Late stages of T cell maturation in the thymus involve NF-κB and tonic type I interferon signaling

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Positive selection occurs in the thymic cortex, but critical maturation events occur later in the medulla. Here we defined the precise stage at which T cells acquired competence to proliferate and emigrate. Transcriptome analysis of late gene changes suggested roles for the transcription factor NF-κB and interferon signaling. Mice lacking the inhibitor of NF-κB (IkB) kinase (IKK) kinase TAK1 underwent normal positive selection but exhibited a specific block in functional maturation. NF-κB signaling provided protection from death mediated by the cytokine TNF and was required for proliferation and emigration. The interferon signature was independent of NF-κB; however, thymocytes deficient in the interferon-α (IFN-α) receptor IFN-αR showed reduced expression of the transcription factor STAT1 and phenotypic abnormality but were able to proliferate. Thus, both NF-κB and tonic interferon signals are involved in the final maturation of thymocytes into naive T cells.

T cell development occurs in the thymus, which provides a unique microenvironment and presents ligands consisting of self peptide and major histocompatibility complex (MHC) molecules to T cell antigen receptors (TCRs). In the cortex of the thymus, low-affinity TCR interactions initiate positive selection signals in CD4⁺CD8⁺ double-positive (DP) immature thymocytes, which supports their survival and differentiation into CD4⁺ or CD8⁺ single-positive (SP) thymocytes. Positively selected cells move to the medullary region and, after several days, emigrate to the periphery. The term ‘positive selection’ is sometimes used to describe the entire process. However, in thinking about molecular mechanisms, it is helpful to break this down into kinetically distinct processes, such as survival, allelic exclusion, lineage commitment and functional maturation.

Cortical DP thymocytes require interaction of the surface TCR with selecting peptide-MHC complexes to induce survival. Expression of the activation marker CD69, the TCR and the anti-apoptotic protein Bcl-2 is rapidly upregulated in cells of this population, which also undergo changes in the expression of many other genes. Genetic deficiency in TCRs, MHC molecules, the co-receptors CD4 or CD8 or molecules in the TCR signaling pathway blocks this process. Recombination-activating genes are rapidly repressed at this stage, which facilitates allelic exclusion. Expression of the chemokine receptor CCR7 is upregulated somewhat later and facilitates the migration of progenitor cells from the cortex to the medulla. Lineage commitment occurs concurrently and involves downregulation of the gene encoding the inappropriate co-receptor and the initiation of genetic remodeling that will ultimately determine if the cell has helper or killer potential. Genetic deficiency in key transcription factors can block commitment to the CD4⁺ or CD8⁺ lineage. Although lineage commitment is mechanistically independent of migration to the medulla, these processes are roughly concurrent. Thus, SP thymocytes reside predominantly in the medulla; however, not all SP thymocytes are equivalent.

CD24hiQa2lo SP thymocytes have been defined as ‘semi-mature’ and have been shown to be susceptible to apoptosis when triggered through the TCR. In contrast, mature SP thymocyte and thymic emigrants proliferate when triggered through the TCR. Over the years, studies have shown that the expression of a small number of other cell-surface proteins, including CD69 and various cytokine and chemokine receptors, changes during maturation. However, the molecular mechanisms that control the maturation of SP thymocytes have remained unclear.

Here we defined steps in the maturation of SP thymocytes through which SP thymocytes became equipped with mature functions, such as proliferation competency, emigration competency and cytokine ‘licensing’. For this, we used comprehensive microarray analysis, quantitative real-time PCR (qPCR) and flow cytometry, along with combinations of several gene-deficient and transgenic mouse models. We found that SP thymocytes received signals via both the cytokine TNF and type I interferons in the thymus and that only TNF-resistant mature thymocytes survived and became emigration competent and licensed to produce cytokines.

RESULTS

Three SP stages defined by function

In this study, we sought to determine the ideal markers for flow cytometry to define SP thymocyte stages by function. The ordered development of SP thymocytes has been characterized by the expression of cell-surface makers (CD24, CD69, CD62L and Qa2), the carbohydrate epitope 6C10 on the glycoprotein Thy-1, recognized by the monoclonal antibody SM6C10 (ref. 9); or chemokine receptors CCR7 and CCR9 (ref. 11). However, these markers have not been well
correlated with functional maturation. For our comprehensive flow cytometry, we used mice with expression of green fluorescent protein (GFP) directed by the promoter of recombination-activating gene 2 (Rag2) via a bacterial artificial chromosome transgene (Rag2-GFP), in which GFP expression (as Rag2-GFP) acts as a ‘molecular timer’ for differentiation events after positive selection and allows the exclusion of re-circulating mature T cells. To focus our analysis on conventional β T cells, we used a ‘dump strategy’ to exclude γδ T cells, invariant natural killer T cells (NKT cells) and regulatory T cells (Treg cells) (Supplementary Fig. 1a). We found that the combination of staining for CD69 and MHC class I (MHCI) precisely defined SP thymocyte stages by function and could be used on cells of both the CD4+ lineage and CD8+ lineage; positively selected medullary thymocytes (TCRβ+CCR7+SM) and CD69+MHCI+ and CD69−MHCI+ cells (M1) cells and CD69−MHCI− (M2) cells (top right), as well as CD4+ and CD8-expression profiles of the populations above (bottom). Numbers adjacent to outlined areas indicate percent cells in each.

Figure 1 Three major SP stages defined by function. (a) Flow cytometry of thymocytes from Rag2GFP mice (n = 4), showing the expression of CCR7 and TCRβ by Rag2-GFP+ thymocytes (dump-gated as in Supplementary Fig. 1a) (top left), gates of three subsets of medullary thymocytes identified by expression of CD69 and MHCI most precisely defined the boundary between proliferation-incompetent cells and proliferation-competent cells. Thus, we designated CD69+MHCI− population as semi-mature (SM) and designated CD69+MHCI+ and CD69−MHCI+ populations as mature 1 (M1) and mature 2 (M2), respectively. To assess emigration and trafficking competence, we assessed expression of the sphingosine 1-phosphate (SIP) receptor S1PR1 (ref. 15) and the transcription factor KLF2, which is required for the expression of S1PR1 and CD62L in thymocytes. There was high expression of S1PR1 and CD62L on M2 cells but not on SM cells or M1 cells (Fig. 1c). Likewise, there was high expression of KLF2 only on M2 cells (Supplementary Fig. 1c). Thus, among proliferation-competent M1 and M2 thymocytes, only the most mature M2 cells were competent to emigrate. Finally, we assessed at which stage SP thymocytes became licensed to produce the cytokine TNF. Only the M2 subset had a substantial population of TNF-producing cells following stimulation via anti-CD3 and anti-CD28 (Fig. 1d), and this fraction continued to increase among recent thymic emigrants (data not shown), consistent with published reports.

Published staining combinations6,9 used to define two populations (CD24hiQa2hi and CD24loQa2hi) or four populations (SP1–SP4) with the combination of CD69, Qa2 and monoclonal antibody SM6C10 directed against Thy-1 did not precisely distinguish between proliferation-incompetent (SM) cells and proliferation-competent (M1) cells, although this nicely distinguished emigration-competent cells from other cells (Supplementary Fig. 1d). The combination of staining for CCR7 and CCR9 together with CD69 on CD4SP thymocytes was found to be specific in distinguishing proliferation-incompetent (CCR9hi) CD4SP cells from proliferation-competent (CCR9int–lo) CD4SP cells (Supplementary Fig. 1d, left), but this staining panel did not separate CD8SP thymocytes very well (Supplementary Fig. 1d, right), as mature CD8SP cells had abundant expression of CCR9. Thus, expression of CD69 and MHC class I was the most effective measure of the late stages of T cell maturation.

To assess proliferation competence, we sorted the three populations, labeled them with the proliferation-detection fluorescent dye CellTrace Violet and stimulated them in vitro with antibody to the invariant signaling protein CD3 (anti-CD3) plus antibody to the coreceptor CD28 (anti-CD28). CD69+MHCI− cells did not proliferate, while CD69−MHCI+ and CD69−MHCI− thymocytes did (Fig. 1b), which suggested that upregulation of the expression of MHCI class I most precisely defined the boundary between proliferation-incompetent cells and proliferation-competent cells. We designated CD69+MHCI− population as semi-mature (SM) and designated CD69+MHCI+ and CD69−MHCI+ populations as mature 1 (M1) and mature 2 (M2), respectively. To assess emigration and trafficking competence, we assessed expression of the sphingosine 1-phosphate (SIP) receptor S1PR1 (ref. 15) and the transcription factor KLF2, which is required for the expression of S1PR1 and CD62L in thymocytes. There was high expression of S1PR1 and CD62L on M2 cells but not on SM cells or M1 cells (Fig. 1c). Likewise, there was high expression of KLF2 only on M2 cells (Supplementary Fig. 1c). Thus, among proliferation-competent M1 and M2 thymocytes, only the most mature M2 cells were competent to emigrate. Finally, we assessed at which stage SP thymocytes became licensed to produce the cytokine TNF. Only the M2 subset had a substantial population of TNF-producing cells following stimulation via anti-CD3 and anti-CD28 (Fig. 1d), and this fraction continued to increase among recent thymic emigrants (data not shown), consistent with published reports.

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Transcriptome analysis indicates a role for NF-κB and IFNs

DP thymocytes have a gene-expression profile substantially different from that of T cells, consistent with the susceptibility of the former to apoptosis and the proliferation competence of the latter. However, our data and published results would suggest that the apoptosis-to-proliferation change occurs at the semi-mature–to–mature stage in the medulla, which we estimate to be at least 24 h after the initiation of positive selection signaling. Thus, we hypothesized that the unique gene changes that occur late in the process might be most relevant to the understanding of this competence transition. To characterize these gene changes, we ‘mined’ microarray data from thymocytes isolated using the previously defined markers Qa2 and CD69 to designate four stages. CD69+ TCRβ+ pre-selection DP (‘pre-DP’) cells and CD69+ TCRβ+ post-selection DP (‘post-DP’) cells (Fig. 2) were sorted from the thymus of B2m−/− mice (data not shown), in which only MHC II–restricted CD4+ T cells undergo positive selection. Qa2+ CD69+ (SM-M1) CD4SP cells and Qa2+ CD69− (M2) CD4SP cells (Fig. 2) were sorted from thymocytes of Rag2−/− mice with a ‘dump’ channel that included the activating NK cell receptor NK1.1, the T cell–activation marker and cytotoxic receptor CD25 and the antibody GL3, directed against TCRβ. By far the largest number of gene expression changes (2,060 genes up- or downregulated) were initiated during the cortical positive selection step from pre-DP to post-DP (Supplementary Fig. 2b). Some of these changes occurred only early (group A), some continued to change at later stages (group B) and a few were transient (group C) (Supplementary Fig. 2b). Notably, a substantial number of gene-expression changes occurred between the post-DP stage and SM stage (1,005 genes; groups D and E), whereas only a small number of genes altered their expression at the very end of medullary maturation (269 genes; group F), including a large number of genes whose expression changes were initiated earlier but then were reversed (Supplementary Fig. 2b). Using these expression-pattern groups, we defined sets of genes whose expression changed early or late during selection (Fig. 2).

To understand which upstream pathways and molecular factors might be acting at early and late stages, we used gene-set–enrichment analysis (GSEA) tools. Distinct stages showed enrichment for the expression of certain sets of genes that share a conserved cis-regulatory motif (called the ‘C3 motif’ in GSEA) in their promoters and 3′ untranslated regions (Table 1). The promoters of genes induced or repressed during positive selection (pre-DP to post-DP) showed enrichment for various transcription factor–binding sites, including those motifs recognized by the transcription factors ATF, EGR, NF-κB, ELK1 or SRF, many of which have already been shown to be involved in positive selection. Genes regulated by the transcription factor E2F showed a negative-enrichment score, indicative of downregulation. Among genes whose expression changed at later time points during the post-DP–to–SM-M1 transition, there was enrichment only for the expression of gene sets regulated by E2F, NF-κB and transcription factors of the IRF family. E2F-regulated genes seemed to be upregulated at this stage, reflective of the transient nature of their repression during positive selection. Among the NF-κB-regulated genes, some changes were initiated earlier, at the pre-DP–to–post-DP transition, and others were initiated later, at the post-DP stage. Substantial enrichment for the expression of IRF-regulated genes was seen only at the post-DP–to–SM-M1 transition. The number of gene changes observed at the latest stage of maturation was small (413), and there was no significant positive enrichment for any transcription factor–binding sites at this stage (normalized enrichment score of ≥1.6 or ≤–1.6). Because this analysis suggested that NF-κB- and IRF-regulated gene changes occurred late after positive selection, we focused on their effects in this study.

Table 1: GSEA of microarray data

| Gene set | # | NES | Gene set | # | NES | Gene set | # | NES |
|----------|---|-----|----------|---|-----|----------|---|-----|
| Pre-DP to post-DP | | | Post-DP to SM-M1 | | | SM-M1 to M2 | | |
| ATF | 3 | 1.97 | E2F | 14 | 1.84 | None | None | ≥1.60 |
| EGR | 6 | 1.86 | NF-κB | 1 | 1.62 | None | None | ≤–1.60 |
| CREB1 | 3 | 1.83 | IRF | 1 | 1.62 | | | |
| E4F1 | 1 | 1.82 | ATF | 1 | 1.60 | | | |
| NF-κB | 4 | 1.78 | EGR | 2 | 1.73 | | | |
| ETS | 3 | 1.73 | PAX6 | 1 | 1.62 | | | |
| ELK1 | 1 | 1.71 | NFAT | 1 | 1.60 | | | |
| SRF | 2 | 1.70 | | | | | | |
| PU.1 | 1 | 1.65 | | | | | | |
| STAT1 | 1 | 1.62 | | | | | | |
| NERF | 1 | 1.61 | | | | | | |
| E2F | 8 | 1.62 | | | | | | |

GSEA of microarray data, showing gene sets whose expression underwent enrichment and that share a conserved transcription factor–binding motif in the promoters and 3′ untranslated regions, among genes whose expression changed (upregulated (bold) or downregulated (not bold)) at the pre-DP–to–post-DP, post-DP–to–SM-M1 and SM-M1–to–M2 transitions (column headings); only gene sets with a normalized enrichment score (NES) of ≥1.6 or ≤–1.6 are included here. #, number of gene sets related to that transcription factor that were labeled.
Figure 3 TAK1 is required for the SM-to-M1 transition. (a) Flow cytometry of total thymocytes from Tak1fl/fl and Tak1fl/flCd4Cre mice (top) and thymocytes in the CD4SP or CD8SP lineage (with the exclusion of CD1d-α-GaICer NKT cells, CD25+ Treg cells, CD44hi recirculating memory cells and GL3+γδ T cells) (below). Numbers adjacent to outlined areas indicate percent CD4+CD8- cells (top left), CD4+CD8+ cells (top right), CD4-CD8- cells (bottom right) or CD4-CD8+ cells (bottom left) (top row), or CD69-MHCII+ (SM) cells (top left), CD69-MHCII+ (M1) cells (top right) or CD69-MHCII- (M2) cells (bottom right) (middle and bottom rows). (b) Quantification of total thymocytes (top) and SM, M1 and M2 CD4SP or CD8SP thymocytes (below) in Tak1fl/fl mice (n = 10) and Tak1fl/flCd4Cre mice (n = 10). Each symbol represents an individual mouse; small horizontal lines indicate the group mean (± s.d.). NS, not significant (P > 0.05); *P < 0.001 (two-tailed, unpaired Student’s t-test). (c) Microarray analysis of 381 genes expressed differentially (twofold or more (red, upregulated; blue, downregulated); P ≤ 0.05) by sorted SM-M1 CD4SP thymocytes from Tak1fl/fl mice versus those from Tak1fl/fl mice (WT vs TAK1; top row) or from Tak1fl/fl mice and Tak1fl/flCd4Cre mice (below). ‘Early’ and ‘Late’ as in Figure 2. (d) qPCR analysis of the expression of select TAK1-dependent genes separately sorted SM-M1 CD4SP thymocytes from Tak1fl/fl and Tak1fl/flCd4Cre mice, to confirm the microarray analysis results; PCR results are normalized to those of the control gene Hprt, and microarray results are those in Tak1fl/fl cells relative to those of Tak1fl/flCd4Cre cells. Each symbol represents an individual gene. Pearson correlation coefficient r = 0.46; P < 0.05 (two-tailed). Data are representative of (a) or pooled from (b) nine independent experiments or are pooled from three independent experiments with biological triplicates (c,d; mean).

Thymocytes and CD8SP thymocytes were lower in Tak1fl/flCd4Cre mice than in their control littermates (Fig. 3a,b). We further investigated the stage at which Tak1 deficiency affected maturation and found a normal number of SM cells but a profound reduction in the number of M1 cells and M2 cells (of 10-fold and 100-fold, respectively) in Tak1fl/flCd4Cre mice, relative to the number of these cells in their control littermates (Fig. 3a,b). This suggested that Tak1-dependent signals were not required for positive selection itself but were required for the ultimate survival and/or maturation of T cells. Mature iNKT cells, Treg cells and intraepithelial lymphocyte precursor thymocytes were almost completely absent from Tak1fl/flCd4Cre mice as well (Supplementary Fig. 3b).

To determine if Tak1 could be a key driver of late gene-expression changes during selection, we performed microarray analysis of purified SM CD4SP thymocytes from Tak1fl/flCd4Cre and control mice. 381 genes were up- or downregulated by Tak1 deficiency (data not shown). Using a heat map to visualize the overall pattern of gene changes, we noted that Tak1-dependent genes were changed mainly at the late stage of maturation, rather than an early stage, in wild-type mice (Fig. 3c,d). Next we used Ingenuity pathway analysis to define potential upstream regulators on the basis of the P value of the overlap between our gene lists and those defined by the literature. The upstream regulators defined for early gene-expression changes included TCR, TGF-β and members of the ‘Id’ family of transcription factors, among others (Supplementary Fig. 4). In contrast, late gene-expression changes overlapped many interferon and IRF pathways (Supplementary Fig. 4), consistent with the enrichment for IRF-binding sites in the promoters at transcripts that underwent late changes in expression (Table 1). Notably, results obtained for the upstream regulators defined for Tak1-dependent genes were very similar to those of transcripts that underwent late changes (yellow or green in Supplementary Fig. 4), which suggested that Tak1-dependent processes were the dominant signals that drove the gene-expression changes late in positive selection.

Restoration of survival but not proliferation by TNF blockade

Because activation of NF-κB protects cells from TNF-induced cell death, we investigated whether the role of Tak1 signaling in positive selection might be mainly to allow survival at the mature stage or if it was required for differentiation and the acquisition of proliferation competence. To study this, we crossed Tak1fl/flCd4Cre mice with TNF-deficient (Tnf−/−) mice. In Tak1fl/flCd4CreTnf−/− mice, the number of M1 cells was completely restored to equivalence to that of Tak1fl/fl mice (Fig. 4a,b), which suggested that one role for Tak1 signals might be to protect cells from TNF-induced death. However, M2 cells were ‘rescued’ only partially, and Tak1fl/flCd4CreTnf−/− mice were profoundly lymphopenic in the periphery (data not shown). The expression of multiple other members TNF receptor family was upregulated during thymic maturation (Supplementary Fig. 5a,b) and, similar to the receptor TNFR1, one of these, DR3, contains a death domain, and cells expressing this receptor might also require NF-κB signals for protection from induced cell death. However, crossing Tak1fl/flCd4Cre mice with mice with transgenic expression of Bcl2 (data not shown) or mice genetically deficient in Bim, a proapoptotic member of the Bcl-2 family (Bcl2l11−/−), yielded a phenotype similar to that of Tak1fl/flCd4CreTnf−/− mice, with low numbers of M2 cells and peripheral lymphopenia (Supplementary Fig. 5c,d). Although TNF deficiency restored the number of mature cells, those cells were unable to proliferate in response to anti-CD3 plus anti-CD28 (Fig. 4c). These data suggested that Tak1 signals might have been required for differentiation, in addition to being required for cell survival.
Restoration of proliferation but not licensing by IKK activity

TAK1 signals result in the activation of both NF-κB and mitogen-activated protein kinases. To determine which TAK1-dependent functions involve NF-κB, we used a transgene encoding constitutively active IKK2 (called 'IKKCA' here) to restore the activation of NF-κB in TAK1-deficient mice. We crossed mice expressing a Cre-inducible IKKCA-encoding transgene to control mice (Fig. 5a). In contrast to the 15–20% of TNF-α stimulated T cells from wild-type mice, IKKCA restored several aspects of maturation in TAK1-deficient SP thymocytes, it did not restore cytokine-production competence or the establishment of T cell populations in the periphery.

Facilitation of IFN signaling by TAK1 independently of NF-κB

To understand which TAK1-dependent gene-expression changes were dependent on NF-κB activity and which were independent of this, we measured the expression of 20 TAK1-dependent genes that were identified by microarray and whose expression changes were confirmed by qPCR (Fig. 3c,d). Thus, we sorted SM and M2 cells from TAK1−/−, TAK1fl/flCd4Cre and TAK1fl/flCd4CreIKKCA mice and measured gene expression by qPCR. The expression of about half (9) of these genes was restored by the IKKCA-encoding transgene and the expression of another half (11) was not (Fig. 6a).

Published studies have shown that interferon-β (IFN-β) is constitutively expressed in thymic medullary epithelial cells from naive mice. Given that an interferon-regulated gene signature was apparent in medullary thymocytes in our microarray analysis, we sought to test the hypothesis that maturing thymocytes might respond to interferon constitutively produced in the thymus. To address this, we studied mice deficient in the receptor for type I interferons (IFN-α/βR) and found that they had improved IFN signaling in M2 thymocytes.
(Ifnar1−/− mice). Indeed, sorted mature thymocytes from Ifnar1−/− mice showed reduced expression of various genes relative to their expression in Ifnar1+/+ mice (Fig. 6c). The genes most affected were all NF-kB-independent interferon-regulated genes noted above (Fig. 6a), which demonstrated that medullary thymocytes responded to constitutively produced interferon. Among the genes with reduced expression were Stat1 and Irf7, which have been shown to be targets of constitutive type I interferon signaling, and Stat1 and Irf7 are essential for ‘priming’ cells for cytokine responsiveness32,33. Thus, next we closely assessed the phenotype and function of SP thymocytes from Ifnar1−/− mice. The number of SM, M1 and M2 CD4SP thymocytes was similar in Ifnar1−/− mice and Ifnar1+/+ mice (Fig. 6d). Many other maturation markers were also expressed normally on CD4SP and CD8SP thymocytes from Ifnar1−/− mice.

**Figure 5** IKK activity restores proliferation and maturation but not licensing or survival. (a) Flow cytometry of thymocytes from Tak1+/+, Tak1+/+ Cd4Cre, Tak1+/+ Cd4CreIKKCA and Cd4CreIKKCA mice, showing CD4 and CD8 profiles (top), and proportion of SM, M1 and M2 CD4SP or CD8SP cells (with exclusion of cells as in Fig. 3a) (below). Numbers adjacent to outlined areas as in Figure 3a. (b) Quantification of total thymocytes (top) or SM, M1 and M2 CD4SP or CD8SP thymocytes (below) from Tak1+/+ mice (n = 10), Tak1+/+ Cd4Cre mice (n = 10), Tak1+/+ Cd4CreIKKCA mice (n = 6) or Cd4CreIKKCA mice (n = 4). (c) Proliferation of SM, M1 and M2 CD4SP thymocytes sorted from Tak1+/+ and Tak1+/+ Cd4CreIKKCA mice and left unstimulated or stimulated for 3 d with anti-CD3 plus anti-CD28 (assessed as in Fig. 1b). (d) TNF production by SM, M1 and M2 CD4SP cells obtained from Tak1+/+ and Tak1+/+ Cd4CreIKKCA mice and stimulated for 4 h anti-CD3 plus anti-CD28. Numbers adjacent to outlined areas indicate percent TNF+ cells. (e) Flow cytometry of total splenocytes from mice as in a. Numbers adjacent to outlined areas indicate percent CD4+ (CD4SP) T cells (top left) or CD8+ (CD8SP) T cells (bottom right). (f) Quantification of total splenocytes (top) and CD4+ or CD8+ T cells (below) in the spleen of Tak1+/+ mice (n = 9), Tak1+/+ Cd4Cre mice (n = 8), Tak1+/+ Cd4CreIKKCA mice (n = 6) or Cd4CreIKKCA mice (n = 4). (g) Flow cytometry of CD4SP thymocytes from mice as in a. Numbers adjacent to outlined areas indicate percent S1PR1+CD62L+ T cells. Each symbol (b,f) represents an individual mouse; small horizontal lines indicate the group mean (± s.d.). *P < 0.05, **P < 0.01 and ***P < 0.001 (two-tailed unpaired Student’s t-test). Data are representative of (a,e,g) or pooled from (b) four independent experiments or are representative of (c,d) or pooled from (f) three independent experiments.
DISCUSSION

Our data have provided a precise analysis of gene-expression changes that occur after positive selection and how they relate to function. While several gating strategies have been proposed for the demarcation of functionally relevant stages, our data suggested that upregulation of MHC class I expression most precisely marked the stage at which cells acquired the competence to proliferate. Other strategies failed in this context, although they were accurate in marking the stage at which cells became emigration competent. The only other cell-surface protein whose expression change correlated with the acquisition of cell division competence was the immunomodulatory receptor GITR. Qa2 is commonly used to mark the most mature thymocytes, but we found that the expression of Qa2 was strongly dependent on type I interferon signaling in SP cells and was not associated with maturation itself. This is consistent with the observation that Qa2 expression is dependent on the transcriptional regulator AIRE, yet T cells in AIRE-deficient mice are not thought to have major maturational defects.

The observations noted above about Qa2 expression would suggest that IFN-β is produced by medullary thymic epithelial cells in the steady state. Some independent evidence supports this hypothesis, yet further investigation is needed, given that constitutive production of IFN-β can also be driven by the microbiota or DNA damage. Regardless of the source of IFN-β, an important question is how exposure to type I interferon during development changes the functional properties of T cells. There is a growing appreciation that constitutive or tonic interferon signaling maintains homeostasis and primes cytokine responsiveness in other hematopoietic cell types. Published analysis of interferon-γ–mixed–bone marrow chimeras suggests an important role for constitutive interferon signals in T cell development and Treg cell homeostasis, so further studies of how interferon alters the gene expression and function of T cells are warranted.

The main finding of our study was that NF-kB signaling was critical for late maturation processes, both for survival at the SP stage and for functional maturation. In terms of survival, it is well established that TNFR signals can trigger both activation of NF-kB and death, through signaling pathways that have been referred to as ‘complex I’ and ‘complex II’. Signaling via complex I results in the activation of NF-kB and protection against complex II–mediated death. Our results established that TNF was present in the thymic environment and was able to mediate cell death, since TNF deficiency was able to rescue...
the number of mature cells in TAK1-deficient mice. Those findings are consistent with the phenotype of mice deficient in the pro-survival factor c-FLIP33, which interferes with apoptotic signaling downstream of death receptors. Although TNF was shown to be a source of the death signal, ‘rescue’ was not complete with TNF deficiency. Thus, it is possible that DR3, another death domain–containing member of the TNFR family expressed in medullary thymocytes, contributes as well.

Our data provided definitive evidence that NF-κB signals were required for late maturation. This is consistent with various reports in the literature showing normal positive selection but peripheral T cell lymphopenia in mice deficient in factors such as IKKγ, IKK1 and IKK2, and Ubc13. Subtle differences in the phenotypes might reflect differences in the rate of protein loss after Cre-mediated deletion in the different models (for example, with IKKγ) and/or redundancies with related components of the pathway (for example, with IKK1 and IKK2). Various receptors can activate the TAK1–NF-κB pathway. The TCR is an obvious candidate, since the groups of genes regulated by NF-κB showed enrichment for genes that changed both at positive selection and into the SM stage. However, the TCR activates TAK1–NF-κB through a Carma1–Bcl-10–Malt1 signalingosome, and mice deficient in those components do not exhibit this phenotype, although they do lack mature Treg cells44,46. We also feel the TCR is unlikely to be the sole source of TAK1 activation in conventional T cells because interaction of the TCR with MHC class II is required for survival from the post-DP stage to the SM stage, but it is not required for survival or maturation from the SM stage to mature stages8 (data not shown), although this is controversial47. Thus, there is a distinction between conventional T cells and Treg cells whereby both require activation of NF-κB through TAK1, but conventional T cells seem to receive sufficient NF-κB stimulation without the TCR. Signaling via the TGF-β receptor TGFβR might provide activation of TAK1 in medullary thymocytes. However, TGFβR was not identified as a potential upstream regulator in our analysis of either genes that underwent late changes in expression or TAK1-dependent genes. Furthermore, mice with TGFβR deficiency in T cells do not show a maturation problem per se, although again they have impaired development of Treg cells and iNKT cells48,49.

We favor the hypothesis that multiple members of the TNFR family can provide TAK1 signals to promote the survival and maturation of developing thymocytes. These include TNRFR1 and TNFR2, which bind to TNF. TNFR1 was expressed constitutively in thymocytes, while TNFR2 expression increased notably from the DP stage to the SP stage. Cells from mice with TNF deficiency alone did not have a maturation defect; thus, we propose that OX40, GITR, CD27 and DR3 provide redundant signals, as these receptors are either constitutively expressed (CD27) or induced by positive selection (OX40, GITR and DR3). The ligands for some of these (OX40L, GITRL and CD70) are known to be expressed in the thymus, particularly the thymic medulla50. This raises the possibility that thymocytes might need to access the medullary environment to receive signals for maturation. Given the data available so far, this seems not to be the case. Thymocytes that lack CCR7 fail to localize to the thymic medulla after positive selection and have defects in central tolerance, but mature and emigrate normally51,52. Likewise, mice that lack an organized medullary environment also have defects in central tolerance, but their thymocytes seem to mature normally51.

In summary, our data have revealed two critical features of T cell maturation that occurred after positive selection but before cells emigrated from the thymus. Activation of NF-κB occurred at the SM stage. NF-κB activity was critical for protecting cells from complex II–mediated death downstream of TNFR1, as it does in many cell types. However, in thymocytes, NF-κB activity was also critical for maturation processes that allowed the cells to mount a proliferative response when stimulated through the antigen receptor. A second critical feature was that thymocytes responded to constitutively produced type I interferon in the medullary environment. This response was dependent on TAK1 but independent of NF-κB. Constitutive type I interferon signaling resulted in upregulation of the expression of STAT1 and IRF7 and primed T cells to respond to inflammatory cytokines.

**METHODS**

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

**Accession codes.** GEO: microarray data, GSE74078.

**Note.** Any Supplementary Information and Source Data files are available in the online version of the paper.

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**AUTHOR CONTRIBUTIONS**

Y.X. designed and performed experiments, analyzed data, and wrote the manuscript; X.W. performed experiments and analyzed data; S.C.J. provided reagents, animals and input for the preparation of the manuscript; and K.A.H. directed the research, analyzed data and wrote the manuscript.

**COMPETING FINANCIAL INTERESTS**

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. C57BL/6 mice were purchased from the National Cancer Institute. H2-Aβ1−/−, B2m−/−, Tmk1fl/fl, Cd4+CD8−R26-Stop31-Ikkb2ca (IKKCA) and Tnf−/− mice were obtained from Jackson Laboratories. Bcl2I1−/− and Ifnar1−/− mice were kindly provided by A. Strauss (Walter and Eliza Hall Institute, Melbourne, Australia) and M. Mescher (University of Minnesota, Minneapolis, MN), respectively. Tmk1fl/flCd4Cre, Tmk1fl/flCd4CreIKKCA, Cd4CreIKKCA, Tmk1fl/flCd4CreTnf−/− and Tmk1fl/flCd4CreBcl2I1−/− mice were generated by crossbreeding at the University of Minnesota. Rag2−/− and Klf2−/− mice were described40,51. Animals were maintained under specific-pathogen-free conditions at the University of Minnesota. All experimental procedures were approved by the institutional animal care and use committee at the University of Minnesota.

Flow cytometry, purification by magnetic-activated cell separation and cell sorting. Single-cell suspensions were stained for 20 min on ice with the indicated antibodies. Antibodies to the following were purchased from BD Biosciences: CD3 (145-2C11), CD4 (GK1.5), CD8a (53-6.7), CD24/HLA (M1/69), CD25 (PC6), CD27 (LG3A10), CD28 (37.51), CD30 (2SH12-SF-2D), CD40 (3/23), CD44 (IM7), CD62L (MEL-14), CD69 (H1.2F3), CD137/41BB (1A9H2), MHCII/H2-Kb (AF6-88.5) and TCRbeta from BD Biosciences: CD3 (145-2C11), CD4 (GK1.5), CD8a (53-6.7), with the indicated antibodies. Antibodies to the following were purchased of Minnesota. All experimental procedures were crossbreeding at the University of Minnesota. Conditions at the University of Minnesota. The microarray data were analyzed using GeneSpring GX following standard procedures. Three independent RNA samples were scanned at the Biomedical Genomic Center (University of Minnesota) and hybridized to Affymetrix murine 430 2.0 gene chips (Affymetrix), and RNA from sorted cells was extracted using RNeasy of the target population.

Intracellular staining of STAT1. We stained freshly isolated thymocytes for surface markers (antibodies identified above), then treated them using Cytofix/Cytoperm (BD Biosciences) for 30 min on ice after surface staining, and then stained for intracellular TNF (MP6-XT22, BD Biosciences).

CellTrace Violet cell proliferation analysis. Sorted cells were labeled with CellTrace Violet (CTV) (Molecular Probes) and cultured in complete medium (RPMI-1640 medium containing 5 mM HEPES pH 7.5, 2 mM L-glutamine, 50 μM 2-mercaptoethanol, 50 μM/mL of penicillin, 50 μg/mL ptreptomycin, 50 μg/mL gentamicin sulfate and 10% FBS) in a 96-well round bottom plate coated with anti-CD3 (145-2C11, BD Bioscience; 10 μg/mL) and anti-CD28 (37.51, BD Bioscience; 20 μg/mL) antibodies. After 3 days, cells were analyzed by flow cytometry after staining with Fixable Viability Dye eFlour 780 (eBioscience), which was used to exclude dead cells. The intensity of CTV dye is diluted by half for every cellular division.

Microarray analysis. RNA from sorted cells was extracted using RNeasy Mini kit (Qiagen) with on column DNase step (Qiagen) per the manufacturer’s instructions. RNA was then quantified using a Nanodrop 2000/2000c spectrophotometer (Thermo Scientific). RNA (150–300 ng) was used for generating biotinylated cRNA through single-round amplification using the MessageAmpIII RNA Amplification kit following the manufacturer’s recommendations. A total of 20 μg of biotinylated cRNA was fragmented and hybridized to Affymetrix murine 430 2.0 gene chips (Affymetrix), and scanned at the Biomedical Genomic Center (University of Minnesota) following standard procedures. Three independent RNA samples were analyzed. The microarray data were analyzed using GeneSpring GX 11 software (Agilent). The normalization was carried out using MA55 algorithm. Probe sets were filtered by flag values (present/marginal as acceptable flags), and probe sets with P ≤ 0.05 (unpaired t-test) were considered statistical significance, then probe sets showing a difference of twofold or more were considered differentially expressed. Gene-expression patterns in Figure 2 were generated using Venn Diagram and Profile Plot operations of GeneSpring software.

GSEA and Ingenuity Pathway Analysis. GSEA was performed on microarray data using GSEA software (Broad Institute) per Broad Institute instructions52–53. C3 transcription factor target gene sets contain genes that share a transcription factor binding site defined in the TRANSFAC (version 7.4, http://www.gene-regulation.com/) database. Each of these gene sets is annotated by a TRANSFAC record. Pathway analysis was performed using Ingenuity Pathway Analysis software (Ingenuity System) according to its instructions.

Quantitative RT-PCR (qPCR). An RNeasy mini kit (Qiagen) and SuperScript III First Strand Synthesis SuperMix for qRT-PCR (Invitrogen) were used for the isolation of RNA and production of cDNA. FastStart Universal SYBR Green Master (Roche) and an ABI PRISM 7900HT sequence detection system (Applied Bioscience) were used for amplification and detection. Hprt (hypoxanthine guanine phosphoribosyl transferase) was used for normalization of samples. Primers were described in Supplementary Table 1.

Statistical analysis. s.d. and P values were determined using Prism software (GraphPad Software, Inc.). P values were calculated using a two-tailed unpaired Student’s t-test with 95% confidence interval.

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