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Both Positive and Negative Effects on Immune Responses by Expression of a Second Class II MHC Molecule

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Abstract

It is perplexing why vertebrates express a limited number of Major Histocompatibility Complex (MHC) molecules when theoretically, having a greater repertoire of MHC molecules would increase the number of epitopes presented, thereby enhancing thymic selection and T cell response to pathogens. It is possible that any positive effects would either be neutralized or outweighed by negative selection restricting the T cell repertoire. We hypothesize that the limit on MHC number is due to negative consequences arising from expressing additional MHC. We compared T cell responses between B6 mice (I-A⁺) and B6.E⁺ mice (I-A⁺, I-E⁺), the latter expressing a second class II MHC molecule, I-E^b, due to a monomorphic *Ed^k* transgene that pairs with the endogenous I-E^b chain. First, the naive T cell V β repertoire was altered in B6.E⁺ thymi and spleens, potentially mediating different outcomes in T cell reactivity. Although the B6 and B6.E⁺ responses to hen egg-white lysozyme (HEL) protein immunization remained similar, other immune models yielded differences. For viral infection, the quality of the T cell response was subtly altered, with diminished production of certain cytokines by B6.E⁺ CD4⁺ T cells. In alloreactivity, the B6.E⁺ T cell response was significantly dampened. Finally, we observed markedly enhanced susceptibility to experimental autoimmune encephalomyelitis (EAE) in B6.E⁺ mice. This correlated with decreased percentages of nTreg cells, supporting the concept of Tregs exhibiting differential susceptibility to negative selection. Altogether, our data suggest that expressing an additional class II MHC can produce diverse effects, with more severe autoimmunity providing a compelling explanation for limiting the expression of MHC molecules.

Keywords

class II MHC; CD4⁺ T cells; viral infection; alloreactivity; autoimmunity

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1. Introduction

The Major Histocompatibility Complex (MHC) functions both in the periphery to present peptides to T cells and in the thymus to select the T cell repertoire (Jensen, 2007). Humans express three types of class I and three class II MHC molecules, while mice have up to three types of class I and only one or two class II MHC (Kelley et al., 2005; Kumanovics et al., 2003; Lawlor et al., 1990). It is a difficult process for thymocytes to make it past selection, and positive selection is particularly stringent with approximately 90% of thymocytes dying by neglect due to an inability to sufficiently bind (pMHC) complexes (Egerton et al., 1990; Jameson et al., 1995; Palmer, 2003; Scollay et al., 1980; Stritesky et al., 2013). Therefore, it is curious why vertebrates do not express more MHC molecules, which would theoretically result in an increase in the mature T cell population that successfully underwent selection as well as a greater diversity of the presented pathogenic peptide repertoire in the periphery. Indeed, mathematical modeling suggests that the number of MHC molecules can be increased significantly without suffering adverse effects on the ability to mount a response to pathogens, with estimates even as high as 1,500 MHC molecules (Borghans et al., 2003; Nowak et al., 1992). The question of what impact expressing additional MHC molecules has on the immune response has intrigued immunologists over the years yet has not been answered thoroughly. Settling this issue is important for increasing our understanding of the factors influencing the nature of the T cell repertoire, which ultimately impacts a wide spectrum of immune responses including reactivity to pathogens, ability to tolerate transplantation, and susceptibility to autoimmune disease.

MHC molecules on medullary thymic epithelial cells and bone marrow derived antigen presenting cells (APCs) induce negative selection, deleting T cells containing high affinity T cell receptors (TCRs) to preserve self-tolerance (Gallegos and Bevan, 2004). This role implies that detrimental effects would ensue from increasing the number of MHC molecules, overly restricting the T cell repertoire and dampening anti-pathogen T cell responses. There is some evidence that the negative selection imparted by adding MHC molecules may be considerable. For instance, it was shown that the expression of I-E deletes the vast majority of V β 17 TCR-expressing T cells (Kappler et al., 1987). Negative selection mediated by a single MHC molecule was demonstrated to account for the elimination of approximately 5% of thymocytes, and it seems likely that each additional MHC expressed would further decrease the T cell repertoire by that substantial percentage (van Meerwijk et al., 1997). And the fact that approximately 1-10% of T cells are alloreactive to non-self MHC molecules supports the notion that a significant portion of T cells would bind to a new MHC molecule encountered in the thymus, possibly too strongly (Bevan, 1977; Sherman and Chattopadhyay, 1993; Suchin et al., 2001). Negative selection by superantigen can also be a mechanism mediating the thymic deletion of particular V β TCR-expressing T cells. To elaborate, mouse mammary tumor virus superantigens bind regions on the MHC and TCR V β , stimulating massive T cell help for infected B cells and establishing infection. However, strains of mice that have integrated superantigen genes into their genome would delete T cells reactive to exogenous infection, a beneficial consequence that would explain the evolutionary preservation of superantigen-mediated elimination of a significant part of the TCR repertoire (Llewelyn and Cohen, 2002).

Knowing whether the introduction of additional MHC molecules results in either a net gain of positive selection or negative selection effects is integral in understanding the ultimate implications on immune responses. Thus far, there are conflicting reports on whether the expression of additional MHC molecules would be beneficial or detrimental to immune responses. MHC heterozygotes – obtained from crossing the F1 progeny of MHC-congenic mice – were shown to have greater pathogen resistance compared to their littermate MHC homozygote mice (McClelland et al., 2003; Penn et al., 2002). On the other hand, studies looking at the F1 progeny of parents with disparate MHC haplotypes have revealed a dampening of T cell reactivity in the context of viral infections. Both naive T cell precursor numbers and T cell cytokine production during infection with influenza and vaccinia viruses were shown to be negatively affected (Day et al., 2011; Flesch et al., 2010). The approach of looking at MHC heterozygote mice, however, is a complicated system that may result in heterogeneous responses in offspring within the same cross due to complex contributions from the varied MHC molecules (Moudgil et al., 1998). An alternative, simpler method is to investigate the effects of adding just one more MHC molecule. Studies employing this method focused on autoimmunity, with most concluding that expressing more MHC is beneficial. Adding I-E expression to the non-obese diabetic (NOD) background protected from diabetes, with I-E-mediated thymic deletion of pathogenic V β 5 TCR-expressing T cells proposed to be a mechanism (Bill et al., 1988; Lund et al., 1990; Nishimoto et al., 1987; Reich et al., 1989). Expression of I-E on other backgrounds including H-2^b and H-2^q likewise resulted in protection from lupus, experimental autoimmune myasthenia gravis, and collagen-induced arthritis (Christadoss et al., 1990; Gonzalez-Gay et al., 1994; Merino et al., 1993). Yet there is a report that the addition of I-E^b actually resulted in detrimental effects, enhancing susceptibility to disease in autoimmune thyroiditis (Brown et al., 2008). Having negative effects from the addition of a second MHC was also corroborated in a different model (viral infection) with a different MHC being investigated (I-A). Here, the CD4⁺ T cell response to influenza peptides was diminished in mice expressing two I-A molecules compared to one (Nayak and Sant, 2012).

The I-E MHC, which has been a useful tool to add back to the mouse MHC repertoire, is puzzling for its disappearance from a substantial population of mouse strains. H-2^b, H-2^s, H-2^f, and H-2^q haplotype containing mouse strains and approximately 20% of wild mice do not express I-E (Mengle-Gaw and McDevitt, 1985). Studies support the idea that there exists some advantage for losing I-E MHC expression. First, it was shown that three distinct mechanisms contribute to the loss of I-E expression (Mathis et al., 1983). Second, one of the mechanisms, a deletion in the *Ea* gene, was found to be identical between wild mice and H-2^b plus H-2^s haplotype mice, suggesting the mutation occurred early and disseminated widely throughout the mouse species (Dembic et al., 1985; Dembic et al., 1984; Tacchini-Cottier et al., 1995). However, it does not make sense why some mice still express both I-A and I-E class II MHC molecules and are capable of surviving normally if I-E is that harmful. Thus, we set out to resolve the important question of what impact does I-E have on immune responses. Although previous studies did reveal insight into the effect of expressing additional MHC molecules, including I-E, each focused only on one disease model. It is unknown whether adding a particular MHC exerts distinct effects on different T cell responses.

In our study, B6.E⁺ mice, which have the monomorphic *Ea^k* transgene thus enabling I-E^b expression, were used to address the impact of having one additional class II MHC on a range of immune responses. We hypothesized that there would be negative consequences to immune responses from adding I-E^b, providing a reason for the limit on the number of MHC molecules expressed. Our results demonstrated diverse effects of I-E^b across multiple immune models. In hen egg-white lysozyme (HEL) protein immunization, we found similar B6 and B6.E⁺ responses. In viral infection with lymphocytic choriomeningitis virus (LCMV), I-E^b elicited subtle differences in CD4⁺ T cell cytokine production. In *in vitro* alloreactivity, there was decreased B6.E⁺ T cell responses compared to B6. Finally, in an autoimmune disease model, experimental autoimmune encephalomyelitis (EAE), expression of I-E^b resulted in detrimental consequences. There was significantly enhanced disease in B6.E⁺ mice compared to B6. This was not attributable to greater numbers of pathogenic T cells or increased effector cytokine production. Instead, B6.E⁺ mice had decreased regulatory T (Treg) cell percentages during EAE, a deficiency related to altered selection mediated by I-E^b. Altogether, the data suggest that the limitation on the number of MHC molecules we express may be to prevent autoimmunity.

2. Materials and Methods

2.1 Mice

Balb/c, C57BL/6 (B6), and SJL mice were purchased from The Jackson Laboratory. B6.E⁺ mice were the kind gift of Chella David (Mayo Clinic), and were derived from insertion of the monomorphic *Ea^k* transgene into C57BL/6 × SJL embryos (Le Meur et al., 1985), which pairs with the endogenous I-E^b chain thus allowing the expression of I-E^b. The mice were extensively backcrossed with C57BL/6 mice onto the H-2^b background, and this was confirmed by analysis of microsatellite markers at the Rheumatic Disease Core Center, Washington University School of Medicine (St. Louis, MO). Mice were bred and housed in specific pathogen-free conditions at the animal facility at the Washington University Medical Center (St. Louis, MO). All use of laboratory animals was approved and performed in accordance with the Washington University Division of Comparative Medicine guidelines.

2.2 Flow cytometry

Analysis of thymocytes and peripheral T cell populations was performed using anti-CD3 (145-2C11)-PE-Cy7, anti-CD25 (eBio3C7)-eFluor 450, anti-Foxp3 (FJK-16s)-PE (eBioscience), anti-Helios (22F6)-Alexa Fluor 647, anti-CD44 (IM7)-Alexa Fluor 700, anti-CD4 (RM4-5)-PerCP, anti-CD8a (53-6.7)-APC-Cy7 (Biolegend), and anti-Vβ TCR-FITC screening panel (BD Biosciences). All samples were analyzed using LSR II or LSR Fortessa cytometers (BD Biosciences) with calculated compensation, and the data were analyzed with FlowJo software (Tree Star).

2.3 Proliferation assay

Mice were immunized sub-cutaneously (s.c.) with 20 nmol hen egg-white lysozyme (HEL) emulsified in complete Freund's adjuvant (CFA) (Difco). After 7 days, draining lymph nodes cells were harvested, and $0.5-1 \times 10^6$ cells were plated in triplicate with medium only,

varying concentrations of HEL, or purified protein derivative (PPD) of *Mycobacterium tuberculosis* for 4 days in RPMI 1640 (Life Technologies) supplemented with 10% fetal bovine serum (PAA), 5×10^{-5} M 2-ME (Sigma-Aldrich), 2mM GlutaMAX (Life Technologies), 1mM sodium pyruvate (Cellgro), 0.1mM non-essential amino acids (Lonza), 25mM HEPES buffer (Cellgro), and 50µg/mL gentamicin (Life Technologies), with 0.4µCi tritiated thymidine ($[^3\text{H}]\text{TdR}$) added for the last 24 h of culture. Assays were harvested on a Skatron cell harvester and counted using a LKB (Pharmacia/LKB) β plate counter.

2.4 Mixed lymphocyte reaction (MLR) and ELISPOT

B6 and B6.E⁺ splenic CD4⁺ T cells were obtained by incubating cells with anti-CD4 microbeads for 30 min. at 4°C and flowing samples through magnetized LS positive selection columns (Miltenyi Biotec). 1.5×10^6 T cells were cultured for 48 h with equal numbers of irradiated Balb/c or SJL splenocytes, 20ng/mL phorbol 12-myristate 13-acetate (PMA) + 1µg/mL ionomycin, or medium only in 96-well MultiScreen-IP plates (Millipore) coated with purified anti-IFN γ (RA-6A2; eBioscience). Plates were then incubated with biotinylated anti-IFN γ (XMG1.2; eBioscience) and streptavidin-conjugated HRP (Southern Biotech), developed with 5-bromo-4-chloro-3-indoyl phosphate/nitro blue tetrazolium (BCIP/NBT; Sigma-Aldrich), read on a CTL ImmunoSpot reader, and analyzed using CTL ImmunoSpot 4.0 (Cellular Technology Limited).

2.5 LCMV infection

Acute infection was performed by intra-peritoneal (i.p.) infection with 4×10^5 pfu LCMV Armstrong, and spleen cells were taken at 8 d post-infection for tetramer and intracellular cytokine stains. Chronic infection was done with intra-venous (i.v.) infection of 2×10^6 pfu LCMV CL13, and at 28 d post-infection spleen cells were taken for intracellular cytokine analysis.

2.6 Tetramer enrichment

LCMV₆₆₋₇₇ (DIYKGVYQFKSV) bound to I-A^b (LCMV/I-A^b)-APC and myelin oligodendrocyte glycoprotein (MOG)₃₈₋₄₉ (GWYRSPFSRVVH) bound to I-A^b (MOG/I-A^b)-PE tetramer were used to analyze antigen-specific T cells. Tetramers were obtained from the NIH Tetramer Core Facility at Emory University (Atlanta, GA). Enrichment was conducted according to published protocols (Moon et al., 2009). In brief, cells were stained with tetramer for 1 h at room temperature, washed, incubated with anti-PE or anti-APC conjugated microbeads for 30 min. at 4°C, and passed over magnetized LS columns for positive selection (Miltenyi Biotec). The enriched cell populations were labeled with antibodies to cell surface markers for 20 min. at 4°C and analyzed by flow cytometry.

2.7 Intracellular cytokine stain

Cells were cultured with the indicated peptide – 10µg/mL for LCMV₆₁₋₈₀ (GLNGPDYKGVYQFKSVEFD) and 10-20µg/mL for MOG₃₅₋₅₅ – for 24 h with 10µg/mL Brefeldin A (Sigma-Aldrich) added during the last 4 h. Live cells were stained using LIVE/DEAD Fixable Blue Dead Cell Stain Kit (Molecular Probes), labeled with surface marker mAbs, fixed with 4% paraformaldehyde (Sigma-Aldrich), permeabilized with 0.5% saponin

buffer, and intracellularly stained with anti-IFN γ (XMG1.2)-FITC, IL-17 (TC11-18H10.1)-PE, and TNF α (MP6-XT22)-PE (Biolegend).

2.8 EAE induction and scoring

EAE was induced by s.c. immunization with 200 μ g MOG₃₅₋₅₅ (MEVGWYRSPFSRVVHLYRNGK) emulsified in 500 μ g CFA (at 10mg/mL *M. tuberculosis* H37Ra, Difco) and i.p. injection of 200ng pertussis toxin (List Biological Laboratories, Inc.). Spleen and draining lymph node cells at 13 d post-immunization were taken and stained for flow cytometry analysis. Mice were clinically scored on a five point scale for signs of disease. 0 = no weakness, 1 = limp tail, 2 = mild hindlimb paresis, 3 = moderate to severe hindlimb paresis, 4 = complete hindlimb paresis, 5 = moribund or dead.

3. Results

3.1 The expression of a second class II MHC molecule, I-E^b, alters the naive T cell repertoire

To understand the impact that increasing the number of MHC molecules has on the naive T cell repertoire, the thymus populations of B6 and B6.E⁺ mice were compared. B6 mice express only one class II MHC molecule, I-A^b, and do not express I-E^b due to a deletion in the *Ea* gene, while B6.E⁺ mice have both I-A^b and I-E^b resulting from transgenic expression of the monomorphic *Ea*^k that pairs with the endogenous I-E β ^b chain. Broad analysis of thymocyte populations revealed similar percentages of B6 and B6.E⁺ CD4⁺ and CD8⁺ double negative (DN) cells (10.1 $\pm$ 1.3% vs. 8.7 $\pm$ 0.4%), double positive (DP) cells (65.9 $\pm$ 3.1% vs. 65.9 $\pm$ 2.7%), CD4⁺ single positive (SP) cells (15.2 $\pm$ 1.7% vs. 16.5 $\pm$ 2.5%), and CD8⁺ SP cells (5.1 $\pm$ 0.4 vs. 5.3 $\pm$ 0.3%) (Fig. 1A).

However, more in-depth examination of the expression patterns of the TCR V β family members revealed differences. V β 5, V β 11, and V β 12 were significantly decreased in the B6.E⁺ CD4⁺ SP populations. V β 5 and V β 11 percentages were also significantly diminished for CD8⁺ SP thymocyte populations in B6.E⁺ mice (Fig. 1B). These results are in line with previous work attributing the I-E molecule to the thymic deletion of these exact V β family members, postulated to be mediated by superantigens (Bill et al., 1988; Dyson et al., 1991; Woodland et al., 1991). What was more interesting and novel is that B6.E⁺ CD4⁺ SP thymocytes had increased percentages of V β 6 and V β 10 compared to B6 (9.3 $\pm$ 0.4% vs. 6.6 $\pm$ 0.3% for V β 6 and 6.3 $\pm$ 0.3% vs. 3.5 $\pm$ 0.2% for V β 10). These differences were not seen with CD8⁺ SP thymocytes, indicating they resulted directly from adding a class II MHC molecule (Fig. 1B). Analysis of DP thymocytes revealed none of the changes in V β staining, suggesting that the altered repertoire in B6.E⁺ mice was due to selection-mediated effects (Fig. 1C). In the periphery, splenic B6.E⁺ CD4⁺ T cells showed increases in the V β 6 and V β 10 TCR-expressing populations compared to B6 (10.5 $\pm$ 0.7% vs. 8.9 $\pm$ 0.4% for V β 6 and 7.1 $\pm$ 0.1% vs. 3.9 $\pm$ 0.1% for V β 10), similar to what was seen in the thymus. Additionally, the significant decreases in V β 5, V β 11, and V β 12 B6.E⁺ populations seen in the thymus were likewise significantly diminished in the periphery for both B6.E⁺ CD4⁺ and CD8⁺ T cells (Fig. 1D). These results demonstrate that an altered TCR repertoire is maintained in the

periphery and present to potentially change the pathogenesis of immune diseases, with our hypothesis predicting a negative impact from I-E^b expression.

3.2 Expression of I-E^b does not change the T cell response to protein immunization with HEL in CFA

We analyzed the T cell response to protein immunization to see if the antigens present in a single protein are sufficient to elicit changes in mice expressing an additional MHC molecule. The HEL protein was chosen because B6 mice exhibit minimal reactivity to it. Out of 11 HEL peptides spanning the entire 129 amino acid sequence of the protein, B6 mice responded to only 3 epitopes (Moudgil et al., 1997; Moudgil et al., 1998). We reasoned that if the addition of I-E^b alters the T cell response, it would be most discernible in a system where the antigen reactivity is moderate to begin with. Additionally, based on a reported I-E^b binding motif (Kong et al., 2011; Kong et al., 2003), there is a putative I-E^b-binding epitope in HEL, increasing the chance that we will detect a different T cell response in B6.E⁺ mice. To test this, B6 and B6.E⁺ mice were immunized in the hind footpads with HEL emulsified in CFA, and 7 days later draining lymph node cells were taken and stimulated *in vitro* with HEL for 4 days. Samples were pulsed with [³H]TdR during the last 24 h and assessed for proliferation. We found that B6 and B6.E⁺ responses to multiple concentrations of HEL were similar, demonstrating that the addition of a second MHC molecule does not affect overall immune reactivity in this situation (Fig. 2A). Furthermore, B6 and B6.E⁺ T cell responses to PPD, containing *Mycobacterium tuberculosis* present in CFA, was compared. The similar proliferative responses to a more complex mixture of antigens underscored the lack of I-E^b-mediated differences in protein immunization (Fig. 2B).

3.3 Addition of I-E^b impacts the quality of the CD4⁺ T cell response to LCMV

We next investigated the response to viral infection, specifically CD4⁺ T cell response so the direct impact of expressing an additional class II MHC could be addressed. B6 and B6.E⁺ mice were infected i.p. with LCMV Armstrong, and 8 days later spleen cells were analyzed for frequency of LCMV-specific T cells by tetramer stain. To narrow our focus, we chose to compare the B6 and B6.E⁺ T cell population specific to a defined I-A^b-restricted LCMV epitope, glycoprotein (GP)₆₆₋₇₇, dominant in the CD4⁺ T cell response (Dow et al., 2008). Upon staining with the LCMV GP₆₆₋₇₇/I-A^b tetramer, we found that B6.E⁺ mice exhibited similar frequencies of LCMV-specific T cells compared to B6 (900.5±177.7 vs. 624.2±78.3 cells/10⁵ CD4⁺ T cells, respectively) (Fig. 3A).

T cell cytokine production was also assessed. To do this, splenic cells 8 d post-infection were stimulated *ex vivo* for 24 h with the I-A^b-restricted LCMV₆₁₋₈₀ peptide containing no predicted I-E^b epitopes according to an I-E^b binding motif previously used for I-E^b peptide identification (Kong et al., 2011; Kong et al., 2003) and then analyzed by flow cytometry. The production of individual cytokines such as TNFα by itself was comparable between B6 and B6.E⁺ CD4⁺ T cells (4.4±0.7% vs. 3.8±0.5%, respectively). But interestingly, the percentage of CD4⁺ T cells co-producing IFNγ and TNFα was reduced in B6.E⁺ mice compared to B6 (0.66±0.06% vs. 0.88±0.09%, respectively) (Fig. 3B). We wondered if the difference in B6.E⁺ CD4⁺ T cell production of IFNγ and TNFα was present only in an acute

infection or if the cytokine decrease would persist throughout a chronic infection. Therefore, we performed chronic infection with LCMV CL13. Our results corroborated the dampened T cell cytokine production seen in the B6.E⁺ mice compared to B6 ($0.16 \pm 0.02\%$ vs. $0.24 \pm 0.02\%$, respectively) at 28 d post-infection (Fig. 3C), demonstrating that the difference is not limited to acute LCMV infection. Altogether, expression of an additional MHC molecule resulted in a subtle impact on T cell immunity by keeping the frequency of antigen-specific CD4⁺ T cells the same but altering the quality of the cytokine-producing T cell repertoire, diminishing the percentage of T cells producing multiple cytokines.

3.4 Against allogeneic stimuli, expression of a second MHC molecule decreases the CD4⁺ T cell response

Thus far, an alteration in the quality of the CD4⁺ T cell response to antigens presented by self MHC has been seen with the addition of I-E^b. We wondered what we would see in response to non-self MHC ligands. An MLR was performed to address this issue. 1.5×10^6 B6 and B6.E⁺ splenic CD4⁺ T cells were cultured with equal numbers of irradiated Balb/c (H-2^d) or SJL (H-2^s) APCs for 48 h, and IFN γ ELISPOT was carried out to determine the frequency of alloreactive T cells. The B6.E⁺ alloreactive T cell frequencies were significantly decreased upon stimulation with both Balb/c and SJL APCs compared to B6 (232.6 ± 72.8 vs. 518 ± 87.8 for Balb/c and 97.9 ± 16 vs. 176.1 ± 30.5 IFN γ spots/well for SJL). B6 and B6.E⁺ samples had similar responses to the nonspecific stimulation PMA + ionomycin (122.8 ± 29.1 vs. 76.3 ± 17.1 IFN γ spots/well, respectively) as well as to no stimulation (20 ± 4.5 vs. 21 ± 4.4 IFN γ spots/well, respectively) (Fig. 4).

3.5 I-E^b increases the onset and severity of EAE due to altered selection of the nTreg population

We were interested in examining B6.E⁺ mice in autoimmunity because there are opposing conclusions about what impact adding I-E has on autoimmune diseases. Though the majority of reports have demonstrated a protective role for I-E in a number of autoimmune models, a thorough study on autoimmune thyroiditis showed increased susceptibility imparted by I-E expression (Brown et al., 2008; Christadoss et al., 1990; Gonzalez-Gay et al., 1994; Lund et al., 1990; Merino et al., 1993; Nishimoto et al., 1987; Reich et al., 1989). Our study provides a unique opportunity to relate the impact of I-E on autoimmunity to its effects on other types of immune responses, a comparison that has not been done before. Here, I-E^b has produced different effects on distinct immune models: it does not change the response to HEL protein immunization but alters the quality of the anti-viral response and diminishes alloreactivity. We wondered what result we would see in autoimmunity and whether it would be completely different or similar to any of our other models. We chose to investigate EAE because the impact of I-E on this autoimmune model has not been examined before and could provide further support for either a protective or detrimental role for I-E. B6 and B6.E⁺ mice were immunized with MOG₃₅₋₅₅ peptide emulsified in CFA and injected with pertussis toxin. Using similar reasoning as in our LCMV infection study, we chose to immunize with an I-A^b-restricted MOG peptide rather than whole protein so that we could perform a direct and focused comparison between the B6 and B6.E⁺ T cell response to a defined antigen.

After immunization, clinical scores were recorded throughout the course of disease. Unexpectedly, results revealed that B6.E⁺ mice had enhanced disease compared to B6, exhibited by earlier days of onset (11.8 ± 0.3 vs. 14.7 ± 1.1 , respectively) as well as higher peak clinical scores (3.25 ± 0.27 vs. 1.72 ± 0.26 , respectively) (Fig. 5A and Table 1). A possible explanation for increased autoimmunity would be an increased pathogenic T cell number or response. We therefore assessed T cell binding to MOG₃₈₋₄₉/I-A^b tetramer and cytokine production. Due to the difference in day of onset, we chose a time-point early in disease when we reasoned differences in lymphoid organs would be most evident. At 13 d post-EAE induction, the frequencies of MOG/I-A^b-specific CD4⁺ T cells extracted from spleen and draining lymph nodes were similar between B6 and B6.E⁺ mice (221.3 ± 41.1 vs. 203.4 ± 24.3 cells/ 10^5 CD4⁺ T cells, respectively) (Fig. 5B). To look at cytokine production, spleen and draining lymph node cells were stimulated *ex vivo* with MOG₃₅₋₅₅, and staining for cytokines relevant to EAE pathogenesis – IFN γ , IL-17, and TNF α – was performed and assessed by flow cytometry (Chitnis and Khoury, 2003; Fletcher et al., 2010). Surprisingly, B6 and B6.E⁺ CD4⁺ T cells had similar production of all three cytokines ($1.2 \pm 0.3\%$ vs. $0.9 \pm 0.1\%$, respectively, for IFN γ , $0.18 \pm 0.03\%$ vs. $0.14 \pm 0.02\%$ for IL-17, and $1.2 \pm 0.2\%$ vs. $1.1 \pm 0.1\%$ for TNF α) (Fig. 5C).

An alternative explanation for enhanced EAE disease is that it resulted not from increased T cell pathogenicity, but from decreased Treg cells. To test this, the percentage of Foxp3⁺ CD4⁺ Treg cells was assessed 13 days after EAE induction. Interestingly, a significantly decreased percentage of Treg cells was seen in B6.E⁺ mice compared to B6 ($8.7 \pm 0.8\%$ vs. $12.7 \pm 1.2\%$, respectively) (Fig. 5D). To determine whether natural Treg (nTreg) cells – which are generated in the thymus – are the subset affected, we stained the cells for Helios, a marker of nTreg cells (Thornton et al., 2010). A significant reduction in the percentage of nTreg cells during EAE was observed in B6.E⁺ mice compared to B6 ($63.9 \pm 2.2\%$ vs. $72.4 \pm 2.5\%$, respectively), implicating a thymic selection-mediated effect of I-E^b on the Treg cell population (Fig. 5E). Analysis of Foxp3 staining in spleen and lymph node cells from naive mice revealed that the decrease in Treg cells was not present in B6.E⁺ mice prior to disease ($9 \pm 0.6\%$ B6 vs. $8.1 \pm 0.9\%$ B6.E⁺) (Fig. 5F). It appears that perturbation of the immune system through autoimmunity was necessary to expose the defect of the B6.E⁺ Treg cell repertoire. Put together, these EAE results provide interesting and novel insight into the differential susceptibility of self-reactive, Treg cells to increased negative selection imparted by the expression of a second class II MHC molecule in autoimmunity.

4. Discussion

Throughout the years, immunologists have been intrigued by the biological implications for the limit on the number of MHC molecules expressed in an individual. Knowing that the role of the MHC is to present peptides to T cells, it is reasonable to think that having additional MHC molecules would result in significantly beneficial consequences; expansion of the T cell repertoire during positive selection as well as an increase in the presented peptide repertoire in the periphery should both enhance T cell responses to pathogens. However, MHC molecules also participate in negative selection, and it is possible this would balance out or even overcome the effects from positive selection. In our study, we compared B6 mice, which have only one class II MHC molecule, I-A^b, to B6.E⁺ mice, which have

both I-A^b and I-E^b, in order to see what effects on T cell responses that expressing a second class II MHC molecule would produce.

Examination of naive B6 and B6.E⁺ mice revealed distinct TCR usage. Specifically, B6.E⁺ mice exhibited higher percentages of CD4⁺ T cells expressing Vβ6 and Vβ10 as well as decreased percentages of Vβ5, Vβ11, and Vβ12 CD4⁺ and CD8⁺ T cells (Fig. 1). Though the changes in Vβ5, Vβ11, and Vβ12 expression have been shown before to be mediated by I-E and superantigen (Bill et al., 1988; Dyson et al., 1991; Woodland et al., 1991), the finding that there is increased Vβ6- and Vβ10-expressing CD4⁺ T cells upon addition of I-E^b is novel. We discovered that the addition of a second MHC molecule resulted in diverse effects. In our first model, protein immunization with HEL in CFA, we saw no net difference in the T cell response to HEL or PPD (Fig. 2). As there is one putative I-E^b peptide epitope in HEL, it is possible that though both I-A^b and I-E^b contribute to the response to HEL in the B6.E⁺ mice, there was no net change in reactivity due to the altered HEL-specific I-A^b and I-E^b T cell repertoires balancing out. However, having additional peptide epitopes present in the more complex disease models resulted in changes to the T cell response, with I-E^b reducing a certain population of T cells in each case. Sequence analysis of naturally occurring peptides eluted from I-A^b and I-E^b revealed distinct sequence motifs between the two MHC molecules (Rudensky et al., 1992). This lends support for the idea of I-E^b introducing a different set of pMHC ligands during thymic selection and altering the resulting T cell repertoire.

In LCMV infection, we saw a subtle alteration in the LCMV-reactive T cell repertoire with decreased percentage of B6.E⁺ CD4⁺ T cells producing IFNγ and TNFα in response to LCMV stimulation (Fig. 3). This would suggest that I-E^b increases the deletion of polyfunctional LCMV-specific T cells. Of course, microbiota and T cell development may differ between mice of two genotypes, so there exists the caveat that the decreased T cell response in B6.E⁺ mice may not be directly due to LCMV antigen. For alloreactivity, we showed that adding I-E^b resulted in a decreased alloreactive T cell repertoire to H-2^d and H-2^s in B6.E⁺ mice (Fig. 4). H-2^d APCs express both I-A and I-E while H-2^s APCs have only I-A. Not unexpectedly, there was a greater magnitude of alloreactivity to the H-2^d APCs compared to H-2^s, demonstrating the contribution of two allogeneic MHC molecules rather than one for stimulation. Compared to B6, B6.E⁺ CD4⁺ T cells exhibited an approximately 50% decrease in alloresponse to both H-2^d and H-2^s APCs. This indicates that I-E^b in B6.E⁺ mice can alter the T cell repertoire to affect the alloresponse to a single allogeneic MHC, I-A^s, and that having two allogeneic MHC molecules, I-A^d plus I-E^d, does not provide enough alloligands to enhance the level of B6.E⁺ alloreactivity. Whether there are similar reductions in the number of B6.E⁺ T cells specific to individual allopeptides could be established in the future using H-2^d or H-2^s pMHC tetramers. For EAE, there was enhanced autoimmunity in B6.E⁺ mice (Fig. 5, Table 1). Unlike the two previous models, we did not detect a change in effector T cells. Rather, I-E^b diminished the nTreg population (Fig. 5). The percentages of T cell effector cytokines produced in our experimental system were different from what has been recorded in the literature. Namely, IL-17 production was lower in our system than others that have found more Th17 than Th1 cells in EAE (Isaksson et al., 2009; Ko et al., 2014). We believe that a potential explanation is the microbiota composition in our mice. It has been shown that lower levels of Th17 cells in mice – for

instance in B6 mice from the Jackson Laboratory – are due to the lack of colonization by segmented filamentous bacteria (Ivanov et al., 2009). As our mouse colony is negative for segmented filamentous bacteria, we think the mice would therefore express the dampened IL-17 seen in our experiments.

We think our results can be reasonably explained by enhanced I-E^b-mediated negative selection dominating the effects from any increase in positive selection. It is known that positive selection results in CD4⁺ T cells that can bind to many different allogeneic MHC molecules, while negative selection establishes MHC restriction and peptide specificity (Huseby et al., 2005). The decreased alloreactive T cells in B6.E⁺ mice would suggest that the effects of additional negative selection from expressing I-E^b outweigh those of increased positive selection. And, we think the observation that nTregs (generated in the thymus) were affected in EAE lends support for I-E^b impacting thymic development to enhance the negative selection of Tregs. The fact that MOG is indeed expressed in medullary thymic epithelial cells (Derbinski et al., 2001) supports this idea. Differentiation of thymocytes into the Treg cell lineage is believed to involve TCRs of high affinity and is a process often characterized as a near-death encounter (Sakaguchi et al., 2008). This allows us to reconcile the idea of I-E^b impacting the Treg cell compartment but not effector T cells in EAE, as the thymocytes with high affinity TCRs may be more likely to be pushed into being negatively selected when a second class II MHC is added. It may also be possible, taking into account work demonstrating a saturable Treg cell selecting niche (Bautista et al., 2009), that Tregs selected on I-E^b compete with and limit Tregs selected on I-A^b. This would lead to a decreased ability for the I-A^b-selected Tregs to expand and function in a MOG₃₅₋₅₅/I-A^b-induced EAE model. Additionally, epitope spreading is known to play a role in EAE, in which T cell reactivity extends to antigens other than what was used to induce disease, which can become accessible through processes such as tissue destruction (Tuohy et al., 1998). It may be that in B6.E⁺ mice, effector T cells specific to I-A^b- or I-E^b-bound self peptides other than MOG₃₅₋₅₅ are impacted, which we did not test in our study.

There is the possibility that Eα₅₂₋₆₈, a peptide derived from the *Ea* gene product, binds to I-A^b and plays a role in dampening immunity. As the peptide is capable of occupying approximately 10-15% of all I-A^b molecules (Brown et al., 2008; Rudensky et al., 1991), it can prevent the selection of certain I-A^b-restricted cells in the thymus or compete with the presentation of pathogenic peptides in the periphery. The Eα peptide along with the I-E^b MHC could both contribute to the altered T cell repertoire and T cell responses in the B6.E⁺ mice. Intrathymic injection of the peptide into B6 mice and addition of the peptide to *in vitro* stimulations for competition with LCMV or MOG would aid in understanding what extent Eα₅₂₋₆₈/I-A^b plays in B6.E⁺ T cell selection and peripheral responses, respectively.

In the models examined in this study, both positive consequences and detrimental effects can result from expression of I-E^b. In LCMV, polyfunctional T cells that produce multiple cytokines have been associated with better protection from parasitic infection and more effective control of viral infection (Ciuffreda et al., 2008; Darrah et al., 2007). Thus, it is possible that the altered cytokine-producing T cell repertoire in B6.E⁺ mice can impact viral load or disease course in subsequent challenges with negative consequences for the host. In alloreactivity, we think the decreased alloreactive T cell repertoire seen upon addition of I-

E^b could have both beneficial and detrimental clinical consequences. It could theoretically lead to less graft-versus-host disease or transplant rejection, but it could also diminish the graft-versus-leukemia effect that eliminates tumors (Bleakley and Riddell, 2004). What we believe is most intriguing is the significantly enhanced autoimmunity seen in B6.E⁺ mice upon EAE induction. This model provides a clear drawback of expressing an additional MHC molecule, which supports our hypothesis that negative consequences on immunity limit the number of MHC molecules expressed.

Though our work would suggest that having more MHC molecules elicits negative consequences, studies looking at the MHC composition in other species and MHC-associated disease susceptibility or resistance might imply a different conclusion. Comparison of mammalian MHC with chicken MHC reveals that the latter is much simpler, with only a minimal essential set of MHC genes within a region approximately 20-fold smaller than the human MHC locus (Kaufman et al., 1999). Interestingly, there are viral diseases clearly associated with the chicken MHC, such as Rous sarcoma virus and Marek's disease. In contrast, there are not many associations of human MHC with infectious disease, with multiple MHC genes allowing every haplotype to give some level of resistance (Kaufman et al., 1995; Trowsdale, 2011). The plethora of MHC-binding epitopes in pathogens and the ability of viruses to mutate would suggest that more MHC molecules and greater polymorphisms are beneficial, resulting in greater resistance to infection at a population level (Trowsdale, 2011). Though we showed decreased cytokine-producing T cells LCMV infection upon expression of an additional MHC, our results are not incompatible with this idea. Our analysis thus far may be limited, and perhaps looking at the T cell response to other LCMV epitopes (we only tested GP₆₁₋₈₀) or assessing the effect of adding I-E^b at a population level (in other strains of mice besides B6) would yield enhanced T cell responses.

However, there are studies that would support our assertion that expressing more MHC is detrimental, or at least that having less MHC is not negative. Though adaptive immunity has given vertebrates advantages such as memory, it has also led to significant drawbacks of immunopathology, hypersensitivity, and autoimmunity that result from heightened responses (Hedrick, 2004). Indeed, in contrast to infectious diseases, there are numerous associations of MHC with autoimmune conditions (Trowsdale, 2011), suggesting that additional expression of MHC molecules would increase the chances of having MHC contributing to autoimmunity. Our work showing enhanced EAE with the additional expression of I-E^b supports this. And, referring back to chickens, their simple and compact MHC still allows for sufficient immune responses (Kaufman et al., 1995), suggesting no functional detriment to having less MHC. It is also possible that having more complex MHC is neither blatantly good nor bad but an effect of lacking evolutionary pressures. For instance, the mole-rat possesses polymorphic MHC while the Syrian hamster, another rodent with similar ecology, is the opposite with monomorphic MHC. The MHC monomorphism of the Syrian hamster can be explained by an absence of disease outbreaks driving the development or maintenance of MHC polymorphism (Nizetic et al., 1984; Streilein and Duncan, 1983). It has also been postulated that a high degree of polymorphism is the result of accumulating alleles over time with no continuous selection eliminating them (Kaufman et al., 1995). All together, it seems that there is no one optimal solution for how many MHC

molecules should be expressed to achieve the best immunity, a reasonable conclusion considering all the disparate species that express MHC and how different the environments in which they reside are. However, we think that a simplified experimental system, such as our model of expressing one additional MHC, I-E^b, can be used to gain valuable information on what impact the MHC composition has on a wide variety of immune responses.

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Abbreviations

[³H]TdR	tritiated thymidine
B6	C57BL/6
DN	double-negative
DP	double-positive
EAE	experimental autoimmune encephalomyelitis
HEL	hen egg-white lysozyme
LCMV	lymphocytic choriomeningitis virus
MOG	myelin oligodendrocyte glycoprotein
PPD	purified protein derivative
Treg	regulatory T cell

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Highlights

- We investigated the impact in mice of expressing 1 class II MHC molecule vs. 2
- There were similar T cell responses to protein immunization with HEL in CFA
- There was a difference in the quality of the CD4 T cell response in viral infection
- We saw decreased CD4 T cell alloreactivity in mice with 2 class II MHC molecules
- Enhanced autoimmunity and diminished Tregs resulted from 2 class II MHC molecules

Figure 1. The expression of one additional class II MHC, I-E^b, results in a distinct naive CD4⁺ T cell repertoire

(A) B6 and B6.E⁺ thymocytes were analyzed by flow cytometry for total number of thymocytes as well as percentage of CD4⁺ and CD8⁺ double negative (DN), double positive (DP), and single positive (SP) cells. Each point represents a single mouse, n = 7-8 from 3 independent experiments, mean ± sem. Mann-Whitney statistical test was performed. (B) The TCR repertoire of B6 and B6.E⁺ thymic CD4⁺ and CD8⁺ T cells was examined by staining for a panel of TCR Vβ family members, n = 8 mice from 3 independent experiments, mean ± sem. 2-way ANOVA test was conducted. (C) The percentage of CD4⁺ CD8⁺ DP thymocytes positive for each Vβ is depicted for B6 and B6.E⁺ samples, n = 8 mice from 3 independent experiments, mean ± sem, 2-way ANOVA test. (D) TCR Vβ staining was also performed on spleen cells with percentages of Vβ positive CD4⁺ and CD8⁺ T cells

shown, $n = 8$ mice from 3 independent experiments. Data are shown as mean $\pm$ sem, with 2-way ANOVA test, **** = $P < 0.0001$, *** = $P < 0.001$, ** = $P < 0.01$, * = $P < 0.05$.

Figure 2. Protein immunization with HEL in CFA elicits similar proliferative T cell responses in mice expressing either one or two class II MHC molecules

T cells from B6 and B6.E⁺ mice immunized with 20 nmol HEL emulsified in CFA were stimulated in culture for 4 d with varying concentrations of (A) HEL and (B) a dilution series of PPD. [³H]TdR was added during the final 24 h of culture, and proliferation was measured. No stimulation (0 μM or No PPD) data points are depicted to show background CPM. Data are shown as mean ± SD and are representative of three experiments.

Figure 3. Upon expression of I-E^b, there are decreased percentages of IFN γ and TNF α double-positive CD4⁺ T cells in response to LCMV infection

(A-B) Mice were infected i.p. with 4×10^5 pfu LCMV Armstrong, and 8 d post-infection splenic T cells were analyzed. (A) Cells were stained with LCMV/I-A^b APC-labeled tetramer, enriched by magnetic selection using anti-APC beads, stained for surface markers, and assessed by flow cytometry. (B) Cells were also stimulated ex vivo with 10 μ g/mL LCMV GP₆₁₋₈₀ peptide for 24 h followed by intracellular cytokine staining for IFN γ and TNF α . (C) Mice were chronically infected i.v. with 2×10^6 pfu of LCMV CL13. At 28 d post-infection, spleen cells were stimulated ex vivo with 10 μ g/mL LCMV GP₆₁₋₈₀ for 24 h, and cytokine production was assessed by flow cytometry. Each point represents one mouse from two independent experiments, and data are shown as mean $\pm$ sem, Mann-Whitney test, * = $P < 0.05$.

Figure 4. The CD4⁺ T cell response to allogeneic stimuli is decreased with expression of I-E^b
 1.5×10^6 B6 and B6.E⁺ splenic CD4⁺ T cells were cultured at a 1:1 ratio with Balb/c (H-2^d) and SJL (H-2^s) APCs. Stimulation with PMA (20ng/mL) + ionomycin (1μM) and medium were used as positive and negative controls, respectively. Data are shown as mean $\pm$ sem, n = 6-9 samples compiled from 2-3 independent experiments, unpaired t test, * = $P < 0.05$.

Figure 5. B6.E⁺ mice exhibit enhanced EAE, and this is associated with decreased percentages of Tregs

EAE was induced with immunization of MOG₃₅₋₅₅ peptide and scored as described in the Materials and Methods. (A) Clinical scores were assessed up to 28 d post-immunization. Results are mean $\pm$ sem at each timepoint and representative of three independent experiments. (B-E) At 13 d post-immunization, spleen and draining lymph node cells were taken for analysis. (B) Cells were incubated with MOG/I-A^b PE-labeled tetramer, enriched with anti-PE magnetic beads, and stained with surface markers for flow cytometry. Results are from four independent experiments and displayed as mean $\pm$ sem, each point is one mouse, Mann-Whitney test. (C) Cells were stimulated with 10-20 μ g/mL MOG₃₅₋₅₅ for 24 h, stained for intracellular cytokines, and analyzed by flow cytometry. Results are shown as mean $\pm$ sem, n = 10 from three independent experiments, Mann-Whitney test. (D) Cells were stained for intracellular Foxp3 expression, with data from three independent experiments displayed as mean $\pm$ sem and each point representing one mouse, Mann-Whitney test, * = $P < 0.05$. (E) Intracellular Helios staining was also performed. Each data point represents one mouse. Results are from three independent experiments and depicted as mean $\pm$ sem, Mann-Whitney test, ** = $P < 0.01$. (F) Intracellular staining of Foxp3 in naive

splenocytes and lymph node cells was done. Each point represents one mouse. Results are from three independent experiments and shown as mean $\pm$ sem, Mann-Whitney test.

Table 1

Compilation of EAE results from four independent experiments

Mouse Genotype	B6	B6.E ⁺
Incidence of disease	20/20	18/18
Day of onset (mean $\pm$ sem) *	14 $\pm$ 0.9	11.2 $\pm$ 0.43
Peak clinical score (mean $\pm$ sem) ***	2 $\pm$ 0.24	3.5 $\pm$ 0.25

* $P < 0.05$ *** $P < 0.001$