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The NF-κB1 transcription factor prevents the intrathymic development of CD8 T cells with memory properties

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The role of specific members of the NF-κB family of transcription factors in CD8 T-cell selection and development is largely unknown. Here, we show that mice lacking NF-κB1 develop a unique population of conventional CD8 single-positive (SP) thymocytes with memory T cell-like properties that populate peripheral immune organs. Development of this memory-like population is not due to PLZF$^+$ thymocytes and instead coincides with changes in CD8 T-cell selection. These include a reduction in the efficiency of negative selection and a dependence on MHC class Ia or Ib expressed by haematopoietic cells. These findings indicate that NF-κB1 regulates multiple events in the thymus that collectively inhibit the excess development of CD8$^+$ thymocytes with memory cell characteristics.

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Introduction

The differentiation of thymocytes that express αβ T-cell receptors (TCRs) is dependent on selection steps that are influenced by the strength and duration of TCR signals (Hogquist, 2001). Initially, pre-TCR signals in CD4$^-$CD8$^-$ (DN) progenitors initiate TCRα gene rearrangement and the subsequent development of CD4$^+$CD8$^+$ (DP) thymocytes. DP thymocytes are then screened by a process of positive and negative selection involving TCR interactions with self-peptide bound to MHC molecules (Bosselut, 2004) that dictate CD4 and CD8 T-cell differentiation and maturation. Cells bearing TCRs that recognise classical MHC class I (class Ia) become CD8$^+$ T cells, while recognition of MHC class II promotes CD4$^+$ T-cell development (Starr *et al*, 2003). For CD4$^+$ and CD8$^+$ T cells, DP thymocytes with TCRs that bind peptide–MHC complexes expressed on thymic epithelial cells (TECs) with low-to-moderate affinity, undergo positive selection. At this stage, cells expressing a non-functional TCR fail to receive a differentiation signal and undergo death by neglect. Thymocytes expressing a TCR that binds peptide–MHC complexes with high affinity are potentially auto-reactive and are targeted by negative selection to undergo apoptosis (von Boehmer and Melchers, 2010). Although DP thymocytes are thought to be first subjected to positive selection, and then in an overlapping manner to negative selection as cells move from the cortex to the medulla, the sequence and timing of these thymic selection events appears to be flexible (von Boehmer and Melchers, 2010).

DP thymocytes also serve as precursors for other T-cell lineages, such as CD4 regulatory T cells and innate T lymphocytes, the latter including CD1d restricted natural killer T (NKT) cells and CD8 T cells that express TCRs specific for non-classical MHC class Ib molecules (Berg, 2007). Innate CD8$^+$ thymocytes typically express high levels of the memory T-cell markers CD44 and CD122, require IL-15 for development as well as for survival, and rapidly produce effector cytokines following activation (Dubois *et al*, 2006; Berg, 2007). Positive selection and differentiation of these cells is dependent on peptides presented by MHC class Ib expressed on haematopoietic cells (Urdahl *et al*, 2002; Kurepa *et al*, 2003). Aside from the importance of MHC class Ia- and Ib-dependent selection by thymic epithelial and haematopoietic cells, respectively, little is known about other mechanisms that shape the distinct phenotypes acquired by conventional and innate CD8 T cells. A recent advance in our understanding of innate/memory-like CD8$^+$ T-cell development has emerged from findings that certain thymic T lymphocytes, including NKT and γδ cells that express the transcription factor PLZF, can promote development of CD8 thymocytes with memory characteristics (Weinreich *et al*, 2010; Lee *et al*, 2011).

Among the transcription factors activated by TCR engagement during thymocyte development and selection are the NF-κB proteins (Gerondakis and Siebenlist, 2010). These transcription factors comprise dimers of related proteins (c-Rel, RelA, RelB, NF-κB1 and NF-κB2), which in most cells remain in a latent state bound to IκB proteins within

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the cytoplasm. Signal-dependent activation of an IκB kinase (IKK), leads to IκB phosphorylation and degradation (Ghosh and Karin, 2002), that results in NF-κB proteins entering the nucleus and controlling the transcription of target genes (Ghosh and Karin, 2002). NF-κB proteins are differentially regulated during thymocyte differentiation (Gerondakis and Siebenlist, 2010). Initially, constitutive NF-κB activation during the late stages of DN thymocyte development (Feuillard *et al*, 2000; Voll *et al*, 2000) precedes a down-regulation of NF-κB in DP thymocytes. Following selection, only mature CD8SP thymocytes have significant levels of NF-κB activity (Hettmann and Leiden, 2000) that comprised RelA/NF-κB1 heterodimers and NF-κB1 homodimers (Moore *et al*, 1995). While the importance of NF-κB in promoting the survival of DN thymocytes is well recognised (Voll *et al*, 2000; Mandal *et al*, 2005), the exact roles of this pathway during and after selection are subject to debate. Although mice expressing T lineage restricted IκB super-repressor transgenes confirm that NF-κB is more important for CD8 than for CD4 T-cell selection and development (Boothby *et al*, 1997; Mora *et al*, 1999), the function of NF-κB during positive and negative selection was confused by variable inhibition of NF-κB in different IκB transgenic mouse strains (Gerondakis and Siebenlist, 2010). Recently, these results were accommodated by a study showing that the level of NF-κB activity corresponded to TCR signal strength that sets the threshold for positive and negative selection of $CD8^+$ T cells (Jimi *et al*, 2008). However, specific roles for the different NF-κB family members in thymocyte differentiation and maturation following TCRαβ repertoire selection remain poorly defined.

Despite functions ascribed to NF-κB for thymocyte survival, selection and differentiation, redundancy limits our understanding of the roles served by each NF-κB transcription factor (Gerondakis *et al*, 2006). The activation of NF-κB1, RelA and c-Rel in thymocytes by signals that impinge on selection and differentiation (Moore *et al*, 1995) highlight the likelihood that different NF-κB proteins engaged by the IKKβ-dependent pathway perform distinct functions during and after selection. Notwithstanding limited information about the role of RelA in thymocyte development due to the embryonic lethality of *rela*$^{-/-}$ mice (Beg *et al*, 1995), to date roles for c-Rel and NF-κB1 in thymocyte development have been restricted to CD4 regulatory T cells (Zheng *et al*, 2003; Isomura *et al*, 2009; Vang *et al*, 2010) and NKT cells (Sivakumar *et al*, 2003; Godfrey and Berzins, 2007; Stankovic *et al*, 2011), respectively.

Here, we report that NF-κB1 regulates the selection and development of conventional TCRαβ CD8SP thymocytes. In naive *nfκb1*$^{-/-}$ mice, elevated numbers of peripheral CD8 memory-like T cells coincide with the development of a unique population of CD8SP thymocytes that possess the phenotypic and functional characteristics of $CD8^+$ memory T cells. Unlike other mouse strains with similar phenotypes (Lee *et al*, 2011), the development of *nfκb1*$^{-/-}$ CD8 memory-like thymocytes is not due to the influence of thymic $PLZF^+$ T cells. Instead, their development coincides with altered patterns of positive and negative selection in NF-κB1-deficient mice. Collectively, our findings indicate that NF-κB1 serves critical functions in the thymus by ensuring CD8SP thymocytes adopt naive characteristics during development and that their selection by MHC class I is restricted to TECs.

Results

CD8 T cells that possess a memory phenotype develop in the thymus of nfκb1$^{-/-}$ *mice*

Despite an increasing appreciation of the importance of NF-κB in thymocyte differentiation, an understanding of the roles served by individual members of this family of transcription factors is limited. In particular, little is known about the non-redundant roles of NF-κB1, prompting a detailed analysis of conventional T-cell development in *nfκb1*$^{-/-}$ mice. Compared with *wt* mice, the thymocyte cellularity was marginally elevated in 7–10-week-old *nfκb1*$^{-/-}$ mice, with significant increases in percentages and absolute numbers of CD4 and CD8 single-positive (SP) thymocytes (Figure 1A and B). A significant change in the CD4:CD8 ratio (*wt* 3.04 ± 0.29 versus *nfκb1*$^{-/-}$ 2.09 ± 0.19; $n = 5$ per genotype; $P = 0.05$) also suggested that T lineage development was altered. Most *nfκb1*$^{-/-}$ CD8SP thymocytes displayed low levels of CD24 and high levels of CD62L (Figure 1C), a phenotype associated with increased maturity. The most striking finding was that ~40% of *nfκb1*$^{-/-}$ CD8SP thymocytes (compared with only 15% of *wt* CD8SP cells) expressed high levels of CD44. While $CD44^{hi}$ T cells are typically activated or memory cells, an absence of CD25 and CD69 expression (data not shown) indicated that *nfκb1*$^{-/-}$ $CD44^{hi}$CD8SP thymocytes were not activated cells. Coupled with the high expression of CD122 (Figure 1D), these cells instead resembled memory T cells that usually only reside in the periphery. Since a hallmark of memory T cells is an ability to rapidly secrete effector cytokines following activation, we assessed IFN-γ production by *nfκb1*$^{-/-}$ CD8 thymic T cells. Following PMA plus ionomycin stimulation *in vitro*, 1% of *wt* $CD44^{hi}$CD8SP thymocytes produced IFN-γ, whereas five- to six-fold more *nfκb1*$^{-/-}$ $CD44^{hi}$CD8SP cells rapidly secreted this cytokine (Figure 1D). In contrast, *nfκb1*$^{-/-}$ CD4SP thymocytes exhibited normal CD44 and CD24 expression (Figure 1C), and failed to produce significant levels of IFN-γ when activated (data not shown).

It remained unclear whether this expanded $CD44^{hi}$CD8 T-cell population developed in the thymus, or instead represented peripheral CD8 memory T cells that had migrated to the thymus. Although a small proportion of activated or memory T cells normally circulate through the thymus (Michie and Rouse, 1989; Agus *et al*, 1991), *nfκb1*$^{-/-}$ $CD8^+$ T cells injected into congenic hosts, while readily detectable in spleen and lymph nodes, were not found in the thymus (Supplementary Figure S1A). This suggested that memory-like CD8SP cells in *nfκb1*$^{-/-}$ mice were not peripheral cells that had entered the thymus, but instead were of thymic origin. This possibility was assessed using FTOC. While *wt* and *nfκb1*$^{-/-}$ thymic precursors readily develop into mature CD4 and CD8 SP thymocytes, a prominent population of $CD44^{hi}$CD8SP thymocytes was only observed in *nfκb1*$^{-/-}$ thymi (Supplementary Figure S1B). Collectively, these results reveal that in the absence of NF-κB1, $CD8^+$ T cells acquire memory-like properties during thymic development.

Development of nfκb1$^{-/-}$ *CD44*^{hi}*CD8 thymocytes coincides with increased Eomes expression*

The memory-like properties of *nfκb1*$^{-/-}$ CD8SP thymocytes are a characteristic shared with innate T cells in the CD8 lineage (Berg, 2007; Veillette *et al*, 2007). These similarities

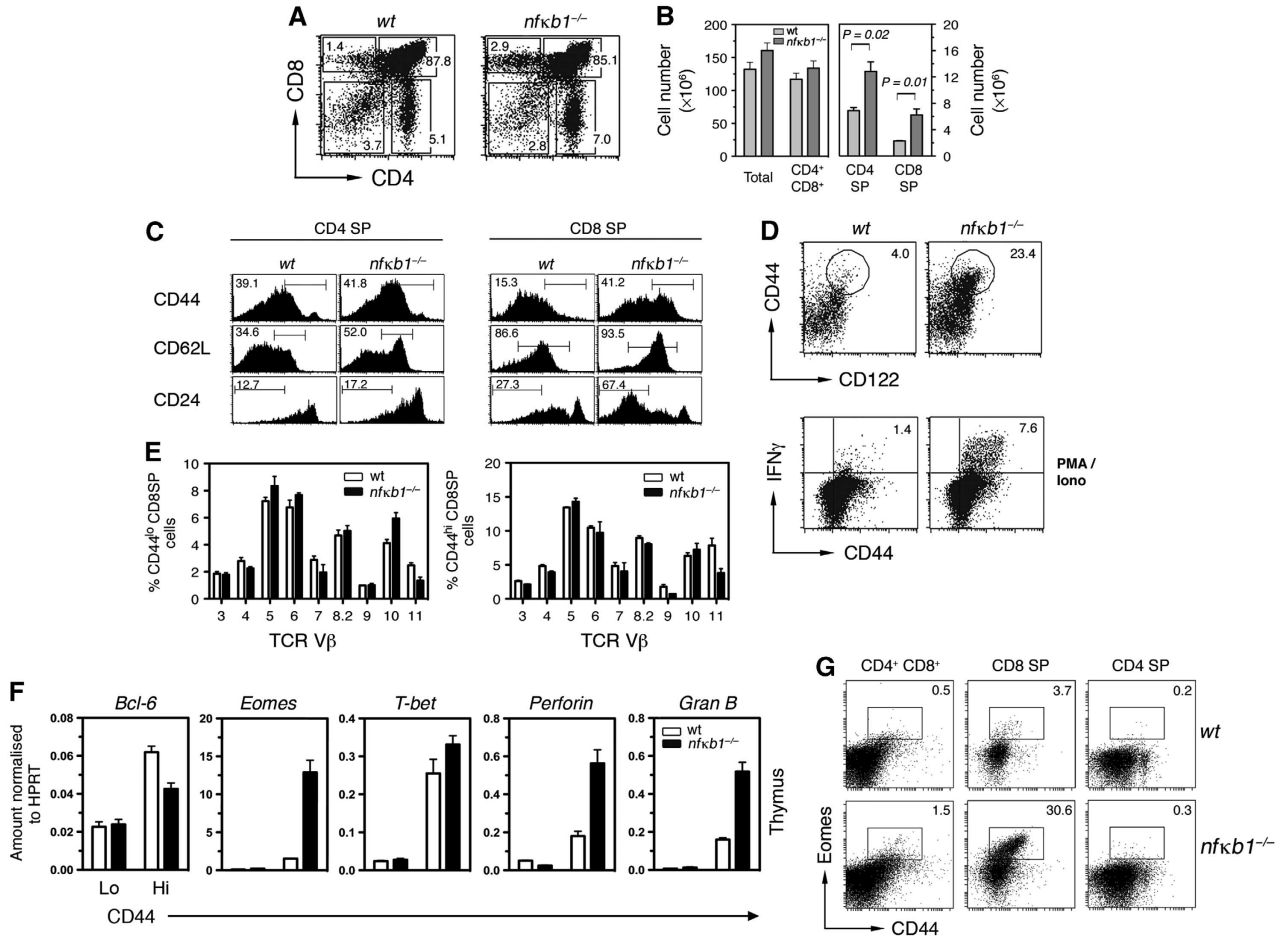


Figure 1 *nfκb1*$^{-/-}$ CD8SP thymocytes display memory characteristics. (**A**) CD4 and CD8 expression by thymocytes from 8-week-old *wt* and *nfκb1*$^{-/-}$ mice with numbers showing percentages of cells in each gate. (**B**) Mean (± s.e.m.) numbers of thymocyte subsets in *wt* and *nfκb1*$^{-/-}$ mice. Data consist of one cohort ($n = 3$ mice per genotype). (**C**) Phenotype of *wt* and *nfκb1*$^{-/-}$ CD4 and CD8 SP thymocytes ($n = 7$ mice per genotype). (**D**) Expression of CD44 and CD122 by *wt* and *nfκb1*$^{-/-}$ CD8SP thymocytes. Values indicate percentages of CD44^{hi}CD122^{hi} cells and data represent seven mice per genotype. IFN-γ and CD44 expression gated on *wt* and *nfκb1*$^{-/-}$ CD8SP thymocytes after 5 h of stimulation with PMA (10 ng/ml) plus ionomycin (1 μg/ml) ($n = 6$ mice per genotype). (**E**) Proportion of Vβ TCRs (*x* axis) gated on CD8SP CD44^{lo} or CD44^{hi} thymocytes for *wt* and *nfκb1*$^{-/-}$ mice (mean ± s.e.m.; $n = 3$ per genotype). (**F**) Relative expression of *Bcl-6*, *Eomes*, *T-bet*, *Perforin* and *Granzyme B* mRNA in *wt* and *nfκb1*$^{-/-}$ CD44^{lo} and CD44^{hi} CD8SP thymocytes (cells were purified from four mice per genotype for each experiment). Graphs show mean ± s.d. from triplicate reactions. (**G**) Expression of Eomes and CD44 by *wt* and *nfκb1*$^{-/-}$ thymocyte subsets ($n = 4$ mice per genotype). Values indicate percentages of cells in each gate. Data shown are representative of seven (**A**), five (**B**), four (**C**, **G**), three (**D**) and two (**E**, **F**) independent experiments. *P*-values were determined by an unpaired two-tailed Student's *t*-test.

prompted a detailed characterisation of the *nfκb1*$^{-/-}$ CD44^{hi}CD8SP thymocyte population. Like *wt* CD44^{lo} and CD44^{hi} CD8SP cells, *nfκb1*$^{-/-}$ CD44^{hi}CD8SP thymocytes utilise a diverse Vβ TCR repertoire (Figure 1E). In addition to rapidly synthesising IFN-γ, *nfκb1*$^{-/-}$ CD44^{hi}CD8SP thymocytes express high levels of *perforin* and *granzyme B* mRNA (Figure 1F). The T-box transcription factors T-bet and Eomesodermin (Eomes), which contribute to a pattern of gene expression that is characteristic of memory CD8 T cells (Pearce *et al*, 2003; Intlekofer *et al*, 2005), were also highly expressed in these cells (Figure 1F). While *T-bet* levels were high in *wt* and *nfκb1*$^{-/-}$ CD44^{hi}CD8SP thymocytes, *Eomes* was selectively elevated in *nfκb1*$^{-/-}$ CD44^{hi}CD8SP cells. Further analysis confirmed that Eomes was expressed in *nfκb1*$^{-/-}$ CD8SP cells, and there was a small but significant increase in the proportion of *nfκb1*$^{-/-}$ DP thymocytes expressing Eomes (Figure 1G). This indicates that the changes in Eomes expression observed in *nfκb1*$^{-/-}$ CD44^{hi}CD8SP thymocytes may have been initiated earlier in DP cells.

Development of CD8 memory-like thymocytes depends on the loss of NF-κB1 transcription factor function and not impaired ERK signalling

In addition to being a precursor for the p50NF-κB1 transcription factor, p105NF-κB1 serves as a scaffold for Tpl2, an MAP3K that controls MEK1-dependent ERK activation (Belich *et al*, 1999). In the absence of p105NF-κB1, Tpl2 is labile (Waterfield *et al*, 2003), resulting in ERK activation defects in various immune cells (Waterfield *et al*, 2003; Banerjee *et al*, 2006). Given that ERK signalling is critical for thymocyte differentiation (Fischer *et al*, 2005), we examined whether development of *nfκb1*$^{-/-}$ CD44^{hi}CD8SP thymocytes was due to the absence of p50NF-κB1, or a failure to activate ERK. These two possibilities were distinguished by examining thymocyte development in *tpl2*$^{-/-}$ mice, which retain normal p50NF-κB1 function (Waterfield *et al*, 2003). CD8SP thymocyte numbers and CD44 expression were normal in *tpl2*$^{-/-}$ mice (Supplementary Figure S1C), establishing that impaired NF-κB activity resulting from the

loss of p50NF-κB1 must be responsible for generating memory-like CD8SP thymocytes.

The development of nfκb1$^{-/-}$ CD44^{hi}CD8SP thymocytes is independent of IL-4 producing PLZF^{+} T cells

Mouse strains with mutations in T-cell signalling molecules, including ITK, KLF2 and Id3, possess CD8SP thymocytes with memory characteristics (Atherly *et al*, 2006; Broussard *et al*, 2006; Verykokakis *et al*, 2010; Weinreich *et al*, 2010), which are dependent on the expanded number of IL-4 producing PLZF^{+} αβ and γδ thymocytes (Verykokakis *et al*, 2010; Weinreich *et al*, 2010; Lee *et al*, 2011). This prompted us to determine if a similar mechanism also accounted for the development of memory-like *nfκb1*$^{-/-}$ CD8SP thymocytes. The proportion of PLZF^{+}TCRαβ^{+} thymocytes in *wt* and *nfκb1*$^{-/-}$ mice was similar (Supplementary Figure S2A), while the analysis of individual thymocyte subsets revealed a small increase in the proportion of *nfκb1*$^{-/-}$ PLZF^{+} DN cells (Figure 2A). While the total number of PLZF^{+} thymocytes was marginally elevated in the *nfκb1*$^{-/-}$ thymus, PLZF^{+}CD4SP cell numbers were comparable with *wt* controls (Supplementary Figure S2B). The proportion of γδ^{+} thymocytes was also examined in *nfκb1*$^{-/-}$ mice. Importantly, the number of *wt* and *nfκb1*$^{-/-}$ TCRγδ^{+}CD4SP cells was equivalent (Figure 2B).

Our findings show that an absence of NF-κB1 does not lead to a pronounced expansion of PLZF^{+} thymocytes. Nevertheless, a small increase in these cells might produce sufficient IL-4 to promote expression of memory markers on CD8 T cells. To test this possibility, *wt* and *nfκb1*$^{-/-}$ thymocytes were stimulated with PMA plus ionomycin and levels of IL-4 plus IFN-γ determined by intracellular FACS staining. Thymic NKT cells (CD1d tetramer^{+} TCRβ^{+}) were used as a positive control for staining, as this population is a potent producer of IL-4 and IFN-γ after stimulation. Importantly, IL-4 production was comparable between *wt* and *nfκb1*$^{-/-}$ thymic NKT cells, while *nfκb1*$^{-/-}$ and *wt* TCRαβ^{+}CD4SP cells produced only barely detectable levels of IL-4 (Figure 2C and D). Collectively, these findings demonstrate that the absence of NF-κB1 does not promote a marked expansion of IL-4 producing PLZF^{+} thymocytes.

Mixed bone marrow (BM) chimeras were generated to determine whether *nfκb1*$^{-/-}$ PLZF^{+} thymocytes are able to promote memory characteristics in bystander *wt* CD8SP cells. Chimeric mice were established by engrafting *wt* hosts with an equal (50:50) or unequal (85:15) mix of *nfκb1*$^{-/-}$ (Ly5.2^{+}) and *wt* (Ly5.1^{+}) BM cells. Analysis of chimeras 5–7 weeks post transplant revealed that *wt* CD8SP thymocytes had a phenotype resembling naive conventional CD8SP cell rather than memory-like cells (Figure 2E; Supplementary Figure S2C). Consistent with our finding in *nfκb1*$^{-/-}$ mice, *wt* and *nfκb1*$^{-/-}$ NKT cells from chimeras produced equivalent levels of IL-4 when stimulated with PMA plus ionomycin, while CD4SP cells of either genotype produced negligible levels of IL-4 (Figure 2F; Supplementary Figure S2D). Overall, these results demonstrate that unlike some mutant strains, such as the *itk*$^{-/-}$ mice (Weinreich *et al*, 2010), the absence of NF-κB1 does not create an expanded population of IL-4 producing PLZF^{+} thymocytes that can confer memory properties on neighbouring *wt* CD8SP cells.

The development of nfκb1$^{-/-}$ CD8 memory-like thymocytes requires the loss of NF-κB1 in the haematopoietic compartment and is independent of MHC class I expressed on TECs

Although *nfκb1*$^{-/-}$ CD44^{hi}CD8SP thymocytes and various innate CD8 thymic T-cell populations have overlapping phenotypes, certain key features distinguish conventional and innate CD8SP thymocytes (Glimcher *et al*, 2004; Berg, 2007). One such property is a dependence of innate CD8 thymocytes on IL-15 for development (Dubois *et al*, 2006; Berg, 2007). The presence of comparable numbers of CD44^{hi}CD122^{hi} CD8SP thymocytes in *nfκb1*$^{-/-}$ and *nfκb1*$^{-/-}$*il15*$^{-/-}$ mice (Figure 3A and B) established that the development of these cells is IL-15 independent. The memory-like thymocytes in *nfκb1*$^{-/-}$ mice like conventional CD8 T cells also express CD8αβ dimers (data not shown), but lack NK1.1 expression (unlike CD1d restricted *nfκb1*$^{-/-}$ NKT cells that are NK1.1^{+}) (Supplementary Figure S3A), a characteristic marker of innate CD8 T cells (Berg, 2007).

Most importantly, the selection and development of conventional CD8 lineage T cells is dependent on MHC class Ia expressed on TECs, whereas innate CD8 lineage T cells typically require MHC class Ib presented by haematopoietic cells (Urdahl *et al*, 2002; Kurepa *et al*, 2003). To determine whether the development of *nfκb1*$^{-/-}$ CD8SP memory-like thymocytes requires MHC class Ia (K^{b}D^{b}), we generated *nfκb1*$^{-/-}$*Kb*$^{-/-}$*Db*$^{-/-}$ mice. A comparison of CD8SP thymocyte populations between *wt*, *nfκb1*$^{-/-}$, *Kb*$^{-/-}$*Db*$^{-/-}$ and *nfκb1*$^{-/-}$*Kb*$^{-/-}$*Db*$^{-/-}$ mice is shown in Figure 4. While *nfκb1*$^{-/-}$ mice had 1.5-fold more CD8SP thymocytes than *wt* controls, with ∼45% of these cells being CD44^{hi} (Figure 4A and D), the absence of MHC class Ia led to a 60% reduction in the entire *nfκb1*$^{-/-}$ CD8SP population, including CD44^{hi} cells (Figure 4A and D). CD24^{lo}CD8SP cells, which normally represent the most mature CD8SP thymocytes, were also markedly reduced in the *nfκb1*$^{-/-}$*Kb*$^{-/-}$*Db*$^{-/-}$ mutants (Figure 4B–D), confirming that the majority of *nfκb1*$^{-/-}$ CD44^{hi}CD8SP cells was lost in these mice. This establishes that a significant proportion of memory-like CD8SP thymocytes that develop in *nfκb1*$^{-/-}$ mice depends on MHC class Ia.

In *Kb*$^{-/-}$*Db*$^{-/-}$ mice, the remaining CD8SP thymocytes are selected by class Ib expressing haematopoietic cells (Urdahl *et al*, 2002; Kurepa *et al*, 2003). Interestingly, the MHC class Ia-independent CD8SP population was 1.5-fold greater in *nfκb1*$^{-/-}$*Kb*$^{-/-}$*Db*$^{-/-}$ mice than in *Kb*$^{-/-}$*Db*$^{-/-}$ mutants (Figure 4A and D), with the remaining CD24^{lo}CD8SP thymocytes that develop in *nfκb1*$^{-/-}$*Kb*$^{-/-}$*Db*$^{-/-}$ mice elevated five-fold (Figure 4B and D). This indicates that the absence of NF-κB1 also increased class Ib-dependent thymocyte differentiation. Further characterisation of these CD24^{lo}CD8SP thymocytes revealed that CD44^{hi}CD122^{hi} and Ly6c^{hi} cells were enriched in *nfκb1*$^{-/-}$*Kb*$^{-/-}$*Db*$^{-/-}$ mice when compared with the equivalent population in *Kb*$^{-/-}$*Db*$^{-/-}$ and *nfκb1*$^{-/-}$ mutants (Figure 4C). This suggests that the loss of NF-κB1 alters the phenotype of the CD44^{hi}CD8 population irrespective of whether their development is dependent on MHC class Ia or Ib.

To determine whether a loss of p50NF-κB1 in haematopoietic cells, thymic epithelium or both compartments promote the abnormally elevated development of memory-like CD8SP thymocytes, embryonic thymic lobes (*wt* or *nfκb1*$^{-/-}$)

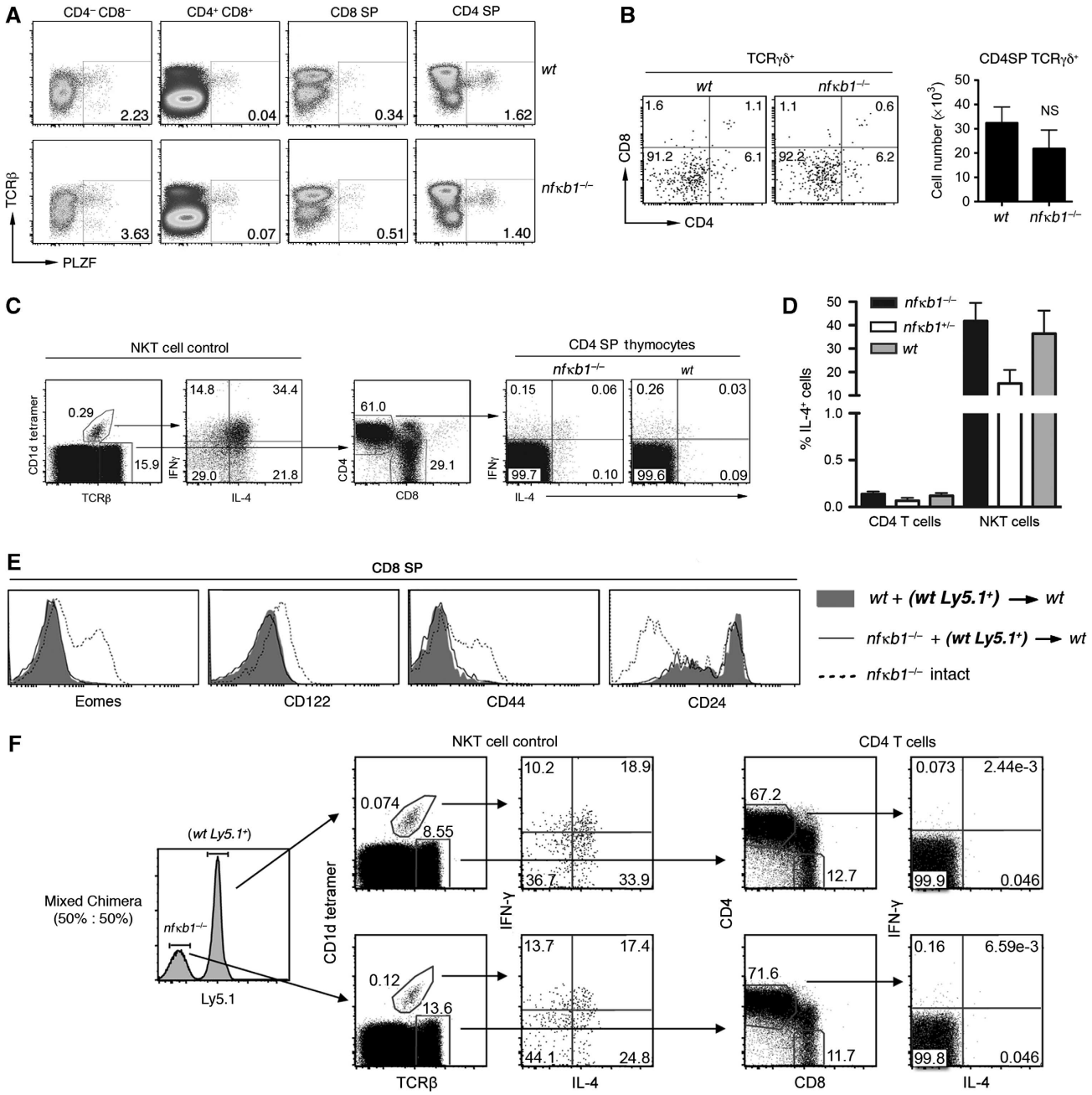


Figure 2 *nfκb1*$^{-/-}$ CD8SP thymocytes acquire memory markers independently of the IL-4 producing PLZF^{+} population. (**A**) Expression of PLZF in *wt* and *nfκb1*$^{-/-}$ thymocyte subsets. Data represent four mice per genotype. (**B**) CD4 and CD8 expression by *wt* and *nfκb1*$^{-/-}$ TCRγδ^{+} thymocytes. Mean (± s.e.m.) numbers of *wt* and *nfκb1*$^{-/-}$ TCRγδ^{+}CD4SP thymocytes, determined by total numbers of TCRγδ^{+} thymocytes and proportion of TCRγδ^{+}CD4SP cells ($n = 3$–4 mice per genotype). (**C**) Expression of IL-4 and IFN-γ in *wt*, *nfκb1*$^{+/-}$ and *nfκb1*$^{-/-}$ thymocytes 2 h after stimulation with PMA plus ionomycin. NKT cells (CD1d tetramer^{+} TCRβ^{+}) stained with CD1d tetramer loaded with PBS-44 were analysed as an internal positive control for IL-4 and IFN-γ (left panel). CD4SP thymocytes gated on TCRβ^{+} cells were examined for IL-4 and IFN-γ (right panel). (**D**) Percentages (mean ± s.e.m.) of IL-4-producing CD4 T cells and NKT cells from thymuses of *wt* ($n = 4$), *nfκb1*$^{+/-}$ ($n = 2$) and *nfκb1*$^{-/-}$ ($n = 7$) mice. Results are representative of three (**B**) and four (**A**, **C**, **D**) experiments. (**E**) Expression of Eomes, CD122, CD44 and CD24 by *wt* (Ly5.1^{+}) CD8SP thymocytes (bold) isolated from wt + (*wt* Ly5.1^{+}) (shaded histograms) or *nfκb1*$^{-/-}$ + (*wt* Ly5.1^{+}) (black lines) chimera mice. Memory phenotype of *nfκb1*$^{-/-}$ CD8SP cells from intact *nfκb1*$^{-/-}$ mice (hatched lines) served as concurrent positive control. Data are representative of three different chimera cohorts ($n > 6$ mice per group). BM chimera mice were established by engrafting *wt* (Ly5.2^{+}) hosts with a mix (50:50) of *nfκb1*$^{-/-}$ and *wt* (Ly5.1^{+}) haematopoietic cells. (**F**) NKT cells (CD1d tetramer^{+} TCRβ^{+}) and CD4 T cells (TCRβ^{+}CD4^{+}CD8^{-}) were assessed for IL-4 and IFNγ expression. Thymocytes were isolated from chimeras and stimulated *in vitro* with PMA and ionomycin for 2 h and then analysed for intracellular levels of IL-4 and IFN-γ by flow cytometry. *Wt* and *nfκb1*$^{-/-}$ thymocytes were defined as Ly5.1^{+} and Ly5.1^{-}, respectively (left panel).

depleted of endogenous thymocytes with 2DG were transplanted under the renal capsule of *wt* or *nfκb1*$^{-/-}$ hosts. In this model, host-derived thymocyte progenitors infiltrate the grafted thymus and develop into mature SP thymocytes by interacting with the stroma of the donor thymus (Jenkinson *et al*, 1992). When *wt* or *nfκb1*$^{-/-}$ thymi were engrafted into

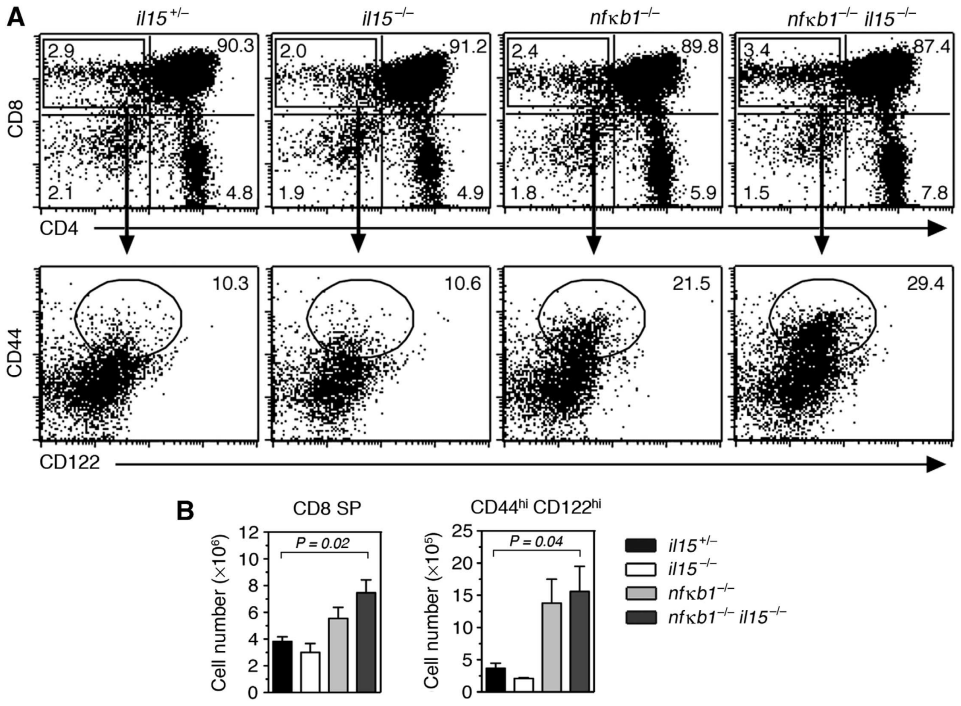


Figure 3 *nfκb1*$^{-/-}$CD8SP thymocytes acquire memory markers independently of IL-15. (**A**) CD4 and CD8 expression by thymocytes from 8-week-old *il15*$^{+/-}$, *il15*$^{-/-}$, *nfκb1*$^{-/-}$ and *nfκb1*$^{-/-}$ *il15*$^{-/-}$ mice. Values represent percentages of cells in each quadrant. Expression of CD44 and CD122 by CD8SP thymocytes (lower dot plots) with numbers representing the percentages CD44^{hi}CD122^{hi} CD8SP cells. (**B**) Mean ± s.e.m. ($n = 6$ mice per genotype) number of CD8SP and CD44^{hi}CD122^{hi} CD8SP thymocytes. Data are representative of five experiments. *P*-values were determined by an unpaired two-tailed Student's *t*-test.

nfκb1$^{-/-}$ hosts, the proportions and numbers of CD8SP thymocytes were slightly elevated in these thymi compared with equivalent grafts in *wt* hosts (Figure 5A and B). While CD44^{hi}CD122^{hi} CD8SP thymocyte numbers were comparable in *wt* and *nfκb1*$^{-/-}$ thymi grafted into *wt* hosts, this population was three- to four-fold higher in *wt* and *nfκb1*$^{-/-}$ thymi engrafted into *nfκb1*$^{-/-}$ mice (Figure 5B). Furthermore, the numbers of these cells appeared to be increased when *nfκb1*$^{-/-}$ thymi were grafted into *nfκb1*$^{-/-}$ hosts. This indicates that the absence of p50NF-κB1 in haematopoietic cells is a prerequisite for the excessive development of memory-like thymocytes, and that the added loss of NF-κB1 in the stroma enhances this phenotype.

Although the development of conventional TCRαβ^{+}CD8SP thymocytes normally depends on MHC class Ia signals delivered by TECs, emerging evidence indicates that strong or sustained TCR signals from antigen–MHC class I complexes expressed on haematopoietic cells are crucial for acquisition of memory properties by innate thymic T cells (Urdahl *et al*, 2002). This raised the intriguing possibility that development of *nfκb1*$^{-/-}$ TCRαβ^{+}CD8 memory-like thymocytes might depend on MHC class I signals presented by haematopoietic cells. This hypothesis was examined by placing 2DG-treated *Kb*$^{-/-}$*Db*$^{-/-}$ thymi under the renal capsule of *wt* or *nfκb1*$^{-/-}$ mice. As expected, CD8SP thymocyte numbers that develop in MHC class Ia-deficient thymi grafted in *wt* mice were markedly reduced (Figure 5C and D), with those remaining CD8SP cells selected by MHC class Ia or Ib expressed on haematopoietic cells. Of these residual cells, ~15% displayed a CD44^{hi}CD122^{hi} phenotype. The two-fold increase in CD8SP thymocytes seen in K^{b}D^{b}-deficient thymi grafted into *nfκb1*$^{-/-}$ mice mainly comprising CD44^{hi}CD122^{hi} cells and represented a six-fold increase in this population (Figure 5D). This shows that the enhanced development of *nfκb1*$^{-/-}$ CD8 memory-like thymocytes can occur in the absence of MHC class Ia expressed on TECs, and regardless of the selecting MHC (Ia or Ib), it is the absence of NF-κB1 in haematopoietic cells that promotes the acquisition of memory characteristics.

Thymocyte negative selection is impaired in nfκb1$^{-/-}$ *mice*

To further understand how the absence of p50NF-κB1 in the haematopoietic compartment promotes the excessive generation of memory-like CD8 thymocytes, we undertook a detailed study of thymocyte development in *nfκb1*$^{-/-}$ mice. At embryonic day 18 and neonatal days 3 and 7, CD8SP thymocyte numbers as well as their expression of CD44, CD122 and CD24 were comparable in *wt* and *nfκb1*$^{-/-}$ mice (Supplementary Figure S3B and data not shown). However, by 2 weeks of age increased numbers of CD44^{hi}CD122^{hi} CD8SP cells were present in *nfκb1*$^{-/-}$ thymi, coinciding with an increased proportion of IFN-γ producing CD8SP thymocytes (Supplementary Figure S3C). In 4–7-week-old *nfκb1*$^{-/-}$ mice, the proportions and absolute numbers of DN and DP thymocytes were normal (data not shown). With the expression of Eomes elevated in *nfκb1*$^{-/-}$ DP cells (Figure 1G), this suggested that CD8 thymocyte development was altered during transition from a DP to CD8SP cell, a phase that coincides with T-cell selection.

To assess whether the loss of NF-κB1 alters positive or negative CD8 T-cell selection, we utilised transgenic mice (HY^{Tg}) expressing a TCR specific for the male HY antigen presented by MHC class I H-2D^{b} (Teh *et al*, 1988). In female

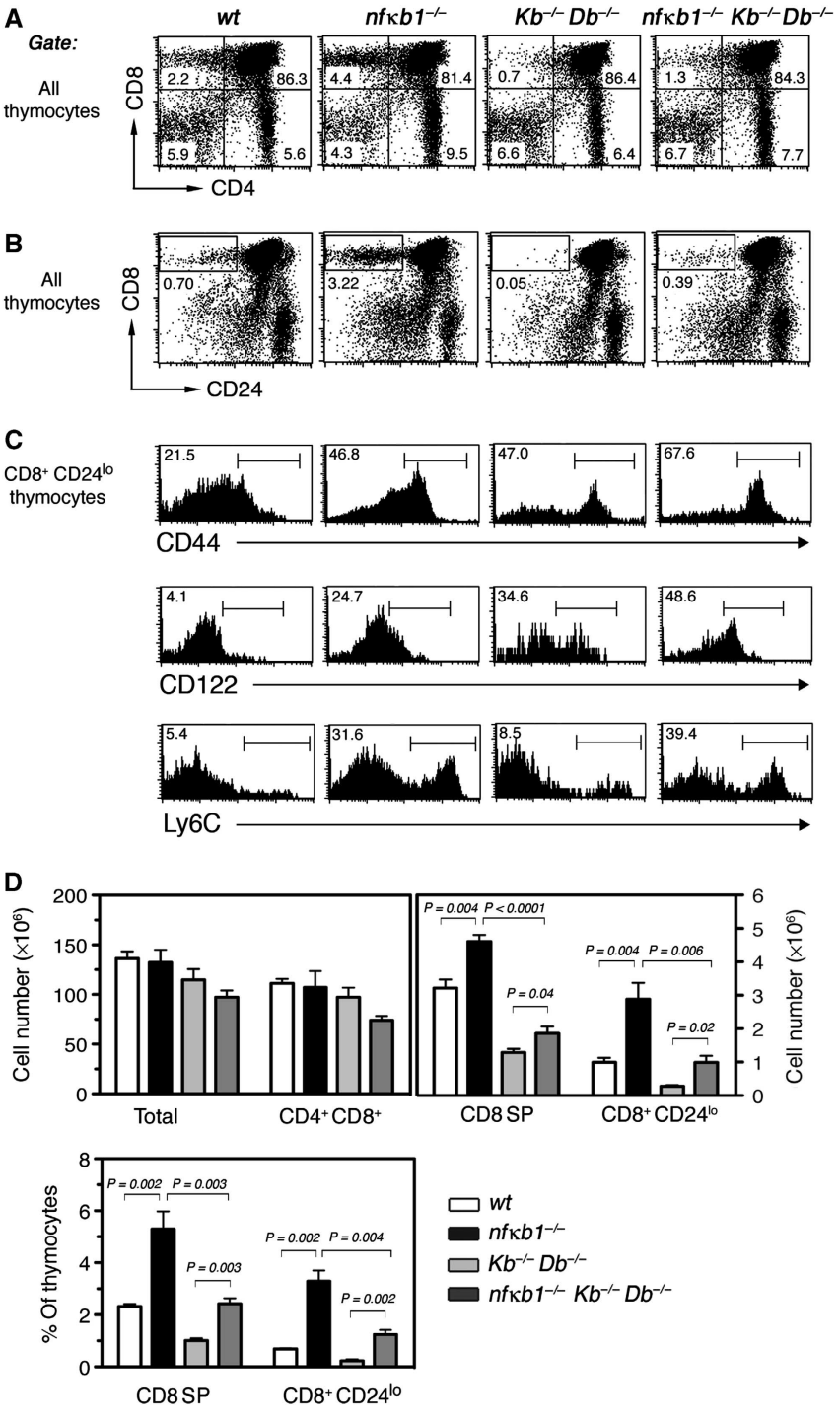


Figure 4 Loss of NF-κB1 promotes memory marker acquisition independently of MHC class Ia. (**A**) CD4 and CD8 profiles for *wt*, *nfκb1*$^{-/-}$, *Kb*$^{-/-}$*Db*$^{-/-}$ and *nfκb1*$^{-/-}$*Kb*$^{-/-}$*Db*$^{-/-}$ thymocytes. Numbers indicate the percent of thymocytes in each quadrant. (**B**) CD8 and CD24 expression by thymocytes from each genotype. Numbers represent percentages of CD8^{+}CD24^{lo} cells in each gate. (**C**) CD44, CD122 and Ly6C expression by CD8^{+}CD24^{lo} thymocytes. Percentages are shown for each gate. (**D**) Absolute numbers (mean ± s.e.m.) of total thymocytes, DP, CD8SP and CD8^{+}CD24^{lo} thymocytes for each genotype; Lower graph shows percentages of CD8SP and CD8^{+}CD24^{lo} thymocytes (mean ± s.e.m.). Data in (**A**–**D**) are representative of four experiments (4–6 mice per genotype). *P*-values were determined by an unpaired two-tailed Student's *t*-test.

mice (on a C57BL/6 background), thymocytes expressing the HY TCR (T3.70) undergo MHC class I restricted positive selection and develop into mature CD8 T cells. While total thymocyte cellularity was slightly elevated (Figure 6A), the loss of NF-κB1 had minimal effects on positive selection. Examination of thymic subsets revealed equivalent profiles in female *wt* and *nfκb1*$^{-/-}$ HY^{Tg} mice, with comparable numbers of HY^{Tg} TCR^{hi} cells (Figure 6A), indicating that NF-κB1 was dispensable for positive selection. In male H-2D^{b} restricted HY^{Tg} mice, thymocytes expressing the HY TCR recognise a self-antigen, resulting in their elimination by negative selection (Teh *et al*, 1988; von Boehmer, 1990).

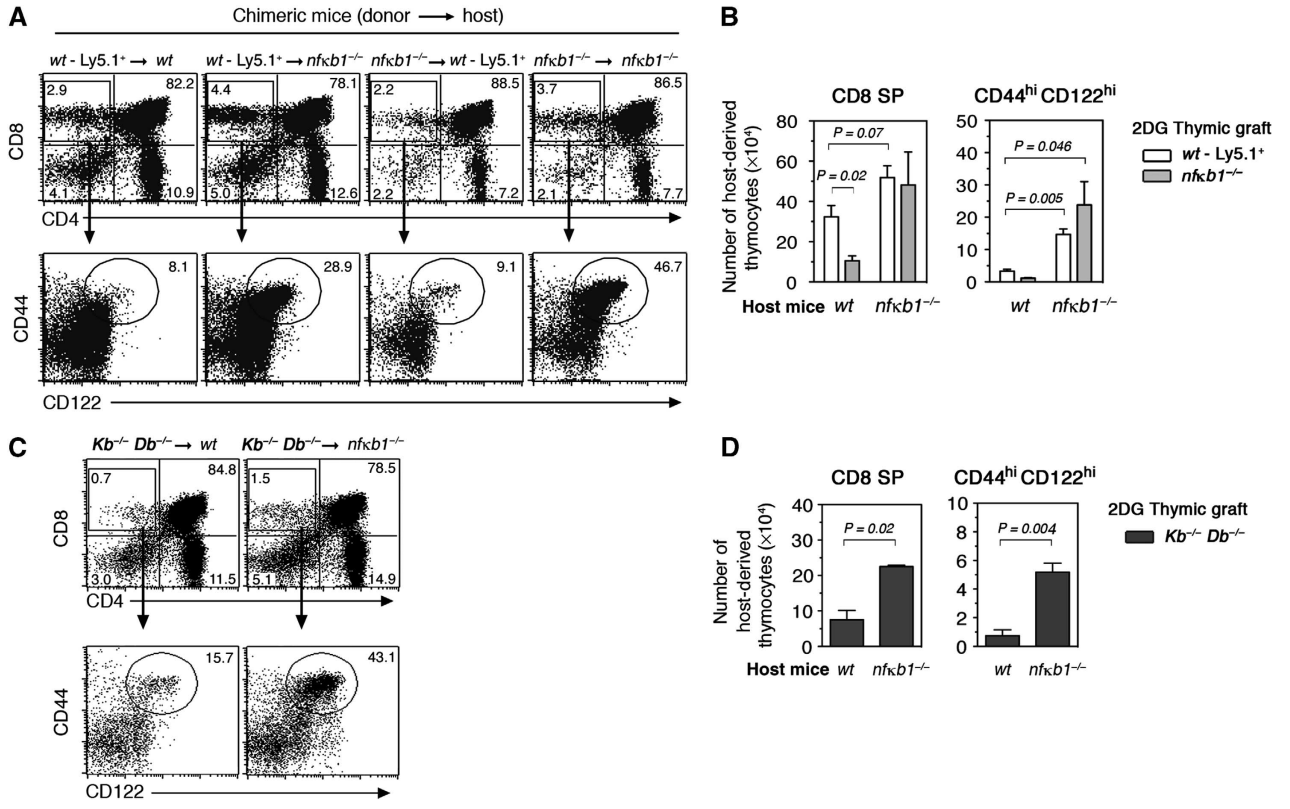


Figure 5 *nfκb1*$^{-/-}$ CD8SP thymocytes acquire a memory phenotype independently of MHC class I expressing TECs. Thymic lobes were harvested from *wt* (Ly5.1^{+}), *nfκb1*$^{-/-}$ (Ly5.2^{+}) and *Kb*$^{-/-}$*Db*$^{-/-}$ day 15.5 embryos and cultured by FTOC in the presence of 2-DG for depletion of endogenous thymocytes. After 7 days in culture, 2-DG-treated thymi were transplanted under the renal capsule of *wt* and *nfκb1*$^{-/-}$ hosts. Grafts were harvested and processed for immunofluorescent staining and cell counting 8 weeks post transplant. (**A**) CD4 and CD8 expression by host-derived thymocytes isolated from thymic grafts. Values represent percentages of each thymocyte subset. CD44 and CD122 expression by CD8SP thymocytes and numbers indicate the percentage of CD44^{hi}CD122^{hi} CD8SP cells. (**B**) Absolute numbers (mean ± s.e.m.) of host-derived CD8SP and CD144^{hi}CD122^{hi} CD8SP thymocytes from 3 to 5 transplanted thymi per host. (**C**) CD4 and CD8 profiles of host-derived thymocytes from *Kb*$^{-/-}$*Db*$^{-/-}$ thymi grafted into *wt* or *nfκb1*$^{-/-}$ mice. CD44 and CD122 phenotype of host CD8SP thymocytes isolated from *Kb*$^{-/-}$*Db*$^{-/-}$ grafts. (**D**) Absolute numbers (mean ± s.e.m.; $n = 4$ grafts per host) of CD8SP and CD144^{hi}CD122^{hi} CD8SP thymocytes isolated from *Kb*$^{-/-}$*Db*$^{-/-}$ grafts. Data in (**A**–**D**) are representative of two independent experiments. *P*-values were determined by an unpaired two-tailed Student's *t*-test.

Negative selection was less efficient in the absence of NF-κB1, with male *nfκb1*$^{-/-}$ HY^{Tg} mice displaying an increase in overall thymocyte cellularity and ~3-fold more DP thymocytes than *wt* male HY^{Tg} controls (Figure 6B). A greater proportion of the remaining male *nfκb1*$^{-/-}$ HY^{Tg} DP and CD8SP thymocytes expressed low levels of the HY TCR (Figure 6B), a finding consistent with reduced TCR expression being one mechanism by which auto-reactive DP thymocytes can escape negative selection (von Boehmer and Melchers, 2010). Interestingly, *nfκb1*$^{-/-}$ CD8SP HY^{Tg} thymocytes in female and male mice developed without acquiring memory markers (Figure 6A and B).

Importantly, features of the *nfκb1*$^{-/-}$ HY^{Tg} model indicative of a defect in negative, but not positive selection were also observed in *nfκb1*$^{-/-}$ mice with a polyclonal TCR repertoire. First, the pattern of CD69 expression on *nfκb1*$^{-/-}$ DP and SP thymocytes was normal (Figure 6C). Second, in *nfκb1*$^{-/-}$ mice reduced TCRβ levels were detected on CD8SP but not on CD4SP thymocytes (Figure 6C). Third, CD5, a cell surface marker whose expression levels on thymocytes reflect the strength of TCR signals received during development and selection (Azzam *et al*, 1998, 2001; Stojakovic *et al*, 2008), differs in NF-κB1-deficient mice. A prominent population of *nfκb1*$^{-/-}$ CD8SP cells expressed higher levels of CD5 (Figure 6C), whereas CD5 expression on *wt* and *nfκb1*$^{-/-}$ DP and CD4SP thymocytes was similar. While high CD5 expression on *nfκb1*$^{-/-}$ CD44^{hi}CD8SP thymocytes supports the notion that this memory-like population had received a stronger TCR signal, elevated CD5 levels were also observed on a prominent population of *nfκb1*$^{-/-}$ CD44^{lo}CD8SP cells (Figure 6D). This suggests that despite *nfκb1*$^{-/-}$ CD8SP thymocytes receiving a stronger TCR signal, this event alone is insufficient to promote the development of a memory phenotype.

To determine whether there is a thymocyte-intrinsic defect in negative selection, the levels of apoptosis were examined in cultures of *wt* and *nfκb1*$^{-/-}$ thymocytes stimulated with plate-bound anti-CD3 antibodies (Figure 6E and F). The absence of NF-κB1 had no impact on the death of DP thymocytes, suggesting that any role of NF-κB1 in this process is not intrinsic to thymocytes undergoing negative selection. This finding prompted an examination of CD11c^{+} CD8^{lo} Sirpα^{+} thymic DCs, a population known to be important in negative selection (Li *et al*, 2009; Proietto *et al*, 2009). We had previously shown that the splenic CD11c population is reduced two- to three-fold in *nfκb1*$^{-/-}$ mice (O'Keeffe *et al*, 2005) and consistent with this result, the *nfκb1*$^{-/-}$ Sirpα^{+} subset of thymic conventional (c) DC was also reduced three-

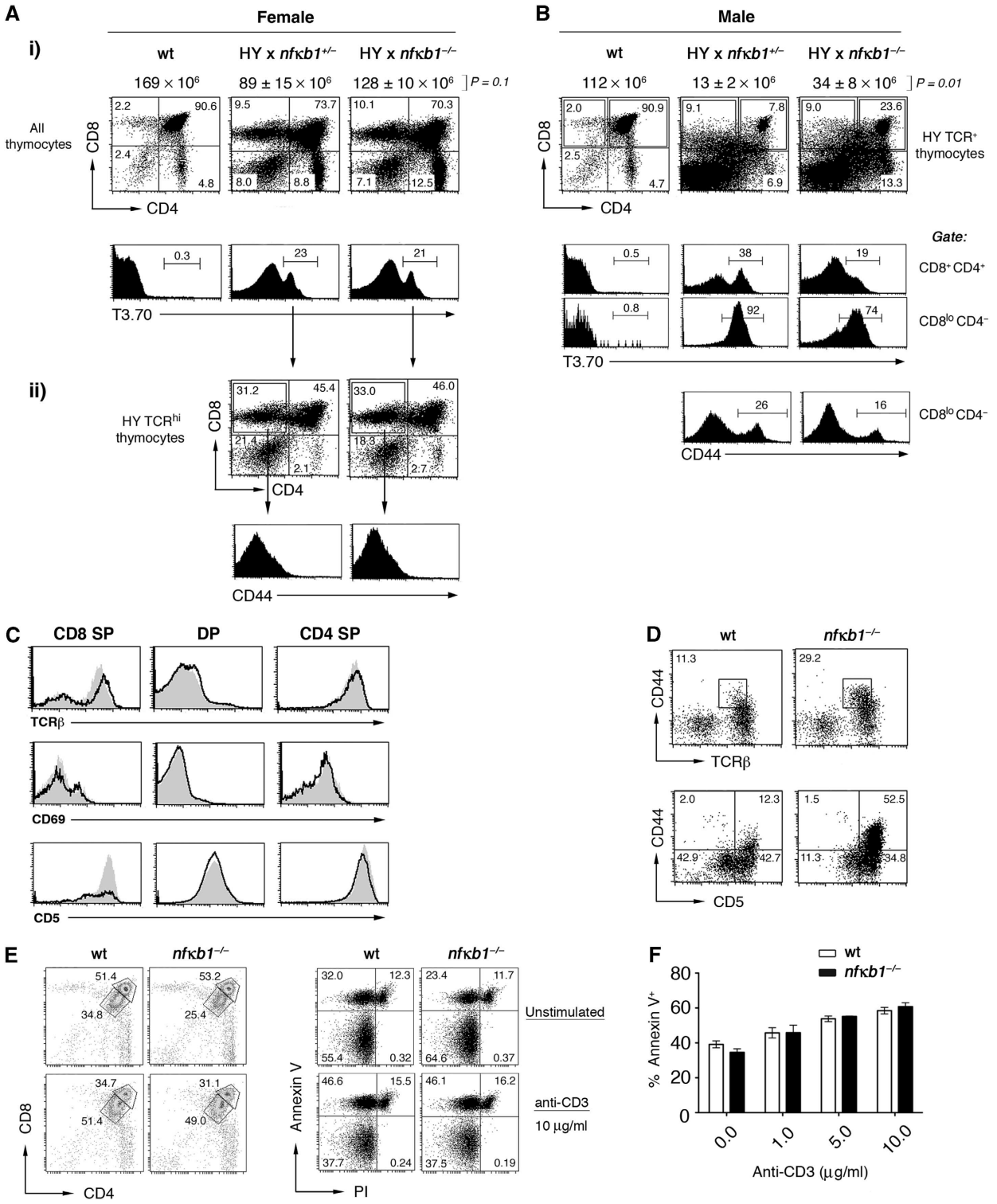


Figure 6 Reduced negative selection in HY TCR transgenic *nfκb1*$^{-/-}$ mice. (**A**) Expression of CD4 and CD8 by thymocytes from female (gate: all thymocytes) and (**B**) male (gate: HY^{+} TCR) *nfκb1*$^{+/-}$ and *nfκb1*$^{-/-}$ HY TCR transgenic mice. Values indicate percentages of cells in each quadrant. Expression of HY TCR (T3.70) is shown for all thymocytes (**Ai**) or individual thymocyte subsets (**B**). *Wt* thymocytes were stained concurrently with T3.70 as a staining control. Values in histograms represent percentages of HY TCR^{hi} thymocytes in gated areas. (**Aii**) CD4 and CD8 profiles by HY TCR^{hi} thymocytes in female transgenic mice. Total thymocyte cellularity is shown for each genotype (above dot plots; mean ± s.e.m.). *P*-values were determined by an unpaired two-tailed Student's *t*-test. (**Aii**, **B**) CD44 expression by HY TCR^{hi}CD8SP thymocytes. Data in (**A**) and (**B**) are representative four experiments ($n > 4$ mice per genotype). (**C**) Phenotype of *wt* (black lines) and *nfκb1*$^{-/-}$ (grey shaded) thymocyte subsets for TCRβ, CD69 and CD5 expression. (**D**) Analysis of CD44 in combination with TCRβ or CD5 gated on *wt* and *nfκb1*$^{-/-}$ CD8SP thymocytes. Data in (**C**) and (**D**) are representative of three experiments ($n = 5$ mice per genotype). (**E**) CD4 and CD8 expression of *wt* and *nfκb1*$^{-/-}$ thymocytes 24 h after *in-vitro* stimulation with anti-CD3 Ab (μg/ml). Values indicate percentages of cells expressing high or low levels of CD4 and CD8. Annexin V and PI staining of *wt* and *nfκb1*$^{-/-}$ thymocytes at the corresponding time point. (**F**) Percentages of Annexin V^{+} cells (mean ± s.e.m.) for *wt* and *nfκb1*$^{-/-}$ thymocytes cultured for 24 h at the indicated concentrations of anti-CD3 (μg/ml) Ab or media alone (>95% of *wt* and *nfκb1*$^{-/-}$ thymocytes were viable at T_0). Data in (**E**) and (**F**) are from three experiments.

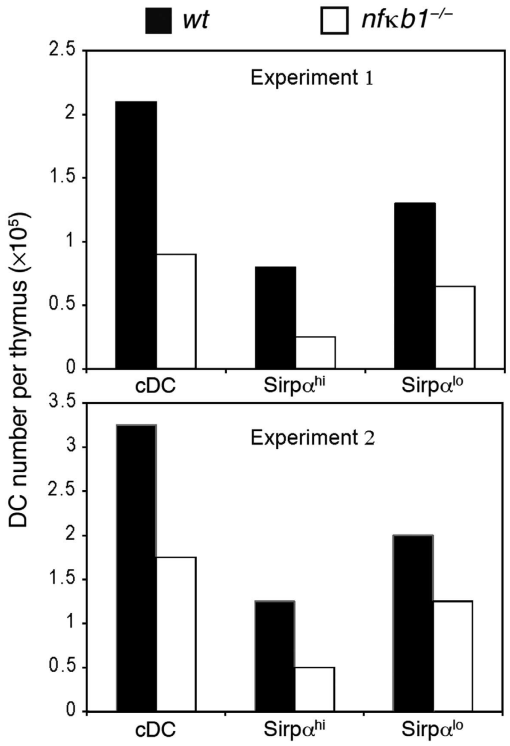


Figure 7 Decreased numbers of Sirpα^{hi} DCs in the thymus of *nfκb1*$^{-/-}$ mice. Comparison of DC numbers (conventional, Sirpα^{hi} and Sirpα^{lo}) from the thymus of *wt* and *nfκb1*$^{-/-}$ mice. DCs were analysed on four pooled thymi per genotype, staining for CD11c, Sirpα and CD45R: cDCs were gated as CD11c^{hi}CD45^{-} cells and analysed for Sirpα expression. Data show two independent experiments using a total of eight mice per genotype.

fold (Figure 7). This indicates that the reduced efficiency of negative selection in *nfκb1*$^{-/-}$ mice is most likely due to a reduction in a specific subset of DCs.

The production of CD8^{+} memory-like T cells in the thymus contributes to the elevated levels of peripheral CD44^{hi} CD8^{+} T cells in nfκb1$^{-/-}$ mice

Although abnormally elevated numbers of CD44^{hi} CD8SP thymocytes were generated in *nfκb1*$^{-/-}$ mice, it was unclear whether these cells could be exported from the thymus and contribute to the peripheral CD8 T-cell pool. An examination of the splenic T-cell population in young *nfκb1*$^{-/-}$ mice revealed that CD44^{hi}CD25^{lo}CD69^{lo} CD8 T-cell numbers were abnormally elevated (Figure 8A and B) and that these cells were capable of rapidly synthesising IFN-γ when activated in culture (Figure 8C). Direct evidence that CD44^{hi}CD8SP thymocytes contribute to the peripheral CD44^{hi} CD8 T-cell population was obtained by showing that FITC-labelled CD44^{hi} CD8 T cells were present in the spleen and lymph nodes of *nfκb1*$^{-/-}$ mice that had been given an intrathymic FITC injection (Figure 8D and E).

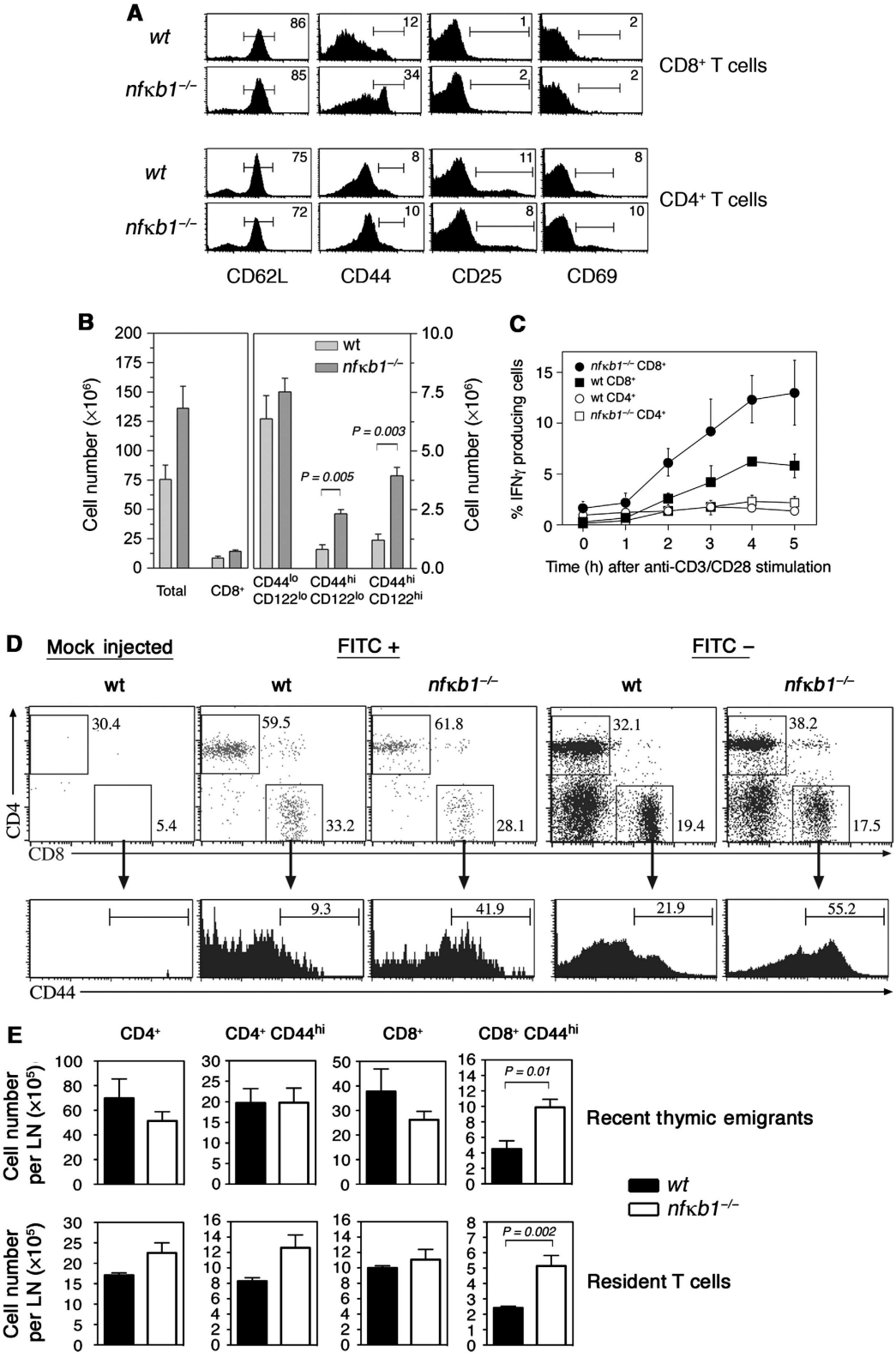
Discussion

The strength and duration of TCR signals delivered to developing thymocytes by antigen/MHC class I complexes expressed on thymic epithelial or haematopoietic cells is thought to be important in determining whether thymocytes acquire naive or memory cell characteristics (Hogquist, 2001; Berg, 2007). Little is known about the signalling pathways that influence CD8 thymocyte acquisition of these distinct properties (Carpenter and Bosselut, 2010). Here, we show that a loss of NF-κB1 function promotes the development of TCRαβ^{+}CD8SP thymocytes with memory properties. Unlike some other mouse strains (*itk*$^{-/-}$ and *klf2*$^{-/-}$) with expanded CD8 memory-like thymocyte populations, the generation of these cells in *nfκb1*$^{-/-}$ mice is not the result of an expanded population of PLZF^{+} thymic T cells. Instead this phenotype in *nfκb1*$^{-/-}$ mice coincided with defects in both positive and negative selection. Importantly, our findings identify a novel NF-κB1 regulated pathway that influences TCRαβCD8 thymocyte differentiation by preventing the acquisition of memory-like characteristics.

To date, non-redundant roles for NF-κB1 in conventional T cells have been confined to mature lymphocyte function (Gerondakis and Siebenlist, 2010). Our finding that *nfκb1*$^{-/-}$ mice had increased numbers of CD4 and CD8 SP thymocytes indicates that NF-κB1 also regulates T-cell development. Although NF-κB activity had previously been linked to thymocyte survival (Voll *et al*, 2000; Mandal *et al*, 2005), the normal size and viability of *nfκb1*$^{-/-}$ DN and DP thymocyte populations suggested that early T-cell differentiation was not perturbed. Instead, NF-κB1 appears to be important in regulating later stages of thymocyte development. While both *nfκb1*$^{-/-}$ CD4 and CD8 SP thymocytes displayed phenotypic changes, the presence of a CD44^{hi}CD24^{lo} subset of TCRαβ^{+}CD8SP thymocytes comprising ∼40% of CD8SP cells was the most striking feature of *nfκb1*$^{-/-}$ mice. These CD44^{hi}CD8SP cells, which develop in the thymus, resembled innate and conventional CD8 memory T cells, possessing the ability to rapidly produce IFN-γ when activated and expressing increased levels of IL-15Rβ, *granzyme B*, *perforin* and *Eomes*. Elevated Eomes expression in these cells is consistent with its role in controlling the transcription of genes expressed in memory T cells (Glimcher *et al*, 2004; Intlekofer *et al*, 2005). Although *nfκb1*$^{-/-}$ CD44^{hi}TCRαβ^{+} CD8SP thymocytes share characteristics with innate CD8 thymic T cells, differences in cell surface marker expression, including NK1.1 and a lack of dependence on IL-15 for their development, instead indicate that *nfκb1*$^{-/-}$ CD44^{hi}CD8SP thymocytes are conventional CD8 T cells that had acquired a memory-like phenotype during differentiation.

Recent studies have shown that the enhanced development of innate/memory CD8SP thymocytes in many mouse mutants is due to increased levels of IL-4 produced by elevated populations of CD4 thymic T cells, NKT and γδ T cells that express PLZF (Verykokakis *et al*, 2010; Weinreich *et al*, 2010). Unlike *itk*$^{-/-}$ and *id3*$^{-/-}$ mice, strains with aberrant PLZF^{+} thymic T cells that can induce a memory phenotype in *wt* CD8 thymocytes, *nfκb1*$^{-/-}$ haematopoietic cells failed to provide such a by-stander effect in mixed BM chimeras. Consistent with this finding PLZF^{+} cells were not markedly elevated in the pool of CD4SP thymocytes that include NKT and γδ T cells. Combined with the observation that the proportion of IL-4 producing CD4 thymic T cells in *nfκb1*$^{-/-}$ mice and mixed BM chimeras was normal, our data indicate that the development of memory-like CD44^{hi}CD8SP thymocytes in NF-κB1-deficient mice was not due to the influence of IL-4 producing PLZF^{+} T cells. However, a potential role for IL-4 cannot be unequivocally ruled out since IL-4 could not be entirely eliminated in these studies.

Our subsequent analysis established that an absence of NF-κB1 in haematopoietic cells was sufficient to generate CD44^{hi}CD8SP thymocytes, although the added loss of NF-κB1 in the thymic stroma augmented their development. One key change in the *nfκb1*$^{-/-}$ haematopoietic compartment that is essential for the development of CD44^{hi}TCRαβ^{+}CD8SP thymocytes is the role of MHC class I-dependent positive selection by haematopoietic cells. Contrary to reports indicating that MHC class Ib selectively promotes the development of innate/memory-like cells, our data indicate that there is nothing unique about MHC class Ib as opposed to class Ia in directing the development of memory properties in con-



ventional TCRαβ$^+$CD8 thymic T cells. While the requirements for MHC class I will need to be assessed further by examining the development of *nfκb1*$^{-/-}$ CD8SP cells on a *B2m*-deficient background. Our findings and those of others (Urdahl *et al*, 2002; Broussard *et al*, 2006) emphasise the importance of haematopoietic cells. Despite this altered mode of CD8 T-cell selection in *nfκb1*$^{-/-}$ mice, *nfκb1*$^{-/-}$ TECs still retain the capacity to promote the MHC class Ia-dependent selection and development of naive CD44^{lo}CD8 thymocytes. This suggests that in the absence of NF-κB1, functional changes in existing haematopoietic APC or the generation of a new haematopoietic APC promotes the development of memory-like CD8SP thymocytes. This interpretation is consistent with the inability of normal thymic haematopoietic APC expressing MHC class Ia to promote the positive selection of conventional T cells (Bix and Raulet, 1992). This differs from NKT and innate CD8 T cells that undergo CD1d and MHC class Ib-dependent selection by haematopoietic cells (Godfrey and Berzins, 2007). In the case of NKT cells, DP thymocytes serve as the APC responsible for positive selection. To overcome any inherent reduced capacity that haematopoietic cells may have in promoting positive selection, the higher affinity Type 1 NKT cells and presumably innate CD8 T cells have for self-ligands are believed to be a prerequisite for their positive selection (Godfrey and Berzins, 2007).

Examination of conventional CD8 T-cell selection in *nfκb1*$^{-/-}$ mice using a transgenic TCR specific for an HY peptide presented by MHC class I revealed that while NF-κB1 is dispensable for positive selection it is required for efficient negative selection. Although the levels of NF-κB activity in DP cells set a threshold for determining whether conventional CD8 T cells undergo positive or negative selection (Jimi *et al*, 2008), a specific contribution by NF-κB1 was unknown. A requirement for NF-κB1 is supported by data showing that *nfκb1* mRNA expression is induced during negative selection (Schmitz *et al*, 2003; Baldwin and Hogquist, 2007) and contrasts with that of c-Rel, which is dispensable for both CD8 positive and negative selection (Strasser *et al*, 1999). While the mechanism(s) by which NF-κB1 promotes negative selection of CD8 T cells remains to be determined, the failure to detect enhanced cell death in cultures of anti-CD3 antibody activated *nfκb1*$^{-/-}$ DP thymocytes indicates that this defect is not intrinsic to thymocytes undergoing selection. Instead, it points to NF-κB1 regulating other aspects of thymic function that influence negative selection. This notion is consistent with a reduction in thymic CD8 Sirpα$^+$ DCs in *nfκb1*$^{-/-}$ mice, an APC population that is important in promoting negative selection (Li *et al*, 2009; Proietto *et al*, 2009). Despite impaired CD8 T-cell selection, *nfκb1*$^{-/-}$ mice have not been reported to be prone to autoimmune disease. This anomaly may reflect a defect in the effector function of *nfκb1*$^{-/-}$ CD8 T cells or that other mechanisms act as a safeguard against autoimmunity.

Our finding that the CD44^{lo}CD8SP thymocyte population in *nfκb1*$^{-/-}$ mice remained intact indicates that additional memory-like CD8 thymic T cells were not generated from the pool of precursors destined to become naive CD8SP thymocytes. Instead, this indicates that these cells emerge from distinct precursors. A clue as to the origin of these cells is that the majority of *nfκb1*$^{-/-}$ CD44^{hi}CD8SP thymocytes have a CD5^{hi} phenotype, indicating that during selection these cells have received a strong TCR signal. Given that the majority of immature thymocytes with high-affinity TCRs are normally deleted during negative selection, our findings raise the intriguing possibility that impaired negative selection in *nfκb1*$^{-/-}$ mice creates an additional pool of thymocytes with higher affinity TCRs that are targeted by haematopoietic APC to differentiate into memory-like CD8SP thymocytes. However, generating CD8 thymic precursors possessing higher affinity TCRs for self-antigens, along with the ability of *nfκb1*$^{-/-}$ haematopoietic cells to promote MHC class Ia-dependent selection, does not guarantee development of memory-like CD8 T cells. Although OT-I TCR transgenic mice represent a model for MHC class I-dependent selection of CD8 T cells with high-affinity TCRs, CD44^{hi}CD8SP thymocytes were absent in *nfκb1*$^{-/-}$ OT-I and HY TCR transgenic mice (Supplementary Figure S3D). Instead, selection of CD8SP T cells expressing these TCRs is thought to require TECs rather than haematopoietic cells. Together, these findings support the notion that certain TCRs are better suited to class I selection by either TECs or haematopoietic cells.

What emerges from our study is that the NF-κB1 transcription factor serves multiple functions in the thymus that include ensuring efficient negative selection and determining which thymic APC can promote MHC Ia-dependent positive selection, events that determine the outcome of conventional CD8 thymocyte development. While altered positive selection due to the loss of NF-κB1 function plays an essential part in the altered development of CD8 T cells, we are currently unable to discern what impact the absence of NF-κB1 might have on the post-selection differentiation of CD8SP thymocytes. Nevertheless, our findings establish that a key role of NF-κB1 in the thymus is to prevent conventional CD8 T cells developing memory-like properties. With our study showing that this phenotype in *nfκb1*$^{-/-}$ mice is independent of the role played by IL-4 producing PLZF$^+$ CD4 T cells, this report

Figure 8 Thymic memory-like CD8$^+$ T cells populate the periphery of *nfκb1*$^{-/-}$ mice. (**A**) Phenotype of *wt* and *nfκb1*$^{-/-}$ CD4$^+$ and CD8$^+$ splenic T cells. Values indicate percentages of cells in gated areas. Data represent at least nine mice per genotype. (**B**) Mean (± s.e.m.) numbers of total splenocytes, CD8$^+$ T cells, naive (CD44^{lo}CD122^{lo}) and memory-like (CD44^{hi}CD122^{lo} and CD44^{hi}CD122^{hi}) CD8$^+$ T cells. Expression of CD44 and CD122 by *wt* and *nfκb1*$^{-/-}$ splenic CD8$^+$ T cells is shown in Supplementary Figure S3E. Data in (**A**) and (**B**) are representative of three experiments ($n>9$ mice per genotype). (**C**) Percentages of IFN-γ producing T cells from *wt* and *nfκb1*$^{-/-}$ mice. Splenocytes were stimulated with plate-bound anti-CD3/anti-CD28 Abs (both 10 μg/ml) and stained for IFN-γ, CD4 and CD8 expression. Stains were performed on duplicate samples every hour and sample flow profiles shown in Supplementary Figure S3E. Data are representative of two independent experiments. (**D**) Memory-like CD8$^+$ T cells home to the periphery of *nfκb1*$^{-/-}$ mice. *Wt* and *nfκb1*$^{-/-}$ mice were injected intrathymically with FITC or PBS (mock injected). After 20 h, mice were euthanised and mesenteric lymph nodes harvested and processed for immunofluorescent staining. FITC$^+$ (Recent Thymic Emigrants) and FITC$^-$ (Resident) T cells were examined for CD4 and CD8 expression. Values indicate percentages of cells in each gated region. CD44 expression gated on Recent Thymic Emigrants or Resident CD8$^+$ T cells from *wt* and *nfκb1*$^{-/-}$ mice. (**E**) Absolute numbers of RTE and resident T cells (mean ± s.e.m.; $n=5$ per genotype) calculated from T-cell percentages and total lymph node cell numbers. *P*-values were determined by an unpaired two-tailed Student's *t*-test.

highlights the existence of a distinct NF-κB1 regulated pathway controlling CD8 thymic T-cell differentiation. Future studies will focus on identifying other components in this NF-κB1 regulated pathway and the specific target genes controlled by NF-κB1 in thymic haematopoietic populations that influence the development of naive CD8 T cells.

Materials and methods

Mouse strains

nfκb1$^{-/-}$ mice (Sha *et al*, 1995) were backcrossed for 10 generations onto a C57BL/6 background. *nfκb1*$^{-/-}$*il15*$^{-/-}$ and *nfκb1*$^{-/-}$*Kb*$^{-/-}$*Db*$^{-/-}$ mice were generated by intercrossing the *nfκb1*$^{-/-}$ mice with *il15*$^{-/-}$ and *Kb*$^{-/-}$*Db*$^{-/-}$ mice (B6 background; Taconic), respectively. The *tpl-2*$^{-/-}$ mice (Dumitru *et al*, 2000) were obtained from Thomas Jefferson University (Philadelphia, PA). The OT-I and HY TCR transgenic mouse lines were crossed to *nfκb1*$^{-/-}$ mice, with transgene-negative littermates used as experimental controls. C57BL/6 (Ly5.1^{+}) congenic mice were obtained from The Walter and Eliza Hall Institute animal facility (Kew, Victoria). All animals were used at 7–10 weeks of age (unless stated otherwise), were bred and housed under SPF conditions at The Walter and Eliza Hall Institute or the Alfred Medical Research and Education Precinct, with all experiments approved by the respective Animal Ethics Committee.

Mixed BM chimeras

BM was flushed from the femur and tibia. BM cells from *nfκb1*$^{-/-}$ (Ly5.2^{+}) and *wt* (Ly5.1^{+}) mice were mixed at an equal (50:50) or unequal (85:15) ratio and injected intravenously into lethally irradiated (2×550 rads) *wt* (Ly5.2^{+}) hosts. Mice were killed and analysed 5–7 weeks after transplant.

Antibodies and flow cytometry

Single cell suspensions were prepared from thymus, lymph node and spleen using standard protocols. Red blood cells in spleen suspensions were lysed by performing a brief incubation with an ammonium chloride-based solution. Immunofluorescent staining was performed as previously described (Pohl *et al*, 2002) with a list of antibodies provided in Supplementary Materials and Methods. For intracellular staining, cells were fixed and permeabilised using the Foxp3 Staining Buffer Set (eBioscience) and stained for 1 h with Alexa647-anti-Eomes (Dan11mag; eBioscience) or Alexa647-anti-PLZF (clone D-9; a gift from Taras Kreslavsky, Daner Faber Cancer Institute, USA) antibodies. Data were acquired on a FACSCalibur, LSR-II or FACS Canto II (BD Biosciences) and analysed with Cell Quest Software (BD Biosciences), Weasel (WEHI) or FlowJo software (Tree Star Inc.).

Stimulation and intracellular cytokine staining

Thymocytes and splenocytes were stimulated *in vitro* for 2–5 h with PMA (10 ng/ml) plus ionomycin (1 μg/ml) or with plate-bound anti-CD3/anti-CD28 antibodies (both 10 μg/ml) respectively. The cells were cultured at 37°C in the presence of Golgi Stop™ (BD Biosciences). Cells were surface stained, fixed and permeabilised using the BD Biosciences Cytofix/Cytoperm Plus Fixation/Permeabilisation Kit, and then stained with PECy7-anti-IFNγ (XMG 1.2) and/or APC-anti-IL-4 (11B11) antibodies.

Thymic transplantation under the renal capsule

wt, *nfκb1*$^{-/-}$ and *Kb*$^{-/-}$*Db*$^{-/-}$ fetal thymi were harvested on day 15 of gestation, cultured for 7 days by FTOC in the presence of 2-Deoxyguanosine (2-DG) (1.3 mg/ml) to deplete endogenous thymocytes and then grafted under the renal capsule of *wt* or *nfκb1*$^{-/-}$ hosts. Grafts were removed and processed for flow cytometric analysis 8 weeks after transplantation.

Real-time quantitative PCR

Total RNA was extracted from 1 to 2×10^6 sorted thymocytes (MoFlo; Cytomation) using the RNeasy Mini-kit (Qiagen) with an on-column DNAse digest was reverse transcribed using SuperScript III RNaseH Reverse Transcriptase (Invitrogen) with random hexamers (Promega). Quantitative RT–PCR was performed using the Roche LightCycler 480 System (primers, Supplementary Table 1). For each sample, the starting quantity of each target gene was normalised to the housekeeping gene *Hprt*.

Dendritic cell isolation

Thymic dendritic cells were isolated using previously established methods (Dakic *et al*, 2004). Purified cell populations were stained for CD11c, CD45R and CD172a (Sirpα). cDC was gated as CD11c^{hi} CD45R cells, and then gated as Sirpα^{lo} or Sirpα^{hi} populations and enumerated.

Examination of thymic emigrants

Wt and *nfκb1*$^{-/-}$ mice were injected intrathymically with FITC as described (Uldrich *et al*, 2006). The animals were anaesthetised by i.p. injection of ~0.3 mg of xylazine hydrochloride (Ilium xylazil; Troy Laboratories) and 1.5 mg of ketamine hydrochloride (Ketalar; Parke-Davis) in 300 μl of PBS, and subsequently administered s.c. with 50 μg/10 g (0.5 μg/kg) body weight of the analgesic carprofen (Rimadyl; Pfizer). The thoracic cavity was opened, each thymic lobe was injected with ~10 μl of an FITC solution (1 mg/ml in sterile PBS) and then the wound was closed with surgical staples. Mice were euthanised 20 h post injection, thymuses, spleens and mesenteric lymph nodes were removed and processed for flow cytometry. Only mice with >70% of FITC-labelled thymocytes were assessed. Thymic emigrants were identified as live-gated FITC^{+} cells expressing either CD4 or CD8 and quantified by analysing the percentage of FITC^{+} cells, total cell counts of immune organs, and the percentage of CD4^{+} and CD8^{+} T cells that were FITC^{+}.

Statistical analysis

All statistical analysis was performed with Prism software (Graphpad) using the unpaired, two-tailed *t*-test to analyse the data and generate *P*-values.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Author contributions: RG designed and performed the experiments, analysed the data and wrote the paper; EH, SAK, FR, DG, MO, GTB and RJG performed the experiments; GG, AB, AS and DG analysed the data and reviewed the paper; AS, DG and PNT provided reagents; SPB designed the experiments, analysed the data and reviewed the paper; SG designed the experiments, analysed the data and wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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