Clonal deletion and the fate of autoreactive thymocytes that survive negative selection

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Clonal deletion of autoreactive thymocytes is important for self-tolerance, but the intrathymic signals that induce clonal deletion have not been clearly identified. We now report that clonal deletion during negative selection required CD28-mediated costimulation of autoreactive thymocytes at the CD4+CD80 intermediate stage of differentiation. Autoreactive thymocytes were prevented from undergoing clonal deletion by either a lack of CD28 costimulation or transgenic overexpression of the antiapoptotic factors Bcl-2 or Mcl-1, with surviving thymocytes differentiating into anergic CD4−CD8− double-negative thymocytes positive for the T cell antigen receptor αβ subtype (TCRαβ) that 'preferentially' migrated to the intestine, where they were sequestered as CD8αα+ intraepithelial lymphocytes (IELs). Our study identifies costimulation by CD28 as the intrathymic signal required for clonal deletion and identifies CD8αα+ IELs as the developmental fate of autoreactive thymocytes that survive negative selection.

Immunocompetent αβ T cells must be reactive to foreign pathogens but tolerant of self-ligands. Those critical features of T cell immunity are imposed by selection events in the thymus that determine the developmental fate of each individual T cell depending on the specificity of its T cell antigen receptor (TCR). Differentiation in the thymus proceeds in an ordered sequence characterized by expression of the coreceptors CD4 and CD8 in which the earliest cells are CD4+CD8− (double-negative (DN)) thymocytes that differentiate into CD4+CD8+ (double-positive (DP)) thymocytes that then terminally differentiate into CD4+ or CD8+ (single-positive (SP)) T cells1,2. Thymocytes at the DP stage of differentiation are the first cells to express endogenous αβ TCR complexes and are the cells subjected to TCR-specific thymic selection. DP thymocytes are intrinsically short-lived cells whose continued survival requires signaling via the TCR by self ligands in the thymic cortex. TCR signaling rescues DP thymocytes from death by neglect and induces either positive or negative selection1,2. Positive selection is induced by low-affinity ligands and results in the differentiation of signaled DP thymocytes into conventional CD4+ SP (SP4) T cells or CD8+ SP (SP8) T cells with helper function or cytotoxic function, respectively, whereas negative selection is induced by high-affinity ligands that prevent DP thymocytes that have received TCR signaling from continuing their differentiation into conventional SP T cells3,4. Thus, the thymus imposes central tolerance by generating mature SP4 and SP8 T cells that express TCRs without substantial autoreactive potential.

The most definitive way of preventing autoreactive TCRs from appearing on mature SP T cells is the clonal deletion of DP thymocytes that bear autoreactive TCRs during negative selection in the thymus5. However, strong TCR signaling of DP thymocytes does not necessarily result in thymocyte death. Indeed, a few DP thymocytes are strongly signaled by agonist ligands to differentiate into specialized SP4 T cell subpopulations with regulatory or natural killer functions6,7, with such specialized differentiation referred to as ‘agonist selection’8. In a similar vein, developing DP thymocytes do not undergo clonal deletion when they receive strong signaling by agonist ligands in the thymic cortex9. Consequently, strong TCR stimulation of DP thymocytes during negative selection seems to be insufficient by itself to induce clonal deletion. However, it is not known what, if any, additional in vivo signals are needed during negative selection to induce thymocytes to undergo clonal deletion.

A potentially useful insight may have been provided by longstanding in vitro studies showing that costimulation by CD28 is needed to induce the death of thymocytes that have received strong TCR signaling10–13. Indeed in vitro costimulation by CD28 blocks upregulation of the antiapoptotic protein Bcl-2 by the TCR14, and transgenic overexpression of Bcl-2 rescues costimulated thymocytes from death induced by TCR signaling in vivo12,14. Although it has been observed only in vitro, a requirement for CD28 costimulation in thymocyte death that results from TCR signaling is potentially consistent with observations that clonal deletion is mediated by thymic dendritic cells and medullary thymic epithelial cells but not by cortical thymic epithelial cells that differ in their expression of the CD80-costimulatory ligands CD80 and CD86 (refs. 9,15–17). However, those in vitro results have been directly contradicted by multiple in vivo studies that investigated a role for CD28 costimulation in clonal deletion. Those studies have shown

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that autoreactive thymocytes are prevented from differentiating into either SP4 or SP8 T cells and that central tolerance is achieved regardless of the presence or absence of in vivo CD28 costimulation18–22.

Nonetheless, we undertook this study to determine if in vivo costimulation by CD28 during negative selection is important for either clonal deletion or central tolerance. Unlike the previously published in vivo studies18–22, we distinguished between negative selection and clonal deletion by considering the possibility that DP thymocytes that have received strong signaling and that bear autoreactive TCRs might survive negative selection even though they do not differentiate into conventional SP4 or SP8 T cells. In fact, we found that in vivo costimulation by CD28 during negative selection was required for autoreactive thymocytes to undergo clonal deletion but that neither costimulation by CD28 nor clonal deletion was critical for central tolerance. We also found that tolerance was induced during negative selection even in the absence of CD28-mediated clonal deletion because strong TCR signaling diverted DP thymocytes from differentiating into conventional SP T cells bearing autoreactive TCRs and directed their differentiation into TCRαβ+ DN thymocytes, a process we refer to as ‘developmental diversion’. Although developmentally diverted thymocytes expressed autoreactive TCRs, they were coreceptor negative and functionally anergic, which diminished their autoreactive potential. Moreover, when developmentally diverted TCRαβ+ cells were transferred into irradiated hosts, they recovered full TCR signaling in vivo and expressed homodimers of CD8α (CD8αα) and were sequestered in the gut as CD8αα+ intraepithelial lymphocytes (IELs). Thus, we have identified CD8αα+ IELs as the fate of autoreactive thymocytes that avoid clonal deletion during negative selection.

RESULTS

Clonal deletion versus rescue of autoreactive thymocytes

We began our assessment of intrathymic costimulation during negative selection by comparing thymocyte populations from wild-type mice and costimulation-deficient mice that lack either the costimulatory receptor CD28 (Cd28−/− mice) or its two costimulatory ligands CD80 (B7-1) and CD86 (B7-2; called ‘B7-deficient’ mice here). In our analysis, we specifically looked for a thymocyte subpopulation that was present in costimulation-deficient (Cd28−/− or B7-deficient) mice but was absent from wild-type mice. Costimulation-deficient mice on two different genetic backgrounds (C57BL/6 and BALB/c) had only modestly altered thymocyte numbers and a slightly greater frequency of DN cells relative to those of wild-type mice (Fig. 1a, top). However, costimulation-deficient mice showed substantial enrichment for a specific subset of DN thymocytes that were TCRαβ+ (Fig. 1a, bottom). Unlike DN thymocytes from wild-type mice, which included few TCRαβ+ cells that were mostly natural killer T cells, as determined by staining with tetramers of the antigen-presenting molecule CD1d, DN thymocytes from costimulation-deficient mice included a high frequency of TCRαβ+ cells that were not natural killer T cells (Fig. 1b,c).

If impaired clonal deletion in costimulation-deficient mice were the basis for greater abundance of TCRαβ+ DN thymocytes, then impairing clonal deletion in costimulation-sufficient mice should also result in more TCRαβ+ DN thymocytes. To test this prediction, we attempted to impair clonal deletion in wild-type mice through the use of transgenes encoding the antiapoptotic proteins Mcl-1 and Bcl-2 (Fig. 2a). Mcl-1-transgenic mice and Bcl-2-transgenic mice had substantially more TCRαβ+ DN thymocytes than did wild-type mice, and these thymocytes were CD5− (Fig. 2a,b), consistent with their having received strong signaling in vivo. Thus, thymocyte expression of transgenes encoding prosurvival molecules in wild-type mice had the same effect as costimulation deficiency—that is, more TCRαβ+ DN thymocytes—which suggested that the TCRαβ+ DN thymocyte subset contained cells that would otherwise have been clonally deleted.

To directly test the possibility that the TCRαβ+ DN subset was enriched for thymocytes bearing autoreactive TCRs, we examined mice expressing endogenous superantigens. BALB/c mice express the proviral proteins Mtv-6, Mtv-8 and Mtv-9, which specifically engage TCRs containing β-chain variable region 3 (Vβ3), Vβ5, Vβ11...
and Vβ12, so BALB/c thymocytes expressing those TCRs undergo clonal deletion. 
Indicative of TCRβ-specific clonal deletion in wild-type BALB/c mice, all TCRαβ+ thymocyte subsets (SP4, SP8 and DN) had considerably fewer thymocytes bearing the superantigen-reactive Vβ3+, Vβ5+ and Vβ11+ TCRs than did preselection DP thymocytes, whereas they had compensatorily more thymocytes bearing unreactive Vβ8+ TCRs (Fig. 3a and Supplementary Fig. 1a). In contrast to wild-type BALB/c thymocytes, costimulation-deficient BALB/c thymocyte populations bearing Mtv-8- and Mtv-9-reactive TCRs (Vβ5+ and Vβ11+) had a uniquely greater frequency and number of TCRαβ+ DN thymocytes but not SP4 or SP8 thymocytes (Fig. 3a). Thus, thymocytes bearing Mtv-8- and Mtv-9-reactive Vβ5+ and Vβ11+ TCRs were not deleted in costimulation-deficient BALB/c mice but instead appeared as TCRαβ+ DN thymocytes.

We additionally analyzed Mtv-6 reactive TCRs but were initially confused by a discrepancy in thymocytes from Cd28−/− mice and those from B7-deficient mice (Supplementary Fig. 1). SP4 and SP8 thymocytes of Cd28−/− BALB/c mice were deleted of Mtv-6 reactive Vβ3+ TCRs (Supplementary Fig. 1a), as noted for other superantigen-reactive TCRs (Fig. 3a), but SP4 and SP8 thymocytes in B7-deficient BALB/c mice were not deleted of these TCRs (Supplementary Fig. 1a), as reported before. Our attempts to understand the basis for this discrepancy led us to discover that the gene encoding Mtv-6 was absent from B7-deficient BALB/c mice (Supplementary Fig. 1b). This turned out to be due to the fact that this gene is on the same chromosomal segment as the genes encoding CD80 and CD86, which in B7-deficient mice was derived from embryonic stem cells of 129 origin that lacked the gene encoding Mtv-6. Consequently, B7-deficient mice did not have the gene encoding Mtv-6 in their genome, which explained the presence of thymocytes bearing the Vβ3+ TCR in all B7-deficient BALB/c thymocyte subsets, including SP4 and SP8 cells.

Having resolved the discrepancy noted above, we then sought to determine if transgenes encoding Mcl-1 and Bcl-2 would prevent the deletion of superantigen-reactive thymocytes in costimulation-sufficient wild-type mice. Both the Mcl-1 transgene and the Bcl-2 transgene did in fact prevent clonal deletion of thymocytes in wild-type mice, as the frequency and number of superantigen-reactive TCRs were greater among TCRαβ+ DN thymocytes in Mcl-1-transgenic mice and Bcl-2-transgenic mice than in nontransgenic wild-type mice, with the Bcl-2 transgene having a greater effect than the Mcl-1 transgene (Fig. 3b). On the basis of these results, we concluded that TCR signaling by high-affinity intrathymic ligands was sufficient to prevent autoreactive thymocytes from becoming SP thymocytes.
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but was not sufficient to induce clonal deletion, which additionally required intrathymic costimulation. In addition, we concluded that transgenes encoding the prosurvival molecules Mcl-1 and Bcl-2 prevented clonal deletion and that thymocytes rescued from clonal deletion appeared in the thymus as TCRαβ+ DN thymocytes.

**Derivation and selection of TCRαβ+ DN thymocytes**

All TCRαβ+ thymocytes are originally derived from TCRαβ- precursors that received signals from the pre-TCR to differentiate into DP thymocytes, and it is in DP thymocytes that endogenously encoded TCRαβ surface complexes are first expressed. Consequently, because TCRαβ+ DN thymocytes bear endogenously encoded TCRαβ surface complexes, it is likely that they are the progeny of DP thymocytes. To determine if TCRαβ+ DN thymocytes are indeed derived from DP thymocytes, we examined the methylation status of their Cd8b1 locus because it remains methylated until it is permanently demethylated when thymocytes first express CD8 to become DP cells. In thymocyte populations from Bcl-2-transgenic mice that were enriched for TCRαβ+ DN thymocytes, the Cd8b1 promoter was methylated in TCR- DN precursor thymocytes but was demethylated in DP thymocytes and in their post-selection SP4 and SP8 progeny (Fig. 4a and Supplementary Fig. 2). The Cd8b1 promoter was also demethylated in TCRαβ+ DN thymocytes (Fig. 4a and Supplementary Fig. 2), which showed that TCRαβ+ DN thymocytes were the progeny of coreceptor-positive (DP) thymocytes.

Next we determined whether the generation of TCRαβ+ DN thymocytes, like that of post-selection SP4 and SP8 thymocytes, required TCR-mediated thymic selection signals. Because TCR signaling in DP thymocytes is strictly dependent on the tyrosine kinase Zap70 (ref. 26), we assessed the effect of Zap70 deficiency on the appearance of TCRαβ+ DN thymocytes (Fig. 4b). B7−/− mice also deficient in Zap70 were devoid of TCRαβ+ DN thymocytes (Fig. 4b), which showed that the generation of TCRαβ+ DN thymocytes was strictly dependent on signals transduced by Zap70. We further determined whether the generation of TCRαβ+ DN thymocytes required intrathymic expression of major histocompatibility complexes (MHCs) that were both expressed in TCR− DN precursor thymocytes but were not sufficient to induce clonal deletion, which additionally required intrathymic costimulation. In addition, we concluded that transgenes encoding the prosurvival molecules Mcl-1 and Bcl-2 prevented clonal deletion and that thymocytes rescued from clonal deletion appeared in the thymus as TCRαβ+ DN thymocytes.

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TCRαβ+ DN required TCR-mediated, MHC-specific thymic selection signals. As thymic selection signals normally induce DP thymocytes to differentiate into coreceptor-positive (SP4 or SP8) thymocytes, we refer to the altered differentiation of DP cells into TCRαβ⁺ coreceptor-negative (DN) thymocytes as ‘developmental diversion’.

Developmental stage at which deletion and diversion occur

During positive selection, DP thymocytes that have received TCR signaling upregulate their surface expression of CD4 and downregulate their surface expression of CD8 to become phenotypically CD69⁺CD4⁺CD8⁻ intermediate thymocytes, and it is in intermediate thymocytes that differentiation into either the CD4⁺ lineage or the CD8⁺ lineage (CD4⁺CD8⁻ lineage choice) occurs. Consequently, we wondered if clonal deletion and developmental diversion also occur in intermediate thymocytes. To assess this possibility, we examined superantigen-reactive Vβ⁺ TCRs in CD69⁺CD4⁺CD8⁻ intermediate thymocytes from wild-type and costimulation-deficient BALB/c mice. Superantigen-reactive TCRs were present on intermediate thymocytes in both wild-type and costimulation-deficient BALB/c mice at frequencies that were essentially equal to those of preselection DP thymocytes (Fig. 5a). In fact, superantigen-reactive TCRs were present at a much greater frequency among intermediate thymocytes that had received TCR signaling than among SP4 or SP8 post-selection thymocytes (Fig. 5a), which indicated that DP thymocytes that had received strong signaling differentiated into intermediate thymocytes before undergoing clonal deletion or developmental diversion (Fig. 5a). That is, in wild-type BALB/c mice, superantigen-reactive TCRs were present in intermediate thymocytes, but all post-selection populations lacked such TCRs (Fig. 5a), which indicated that autoreactive thymocytes did not survive beyond the intermediate stage of differentiation. In costimulation-deficient BALB/c mice, superantigen-reactive TCRs were present in both intermediate and TCRαβ⁺ DN thymocytes, but SP4 and SP8 post-selection thymocytes lacked such TCRs (Fig. 5a), which indicated that autoreactive intermediate thymocytes were developmentally diverted into TCRαβ⁺ DN thymocytes. These results indicated that signals for both clonal deletion and developmental diversion were provided during negative selection at the intermediate stage of differentiation, but they do not formally exclude the possibility that some DP thymocytes might become DN cells directly.

For intermediate thymocytes to receive costimulation-dependent deletional signals, intermediate thymocytes must be in contact with B7-expressing cells. To determine if intermediate thymocytes were in fact in contact with B7-expressing cells in the thymus, we made use of the fact that CD28-B7 interactions specifically downregulate CD28 surface expression. In fact, surface expression of CD28 was upregulated during the differentiation of DP cells into intermediate and TCRαβ⁺ DN thymocytes in B7-deficient mice, whereas it was downregulated on cells from B7-sufficient mice (Fig. 5b and Supplementary Fig. 3), which showed that both intermediate thymocytes and TCRαβ⁺ DN thymocytes in B7-sufficient mice (which survived because of transgenic overexpression of Bcl-2) were in contact with B7-expressing cells in the thymus and received strong costimulation in vivo.

We concluded that CD4⁺CD8⁻ intermediate thymocytes were located in an area of the thymus where they were able to contact B7 costimulatory ligands. Furthermore, the intermediate stage of differentiation was a point in thymocyte development where clonal deletion and developmental diversion both occurred. Because the intermediate stage of differentiation is also the point in thymocyte development at which CD4⁺CD8⁻ lineage choice occurs, we suggest that it was in intermediate thymocytes that different TCR signals were ‘translated’ into different lineage fates, depending on the intensity and duration of TCR signaling as well as the presence or absence of costimulation (Supplementary Fig. 4).

Characterization of developmentally diverted thymocytes

Next we analyzed the phenotype of developmentally diverted TCRαβ⁺ DN thymocytes. This showed that during the differentiation of DP cells into developmentally diverted TCRαβ⁺ DN thymocytes, a variety of molecules were upregulated (TCRβ, PD-1, CD5, CD69, CD122, Bcl-2 and αβ), whereas other molecules were either unchanged or
Findings: their TCRs did not induce calcium mobilization (Fig. 5c and Supplementary Fig. 6a), they were unable to produce their own interleukin 2 (IL-2; Supplementary Fig. 6b) and they required the addition of exogenous IL-2 to proliferate in response to stimulation with antibody to TCR (anti-TCR) and anti-CD28 (Fig. 5d). We then considered whether PD-1 expression was required for developmental diversion. However, germline deletion of the gene encoding PD-1 did not affect the generation of developmentally diverted TCRαβ+ DN thymocytes in B7-deficient mice (Supplementary Fig. 6c). In addition, little or no surface expression of CD127 (IL-7 receptor α-chain) indicated that developmentally diverted TCRαβ+ DN thymocytes were probably not dependent on IL-7 for their in vivo survival (Supplementary Fig. 5a), unlike mature SP thymocytes and T cells. Indeed, despite their almost complete lack of CD127 expression, developmentally diverted TCRαβ+ DN thymocytes had high expression of Bcl-2 and were not apoptotic (< 0.05 and **P < 0.01 (Student’s two-tailed t-test)). (d) Proliferation of sorted TCRβ+ CD8αα+ IELs and SP4 lymph node T cells (LNT; 2.5 × 10^4 cells per well) stimulated with soluble monoclonal anti-CD3 (5 μg) and irradiated syngeneic antigen-presenting cells in the presence of medium alone or recombinant IL-2 (200 U/ml) or IL-15 (100 ng/ml), assessed as incorporation of [3H]thymidine. Data represent three independent experiments (mean and s.e.m.).

Developmentally diverted thymocytes become CD8αα+ IELs

To determine their in vivo developmental potential, we adoptively transferred TCRαβ+ DN thymocytes from B7-deficient donor mice...
into host mice deficient in recombination-activating gene 2 (Fig. 6a). At 5 weeks after transfer, most developmentally diverted TCRβ+ DN thymocytes had homed to the small intestine and differentiated into TCRβ+CD8αα+ IELs, whereas SP8 T cells remained mainly TCRβ+CD8αβ+ T cells regardless of their homing destination (Fig. 6a). Because IL-15 is present in the gut and because TCRβ+ DN thymocytes are potentially responsive to IL-15 because of their expression of CD122 and CD132, we wondered if IL-15 might contribute to the differentiation of TCRβ+ DN thymocytes into TCRβ+CD8αα+ cells. To assess this, we stimulated developmentally diverted TCRβ+ DN thymocytes in vitro with anti-TCR and IL-15 (Fig. 6b). Indeed, by day 4 of culture, most TCRβ+ DN thymocytes that received such signaling had differentiated into TCRβ+CD8αα+ cells (Fig. 6b).

The results presented above showed that developmentally diverted TCRβ+ DN thymocytes had the potential to become TCRβ+CD8αα+ IELs, and they resembled results obtained with wild-type TCRβ+ DN thymocytes9, but we did not know if developmentally diverted TCRβ+ DN thymocytes actually differentiated into TCRβ+CD8αα+ IELs in vivo. To test this, we took advantage of the finding that TCRβ+CD8αα+ IELs were nearly completely absent from C57BL/6 mice deficient in β2-microglobulin (B2m−/− mice; Fig. 6c). We crossed B2m−/− mice with B7-deficient or Bcl-2-transgenic mice, each of which had a substantial frequency of developmentally diverted thymocytes (Figs. 1 and 2). TCRβ+CD8αα IELs were present in the B7-deficient or Bcl-2-transgenic B2m−/− progeny of each cross (Fig. 6c), which demonstrated that developmentally diverted T cells did in fact differentiate in vivo into TCRβ+CD8αα+ IELs. To confirm this, we sought to determine if TCRβ+CD8αα+ IELs displayed the same TCR Vβ repertoire as that displayed by developmentally diverted TCRβ+ DN thymocytes. Indeed, in B7-deficient BALB/c mice, TCRs expressing superantigen-reactive Vβ molecules were over-represented at essentially identical frequencies in both TCRβ+CD8αα+ IELs and TCRβ+ DN thymocytes (Fig. 7a). We concluded that developmentally diverted TCRβ+ DN thymocytes differentiated in vivo into TCRβ+CD8αα+ IELs.

Developmental diversion and the origin of CD8αα+ IELs

Having shown that developmentally diverted TCRβ+ DN thymocytes differentiated in vivo into TCRβ+CD8αα+ IELs, we wondered if all TCRβ+CD8αα+ IELs might be derived from cells that had undergone developmental diversion during negative selection in the thymus. If so, mice in which thymic clonal deletion was absent would have more TCRβ+CD8αα+ IELs than would wild-type mice. In fact, costimulation-deficient mice had many more TCRβ+CD8αα+ IELs than wild-type mice had (about ten times more; Fig. 7b), which suggested that developmental diversion was the main origin of TCRβ+CD8αα+ IELs. We then wondered if the TCRβ+CD8αα+ IELs present in normal wild-type mice were also the progeny of thymocytes that had survived negative selection and had undergone developmental diversion. In fact, we found that even in wild-type mice, TCRβ+CD8αα+ IELs specifically overexpressed superantigen-reactive TCRs, but other T cell populations did not (Fig. 7c). That finding suggested that in normal wild-type mice, substantial numbers of autoreactive T cells avoided clonal deletion and underwent developmental diversion, a conclusion that was also consistent with our observation that the frequency of superantigen-reactive TCRs in wild-type mice was always greater in TCRβ+ DN thymocytes than in SP4 or SP8 thymocytes (Figs. 3a and 5a and Supplementary Fig. 1a).

The over-representation of autoreactive TCR specificities in CD8αα+ IELs in normal and experimental mice, none of which were auto-immune, made us question the TCR responsiveness of CD8αα+ IELs. In fact, CD8αα+ IELs resembled TCRβ+ DN thymocytes in being unresponsive to stimulation with anti-TCR and antigen-presenting cells in vitro without exogenous cytokines (Fig. 7d). We concluded that TCRβ+CD8αα+ IELs were the progeny of cells that survived negative selection and underwent developmental diversion in the thymus.

Role of the transcription factor Runx3

Finally, we sought to gain molecular insight into the differentiation of developmentally diverted TCRβ+ DN thymocytes into CD8αα+ IELs. Because such differentiation requires reactivation of Cd8α expression, which is a known function of the transcription factor Runx3 (ref. 30), we considered that Runx3 might be required for this differentiation. To analyze this possibility, we used Bcl-2-transgenic mice also heterozygous for an endogenous Runx3 allele re-engineered to encode yellow fluorescent protein (YFP) instead of Runx3 (Runx3+YFP mice)31. Developmentally diverted TCRβ+ DN thymocytes in these Bcl-2-transgenic Runx3+YFP mice were YFP+, whereas TCRβ+CD8αα+ IELs were YFP− (Fig. 8a), which indicated that Runx3 expression was upregulated at some point during the differentiation of TCRβ+ DN thymocytes into TCRβ+CD8αα+ IELs.

To determine if that point was related to the reactivation of Cd8α, we stimulated TCRβ+ DN thymocytes in vitro with anti-TCR and IL-15 (Fig. 8b). After that in vitro stimulation, TCRβ+ DN thymocytes expressed Runx3 and differentiated into CD8αα+ cells (Fig. 8b). To determine if their differentiation into CD8αα+ cells required Runx3, we used Bcl-2-transgenic Runx3+YFP mice, which were Runx3−/− because they had two Runx3 alleles re-engineered to encode YFP instead of Runx3 (Fig. 8b). In vitro stimulation of TCRβ+ DN thymocytes that were Runx3−/− did not induce their differentiation into CD8αα+ cells, although it did induce YFP expression (Fig. 8b). Thus, Runx3 was required for the reactivation of Cd8α expression by developmentally diverted TCRβ+ DN thymocytes and for their differentiation into CD8αα+ cells in vitro. Applying those in vitro observations to the developmental fate of TCRβ+ DN thymocytes in vivo, we wondered if the differentiation of developmentally diverted TCRβ+ DN thymocytes into TCRβ+CD8αα+ IELs would be impaired in Runx3−/−/− mice because they were Runx3−/−. Indeed, Bcl-2-transgenic Runx3−/−/− (Runx3−/−) mice had significantly fewer TCRβ+CD8αα+ IELs than did Bcl-2-transgenic Runx3−/−/− (Runx3−/−) mice, even though they had equal numbers of developmentally diverted TCRβ+ DN thymocytes (Fig. 8c). We concluded that TCRs and IL-15 stimulated developmentally diverted TCRβ+ DN thymocytes to express Runx3, which promoted the reactivation of Cd8α and the differentiation of developmentally diverted TCRβ+ DN thymocytes into TCRβ+CD8αα+ IELs. Thus, we were able to integrate lineage choice during positive selection and lineage fate during negative selection into a unified picture of thymic development in which the developmental fate of TCR-signalized DP thymocytes is determined by TCR and costimulatory signals (Supplementary Fig. 7).

DISCUSSION

Here we have identified costimulatory signals from CD28 as being necessary for thymocytes to undergo clonal deletion during negative selection in vivo and have demonstrated that neither costimulation by CD28 nor clonal deletion was required for self-tolerance. Regardless of the presence or absence of CD28-mediated clonal deletion, strong TCR signaling prevented DP thymocytes bearing autoreactive TCRs from differentiating into conventional SP4 or SP8 T cells, so the absence of autoreactive TCRs on SP T cells was indicative of in vivo
negative selection but was not necessarily indicative of in vivo clonal deletion. Indeed, DP thymocytes that had received strong signaling and bore autoreactive TCRs that survived negative selection did not differentiate into conventional SP T cells but instead underwent developmental diversion and differentiated into TCROβ+ DN thymocytes. Developmentally diverted TCROβ+ DN thymocytes, after leaving the thymus, migrated mainly to the intestine and received signaling via IL-15 to express Runx3 and to further differentiate into Runx3+CD8αα+ IELs. Clonal deletion and developmental diversion occurred during negative selection at the intermediate CD4hiCD8lo thymocyte stage of differentiation, the same point in thymocyte development at which lineage choice occurs during positive selection5.

Thus, our study has distinguished in vivo negative selection from in vivo clonal deletion, has identified costimulation by CD28 as the in vivo signal required for clonal deletion during negative selection, and has identified TCROβ+CD8αα+ IELs as the ultimate fate of autoreactive cells that survive negative selection in the thymus.

During thymic selection, DP thymocytes that have received weak TCR signaling undergo positive selection into SP4 or SP8 T cells, whereas most DP thymocytes that have received strong TCR signaling undergo negative selection, which prevents them from differentiating into SP4 or SP8 T cells. Notably, as we have shown here, the absence of autoreactive TCR specificities among in vivo SP T cells was indicative of negative selection but was not necessarily indicative of clonal deletion. Consequently, our study has provided a new conceptual model of thymic selection in which ‘positive selection’ is the differentiation of signaled DP thymocytes into SP T cells and ‘negative selection’ is the prevention of their differentiation into SP T cells; ‘developmental diversion’ refers to their differentiation into mature T cells that are neither SP4 nor SP8 but are DN; and ‘clonal deletion’ refers to their death before maturation.

We used specific Vβε TCRs to monitor the developmental fate of DP thymocytes that had received strong signaling by endogenously encoded proviral antigens of the Mtv family. DP thymocytes are the first cells in the thymus to express endogenously encoded TCROβ complexes and are the cells that are subjected to thymic selection. As observed before23, DP thymocytes bearing superantigen-reactive TCRs underwent negative selection, as they did not differentiate into SP4 or SP8 T cells. However, our study has documented that clonal deletion of superantigen-reactive thymocytes during negative selection additionally required costimulation by CD28. In the absence of CD28 costimulation, superantigen-reactive thymocytes survived negative selection and underwent developmental diversion to TCROβ+ DN thymocytes. Similarly, superantigen-reactive thymocytes that were prevented from undergoing clonal deletion by the expression of a transgene encoding Bcl-2 or Mcl-1 also survived negative selection and underwent developmental diversion into TCROβ+ DN thymocytes. Thus, costimulation by CD28 during negative selection was required for in vivo clonal deletion, which was prevented by transgenic expression of either Bcl-2 or Mcl-1.

The developmental diversion of surviving autoreactive thymocytes to TCROβ+ DN thymocytes precluded autoreactivity by removing the contribution of the coreceptors CD4 and CD8 to TCR signaling, which lessened their in vivo autoreactive potential, as CD8αα surface complexes have been suggested to sequester the signaling kinase Lck away from the TCR32. Indeed, costimulation-deficient mice were free of autoimmunity despite our finding that they had many CD8αα+ IELs bearing autoreactive TCR specificities and despite their lack of functionally suppressive Foxp3+ regulatory T cells33.

Developmental diversion occurs in TCR-signaled CD4hiCD8lo intermediate thymocytes that transcribe Cd4 but lack transcription of Cd8, with the result that differentiation into TCROβ+ DN thymocytes during negative selection requires only the termination of Cd4 expression. In fact, in intermediate thymocytes that transcribe Cd4 but lack transcription of Cd8, such termination occurs during MHCI class I-specific positive selection into SP8 T cells, but in that case it is mediated by the transcription factor Runx3 (refs. 30,34), which additionally induces reactivation of Cd8 to result in ‘coreceptor reversal’35. In contrast, developmental diversion into TCROβ+ DN thymocytes did not involve or require Runx3, which suggested that strong TCR stimulation of intermediate thymocytes during negative selection terminates Cd4 transcription independently of Runx3 and without reactivation of Cd8. Notably, Runx3-independent termination of Cd4 transcription by strong TCR signaling in intermediate thymocytes that transcribe Cd4 but lack transcription of Cd8 would also explain why negatively selected thymocytes become DN cells instead of SP4 T cells.

By identifying developmental diversion and clonal deletion as alternative outcomes of negative selection, our study has resolved many longstanding experimental contradictions. Published in vitro experiments have demonstrated that thymocyte death as a result of TCR signaling requires CD28 costimulation10–14 and can be prevented by transgenic Bcl-2 expression12,14, whereas published in vivo experiments have arrived at opposite conclusions18–22. Our study here has shown that those contradictory observations were due mainly to the presumption that autoreactive thymocytes had been clonally deleted in vivo if they failed to differentiate into either SP4 or SP8 T cells. In fact, here we have documented that the absence of autoreactive TCR specificities on SP T cells in vivo indicated that autoreactive thymocytes had undergone negative selection but did not indicate that they had undergone clonal deletion. Our study has also resolved the discrepant observations that genetic deletion of the proapoptotic protein Bim interferes with clonal deletion36, whereas transgenic overexpression of the antiapoptotic protein Bcl-2 does not20. In fact, many features of Bim-deficient mice37–39 resemble those of the Bcl-2-transgenic and Mcl-1-transgenic mice we studied here; thus, the results of our study would explain the high frequency of TCROβ+ DN thymocytes in mice deficient in Bim or the proapoptotic protein Puma37,40,41 as resulting from their being autoreactive thymocytes that survived negative selection and underwent developmental diversion. Our study has also explained why SP4 and SP8 T cells bearing Mtv-6 reactive Vβε TCRs have been noted before in B7-deficient BALB/c mice but not Cd28−/− BALB/c mice24. The discrepancy between these mice does not indicate an as-yet-unknown B7-specific receptor that signals clonal deletion in Cd28−/− BALB/c mice24 but is instead due to the fact that Mtv-6 is not encoded by the genome of B7-deficient BALB/c mice.

Although it has resolved many longstanding experimental contradictions, our study does contradict the experimental finding obtained with perinatal mice showing that blockade of costimulation by in vivo injection of anti-B7 rescues superantigen-reactive TCRs that are then expressed on SP4 T cells42. In our study here, genetic deletion of B7 ligands in B7-deficient mice also rescued superantigen-reactive TCRs; however, the rescued TCRs were not expressed on SP4 T cells but instead were expressed only on developmentally diverted TCROβ+ DN thymocytes.
DN thymocytes and CD8α+ IELs. We think the explanation for this disparity is that injection of anti-B7 depletes the thymus of superantigen-bearing B7+ cells and thus eliminates intrathymic expression of the negative selecting (superantigen) ligand.

Does developmental diversion occur in normal wild-type (costimulation-sufficient) mice? Costimulation-sufficient TCR-transgenic mice have a substantial number of TCRαβ+ DN thymocytes47,48 that can become CD8α+ high-affinity ligands in the thymic cortex47,48. Notably, we found that mentally arrested because their transgenically expressed TCR engaged TCR-transgenic mice are post-selection cells that result from developmentally directed or are preselection DN thymocytes that were developmentally arrested because their transgenically expressed TCR engaged high-affinity ligands in the thymic cortex47,48. Notably, we found that in normal, nontransgenic mice, many or all TCRαβ+ IELs were the progeny of thymocytes that survived negative selection and underwent developmental diversion in the thymus. Indeed, normal wild-type mice had a substantial number of TCRαβ+CD8α+ IELs (~5 x 10^5) in which TCRs with autoreactive specificities were over-expressed, which indicated that a substantial number of autoreactive T cells avoided clonal deletion and underwent developmental diversion in normal mice.

In conclusion, by distinguishing in vivo negative selection from in vivo clonal deletion, we have identified costimulation by CD28 as being critical for clonal deletion and have identified developmental diversion as an alternative outcome of negative selection. Moreover, we have identified TCRαβ+CD8α+ IELs as the ultimate fate of autoreactive T cells that survived negative selection and underwent developmental diversion in the thymus. Also, by showing that the fate of thymocytes undergoing negative selection was determined at the same point in differentiation as that of thymocytes undergoing positive selection, our study has integrated negative selection and positive selection into a unified picture of thymic selection.

METHODS
Methods and any associated references are available in the online version of the paper.

Note: Supplementary information is available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
L.A.P. designed the study, did experiments, analyzed data and contributed to the writing of the manuscript; G.S.A., X.T., S.J. and E.V.L. did experiments and analyzed data; J.-H.P. and L.E. generated transgenic mice, and A.S. designed the study, analyzed data and wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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Animals. BALB/c, C57BL/6, Cd28−/−, B2m−/−, Ab−/− b2m−/−, Zap70−/−, Rag2−/− and human Bcl-2–transgenic mice were maintained in our own colony at the US National Institutes of Health. Cd80−/−Cd86−/− (B7-deficient) mice were provided by A. Sharpe. Runx3-YFP reporter mice were provided by D. Littman and Pdcd1−/− mice were provided by T. Honjo. The Mcl-1–transgenic construct was made by ligation of cDNA encoding Mcl-1 into a vector based on the human CD2 enhancer-promoter and was injected into fertilized C57BL/6 oocytes for the generation of Mcl-1–transgenic mice. All mice were cared for in accordance with the guidelines of the US National Institutes of Health.

Flow cytometry. Monoclonal antibodies with the following specificities were used: CD4 (GK1.5 and RM4.5), CD5 (53-7.3), CD8α (53-6.7), CD8β (H35-17.2), TCRβ (GL3), TCRβ (H57-597), Vβ3 (KJ25), Vβ4 (KT4), Vβ5.1-5.2 (MR9-4), Vβ6 (RR4-7), Vβ8 (F23.1), Vβ10 (B2.15), Vβ11 (RR3-15), Vβ12 (MR11-1), NK.1.1 (PK136), PD-1 (J43), CD132 (4G3), CD122 (TM-β1), CD44 (IM7), CD69 (H1.2F3), Bcl-2 PE-set (3F11), IL-4Rα (mL4R-M1), IL-7Rα (SB/199), CD25 (PC61) or CD28 (37.51; all from BD Pharmingen); Bcl-XL (54H6) or CD8α (5H10; Invitrogen). The Annexin-V FLUOS staining kit was from Roche Applied Science. PBS57–loaded or unloaded CD1d tetramers (5-10 μg/ml) and monoclonal anti-CD1d were provided by A. Sharpe. Runx3-YFP reporter mice were provided by T. Honjo. The Mcl-1–transgenic construct was made by ligation of cDNA encoding Mcl-1 into a vector based on the human CD2 enhancer-promoter and was injected into fertilized C57BL/6 oocytes for the generation of Mcl-1–transgenic mice. All mice were cared for in accordance with the guidelines of the US National Institutes of Health.

Calcium mobilization. Cells were loaded at 31 °C with the calcium-sensitive dye Indo-1 (1.8 μM; Invitrogen) and then coated at 4 °C with biotinylated anti-TCR. Cells were warmed for 2 min before stimulation and then applied to the flow cytometer. Antibody crosslinking was induced with avidin (4 μg/ml, Sigma) and data acquisition was recorded for 5 min.

Adoptive transfer. Purified TCRβ+ DN thymocytes or CD8+CD5+ lymph node T cells (0.8 × 10⁶) were injected into host mice deficient in recombination-activating genes. Five weeks after transfer, cells from the thymus, peripheral lymphoid organs and small intestine were isolated and analyzed by flow cytometry.

In vitro T cell proliferation and differentiation cultures. For in vitro differentiation, purified TCRβ+ DN thymocytes were cultured for 4–5 d in medium only or were stimulated for 4–5 d with immobilized monoclonal anti-TCRβ (5-10 μg/ml) in the presence of IL-15 (100 ng/ml; R&D Systems), then were collected and analyzed by flow cytometry. For in vitro proliferation, sorted TCRβ+ DN and SP8 thymocytes from B7-deficient mice were stimulated for 72 h with immobilized monoclonal anti-TCRβ (5 μg/ml) and monoclonal anti-CD28 (10 μg/ml) in the presence or absence of recombinant IL-2 (200 U/ml). For in vitro proliferation of IELs, sorted TCRβ+CD8α+ and SP4 lymph node T cells were stimulated for 48 h with anti-CD3 (5 μg/ml) in the presence of 1 × 10⁶ antigen-presenting cells (syngenic splenocytes irradiated with 3,000 rads) in the presence or absence of recombinant IL-2 (200 U/ml) or IL-15 (100 ng/ml). Cultures were pulsed with [3H]thymidine (1 μCi) 8 h before collection. For analysis of intracellular IL-2 production, T cells that had been stimulated for 4 d were treated for 4 h with phorbolester 12-myristate 13-acetate (50 ng) and ionomycin (500 nM) in the presence of protein-transport inhibitor (BD Biosciences), followed by intracellular staining for IL-2.

Genomic PCR. PCR analysis of DNA from the mouse tail was used for the detection of genomes of integrated Mtv-6 and Mtv-9 proviruses with the following primers: Mtv-6 forward, 5′-GCTGGCTATCATCACAAGGCG-3′, and reverse, 5′-GGAGTTCAACCATTTCTGCTGC-3′; Mtv-9 forward, 5′-GCCAGTCAAGAACAGGTC-3′, and reverse, 5′-CAGGAAGACCCTTGTCTCACATCC-3′. DNA-methylation analysis. Methylation was analyzed as described50. DNA was isolated with the ZR genomic DNA II kit (Zymo Research). Bisulfite conversion of total DNA was achieved with the EZ-DNA methylation-Gold kit (Zymo Research). PCR products were purified and cloned with the TOPO-TA cloning kit. Plasmid DNA from MiniPrep (Qiagen) was sequenced.

Statistical analysis. Statistical significance was determined by Student’s t-test with two-tailed distribution.