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Rescue of Cytotoxic Function in the CD8 α Knockout Mouse by Removal of MHC II

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Summary

CD8 plays an important role in the activity of cytolytic T cells (CTL). However, whether or not CD8 is required for the development of CTL has not been clearly determined. Cytotoxic activity in the CD8 α knockout mouse is difficult to induce, and has only been demonstrated against allogeneic MHC targets. The lack of cytotoxicity may result from impaired lineage commitment of CTL in the absence of CD8, or diminished competitiveness during selection against (unimpaired) development of CD4⁺ T cells on MHC II. To differentiate between these possibilities, we have generated a double knockout mouse, CD8 $\alpha^{-/-}$ $\times$ MHC II^{-/-} (II-/8-). In II-/8- mice, developing MHC I-reactive thymocytes cannot rely upon CD8 for selection, but they also cannot be overwhelmed by efficient selection of MHC II-reactive thymocytes. In this mouse, a large, heterogeneous population of peripheral coreceptor double negative (DN) and CD4 positive T cells develops. Peripheral DN T cells are fully functional CTL. They display cytolytic activity against allogeneic MHC, and against syngeneic MHC following LCMV infection. Cells from LCMV-infected mice bind more MHC I tetramer at lower concentrations than their wild type CTL counterparts. These results demonstrate unequivocally that CD8 is not required for commitment of thymocytes to the CTL lineage.

Keywords

T cell selection; CD8; MHC II; knockout

Introduction

T cell lineage commitment is the process by which a developing thymocyte commits to the helper (Th) or cytolytic T cell (CTL) lineage. Lineage commitment is a multi-step process that results in singular expression of either CD4 or CD8, and differentiated function. The strict association between coreceptor identity and MHC recognition by TCR (i.e. that CD4 T cells recognize MHC II and CD8 T cells recognize MHC I) suggests that the TCR being tested on a developing thymocyte has an inherent preference for one MHC allele over the others, and therefore guides coreceptor choice. Indicative of this, T cells that develop in TCR transgenic mice show strong bias toward use of the original coreceptor, e.g. [1-4].

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Understanding how coreceptor contributes to lineage commitment has been one of the hallmark issues addressed by the two models of lineage commitment historically cited (reviewed in [5,6]). In the instructive model, TCR/coreceptor sends a unique signal that guides coreceptor and lineage choice. In the stochastic-selective model, coreceptor and lineage choice are made arbitrarily, so that TCR/coreceptor signals serve only to promote survival of a committed thymocyte. Both of these models rely on the fact that at some point in thymocyte development, coreceptors contribute to signaling, and therefore seek to discern when coreceptors play a role in lineage commitment. The models do not address the question of whether coreceptor is strictly required for the process.

The possibility that coreceptor was not required for lineage commitment was discussed early in the subject's history (e.g. [7]), but the model systems used to study lineage commitment have led to its abandonment. In this study we revisited the possibility by determining if CD8 was required for commitment to the CTL lineage. There have been several investigations that have addressed some aspects of this problem. For example, the dramatic loss of CTL in CD8 α knockout (8-) mice [8,9] suggested that CD8 was required for a thymocyte to commit to the CTL lineage. However, the recovery of coreceptor negative T cells in 8- mice following priming with allogeneic skin grafts [10], and the existence of alloreactive cytolytic cells in CD4/CD8 double knockout (4-/8-) mice [11], suggested that development of cytotoxic cells without CD8 was possible, but very inefficient in the context of normal Th phenotype development driven by CD4 and MHC II. Nonetheless, because 8- mice were not able to reject minor antigen-mismatched skin, and because they were not able to produce an anti-viral CTL response [8,10,11], it is unclear if these coreceptor negative cytolytic cells represented successful CTL lineage commitment without CD8, or an alternate developmental pathway [12,13].

Attempts to force CTL development in the absence of CD8 by crossing mice transgenic for an MHC I-reactive TCR onto 8- mice have failed for the H-Y, P14, 2C and OT-I TCRs [14,15]. Nonetheless, when peptide agonist was provided to thymic lobes from the 8-(P14) and 8-(OT-I) mice *in vitro*, CTL development was restored, suggesting that a strong signal can compensate the loss of CD8 during development [15,16]. However, the use of a transgenic TCR expressed in 8- mice is not an unprejudiced tool to study the influence of CD8 on CTL lineage commitment, since transgenic TCR are derived from T cells originally selected with CD8. In 8- mice, thymocytes forced to express an MHC I-reactive TCR that was originally selected in the context of CD8 binding to MHC I are at a severe disadvantage to be positively selected, since the ability of their TCR to successfully interact with MHC I is likely to be CD8-dependent – especially during selection on low affinity “self” antigen. Furthermore, this approach leaves unanswered the question: “Can the process of thymic selection, including TCR generation, yield lineage-committed CTLs without any contribution from the CD8 coreceptor?”

To directly answer this question, we tested for CTL development in a mouse where TCR/CD8-MHC I co-engagement could not occur, and where selection of thymocytes by CD4/MHC II could not interfere with their possible development. We crossed the 8- mouse [8] with the MHC II knockout mouse [17] (hereafter referred to as II-/8- mice). Indeed, in II-/8- mice, there is a large population of CD4 and DN α/β TCR positive T cells. All peripheral T cells in these mice are predicted to be MHC I selected without any contribution from CD8 or CD4 binding. Importantly, thymocyte selection in II-/8- mice truly reflects the genesis of a population of MHC I-reactive TCR that do not rely upon CD8 contribution, which is distinct from constrained development of a population of thymocytes expressing a single MHC I-reactive TCR that was originally selected with CD8. Functional, peripheral CTLs are clearly present in II-/8- mice, and DN T cells respond as do “normal” CTLs following viral infection. Our results show conclusively that CTL lineage commitment can occur without

TCR/CD8-MHC I co-engagement during thymic selection, suggesting that the lack of CTL lineage commitment in 8- mice was likely due to competition by uninhibited CD4 T cell development.

Results

T cell development in II-/8- mice

The II-/8- knockout mouse was generated by crossing, intercrossing, and selecting F2 progeny from the MHC II single KO mouse [17] and the CD8 α single KO mouse [8]. Both mice are on the C57Bl/6 background. Figure 1a shows confirmation of the double knockout phenotype of splenocytes by flow cytometry. The frequency of T cells observed in the spleens of II-/8- mice was roughly 1/2 of T cell frequency found in B6 mice (13% in II-/8- versus 30% in B6, Figure 1b). Interestingly, despite the absence of MHC II as a selecting ligand, there was a large population of CD4⁺ T cells in the periphery (Figure 1c). The percentages of NK T cells expressing CD49 and NK1.1 were comparable between the II-/8- and B6 mice (data not shown), while the percentage of those staining positive for the α GalCer tetramer was slightly elevated in II-/8- mice (data not shown; percentage of TCR⁺CD4⁺ cells that were α GalCer tetramer⁺: 11%, II-/8-; 3%, B6).

At four weeks of age, the cellularity of the II-/8- thymus was much smaller than the B6 thymus (Figure 2a, B6 = 4.1×10^8 cells versus II-/8- = 2.4×10^7 cells; $p=0.029$). However, by eight weeks, the cellularity was similar between II-/8- and B6 mice thymi (Figure 2a). H&E staining of thymic sections (from eight week old mice) showed normal thymic architecture with a cortex (blue) and medulla (pink) in the II-/8- mouse (Figure 2b). These data suggested that there was a delay in the development of the T cell population, but that the basic thymic architecture developed normally.

II-/8- thymi contained mature thymocytes, defined by TCR^{HI}HSA^{NEG} staining (Figure 2c). Due to the absence of CD8 in the II-/8- mouse, we used a CD4 vs. TCR co-stain to assess the DN/DP/SP immature thymocyte population distribution. Because expression levels of CD4 and TCR are unique when comparing B6, II-, 8-, and II-/8- thymocytes, fixed quadrant assignment could not be used to delineate each thymocyte population cleanly. As expected, both the Th precursor (CD4^{POS}/TCR^{POS}) and CTL precursor (CD4^{NEG}/TCR^{POS}) populations were dramatically diminished in II-/8- mice, akin to the populations diminished in the respective MHC II and CD8 single knockout mice (Figure 2d). There is no change in the size of the CTL precursor population (CD4^{NEG}/TCR^{POS}) between 8- mice and II-/8- mice (cell population constitutes 1.2% of all thymocytes in both mice, derived from Figure 2d, gate not shown). Interestingly, the population equivalent to DP thymocytes in the II-/8- thymus expresses higher levels of both CD4 and TCR than DP thymocytes in B6 mice. This observation appears to be due mostly to the effects of MHC II elimination (Figure 2d, and [18]), and suggests that immature thymocytes are able to increase expression levels of TCR in order to compensate for the weaker TCR signal found in the absence of a coreceptor contribution, and to increase CD4 coreceptor expression in response to low signal strength in an attempt to be positively selected. In addition, although diminished, each Th and CTL precursor population appears to be present in the thymus.

T cell function in II-/8- mice

One possible outcome of coreceptor-less thymic selection is that it would be so inefficient that only a limited number of mature T cells would emerge from the thymus and expand in the periphery by homeostatic proliferation. This would be readily apparent as a restricted TCR V gene usage in peripheral T cells. Furthermore, if the population were dramatically reduced, there would be an apparent reduction in the plasticity of antigen recognition. For

example, if very few T cells were to develop in II-/8- mice, we would not expect full allo-recognition, or a complex, anti-viral CTL response to multiple viral antigens.

TCR variable gene usage is diverse in II-/8- mice

In order to examine V β usage, we sorted individual T cells and sequenced the TCR β chain of 54 individual splenic II-/8- T cells by PCR. As shown in Figure 3, TCR β chain usage is diverse in the II-/8- mouse, drawing from 16 different V β gene segments. Analysis of the sequence of the third complementarity-determining region (CDR3) confirmed that no TCRs sharing the same V β also shared the same CDR3 sequence. Therefore, all 54 T cells sequenced from a single II-/8- spleen were different clonotypes.

This is a relatively small sample size of potential TCR in the full repertoire. These data do not allow us to determine 1) the size of the II-/8- TCR repertoire, or 2) if the size of the naive T cell repertoire is similar between II-/8- and wild type B6 mice. However, using even the small number of B6 and II-/8- TCR sequences that were determined, we can assess the extent of TCR V β usage diversity between the two strains of mice. The Shannon entropy has been widely used as an index of sample diversity [19]. We have developed a method for estimating the posterior distribution of the entropy conditional on observed species abundances (Kepler, in preparation). This method accounts for species that may be present in the population, but are not present in the sample. The posterior means and 95% credible intervals for the TCR V β entropies for the II-/8- and B6 mice are 2.76 $\pm$ 0.14 and 2.56 $\pm$ 0.18, respectively, and are not statistically different (Figure 3 inset). This indicates that the altered conditions in the II-/8- thymus do not observably affect the TCR V β that may be selected. Thus, with TCR V β gene usage as a metric, the II-/8- T cell population is not measurably less diverse than the B6 T cell population.

Alloreactivity is present in II-/8- mice

We next turned to examining function of the T cells that are selected in II-/8- mice. It has already been determined that 8- mice could reject skin grafts, but 8- splenocytes could not mount an *in vitro* alloreactive response [8,11]. To determine if II-/8- splenocytes could lyse alloreactive targets *in vitro*, we first utilized an alloreactive mixed lymphocyte culture (MLC). II-/8- splenocytes were stimulated *in vitro* with BALB/c (H2^d, MHC and minor mismatched) splenocytes for three days, then assayed for lysis against ⁵¹Cr labeled BALB/c targets. Splenic T cells from the II-/8- mouse were as efficient at lysis as those from both the B6 and II- mice (Figure 4a). Importantly, this contrasts with the complete lack of *in vitro* cytotoxicity demonstrated by splenocytes from the 8- mouse.

By definition, II-/8- CTL are CD8-independent and selected on H-2^b MHC I. However, it was possible in this MLC that the II-/8- responder cells recognized and responded to both the H-2^d MHC II in addition to MHC I expressed on BALB/c splenocytes. To more specifically assess the ability of II-/8- CTL to respond to allo MHC II without the influence of CD4, we set up another MLC using B6 splenocytes as stimulator cells and as targets in the ⁵¹Cr release assay. Since II-/8- and B6 mice share the same MHC haplotype (H-2^b), II-/8- T cells should not respond to B6 MHC I as allogenic (nor should MHC I-associated minor antigens be targeted). However, the absence of H-2^b MHC II (I-A^b) during selection in II-/8- thymus defines the I-A^b expressed on B6 splenocytes as “allogenic.” Figure 4b shows that, in fact, I-A^b-expressing targets were lysed. The reaction was MHC II-specific because antibody to MHC II completely inhibited lysis, and it did not depend upon CD4 binding to MHC II because antibody to CD4 had no effect on lysis (Figure 4b).

To confirm our *in vitro* results, we performed allogeneic skin grafts. Grafts from BALB/c and wild type B6 were transplanted onto II-/8- and B6 mice. There was no significant

difference in graft survival between groups receiving the BALB/c grafts (Figure 4c, median survival on II-/8-: 13 days, on B6: 15 days; $p=0.52$). As expected, the B6 mice did not reject the B6 grafts. Confirming the recognition of MHC II by II-/8- CTL in the MLC, the II-/8- mice also rejected the B6 grafts rapidly. Interestingly, there was no significant difference in graft survival between II-/8- rejection of the BALB/c (MHC I and II as antigen) and B6 (MHC II only as antigen) grafts (Figure 4c, median survival of BALB/c graft: 13 days, of B6 graft: 15 days; $p=0.24$). Therefore, II-/8- mice are fully able to mount alloreactive responses as do B6 mice.

Cytolytic LCMV response is present in the II-/8- mouse

While alloreactivity is an interesting function of cytolytic T cells, their *raison d'être* is to lyse infected cells. Therefore, we tested the ability of T cells in the II-/8- mouse to respond to peptide presented by self MHC I in response to a viral infection. LCMV was chosen because we were unsure if the CD4 cells in II-/8- mice could function as helpers, and the primary CTL response to LCMV has been shown to be helper-independent [20]. II-/8- mice were infected intraperitoneally with LCMV and remained healthy and active until day 8 following infection, when splenocytes were harvested for CTL analysis. The cells were stimulated *in vitro* for three days with one of three well-characterized LCMV MHC I-associated peptide epitopes: gp33(C9M), gp276, or np396. Following stimulation, the cells were tested for lytic capacity against the stimulating peptide pulsed onto ^{51}Cr labeled ANA-1 cells (H2^b) at a variety of effector:target ratios. For all three LCMV peptides tested, peptide-reactive CTL were present in II-/8- mice (Figure 5). Furthermore, the DN T cells appeared to be responsible for lysis, as the percentage of CD4⁺ T cells was low after peptide stimulation, and selective depletion of CD4 T cells had no effect on lysis (Figure 5). Thus, in contrast to studies with the CD8 α single knockout and CD4/CD8 α double knockout mice, II-/8- mice can successfully mount a complex, MHC-restricted CTL response to viral infection.

II-/8- TCR bind MHC tetramer with greater avidity than B6 TCR

We anticipated as a consequence of the absence of CD8 binding during T cell selection, CTL from the II-/8- mice might express TCR with a higher affinity for their MHC I target. A metric commonly used to estimate TCR affinity differences between two T cell populations (without measuring actual TCR-pMHC affinities) is MHC tetramer binding [21]. Therefore, we measured binding of the D^b -gp33(C9M) tetramer to T cells responding to LCMV infection in II-/8- and B6 mice. As shown in Figure 6, T cells from II-/8- mice have a much higher avidity to the tetramer than do equivalent cells from B6 mice. The median fluorescence intensity of tetramer staining at a range of concentrations reveals that more of the tetramer binds to T cells from II-/8- mice at lower concentrations than to those of B6 mice. The D227K mutation of D^b , which inhibits CD8 binding to D^b , did not diminish tetramer binding to II-/8- T cells, but greatly reduced tetramer binding to B6 T cells (data not shown). Finally, it is noteworthy that in the three II-/8- mice tested, only DN (and not CD4) T cells stained positive for tetramer (data not shown).

Discussion

Here we describe CTL development where TCR was forced to bind to MHC I without the benefit of CD8 binding. Despite this constraint, a population of heterogeneous, functional cytotoxic T cells developed. The rescue of potent CTL activity in II-/8- mice, when compared to 8- mice, suggests that development along the MHC II pathway completely overwhelmed CTL development in 8- mice, providing a simple explanation for the apparent absence of CTL in those mice.

Although functional CTL development occurred in II-/8- mice, T cell development was clearly impaired. There seemed to be a bottleneck to development as cellularity of the thymus increased more slowly, and there were fewer peripheral T cells in II-/8- mice, compared to B6 mice. However, the diversity of TCR V gene usage, and the ability to respond to multiple LCMV antigens in a polyclonal fashion (data not shown) argues against the possibility that selection in this environment was so restrictive that only a few thymocytes managed to escape the normal control mechanisms that prevent emigration of immature thymocytes. Furthermore, the presence of mature thymocytes and the normal architecture that was established in the II-/8- thymus are indicators that the progression from thymocyte entry, positive and negative selection, to thymus exit is occurring, despite quantitative limitations [22].

The avidity model for T cell selection [23,24] provides one possible explanation for how the absence of CD8 binding to MHC I might impair thymic development in II-/8- mice. This model suggests that there are thresholds on the magnitude of TCR-originating signal in the thymocyte, above which negative selection occurs, below which thymocytes die by neglect. It is likely that participation of coreceptor during selection lessens the burden of binding energy placed on the TCR [25,26]. One effect of this coreceptor “crutch” would be that TCRs with lower affinities (in the μ M range) are selected, rather than those with higher affinities (Figure 7), as is frequently observed for the variable regions of antibodies. In the absence of coreceptor, the TCR must bear the binding burden of selection alone. Since CDRs 1 and 2 have limited combinatorial diversity and do not interact with the MHC strongly, the burden of binding is transferred mostly to CDR3. Thus, the absence of coreceptor diminishes the percentage of CDR3s amenable to selection so that fewer TCRs survive positive selection (Figure 7). Fewer selectable TCRs translates to smaller thymi in young mice. Interestingly, the variability in TCR V β usage suggests that the absence of MHC II does not dramatically skew the population toward or away from a set of V β . While the Db-gp33(C9M) tetramer titration shows that II-/8- T cells bind MHC with higher avidity than their B6 counterparts, ongoing studies with individual TCRs obtained from LCMV-reactive DN T cells will reveal if higher TCR-pMHC affinities are indeed generated in II-/8- mice.

Nonetheless, this explanation does not account for the absence of normal CTL activity in the seemingly similar 4-/8- mouse. In that mouse, allo-restricted cytotoxicity was observed from a population of DN T cells, but no self MHC (anti-viral) response could be induced [11]. Clearly selection of CD8-independent CTL in the absence of MHC II is fundamentally different from selection in the absence of CD4. We postulate that lck distribution between II-/8- and 4-/8- mice would be dramatically different. In the absence of MHC II, most of the lck available for TCR signaling is likely to be CD4-associated, even more so than in B6 mice, since CD4-lck association is increased in II- mice compared to B6 mice [27]. By contrast, in the absence of CD4, more “free” lck is likely to be available for TCR signaling, which may enhance negative selection. A recent report describing thymic selection in quad knockout (4-/8-/I-/II-) mice supports this idea, indicating that too much “free” lck may be detrimental to thymocyte development [28].

The prevalent view of T cell lineage commitment may be summarized by the tenets of a quantitative instruction model. The idea is that the signal emanating from the TCR when in complex with CD4 and MHC II differs from when it is in complex with CD8 and MHC I, and therefore is able to “instruct” the choice of cellular function (helper vs. cytolytic), and thus coreceptor (CD4 vs. CD8). The ability of these complexes to signal uniquely has been explained by the facts that 1) lck associates more strongly with CD4 than with CD8 [29], and 2) the coreceptors are differentially expressed during thymocyte development [30]. However, neither of these two mechanisms can be responsible for lineage commitment in

II-/8- mice: that lck associates more strongly with CD4 is irrelevant, since CD4 has no binding partner without MHC II, and that CD8 can be transiently downregulated to test for MHC I reactivity at the double positive thymocyte stage is similarly irrelevant, since CD8 is not expressed in these mice. In fact, the strength of signal model would predict that all thymocytes would develop as CTL, given the low lck activity (no CD4 involvement), while the kinetic signaling model would predict that all thymocytes would develop as Th, given the uninterrupted lck activity (without CD8 downregulation). In fact, there is no coreceptor-dependent mechanism in II-/8- mice for lck modulation at the immunological synapse during thymocyte development. The lck used by the developing II-/8- thymocyte is likely to be membrane-associated (palmitoylation, myristylation [31]), whether it is “free” lck, or is co-localized with CD4 or other receptors ([32-34]). Thus, only differences in the strength of the TCR/MHC interaction can be responsible for directing lineage choice in the II-/8- mouse, a mechanism that may have a role in normal thymocyte development [35]. The result of these stronger interactions (relative to development in B6 mice) may be that TCRs form larger clusters, maintain contacts for longer time periods, or both. It may also be that other protein tyrosine kinases function in lieu of coreceptor-associated lck to facilitate T cell selection and function.

How CD8 contributes to CTL development in humans is also unclear. A naturally-occurring (human) familial CD8 deficiency leads to mild symptoms of immunodeficiency [36]. Individuals with this deficiency have an increased population of peripheral DN T cells that express heterogeneous TCR (eight different TCR V β by flow cytometry), but no demonstrable *in vitro* CTL activity. Nonetheless, these DN T cells express CD8 α (mutant) and CD8 β message and additional surface markers that suggest they may be committed to the CTL lineage, but unable to function normally.

If CD8 is not required for CTL development, what is its function under normal circumstances? As mentioned, transport of lck to the immunological synapse is likely to be enhanced when CD8 is available. Also, CD8 increases the avidity of the TCR/MHC interaction, which may be responsible for the low affinity interactions observed between TCR and MHC. Even so, the fact that CTL can develop without CD8 in the II-/8- mouse suggests that neither of these functions is absolutely indispensable. The levels of lck in the immunological synapse without directed coreceptor transport *are* sufficient for T cell selection and function. VDJ recombination *can* yield TCRs that interact as strongly with their MHC targets as the TCR/coreceptor pairs in wild type mice. The presence of functional CTL in II-/8- mice indicates that CTL lineage commitment does not require CD8 to provide a boost to the strength of the lck signal or to the strength of the TCR/MHC interaction. The diminished number of peripheral T cells in the II-/8- mouse suggests that thymic positive selection is not as frequent in the absence of coreceptor. Therefore, a major function of CD8 may be to facilitate the positive selection of greater numbers of thymocytes by lowering the TCR affinity requirement, ensuring that a greater percentage of thymocytes that audition actually make the cut. CD8 has been shown to perform a similar task in the post-selected repertoire, ensuring that low-affinity TCR can serve as effectors in the periphery during an anti-viral response [37].

We have shown that the canonical diagonal docking orientation of TCR onto its pMHC I target is not adopted when the TCR is responding in a CD8-independent fashion [38]. We suggested at that time that CD8 operates during thymic selection to impose diagonal docking, resulting from spatial constraints associated with the interactions between CD8 and its two binding partners, MHC (extracellular) and lck (intracellular). During thymic selection in II-/8- mice, however, this constraint is absent. Therefore, TCR from II-/8- mice may dock onto their MHC I targets in non-canonical orientations. Our ongoing investigations will examine this directly. Or, taking this idea one logical step further, the

inability of either TCR coreceptor to contribute to thymocyte development in II-/- mice may result in positive selection on other (non-MHC) surface antigens expressed on the thymic epithelium, an idea supported by the development of $\alpha\beta$ T cells in quad (4-/-I-/II-) knockout mice [28]. That CD8 imposes “rules” for how TCR can bind to MHC I, yet is not required for thymocyte survival or lineage commitment, suggests that CD8 functions to encourage MHC I specificity, but plays no role in dictating the CTL fate.

Materials and Methods

Animals

C57Bl/6J and BALB/cJ were obtained from the Jackson Laboratory (Bar Harbor, ME). The B6.129S2-CD8 $\alpha^{tm1Mak/J}$ (Jackson SN 002665) and B6.129-H2 $^{dIAb1-Ea/J}$ (Jackson SN 003584) mice were maintained at UNC. To generate the II-/- mice, B6.129S2-CD8 $\alpha^{tm1Mak/J}$ and B6.129-H2 $^{dIAb1-Ea/J}$ mice were crossed, intercrossed, and the desired phenotype was confirmed by surface analysis for simultaneous knockout of CD8 and MHC II expression in the F2 progeny. Thymi were harvested and preserved in a neutral phosphate-buffered 10% formalin solution. Thymi were sectioned and H&E stained by American HistoLabs (Gaithersburg, MD), and images were captured on a Nikon Microphot FXA microscope. For quantitation of thymic cellularity, thymi were harvested, made into single cell suspensions by mechanical disruption between two glass microscope slides, and viable cells counted by Trypan blue exclusion. Statistical significance was calculated using the Mann-Whitney rank test.

For all experiments that use splenocytes, the cells were generated according to the following protocol. Spleens were harvested and mechanically disrupted into single cells by crushing between two glass microscope slides. Red blood cells were lysed by treatment with hypotonic solution (ACK lysis buffer) and connective tissue was removed by filtering through a 40 μ m nylon cell strainer. Splenocytes were maintained in RPMI + 10% FCS on ice, or as indicated below.

All experiments performed on mice complied with IACUC policies and procedures, and were approved by IACUC, UNC Chapel Hill.

Cell phenotype

Antibodies I-A b -FITC, CD8-PE, H57-FITC, HSA-PE, CD11b-PE-Cy7, CD11c-PE-Cy7, CD19-PE-Cy7, NK1.1-PE-Cy7, and H57-APC were obtained from eBioscience (San Diego, CA). PE- α -galactosylceramide-loaded CD1d tetramer (α GalCer) was a gift from the laboratory of Dr. Roland Tisch. For FACS analyses, splenocytes were treated with Fc block (2.4G2 supernatant), stained, washed, and fixed, then analyzed on a Cyan flow cytometer (Dako Cytomation, Fort Collins, CO). Data were processed using Summit software (Dako Cytomation, Fort Collins, CO). For all FACS analyses, single events are gated by their location on a plot of forward scatter linear versus forward scatter area; lymphocytes are gated by their location on a plot of forward scatter linear versus side scatter linear.

Single cell sequencing

Splenocytes from C57Bl/6J and II-/- mice were incubated with Fc block, then stained with H57-APC, NK1.1-PE-Cy7, CD19-PE-Cy7, CD11b-PE-Cy7, and CD11c-PE-Cy7. Single T cells (APC-positive, PE-Cy7-negative) were sorted on a MoFlo (Dako Cytomation, Fort Collins, CO) into 96 well PCR plates containing lysis buffer. In order to amplify TCR α and TCR β chain genes from the single cells, the lysate was split into two 96 well plates, and combined cDNA synthesis/first step PCR amplification was performed according to manufacturer's instructions (Superscript III One-step RT-PCR with Platinum Taq,

Invitrogen, Carlsbad, CA) using external primer sets specific for all known TCR α or TCR β variable genes and TCR C α or C β region primers [39]. This reaction was followed by nested PCR using internal primer sets for TCR α or TCR β variable genes and TCR C α or C β region primers [39]. PCR products were treated with Exonuclease I (New England Biolabs, Ipswich, MA) and shrimp alkaline phosphatase (Roche Diagnostics, Corp., Indianapolis, IN) to eliminate interference by unused primers. Sequencing was done by the UNC sequencing core facility. Sequences were aligned using Sequencher software version 4.5 (Gene Codes Corp., Ann Arbor, MI), and TCR gene identification made using SoDA [40].

The population entropy, a measure of the population diversity, is estimated using a Bayesian method we have developed (Kepler, manuscript in preparation). Rather than using the sample entropy directly as a measure of the population entropy (a procedure known to produce very biased results when the sample size is not much greater than the number of species), we compute the complete posterior distribution of the entropy conditional on the observed data. We furthermore integrate over any uncertainty in the total number of distinct species. This method allows us to compare the diversity between two T cell populations rigorously and reliably, even with limited sampling from each population.

Mixed Lymphocyte Culture

Splenocytes were harvested from the II-/8-, II KO, 8 KO, and wild type B6 mouse (H2^b), and co-cultured with BALB/c splenocytes (H2^d) as stimulator cells. After three days, cultured cells were used in a four-hour ⁵¹Cr release assay against 24 hour ConA treated BALB/c splenocyte targets. To show B6 reactivity in the II-/8- splenocytes, the same protocol was used, except B6 splenocytes were used as stimulator cells in the three day co-culture, and 24 hour LPS treated B6 splenocytes were used as targets in the ⁵¹Cr release assay, with or without blocking MHC II antibody (mouse IgM, clone 28-16-8S). ⁵¹Cr release into culture supernatant was detected by gamma count (Cobra II, Packard Instrument Company, Downers Grove, IL.). Percent specific lysis was calculated as $100 \times (\text{sample} - \text{spontaneous}) / (\text{maximum} - \text{spontaneous})$.

Skin grafts

Tail skin grafts were prepared from either B6 or BALB/c mice using an 11 blade, and sections measuring 1 to 2 mm by 5 to 7 mm were stored briefly on PBS-soaked 3MM paper. Graft beds were prepared in the same fashion on II-/8- and B6 recipient mice. Grafts were placed and adhered by pressing briefly with bibulous paper. The graft was shielded to prevent the mice from removing it, and graft adherence was monitored approximately every two days. Rejection was defined as scaling and lifting at the edges of the graft, along with the death of graft hairs, which generally precede sloughing of the graft itself.

LCMV Response

Mice were infected intraperitoneally with 500 pfu LCMV Armstrong in 150 μ L PBS. Eight days later, splenocytes were harvested and cultured in complete medium (RPMI with 10% calf serum, β -mercaptoethanol, essential and non-essential amino acids, and antibiotic/antimycotic agents) plus 10 U/mL rhIL-2 (Proleukin, Chiron Corp., Emeryville, CA.) at 2×10^6 cells/mL in the presence of one of the following MHC I-binding LCMV peptides (at 1 μ g/mL): gp33(C9M) KAVYNFATM, gp276 SGVENGPPYCL, or np396 YTVKYPNL. Following three days of *in vitro* stimulation, cells were purified by lymphocyte separation medium, then tested for their ability to lyse targets pulsed with the stimulating peptide. Target cells (ANA-1, H2^b) were labeled with ⁵¹Cr, pulsed with peptide, and used in a lysis assay with the cultured LCMV-responsive T cells. ⁵¹Cr release into culture supernatant was detected by gamma count (Cobra II, Packard Instrument Company, Downers Grove, IL.). Percent specific lysis was calculated as $100 \times (\text{sample} - \text{spontaneous}) / (\text{maximum} - \text{spontaneous})$.

spontaneous). CD4 depletion was accomplished using the CD4⁺ T cell isolation kit (Miltenyi Biotec, Auburn, CA.).

Tetramer Titration

D^b-gp33(C9M) and D^b(D227K)-gp33(C9M) tetramers were made as described [26] using streptavidin-APC (eBioscience, X, CA). B6 and II-/8- mice were infected intraperitoneally with 500 pfu LCMV Armstrong in 150 µL PBS. Eight days later, splenocytes were harvested and stained for two hours on ice for H57, CD4, CD19, CD11b, CD11c, and a range of tetramer concentrations from 1.4 nM to 2.8 µM. The median fluorescence intensity of APC-tetramer staining was determined for T cells (H57 positive, CD19/11b/11c-negative) by flow cytometry. Plot of MFI tetramer versus tetramer concentration yielded a standard binding isotherm, and nonlinear curve fit to these data was done using GraphPad Prism Software.

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Abbreviations

8-	CD8 α knockout mouse
II-/8-	MHC II $\times$ CD8 α double knockout mouse
DN	double negative
DP	double positive
SP	single positive
HSA	Heat-stable antigen
LCMV	lymphocytic choriomeningitis virus
lck	tyrosine kinase p56lck

Figure 1. Phenotype of splenic lymphocytes in II-/8- mice

The II-/8- mouse was generated by intercrossing the MHC II knockout mouse with the CD8 α knockout mouse. Both CD8⁺ and MHC II⁺ cells are absent in the II-/8- mouse, but there is a large population of TCR⁺ cells present. Splenocytes from the II-/8- and B6 mice were stained for CD8 α and I-A^b (A) and TCR β and CD19 (B), then analyzed by flow cytometry. CD19- lymphocytes were stained for CD2 and CD4, then analyzed by flow cytometry (C).

Figure 2. Characterization of the II-/8- thymus

Concomitant elimination of MHC II and CD8 alters the population balance of thymocytes, but thymic architecture remains unchanged. A) The thymi of four, eight, and twelve week old II-/8- and B6 mice were harvested. The organs were mechanically disrupted into single cell suspensions, and viable cell counts were performed using Trypan blue. In each graph, a point represents one mouse, and the bar represents the average (four mice each group). B) Thymi from eight week old II-/8- and B6 mice were sectioned and H&E stained. Metric is 100 μ m (left panels) and 10 μ m (center and right panels). Arrows indicate cortical macrophages (green) and thymic epithelial cells (yellow). C&D) Thymocytes from eight week old mice (II-/8-, II-, 8-, and B6 as labeled) were stained for TCR β and HSA (C) or TCR β and CD4 (D), then analyzed by flow cytometry.

Figure 3. TCR variable gene usage is diverse in II-/8- mice

Splenocytes were harvested from B6 and II-/8- mice and stained for surface TCR expression. Individual T cells were sorted into 96 well plates (n=54 for the II-/8- mouse, n=59 for the B6). TCR β chain was amplified using a two-step PCR process (RT-PCR, followed by nested PCR) and identified by sequence comparison with known TCR V β genes. The percentage of cells expressing each V β gene is shown. The inset shows posterior mean entropy values with 95% credible intervals calculated on V β usage for the II-/8- and B6 T cell populations.

Figure 4. II-/8- splenocytes respond to allogeneic MHC targets

A) Naïve splenocytes from a II-/8- mouse (and B6, II-, and 8-, as labeled) were used in a mixed lymphocyte culture (MLC) assay. Spleens were harvested, made into single cell suspensions, and co-cultured with lethally irradiated, MHC-mismatched BALB/c splenocytes (H2^d). Three days later, the stimulated splenocytes were used in a ⁵¹Cr release assay against Con A treated BALB/c splenocytes. B) MLC is identical to A), except splenocytes from wild type B6 (expressing I-A^b) were used for the three day co-culture. Targets in the ⁵¹Cr release assay were LPS-treated B6 splenocytes, without antibody, with blocking antibody to CD4, or with blocking antibody to MHC II, as labeled. C) The tails of II-/8- or B6 mice were grafted with skin from the tail of either BALB/c or B6 mice, as indicated. Each trace represents graft survival on the indicated group of mice; each group consisted of 5 mice.

Figure 5. II-/8- T cells respond to LCMV peptide antigens presented by self MHC I
 B6 and II-/8- mice were infected with 500 pfu LCMV Armstrong i.p. Eight days later, splenocytes were harvested and stimulated *in vitro* in the presence of one of three D^b-restricted LCMV viral peptides (gp33(C9M), gp276, or np396, as labeled). Following three days in culture, untouched or CD4-depleted splenocytes were used in a ⁵¹Cr release assay at a variety of E:T ratios. Targets were ⁵¹Cr-labeled ANA-1 cells (H-2^b) pulsed with either the stimulating LCMV peptide (solid traces), or an irrelevant D^b-binding peptide (dashed traces). Some of the splenocytes on the day of harvest, and after *in vitro* stimulation were collected, stained for TCR and CD4, and analyzed by flow cytometry.

Figure 6. D^b-gp33(C9M) tetramer binds more tightly to II-/8- T cells than B6 T cells
 II-/8- and B6 mice were infected with 500 pfu LCMV Armstrong i.p. Eight days later, splenocytes were harvested and stained across a range of concentrations of APC-D^b-gp33(C9M), as indicated. Intensity of tetramer staining was determined by flow cytometry (gated on T cells), and was plotted versus tetramer concentration (closed circles, II-/8-; open triangles, B6). Data were modeled by nonlinear regression curve fit (GraphPad Prism software).

Figure 7. CD8 lowers the TCR affinity requirement for positive selection

Our data demonstrate that CD8 is not necessary for CTL lineage commitment. The TCRs which facilitate positive selection in the absence of CD8 (II-/8- mice, solid trace) lie within an affinity range that is underrepresented in the TCR population. Thus, II-/8- mice have smaller thymi, and fewer peripheral T cells. The effect of CD8 coreceptor during normal thymic development (B6 mice, dashed trace) is to permit positive selection of TCR across a range of affinities that is better represented in the TCR population, generating a larger pool of T cells.