NKAP is required for T cell maturation and acquisition of functional competency

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Newly generated T cells are unable to respond to antigen/MHC. Rather, post-selection single-positive thymocytes must undergo T cell maturation to gain functional competency and enter the long-lived naive peripheral T cell pool. This process is poorly understood, as no gene specifically required for T cell maturation has been identified. Here, we demonstrate that loss of the transcriptional repressor NKAP results in a complete block in T cell maturation. In CD4-cre NKAP conditional knockout mice, thymic development including positive selection occurs normally, but there is a cell-intrinsic defect in the peripheral T cell pool. All peripheral naive CD4-cre NKAP conditional knockout T cells were found to be functionally immature recent thymic emigrants. This defect is not simply in cell survival, as the T cell maturation defect was not rescued by a Bcl-2 transgene. Thus, NKAP is required for T cell maturation and the acquisition of functional competency.

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Abbreviations used: cKO, conditional KO; DN, double negative; DP, double positive; Q-PCR, quantitative PCR; RTE, recent thymic emigrant; SP, single positive.

The completion of development of B cells within the bone marrow, or T cells within the thymus, does not result in the generation of lymphocytes that are ready to participate in an immune response (Thomas et al., 2006; Fink, 2009). B and T cells that are newly produced are not yet competent, and must undergo a further maturation step that is initiated in the primary lymphoid organs and completed in the secondary. Peripheral B cell maturation is well defined. Immature, newly produced B cells enter the spleen and undergo a transition to become competent mature peripheral B cells; defined phenotypically as (transitional) T1, T2, and T3 populations (Allman et al., 2001). This is dependent on the cytokine BAFF/BLyS and its receptor BAFF-R/BLyS receptor, as well as signals through the BCR (Hsu et al., 2002; Wang and Clarke, 2003). Typically, B cells stay in transition for only a few days, at which point they either mature into the long-lived pool or die (Allman et al., 1993). T cells undergo a similar maturation (Boursalian et al., 2004). As T cells gain functional competence, they express the marker Qa2 and down-regulate expression of CD24/HSA (Vernachio et al., 1989; Ramsdell et al., 1991). Maturation initiates postselection in the thymic medulla and is completed in the periphery (Houston et al., 2008), with recent thymic emigrants (RTEs) having a more mature phenotype than medullary single-positive (SP) thymocytes (Gabor et al., 1997). RTEs are not fully mature, as they have a defect in proliferation and cytokine production in response to CD3 stimulation (Boursalian et al., 2004). No gene has yet been isolated that specifically regulates T cell maturation.

On a genetic complementation screen to identify novel regulators of T cell activation, we identified NKAP (Pajerowski et al., 2009). NKAP is a transcriptional repressor that associates with DNA, although likely in an indirect manner as it lacks any previously characterized DNA-binding domain (Pajerowski et al., 2009). NKAP binds to HDAC3, as well as CIR, which is a component of the Notch co-repressor complex (Pajerowski et al., 2009). Loss of NKAP early in T cell development, using Lck-cre NKAP conditional KO (cKO) mice, led to a severe block in αβ T cell development at the double-negative 3 (DN3) to double-positive (DP) transition, whereas γδ T cell development was

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unaffected (Pajerowski et al., 2009). In addition, NKAP is also absolutely required for the maintenance and survival of hematopoietic stem cells, as conditional deletion of NKAP results in hematopoietic failure, severe anemia, and lethality (Pajerowski et al., 2010).

To understand if NKAP has additional roles in T cell development after the DN3-DP transition, we generated CD4-cre NKAP cKO mice. These mice have a severe defect in peripheral T cell numbers, although thymic cellularity and development are normal. Rather, we observe a defect in the expression of Qa2, indicating that T cell maturation may require NKAP. CD4-cre NKAP cKO thymocytes undergo normal positive selection, and the defect in T cell maturation cannot be rescued by expression of the OT-II transgenic TCR (Barnden et al., 1998). Using mixed stem cell chimeras, the block in T cell maturation was found to be cell intrinsic. To prove that the defect in CD4-cre NKAP cKO mice is caused by a failure in T cell maturation, we crossed them to a Rag1-GFP reporter transgenic mouse (Kuwata et al., 1999). We demonstrate that all the naive T cells in the periphery of CD4-cre NKAP cKO mice are RTEs that are functionally immature, as demonstrated by failure to produce IL-2 upon TCR/CD28 stimulation. The paucity of peripheral T cells is not caused by a defect in thymic egress, as Rag1-GFP^{lo} cells do not accumulate in the thymus of CD4-cre NKAP cKO mice. In addition, expression of CCR7, KLF2, and S1P1 is unaffected. The defect is not caused by loss of IL-7Rα expression, and IL-7 stimulation leads to appropriate phosphorylation of Stat5 and up-regulation of Bcl-2. The block in T cell maturation cannot be rescued by a Bcl-2 transgene, indicating that the defect is not simply caused by a block in IL-7Rα signaling or decreased survival. Mechanistically, this effect of NKAP deficiency on T cell maturation is independent of Notch, as the failure to mature was not eliminated upon RBP-Jκ deletion. Therefore, NKAP has a critical and specific role in T cell maturation, and the defect in T cell maturation caused by NKAP deletion is not caused by a defect in positive selection, thymic egress, or IL-7Rα signaling.

RESULTS

CD4-cre NKAP cKO mice have normal thymic development but severe defects in peripheral T cells resulting from a defect in T cell maturation

Previously, we identified NKAP as a transcriptional repressor and found that loss of NKAP early in T cell development resulted in a severe block at the DN3 to DP transition (Pajerowski et al., 2009). To determine whether NKAP has additional roles in T cell development and function downstream of this block, we crossed our floxed NKAP mice with CD4-cre transgenic mice (Lee et al., 2001). No gross alterations in T cell development were observed, as demonstrated by similar proportions and absolute numbers of CD4$^-$CD8$^-$ DN, CD4$^+$CD8$^+$ DP, CD4$^+$ SP, or CD8$^+$ SP thymocytes (Fig. 1 A). However, in the periphery, defects in both the proportion and absolute number of peripheral CD4 and CD8 T cells were observed (Fig. 1 B and unpublished data). Interestingly, in contrast to WT in which the majority of peripheral cells were naive (CD62L^{high}CD44^{low}), the majority of T cells in the periphery of CD4-cre NKAP cKO mice had a memory (CD62L^{low}CD44^{high}) phenotype. There was no statistical difference in the absolute number of memory CD4 T cells in the spleen between CD4-cre NKAP cKO mice and WT littermates, although the absolute number of naive CD4 T cells was decreased by ∼20-fold (Fig. 1 B). Examination of deletion efficiency of the floxed NKAP allele demonstrated that NKAP was not deleted in the splenic CD4 memory population, which explains why no defects in cell numbers were observed in this population (Fig. 1 C). However, in DP T cells in the thymus, NKAP was deleted with a >95% efficiency, whereas partial deletion was observed in peripheral CD4 naive T cells. This suggested that there was selective pressure against cells that had deleted NKAP and that the memory phenotype observed in the periphery was likely caused by homeostatic expansion of T cells in which NKAP was not deleted. After completing T cell development in the thymus, newly produced T cells exit into the periphery. These RTEs are not competent to initiate an immune response (Boursalian et al., 2004). Rather, they must undergo T cell maturation to gain functional competence and enter the long-lived peripheral T cell pool. This process initiates in the thymus and is completed in the periphery (Houston et al., 2008). The acquisition of functional competence and completion of T cell maturation correlates with the up-regulation of the marker Qa2 and down-regulation of the marker CD24/HSA (Vernachio et al., 1989; Ramsdell et al., 1991). Qa2 expression is significantly decreased in CD4 SP thymocytes and peripheral naive CD4 T cells from CD4-cre NKAP cKO mice, as compared with WT mice (Fig. 1 D). Similar results were observed in the CD8 lineage (unpublished data); therefore, CD4-cre NKAP cKO mice have a defect in T cell maturation.

The T cell defect is not caused by altered selection and cannot be rescued by the OT-II TCR transgene

To exclude the possibility that the paucity of peripheral T cells from CD4-cre NKAP cKO mice is caused by altered positive selection during T cell development, the expression of CD5, CD69, and Nur77 were examined. No differences were observed in the cell surface expression of CD5 or CD69 in either DP or CD4$^+$ SP thymocytes (Fig. 2 A). Expression of Nur77 mRNA by quantitative PCR (Q-PCR) in semi-mature CD4 SP thymocytes was similarly unaltered in CD4-cre NKAP cKO mice as compared with WT (Fig. 2 B). NKAP mRNA expression was also examined by Q-PCR in semi-mature CD4 SP thymocytes and was decreased by approximately fivefold (Fig. 2 B) in CD4-cre NKAP cKO mice, demonstrating that loss of NKAP expression does not lead to alterations in the expression of CD5, CD69, or Nur77. To determine whether the defect in T cell maturation could be rescued by expression of a TCR transgene, we crossed our mice to OT-II TCR transgenics (Barnden et al., 1998). Thymic development of OT-II T cells effectively produced CD4 SP T cells in CD4-cre NKAP cKO mice (Fig. 2 C). However, the

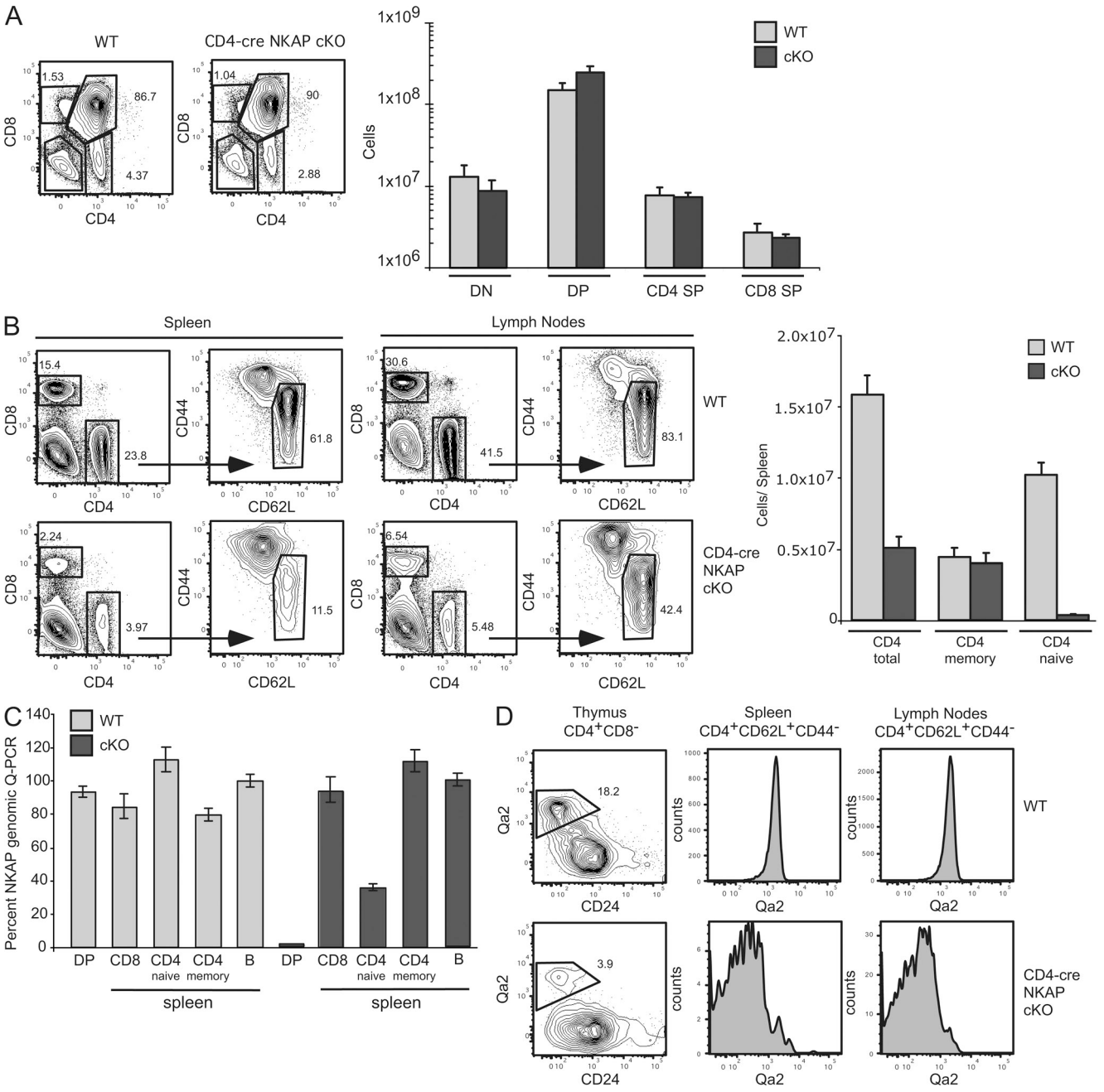


Figure 1. Thymic development is normal in CD4-cre NKAP cKO mice, but there is a defect in T cell maturation. (A, left) A representative FACS analysis of T cell development in a CD4-cre NKAP cKO mouse and WT littermate. (right) Absolute number of thymic populations in CD4-cre NKAP cKO and WT littermate controls. The absolute number is the mean of four CD4-cre NKAP cKO and three WT littermates from at least two independent experiments. There is no statistically significant difference in the absolute number of DN, DP, or SP thymocytes between CD4-cre NKAP cKO and WT littermate controls. (B, left and middle) A representative FACS analysis of splenic and lymph node T cell populations in a CD4-cre NKAP cKO mouse and WT littermate. (right) Absolute number of splenic total CD4, memory CD4, and naive CD4 T cell populations in CD4-cre NKAP cKO and WT littermate controls. The absolute number is the mean of 9 CD4-cre NKAP cKO and 10 WT littermates from 6 independent experiments. The differences in splenic total CD4 numbers and in the number of naive CD4 T cells is statistically significant by Student's *t* test ($P < 0.001$). (C) CD4/CD8 DP thymocytes, or splenic $CD19^+$ B cells, total $CD8^+$ T cells, and naive or memory CD4 T cells from one CD4-cre NKAP cKO mouse and one WT littermate were FACS sorted and examined for NKAP deletion efficiency by Q-PCR (Pajerowski et al., 2009). All samples were normalized to expression of undeleted NKAP allele in WT B cells (100%). (D) CD4 SP thymocytes or naive $CD4^+$ $CD62L^+$ $CD44^-$ splenic or lymph node T cells were examined for T cell maturation using the markers CD24/HSA and Qa2 from WT and CD4-cre NKAP cKO mice. Data shown are representative of at least five mice in each group from five independent experiments. Error bars represent the SEM.

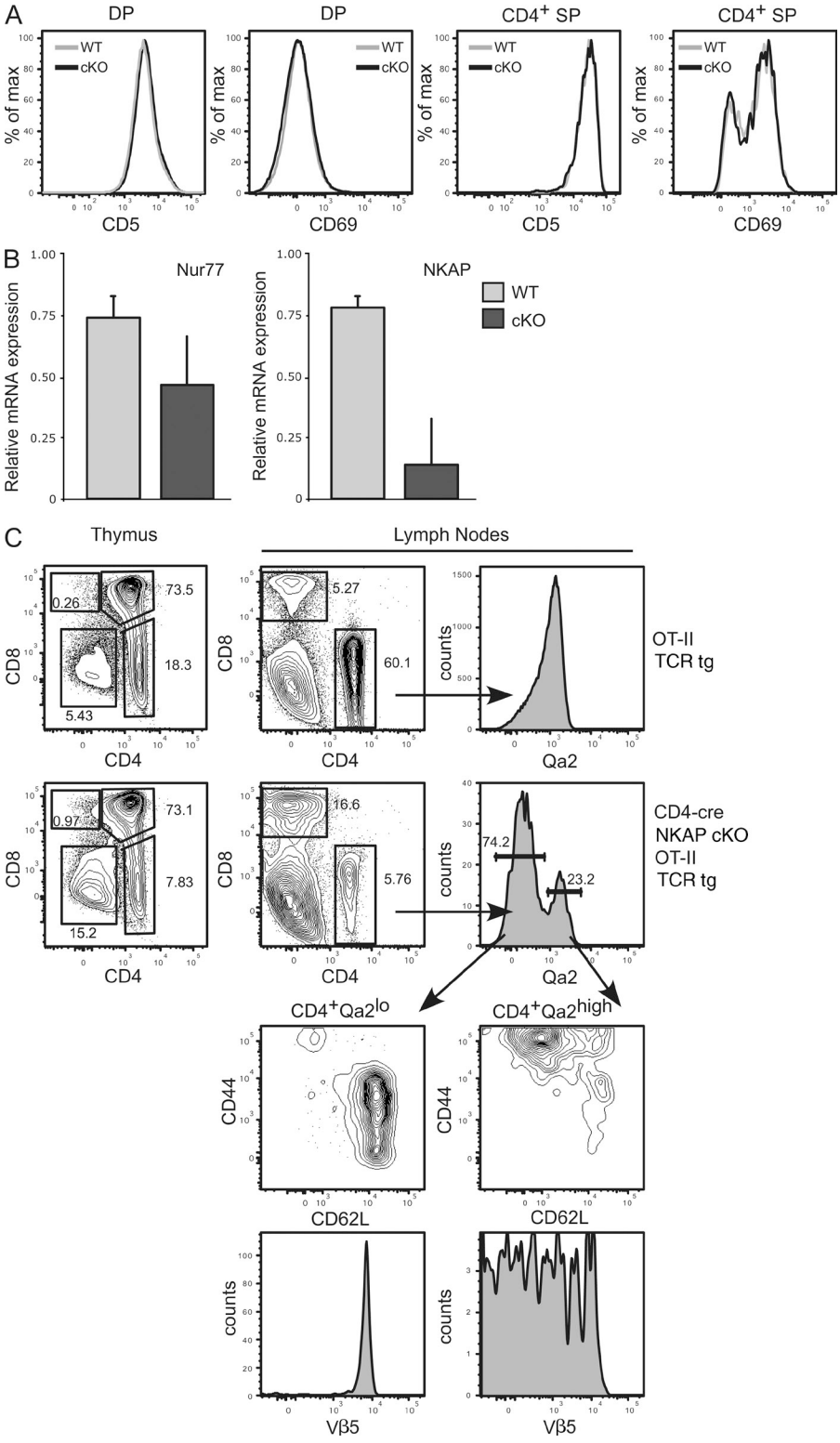


Figure 2. The defect in peripheral maturation in CD4-cre NKAP cKO mice cannot be rescued by the OT-II transgene. (A) Expression of CD5 and CD69 on DP and CD4 SP thymocytes from WT and CD4-cre NKAP cKO mice was examined. Representative data from four WT and three cKO from two independent experiments are shown. (B) The relative mRNA expression of Nur77 and NKAP in semimature CD4 SP thymocytes from WT and CD4-cre NKAP cKO mice was examined by Q-PCR. All data were normalized to the expression in one of the WT samples. Data are the mean from independent sorts from four WT and three CD4-cre NKAP cKO mice from two independent sorting experiments. Error bars represent the SEM. There is no statistically significant difference between expression of Nur77 in WT and CD4-cre NKAP cKO mice. The difference in NKAP expression between WT and CD4-cre NKAP cKO mice is statistically significant by Student's *t* test ($P < 0.01$). (C) FACS analysis of thymic T cell development and T cell maturation was performed as described in Fig. 1, using WT and CD4-cre NKAP cKO mice crossed to OT-II TCR transgene. The data shown are representative of at least four mice in each group from four independent experiments.

peripheral maturation defect was maintained. Interestingly, if we examined expression of the OT-II transgenic TCR using antibodies against Vβ5, the peripheral T cells that expressed the OT-II TCR were $Qa2^{low}$ and had a naive phenotype, whereas those that did not express Vβ5 were $Qa2^{high}$ and had a memory phenotype. Although many transgenic TCRs have the ability to homeostatically proliferate under lymphopenic conditions, a few, including those expressing the OT-II TCR, cannot (Ernst et al., 1999). Therefore, only rare NKAP nondeleters that do not express the OT-II TCR would be able to homeostatically proliferate, and the small number of T cells that expressed high levels of Qa2 were comprised almost exclusively of T cells that endogenously rearranged and expressed a TCR other than OT-II, as demonstrated by reduced expression of Vβ5. Thus, the T cell maturation defect upon loss of NKAP cannot be rescued by a transgenic TCR, and these data also support the hypothesis that the mature cells present in the periphery represent a homeostatic expansion.

The T cell maturation defect is cell intrinsic

The maturation of T cells requires entrance into the peripheral lymphoid organs and interactions with MHC (Houston et al., 2008). Therefore, because additional signals must be

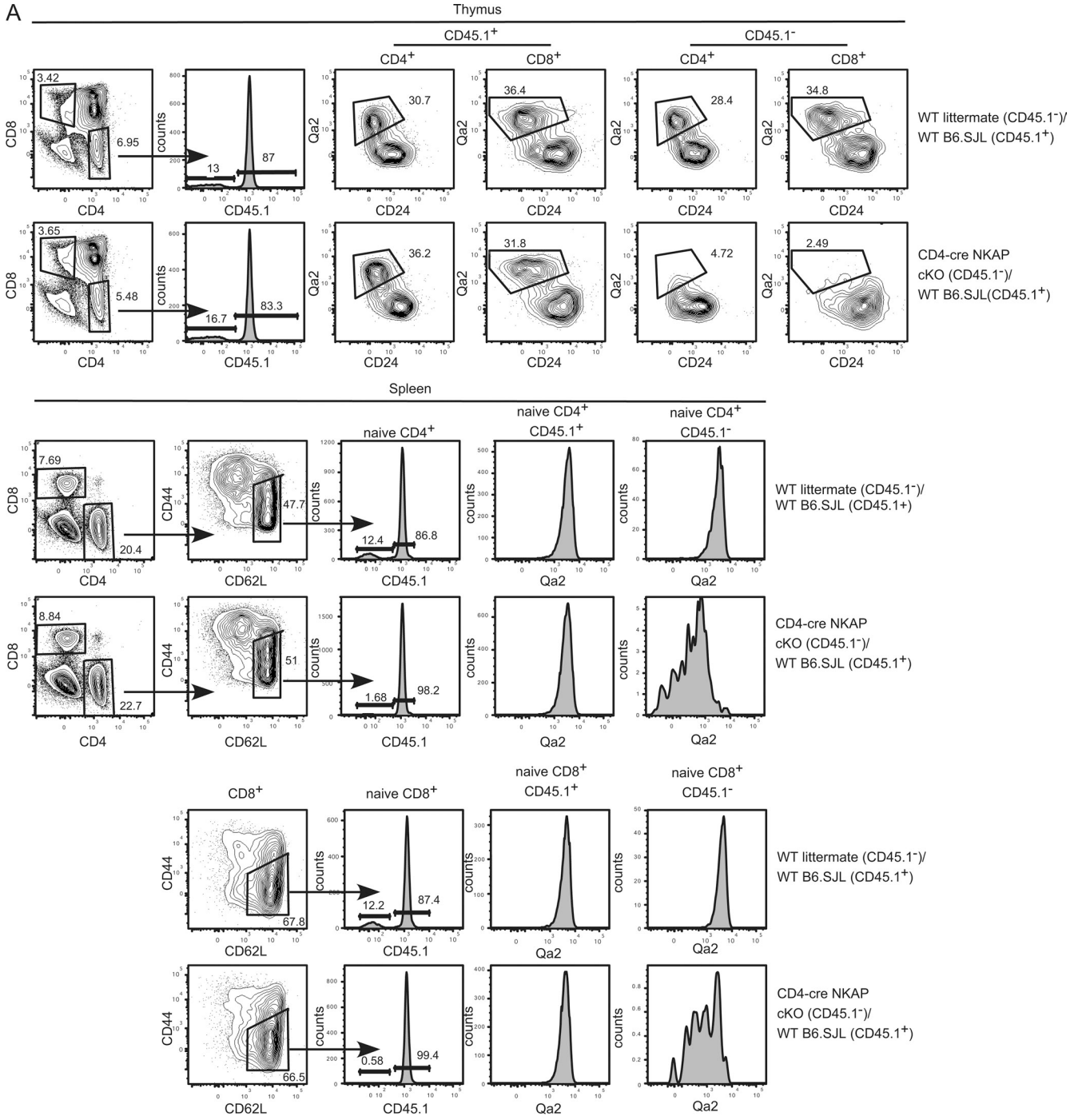
received by an RTE, it is possible that the defect in T cell maturation in CD4-cre NKAP cKO mice may not be cell intrinsic. Therefore, we generated mixed stem cell radiation chimeras using bone marrow from either CD4-cre NKAP cKO mice or WT littermates, along with B6.SJL congenic bone marrow. The donors express different alleles of CD45 which can be differentiated by flow cytometry, to determine the contribution of each donor to the thymic and peripheral lymphocyte pools (B6.SJL congenic mice express CD45.1 although WT littermate/CD4-cre NKAP cKO mice express CD45.2). Induction of Qa2 was normal in both the thymus and spleen of control WT littermate/B6.SJL chimeras (Fig. 3 A). However, no up-regulation of Qa2 was observed in either the CD4 or CD8 lineage in T cells derived from CD4-cre NKAP cKO bone marrow progenitors, whereas Qa2 expression was normal in T cells derived from B6.SJL progenitors within the same mixed chimera. Therefore, the block in T cell maturation caused by loss of NKAP is cell intrinsic. Additionally, the severity of the peripheral T cell phenotype was exacerbated in the mixed chimeras. The percentage of chimerism was stable in the control WT littermate/B6.SJL chimera from the CD4 and CD8 SP populations to the peripheral naive CD4 and CD8 T cell pools (Fig. 3 B). However, there was a severe alteration in the relative chimerism in the CD4-cre NKAP cKO/B6.SJL chimera, resulting in a >95% drop in the contribution of CD4-cre NKAP cKO T cells to the naive CD4 or CD8 peripheral pool as compared with the chimerism in the thymic SP populations. Because the peripheral T cell pool is comprised of normal B6.SJL-derived naive T cells, there was no lymphopenia to cause the homeostatic expansion of non–NKAP-deleted T cells, as was observed in CD4-cre NKAP cKO mice. Therefore, the block in T cell maturation is intrinsic to both CD4 and CD8 RTEs in CD4-cre NKAP cKO mice.

All naive peripheral CD4 T cells in CD4-cre NKAP cKO mice are functionally immature, RTEs

If the defect in CD4-cre NKAP cKO mice is caused by a failure in T cell maturation, then all peripheral naive CD4 T cells would be expected to be functionally immature RTEs. RTEs in the periphery can be identified using a Rag-GFP reporter (Boursalian et al., 2004). GFP expression is induced concurrently with Rag gene expression during T cell development. Although GFP expression ceases after Rag genes are turned off after TCR rearrangement is complete, the stability of GFP protein enables GFP expression to be maintained for 2–3 wk after T cell development is complete and these newly produced T cells exit the thymus (Boursalian et al., 2004). GFP expression is slowly extinguished in the periphery, with expression decreasing as the length of time increases from thymic egress. Thus, by use of a Rag-GFP reporter, RTEs can be distinguished from naive T cells that are in the long-lived peripheral pool. Expression of the Rag1-GFP reporter was similar throughout T cell development in WT and CD4-cre NKAP cKO mice (Fig. 4 A). In particular, the expression of Rag1-GFP was similar in both CD4 and CD8 SP populations. If the loss of peripheral T cells was caused by a defect in appropriate developmental migration of DP thymocytes to the medulla, or in thymic egress, then an accumulation of SP cells that expressed lower levels of Rag-GFP reporter would have been predicted, but this was not observed. In addition, no differences were found in the expression of KLF2 and S1P1, which regulate thymic egress (Carlson et al., 2006; Zachariah and Cyster, 2010), or in CCR7, which regulates thymic cortex to medulla migration (Kurobe et al., 2006), in either $CD4^+$ semimature ($CD4^+CD8^-CD69^+Qa2^{lo}$) thymocytes or RTEs from CD4-cre NKAP cKO mice (Fig. 4 B). Therefore, the defect in peripheral T cell numbers is not caused by a defect in thymic migration or egress. In the periphery, in Rag1-GFP knock-in mice (Kuwata et al., 1999), a minority of naive T cells in the spleen were GFP^+, with a small fraction with the highest expression of GFP detected as a broad shoulder of cells that exited from the thymus at progressively earlier time points (Fig. 4 C). However, in CD4-cre NKAP cKO mice that were crossed to Rag1-GFP knock-in mice, all naive CD4 T cells expressed high levels of GFP, indicating that they all were very RTEs. To examine the functional competency of these RTEs, splenocytes from Rag1-GFP knock-in mice crossed to either WT or CD4-cre NKAP cKO mice were stimulated overnight with plate-bound anti-CD3 and soluble anti-CD28 (Fig. 4 D). The following day, expression of IL-2 was measured by intracellular flow cytometry. Although $CD4^+GFP^+$ WT RTEs produced IL-2, RTEs from CD4-cre NKAP cKO mice did not. Therefore, the naive peripheral pool of CD4-cre NKAP cKO mice is comprised entirely of RTEs that lack functional competency, demonstrating that NKAP is critical for T cell maturation, and that the defect is not caused by a failure in thymocyte migration or egress.

CD4-cre NKAP cKO mice do not have a defect in IL-7Rα expression or function

An alternative hypothesis for the paucity of peripheral T cell may lie in a defect in their survival. For example, Foxo1-deficient (Kerdiles et al., 2009) or Runx1-deficient (Egawa et al., 2007) T cells have a defect in peripheral T cell numbers, but not thymocyte numbers, caused by defective IL-7Rα expression leading to decreased survival. The expression of IL-7Rα was examined in $CD4^+$ semimature thymocytes and splenic RTEs (Fig. 5 A). There was a small decrease in IL-7Rα expression in semimature $CD4^+$ thymocytes, which is not statistically significant. There was a twofold decrease in IL-7Rα expression in splenic $CD4^+$ RTEs between WT and CD4-cre NKAP cKO. Although this result is statistically significant ($P < 0.01$, Student's *t* test), it is unlikely to be biologically meaningful, as IL-7Rα heterozygous mice do not have a peripheral T cell defect (Peschon et al., 1994; Akashi et al., 1997). In addition, stimulation of CD4 SP thymocytes with IL-7 led to similar up-regulation of Bcl-2 expression and Stat5 phosphorylation in WT and CD4-cre NKAP cKO mice (Fig. 5, B and C), indicating that IL-7R responses are unaltered in CD4-cre NKAP cKO mice.


A
Thymus
CD45.1+
CD45.1-
CD4+
CD8+
CD4+
CD8+
3.42
6.95
13
87
30.7
36.4
28.4
34.8
WT littermate (CD45.1-)/
WT B6.SJL (CD45.1+)
3.65
5.48
16.7
83.3
36.2
31.8
4.72
2.49
CD4-cre NKAP
cKO (CD45.1-)/
WT B6.SJL(CD45.1+)
CD8
CD4
counts
CD45.1
Qa2
CD24
Spleen
naive CD4+
naive CD4+
CD45.1+
naive CD4+
CD45.1-
7.69
20.4
47.7
12.4
86.8
WT littermate (CD45.1-)/
WT B6.SJL (CD45.1+)
8.84
22.7
51
1.68
98.2
CD4-cre NKAP
cKO (CD45.1-)/
WT B6.SJL (CD45.1+)
CD44
CD62L
CD8+
naive CD8+
naive CD8+
CD45.1+
naive CD8+
CD45.1-
67.8
12.2
87.4
WT littermate (CD45.1-)/
WT B6.SJL (CD45.1+)
66.5
0.58
99.4
CD4-cre NKAP
cKO (CD45.1-)/
WT B6.SJL (CD45.1+)
B
Ratio of chimerism in
WT
cKO
CD4
CD8

Therefore, unlike Foxo1- or Runx1-deficient mice, the reduction in peripheral T cell numbers in CD4-cre NKAP cKO mice is not caused by the loss of expression or function of the IL-7R.

Overexpression of Bcl-2 does not rescue the T cell maturation defect in CD4-cre NKAP cKO mice

During peripheral B cell maturation, transitional B cells must receive a maturation signal before progressing to the long-lived follicular or marginal zone B cell pool (Thomas et al., 2006). Through modulation of the cytokine BAFF/BLyS, or overexpression of a Bcl-xl transgene, the length of time these newly produced B cells are maintained in the transitional B cell pool is expanded, allowing for a greater percentage of transitional B cells to receive the maturation signal and enter the long-lived peripheral pool (Hsu et al., 2002; Amanna et al., 2003). By extension, one explanation for the failure of T cell maturation in CD4-cre NKAP cKO mice may be caused by decreased survival, which prevents these RTEs from receiving the maturation signal. If so, then overexpression of a Bcl-2 transgene would be able to rescue the T cell maturation defect in the absence of NKAP. CD4-cre NKAP cKO mice were crossed with a human Bcl-2 transgene under the control of the *lck* promoter (Sentman et al., 1991). As shown in Fig. 6 A, the T cell maturation defect was not rescued by overexpression of Bcl-2. Although Bcl-2 overexpression increased the size of the naive peripheral pool in both WT and CD4-cre NKAP cKO mice, there was still a 10-fold decrease in the absolute number of splenic naive $CD4^+$ T cells in WT mice as compared with Bcl-2 transgenic CD4-cre NKAP cKO mice (Fig. 6 B). As a control, we found that the Bcl-2 transgene was expressed at similar levels in both $CD4^+$ semimature thymocytes and splenic RTEs (Fig. 6 C). Therefore, the failure of Bcl-2 to rescue peripheral T cell cellularity indicates that the block in T cell maturation is not secondary to a defect in RTE survival.

The defect in T cell maturation in CD4-cre NKAP cKO mice is independent of Notch activation

In our initial description of NKAP, we identified it as a novel HDAC3-associated transcriptional repressor that functions as a negative regulator of the Notch pathway (Pajerowski et al., 2009). However, this does not exclude the possibility that NKAP modulates additional transcriptional pathways. Both the Notch co-repressor complex and the activation complex, which includes intracellular Notch and the co-activator MAML, function through the transcription factor RBP-Jκ (also known as CSL; Maillard et al., 2005). In the absence of the Notch co-repressor complex, the Notch activation complex can proceed unabatedly to induce target genes, and the loss of NKAP early in T cell development leads to substantial potentiation of several Notch-regulated genes (Pajerowski et al., 2009). However, in work by other groups, transduction of hematopoietic progenitors with constitutively activated truncated Notch did not cause any alterations in peripheral T cell numbers before the development of leukemia (Pear et al., 1996). Thus, the defect in T cell maturation in CD4-cre NKAP cKO mice could be independent of its regulation of the Notch pathway. To determine this, we crossed CD4-cre NKAP cKO mice to mice with floxed RBP-Jκ (Tanigaki et al., 2004). It has been previously shown that conditional ablation of RBP-Jκ using CD4-cre did not lead to a block in T cell maturation, as peripheral T cell numbers were normal (Tanigaki et al., 2004). In the absence of RBP-Jκ, Notch cannot bind to DNA and activate target gene expression, and therefore would blunt the super-activation of Notch target genes in the absence of NKAP. Therefore, if the block in T cell maturation was maintained upon deletion of both RBP-Jκ and NKAP, then it would indicate that the block in T cell maturation in the absence of NKAP is independent of Notch. Consistent with previous studies (Tanigaki et al., 2004), there was no block in T cell maturation in CD4-cre RBP-Jκ cKO mice (unpublished data). However, the block in T cell maturation remained in CD4-cre NKAP/RBP-Jκ double-cKO mice (Fig. 7). Thus, the effect on NKAP deficiency on T cell maturation occurs via a separate and distinct mechanism from disruption of the Notch co-repressor complex.

DISCUSSION

We have shown that NKAP is absolutely required for T cell maturation. Although T cell development proceeds unimpeded upon loss of NKAP at the DP stage, the newly produced thymocytes do not complete T cell maturation, gain functional competency, or enter the long-lived naive T cell pool. A defect in maturation was indicated by a failure to down-regulate CD24/HSA and up-regulate Qa2 markers, which distinguish

Figure 3. The defect in T cell maturation is cell intrinsic in CD4-cre NKAP cKO mice. (A) Mixed radiation chimeras were generated by injecting lethally irradiated B6.SJL mice ($CD45.1^+$) with a combination of either CD4-cre NKAP cKO ($CD45.1^-$)/B6.SJL ($CD45.1^+$) bone marrow or a combination of WT littermate ($CD45.1^-$)/B6.SJL ($CD45.1^+$) bone marrow. 10 wk later, T cell development and maturation in both the CD4 and CD8 populations was analyzed as in Fig. 1. Within each mouse, the CD45.1 allelic marker was used to differentiate T cells derived from B6.SJL ($CD45.1^+$) from either WT or CD4-cre NKAP cKO mice ($CD45.1^-$). The data shown are representative of eight WT mixed chimeras and six cKO mixed chimeras from three independent experiments. (B) Shown is a comparison of relative $CD45.1^-$ chimerism, comparing the percentage of chimerism in splenic naive T cells to the percentage of chimerism in SP thymocytes for both CD4 and CD8 T cells, from either CD4-cre NKAP cKO or control WT littermate mixed radiation chimeras described in A. The data are the mean from eight control WT littermate mixed chimeras and six CD4-cre NKAP cKO mixed chimeras from three independent experiments. For each mouse, the data are calculated by dividing the $CD45.1^-$ chimerism in spleen by the relative chimerism in thymus. The mean relative chimerism for each population is shown, and error bars reflect the standard deviation.

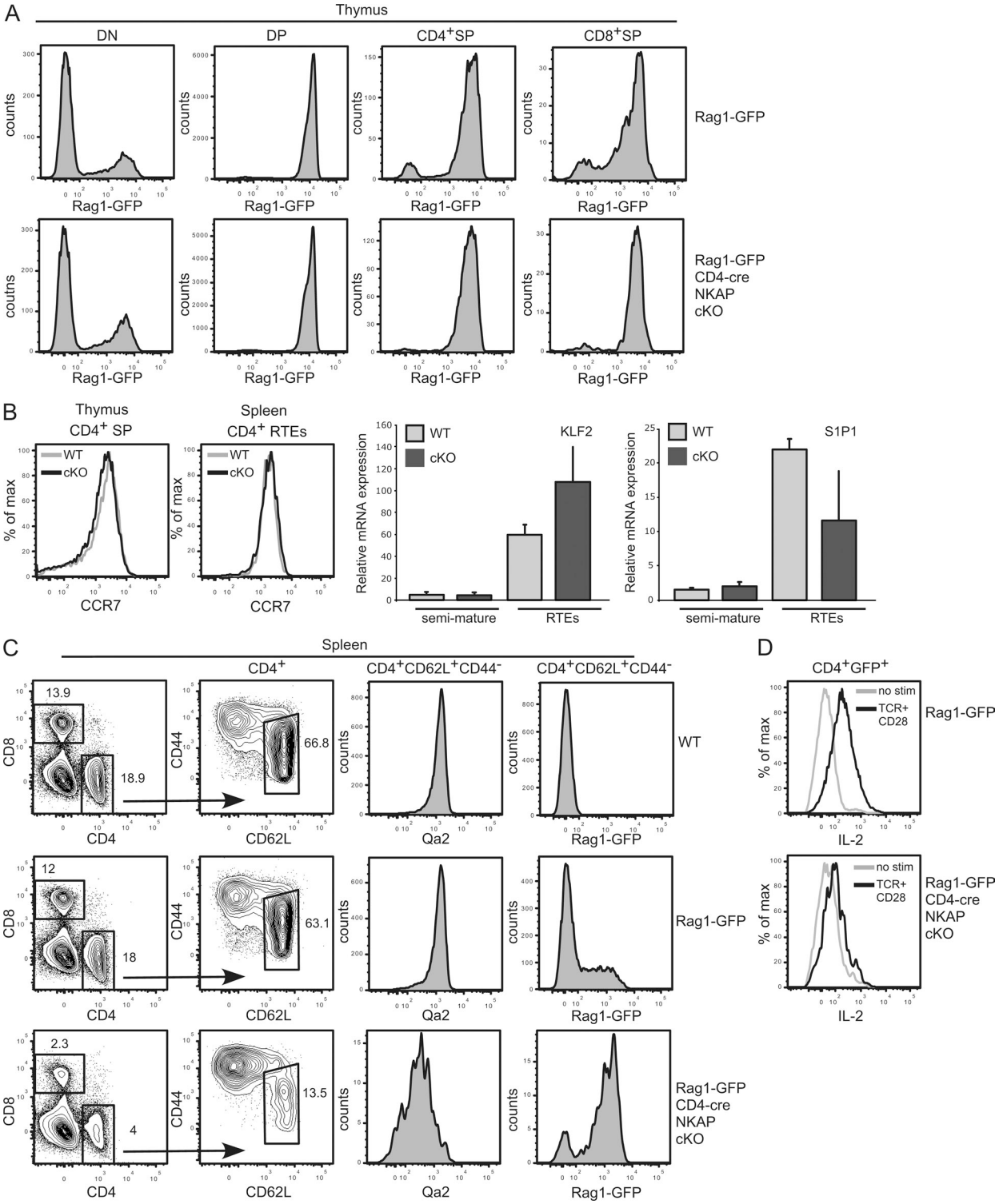


Figure 4. Nearly all naive peripheral CD4 T cells in CD4-cre NKAP cKO mice are RTEs. (A) Expression of the knock-in Rag1-GFP reporter was examined in DN, DP, CD4 SP, and CD8 SP thymocyte populations in WT and CD4-cre NKAP cKO mice. Data shown are representative of at least six mice per group from five independent experiments. (B) The expression of CCR7, KLF2, and S1P1 was examined in semimature CD4 SP thymocytes and CD4 RTEs. No differences were observed in the expression of CCR7 on the cell surface of WT and CD4-cre NKAP cKO semimature CD4 SP thymocytes and CD4 RTEs. A representative FACS plot of at least four mice examined per group from four independent experiments is shown. The relative mRNA expression of KLF2 and S1P1 in semimature CD4 SP thymocytes from WT and CD4-cre NKAP cKO mice was examined by Q-PCR. All data were normalized to the expression in one of the WT samples. Data are the mean from four WT, three CD4-cre NKAP cKO, six Rag1-GFP mice, and at least four Rag1-GFP CD4-cre

semimature T cells that have completed T cell development from fully mature T cells that can participate in an immune response. A functional defect in maturation was exhibited by naive CD4 T cells from CD4-cre NKAP cKO mice, which did not produce IL-2 upon TCR/CD28 stimulation. The defect in maturation was cell intrinsic, as demonstrated using mixed radiation chimeras, and could not be rescued by expression of the OT-II transgenic TCR. Consistent with a block in T cell maturation, NKAP-deficient CD4 peripheral T cells did not enter the long-lived T cell pool and were almost exclusively RTEs, as demonstrated by residual expression of a Rag1-GFP reporter. Thus, NKAP is required for T cell maturation.

Although other KO animals display defects in peripheral T cell numbers, such as Foxo1-deficient (Kerdiles et al., 2009), Runx1-deficient (Egawa et al., 2007), KLF2-deficient (Carlson et al., 2006), and Foxp1-deficient (Feng et al., 2010) mice, the specific defect in T cell maturation observed upon NKAP deletion is unique. Conditional deletion of Foxo1 (Kerdiles et al., 2009) or Runx1 (Egawa et al., 2007) led to down-regulation of IL-7R expression, and consequently altered thymocyte and peripheral T cell survival. The differences in expression of IL-7Rα was twofold or less in $CD4^+$ semimature or splenic RTEs from CD4-cre NKAP cKO mice as compared with WT. As IL-7Rα heterozygous mice do not have a defect in the number of peripheral T cells (Peschon et al., 1994; Akashi et al., 1997), it is unlikely that this difference is the cause of the effect on the naive T cell pool. In addition, thymocytes from CD4-cre NKAP cKO mice respond normally to IL-7 stimulation by up-regulating Bcl-2 expression and phosphorylating Stat5, indicating that there is also no defect in IL-7R signaling. Mice deficient in KLF2 (Carlson et al., 2006) have a defect in thymic egress stemming from defects in S1P1 expression (Zachariah and Cyster, 2010), leading to an accumulation of CD4 and CD8 SP T cells in the thymus. CD4-cre NKAP cKO mice have similar CD4/CD8 FACS profiles and numbers of CD4 and CD8 SP cells in the thymus as compared with WT, indicating that there is no accumulation of SP thymocytes. By crossing the mice to Rag1-GFP knock-in mice (Kuwata et al., 1999), we demonstrate that the naive peripheral T cell pool in CD4-cre NKAP cKO mice is comprised almost entirely of GFP^+ RTEs, demonstrating that these cells can egress from the thymus and migrate appropriately to the periphery, despite subsequently failing to complete maturation and enter the long-lived T cell pool. CD4-cre Foxp1 cKO mice have a peripheral T cell defect, but are capable of appropriately down-regulating CD24/HSA and up-regulating Qa2 (Feng et al., 2010), indicating that the defect is not in T cell maturation. Expression of Runx1, Foxo1, Foxp1, KLF2 and S1P1 were not altered in $CD4^+$ semimature thymocytes or $CD4^+$ RTEs from Rag1-GFP CD4-cre NKAP cKO mice as compared with Rag1-GFP WT mice as measured by Q-PCR (Fig. 5 and Fig. S1). Therefore, the phenotype of the CD4-cre NKAP cKO mice, a specific block in T cell maturation, is unlike those of other mice previously examined with peripheral T cell defects.

Similarly, several lines of mice that alter NF-κB signaling have been generated that lead to a severe deficiency in peripheral T cells: Tak1 cKO mice (Liu et al., 2006; Sato et al., 2006; Wan et al., 2006), c-FLIP cKO mice (Chau et al., 2005; Zhang and He, 2005), NEMO/IKKγ cKO mice (Schmidt-Supprian et al., 2003), and mice with conditional ablation of the kinase domain of IKK2 (IKK2ΔK; Schmidt-Supprian et al., 2003). CD4-cre NEMO/IKKγ cKO mice and CD4-cre IKK2ΔK have normal numbers of thymocytes, but a dramatic decrease in peripheral T cell numbers. Examination of CD24/HSA in SP thymocytes indicated that both of these lines had defects in down-regulation of CD24/HSA, implying that there may be a defect in maturation. Similar results were observed in Lck-cre Tak1 and Lck-cre c-FLIP cKO mice (Zhang and He, 2005; Liu et al., 2006; Sato et al., 2006). However, expression of CD24/HSA or Qa2 was not examined in peripheral populations to determine whether there was a defect in T cell maturation, nor were these lines crossed to a Rag-GFP reporter strain to indicate that the peripheral T cells present were all RTEs. It may be that defects in the NF-κB pathway lead to alterations in the survival of mature peripheral T cells rather than a defect in T cell maturation. CD4-cre TAK1 cKO mice have normal expression of IL-7Rα, but have defects in IL-7–mediated survival (Wan et al., 2006). Enhanced apoptosis was also observed in SP thymocytes from CD4-cre NEMO/IKKγ cKO mice and Lck-cre c-FLIP cKO mice (Schmidt-Supprian et al., 2003; Zhang and He, 2005), which is not surprising as the NF-κB pathways regulate cell survival. In contrast, NKAP-deficient thymocytes responded normally to IL-7 stimulation and the defect in T cell maturation could not be rescued by overexpression

NKAP cKO mice from four independent sorting experiments. Error bars reflect the SEM. No differences in expression were statistically significant between WT and CD4-cre NKAP cKO mice. (C) Analysis of peripheral T cell populations in Rag1-GFP or Rag1-GFP CD4-cre NKAP cKO mice was performed as in Fig. 1. Although transcription of GFP under the control of Rag1 ceases at the $CD4^+/CD8^+$ DP stage, GFP expression is maintained throughout subsequent T cell development and for ∼2-3 wk in the periphery, marking cells as recent RTEs. WT mice without the Rag1-GFP transgene is shown for comparison. The data shown are representative of six mice in each group from five independent experiments. (D) Splenocytes from Rag1-GFP or Rag1-GFP CD4-cre NKAP cKO mice were left unstimulated (gray histogram) or stimulated overnight with 10 µg/ml anti-CD3 and 1 µg/ml anti-CD28 (black histogram). The following day, cultures were harvested and surface stained for CD4 and CD8, and then stained intracellularly for IL-2. For IL-2 histograms, cells were gated on $CD4^+CD8^-GFP^+$ RTEs. The data shown are representative of four Rag1-GFP CD4-cre NKAP cKO mice and two Rag1-GFP mice examined from two independent experiments.

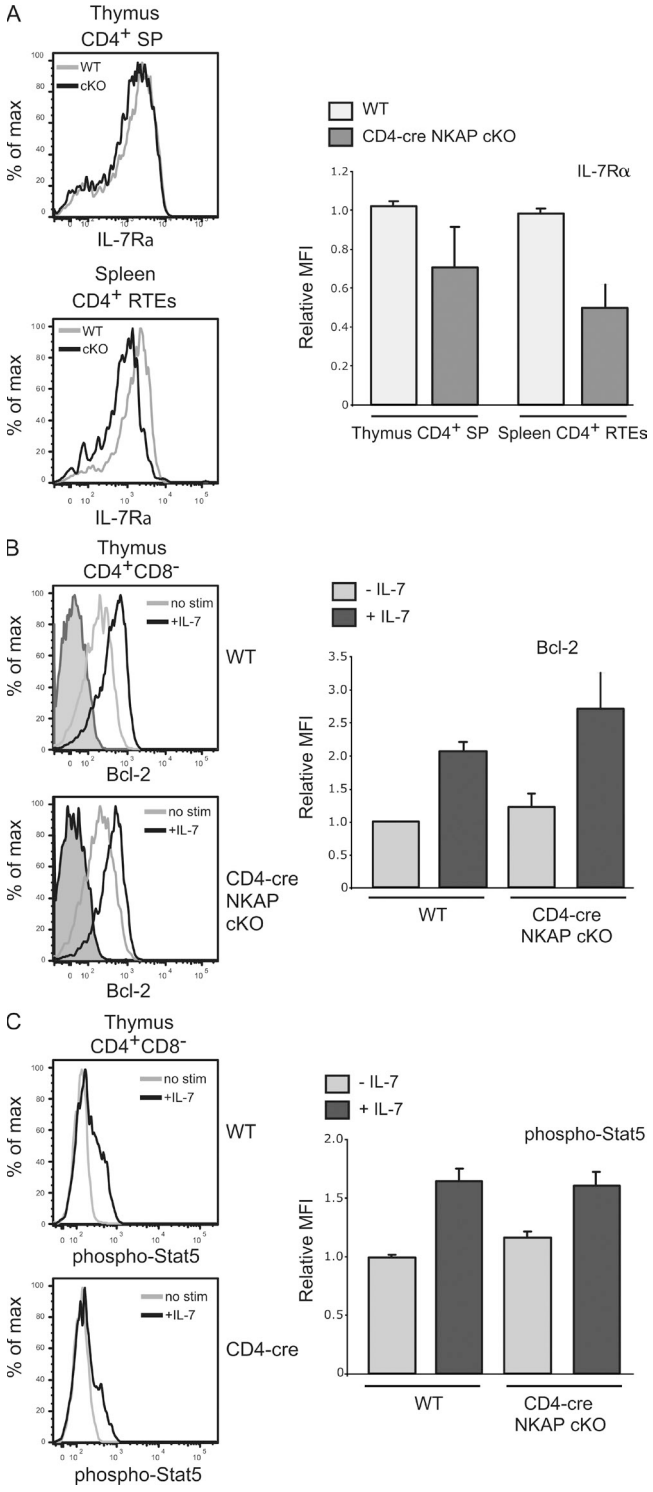


Figure 5. The defect in peripheral T cells is not caused by loss of in IL-7R expression or in failure to respond to IL-7. (A) IL-7Rα chain expression on CD4+ SP thymocytes (CD4+CD8−Rag1-GFP+) and CD4+CD8−GFP+ RTEs from spleen from WT and CD4-cre NKAP cKO mice was examined. The relative mean fluorescence intensity was examined. Within each experiment, the expression of IL-7Rα was normalized to that in WT CD4+ SP thymocytes (= 1). The difference in IL-7Rα expression between WT and CD4-cre NKAP cKO CD4+ SP thymocytes is not statistically significant. The twofold difference in expression in CD4+ RTEs between WT and CD4-cre NKAP cKO mice is significant ($P < 0.01$). A total of four Rag1-GFP+ and three Rag1-GFP+ CD4-cre NKAP cKO mice were examined in three independent experiments. (B) Thymocytes from WT and CD4-cre NKAP cKO mice were stimulated overnight with 10 ng/ml IL-7. The cultures were surface stained for CD4, CD8, and IL-7R expression, and then stained intracellularly for expression of Bcl-2. The difference in Bcl-2 expression in IL-7–stimulated WT and CD4-cre NKAP cKO CD4+ SP thymocytes is not statistically significant. The data shown are representative of three mice in each group from three independent experiments. (C) Thymocytes from WT and CD4-cre NKAP cKO mice were stimulated overnight with 10 ng/ml IL-7. The cultures were surface stained for CD4, CD8, and IL-7R expression, and then stained intracellularly for expression of phospho-Stat5. The difference in phospho-Stat5 expression in IL-7–stimulated WT and CD4-cre NKAP cKO CD4+ SP thymocytes is not statistically significant. The data shown are representative of six mice in each group from five independent experiments.

of a Bcl-2 transgene, indicating that the paucity of peripheral T cells in CD4-cre NKAP cKO mice is not simply caused by increased apoptosis. Expression of Tak1, NEMO, or cFLIP as measured by Q-PCR was not altered in CD4+ semimature thymocytes or CD4+ RTEs from Rag1-GFP CD4-cre NKAP cKO mice as compared with Rag1-GFP WT mice (Fig. S1). Thus, conditional deletion of NKAP in DP thymocytes leads to a unique and specific block in T cell maturation, unlike other previously described mice with peripheral T cell defects.

Our initial biochemical work demonstrated that NKAP functions as a transcriptional repressor, and, at least in part, as a negative regulator of Notch signaling through a direct association with CIR and the Notch co-repressor complex (Pajerowski et al., 2009). Upon NKAP deletion at the DN3 stage in Lck-cre NKAP cKO mice, expression of three Notch-regulated genes, Hes1, Deltex, and CD25, increased from 8- to 20-fold, indicating that NKAP functions as a negative regulator of Notch signaling in vivo. To determine whether the defect in T cell maturation in the CD4-cre NKAP cKO mice was caused by altered regulation of the Notch pathway, we crossed the mice to RBP-Jκ floxed mice (Tanigaki et al., 2004) to eliminate the transcription factor through which intracellular Notch activates transcription. CD4-cre RPB-Jκ cKO mice had normal numbers of peripheral T cells, demonstrating that T cell maturation was intact. However, the defect in T cell maturation was still observed in CD4-cre RBP-Jκ/NKAP double deficient mice, indicating that altered Notch pathway activation is not the cause of the T cell maturation defect. Therefore, NKAP must modulate transcription pathways in addition to Notch to regulate T cell maturation, which is currently under investigation.

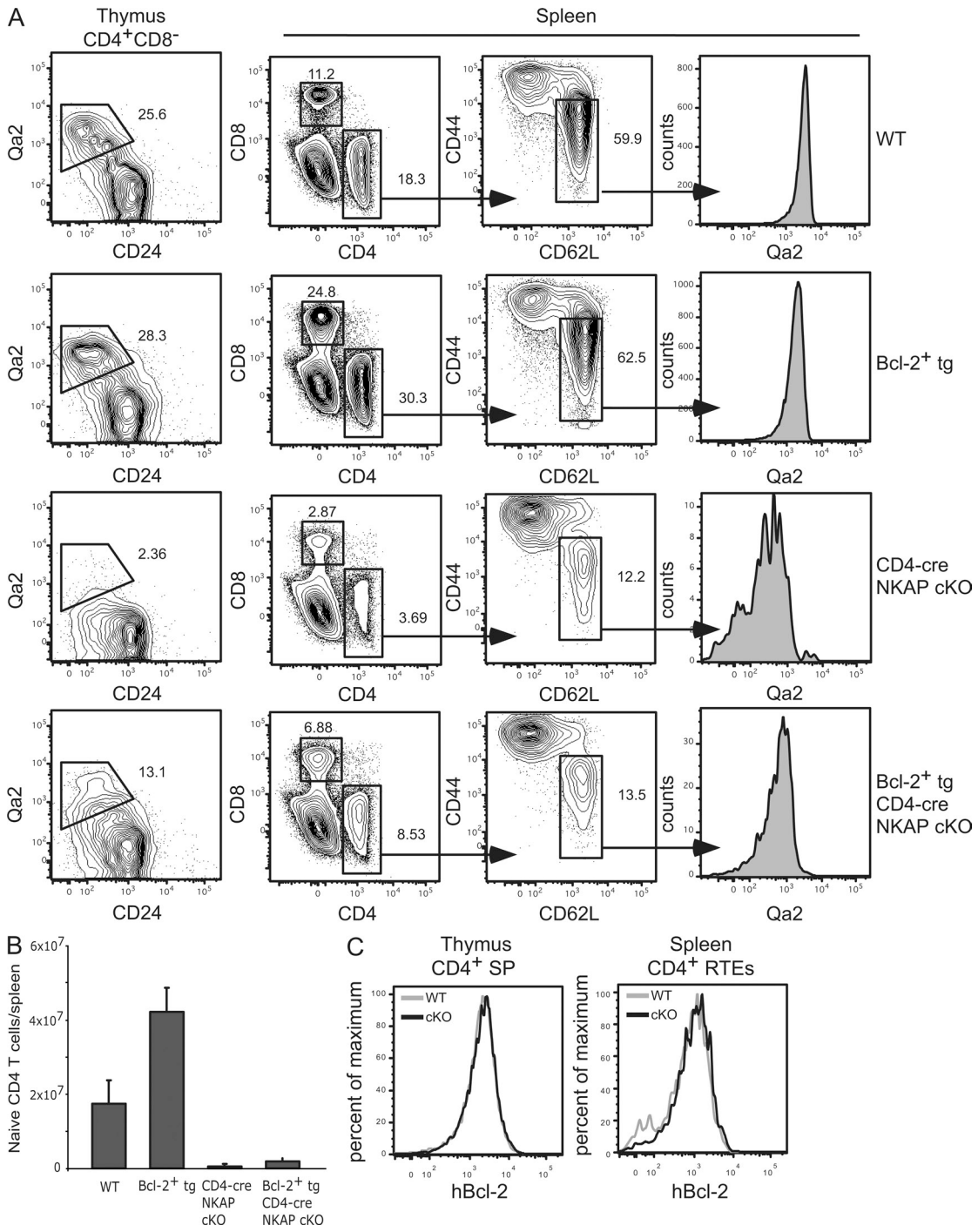


Figure 6. The block in T cell maturation cannot be rescued by a Bcl-2 transgene. (A) Analysis of thymic T cell development and T cell maturation as in Fig. 1 in WT, CD4-cre NKAP cKO mice, Bcl-2 transgenic, and Bcl-2 transgenic/CD4-cre NKAP cKO mice. Data shown are representative of two to three mice per group from two independent experiments. (B) Absolute number of naive CD4 splenocytes from WT, Bcl-2-transgenic, CD4-cre NKAP cKO, and Bcl-2-transgenic CD4-cre NKAP cKO mice is shown. Data are the mean of 2–3 mice per group from two independent experiments. Error bars represent SEM. The 10-fold difference in cellularity of naive CD4 splenocytes between WT and Bcl-2-transgenic CD4-cre NKAP cKO mice is statistically significant by Student's *t* test ($P < 0.02$). (C) Human Bcl-2 expression on $CD4^+$ SP thymocytes ($CD4^+CD8^-Rag1\text{-}GFP^+$), and $CD4^+CD8^-GFP^+$ RTEs from spleen from Bcl-2-transgenic and Bcl-2-transgenic CD4-cre NKAP cKO mice was examined. The expression of the Bcl-2 transgene is similar between WT and CD4-cre NKAP cKO mice. Data shown are representative of four mice per group from three independent experiments.

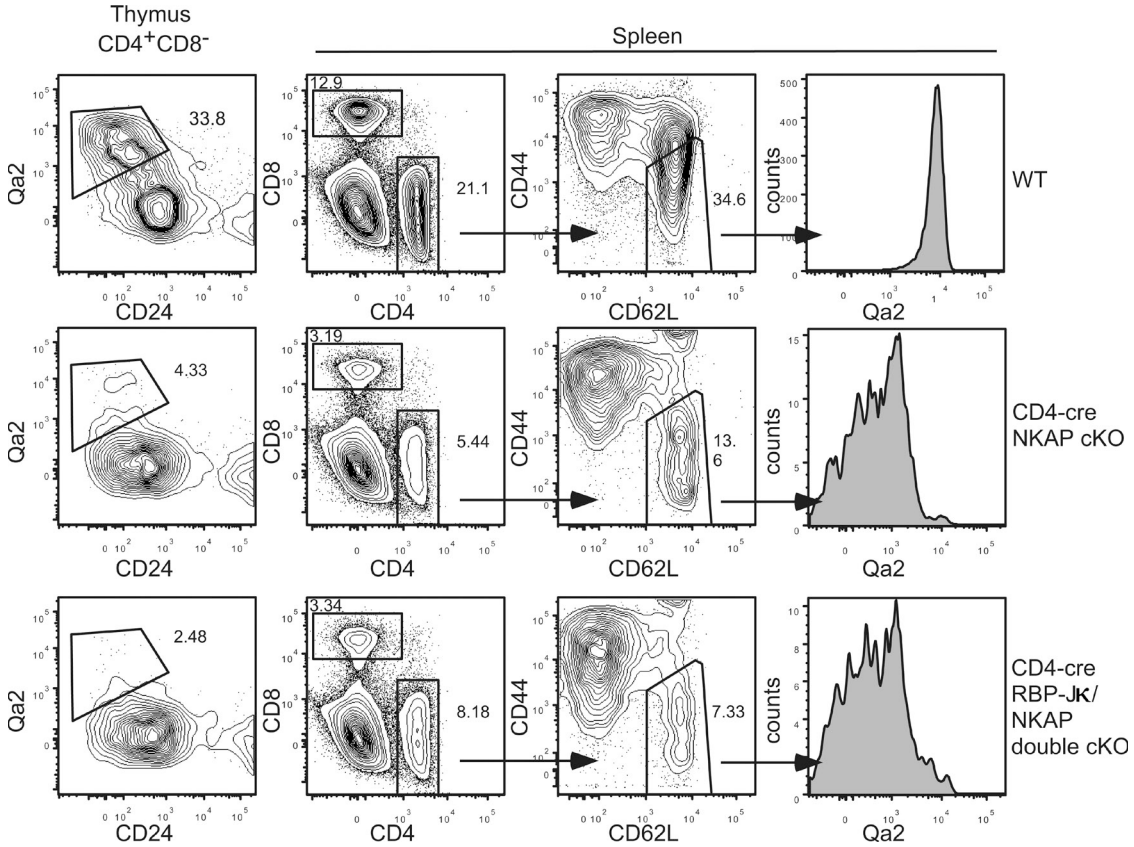


Figure 7. The block in T cell maturation is independent of the Notch pathway. Analysis of thymic T cell development and T cell maturation as in Fig. 1 in WT, CD4-cre NKAP cKO mice, and CD4-cre RBP-Jκ/NKAP double cKO mice. Data shown are representative of at least three mice per group from two independent experiments.

MATERIALS AND METHODS

Floxed and transgenic mice. The generation of floxed NKAP mice was previously described (Pajerowski et al., 2009). RBP-Jκ floxed mice were generated and provided by T. Honjo (Kyoto University, Kyoto, Japan; Tanigaki et al., 2004). Rag1-GFP mice were generated and provided by N. Sakaguchi (Kumamoto University, Kumamoto, Japan; Kuwata et al., 1999). Human Bcl-2 transgenic mice were generated by S. Korsmeyer (Dana-Farber Cancer Institute, Boston, MA; Sentman et al., 1991) and provided by A. Singer (National Institutes of Health, Bethesda, MD). CD4-cre (Lee et al., 2001) and OT-II TCR transgenics (Barnden et al., 1998) were purchased from Taconic through the National Institute of Allergy and Infectious Disease emerging models program. Mice were housed in a barrier facility and all experiments were performed under the guidelines and with the approval of the Mayo Clinic institutional animal care and use committee. All animals were analyzed between 8–12 wk of age. All CD4-cre NKAP cKO mice were examined with littermates as controls.

Generation of radiation chimeras. B6.SJL donors and recipients were purchased from National Cancer Institute Frederick, and radiation chimera generation was performed as previously described (Pajerowski et al., 2010).

Flow cytometry. Analysis was performed on a LSR II Flow cytometer (BD). Experiments were analyzed using FlowJo (Tree Star). All data were doublet excluded using FSC-H/FSC-W and SSC-H/SSC-W before analysis. In all experiments, except intracellular staining, dead cells were excluded from analysis by staining with DAPI.

Antibodies. All antibodies for flow cytometry were purchased from either BD, BioLegend, or eBioscience. 2C11 anti–mouse CD3 and 37.51 anti–mouse CD28 were purchased from Bio-X-Cell.

Stimulations. Recombinant mouse IL-7 was purchased from PeproTech. Thymocytes from WT or CD4-cre NKAP cKO mice were left unstimulated or stimulated overnight with 10 ng/ml IL-7. The following day, thymocytes were surface stained for CD4, CD8, and/or IL-7R, or stained intracellularly for Bcl-2 or phospho-Stat5. For intracellular IL-2, splenocytes from Rag1-GFP WT or Rag1-GFP CD4-cre NKAP cKO mice were left unstimulated or stimulated overnight with 10 µg/ml anti-CD3 (clone 2C11) and 1 µg/ml anti-CD28 (clone 37.51).

Analysis of deletion efficiency of NKAP. NKAP deletion efficiency was analyzed by genomic Q-PCR as previously described (Pajerowski et al., 2009).

FACS sorting. To isolate $CD4^+$ semimature T cells, thymocytes from Rag1-GFP or Rag1-GFP CD4-cre NKAP cKO mice were harvested and negatively selected for $CD8^+$ cells using magnetic bead selection. Cells were sorting used antibodies to CD4, CD8, TCRβ, Qa2, CD69, and a "dump" cocktail containing CD25, NK1.1, and PBS-57/CD1d tetramers to exclude T reg cells, NK cells, and invariant NKT cells. PE-labeled PBS-57/CD1d tetramer was obtained through the National Institutes of Health Tetramer Facility. $CD4^+$ semimature T cells were selected for $CD4^+CD8^-TCR\beta^+Qa2^{lo}CD69^{hi}GFP^+$ expression. $CD4^+$ RTEs were sorted from Rag1-GFP or Rag1-GFP CD4-cre NKAP cKO mice splenocytes based on $CD4^+CD8^-GFP^+$ expression. All sorting was performed on a FACSAria (BD).

Real-time Q-PCR analysis of relative mRNA expression. mRNA was isolated from $CD4^+$ RTEs or $CD4^+$ semimature cells (QIAGEN). cDNA was generated with Superscript III (Invitrogen), amplified using an Ovation PicoSL WTA (whole transcript amplification) kit (NuGen), and detected using TaqMan probes (Applied Biosystems) for Nur77, NKAP, KLF2, Runx1, Tak1, cFLIP, Foxo1, Foxp1, and NEMO, using 18S rRNA as an internal control. S1P1 expression was measured as previously described (Maeda et al., 2007). An ABI RT-PCR StepOne Plus System (Applied Biosystems) was used, and relative expression was calculated via the 2-ΔΔCT method (Livak and Schmittgen, 2001).

Online supplemental material. Fig. S1 shows expression of Runx1, Tak1, cFLIP, Foxo1, Foxp1, and NEMO by Q-PCR in semimature cells and RTEs from WT and cKO mice. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20101874/DC1.

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REFERENCES

Akashi, K., M. Kondo, U. von Freeden-Jeffry, R. Murray, and I.L. Weissman. 1997. Bcl-2 rescues T lymphopoiesis in interleukin-7 receptor-deficient mice. *Cell*. 89:1033–1041. doi:10.1016/S0092-8674(00)80291-3

Allman, D.M., S.E. Ferguson, V.M. Lentz, and M.P. Cancro. 1993. Peripheral B cell maturation. II. Heat-stable antigen(hi) splenic B cells are an immature developmental intermediate in the production of long-lived marrow-derived B cells. *J. Immunol*. 151:4431–4444.

Allman, D., R.C. Lindsley, W. DeMuth, K. Rudd, S.A. Shinton, and R.R. Hardy. 2001. Resolution of three nonproliferative immature splenic B cell subsets reveals multiple selection points during peripheral B cell maturation. *J. Immunol*. 167:6834–6840.

Amanna, I.J., J.P. Dingwall, and C.E. Hayes. 2003. Enforced bcl-xL gene expression restored splenic B lymphocyte development in BAFF-R mutant mice. *J. Immunol*. 170:4593–4600.

Barnden, M.J., J. Allison, W.R. Heath, and F.R. Carbone. 1998. Defective TCR expression in transgenic mice constructed using cDNA-based alpha- and beta-chain genes under the control of heterologous regulatory elements. *Immunol. Cell Biol*. 76:34–40. doi:10.1046/j.1440-1711.1998.00709.x

Boursalian, T.E., J. Golob, D.M. Soper, C.J. Cooper, and P.J. Fink. 2004. Continued maturation of thymic emigrants in the periphery. *Nat. Immunol*. 5:418–425. doi:10.1038/ni1049

Carlson, C.M., B.T. Endrizzi, J. Wu, X. Ding, M.A. Weinreich, E.R. Walsh, M.A. Wani, J.B. Lingrel, K.A. Hogquist, and S.C. Jameson. 2006. Kruppel-like factor 2 regulates thymocyte and T-cell migration. *Nature*. 442:229–302. doi:10.1038/442229a

Chau, H., V. Wong, N.-J. Chen, H.-L. Huang, W.-J. Lin, C. Mirtsos, A.R. Elford, M. Bonnard, A. Wakeham, A.I. You-Ten, et al. 2005. Cellular FLICE-inhibitory protein is required for T cell survival and cycling. *J. Exp. Med*. 202:405–413. doi:10.1084/jem.20050118

Egawa, T., R.E. Tillman, Y. Naoe, I. Taniuchi, and D.R. Littman. 2007. The role of the Runx transcription factors in thymocyte differentiation and in homeostasis of naive T cells. *J. Exp. Med*. 204:1945–1957. doi:10.1084/jem.20070133

Ernst, B., D.S. Lee, J.M. Chang, J. Sprent, and C.D. Surh. 1999. The peptide ligands mediating positive selection in the thymus control T cell survival and homeostatic proliferation in the periphery. *Immunity*. 11:173–181. doi:10.1016/S1074-7613(00)80092-8

Feng, X., G.C. Ippolito, L. Tian, K. Wiehagen, S. Oh, A. Sambandam, J. Willen, R.M. Bunte, S.D. Maika, J.V. Harriss, et al. 2010. Foxp1 is an essential transcriptional regulator for the generation of quiescent naive T cells during thymocyte development. *Blood*. 115:510–518. doi:10.1182/blood-2009-07-232694

Fink, P.J. 2009. RTEs: lazy T-cell teenagers. *Blood*. 113:5374–5375. doi:10.1182/blood-2009-03-207308

Gabor, M.J., D.I. Godfrey, and R. Scollay. 1997. Recent thymic emigrants are distinct from most medullary thymocytes. *Eur. J. Immunol*. 27:2010–2015. doi:10.1002/eji.1830270827

Houston, E.G. Jr., R. Nechanitzky, and P.J. Fink. 2008. Cutting edge: contact with secondary lymphoid organs drives postthymic T cell maturation. *J. Immunol*. 181:5213–5217.

Hsu, B.L., S.M. Harless, R.C. Lindsley, D.M. Hilbert, and M.P. Cancro. 2002. Cutting edge: BLyS enables survival of transitional and mature B cells through distinct mediators. *J. Immunol*. 168:5993–5996.

Kerdiles, Y.M., D.R. Beisner, R. Tinoco, A.S. Dejean, D.H. Castrillon, R.A. DePinho, and S.M. Hedrick. 2009. Foxo1 links homing and survival of naive T cells by regulating L-selectin, CCR7 and interleukin 7 receptor. *Nat. Immunol*. 10:176–184. doi:10.1038/ni.1689

Kurobe, H., C. Liu, T. Ueno, F. Saito, I. Ohigashi, N. Seach, R. Arakaki, Y. Hayashi, T. Kitagawa, M. Lipp, et al. 2006. CCR7-dependent cortex-to-medulla migration of positively selected thymocytes is essential for establishing central tolerance. *Immunity*. 24:165–177. doi:10.1016/j.immuni.2005.12.011

Kuwata, N., H. Igarashi, T. Ohmura, S. Aizawa, and N. Sakaguchi. 1999. Cutting edge: absence of expression of RAG1 in peritoneal B-1 cells detected by knocking into RAG1 locus with green fluorescent protein gene. *J. Immunol*. 163:6355–6359.

Lee, P.P., D.R. Fitzpatrick, C. Beard, H.K. Jessup, S. Lehar, K.W. Makar, M. Pérez-Melgosa, M.T. Sweetser, M.S. Schlissel, S. Nguyen, et al. 2001. A critical role for Dnmt1 and DNA methylation in T cell development, function, and survival. *Immunity*. 15:763–774. doi:10.1016/S1074-7613(01)00227-8

Liu, H.-H., M. Xie, M.D. Schneider, and Z.J. Chen. 2006. Essential role of TAK1 in thymocyte development and activation. *Proc. Natl. Acad. Sci. USA*. 103:11677–11682. doi:10.1073/pnas.0603089103

Livak, K.J., and T.D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*. 25:402–408. doi:10.1006/meth.2001.1262

Maeda, Y., H. Matsuyuki, K. Shimano, H. Kataoka, K. Sugahara, and K. Chiba. 2007. Migration of CD4 T cells and dendritic cells toward sphingosine 1-phosphate (S1P) is mediated by different receptor subtypes: S1P regulates the functions of murine mature dendritic cells via S1P receptor type 3. *J. Immunol*. 178:3437–3446.

Maillard, I., T. Fang, and W.S. Pear. 2005. Regulation of lymphoid development, differentiation, and function by the Notch pathway. *Annu. Rev. Immunol*. 23:945–974. doi:10.1146/annurev.immunol.23.021704.115747

Pajerowski, A.G., C. Nguyen, H. Aghajanian, M.J. Shapiro, and V.S. Shapiro. 2009. NKAP is a transcriptional repressor of notch signaling and is required for T cell development. *Immunity*. 30:696–707. doi:10.1016/j.immuni.2009.02.011

Pajerowski, A.G., M.J. Shapiro, K. Gwin, R. Sundsbak, M. Nelson-Holte, K.L. Medina, and V.S. Shapiro. 2010. Adult hematopoietic stem cells require NKAP for maintenance and survival. *Blood*. 116:2684–2693. doi:10.1182/blood-2010-02-268391

Pear, W.S., J.C. Aster, M.L. Scott, R.P. Hasserjian, B. Soffer, J. Sklar, and D. Baltimore. 1996. Exclusive development of T cell neoplasms in mice transplanted with bone marrow expressing activated Notch alleles. *J. Exp. Med*. 183:2283–2291. doi:10.1084/jem.183.5.2283

Peschon, J.J., P.J. Morrissey, K.H. Grabstein, F.J. Ramsdell, E. Maraskovsky, B.C. Gliniak, L.S. Park, S.F. Ziegler, D.E. Williams, C.B. Ware,

et al. 1994. Early lymphocyte expansion is severely impaired in interleukin 7 receptor–deficient mice. *J. Exp. Med.* 180:1955–1960. doi:10.1084/jem.180.5.1955

Ramsdell, F., M. Jenkins, Q. Dinh, and B.J. Fowlkes. 1991. The majority of CD4+8- thymocytes are functionally immature. *J. Immunol.* 147:1779–1785.

Sato, S., H. Sanjo, T. Tsujimura, J. Ninomiya-Tsuji, M. Yamamoto, T. Kawai, O. Takeuchi, and S. Akira. 2006. TAK1 is indispensable for development of T cells and prevention of colitis by the generation of regulatory T cells. *Int. Immunol.* 18:1405–1411. doi:10.1093/intimm/dxl082

Schmidt-Supprian, M., G. Courtois, J. Tian, A.J. Coyle, A. Israël, K. Rajewsky, and M. Pasparakis. 2003. Mature T cells depend on signaling through the IKK complex. *Immunity.* 19:377–389. doi:10.1016/S1074-7613(03)00237-1

Sentman, C.L., J.R. Shutter, D. Hockenbery, O. Kanagawa, and S.J. Korsmeyer. 1991. bcl-2 inhibits multiple forms of apoptosis but not negative selection in thymocytes. *Cell.* 67:879–888. doi:10.1016/0092-8674(91)90361-2

Tanigaki, K., M. Tsuji, N. Yamamoto, H. Han, J. Tsukada, H. Inoue, M. Kubo, and T. Honjo. 2004. Regulation of alphabeta/gammadelta T cell lineage commitment and peripheral T cell responses by Notch/RBP-J signaling. *Immunity.* 20:611–622. doi:10.1016/S1074-7613(04)00109-8

Thomas, M.D., B. Srivastava, and D. Allman. 2006. Regulation of peripheral B cell maturation. *Cell. Immunol.* 239:92–102. doi:10.1016/j.cellimm.2006.04.007

Vernachio, J., M. Li, A.D. Donnenberg, and M.J. Soloski. 1989. Qa-2 expression in the adult murine thymus. A unique marker for a mature thymic subset. *J. Immunol.* 142:48–56.

Wan, Y.Y., H. Chi, M. Xie, M.D. Schneider, and R.A. Flavell. 2006. The kinase TAK1 integrates antigen and cytokine receptor signaling for T cell development, survival and function. *Nat. Immunol.* 7:851–858. doi:10.1038/ni1355

Wang, H., and S.H. Clarke. 2003. Evidence for a ligand-mediated positive selection signal in differentiation to a mature B cell. *J. Immunol.* 171:6381–6388.

Zachariah, M.A., and J.G. Cyster. 2010. Neural crest-derived pericytes promote egress of mature thymocytes at the corticomedullary junction. *Science.* 328:1129–1135. doi:10.1126/science.1188222

Zhang, N., and Y.-W. He. 2005. An essential role for c-FLIP in the efficient development of mature T lymphocytes. *J. Exp. Med.* 202:395–404. doi:10.1084/jem.20050117