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Self-pMHCII complexes are variably expressed in the thymus and periphery independent of mRNA expression but dependent on the activation state of the APCs

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Abstract

Self-peptide MHCII ligands are critical for selection of CD4⁺ T cells in the thymus, and maintenance in the periphery. To date, no investigation as to the exact thymic and peripheral expression of a naturally occurring positive selecting self-peptide MHCII (self-pMHCII) complex has taken place. We have generated a sensitive T cell hybridoma to functionally detect the endogenous presentation of a confirmed positive selecting self-pMHCII complex for a CD4⁺ transgenic T cell. Using this tool to survey and quantify the expression selecting self-pMHCII, we have shown unequivocal proof that a known CD4⁺ selecting ligand can be presented on both positive and negative selecting thymic APCs. We also show that peripheral presentation of this same selecting ligand is affected by activation state of the APCs. Furthermore, discrepancies between the gene expression and self-pMHCII complex presentation of this bona fide selecting ligand suggest that functional detection self-ligand complexes will be required to establish a complete view of the naturally presented endogenous self-pMHC landscape.

Keywords

CD4⁺ T cell; self-pMHCII; positive selection; negative selection

1. Introduction

The development and maintenance of a functional and self-tolerant T cell repertoire depends on the continued interaction between TCR: self-pMHC (Morris and Allen, 2012). It is well established that these self-peptides are constitutively processed and presented (Lorenz and

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Allen, 1988; Veeraswamy et al., 2003; Wilson et al., 2004). Much progress has been made toward elucidating the key mechanisms of thymic selection that result in the production of a mature T cell pool that not only effectively detects self-peptides presented on MHC molecules (positive selection), but also avoids autoreactivity to self-peptides (negative selection). The undisputed requirement for recognition of self-pMHC at both the positive selection and self-tolerizing stages of thymocyte development appears paradoxical, and a variety of non-mutually exclusive models have been proposed to explain how the recognition of self-peptides can be both essential for survival but also potentially deadly to developing thymocytes (Goldrath and Bevan, 1999; Starr et al., 2003). Decades of investigation have suggested myriad models hypothesizing how this might be. Early work suggested that positive selecting cortical thymic epithelial cells (cTECs) display unique peptides for positive selection that are not duplicated among APCs involved with tolerance induction in the thymus or periphery. However subsequent work showing overlap between cTEC and splenic eluted peptide (Marrack et al., 1993), and *in vitro* assays suggesting the ability of low amounts of agonist ligand or antagonist and partial agonist ligands to induce positive selection have led to more current models emphasizing the avidity and affinity of TCR: selecting self-pMHC interaction (Hogquist et al., 1994; Marrack et al., 1993; Sebzda et al., 1994).

Currently, intrathymic selection models are evolving with our understanding of the thymic microenvironment, and not only account for the affinity of the TCR: selecting self-pMHC interaction, but the quantity, quality, spatial and temporal characteristics of the selecting ligands and the APCs that present them (Klein et al., 2009). Interestingly, a revival of the discrete peptide model is ongoing as it becomes clear that the APCs vital for positive selection and central tolerance are differentially equipped to process and present unique peptides (Derbinski et al., 2005). Though elucidation of the exact mechanisms that generate self-pMHC able to mediate thymic selection is ongoing, it is clear they are complicated in nature and challenge our current understanding of antigen processing. Involvement of antigen processing machinery outside of the “classical” pathways may prove to play a vital role in establishing the selecting ligandome that leads to functional and self-tolerant CD4 and CD8 T cell repertoires (Miller et al., 2013). Unequivocal determination of which model, or more likely, combination of models, most accurately reflects *in vivo* thymocyte selection depends intimately upon gaining an understanding of the landscape of selecting self-pMHC complex expression. Specifically, a clear understanding of if and how the ligand complexes mediating positive selection differ from those orchestrating negative selection will bring clarity to the debate (Derbinski and Kyewski, 2010; Schneider and Sercarz, 1997).

Elucidation of the selecting self-pMHC ligandome has proven difficult for a variety of technical reasons (Klein et al., 2014). First, very few naturally occurring TCR: selecting self-pMHCII pairs have been identified and validated (Ebert et al., 2009). Given the clear differences between CD4 and CD8 T cell development and maintenance (Seder and Ahmed, 2003; Surh and Sprent, 2005) it is critical to evaluate these two systems independently. Second, the rare abundance of individual thymic APC subsets compounded with the low concentration of any one unique selecting self-pMHC complex makes *ex vivo* biochemical analysis of these complexes challenging, and skewed toward the few most abundant self-

peptides (Adamopoulou et al., 2013). Alternative routes to investigate the self-peptide repertoire have focused on evaluating mRNA levels (Fortier et al., 2008). Unfortunately, surveillance of eluted peptides and message levels do not provide any insight as to the spatial and temporal nature of the functional unit of selecting self-peptide in complex with MHC. Recent discoveries of TCR: selecting self-pMHCII pairs *in vitro* have opened the field for investigation of naturally occurring selecting ligands, however to date no studies have shown directly the presence of these ligand complexes on the selecting thymic APCs.

It is clear that a T cell's dependence on self-pMHC recognition does not end in the thymus. In addition to the clear intrathymic requirements for self-pMHC expression, continued engagement of TCR by self-pMHCII is required for the homeostatic maintenance of mature T cells, the mechanisms of which are not yet completely understood (Jameson, 2002; Takada and Jameson, 2009). Studies suggest that the same ligands present in the thymus are utilized in the periphery for maintaining the T cell repertoire (Ernst et al., 1999). Given that the entire life of a T cell depends on continued recognition of (selecting) self-pMHC, it is critical to understand the natural landscape of these complexes in the thymus and periphery. The technical hurdles involved with *ex vivo* analysis of the peptide repertoire indicate a need to functionally detect these selecting self-pMHC complexes in their natural environments.

Our laboratory has recently identified a naturally occurring positive selecting self-pMHC for the ANDtg T cell, called gp250/I-E^k (Lo et al., 2009). The gp250 self-peptide was isolated from self-peptides bound to I-E^k on the B cell line CH27. In the present report, we have generated a T cell hybridoma specific for gp250/I-E^k. Using this hybridoma, we have functionally analyzed the expression of naturally occurring selecting self-pMHCII complexes on thymic and peripheral APCs. Because T cell hybridoma responses rely solely on pMHC engagement and are not affected by co-stimulatory molecules, this tool will only reflect the presence of gp250/I-E^k complexes on surveyed APCs. The results indicate that naturally occurring selecting ligand gp250/I-E^k is ubiquitously presented by positive selecting cTECs, negatively selecting mTECs and thymic DCs, as well as peripheral MHCII expressing APCs. These data show for the first time the presence of a naturally occurring selecting self-pMHCII on both cTEC and mTECs, suggesting that despite unique processing capabilities, positive and negative selecting thymic APCs do functionally present overlapping pMHC complexes. Additionally, we observed discrepancies between gene expression and functional complex presentation. Furthermore, the peripheral presentation of this selecting complex changes with recent immune status, suggesting the existence of a dynamic self-pMHC landscape with potential consequences to the existing mature T cell repertoire. These findings support a thymic selection model that merges, not excludes, currently proposed systems, and suggests that investigation of the self-pMHC landscape must include careful functional evaluation of pMHC complexes in addition to broadly visualizing the gene expression and eluted peptide profiles.

2. Materials and Methods

2.1 Mice

gp250 deficient mice (Jiang et al., 2008) were bred to the B6.K (H-2^k restricted) line (JAX 001148). The mice were extensively backcrossed to B6.K, and were monitored by the

analysis of micro satellite markers at the Rheumatic Disease Core Center, Washington University School of Medicine (St. Louis, MO). The gp250 deficient mice are immunologically normal. gp250 is derived from the sortilin-related LDL endocytic receptor LR11, and while primary expression is in the cortical brain, its transcripts are expressed to varying degrees in all known APC subsets. gp250 has no sequence homology to ANDtg CD4 T cells' agonist peptide MCC (Kaye et al., 1989; Lo et al., 2009). The MCC-I-E^k specific AND TCRtg (Kaye et al., 1989; Kaye et al., 1992), B6.K (H-2^k), C57BL/6 (B6, H-2^b) (JAX 000664) and B6.*Rag1*^{-/-} (JAX 002096) mice were purchased from The Jackson Laboratory. The AND TCRtg line was bred onto *Rag1*^{-/-} background: AND.*Rag1*^{-/-}-H-2^k. The LLO TCRtg mice were generated in our laboratory as previously described (Lo et al., 2014; Weber et al., 2012). All mice were bred and housed in specific pathogen-free conditions of the animal facility at the Washington University Medical Center. The use of all laboratory animals was approved and performed in accordance with the Washington University Division of Comparative Medicine guidelines.

2.2 Development of gp250-Specific T cell hybridomas

gp250 specific hybridomas were generated by using a standard fusion protocol (Lorenz and Allen, 1988). Briefly, popliteal lymph nodes were taken 7 to 10 days after immunization of gp250 deficient mice in the hind footpads with 40 nMole of gp250 peptide emulsified in complete Freund's adjuvant H37Ra (BD Difco). A single-cell suspension was prepared and these bulk cultures were fused with the BW5147α-β⁻ fusion partner. Successful fusions were enriched by hypoxanthine-aminopterin-thymidine (HAT) selection. The Ga2.6 hybridoma was subcloned three times by limiting dilution analysis to ensure clonality of the line.

2.3 Flow cytometry and APC isolation

For the enrichment and purification of thymic APC populations, 5–30 thymi were digested as previously described (McLelland et al., 2011; Williams et al., 2009). Briefly, thymi were isolated into CO₂ independent media (Life Technologies) supplemented with 10% FCS, thymocytes were released and thymic remnants were digested by either stirring at 1200 rpm for 30 minutes in 0.125% w/v Collagenase D (Roche) at 37°C or with 1.8mg/mL Liberase TH using the gentle MACS dissociator Spleen_01 program, followed by a 15 minute rotation at 37° and final dissociation with program Spleen_02. (Miltenyi). Each digest was supplemented with 0.025% w/v DNaseI (Roche). For the enrichment and purification of splenic B cell and DC populations, 5–10 spleens were digested as with the Collagenase D digested thymi above. CD45, CD11c or CD19 positive and negative populations were separated by magnetic bead positive selection using the respective microbeads (Miltenyi). CD45⁻ and CD45⁺ thymic populations were stained for cTECs, mTECs and CD11c⁺ DCs using the following antibodies: UEA-1 FITC (Vector Laboratories), EPCAM (G8.8) PE, MHCII (M5) PECy7, Ly51 (6C3) APC, CD45.1 (A20) or CD45.2 (104) APCCy7, and CD11c (N418) PacBlue (eBioscience or BioLegend). Splenic populations were stained for B cell and DC populations using CD19 (6D5), B220 (RA3-6B2), CD11c and MHCII. APCs were sorted on the FACS AriaII into serum and plated at the indicated concentrations in 96-well flat bottom plates.

2.4 Cell lines, peptides and monoclonal antibodies

The CH27 (H-2^a, I-E^k) B cell line, the MHCII I-E^k transfected Chinese hamster ovary (CHO-E^k) cell line, and the BW5147 $\alpha^{-}\beta^{-}$ cell line, have been described (Ehrich et al., 1993; Evavold et al., 1992). The CHO-E^k cells were cultured in MEM- α containing 5% (vol/vol) bovine growth serum (Hyclone) and 0.5 mg/ml of G418, and 50 μ M 2-mercaptoethanol. BW5147 $\alpha^{-}\beta^{-}$ cells and the Ga2.6 hybrid generated here were grown in Iscove's medium containing 20% (vol/vol) FCS. Hybrids were subcloned and maintained in IMDM medium containing 10% (vol/vol) FCS, as were the CH27 cells. CTLL cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) containing 5% (vol/vol) FCS. Media were supplemented with 2 mM Glutamax (Invitrogen) and 50 μ g/ml of gentamicin. Monoclonal antibody 14.4.4S, specific for the I-E α -chain, was obtained from the American Type Culture Collection and was purified from ascites fluid and used at the indicated concentrations (Felix et al., 2007). All peptides were synthesized, purified, and analyzed as previously described (Felix et al., 2007). gp250 (SAPGLIATGSGVGK) and CLiP (PVSKMRMATPLMQA) I-E^k biotinylated monomers were obtained from the NIH tetramer facility.

2.5 Hybridoma stimulation assays

This assay system examines the *in vivo* presentation of gp250 by APCs to the gp250-specific T-cell hybridoma Ga2.6. APCs used were the CH27 B cell line, CHO-E^k cell line (10^5 /well), or primary APCs isolated from enzymatically digested spleen or thymus. In the case of plate-bound monomer stimulations, 96-well round bottom plates were first coated overnight at 4°C with 1 μ g/well streptavidin. Plates were then washed and coated with biotinylated monomer at the indicated concentrations, overnight at 4°C. To these was added the T-cell hybridoma Ga2.6 (10^5 cells per well) in the presence or absence of exogenous gp250 peptide. APCs and hybridoma cells were incubated for 15–20 hours at 37°C in 96-well flat-bottom plate. T-cell stimulation was measured by quantifying the release of interleukin 2 (IL-2) by the T-cell hybridoma. IL-2 in the supernatant was measured by IL-2 ELISA, or by measuring [³H]thymidine incorporation by the IL-2-dependent CTLL line (Allen and Unanue, 1984; Lynch et al., 2013). ELISA was performed using 100 μ g/well capture anti-IL-2 mAb (JES6- 1A12; Biolegend), 50 μ g/well biotinylated-anti-IL-2 detection mAb (JES6-5H4; Biolegend), 100 μ l/well 1/10,000 dilution streptavidin-HRP (Southern Biotech), and developed using 100 μ l/well 1-Step-Ultra tetramethylbenzidine substrate (Thermo Scientific). The reaction stopped at 15 min by the addition of 100 μ l/well 2 M sulfuric acid, and assessed using a Victor3 plate reader (PerkinElmer). Regression analysis of IL-2 standards using GraphPad Prism 6 (GraphPad Software) was used to convert absorbance into amount of IL-2. Alternatively, CTLL cells (10^4 in 100 μ l) were added to the 100 μ l of supernatant and were cultured for 24–32 hours. Each well was pulsed with 0.2 μ Ci of [³H]thymidine during the final 6 hr of culture, and plates were harvested and [³H]thymidine incorporation was measured.

2.6 T cell receptor sequencing and quantitative PCR

RNA was isolated with an RNeasy kit (Qiagen) from cell lines and splenic or thymic APCs enriched through the use of magnetic-activated cell sorting beads and purified by flow

cytometric sorting. cDNA was synthesized using random hexamer primers (SuperScript II kit; Invitrogen). TCR were amplified as previously described (Morris et al., 2011). Briefly, nested PCR was used to amplify TCR α and β transcripts (Baker et al., 2002), which were bulk ligated into pCR2.1 TOPO sequencing vector, sequenced, and analyzed using Lasergene 8 (Invitrogen, Genewiz, DNASTAR). For quantitative PCR analysis, SYBR Green PCR master mix and ABI7000 machinery (Applied Biosystems) were used as previously described (Weber et al., 2010). Cycling conditions were 50 °C for 2 min, followed by 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and a dissociation stage (95 °C for 15 s, 60 °C for 20 s and 95 °C for 15 s); β -actin was included as internal control for all samples. Changes were calculated by the C_T method with CH27 B cell line samples as the baseline sample and *Actb* as the reference gene. The following primers (5' to 3'; forward and then reverse) were used: *LR11*, GGGAATTTCTTCAGGCCCA and TCCGAAGTTGGAGGTTGAGC; β -actin, CTAAGGCCAACCGTGAAAAG and ACCAGAGGCATACAGGGACA.

2.7 Bacterial infections and in vitro activation

The *Listeria monocytogenes* strain 10403S was used in this study. Frozen stocks of *L. monocytogenes* in PBS plus 20% glycerol were thawed and serially diluted to a density of 1×10^4 colony-forming units (CFU) per ml in PBS. Mice were infected three days prior to APC isolation retro-orbitally with 10^3 CFU *L. monocytogenes* (Persaud et al., 2014). For *ex vivo* activation, enriched splenic B cells were stimulated in 6-well plates with 10 μ g/mL anti-CD40 and 10 μ g/mL anti-IgM F(ab)2, or 20 μ g/mL LPS for 48 hours at 37°C (Allman and Pillai, 2008).

2.8 Analysis of Listeria specific response

For analysis of Listeria specific response, LLO56 CD4+ T cells purified by magnetic bead positive selection were cultured with varying amounts of sorted CD11c+ DCs or CD19+ B cells from the spleens of naive or Listeria infected mice in triplicate, in 96 well plates. After 15–20 hours at 37°C in 5% CO₂ in Iscoves medium containing 10% FCS, CD4+ T Cells were analyzed for upregulation of CD69 by flow cytometry.

3. Results

3.1 Generation of a sensitive T cell hybridoma specific for the positive selecting ligand gp250/I-E^k

We generated a sensitive T cell hybridoma to investigate the constitutive expression of the naturally occurring gp250/I-E^k positive selecting protein complex by various APCs. Attempts to analyze the self-peptide landscape throughout various tissues have previously been performed on a transcriptional level. However, mRNA expression does not always correlate with protein expression, and this correlation is further complicated when focusing on functional pMHC complexes. We sought to use a T cell hybridoma as a tool to functionally detect selecting self-pMHCII complexes. The inability of co-stimulation to affect a hybridoma response means that this tool will solely reflect the presence of self-pMHC presented by the surveyed APCs. A panel of hybridomas from H-2^k gp250 deficient mice, primed with gp250 peptide in CFA, were tested for activity against the CH27 B cell

line from which the gp250/I-E^k selecting complexes were identified biochemically. Reactive hybridomas, identified by IL-2 production after co-culture with the CH27 APC line, were subcloned by limiting dilution three times to achieve clonality. Hybrid specificity was tested using gp250 peptide pulsed CHO-E^k APCs. Because this I-E^k restricted cell line very poorly presents endogenously processed peptides, we are able to confirm that hybridoma reactivity is specific to our peptide of interest, and not an unknown peptide entity. The chosen gp250/I-E^k specific T cell hybridoma (out of ~100), herein termed Ga2.6, expresses the V α 4 (TRAV4), V β 1 (TRBV1) TCR with CDR3 regions that are 10 and 12aa long, respectively (data not shown).

Despite the low concentration of a given self-pMHC on an APC, this small amount of complex is biologically relevant, providing essential stimuli to both developing thymocytes and mature T cells (Davey et al., 1998; Dorfman et al., 2000; Lo and Allen, 2012). Because levels of most endogenously presented self-pMHC are very low, it was important that the chosen gp250/I-E^k specific T cell hybridoma could sensitively and specifically detect physiological levels of the selecting self-pMHCII complex gp250/I-E^k. The Ga2.6 hybridoma proved extremely sensitive, producing detectable amounts of IL-2 when activated by as few as 1,000 cells from the CH27 B cell line, and 0.05 μ M peptide on pulsed CHO-E^k cells (Fig. 1A, B). Ga2.6 was tested for broad recognition of gp250 altered peptide ligands (APLs) at the TCR contact residues, and was shown to be completely intolerant to alterations at three out of four contact locations, producing no IL-2 when stimulated with respective APL pulsed CHO-E^k cells. Ga2.6 exhibited flexibility in peptide recognition exclusively at the P8 position, thus exhibiting exquisitely specific pMHCII recognition, as was described for the primary ANDtg CD4 T cell selected by gp250/I-E^k (Lo et al., 2009) (Fig. 1C). It is known that T cell hybridomas do not depend on adhesion molecules or co-stimulation to become activated (Harding and Unanue, 1990). To verify that Ga2.6 works independently from these factors, and to survey the presentation of selecting self-pMHCII in the absence of adhesion or co-stimulatory molecules, we stimulated Ga2.6 with a soluble gp250/I-E^k monomer and noted a sensitive response to this stimulus (Fig. 1D). To confirm that recognition by Ga2.6 is I-E^k restricted, we used anti-I-E^k monoclonal antibodies to block Ga2.6 stimulation. The ability of anti I-E^k 14.4.4 antibody to block Ga2.6 activation indicates that recognition by Ga2.6 is MHCII I-E^k restricted (Fig. 1E). Together, these characteristics show that the Ga2.6 T cell hybridoma is an ideal functional probe for the detection of endogenously processed and presented gp250/I-E^k.

3.2 Characterization of a sensitive T cell hybridoma specific for the positive selecting ligand gp250/I-E^k

Detection of endogenous gp250/I-E^k was closely investigated using primary splenic or thymic single cell preparations from either wild type H-2^k restricted or gp250 deficient H-2^k restricted mice. Using Ga2.6, we were able to functionally detect expression of gp250/I-E^k protein complexes in both whole thymus and spleen from low numbers of primary cells. As expected based on its role in the selection of the ANDtg T cell, selecting self-pMHCII complexes were detected within whole thymic preparations from gp250 sufficient mice (Fig 2A). Though peripheral expression of self-pMHC is a well-described phenomenon, the purpose of this peripheral expression is hitherto unknown. Using Ga2.6 as a functional tool,

we sought to survey the peripheral expression of selecting ligand gp250/I-E^k within splenic preparations. Ga2.6 can detect gp250/I-E^k complexes presented by as few as 5,000 primary splenic APCs (Fig 2B). Importantly, single cell preparations from gp250KO/H-2^k mice were unable to promote IL-2 production by Ga2.6. Thymic and splenic preparations from B6 (I-E-negative) mice failed to activate Ga2.6, confirming the I-E^k specific recognition of this hybridoma (Fig. 2). The intermediary response produced upon stimulation with gp250 heterozygote splenic and thymic preparations, compared to B6.K mice, indicates that Ga2.6 can detect two fold differences in gp250/I-E^k expression (Supplemental Fig. 1). Together, these data show that endogenously processed and presented selecting ligand gp250/I-E^k is present in both the thymus and periphery, and can be detected by the T cell hybridoma Ga2.6 in a dose dependent manner.

3.3 Transcript analysis predicts variable expression of the selecting ligand gp250 among APC subsets

Data repositories such as NCBI Geoprofile (Barrett et al., 2013) and ImmGen (Ergun et al., 2013) are excellent resources for gaining an understanding of organism-wide gene expression. Due to the technical limitations in peptide analysis from small numbers of cells, transcriptome analysis is often the best available indicator of potential self-peptide expression. Databases indicate that *LR11* mRNA, from which gp250 is derived, can be detected in various APC populations, including cortical and medullary thymic epithelial cells; notably, *LR11* message is minuscule in dendritic cells from both the thymus and spleen (GEO: GDS4303 (McGuire et al., 1992), GEO: GDS1655 (Derbinski et al., 2005), GEO: GDS297). In our hands, quantitative PCR analysis of *LR11* expression in sorted thymic and splenic APC populations (Supplemental Fig. 2) confirms the expression profile described in available databases. Specifically, positive selecting cTECs contain abundant levels of *LR11* message; negatively selecting thymic DCs express very low amounts while expression in mTECs is intermediary (Fig. 3A). *LR11* mRNA was also detected in sorted peripheral APCs. While high expression of *LR11* was detected in B cells, much lower levels were observed in splenic DCs (Fig. 3B). *LR11* message was undetected in gp250 deficient spleen and thymus.

3.4 The positive selecting gp250/I-E^k complex is constitutively and efficiently presented by thymic APCs

Using the Ga2.6 hybridoma, we surveyed FACS sorted thymic APCs for functional expression of the positive selecting self-pMHCII, gp250/I-E^k protein complexes. We detected gp250/I-E^k on all three thymic APC populations, cTECs, mTECs and DCs, providing a picture of the naturally occurring selecting self-pMHCII expression landscape (Fig. 4A). To our knowledge, this is the first demonstration of a validated and endogenous positive selecting ligand being expressed on both the positive selecting cTECs as well as tolerance inducing mTECs and thymic DCs. This close investigation of selecting ligand presentation within the thymus showed that the hierarchy of *LR11* transcript abundance among the cells responsible for intrathymic selection (Fig. 3) is not reflected in gp250/I-E^k protein complex expression, as detected by Ga2.6. Despite *LR11* transcript expression being most abundant in cTECs, significantly diminished in mTECs, and nearly undetectable in thymic CD11c+ DCs, the Ga2.6 hybridoma detected the most efficient presentation of

selecting ligand gp250/I-E^k on the thymic APCs with the lowest *LR11* gene expression. Specifically, CD11c+ DCs exhibited more potent and sensitive stimulation of Ga2.6 than did mTECs. Meanwhile, the expression of gp250/I-E^k complexes on positive selecting cTECs was present, but low, as detected by the Ga2.6 hybridoma. To elucidate if differences in Ga2.6 stimulation by thymic APCs were due to inherent differences in peptide processing or simply a result of differing pMHCII expression, exogenous gp250 peptide was added to each cell type, such that differences in processing would be overridden by direct loading of gp250 peptide onto MHCII I-E^k molecules. Ga2.6 responds similarly to peptide pulsed primary cTECs, mTEC and DCs, with the DCs tending to be insignificantly yet reproducibly elevated in their self-pMHCII expression (Fig. 4B). This equivalent presentation of exogenous peptide indicates that differences in gp250/I-E^k complex expression likely arise during the endogenous processing of this naturally occurring ligand, and are not due to differences in pMHCII abundance. The hybridoma's co-stimulation independent functionality further ensures that the differences we see are not due to the variable co-stimulatory capacity of the APC subsets. Of note, all three main thymic APC subsets express similar levels of MHCII (data not shown). These results suggest that the unique endogenous processing machinery of each thymic APC subset dictates the efficiency with which positive selecting ligand gp250/I-E^k is expressed. Overall, Ga2.6 was able to functionally detect selecting self-pMHCII gp250/I-E^k complexes on all thymic APCs involved in intrathymic selection. Notably, RNA expression did not correlate with the functional expression of pMHCII complexes as evidenced by Ga2.6.

3.5 Constitutive presentation of the positive selecting ligand gp250/I-E^k in the periphery is affected by the activation state of the splenic APCs

Self-peptides, including positive selecting ligands, are known to be expressed in the peripheral lymphoid tissues and play essential roles in the survival and activation of peripheral T cells. The precise roles of positive selecting self-pMHCII in the periphery have yet to be unequivocally determined. Understanding the peripheral APC network that expresses these selecting ligands will be critical in elucidating their peripheral function. Immune responses occur in the periphery and lead to explicit changes in the APC network toward the elimination of pathogens. Of interest is how the landscape of selecting self-pMHCII expression changes in response to unrelated antigenic stimulation. Having already established the detection of gp250/I-E^k within whole splenic preparations (Fig. 2), we used the Ga2.6 hybridoma to assess the peripheral expression of positive selecting ligand gp250/I-E^k on specific APC subsets. B cell and DC populations were enriched from whole spleen by magnetic bead enrichment and FACS (Supplemental Fig. 2). gp250/I-E^k selecting complexes were detected on both B cell and DC enriched and sorted populations (Fig 5). Similar to the thymic APC populations, the hierarchy of gp250/I-E^k protein complex expression did not reflect the *LR11* gene expression hierarchy (Fig. 3). CD11c+ DCs more efficiently present the selecting ligand, despite CD11c+ cells having dramatically less expression of the *LR11* gene. This confirms the benefit of a system that functionally detects naturally occurring selecting self-pMHCII protein complexes. Because the proposed roles of self-pMHC in the periphery include augmenting an immune response to foreign antigen, we sought to investigate any changes in the expression of selecting ligand gp250/I-E^k that occur as the activation state of the APCs change. A close look at the expression of the selecting

ligand on peripheral B cells shows that *ex vivo* activation leads to decreased presentation of gp250/I-E^k. Activating B cells using a regimen that mimics immune complex activation, anti-CD40/anti-IgM, reduces the efficiency of self-pMHCII expression. This efficiency is even more dramatically reduced upon activation through TLR 4 using LPS (Fig 6A). We next sought to investigate any changes in the self-pMHCII landscape during the course of an *in vivo* infection. Three days post infection with *Listeria monocytogenes* B cells (Fig. 6A) and DCs (Fig. 6B) were enriched from the spleen and surveyed for gp250/I-E^k expression. In both B cell and DC compartments, activation of Ga2.6 was reduced during bacterial infection. Despite dramatic reductions in the maximal IL-2 production generated in response to stimulation with *Listeria* infected APCs, the half-maximal cell dose was similar to that of steady-state APCs. Given the well-established role for dendritic cells during the course of a *Listeria* infection, it was not surprising to see changes in the landscape of presented peptides within this APC subset. However, the reduced presentation of gp250/I-E^k by B cells was curious, as these cells are not known to play a role in the direct presentation of *Listeria* antigen on MHCII. To investigate the extent of *Listeria* pMHCII expression by B cell and DC subsets, *Listeria* specific TCR transgenic CD4+ T cells (LLO56) were co-cultured with APCs enriched as above. CD69 upregulation was assessed 15–20 hours later as a measure of T cell activation (Fig. 6C). As expected, DC enriched populations were efficiently presenting *Listeria* antigens as indicated by CD69 upregulation. Meanwhile, B cell enriched populations appeared to be presenting suboptimal levels of *Listeria* pMHCII. These results suggest that the landscape of selecting self-pMHC is a dynamic entity, experiencing antigen nonspecific changes in expression patterns throughout the course of immune responses.

4. Discussion

There are several technical hurdles involved in evaluating the endogenous self-peptide landscape. To date, the most common insight into self-ligand expression is gained through transcriptional analysis. The discrepancy viewed herein between endogenous mRNA and self-pMHCII complex expression is just one clear example of the caution required when evaluating the functional self-pMHC repertoire. The mechanisms behind this modulation may have a direct impact on the overall peptide landscape. Only recently has the presence and impact of post-translational modifications (PTMs) on presented pMHC complexes become clear (Engelhard et al., 2006). Some of the PTMs identified are regulated by immune response factors, such as inducible nitric oxide synthase (iNOS) (Birnboim et al., 2003) and have direct impacts on the immunogenicity of presented peptide epitopes. As such, the link between self-pMHC modifications and autoimmunity has been previously described (Herzog et al., 2005), suggesting that PTMs may explain the differences between the expression of mRNA and self-pMHCII complexes observed here. It is also possible that the variation between APC subsets can be attributed to their unique processing machinery. Increasingly clear is that PTMs and MHC processing are not mutually exclusive, and that PTMs can directly and indirectly influence the pMHC landscape.

We were able to functionally detect differences in the peripheral presentation of a single natural positive selecting ligand in response to a bacterial infection. Given the intricate regulation of the pMHC-ligandome, particularly as a result of an immune response, this is not entirely surprising. This suggests that peripheral self-peptide landscape is dynamic, and

has implications for the maintenance of the mature T cell pool. Assuming the widely held paradigm that self-pMHC, in particular selecting ligands, are essential for the peripheral maintenance of T cells holds true, dramatic reductions in the expression of a given self-pMHC might be expected to result in the loss of T cells dependent on that homeostatic signal. In the case of immune response, there exists the potential to lose T cell pools completely unrelated to the ongoing reactions. In practice, this would likely not be a completely detrimental consequence. Studies with single peptide and selecting peptide deficient mice suggest that a single peptide or peptide family can support multiple T cell clones, and that non-dominant self-pMHC exist that can select a single T cell clone in the absence of the dominant selecting ligand (Ignatowicz et al., 1996). This would imply that peripheral T cells ought to receive homeostatic signals from more than one specific self-pMHC. Further work is needed to determine how the self-pMHC repertoire contributes to the maintenance of the mature T cell pool and fits into the models of immune response driven T cell repertoire fluctuations (Singh et al., 2012).

To our knowledge, this is the first description of the presence of a known MHCII positive selecting ligand that is naturally occurring on both positive and negative selecting thymic APCs. These data support the affinity model for how a CD4⁺ T cell can be positively selected on a "public" ligand, but not be negatively selected by the same ligand (Klein et al., 2014). The non-mutually exclusive possibility that some CD4⁺ T cells are selected by "private" class II bound peptides, uniquely generated by Cathepsin L or TSSP mechanisms, is not excluded by these data. How our findings relate to the positive selection of CD8⁺ T cells was not established in this study. The thymoproteasome, containing the $\beta 5t$ subunit, has been shown to generate "private" peptides in cTECs in addition to the "public" peptides concurrently generated. The currently growing list of proteases being associated with antigen processing suggests we are only beginning to understand the intricate mechanisms and regulation of pMHC generation (Burster et al., 2010). Given the inefficiency of positive selection it is likely that both "public" and "private" peptides are involved in the positive selection of a broad T cell repertoire. It remains to be determined if there are functional differences between such T cells selected on "private" or "public" peptides. "Public" peptides may have a specific role in the peripheral maintenance of T cells, which requires re-recognition of thymic self-pMHC in the periphery. What is clear from this study is that the naturally occurring self-pMHC landscape is intricately modulated. Complete understanding of the self-landscape will be critical in elucidating the exact mechanisms of thymic development and how this process and the ligands involved impact the T cell for life.

5. Conclusion

We have generated a sensitive T cell hybridoma that is specific for the bona fide, naturally occurring gp250/I-E^k positive selecting ligand. This hybridoma is I-E^k MHCII restricted and specific for the gp250 epitope identified as the selecting ligand for the ANDtg CD4 T cell. Using this tool, we were able to assess the expression of gp250/I-E^k protein complexes in thymus and periphery. The efficiency of selecting self-pMHCII expression did not correlate with the levels of *LR11* gene expression in either location. Despite nearly absent gene expression in the CD11c compartments of the thymus and spleen, Ga2.6 detected the most efficient presentation of gp250/I-E^k within these populations. To our knowledge, the

detection of this naturally occurring selecting ligand on cTECs as well as mTECs and thymic DCs is the first unequivocal proof that the same self-ligand is present on both positive selecting and tolerance inducing thymic APCs. In the periphery, the extent of gp250/I-E^k presentation was dependent on the activation state of the CD19⁺ B cells and CD11c⁺ DCs, with B cells predominating in the naive setting and the DCs playing a bigger role in self-pMHCII presentation while in the mature state. These findings suggest that despite the unique processing capabilities of the thymic APCs, they do present overlapping functional self-pMHCII complexes. In regards to the peripheral presentation of these complexes, the landscape is dynamically regulated, and depends on the recent immune status. In either location, evaluation of the self-ligandome should include functional detection self-pMHC complexes in addition to transcriptional analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

APL	altered peptide ligand
cTEC	cortical thymic epithelial cell
mTEC	medullary thymic epithelial cell
PTM	post translational modification

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Highlights

- A T cell hybridoma detecting endogenous positive selecting self-pMHCII was generated
- Positive and negative selecting thymic APCs can present the same self-pMHCII complex
- Peripheral expression of self-pMHCII is affected by the APC activation state
- Thymic and peripheral expression of self-pMHCII complex did not reflect mRNA levels

Figure 1. Ga2.6 hybridoma response is specific for gp250/I-E^k

Enzyme-linked immunosorbent assay (ELISA) of IL-2 produced by Ga2.6 detecting gp250/I-E^k in overnight culture with the indicated number of (A) CH27 B cell lymphoma line (B) exogenous gp250 peptide presented by 10⁵ CHO-E^k cells or (C) gp250 altered peptide ligands with proline mutations at TCR contacts presented by CHO-E^k. (D) IL-2 production by Ga2.6 stimulated overnight with plate-bound gp250/I-E^k monomer. (E) 5×10⁵ whole splenocyte or thymic cells cultured with (black bars) or without (white bars) anti-MHCII clone 14.4.4; Ga2.6 activation was measured by IL-2 response. N.D. = not detected. Data are representative of at least 3 experiments and depict mean ± SEM.

Figure 2. Ga2.6 sensitively detects endogenous gp250/I-E^k complexes

Detection of gp250/I-E^k complexes from titrated doses of single cell (A) thymic or (B) splenic preparations by IL-2 ELISA of Ga2.6 culture supernatant. Mice expressed gp250/I-E^k (B6.K-black circles), I-E^k without gp250 (gp250 KO-triangles) or gp250/I-A^b (B6-crosses). Data are representative of at least 3 experiments and depict mean $\pm$ SEM.

Figure 3. Variable LR11 gene (encoding gp250) expression in thymic and splenic APCs
 Quantitative RT-PCR analysis of *LR11* mRNA expression in various FACS sorted (A) thymic and (B) splenic APC populations; results are presented as C_T fold change relative to *Actb* (encoding β -actin) with CH27 as the baseline sample. tDC = thymic CD11c+ DC, sDC = splenic CD11c+ DC. Data are representative of two-three experiments and depict mean $\pm$ SEM.

Figure 4. Ga2.6 detects endogenous gp250/I-E^k complexes on positive and negative selecting thymic APCs

IL-2 production by Ga2.6 after overnight culture with FACS sorted thymic APCs (equivalent numbers of DC, mTEC, or cTEC) in the (A) absence or (B) presence of exogenous gp250 peptide. gp250/I-E^k detection is measured via [³H]thymidine incorporation during the IL-2 dependent proliferation of CTLL cells. To accommodate for experimental variation in maximal cTEC recovery and IL-2 production, equal numbers of all thymic APCs, ranging from 4,000–15,000, were utilized within each assay. Data were normalized to the maximal [³H]thymidine incorporation in each experiment. Data are a compilation of five experiments and depict mean ± SEM. One-way Anova, **P<0.01, ***P<0.0001.

Figure 5. Ga2.6 detects gp250/I-E^k complexes on peripheral splenic APCs

(A) IL-2 production by Ga2.6 measured after overnight culture with the indicated number of FACS sorted splenic B cells and DCs. (B) EC_{50} indicates the number of APCs required to produce a half-maximal IL-2 response. Data are representative of three experiments and depict mean $\pm$ SEM. Mann-Whitney test, ** $p < 0.01$.

Figure 6. Expression of gp250/I-E^k is affected by the recent immune status

IL-2 production by Ga2.6 in response to the indicated number of (A) B cells activated *ex vivo* using anti-CD40 and anti-IgM or LPS for 48 hours prior to co-culture with Ga2.6. Alternatively, *in vivo* activated B cells and (B) DCs were harvested from the spleens of B6.K mice infected with *Listeria monocytogenes* three days prior to co-culture with Ga2.6. (C) Presentation of Listeria-pMHCII as measured by CD69 upregulation (mean fluorescence intensity-MFI) on LLO specific CD4 TCRtg T cells stimulated with B cells or DCs from Listeria mice. Data are representative of at least three experiments and depict mean $\pm$ SEM.