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

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Summary

Clonal deletion of autoreactive thymocytes is important for self-tolerance, but the intra-thymic signals that induce clonal deletion have not been clearly identified. We now report that clonal deletion during negative selection requires CD28 costimulation of autoreactive thymocytes at the CD4^{+}CD8^{lo} intermediate stage of differentiation. Autoreactive thymocytes were prevented from undergoing clonal deletion by either absent CD28 costimulation or transgenic over-expression of the anti-apoptotic factors Bcl-2 or Mcl-1, with surviving thymocytes differentiating into anergic T cell receptor αβ^{+} double negative thymocytes that preferentially migrated to the intestine where they re-expressed CD8α and were sequestered as CD8αα intraepithelial lymphocytes (IELs). This study identifies CD28 costimulation as the intrathymic signal required for clonal deletion and identifies CD8αα IELs as the developmental fate of autoreactive thymocytes that survive negative selection.

Keywords

tolerance; autoimmunity; CD8αα; IEL; Runx3

Immunocompetent αβ T cells must be reactive to foreign pathogens but tolerant to self ligands. These 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 CD4 and CD8 coreceptor expression 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 cells[1, 2]. Thymocytes at the DP stage of differentiation are the first

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cells to express endogenous αβTCR complexes and are the cells subjected to TCR-specific thymic selection. DP thymocytes are intrinsically short-lived cells whose continued survival requires TCR signaling by self-ligands in the thymic cortex. TCR signaling rescues DP thymocytes from 'death-by-neglect' and induces either positive or negative selection [1, 2]. Positive selection is induced by low affinity ligands and results in the differentiation of TCR-signaled DP thymocytes into conventional SP4 or SP8 T cells possessing helper or cytotoxic function respectively, whereas negative selection is induced by high affinity ligands that prevent TCR-signaled DP thymoctyes from continuing their differentiation into conventional SP T cells[3, 4]. Thus, the thymus imposes central tolerance by generating conventional SP4 and SP8 mature T cells that express TCRs lacking significant autoreactive potential.

The most definitive way of preventing autoreactive TCRs from appearing on mature SP T cells is the clonal deletion of DP thymocytes bearing autoreactive TCRs during negative selection in the thymus[5]. 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 possessing regulatory or NK functions [6, 7], with such specialized differentiation referred to as 'agonist selection'[8]. In a similar vein, developing DP thymocytes do not undergo clonal deletion when strongly signaled by agonist ligands in the thymic cortex [9]. Consequently, strong TCR signaling of DP thymocytes during negative selection appears 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 which demonstrated that CD28 costimulation was required to induce strongly TCR-signaled thymocytes to die[10–13]. Indeed *in vitro* CD28 costimulation blocked TCR up-regulation of the anti-apoptotic protein Bcl-2[14], and transgenic Bcl-2 (Bcl-2 TG) over-expression rescued costimulated thymocytes from TCR-signaled death *in vitro* [12, 14]. Although only observed *in vitro*, a requirement for CD28 costimulation in TCR-signaled thymocyte death was potentially consistent with observations that clonal deletion was mediated by thymic dendritic cells and medullary thymic epithelial cells which differed from non-deleting cortical thymic epithelial cells in high expression of the CD28 costimulatory ligands CD80 and CD86 [9, 15–17]. However, these *in vitro* results were directly contradicted by multiple *in vivo* studies that examined a role for CD28 costimulation in clonal deletion. These studies showed that autoreactive thymocytes were prevented from differentiating into either SP4 or SP8 SP T cells and that central tolerance was achieved regardless of the presence or absence of *in vivo* CD28 costimulation[18–22].

Nonetheless, we undertook the present study to determine if *in vivo* CD28 costimulation during negative selection was important for either clonal deletion or central tolerance. Unlike previous *in vivo* studies, we distinguished between negative selection and clonal deletion by considering the possibility that strongly signaled DP thymocytes bearing autoreactive TCRs might survive negative selection even though they do not differentiate into conventional SP4 or SP8 T cells. In fact we now report that *in vivo* CD28 costimulation during negative selection is required for autoreactive thymocytes to undergo clonal deletion,

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but that neither CD28 costimulation nor clonal deletion is critical for central tolerance. We report that tolerance can be induced during negative selection even in the absence of CD28-mediated clonal deletion because strong TCR signaling diverts DP thymocytes from differentiating into conventional SP T cells bearing autoreactive TCRs and directs their differentiation into TCRαβ$^+$DN thymocytes, a process we refer to as 'developmental diversion.' Although developmentally diverted thymocytes express autoreactive TCRs, they are coreceptor-negative and functionally anergic which diminishes their autoreactive potential. Moreover, when developmentally diverted TCRαβ$^+$DN thymocytes leave the thymus, they preferentially migrate to the intestine where they are signaled to express CD8αα coreceptor homodimers and are sequestered in the gut as CD8αα intra-epithelial lymphocytes (IELs). Thus, this is the first study to identify CD28 as critical for inducing autoreactive thymocytes to undergo clonal deletion during *in vivo* negative selection and additionally identifies 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 wildtype and costimulation-deficient mice that lacked either the costimulatory receptor CD28 or its two costimulatory ligands CD80 and CD86 (also known as B7.1 and B7.2). We refer to mice genetically deficient in CD28 as *Cd28*$^{-/-}$ mice and to mice genetically deficient in both CD80 and CD86 as B7-deficient (B7-DKO) mice. In our analysis, we specifically looked for a thymocyte subpopulation that was present in costimulation-deficient (*Cd28*$^{-/-}$ and B7-DKO) mice but was absent in wildtype mice. Costimulation-deficient mice on two different genetic backgrounds (C57BL/6 and BALB/c) had only modestly changed thymocyte numbers and modestly increased frequencies of CD4$^-$CD8$^-$ (DN) cells compared to wildtype mice (Fig. 1a top panels). However, costimulation-deficient mice were substantially enriched in a specific subset of DN thymocytes that were TCRαβ$^+$ (Fig. 1a bottom panels). Unlike DN thymocytes from wildtype mice which contained few TCRαβ$^+$ cells that were mostly NKT cells as determined by CD1d tetramer staining, DN thymocytes from costimulation-deficient mice contained high frequencies of TCRαβ$^+$ cells that were not NKT cells (Fig. 1b,c).

If impaired clonal deletion in costimulation-deficient mice were the basis for increased TCRαβ$^+$DN thymocytes, then impairing clonal deletion in costimulation-sufficient mice should also increase TCRαβ$^+$DN thymocytes. To test this prediction, we attempted to impair clonal deletion in wildtype mice with transgenes encoding anti-apoptotic proteins Mcl-1 and Bcl-2 (Fig. 2a). Mcl-1 and Bcl-2 transgenes substantially increased TCRαβ$^+$DN thymocytes in wildtype mice and these thymocytes were CD5^{hi} (Fig. 2a,b), consistent with their having been strongly signaled *in vivo*. Thus, thymocyte expression of pro-survival transgenes in wildtype mice had the same effect as costimulation-deficiency in increasing TCRαβ$^+$DN thymocytes, suggesting that the TCRαβ$^+$DN thymocyte subset contained cells that would otherwise have been clonally deleted.

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To directly test the possibility that the TCRαβ^{+}DN subset was enriched in thymocytes bearing autoreactive TCRs, we examined mice expressing endogenous super-antigens (SAg). BALB/c mice encode proviral proteins Mtv-6, -8, and -9 which specifically engage Vβ3, Vβ5, Vβ11, and Vβ12 TCRs so that BALB/c thymocytes expressing these TCRs undergo clonal deletion[23]. Indicative of TCRβ-specific clonal deletion in wildtype BALB/c mice, thymocytes bearing the SAg-reactive TCRs Vβ3, Vβ5, and Vβ11 were substantially reduced in all TCRαβ^{+} thymocyte subsets (SP4, SP8, and DN) compared to pre-selection DP thymocytes, whereas thymocytes bearing unreactive Vβ8 TCRs were compensatorily increased (Fig. 3a, and Supplementary Fig. 1a). In contrast to wildtype BALB/c thymocytes, costimulation-deficient BALB/c thymocytes bearing Mtv-8, 9-reactive TCRs (Vβ5 and Vβ11) were uniquely increased in both frequency and number in TCRαβ^{+}DN thymocytes, though not in SP4 or SP8 thymocytes (Fig. 3a). Thus, thymocytes bearing Mtv-8, 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 between thymocytes from *Cd28*$^{-/-}$ and B7-deficient mice (Supplementary Fig. 1). In *Cd28*$^{-/-}$ BALB/c mice, the frequency and number of thymocytes bearing Mtv-6 reactive Vβ3 TCRs were depleted in SP4 and SP8 thymocytes (Supplementary Fig. 1a), as we observed with other SAg-reactive TCRs (Fig. 3a), but they were not depleted in B7-deficient BALB/c mice (Supplementary Fig. 1a), as was previously reported[24]. Our attempts to understand the basis for this discrepancy led us to discover that the gene encoding Mtv-6 was absent in B7-deficient BALB/c mice (Supplementary Fig. 1b). This turned out to be due to the fact that the Mtv-6 gene is on the same chromosomal segment as genes encoding CD80 and CD86 which, in B7-deficient mice, was derived from embryonic stem cells of 129 origin that lacked the proviral Mtv-6 gene. Consequently, B7-deficient mice do not contain the Mtv-6 gene in their genome, explaining the presence of TCR-Vβ3 thymocytes in all B7-deficient BALB/c thymocyte subsets, including SP4 and SP8.

Having resolved this discrepancy, we then wondered if Mcl-1 and Bcl-2 transgenes would prevent deletion of SAg-reactive thymocytes in costimulation-sufficient wildtype mice. Both Mcl-1 and Bcl-2 transgenes did in fact prevent clonal deletion of thymocytes in wildtype mice, as the frequency and number of SAg-reactive TCRs were increased among TCRαβ^{+}DN thymocytes, with the Bcl-2 transgene having a greater effect than the Mcl-1 transgene (Fig. 3b).

Based on these results, we conclude that TCR signaling by high affinity intrathymic ligands is sufficient to prevent autoreactive thymocytes from becoming SP thymocytes, but it is not sufficient to induce clonal deletion which additionally requires intrathymic costimulation. In addition we conclude that Mcl-1 and Bcl-2 pro-survival transgenes prevent clonal deletion, and that thymocytes rescued from clonal deletion appear in the thymus as TCRαβ^{+}DN thymocytes.

Derivation and selection of TCRαβ^{+} DN thymocytes

All TCRαβ^{+} thymocytes originally derive from TCR^{-}DN precursor thymocytes that were signaled by pre-TCR to differentiate into DP thymocytes, and it is in DP thymocytes that

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endogenously encoded TCRαβ surface complexes are first expressed. Consequently, because TCRαβ^{+}DN thymocytes bear endogenously encoded TCRαβ surface complexes, it was likely that they were the progeny of DP thymocytes. However, to assess if TCRαβ^{+}DN thymocytes were indeed derived from DP thymocytes, we examined the methylation status of their *Cd8b* gene locus because it remains methylated until it is permanently de-methylated when thymocytes first express CD8 to become DP[25]. In thymocytes from Bcl-2 transgenic mice that were enriched in TCRαβ^{+}DN thymocytes, the *Cd8b* promoter was methylated in TCR^{-}DN precursor thymocytes but de-methylated in DP thymocytes and in their post-selection SP4 and SP8 progeny (Fig. 4a and Supplementary Fig. 2). The *Cd8b* promoter was also de-methylated in TCRαβ^{+}DN thymocytes (Fig. 4a and Supplementary Fig. 2), revealing that TCRαβ^{+}DN thymocytes were the progeny of coreceptor-positive, i.e. DP, thymocytes.

Next, we assessed if 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 protein tyrosine kinase ZAP70[26], we examined the impact of ZAP70-deficiency on the appearance of TCRαβ^{+}DN thymocytes (Fig. 4b left panels). Costimulation-deficient and ZAP70-deficient (B7-DKO *Zap70*$^{-/-}$) mice were devoid of TCRαβ^{+}DN thymocytes (Fig. 4b left panels), documenting that the generation of TCRαβ^{+}DN thymocytes was strictly dependent on signals transduced by ZAP70. We further assessed if generation of TCRαβ^{+}DN thymocytes required intrathymic MHC expression because TCR-signaled thymic selection is MHC-specific. Costimulation-deficient *Cd28*$^{-/-}$ mice that were additionally MHC-KO (*Cd28*$^{-/-}$*B2m*$^{-/-}$*H2-Ab1*$^{-/-}$) were essentially devoid of TCRαβ^{+}DN thymocytes, revealing that both MHC and ZAP70 expression was required for *in vivo* generation of TCRαβ^{+}DN thymocytes (Fig. 4b right panels).

We conclude that TCRαβ^{+}DN thymocytes are the progeny of DP thymocytes and that differentiation of DP into TCRαβ^{+}DN thymocytes requires TCR-mediated, MHC- specific thymic selection signals. Since thymic selection signals normally induce DP thymocytes to differentiate into coreceptor-positive SP4 or SP8 thymocytes, we refer to the altered differentiation of strongly signaled DP into coreceptor-negative TCRαβ^{+}DN thymocytes as ‘developmental diversion’.

Developmental stage that deletion and diversion occur

During positive selection, TCR-signaled DP thymocytes upregulate surface CD4 and downregulate surface CD8 to phenotypically become CD69^{+}CD4^{hi}CD8^{lo} intermediate (INT) thymocytes, and it is in INT thymocytes that CD4-CD8 lineage choice occurs [1]. Consequently, we wondered if clonal deletion and developmental diversion also occurred in INT thymocytes. To assess this possibility, we examined SAg-reactive TCR-Vβs in CD69^{+}CD4^{hi}CD8^{lo} INT thymocytes from both wildtype and costimulation-deficient BALB/c mice. SAg-reactive TCRs were present in INT thymocytes in both wildtype and costimulation-deficient BALB/c mice in frequencies that were essentially equal to those in pre-selection DP thymocytes (Fig. 5a). In fact, SAg-reactive TCRs were present in substantially higher frequencies among TCR-signaled INT thymocytes than among SP4 or SP8 post-selected thymocytes (Fig. 5a), indicating that strongly signaled DP thymocytes

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differentiated into INT thymocytes before undergoing clonal deletion or developmental diversion (Fig. 5a). That is, in wildtype BALB/c mice, SAg-reactive TCRs were present in INT thymocytes but were depleted in all post-selection populations (Fig. 5a left panels), indicating that autoreactive thymocytes did not survive beyond the INT stage of differentiation; and, in costimulation-deficient BALB/c mice, SAg-reactive TCRs were present in both INT and TCR$\alpha\beta^+$DN thymocytes but were depleted in SP4 and SP8 post-selection thymocytes (Fig. 5a middle and right panels), indicating that autoreactive INT thymocytes were developmentally diverted into TCR$\alpha\beta^+$DN cells. These results indicate that clonal deletion and developmental diversion are both signaled during negative selection at the INT stage of differentiation, but they do not formally exclude the possibility that some DP thymocytes might become DN cells directly.

For INT thymocytes to receive costimulation-dependent deletional signals, INT thymocytes must be in contact with B7 expressing cells. To assess if INT 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[27]. In fact, CD28 surface levels were up-regulated during differentiation of DP into INT and TCR$\alpha\beta^+$DN thymocytes in B7-deficient mice, whereas CD28 surface levels were down-regulated in B7 wildtype mice (Fig. 5b and Supplementary Fig. 3), revealing that INT and TCR$\alpha\beta^+$DN thymocytes in wildtype mice (which survived because of the Bcl-2 transgene) were both in contact with B7 expressing cells in the thymus and were both strongly costimulated *in vivo*.

We conclude that CD4$^+$CD8^{lo} INT thymocytes are localized in the thymus where they can contact B7 costimulatory ligands. Furthermore, the INT stage of differentiation is a point in thymocyte development during which clonal deletion and developmental diversion can both occur. Because the INT stage of differentiation is also the point in thymocyte development that CD4–CD8 lineage choice occurs, we suggest that it is in INT thymocytes that different TCR signals are translated into different lineage fates, depending on the intensity and duration of TCR plus coreceptor signals as well as the presence or absence of intrathymic costimulation (schematized in Supplementary Fig. 4).

Characterization of developmentally diverted thymocytes

Next we performed a phenotypic analysis of developmentally diverted TCR$\alpha\beta^+$DN thymocytes which revealed that, during differentiation of DP into developmentally diverted TCR$\alpha\beta^+$DN thymocytes, a variety of molecules were up-regulated (TCRβ, PD-1, CD5, CD69, CD122, Bcl-2 and $\alpha_4\beta_7$) and other molecules were either unchanged or down-regulated (CD25, CD44, CD124, CD127, CD132, Bcl-XL) (Supplementary Fig. 5 a and 5b). The expression pattern of these surface molecules provided us with some potentially useful insights into the generation and fate of TCR$\alpha\beta^+$DN thymocytes.

First, TCRβ, CD5 and CD69 upregulation suggested that TCR$\alpha\beta^+$DN thymocytes had been TCR signaled, and their high level expression of PD-1 indicated that those *in vivo* TCR signals had been strong and persistent as would be expected of autoreactive TCRs (Supplementary Fig. 5a). Because surface PD-1 molecules dampen TCR signal transduction [28], we then assessed the *in vitro* reactivity of TCR$\alpha\beta^+$DN thymocytes. Developmentally diverted TCR$\alpha\beta^+$DN thymocytes were determined to be anergic cells

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because (1) their TCR could not induce calcium mobilization (Fig. 5c and Supplementary Fig. 6a), (2) they were unable to produce their own IL-2 (Supplementary Fig. 6b), and (3) they were unable to proliferate in response to anti-TCR-CD28 stimulation without addition of exogenous IL-2 (Fig. 5d). We then considered whether PD-1 expression was required for developmental diversion. However, germline deletion of the *Pdcd1* gene which encodes PD-1 protein did not affect generation of developmentally diverted TCRαβ$^+$DN thymocytes in B7-deficient mice (Supplementary Fig. 6c). In addition, little or no surface expression of CD127 (IL-7Rα) 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 virtually absent CD127 expression, developmentally diverted TCRαβ$^+$DN thymocytes contained high levels of Bcl-2 and were not apoptotic *in vivo* (as revealed by negative Annexin V staining and absent active caspase-3) (Supplementary Fig. 5b), possibly because they expressed CD122 (IL-2Rβ) and CD132 (common γ chain) which conferred the potential to respond *in vivo* to IL-2 and IL-15 (Supplementary Fig. 5a). Moreover, developmentally diverted TCRαβ$^+$DN thymocytes expressed $\alpha_4\beta_7$ integrin, indicating that they could potentially leave the thymus and migrate to the intestine (Supplementary Fig. 5a).

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 *Rag2*$^{-/-}$ host mice (Fig 6a). Five weeks after transfer, most developmentally diverted TCRαβ$^+$DN thymocytes had homed to the small intestine and differentiated into TCRαβ$^+$CD8αα IELs, whereas SP8 T cells remained primarily TCRαβ$^+$CD8αβ T cells regardless of where they had homed (Fig. 6a). Because IL-15 is present in the gut and because TCRαβ$^+$DN thymocytes are potentially responsive to IL-15 by virtue of their expression of CD122 and CD132, we wondered if IL-15 might contribute to the differentiation of TCRαβ$^+$DN thymocytes into TCRαβ$^+$CD8αα cells. To test this possibility, we signaled developmentally diverted TCRαβ$^+$DN thymocytes *in vitro* with anti-TCRβ and IL-15 (Fig. 6b). Indeed, by culture day 4, most TCR+IL-15 signaled TCRαβ$^+$DN thymocytes had differentiated into TCRαβ$^+$CD8αα cells (Fig. 6b).

These results revealed that developmentally diverted TCRαβ$^+$DN thymocytes possessed the potential to become TCRαβ$^+$CD8αα IELs and resembled observations made with wildtype TCRαβ$^+$DN thymocytes[29], but we did not know if developmentally diverted TCRαβ$^+$DN thymocytes actually differentiated into TCRαβ$^+$CD8αα IELs *in vivo*. To assess this possibility, we took advantage of the fact that TCRαβ$^+$CD8αα IELs were nearly absent from B6 mice that were β2m-deficient (Fig. 6c left panels). We crossed *B2m*$^{-/-}$ mice with costimulation-deficient or Bcl-2 transgenic mice, both of which contained substantial frequencies of developmentally diverted thymocytes (Fig. 1 and Fig. 2). TCRαβ$^+$CD8αα IELs were present in both B7-DKO*β2m*$^{-/-}$ mice and Bcl-2 TG*β2m*$^{-/-}$ mice (Fig. 6c right panels), demonstrating that developmentally diverted T cells do in fact differentiate *in vivo* into TCRαβ$^+$CD8αα IELs. To confirm this finding, we asked if TCRαβ$^+$CD8αα IELs displayed the same TCR-Vβ repertoire as developmentally diverted TCRαβ$^+$DN thymocytes. Indeed, in B7-deficient BALB/c mice, SAg-reactive TCR-Vβ's were over-represented in essentially identical frequencies in TCRαβ$^+$CD8αα IEL's and TCRαβ$^+$DN

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thymocytes (Fig. 7a). We conclude that developmentally diverted TCRαβ$^+$DN thymocytes differentiate *in vivo* into TCRαβ$^+$CD8αα IELs.

Development diversion and the origin of CD8αα IELs

Having found that developmentally diverted TCRαβ$^+$DN thymocytes can differentiate *in vivo* into TCRαβ$^+$CD8αα IELs, we wondered if all TCRαβ$^+$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 contain more TCRαβ$^+$CD8αα IELs than wildtype mice. In fact, costimulation-deficient mice contained remarkably more (~10 times more) TCRαβ$^+$CD8αα IELs than wildtype mice (Fig. 7b), suggesting that developmental diversion was the predominant origin of TCRαβ$^+$CD8αα IELs. We then wondered if the TCRαβ$^+$CD8αα IELs present in normal wildtype mice were also the progeny of thymocytes that had survived negative selection and undergone developmental diversion. In fact we found that, even in wildtype mice, SAg-reactive TCRs were specifically over-expressed in TCRαβ$^+$CD8αα IELs but were not over-expressed in other T cell populations (Fig. 7c). This finding suggests that, in normal wildtype mice, significant numbers of autoreactive T cells avoid clonal deletion and undergo developmental diversion, a conclusion which is also consistent with our observation that the frequency of SAg-reactive TCRs in WT mice is always higher among TCRαβ$^+$DN thymocytes than among SP4 or SP8 thymocytes (see Figs. 3a, 5a and Supplementary Fig. 1a).

The over-representation of autoreactive TCR specificities among CD8αα IELs in normal and experimental mice, none of which were autoimmune, made us question the TCR responsiveness of CD8αα IELs. In fact, CD8αα IELs resembled TCRαβ$^+$DN thymocytes in being unresponsive to anti-TCR+APC stimulation *in vitro* without exogenous cytokines (Fig. 7d).

We conclude that TCRαβ$^+$CD8αα IELs are the progeny of cells that survived negative selection and underwent developmental diversion in the thymus.

Effect of Runx3 on developmentally diverted thymocytes

Finally, we wished to gain molecular insight into the differentiation of developmentally diverted TCRαβ$^+$DN thymocytes into CD8αα IELs. Because differentiation of TCRαβ$^+$DN thymocytes into CD8αα IELs requires re-activation of *Cd8a* gene expression which is a known function of the transcription factor Runx3[30], we considered that Runx3 might be required for differentiation of TCRαβ$^+$DN thymocytes into CD8αα IELs.

To analyze this possibility, we utilized Bcl-2 transgenic Runx3$^{+/YFP}$ heterozygous mice because their cells contained an endogenous *Runx3* allele that had been re-engineered to encode YFP instead of Runx3 proteins[31]. Developmentally diverted TCRαβ$^+$DN thymocytes in these mice were YFP^{low} whereas TCRαβ$^+$CD8αα IELs were YFP^{hi} (Fig. 8a), indicating that *Runx3* gene expression was up-regulated at some point during differentiation of TCRαβ$^+$DN thymocytes into TCRαβ$^+$CD8αα IELs. To determine if that point was related to *Cd8a* gene re-activation, we stimulated TCRαβ$^+$DN thymocytes *in vitro* with anti-TCR and IL-15 (Fig. 8b). Upon *in vitro* stimulation, TCRαβ$^+$DN thymocytes expressed *Runx3*

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and differentiated into CD8αα cells (Fig. 8b upper panels). To determine if their differentiation into CD8αα cells required Runx3, we utilized Bcl-2 transgenic Runx3$^{YFP/YFP}$ homozygous mice which were Runx3-deficient because they contained two *Runx3*-YFP alleles (Fig. 8b, lower panels). *In vitro* stimulation of TCRαβ$^+$DN thymocytes that were Runx3-deficient failed to induce differentiation into CD8αα cells even though it did induce YFP expression (Fig. 8b lower panels). Thus, Runx3 proteins were required for developmentally diverted TCRαβ$^+$DN thymocytes to re-activate *Cd8a* gene expression and to differentiate into CD8αα cells *in vitro*.

Applying these *in vitro* observations to the developmental fate of TCRαβ$^+$DN thymocytes *in vivo*, we wondered if differentiation of developmentally diverted TCRαβ$^+$DN thymocytes into TCRαβ$^+$CD8αα IELs would be impaired in Runx3$^{YFP/YFP}$ homozygous mice because they were Runx3-deficient. Indeed, Runx3-deficient (Runx3$^{YFP/YFP}$) Bcl-2 transgenic mice contained significantly fewer TCRαβ$^+$CD8αα IELs than Runx3-sufficient (Runx3$^{+/YFP}$) Bcl-2 transgenic mice, even though they contained equal numbers of developmentally diverted TCRαβ$^+$DN thymocytes (Fig. 8c).

We conclude that TCR and IL-15 stimulate developmentally diverted TCRαβ$^+$DN thymocytes to express Runx3 which promotes *Cd8a* gene re-activation and differentiation of developmentally diverted TCRαβ$^+$DN thymocytes into TCRαβ$^+$CD8αα IELs. As a result, lineage choice during positive selection and lineage fate during negative selection can be integrated into a unified picture of thymic development in which the developmental fate of TCR-signaled DP thymocytes is determined by TCR and costimulatory signals (schematized in Supplementary Fig. 7).

Discussion

The present study identifies CD28 costimulatory signals as necessary for thymocytes to undergo clonal deletion during negative selection *in vivo*, and demonstrates that neither CD28 costimulation nor clonal deletion is 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 that 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, strongly signaled DP thymocytes bearing autoreactive TCRs that survived negative selection did not differentiate into conventional SP T cells, but instead underwent developmental diversion and differentiated into TCRαβ$^+$DN thymocytes. Developmentally diverted TCRαβ$^+$DN thymocytes, upon leaving the thymus, preferentially migrated to the intestine and were signaled by IL-15 to express Runx3 and to further differentiate into Runx3$^+$CD8αα IELs. Clonal deletion and developmental diversion occurred during negative selection in cells at the intermediate CD4^{hi}CD8^{lo} thymocyte stage of differentiation, the identical point in thymocyte development that CD4 and CD8 lineage choice occurs during positive selection [1]. Thus, this study distinguishes *in vivo* negative selection from *in vivo* clonal deletion, identifies CD28 costimulation as the *in vivo* signal required for clonal deletion during negative selection, and identifies TCRαβ$^+$CD8αα IELs as the ultimate fate of autoreactive cells that survive negative selection in the thymus.

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During thymic selection, weakly signaled DP thymocytes undergo positive selection into SP4 or SP8 T cells, whereas strongly signaled DP thymocytes undergo negative selection which prevents them from differentiating into either SP4 or SP8 T cells. Importantly, as shown in this study, the absence of autoreactive TCR specificities among *in vivo* SP T cells is indicative of negative selection but it is not necessarily indicative of clonal deletion. Consequently, this study provides a new conceptual model of negative selection in which 'positive selection' is the differentiation of TCR-signaled DP thymocytes into SP T cells; 'negative selection' is the prevention of TCR-signaled DP thymocytes from differentiating into SP T cells; 'developmental diversion' refers to the differentiation of TCR-signaled DP thymocytes into mature T cells that are neither SP4 nor SP8 but are DN; and 'clonal deletion' refers to the death of TCR-signaled DP thymocytes prior to maturation.

We used specific TCR-Vβ's to follow the developmental fate of DP thymocytes strongly signaled by endogenously encoded Mtv proviral antigens. DP thymocytes are the first cells in the thymus to express endogenously encoded TCRαβ complexes and are the cells that are subjected to thymic selection. As previously observed, DP thymocytes bearing SAg-reactive TCRs underwent negative selection as they did not differentiate into SP4 or SP8 T cells[23]. However, our study now documents that clonal deletion of SAg-reactive thymocytes during negative selection additionally required CD28 costimulation. In the absence of CD28 costimulation, SAg-reactive thymocytes survived negative selection and underwent developmental diversion into $TCR\alpha\beta^+$DN thymocytes. Similarly, SAg-reactive thymocytes that were prevented from undergoing clonal deletion by Bcl-2 or Mcl-1 transgenes also survived negative selection and underwent developmental diversion into $TCR\alpha\beta^+$DN thymocytes. Thus, contrary to current concepts, CD28 costimulation during negative selection is required for *in vivo* clonal deletion and is prevented by transgenic expression of either Bcl-2 or Mcl-1.

Developmental diversion of surviving autoreactive thymocytes into $TCR\alpha\beta^+$DN thymocytes avoids autoreactivity by removing the contribution of CD4 and CD8 coreceptor proteins to TCR signaling which lessens their *in vivo* autoreactive potential, as does their expression of negative costimulatory receptors such as PD-1 [28]. In addition, upon leaving the thymus, developmentally diverted $TCR\alpha\beta^+$DN thymocytes migrate to the intestine where they are sequestered away from the rest of the body and signaled by IL-15 to express Runx3, which re-activates *Cd8a* gene expression and promotes further differentiation into CD8αα IELs that are hyporesponsive to TCR stimulation. Their CD8αα phenotype may further reduce their autoreactive potential, as CD8αα surface complexes have been suggested to sequester Lck signaling kinases away from the TCR[32]. Indeed, costimulation-deficient mice are free of autoimmunity despite our finding that they contain huge numbers of CD8αα IELs bearing autoreactive TCR specificities and despite lacking functionally suppressive $Foxp3^+$ regulatory T cells[33].

Developmental diversion occurs in TCR signaled $CD4^{hi}CD8^{lo}$ intermediate thymocytes that are transcriptionally $Cd4^+Cd8^-$, with the result that differentiation into $TCR\alpha\beta^+$DN thymocytes during negative selection only requires termination of *Cd4* gene expression. In fact *Cd4* gene termination occurs in $Cd4^+Cd8^-$ intermediate thymocytes during MHC class I-specific positive selection into SP8 T cells, but in that case it is mediated by the

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transcription factor Runx3 [30, 34] which additionally induces *Cd8* gene reactivation, events referred to as 'coreceptor reversal'[35]. In contrast to MHC class I selected SP8 T cells which are dependent on Runx3, developmental diversion into TCRαβ$^+$DN thymocytes did not involve or require Runx3, suggesting that strong TCR signaling of intermediate thymocytes during negative selection terminates *Cd4* gene transcription independently of Runx3 and without *Cd8* gene re-activation. Interestingly, Runx3-independent termination of *Cd4* gene transcription in *Cd4*$^+$*Cd8*$^-$ intermediate thymocytes by strong TCR signaling would also explain why negatively selected thymocytes become DN instead of SP4 T cells.

By identifying developmental diversion and clonal deletion as alternative outcomes of negative selection, the present study resolves a number of longstanding experimental contradictions. Previous *in vitro* experiments demonstrated that TCR signaled thymocyte death required CD28 costimulation[10–14] and could be prevented by Bcl-2 transgene expression[12, 14], whereas previous *in vivo* experiments came to opposite conclusions[18–22]. Our present study reveals that these contradictory observations were primarily due to the presumption that autoreactive thymocytes had been clonally deleted *in vivo* if they failed to differentiate into either SP4 or SP8 Tcells. In fact our current study documents 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 present study also resolves the discrepant observations that genetic deletion of the pro-apoptotic protein Bim interfered with clonal deletion[36] while transgenic over-expression of the anti-apoptotic protein Bcl-2 did not[20]. In fact many features of Bim-deficient mice[37–39] resemble those of Bcl-2 transgenic and Mcl-1 transgenic mice in our current study, so that our study explains the high frequency of TCRαβ$^+$DN thymocytes in Bim- and Puma-deficient mice[37, 40, 41] as autoreactive thymocytes that survived negative selection and underwent developmental diversion. Our present study also explains why SP4 and SP8 T cells bearing Mtv-6 reactive Vβ3$^+$ TCRs were previously observed in B7-deficient but not *Cd28*$^{-/-}$ BALB/c mice[24]. The discrepancy between B7-deficient and *Cd28*$^{-/-}$ BALB/c mice did not indicate an as yet unknown B7-specific receptor that signaled clonal deletion in *Cd28*$^{-/-}$ BALB/c mice[24], but was instead due to the fact that Mtv-6 was not encoded in the genome of B7-deficient BALB/c mice.

While resolving many longstanding experimental contradictions, our present study does contradict an experimental finding in perinatal mice in which costimulation blockade by *in vivo* anti-B7 antibody injections rescued SAg-reactive TCRs which were then expressed on SP4 T cells[42]. In our current study, genetic deletion of B7 ligands in B7-deficient mice also rescued SAg-reactive TCRs but the rescued TCRs were not expressed on SP4 T cells, only on developmentally diverted TCRαβ$^+$DN thymocytes and CD8αα IELs. We think the explanation for this disparity is that anti-B7 antibody injections depleted SAg-bearing B7$^+$ cells from the thymi of injected mice and so eliminated intra-thymic expression of the negative selecting (SAg) ligand.

Does developmental diversion occur in normal wildtype (i.e. costimulation-sufficient) mice? Costimulation-sufficient TCR transgenic mice contain substantial numbers of TCRαβ$^+$DN thymocytes[37, 41, 43] that can become CD8αα IELs [29, 32, 43–46]. However, transgenic TCRs differ from endogenous TCRs in that they are expressed by early DN thymocytes prior to the

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DP stage of differentiation, so it is difficult to know if TCRαβ$^+$DN thymocytes in TCR transgenic mice are post-selection cells resulting from developmental diversion or are pre-selection DN thymocytes that were developmentally arrested because their transgenic TCR engaged high affinity ligands in the thymic cortex[47, 48]. Importantly, the present study found in normal, non-transgenic mice that many or all TCRαβ$^+$CD8αα IELs were the progeny of thymocytes that survived negative selection and underwent developmental diversion in the thymus. Indeed normal wildtype mice contained substantial numbers (~5 million) of TCRαβ$^+$CD8αα IELs in which autoreactive TCR specificities were over-expressed, indicating that substantial numbers of autoreactive T cells avoid clonal deletion and undergo developmental diversion in normal mice.

In conclusion, by distinguishing *in vivo* negative selection from *in vivo* clonal deletion, the present study identified CD28 costimulation as critical for clonal deletion and identified developmental diversion as an alternative outcome of negative selection. Moreover, this study identified TCRαβ$^+$CD8αα IELs as the ultimate fate of autoreactive T cells that survive negative selection and undergo developmental diversion in the thymus. And, by discovering that the fate of thymocytes undergoing negative selection was determined at the same point in differentiation as thymocytes undergoing positive selection, this study integrates negative and positive selection into a unified picture of thymic selection.

METHODS

Animals

BALB/c, C57BL/6 (B6), *Cd28*$^{-/-}$, *B2m*$^{-/-}$, *Ab*$^{-/-}$*b2m*$^{-/-}$, *Zap70*$^{-/-}$, *Rag2*$^{-/-}$, and human Bcl-2 transgenic mice[20] (Bcl-2 TG) were maintained in our own colony. *Cd80*$^{-/-}$*Cd86*$^{-/-}$ mice were referred to as B7-deficient and were provided by Arlene Sharpe (Harvard University); RUNX3-YFP reporter mice were provided by Dan Littman (NYU) [31]; *Pdcd1*$^{-/-}$ mice was provided by Tasuku Honjo (Kyoto University). The Mcl-1-transgenic construct was made by ligating Mcl-1 cDNA into a human CD2 (hCD2) enhancer-promoter-based vector and injected it into fertilized B6 oocytes to generate Mcl-1 transgenic mice. All mice were cared for in accordance with National Institutes of Health (NIH) guidelines.

Flow cytometry

Monoclonal antibodies with the following specificities were used in this study: CD4 (GK1.5 and RM4.5), CD5 (53-7.3), CD8α (53-6.7), CD8β (H35-17.2), TCRγδ (GL3), TCRβ (H57-597), Vβ3 (KJ25), Vβ4 (KT4), Vβ5.1-5.2 (MR9-4), Vβ6 (RR4-7), Vβ8 (F23.1), Vβ10 (B21.5), Vβ11 (RR3-15), Vβ12 (MR11-1), NK1.1 (PK136), PD-1 (J43), CD132 (4G3), CD122 (TM-beta1), CD44 (IM7), CD69 (H1.2F3), Bcl-2 PE-set (3F11), IL4Rα (mIL4R-M1), IL7Rα (SB/199), CD25 (PC61), CD28 (37.51), were obtained from BD Pharmingen; Bcl-XL (54H6) and the cleaved form of Caspase-3 (D175) were obtained from Cell Signaling; IL-2 (JES-5H4) was from eBioscencie; CD8α (5H10) was from Invitrogen. Annexin-V FLUOS staining kit was obtained from Roche Applied Science. PBS57 loaded CD1d tetramers or unloaded CD1d control tetramers were provided by the NIH Tetramer Core Facillity. Cells were analyzed either on a FACSVantage SEM or LSRII (BD). Doublets and dead cells were removed from data analysis. For intracellular staining, live cells were

first stained for surface proteins, then fixed and permeabilized, and then stained for intracellular proteins. Data were analyzed with software designed by the Division of Computer Research and Technology of National Institutes of Health or with FlowJo software by TreeStar.

Cell sorting and purification

$CD8^+$ and LNT cells were isolated by antibody-mediated magnetic bead depletion of $CD4^+$ and Ig^+ cells (BioMag Qiagen). For purification of $TCR\alpha\beta^+DN$ thymocytes, thymocytes were depleted of $CD4^+$ and $CD8^+$ cells by antibody-mediated magnetic bead depletion and then electronically sorted for either $CD5^+CD4^-CD8\beta^-B220^-GL3^-NK1.1^-$ or $CD5^+CD4^-CD8\beta^-B220^-GL3^-CD1dPBS57^-$ cells. Isolation of IELs was performed as described[49]. For purification of $TCR\alpha\beta^+CD8\alpha\alpha$ IELs, IELs were electronically sorted for $CD45^+CD8\alpha^+CD8\beta^-CD4^-GL3^-$ cells.

Calcium mobilization

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

Adoptive transfer

0.8×10^6 purified $TCR\alpha\beta^+DN$ thymocytes or $CD8^+CD5^+$ LNT cells were injected into $Rag^{-/-}$ host mice. Five weeks after transfer, cells from thymi, peripheral lymphoid organs, and the small intestine were isolated and analysed by flow cytometry.

In vitro T cell proliferation and differentiation cultures

For *in vitro* differentiation, purified $TCR\alpha\beta^+DN$ thymocytes were cultured in medium only or stimulated with immobilized anti-TCRβ mAb (5–10 μg/ml) in the presence of IL-15 (100 ng/ml, R&D Systems) for 4–5 days at which time cells were harvested and analyzed by flow cytometry. For *in vitro* proliferation, sorted $TCR\alpha\beta^+DN$ and SP8 thymocytes from B7-deficient mice were stimulated for 72h with immobilized anti-TCRβ (5μg/ml) and anti-CD28 mAbs (10μg/ml) in the presence or absence of recombinant IL-2 (200 U/ml). For *in vitro* proliferation of IELs, sorted $TCR\alpha\beta^+CD8\alpha\alpha$ and SP4 LN T cells were stimulated for 48h with anti-CD3 (5μg/ml) in the presence of 10^5 APCs (3000R irradiated syngenic splenocytes) in the presence or absence of recombinant IL-2 (200 U/ml) or IL-15 (100ng/ml). Cultures were pulsed with [^{3}H]-thymidine (1μCi) 8h before collection. For intracellular IL-2 production, T cells that had been stimulated for 4d were treated with PMA (50ng) and ionomycin (500nM) for 4h in the presence of protein transport inhibitor (BD Biosciences) prior to intracellular staining for IL-2.

Genomic-PCR

PCR reactions were performed using mouse tail DNA to detect genomes of integrated *Mtv-6* and *Mtv-9* proviruses. Primers for: *Mtv-6* forward 5′-GCTGGCTATCATCACAAGAGCG-3′, reverse 5′-GGAGTTCAACCATTTCTGCTGC-3′;

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Mtv-9 forward 5′-ACCGCAGTCAAAGAACAGGTGC-3′ reverse 5′-CAGGAAACCACTTGTCTCACATCC-3′.

DNA Methylation analysis

Methylation analysis was performed as described[50]. Briefly, DNA was isolated using the ZR genomic DNA II kit (Zymo Rearch). Bisulfite conversion of total DNA was made using the EZ-DNA methylation-Gold kit (Zymo Research). The following PCR primers were used: CD8β promoter forward 5′-TTGAAAAGTTAAGGTTTTGATGTT-3′ and reverse 5′-AAACACTATTCCCCTCAATACTCTATC-3′. PCR products were purified and cloned using 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 tail distribution.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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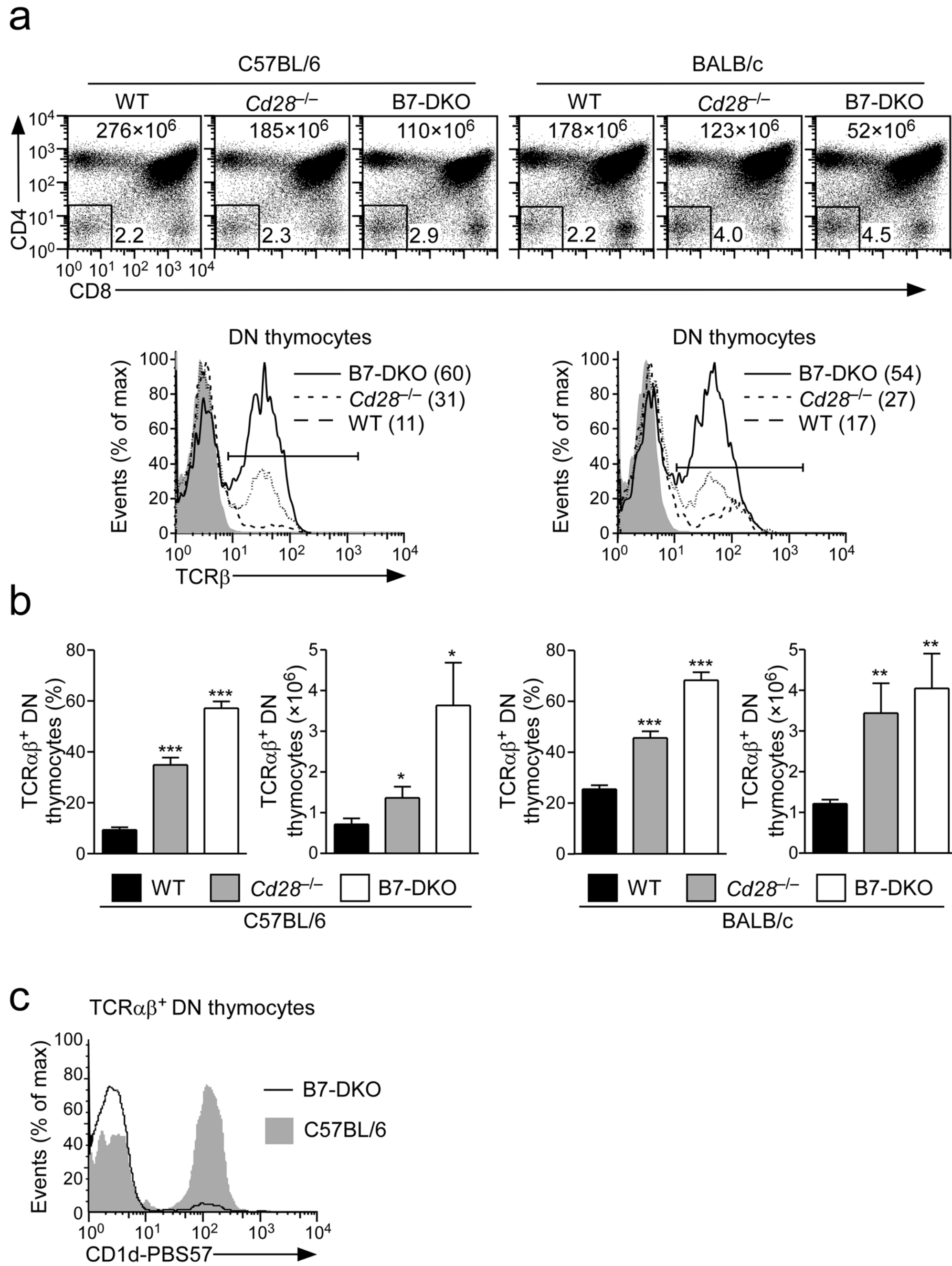

Figure 1.

TCRαβ+DN thymocytes are increased in CD28 costimulation-deficient mice. **(a)** CD4 versus CD8 staining of thymocytes from wildtype and costimulation-deficient (*Cd28*$^{-/-}$ and B7-deficient) mice on C57BL/6 (left) and BALB/c (right) backgrounds (top panels). Numbers of total thymocytes and frequencies of DN cells are indicated. Surface TCRβ expression on gated DN thymocytes is also shown (lower panels). **(b)** Quantitation (frequency and number) of TCRαβ+DN thymocytes in wildtype and costimulation-deficient mice. Displayed are the mean + S.E. of each group which was compared to that from WT mice. *p*-values were obtained by Student's two-tailed *t*-test. *, $p<0.01$; **, $p<0.001$; ***,

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p<0.0001. Data represent at least five independent experiments. (**c**) Profile of CD1d-PBS57 tetramer staining of TCRαβ$^+$DN thymocytes from wildtype (shaded curve) and B7-deficient (bold line) mice.

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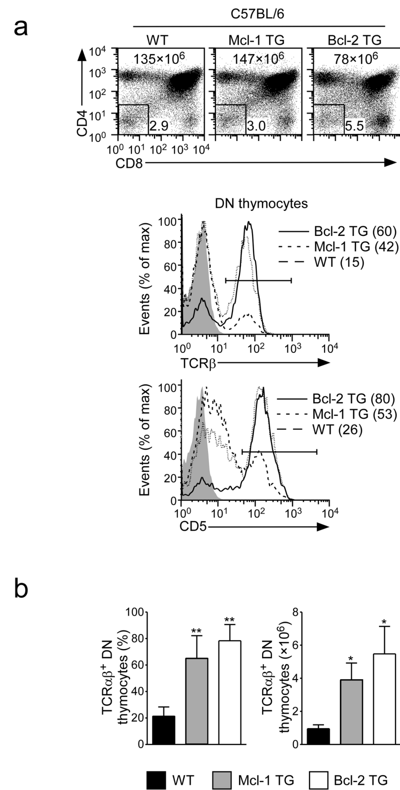

Figure 2.

Effect of Bcl-2 and Mcl-1 pro-survival transgenes on the appearance of TCRαβ^{+}DN thymocytes. **(a)** CD4 versus CD8 staining of thymocytes from wildtype and transgenic mice (top panels). Numbers of total thymocytes and frequencies of DN cells are indicated. Surface TCRβ and CD5 expression on gated DN thymocytes is also shown (middle and lower panels). **(b)** Quantitation of TCRαβ^{+}DN thymocytes in wildtype and transgenic mice. Displayed are the mean + S.E. of each group which was compared to that from WT mice. *, $p<0.05$; **, $p<0.001$; Data represent four independent experiments.

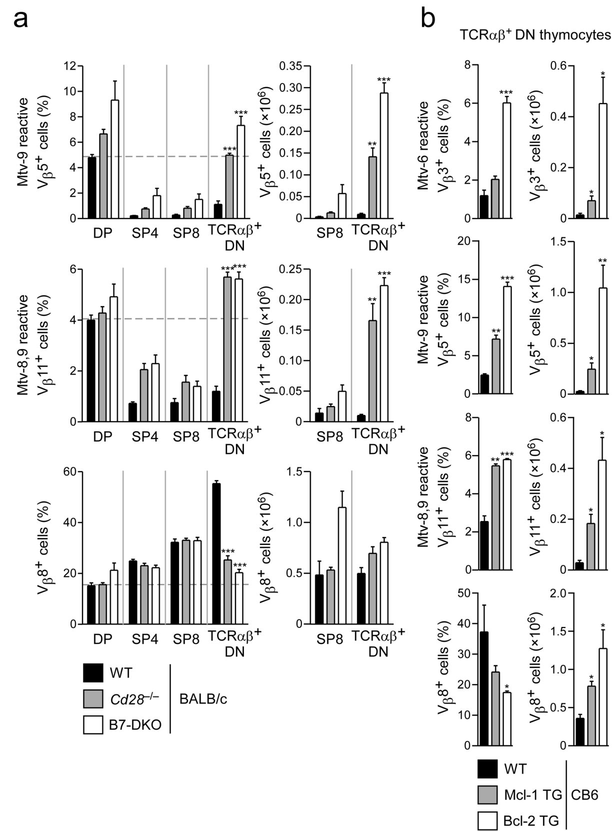

Figure 3.

Expression of Mtv-reactive TCR-Vβs in pre- and post-selection thymocyte subsets. **(a)** Expression of Mtv-reactive TCR-Vβs in different thymocyte subsets from wildtype (WT) and costimulation-deficient mice on the BALB/c background. Displayed are the mean + S.E. of each group which was compared within each group to that from WT mice. The horizontal dashed line indicates the frequency of pre-selection DP thymocytes in WT mice expressing the particular TCR-Vβ specificity. **(b)** Quantitation (frequency and number) of specific TCR-Vβs expressed by TCRαβ^{+}DN thymocytes from BALB/c crossed with C57BL/6 (CB6) mice that were either WT or transgenic for Mcl-1 or Bcl-2. Displayed are the mean + S.E. of each group which was compared within each group to that from WT mice. Data represent at least three independent experiments with at least five mice per group. *, $p<0.01$; **, $p<0.001$; ***, $p<0.0001$.

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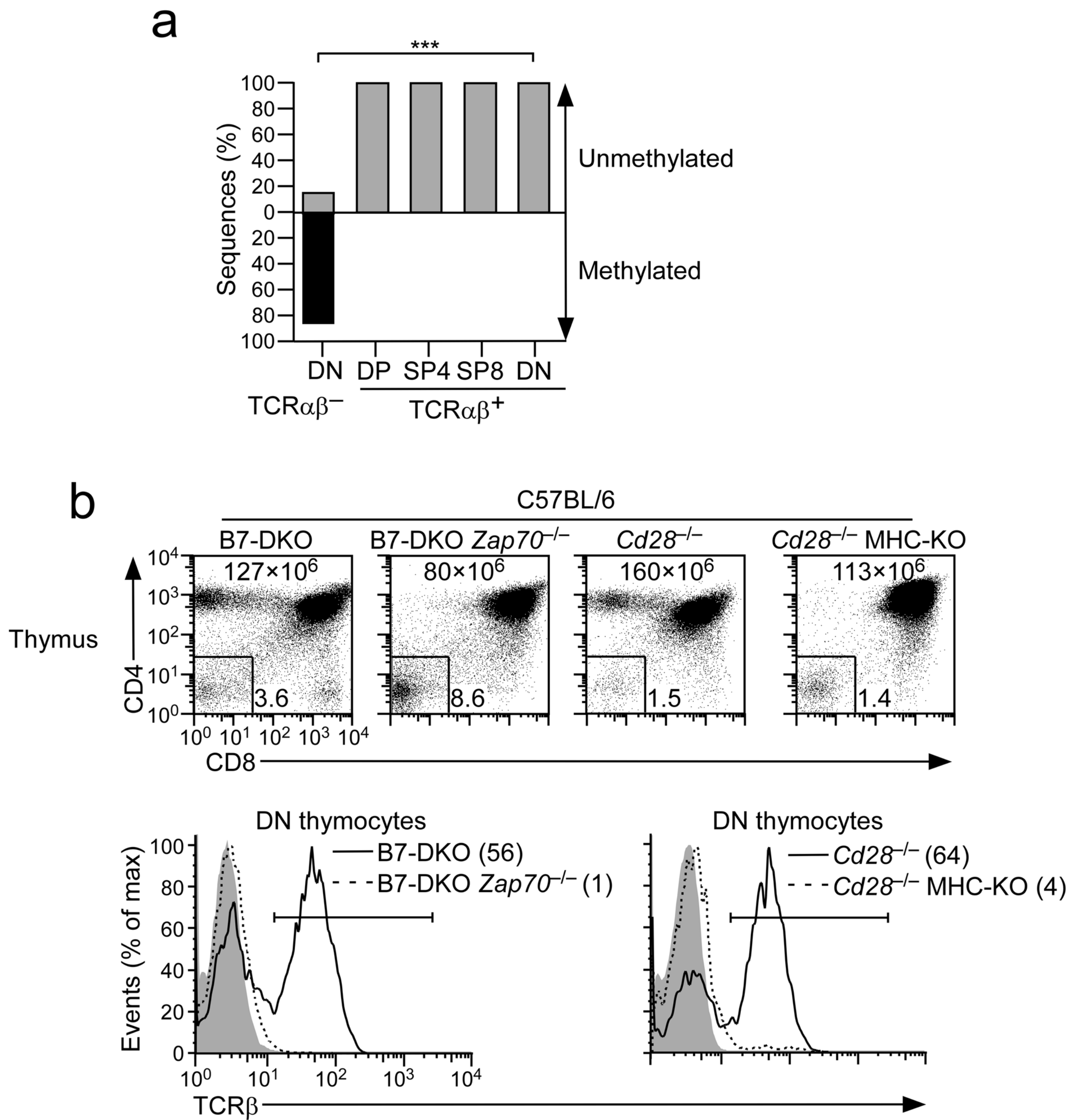

Figure 4.

TCRαβ+DN thymocytes are the progeny of DP thymocytes. **(a)** The methylation status of the *Cd8b* promoter in Bcl-2 transgenic thymocytes was determined by bisulfite conversion and sequencing of genomic DNA from sorted thymocyte subpopulations (n=3). Data are presented as the frequency of *Cd8b* promoter sequences isolated from each cell population that were methylated or unmethylated as described and displayed in Supplementary Figure S2. **(b)** CD4 versus CD8 staining of thymocytes from the indicated mouse strains (top panels). Numbers of total thymocytes and frequencies of DN cells are indicated. Surface TCRβ expression on gated DN thymocytes is also shown (lower panels). Data are from two independent experiments.

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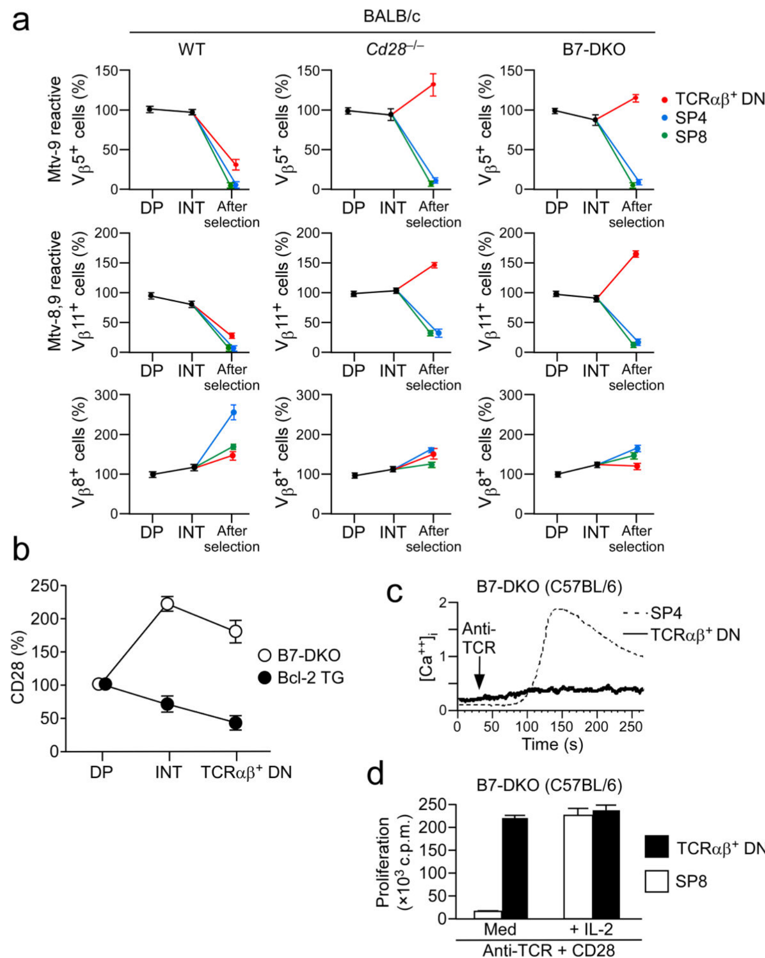

Figure 5.

Impact of thymic selection on expression of Mtv-reactive TCR-Vβs. **(a)** The frequency of cells expressing Mtv-reactive TCR-Vβs among post-selection ($CD69^+CD4^+CD8^{lo}$ intermediate (INT) and SP) thymocytes was expressed relative to that among pre-selection DP thymocytes which was set = 100%. Displayed are the mean + S.E. of each group. Data are summaries of five independent experiments. **(b)** Surface CD28 expression on INT and $TCR\alpha\beta^+$DN thymocyte subsets from each mouse strain was quantified as mean fluorescence intensity and was expressed relative to that of preselection DP thymocytes which was set = 100%. Data are representative of two independent experiments. **(c)** TCR induced calcium mobilization was assessed in CD8- depleted thymocytes. Biotinylated anti-TCR mAb (5 μg/ml) was crosslinked by avidin as indicated by arrows. Data are representative of three independent experiments. **(d)** Purified $TCR\alpha\beta^+$DN and SP8 thymocytes (2×10^4/well) were stimulated with plate-bound anti-TCR+CD28 (5μg +10μg) in the presence or absence of exogenous IL-2 (200 U/ml). Data represent mean c.p.m. + S.E. of triplicate cultures.

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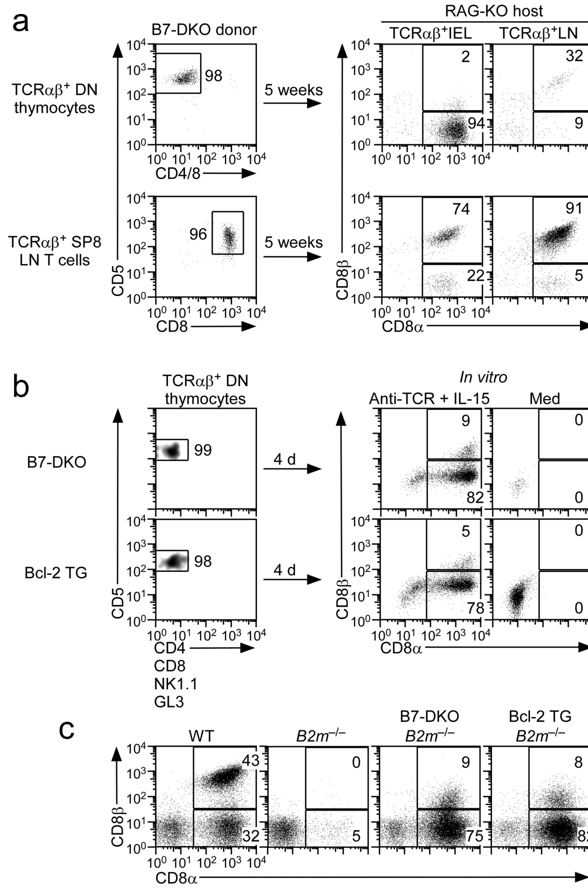

Figure 6.

Developmentally diverted TCRαβ+DN thymocytes migrate to the intestine where they become CD8αα+ IELs. (**a**) Homing and fate of developmentally diverted TCRαβ+DN thymocytes. 0.8×10^6 sorted CD5+DN thymocytes or CD5+CD8+ LN T cells from B7-deficient mice (left panels) were adoptively transferred into *Rag2*−/− recipients. Five weeks later lymph nodes and small intestine of recipient animals were evaluated for the presence of TCRαβ+ T cells (right panels). The frequency of cells in each gate is indicated. (**b**) Effect of *in vitro* TCR+IL-15 stimulation of developmentally diverted TCRαβ+DN thymocytes from B7-deficient and Bcl-2 transgenic mice. 2×10^4 sorted CD5+DN thymocytes/well (left panels) were cultured for 4d in either medium alone or with platebound anti-TCRβ mAb and soluble IL-15 (100ng/ml) and then analyzed. (**c**) CD8α versus CD8β expression is shown on TCRβ+ cells from the small intestine of the indicated mouse strains. The frequency of cells in each gate is shown. Data are representative of two independent experiments.

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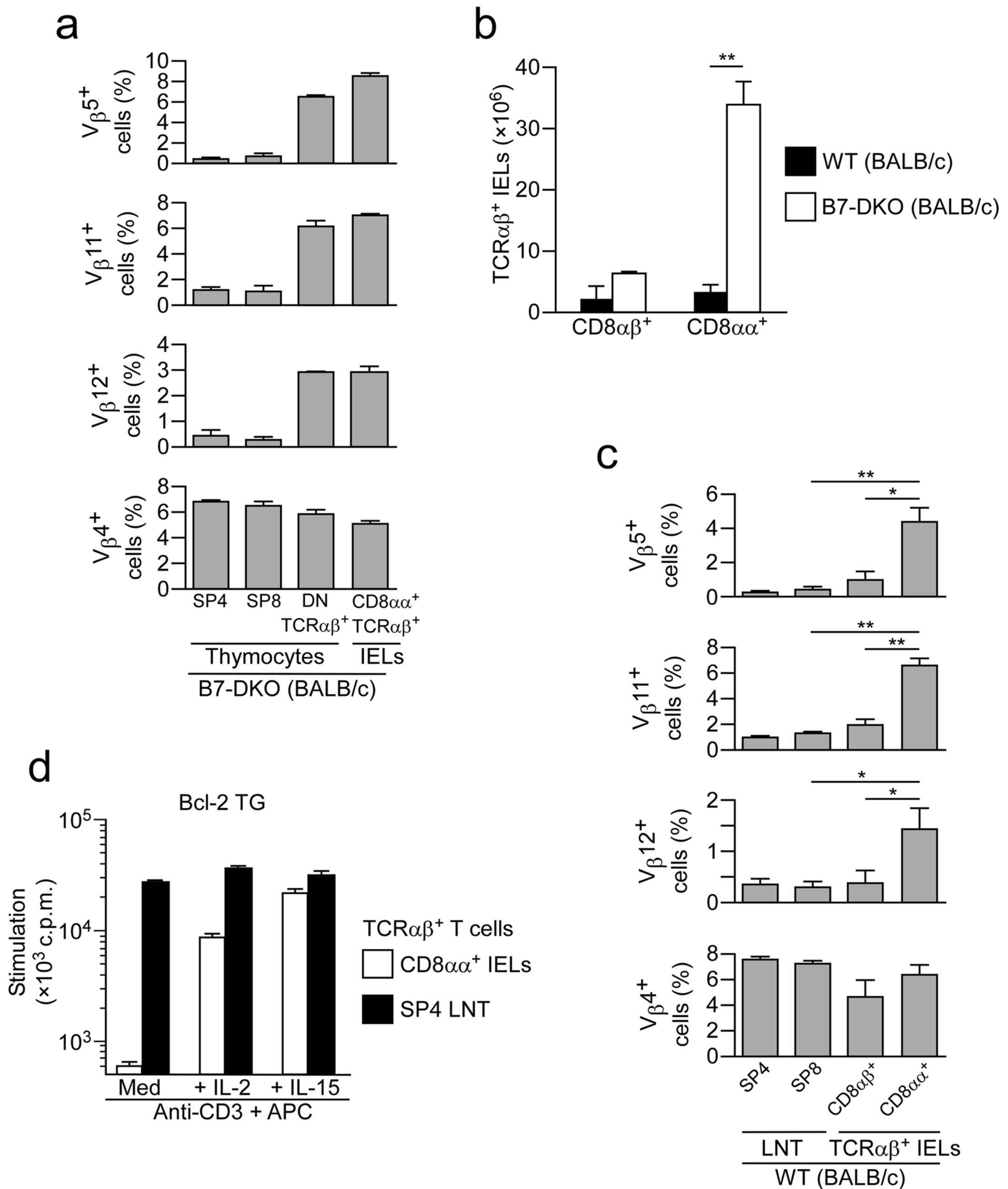

Figure 7.

Developmentally diverted TCRαβ+DN thymocytes become CD8αα IELs. **(a)** Expression of Mtv-reactive TCR-Vβ5,11, and 12 in different thymocyte subsets and IELs from B7-deficient (BALB/c) mice. Displayed are the mean + S.E. of each group. Data represent three independent experiments. **(b)** Total number of TCRαβ+CD8αβ and CD8αα IELs in BALB/c and B7-deficient (BALB/c) mice. Displayed are the mean + S.E. of each group from three independent experiments. **, $p<0.002$. **(c)** Expression of Mtv-reactive TCR-Vβ5,11, and 12 in different T cell subsets in BALB/c mice. Displayed are the mean + S.E. of each group from three independent experiments. *, $p<0.05$; **, $p<0.01$. **(d)** Sorted TCRαβ+CD8αα+

IELs and SP4 LNT cells (2.5×10^4/well) were stimulated with soluble anti-CD3 mAb (5 μg) and irradiated syngenic APC in the presence or absence of recombinant IL-2 (200 U/ml) or IL-15 (100ng/ml). Proliferation was assessed by [^{3}H]-thymidine incorporation and data represent mean c.p.m. + S.E. of triplicate wells.

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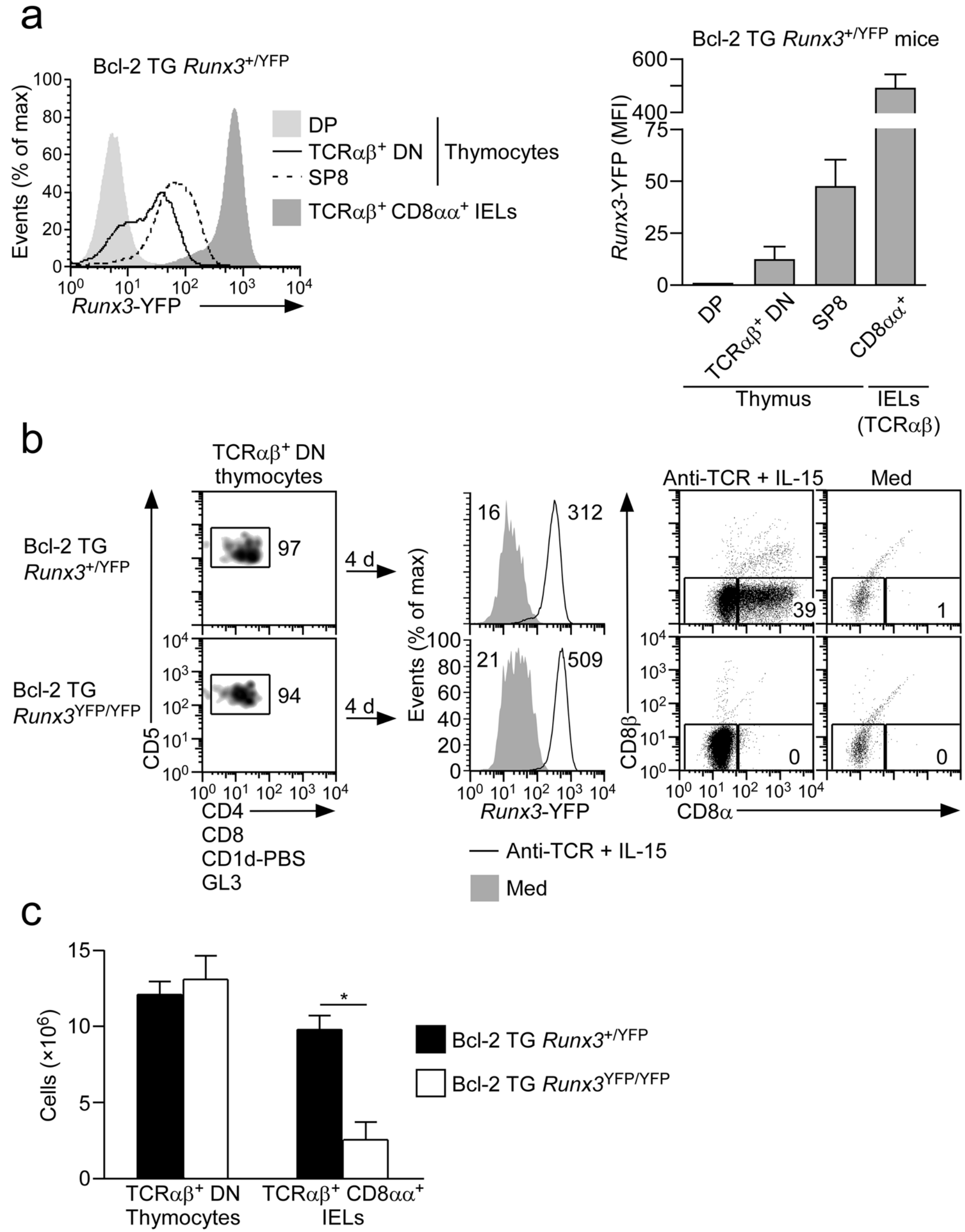

Figure 8.

Runx3 is required for differentiation of TCRαβ$^+$DN thymocytes into developmentally diverted CD8αα$^+$ IELs. (**a**) Runx3d reporter expression in thymocyte subsets and IELs. Runx3-YFP reporter expression was assessed in the indicated T cell populations from Bcl2 transgenic mice (left panel). Displayed are the mean + S.E. of Runx3-YFP expression in each group (right panel). Data are representative of three independent experiments. **(b)** Effect of *in vitro* TCR+IL-15 stimulation of developmentally diverted TCRαβ$^+$DN thymocytes from Runx3-sufficient (*Runx3$^{+/YFP}$*) and Runx3-deficient (*Runx3$^{YFP/YFP}$*) Bcl-2 transgenic mice. 2×10^4 sorted CD5$^+$DN thymocytes/well (left panels) were cultured for 4d in either medium alone or with plate- bound anti-TCRβ mAb and soluble IL-15 (100 ng/ml) and then analyzed. Runx3d reporter expression was quantified as mean fluorescence

intensity of YFP and is indicated. **(c)** Total number of $TCR\alpha\beta^+$DN thymocytes and $TCR\alpha\beta^+CD8\alpha\alpha^+$ IELs in Runx3-sufficient and Runx3-deficient Bcl2 transgenic mice. Displayed are the mean + S.E. of each group. Data are representative of three independent experiments. *, $p<0.01$.

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