation of type B pMHC from membrane-bound "self" protein is significant as each time a DC encounters TLR ligands in the context of an infection, there is the potential to present self pMHC whose cognate T cells are typically not deleted during negative selection. We have previously shown that type B insulin-reactive T cells are capable of initiating disease in the NOD background (Mohan, Levisetti et al. 2010). In our system, there was no overt disease caused following priming of auto-reactive type B T cells, indicating that other mechanisms of peripheral tolerance were still intact. This indicates that in non-autoimmune-prone backgrounds, potentially auto-reactive type B T cells can be controlled even if they encounter their cognate antigens during states of inflammation. However, there is a well established link between TLR exposure and infection and human autoimmune disease (Mills 2011) and it is possible that when these control mechanisms fail, type B T cells become participants or drivers in autoimmunity.

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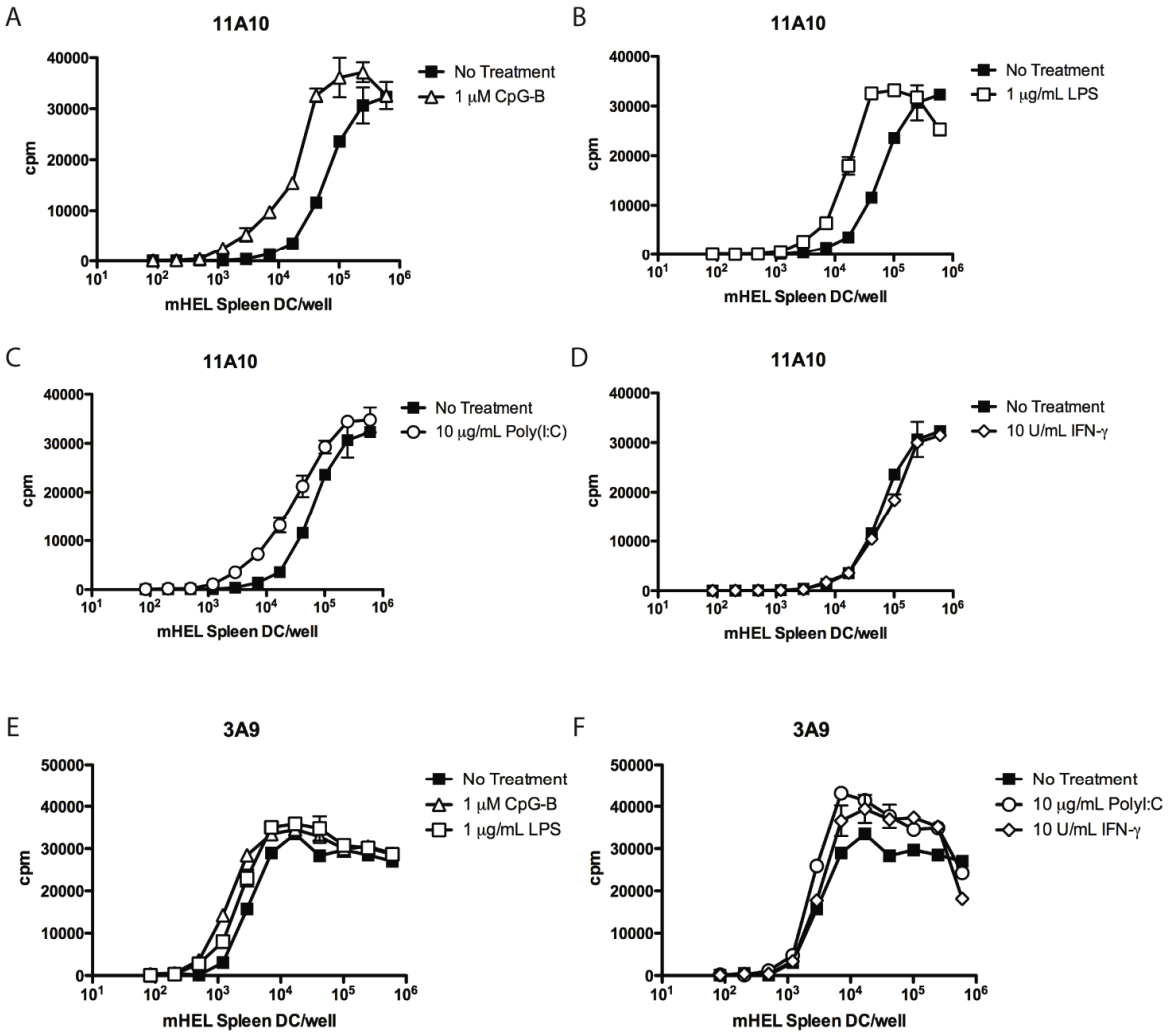

Figure 1. ***in vitro*** **stimulation of mHEL DC with TLR ligands induces presentation of type B pMHC.** (A, B) Presentation to type B 11A10 (A) or type A 3A9 (B) T cell hybridomas by mHEL spleen DC incubated with or without stimulants. Data representative of two independent experiments. Error bars represent SD.

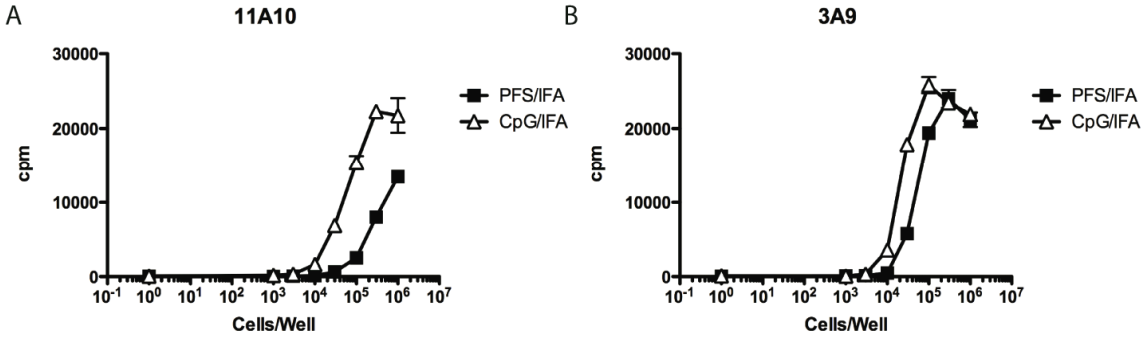

Figure 2. *in vivo* Administration of CpG-B induces Presentation of type B pMHC by mHEL Lymph Node DC. Presentation to type B 11A10 (A) or type A 3A9 (B) T cell hybridomas by fixed LN cells isolated 24 hours after immunization of mHEL mice in the footpads with Saline/IFA or 50 μg CpG-B/IFA. Data representative of one experiment. Error bars represent SD.

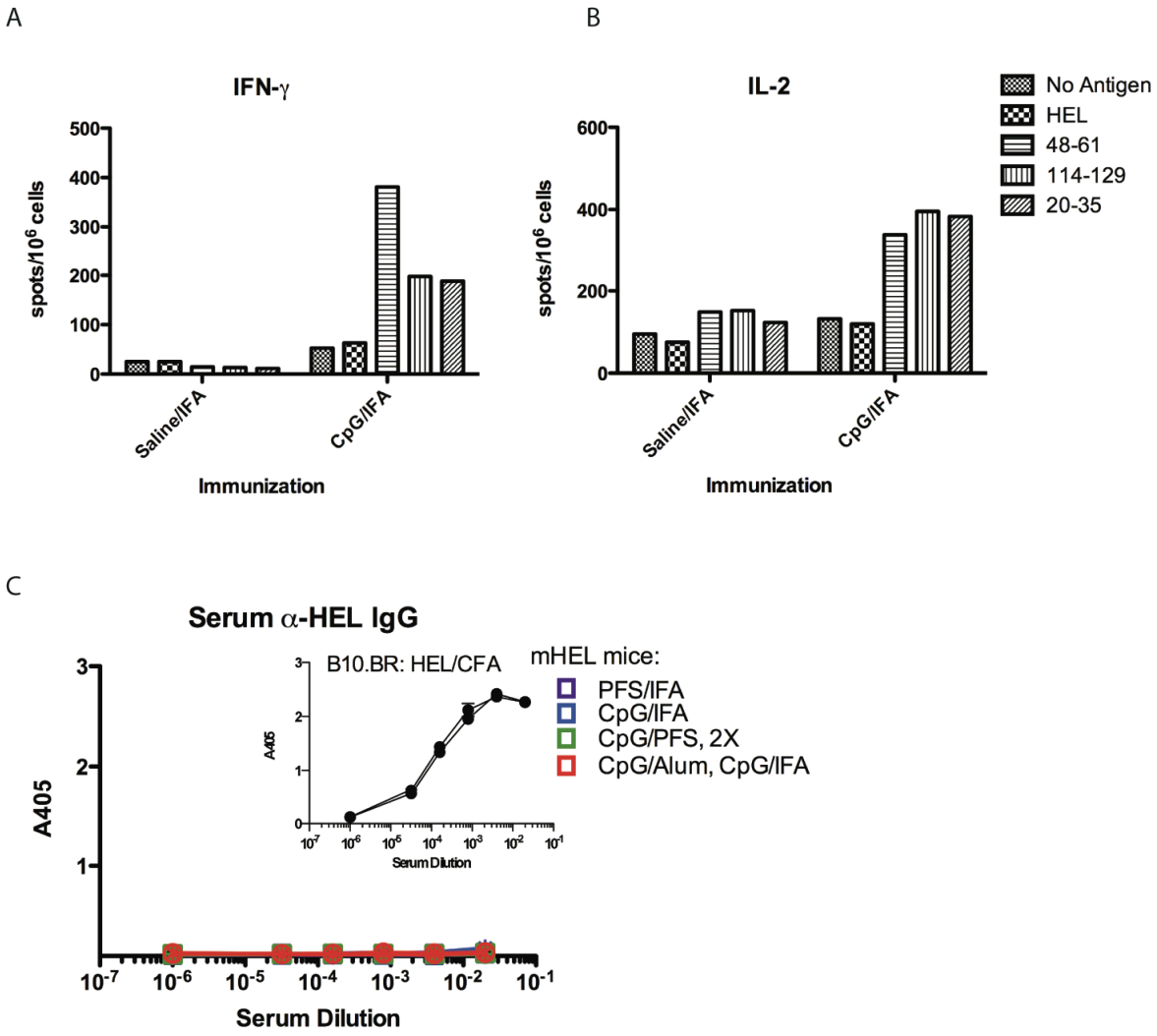

Figure 3. Administration of CpG-B Primes Self-Reactive Type B T cells but not B Cells. (A) mHEL mice immunized with Saline/IFA or CpG-B/IFA. Draining lymph nodes recalled in ELISPOT assay seven days after immunization. Data representative of three independent experiments. (B) mHEL mice immunized as indicated in figure were monitored weekly for serum HEL-specific IgG (four mice per group.) Inset graph: non-transgenic B10.BR mice immunized with 10 nmol HEL in CFA (two mice per group.) Data shown is from fourteen days post-immunization. Additional plots shown in Figure 4.

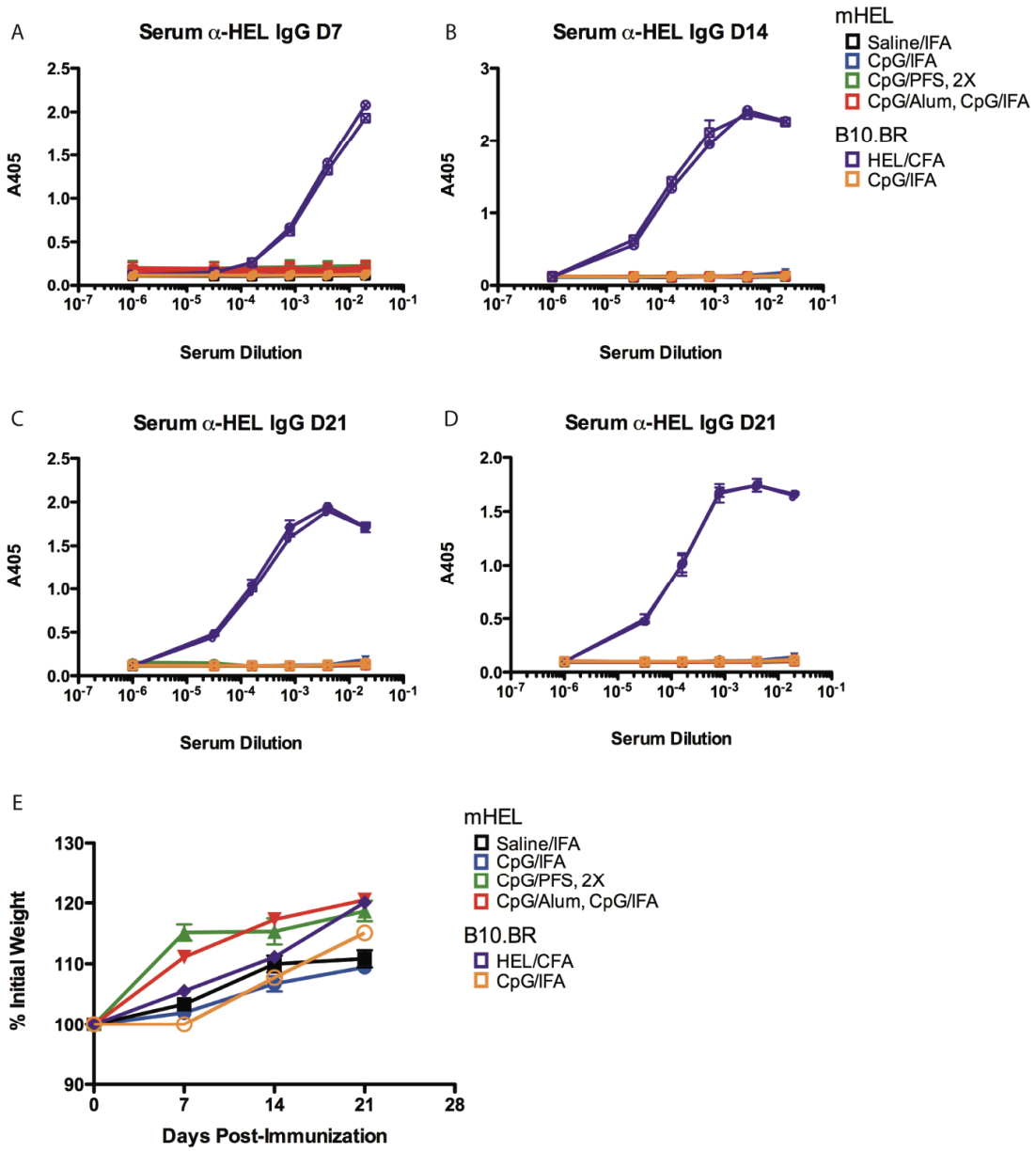

Figure 4. Serum anti-HEL Antibody and Weight Gain of Animals Immunized with CpG-B. (A-D) mHEL mice immunized as indicated in figure were monitored weekly for serum HEL-specific IgG (four mice per group.) (E) Animal weight plotted as percentage of initial weight.

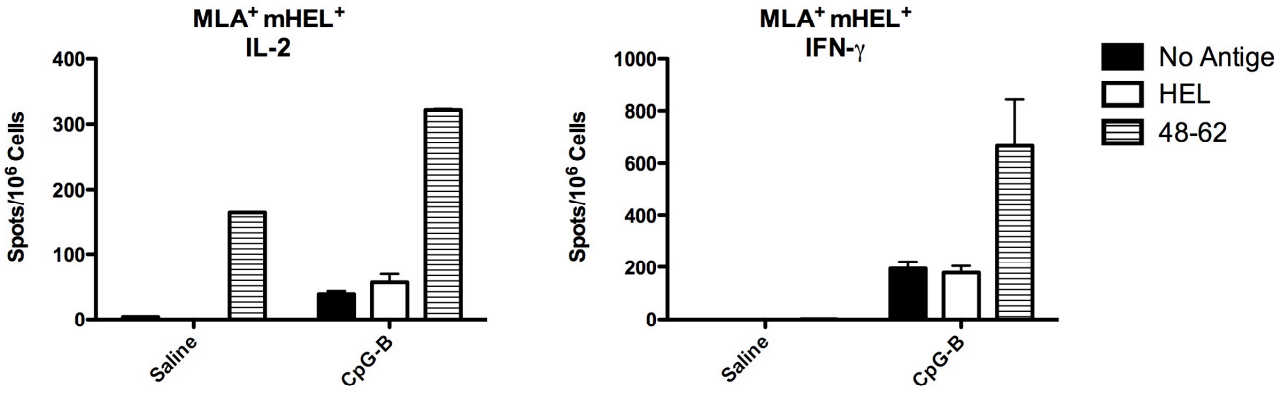

Figure 5. Administration of CpG-B in MLA x mHEL Mice Primes Self-Reactive Type B T cells. MLA x mHEL dual-transgenic mice immunized with Saline or CpG-B. Draining lymph nodes recalled in ELISPOT assay seven days after immunization. Data representative of two independent experiments.

Chapter 6
Discussion and Future Directions

Mechanism of Inflammation-Associated Type B Presentation

While I have not elucidated the subcellular mechanism of inflammation associated type B pMHC generation from HEL protein, we have learned many things about this phenotype. The first is that TLR ligands and type I IFN act directly on cDC allowing these activated APC to present type B pMHC from HEL. I also have found that generation of the type B pMHC conformers from HEL is a very slow event, requiring a minimum of eighteen hours for a detectable number of complexes to be present at the surface. Additionally, I have found that even though splenic cDC are much more efficient at the process, macrophages, B cells, and GM-CSF BMDC can all present type B pMHC from HEL when incubated with TLR ligands for the proper amount of time required to generate the complexes. Additionally, I have found that *in vivo* the same DC present both type A and B conformers, indicating that inducible presentation of type B is not at the expense of generation of type A pMHC. While these findings are important and informative, they have not yet provided a clear picture of what is occurring inside the activated DC.

The experiments with H2-DM-deficient DC are critical in understanding the regulation of type B presentation from HEL protein. If H2-DM were the only mechanism that prevented type B pMHC from being generated from HEL, as we previously assumed, it is expected that H2-DM-deficient DC would spontaneously present type B pMHC from HEL. However, this was not the case—like wild type DC, H2-DM-deficient DC required TLR stimulation to generate type B pMHC conformers from HEL protein. This indicates

two things—first, that H2-DM is not the only mechanism of preventing generation of type B conformers by resting DC and second, that regulation of H2-DM is not the mechanism of TLR-induced type B presentation since these cells lack H2-DM. These results indicate that there is some other feature or regulatory molecule in resting DC that is more important than H2-DM in preventing type B conformers from being presented from HEL. This feature is likely regulated by TLR ligands and type I IFN to allow type B pMHC to be generated from HEL.

It is important to note that these results are not contradictory to our previous data showing that H2-DM is capable of eliminating type B pMHC formation. Cell-free assays showed that both type A and B conformers form when recombinant, plate-bound I-A^k is incubated with peptide (Pu, Lovitch et al. 2004). However, when recombinant H2-DM is added to the reaction, only type A conformers are generated, indicating that H2-DM strongly favors the loading of type A over type B conformers. Additionally, experiments were performed targeting peptide to early versus late endosomes using liposomes (Pu, Lovitch et al. 2004). These experiments showed that type B pMHC form preferentially in early endosomes, where H2-DM is not present or active. When peptide was targeted to the late endosomes of H2-DM-deficient DC, type B pMHC were now able to form. This indicates that when there is a large pool of available peptide, H2-DM selects for type A pMHC generation. However, the new data indicates that when intact protein is given to a DC there are additional factors other than H2-DM that prevent type B pMHC from being presented effectively.

There are numerous changes that occur in DC following stimulation through TLR. Three relevant changes to the MHC II pathway that we have considered are increased levels of MHC II molecules, increased surface half-life of pMHC II, and increased uptake of antigen. It is possible that any or all three of these aspects could participate in type B pMHC generation. As reported by others (Cella, Engering et al. 1997), we observe a transient increase in MHC II synthesis and a small but reproducible increase in surface levels of MHC II molecules (Chapter 3, Figure 1A, 6D.) This modest change in surface levels of MHC II seems unlikely to be the most critical contributor to induced type B presentation, though it is possible but difficult to prove that it contributes to the phenotype.

It is also possible that increased surface half-life of pMHC II could be a significant mechanism of induced type B pMHC (Cella, Engering et al. 1997; Shin, Ebersold et al. 2006; van Niel, Wubbolts et al. 2006). Since these complexes are much less stable than their type A counterparts, any increased stability at the surface could allow complexes that might be generated in resting DC to now remain at the surface long enough to reach detectable levels. I have attempted to set up a cell-based assay for detecting pMHC half-life on DC using T cell hybridomas as detection. These protocols included variations of peptide pulsed resting and CpG-activated DC followed by varying chase times, fixation, and detection with T cell hybridomas. However, I am not convinced that the results are reproducible enough to address this question (the peptide does not always fall off or does not fall off to the same degree between experiments.) I think these assays are complicated by the longevity of the DC in culture, the ability of

peptide to fall off and rebind to recycling MHC II, and the added variability involved with fixation.

It is also possible that increased uptake of antigen could contribute to TLR-induced type B presentation. This could occur by simply overwhelming the normal mechanisms that prevent type B pMHC from being generated from HEL in resting DC. Resting mHEL DC, which express large amounts of membrane-bound HEL under the MHC II promoter express low levels of type B pMHC. This indicates that at least in the mHEL system, it is possible to generate some type B pMHC complexes from HEL in a resting APC when large amounts of protein are present. We examined the effect of TLR ligands and IFN-beta on uptake of fluorescently labeled HEL protein at times ranging between one and twenty-four hours (only 6 hour time point shown.) While CpG-B and zymosan A both significantly increase the amount of HEL protein taken up by DC, the other stimulants have a much more modest effect which is only seen as the six hour time point (Chapter 3, Figure 6A), making this aspect of TLR-activation unlikely to be the primary driver of type B presentation.

One phenotype that we have seen with almost all of the stimulants tested is increased generation of pMHC II complexes. All TLR ligands and IFN-beta increase generation of the 48-62-I-A^k complex from both peptide and HEL protein (Chapter 3, Figure 6 B, C.) The observation that increased complexes are seen from both peptide and protein argues against this being a result from changes in processing of protein since the peptide does not require processing. It suggests that this is due to increased levels of MHC II, increased surface half-life, or potentially increased efficiency of MHC II

loading. Along similar lines, we have observed a modest increase in catabolism of HEL with CpG-activation (Chapter 3, Figure 8) but when combined with these results, it seems unlikely that the modest increase in catabolism is the main mechanism.

Another theory that we have favored is that TLR-stimulation changes the location of HEL processing and/or presentation such that it occurs in a site of the endocytic pathway that allows type B pMHC generation. The liposome experiments performed by Lovitch clearly showed that peptide targeted to early endosomes generated type B pMHC conformers much more effectively than peptide targeted to late endosomes (Pu, Lovitch et al. 2004). While we previously ascribed these results exclusively to the role of H2-DM in the late endosomes, it is possible that there are other features of late endosomes that are refractory to generation of type B pMHC. Additionally, one paper indicated that CpG-B treatment of GM-CSF BMDC is capable of inducing a brefeldin A-resistant pathway of MHC II presentation (Askew, Chu et al. 2000). This indicates that at least in these cells, TLR activation can shift presentation to an early compartment where newly processed antigen is loaded onto recycling MHC II molecules.

I invested considerable time attempting to locate the site of type B pMHC generation within the endocytic pathway using subcellular fractionation. While this technique would have been invaluable if it had worked with the type B system, I think the level of type B pMHC complexes is too low to detect using T cell hybridoma reactivity to subcellular fractions. While type A pMHC localization is easily detectable using T cell hybridomas, type B pMHC appear to be below the limit of detection at all times tested between 90 min and 36 hours. We have done many manipulations of the system

including increasing antigen dose, varying the time of antigen incubation, increasing the number of DC fractionated per sample, and increasing the amount of each fraction that is incubated with the T cell hybridomas. The only scenario where we are able to detect type B pMHC is if DC are pulsed with peptide or if DC are incubated for approximately forty-two hours with HEL and CpG. At this time point, the level of type B pMHC is very low and the DC are beginning to have lower viability. Due to the low levels and the extremely late timing of type B pMHC detection with this protocol, we are not confident that we can use this system to identify the subcellular compartment where type B pMHC is generated. It seems likely that this is due to low density of the type B pMHC complexes generated from DC incubated with HEL and CpG. We can reproducibly detect type B pMHC in mHEL DC, however this system is not idea for elucidating the mechanism.

Aside from direct detection of pMHC within the endocytic pathway, there are other means of testing this theory. I have attempted to look at global changes in proteolytic activity and have not seen differences between resting and CpG-B-activated DC (data not shown.) This was performed using EnzChek, a BODIPY-FL-labeled casein molecule that changes fluorescence when cleaved by a range of proteases. This detection molecule was added to fractions generated from resting and CpG-B-activated DC, but I did not observe a difference in the location of general proteolytic activity (data not shown.) I have also attempted to look at processed protein using DQ-ovalbumin. This protein conjugate also fluoresces when cleaved. Again, using subcellular fractionation, I was unable to detect a difference in localization of where ovalbumin was being cleaved

when DQ-ovalbumin was incubated with resting and CpG-B-activated DC (data not shown.) Additionally, I used fluorescently labeled HEL in combination with the subcellular fractionation protocol to assess where the protein traffics within resting and CpG-activated DC. While the CpG-activated DC took up remarkably higher levels of antigen, there was a modest peak in the lysosomal fractions and a large peak in the early endosomal fractions of both populations. In sum, I have been unable to find evidence that the location of processing and presentation of soluble protein is altered in CpG-activated DC. However, I do not think this lack of evidence has conclusively eliminated the possibility that TLR activation alters the location of HEL processing and/or presentation.

I have also used confocal microscopy to attempt to localize HEL protein in resting and activated DC. I first used fluorescently labeled HEL, lysotracker, and labeled transferrin to localize the protein to lysosomes versus recycling endosomes, respectively. However, the splenic DC appeared to largely take up either HEL protein or the label but rarely both, making it difficult to draw conclusions from the data. I next used fluorescently labeled HEL and stained DC for lamp1 and EEA1, markers of lysosomes and early endosomes, respectively. While HEL appears in a vesicular pattern in DC from as early as one hour to as late as twenty-four hours after exposure, the vast majority of the protein does not co-localize with either lamp1 or EEA1, making it difficult to ascertain the location of the protein. I also incubated DC with fluorescently labeled HEL with or without HEL for twenty hours, then fractionated the cells. While the CpG-treated DC

took up considerably more protein, HEL appeared to be concentrated in the same fractions as in resting DC.

I have not found evidence that HEL protein, processing activity, or pMHC are shifted to a different location within the endocytic pathway, but I also have not disproved the theory either. It is possible that the accumulation of several small changes may account for TLR-induced type B pMHC presentation from HEL. It is possible that with modestly increased levels of surface MHC II molecules and increased amount of HEL protein taken up by DC that there are higher levels of type B complexes generated. Additionally, with increased surface pMHC half-life, these unstable complexes that do form may be able to remain at the surface for a longer period of time. These factors together may allow enough complexes to remain at the surface long enough at a high enough density to be detectable by T cell hybridomas. While this is a likely explanation for TLR-induced type B presentation, it is difficult to directly test since it is the accumulation of small related but separate changes in the cell.

Type B T Cells *in vivo*

The *in vivo* findings with mHEL mice showed that exposure to TLR ligands can increase presentation of type B pMHC derived from membrane-bound self-proteins. While the data indicating that this leads to T cell priming but not disease are preliminary, they indicate that in normal genetic backgrounds, this presentation of type B pMHC may not be harmful. It will be informative to determine what regulatory mechanisms function to control the priming of endogenous type B T cells that I observed. With the MLA x mHEL system it would be possible to determine if priming of these cells leads to

conversion into regulatory T cells or simply abortive proliferation. Further experimentation could help elucidate checkpoints that may fail, allowing progression from mere auto-reactivity to autoimmune disease.

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