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An endogenous peptide positively selects and augments peripheral CD4+ T cell activation and survival

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Abstract

Although $CD4^+$ and $CD8^+$ T cells differ in their positively selecting signal strength, endogenous positively selecting ligands have only been identified for MHC class I-restricted T cell receptors (TCRs). Here we screened for ligands that can positively select MHC class II-restricted TCRs, using thymocytes from four I-E^k restricted TCR transgenic mice and a large panel of self peptides. One peptide, gp250, induced positive selection of AND $CD4^+$ T cells, had no homology with the AND TCR agonist ligand, and was recognized with a high degree of specificity. gp250 acted as a co-agonist to initiate activation and enhance survival of peripheral AND $CD4^+$ T cells. Thus, positively selecting ligands play critical roles in thymocyte development and in the activation and maintenance of peripheral T cells.

Introduction

Self peptides are required during positive and negative selection in the thymus. Weak interactions between T cell receptors (TCRs) on double positive (DP) thymocytes and self peptide-MHC (pMHC) complexes on cortical thymic epithelial cells (cTECs) induce positive selection and prevent death by neglect1-4. Positive selection ensures that TCRs are restricted by the self MHC alleles present in the thymus. MHC class I and MHC class II-restricted T cells share many common features but do have some marked differences. The processes of positive selection and commitment of DP thymocytes to CD4 single positive (CD4SP) and CD8 single positive (CD8SP) lineages are intricately linked. Stronger signals lead to the development of CD4SP cells, whereas weaker signals lead to CD8SP cells3, 4. CD4 and CD8 co-receptors are obvious candidates to influence the positive selecting signal

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Author Contributions: W.-L.L., N.J.F., and P.M.A. designed the study and wrote the manuscript; J.J.W., H.R., and M.L.G. identified by LC tandem mass spectrometry all of the self peptides and analyzed the synthetic peptides; W.-L.L. and N.J.F. did all of the *in vitro* and *in vivo* experimental work.

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strength, as these co-receptors differ in their subunit composition, binding affinity for MHC molecules, and signaling capacity. Alternatively and/or in combination, the interactions between TCRs and positively selecting self pMHCI versus self pMHCII complexes may differ in kinetics and/or affinity. However, the current lack of any naturally occurring self peptides identified to positively select MHC class II-restricted T cells precludes a direct comparison.

The nature of TCR ligand(s) involved in positive selection has long been an area of intense interest, with the initial contribution of self peptides being suggested by several compelling studies5, 6. The self peptide(s) that positively select an individual TCR is rare in the universe of self peptides, implying a certain degree of specificity of positively selecting ligands7-12. The naturally occurring positively selecting self peptides that have been identified to date are all MHCI-restricted. Although a single pMHCII molecule can positively select a large and broadly-reactive TCR repertoire13-18, almost all of these T cells were negatively selected when exposed to antigen-presenting cells (APCs) expressing a normal self peptide repertoire. These reductionist studies showed that one pMHCII species was capable of positively selecting a significant number of T cells, but did not identify an endogenous peptide capable of positively selecting a MHCII-restricted TCR.

Self peptides also participate in mature T cell activation19, and can co-localize with agonist peptides at the immunological synapse20. Co-localization of self peptides with agonist peptides has functional relevance, as some self peptides augment the initiation of T cell activation induced by a low amount of agonist peptide. The pseudodimer model supports a mechanism whereby the monomeric CD4 co-receptor allows certain self pMHC complexes (termed co-agonists) to contribute to T cell activation. For example, several self peptides act as co-agonists and enhance activation of MCC–I-E^k specific 5C.C7 T cells21, 22. However, a relationship between positively selecting peptides and co-agonists has not been established.

Thymic positively selecting self peptides have also been hypothesized to influence peripheral T cell survival. The possibility that presentation of self peptides by peripheral APCs generates sufficient tonic TCR signaling to maintain peripheral T cell survival and render T cells sensitive to foreign antigen is an appealing hypothesis23 with little experimental support. Some evidence indicates that peptides mediating positive selection can also facilitate homeostatic proliferation of naive T cells24-28. For example, naive $CD4^+$ cells from B6 mice failed to proliferate in T cell-depleted H2-DM-deficient hosts, whereas naive $CD4^+$ cells from H2-DM-deficient mice proliferated strongly in H2-DM-deficient hosts24, 25. Similarly, OVA-specific $CD8^+$ T cells failed to proliferate when transferred into transporter associated with antigen processing (TAP)-deficient mice, but did proliferate when transferred into mice expressing a transgene encoding a positively selecting altered peptide ligand (APL) of OVA26. Further support of the hypothesis that positively selecting self peptides can influence the proliferation and survival of naive T cells awaits identification of a positively selecting self peptide for MHC class II-restricted T cells.

To gain insight into the nature of self peptide(s) capable of positively selecting MHC class II-restricted TCRs, the potential differences in signal strength facilitating positive selection

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of $CD4^+$ and $CD8^+$ T cells, the relationship between positive selecting self peptides and co-agonists, and the role of positively selecting self peptides in peripheral T cell survival, we sought to identify a naturally occurring self peptide capable of positively selecting a MHC class II-restricted TCR. We tested a panel of 95 self peptides for the ability to positively select four different TCRs (AND, N3.L2, 2.102 and A1). We identified one self peptide from the protein gp250 (hereafter the peptide is referred to as gp250), which supported positive selection of AND T cells. No peptides were capable of positively selecting the other tested TCRs. The gp250 peptide has no homology with the AND TCR agonist peptide MCC, but single amino acid substitutions of gp250 disrupted its ability to mediate positive selection; these findings indicate TCR specificity for the positively selecting self peptide. Importantly, the positively selecting self peptide acted as a co-agonist for peripheral AND T cells and enhanced their survival, thereby establishing a link between positive selection and peripheral T cell activation and survival.

Results

A peptide that positively selects the AND TCR

To establish a system to screen for positively selecting self peptides, we tested the ability of a large panel of I-E^k-binding self peptides to induce CD69 upregulation on DP thymocytes. Four I-E^k-restricted TCR transgenic (TCRtg) mouse lines were bred onto non-selecting MHC and Rag-1-deficient backgrounds (AND.*Rag1*$^{-/-}$H-2^d (MCC–I-E^k specific); N3L2.*Rag1*$^{-/-}$H-2^b (Hb–I-E^k specific); 2.102.*Rag1*$^{-/-}$H-2^b (Hb–I-E^k specific); A1.*Rag1*$^{-/-}$H-2^b (HY–I-E^k specific)). The thymocytes from each of the TCRtg mice were primarily DP with no SP cells, indicating the absence of positive selection (Supplementary Fig. 1). A panel of 95 unique endogenous self peptides from the CH27 B cell line was previously identified, characterized, and confirmed to bind to I-E^k molecules29. This panel represented approximately 5% of the different peptide species presented by I-E^k molecules on the APC surface30. Each of the peptides was individually loaded onto I-E^k-Ig dimers and tested for the ability to induce CD69 upregulation on DP thymocytes from each of the TCRtg mice. One peptide, gp250, strongly stimulated CD69 upregulation on AND thymocytes (Fig. 1). None of the peptides stimulated CD69 expression for the N3L2, 2.102, or A1 TCRs, while the corresponding agonist peptide for each TCR strongly stimulated CD69 expression (Fig. 1 and Supplementary Fig. 2 online). Thus, gp250 was a candidate positively selecting self peptide for AND TCRtg cells.

Having established that gp250 upregulated CD69 on AND thymocytes, we wanted to determine directly its ability to positively select CD4SP T cells in thymocyte reaggregate cultures. The cTEC cell line, ANV41.2 transfected with I-E^k is incapable of promoting positive selection because of inefficient MHC class II processing and presentation13. However, the addition of low concentrations of agonist peptide or APLs to ANV41.2 cells can facilitate positive selection of AND TCRtg T cells13. Similarly, treatment of the ANV41.2 cells with interferon (IFN)-γ restores MHC class II processing and presentation and facilitates positive selection in the absence of exogenous peptides (13 and unpublished observations). We incubated DP thymocytes from newborn AND.*Rag1*$^{-/-}$H-2^d mice with ANV41.2 I-E^k cells (not IFN-γ treated) and gp250 peptide for four days and analyzed the

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cultures for the presence of CD4SP T cells (Fig. 2). The addition of gp250 induced a significant population of CD4SP thymocytes, whereas untreated ANV41.2 I-E^{k} cells (data not shown) or ANV41.2 I-E^{k} cells pulsed with control peptide did not (Fig. 2a). In five experiments, gp250 consistently induced a CD4SP population of AND TCRtg cells, ranging from 2.8% to 7.3%. However, ten pools each containing ten of the 95 I-E^{k} self peptides in reaggregate culture all failed to induce CD4SP cell differentiation in thymocytes from the three other TCRtg lines (data not shown).

In addition to commitment into the CD4SP lineage, the CD4SP cells in gp250-treated cultures also expressed markers of positive selection including upregulation of CD5, CD69 and TCR (Fig. 3a,b). Unlike DP thymocytes but like mature CD4SP T cells, the gp250-selected CD4SP T cells produced cytokines, including IL-2 and IFN-γ, in response to stimulation with phorbol 12-myristate 13-acetate (PMA) and ionomycin stimulation (Fig. 3c,d). This finding is consistent with published results31 indicating that only committed CD4SP acquire the ability to produce IL-2 and IFN-γ, and suggests that gp250-selected CD4SP were both phenotypically and functionally mature.

The gp250 peptide is derived from a sortilin-related multifunctional endocytic receptor protein that is constitutively expressed in dendritic cells (DCs), macrophages, B cells, and cortical and medullary thymic epithelial cells32. As a membrane protein in the endocytic pathway, gp250 is readily accessible to the MHC class II processing pathway, consistent with our identification of the gp250 peptide bound to I-E^{k} on a B cell line. The anatomy of I-E^{k} restricted T cell epitopes has been extensively studied structurally and functionally33-36. There are four TCR contact residues (P2, P3, P5 and P8), and four I-E^{k}-binding residues (P1 and P9 the main anchors, and P4 and P6 the secondary anchors). gp250 differed with MCC at all of the predicted TCR contact residues (Fig. 4a). The P1 and P9 MHC anchor residues were identical in gp250 and MCC, but the two peptides differed at the P4 and P6 MHC anchor residues. Nevertheless, the reaggregate cultures established that gp250 was a *bona fide* positively selecting self peptide for AND TCRtg cells.

Positive selection by gp250 has specificity

From our screen of 95 self peptides and four I-E^{k} restricted TCRs, gp250 was the only self peptide capable of positively selecting CD4SP thymocytes, implying that the process of positive selection involves a high degree of peptide specificity. To examine the degree of peptide specificity of gp250-mediated positive selection in more detail, we tested the sensitivity of positive selection to single conservative amino acid substitutions at each of the four predicted TCR contact residues of gp250. We altered P2 (I to A), P3 (A to I), P5 (G to A) and P8 (G to I) (Fig. 4b). Notably, none of the mutant peptides promoted positive selection (Fig. 4c), even at concentrations up to 50 μM. We also tested the involvement of the secondary MHC anchor residues in the specificity of positive selection by introducing the MCC peptide MHC anchor residues (at P4 and P6) into gp250. Subtle changes at the P6 anchor residue can have significant effects on the recognition of peptides by T cells34. Indeed, the gp250-MCC chimeric peptide (gp250-MCC) did not positively select AND T cells, revealing an important role of the MHC anchor residues in generating a positively

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selecting self peptide (Fig. 4d). These findings reveal a high degree of specificity of positive selection of the AND TCR by gp250.

gp250 acts as a co-agonist for peripheral T cells

Most peripheral T cells frequently encounter self pMHC ligands, but never encounter cognate foreign antigen(s). An important but unanswered question is whether positively selecting self peptides have a role in the maintenance and/or activation of peripheral T cells. To confirm that the gp250 peptide does not directly activate peripheral AND TCRtg T cells, we cultured naive AND TCRtg T cell (Fig. 5a) or primary AND TCRtg T cell blasts (Fig. 5b) with gp250 and CH27 cells as APCs. Even high concentrations of gp250 did not stimulate the proliferation of AND T cells.

To determine if gp250 could function as a co-agonist for AND TCRtg T cells, we utilized I-E^k-Ig dimers loaded with MCC and either gp250 or a control peptide (Hb). Importantly, the three peptides bound with identical affinities to I-E^k molecules (Supplementary Fig. 3). I-E^k-Ig dimers were loaded with varying ratios of MCC and gp250 or MCC and Hb (1:10 to 1:1,000), with the goal of identifying a MCC:Hb ratio that caused minimal stimulation, permitting the assessment of the co-agonist activity of gp250 (Supplementary Fig. 4). Hb did not upregulate CD69 and did not positively select AND TCRtg T cells in thymic reaggregate culture (Fig. 2,3). Ratios of 1:80 and 1:100 of the control MCC:Hb heterodimers resulted in minimal stimulation of AND T cells (Fig. 6a). In marked contrast, MCC:gp250 heterodimers at these ratios strongly stimulated AND TCRtg T cell proliferation, suggesting that gp250 exerts co-agonist activity (Fig. 6a). The 1:20 ratio of MCC:peptide was stimulatory for both gp250 and Hb because of the existence of substantial amounts of MCC:MCC homodimers in these preparations. Conversely, the 1:1,000 ratio was non-stimulatory for both gp250 and Hb, as these preparations were comprised mostly of gp250:gp250 homodimers, and contained very few MCC:MCC homodimers or MCC:gp250 heterodimers.

The initial report of co-agonists demonstrated that several self peptides could act as co-agonists for 5C.C7 T cells, implying a modest degree of peptide specificity. To examine the specificity of the gp250 co-agonist activity, we tested the single amino acid substituted peptides of gp250 for their co-agoinst activity. Two of the substituted gp250 peptides (P2A and P3I) were able to act as co-agonists, whereas two were not (P5A and P8I) (Fig. 6b). In contrast, none of the mutant peptides induced positive selection. Thus, a positively selecting self peptide can act as a co-agoinst for a peripheral T cell, and co-agonism may require less peptide specificity than positive selection.

gp250 enhances survival of peripheral T cells

Newly generated T cells egress from the thymus and seed in the periphery to form the large pool of long-lived circulating naive T cells. Self pMHC complexes in general are critical for the survival and homeostatic proliferation of T cells transferred into lymphopenic hosts37, 38, but the nature and specificity of the self pMHC ligands mediating this effect has not been established. We tested the effect of gp250 on the homeostatic proliferation of AND TCRtg cells *in vivo* (Fig. 7). We injected B6.K.*Rag1*$^{-/-}$ mice with 0.5 mg of gp250 or Hb on days -1 and +1, and CFSE-labeled AND TCRtg T cells on day 0. In both the gp250 and Hb-

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treated mice, we observed proliferation of the AND TCRtg T cells (Fig. 7a); however, we recovered 2-9-fold more AND TCRtg $CD4^+$ T cells in the gp250-injected mice (Fig. 7b). We also observed similar findings using a lower peptide dose (0.1 mg) of gp250 (Supplementary Fig. 4). These findings show that the administration of additional gp250 enhanced the survival of AND TCRtg T cells undergoing homeostatic proliferation. None of the gp250 TCR contact residue mutants enhanced the survival of AND TCRtg $CD4^+$ T cells (Fig. 7c), demonstrating a degree of specificity identical to what we observed for positive selection (Fig. 4d). Thus, the positively selecting self peptide, gp250, also significantly influences peripheral T cells by enhancing their survival.

Discussion

Here we showed that the recognition of positively selecting self peptides has an unexpectedly high degree of specificity, and that in the periphery these self peptides can act as co-agonists and also enhance the survival of T cells. The study of self peptides in positive selection of $CD4^+$ T cells has lagged behind that of $CD8^+$ T cells because of a lack of suitable genetic models that limit the display of endogenous self peptides. Our approach was to utilize a library of 95 I-E^k self peptides to examine the positive selection of four different I-E^k restricted transgenic TCRs that are specific for three different antigens and were bred onto a Rag-1-deficient non-selecting genetic background. In the initial screening, we observed a surprising degree of specificity; only one peptide (gp250) positively selected one TCR (AND). The 379 other combinations of self peptides and TCRs did not result in positive selection. Additionally, positive selection was highly specific and depended on each TCR contact residue, as single TCR contact residue substitutions of the gp250 peptide abolished the capacity of this peptide to positively select AND TCRtg T cells. This specificity also extended to the MHC anchor residues, which influence the overall conformation of the peptide in the MHC binding groove. Taken together, these results reveal a similar degree of peptide specificity during positive selection as is observed during peripheral T cell recognition of foreign peptides.

Previous studies of the positive selection of $CD8^+$ T cells also support the concept of peptide specificity in positive selection. The seminal study by Hogquist *et al*. revealed that APLs of the OVA peptide (SIINFEKL) had a differential ability to positively select OT-I T cells, and that only APLs with antagonist, but not agonist activity could promote positive selection7-9. Subsequent work identified only two naturally occurring H-2K^b-bound self peptides (CPα1 and β-catenin) that could promote the positive selection of OT-1 CD8SP cells 10. These and our studies utilized *in vitro* fetal thymic organ cultures or reaggregate cultures, which produce limited numbers of positively selected SP T cells and require relatively high concentrations of peptides. It is possible that these *in vitro* culture models complicate our calculation of the number of peptides that are able to positively select MHC class II-restricted TCRs, but *in vivo* experiments manipulating the peptide repertoire without reducing it to a single species are not available. A recent study reported that the knockout of an immunoproteosome subunit specifically expressed in cortical thymic epithelial cells reduced the positive selection of some but not all tested MHC class I-restricted TCRtg39. These findings may indicate that the peptide(s) involved in the positive selection of these

individual cells are only generated in the presence of this immunoproteosome subunit, implying a substantial degree of specificity.

Do CD4$^+$ and CD8$^+$ T cells interact with endogenous peptides in an identical manner, or in a similar but distinct way? Our identification of an endogenous MHC class II positively selecting self peptide will now permit the direct comparison of the positive selection of CD4$^+$ and CD8$^+$ T cells, as well as the testing of the hypothesis that positive selection of CD4$^+$ T cells requires a stronger signal through the TCR. The relative inefficiency in generating large numbers of cells in the *in vitro* positive selection assays makes biochemical studies difficult, but GFP-based reporter assays or single cell FACS assays, will greatly facilitate these important studies. For CD8$^+$ T cells, a pseudodimer model does not have to be evoked, given the dimeric nature of CD8 molecules; however, self peptides have also been shown to function as co-agonists in MHC class I-restricted responses40. Thus, it remains to be determined how endogenous peptides augment CD4$^+$ and CD8$^+$ peripheral T cell activation, and if there is a difference in the degree of peptide specificity of co-agonists for CD4$^+$ and CD8$^+$ T cells. According to the pseudodimer model, CD4 monomers, unlike CD8 heterodimers, are able to crosslink two TCRs to form a pseudodimer with one TCR bound to an agonist peptide and the other one bound to a self peptide41. The recognition requirements for co-agonists have not been established, but it appears there is flexibility given that three of seven tested peptides were active co-agonists for 5C.C7 T cells22, and our findings that two separate TCR contact residue substitutes of gp250 still retained co-agonist activity, whereas these same mutants were unable to mediate positive selection.

There is mounting evidence that interactions with self pMHC ligands are important for the long-term maintenance of T cells37, 38. Here we studied homeostatic proliferation as a surrogate assay for the effect of gp250 on the maintenance of peripheral T cells. We recovered many more AND cells from gp250-injected than from control peptide-injected Rag-1-deficient mice, indicating that the presence of the positively selecting self peptide enhanced the survival of peripheral T cells. The results of the single substituted gp250 peptides indicated that the peptide-specificity involved in homeostatic proliferation was similar to positive selection; the TCR single contact residue substitutions that functioned as co-agonists but not as positively selecting ligands did not enhance the homeostatic proliferation of peripheral CD4$^+$ T cells. Overall, our findings reveal that the interaction of a T cell with a positive selecting self peptide MHC ligand is not only a developmental process in the thymus, but is a life-long interaction, critical for its activation and survival.

Our demonstration of a high degree of peptide specificity raises the key question of how many different T cells can be positively selected by one pMHC species. Obviously there is not a monogamous relationship between a pMHC and a single TCR. It was estimated that an APC displays approximately 2,000 different peptide species on class II molecules30, and that the mouse contains a total number of 3×10^7 peripheral CD4$^+$ T cells. Thus a single peptide species must select several thousand different T cells. Even if our estimate of the number of different peptide species on an APC is low by 10-fold or even 100-fold due to the existence of many very low abundance peptides, each peptide would still have to select more than one T cell. The finding that mice expressing a single pMHC complex are able to positively select substantial populations of T cells supports this conclusion14-18. There

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could also be a spectrum of peptide ability to positively select, with some being dominant selectors; however, this would not obviate the need for a single pMHC to select more than one T cell. The concept that emerges from our studies is that there is a dynamic network of selecting interactions in the thymus involving the self pMHC ligands displayed on the surface of cortical thymic epithelial cells. A single DP thymocyte must scan the surface of a cortical thymic epithelial cell to find an appropriate self pMHC complex that provides the strength of signal required for positive selection. However, only a few different self pMHC complexes would be able to select each T cell, and most DP thymocytes would fail to be selected and die from neglect, as is known to occur.

In this issue of *Nature Immunology*, Ebert *et al*.42 also reported a high degree of peptide specificity in positive selection. Using the same set of I-E^k self peptides, they identified only one peptide, gag-pol, capable of positively selecting the 5C.C7 MCC–I-E^k specific TCR. The remarkable finding from these two studies is that two different mutually exclusive peptides were able to positively select two MCC–I-E^k specific TCRs. However, the gag-pol peptide did not positively select AND TCRtg T cells (Fig. 1), and study, the gp250 peptide did not select 5C.C7 TCRtg T cells42. The gp250 and gag-pol peptides do not have any similarities in their TCR contact residues with each other or with MCC. However, the AND and 5C.C7 TCRs are highly similar; both utilize $V_{\alpha}11$ and $V_{\beta}3$ segments, and they only differ by four amino acids in the CDR3α chain43. Thus, these two very similar TCRs, which recognize the same antigen, are positively selected by different self peptides. Together, these findings suggest that there is not a common positively selecting self peptide for a set of closely related TCRs recognizing the same antigen, and imply that there is a unique relationship between a given TCR and a self pMHC.

Methods

Cells and mice

The CH27 (H-2^a, I-E^k) B cell line29 was cultured in RPMI 1640 medium containing 10% (vol/vol) FCS (Hyclone). The cortical epithelial cell line, ANV41.213, transfected with I-E^k, was kindly provided by A.G. Farr and was cultured in I-10 plus medium (Iscove's DMEM medium containing 10% FCS (Hyclone), 5×10^{-5} M 2-mercaptoethanol (2-ME)) and 0.5 mg/ml of G418. The MCC–I-E^k specific AND TCRtg44, 45, B6.K (H-2^k), B6.D (H-2^d) and B6.*Rag1*$^{-/-}$ mice were purchased from The Jackson Laboratory. The Hb–I-E^k specific TCRtg lines N3L2, 2.102, were produced in our laboratory46, 47, and the H-Y–I-E^k specific A1 TCRtg line was kindly provided by B. Stockinger48. The four TCRtg lines were bred onto non-selecting *Rag1*$^{-/-}$ backgrounds: AND.*Rag1*$^{-/-}$H-2^d, N3L2.*Rag1*$^{-/-}$H-2^b, 2.102.*Rag1*$^{-/-}$H-2^b and A1.*Rag1*$^{-/-}$H-2^b. All mice were bred and housed in specific pathogen–free conditions of the animal facility at the Washington University Medical Center. All the use of laboratory animals was approved and performed in accordance with the Washington University Division of Comparative Medicine guidelines.

I-E^k self peptides

A set of 95 self peptides naturally bound to I-E^k molecules from CH27 B cells were identified by liquid chromatography tandem-mass spectrometry (LC-MS) as described in

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detail29. Eighty-three of these peptides were previously reported29. In this study an additional 12 peptides (the last 12 listed in Supplementary Fig. 2) that were identified in the original LC-MS analysis were added. All 95 peptides were selected to possess canonical I-E^k anchor residues at P1 and P9, and were confirmed to bind to I-E^k. The self peptides bound to MHCII are often found in peptide families, which have common 9 amino acid core but differ in length at both the amino and carboxy termini. One representative for each peptide family, 13-16 amino acids in length, was selected.

Peptide synthesis and I-E^k-Ig dimers

All of the peptide utilized in this study were synthesized, purified, and analyzed as previously described29. I-E^k-Ig dimers were produced in insect S2 cells and purified using a Protein A column49. Each of the 95 self-peptides was loaded onto I-E^k Ig dimers in 0.2 M pH 5.0 Citrate–PO_4 buffer at 37°C for 72 hours, followed by neutralization with 1M pH 9.0 Citrate–PO_4 buffer.

Flow cytometry

Fluorescently conjugated antibodies purchased from commercial sources were FITC-anti-CD4, APC-anti-CD4, APC–Cy5-anti-CD4 (GK1.5; BioLegend PE–Cy5-anti-CD8 (53-6.7; BioLegend), PE-anti-CD69 (1-11.2F3; BioLegend), PE-anti-CD5 Ly-1 (53-7.3; BD Pharmingen), FITC-anti-V_α11 TCR, biotinylated-anti-V_α11 (RR8-1; BD Pharmingen) in conjunction with streptavidin-PE–Cy7 (BD Pharmingen), IL-2 detection antibody conjugated to PE or to APC (Miltenyi Biotec), and IFN-γ detection antibody conjugated to PE (Miltenyi Biotec). All samples were analyzed on a FACScan, FACSCalibur, or FACSCanto, and data were analyzed using FlowJo software (Tree Star).

CD69 upregulation Assay

Each well of a 96-well plate was coated with 1 μg/well of each of the 95-self peptide-loaded dimers and 10 μg/well anti-CD28 (37.51; BioLegend) in 200 μl PBS overnight at 4°C. Thymocytes from AND.*Rag1*$^{-/-}$H-2^d, N3L2.*Rag1*$^{-/-}$H-2^b, 2.102.*Rag1*$^{-/-}$H-2^b and A1.*Rag1*$^{-/-}$H-2^b TCRtg mice were added (1×10^6 cells/well) and cultured at 37°C overnight in I-10 plus medium. CD69 upregulation was examined by flow cytometry.

Thymic reaggregate culture

Thymic reaggregation assays were performed as described13, 50. DP thymocytes were purified from thymi of newborn mice (3-14 days old) by $CD8^+$ MicroBeads (Miltenyi Biotec). Thymic reaggregate culture was established in 24-well plates inserted with transwells (0.4 μm pore size; Costar). A mixture of 1×10^6 DP thymocytes and 5×10^5 ANV41.2 I-E^k cell lines was centrifuged, and the resulting cell pellet was placed as standing drops on the upper well of the transwell. The desired concentration of peptide was added in the bottom well in I-20 plus medium. The thymic reaggregate culture was incubated at 37°C for 96 hours. Cells were recovered by re-suspending the reaggregated lobes, and were then stained with indicated antibodies and examined by flow cytometry.

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Cytokine secretion assays

The 96-hour reaggregate culture was resuspended to single cell suspension in culture medium and stimulated with 50 ng/ml PMA and 500 ng/ml ionomycin for 3 hours at 37°C. The IL-2 and IFN-γ cytokine capture assays were done according to the manufacturer's protocol (Miltenyi-Biotect). The dead cells were excluded by PI staining. The cytokine secretion assays were analyzed by flow cytometry.

Proliferation assay

Naive T cells from spleens and lymph nodes of AND TCRtg (H-2^b) mice were purified. Primary T cell blasts were generated by incubating naive AND TCRtg $CD4^+$ T cells (H-2^b) with 10 μM MCC_{88-103} and irradiated B6.K (H-2^k) spleen cells for three days, followed by a 1:1 split and culture for another two days. Naive T cells or primary T cell blasts were (re)-stimulated with CH27 B cells and a range of concentrations of gp250 or MCC_{88-103} peptide in triplicate. The proliferation was assessed by ^{3}H-TdR incorporation.

Peptide binding assay

The peptide binding to I-E^k was determined using a competition binding assay as previously described29 using CLIP-loaded I-E^k and biotin-MCC as the reference peptide.

Co-agonist assay

Each well of a 96-well plate was coated in triplicate with 1 μg/well gp250:MCC or Hb:MCC-loaded I-E^k Ig dimers of various ratios. One-2 × 10^6 purified AND $CD4^+$ peripheral T cells (H-2^b) were added to each well and the co-agonist activity was assessed by ^{3}H-TdR incorporation (Supplementary Fig. 4).

Homeostatic proliferation assay

Homeostatic proliferation of AND TCRtg T cells was analyzed by transfer of CFSE labeled T cells into lymphopenic recipients24, 27. Purified $CD4^+$ AND TCRtg T cells were labeled with 5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE, Molecular Probes). One-10^6 CFSE-labeled cells/mouse were adoptively transferred into B6.K.*Rag1*$^{-/-}$ mice by retroorbital injection on day 0. The B6.K.*Rag1*$^{-/-}$ mice were injected intraperitoneally with gp250 or control peptide (Hb_{64-76}) of indicated amount on days -1 and +1. After four days, cells were recovered from inguinal and auxiliary lymph nodes, and expression of CD4, $V_\alpha 11$ and CFSE was analyzed by flow cytometry.

Statistics

All data were analyzed nonparametrically by Mann-Whitney *U* test using Prism 4 software (Graph Pad). Values of $P < 0.05$ were considered significant.

Supplementary Material

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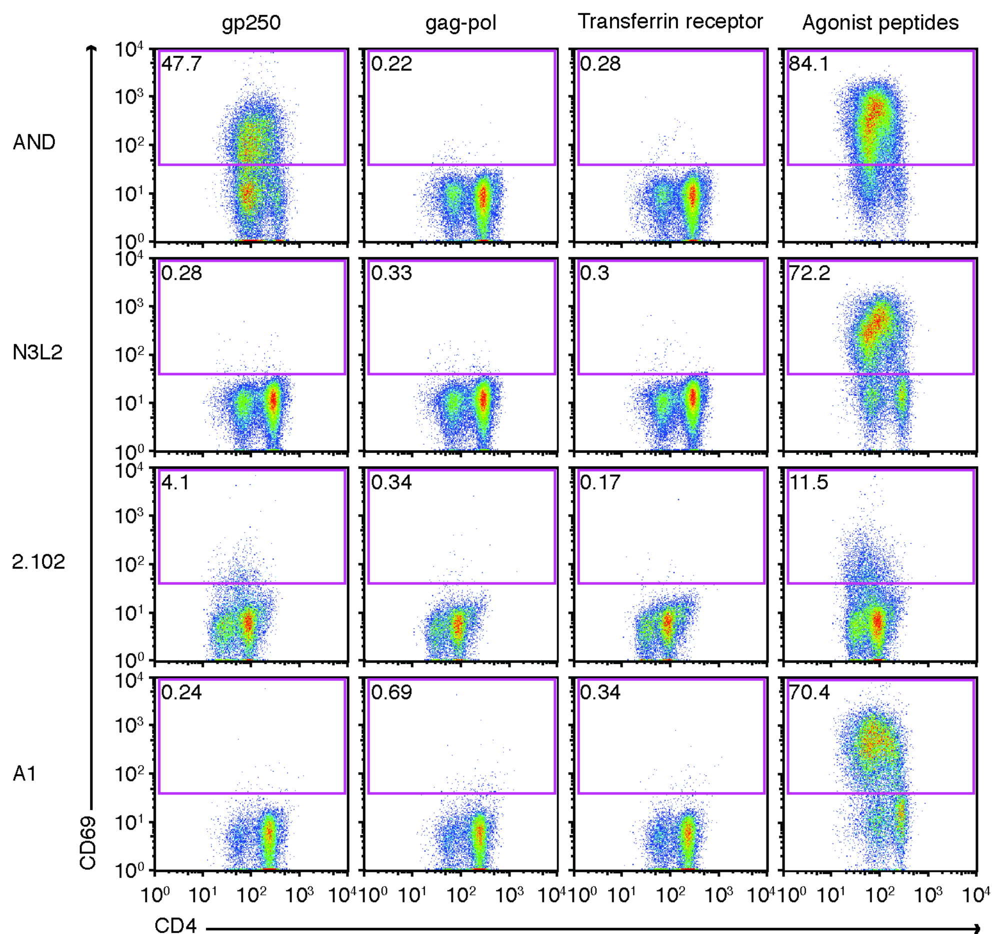

Figure 1.

gp250 is identified as a candidate positively selecting ligand. The 95 endogenous I-E^k self peptides were individually loaded onto I-E^k-immunoglobulin (I-E^k-Ig) dimers, which were immobilized along with anti-CD28 on plates. Thymocytes from AND, N3L2, 2.102 or A1 TCRtg mice were incubated in plates overnight and then CD69 surface expression was analyzed by flow cytometry. Representative dot plots of CD69 expression induced by three peptides (gp250, gag-pol and Transferrin receptor) are shown. The corresponding agonist peptide for each TCR was used as a positive control, and thymocytes incubated with medium alone served as a negative control (data not shown). A complete list of the 95 peptides and their capacity to induce CD69 expression can be found as Supplementary Fig. 2. Data are representative of two independent experiments for each TCRtg mouse strain.

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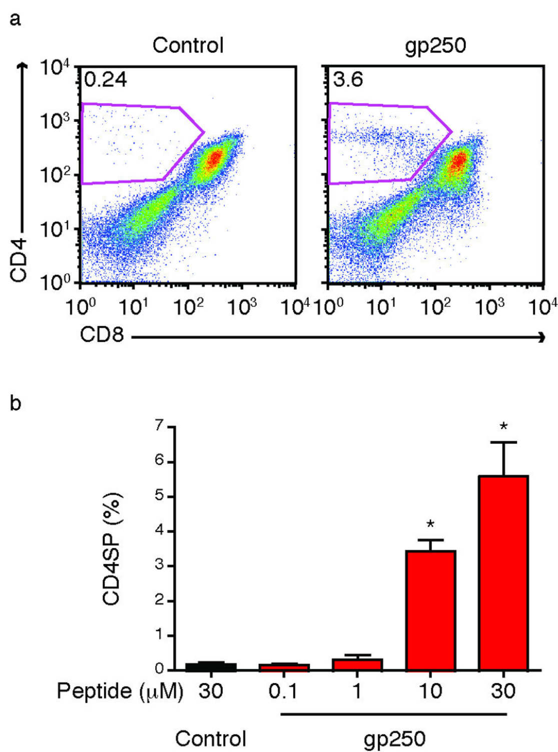

Figure 2.

gp250 positively selects AND TCRtg thymocytes in reaggregate culture. DP thymocytes from newborn AND.*Rag1*$^{-/-}$H-2^{d} mice were cultured with the I-E^{k} transfected cTEC cell line ANV41.2 I-E^{k} along with gp250 peptide or control peptide (Hb: GKKVITAFNEGLK) for 96 hours. CD4 and CD8 expression was then analyzed by flow cytometry. (a) Representative dot plots of thymocytes cultured with 30 μM of gp250 or Hb, with the percentage of positively selected CD4SP cells shown. Data are representative of five independent experiments. (b) CD4SP differentiation induced by indicated doses of the gp250 peptide compared to 30 μM of the Hb control peptide. Results are the mean ± s.e.m of 5 independent experiments. $*P < 0.05$ by two-tailed Mann-Whitney test.

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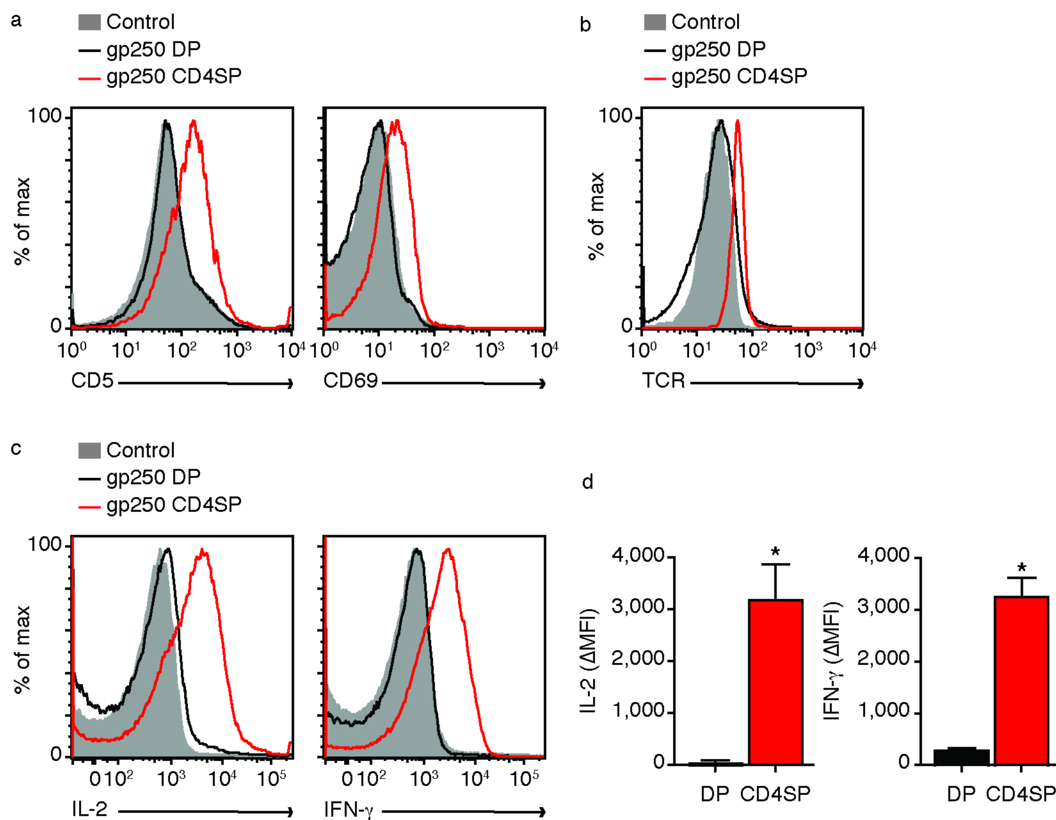

Figure 3.

gp250-selected CD4SP AND TCRtg T cells are phenotypically and functionally mature. (a,b) Expression of markers of positive selection on CD4SP and DP cells in reaggregate cultures pulsed with 30 μM gp250 or 30 μM Hb control peptide. Data are representative of five independent experiments. (c,d) Reaggregate cultures in (a,b) were resuspended and stimulated with PMA and ionomycin for 3 hours. IL-2 and IFN-γ were detected with cytokine capture reagents followed by flow cytometry analysis. The dead cells were excluded by PI staining. (c) Representative histograms. (d) MFI of the gp250-selected CD4SP and DP compared to that of cells selected with Hb control peptide. n = 6 samples in duplicate in three independent experiments. *$P < 0.05$ by two-tailed Mann-Whitney test).

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Figure 4.

Positive selection by gp250 has a high degree of specificity. (a) The aligned amino acid sequences of gp250 and MCC. The P2, P3, P5 and P8 residues are predicted TCR contact residues (indicated by upward arrows), and the P1, P4, P6, and P9 residues are I-E^k MHC anchor residues (indicated by downward arrows). (b) gp250 peptides containing single conservative amino acid substitutions of individual TCR contact residues. The substituted peptides were 14 amino acids long (P-4 to P9, see (a)), but only the P1 to P9 residues are shown for clarity. (c) The percentage of CD4SP cells positively selected in reaggregate culture by gp250, control peptide (Hb), or a gp250-MCC chimeric peptide (gp250-MCC) with MCC residues substituted at P4 and P6 (30 μM). Data show mean ± s.e.m of 3 independent experiments. *$P < 0.05$ by two-tailed Mann-Whitney test. (d) The percentage of CD4SP positively selected in reaggregate culture by 30 μM gp250, the single residue substitution peptides, or the control peptide (Hb). The percentages of CD4SP of total thymocytes are shown as the mean ± s.e.m. of 4 independent experiments. *$P < 0.05$ by Mann-Whitney test).

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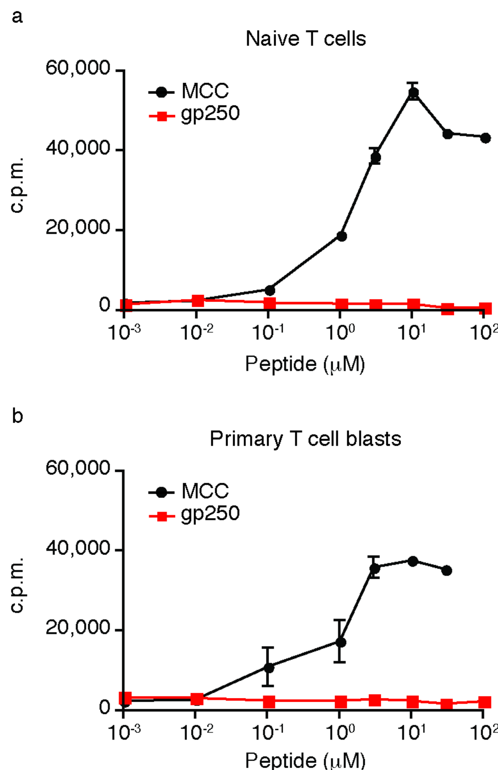

Figure 5.

gp250 does not stimulate proliferation of peripheral AND TCRtg T cells *in vitro*. (a) Naive $CD4^+$ T cells were isolated from AND TCRtg ($H\text{-}2^b$) spleen cells and cultured with CH27 cells and the indicated concentrations of gp250 or MCC peptide. (b) Primary T cell blasts were generated by stimulation of AND TCRtg ($H\text{-}2^b$) T cells with 10 μM MCC and irradiated B6.K ($H\text{-}2^k$) spleen cells for three days. The AND blasts were isolated and restimulated with CH27 B cells and the indicated concentrations of gp250 or MCC. The cultures were pulsed with ^{3}H-TdR (0.4 μCi/well) on (a) day 3 or (b) day 2, and the cultures were harvested 18-24 hours later. The values represent the mean ± s.d. of triplicate determinations. Data are a representative of three independent experiments.

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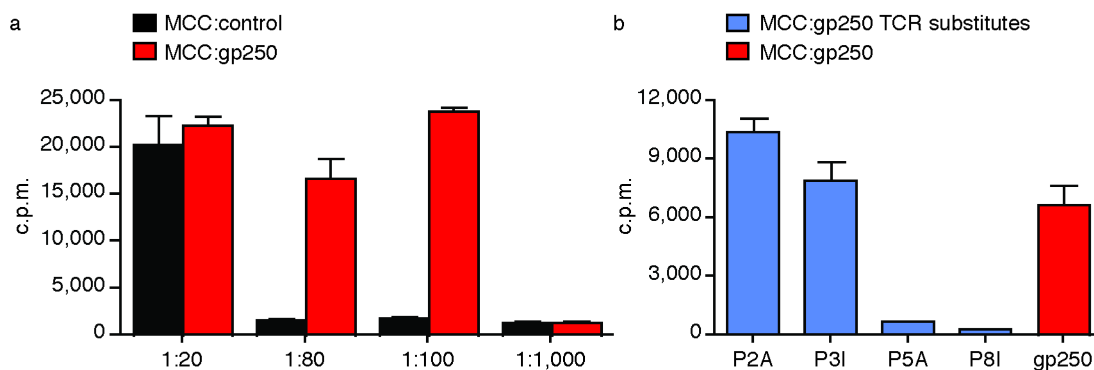

Figure 6.

gp250 acts as a co-agonist for peripheral AND TCRtg T cells. (a) The co-agonist ability of control peptide (Hb), gp250 (a) or gp250 single substituted peptides shown in Fig. 4b (b) was tested. I-E^k Ig heterodimers loaded with the indicated ratios of peptides were coated onto triplicate wells of a 96 well tissue culture plate. $CD4^+$ peripheral T cells were isolated from AND TCRtg (H-2^b) spleen cells, cultured for three days in the plates, and pulsed with ^{3}H-TdR for the last 18-24 hours of culture. The data in (a) are representative of five independent experiments with the values representing the mean ± s.d. of triplicate determinations. The data in (b) are the mean ± s.e.m of 3 independent experiments, with triplicate wells for each experiment.

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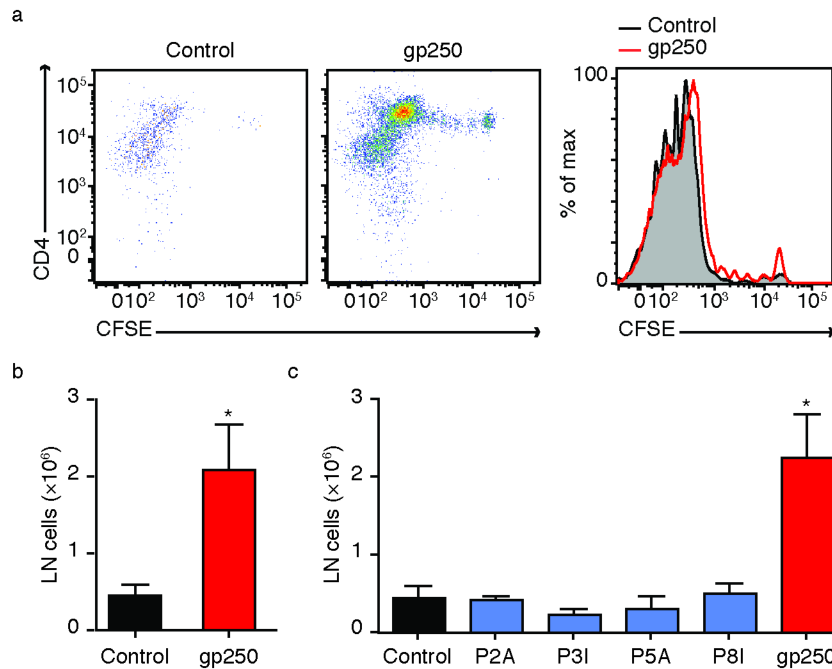

Figure 7.

gp250 enhances survival of homeostatically proliferating AND TCRtg T cells. $CD4^+$ peripheral T cells were isolated from AND.*Rag1*$^{-/-}$H-2^k spleen cells, CFSE labeled and transferred to B6.K.*Rag1*$^{-/-}$ mice by retro-orbital injection on day 0. The B6.K.*Rag1*$^{-/-}$ mice were injected intraperitoneally with 0.5 mg gp250 or control peptide (Hb) on days -1 and +1. CD4 and CFSE were analyzed by flow cytometry. (a) The dot plots (left) and CFSE histogram (right) are representative of eight individual mice from a total of five separate experiments. (b) The total number of T cells recovered from pooled inguinal and auxiliary lymph nodes (mean ± s.e.m.) from five separate experiments from mice receiving either control (Hb, n = 8) or gp250 (n = 8) peptide. *$P < 0.05$ by two-tailed Mann-Whitney test. (c) The total number of T cells recovered from pooled inguinal and auxiliary lymph nodes (mean ± s.e.m.) from five separate experiments from peptide treated mice. Hb, n = 5; gp250, n = 5; P2A, n = 5; P3I, n = 3; P5A, n = 3; P8I, n = 2. *$P < 0.05$ by two-tailed Mann-Whitney test.