A central role for Notch in effector CD8+ T cell differentiation

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Activated CD8+ T cells choose between terminal effector cell (TEC) or memory precursor cell (MPC) fates. We found that the signaling receptor Notch controls this ‘choice’. Notch promoted the differentiation of immediately protective TECs and was correspondingly required for the clearance of acute infection with influenza virus. Notch activated a major portion of the TEC-specific gene-expression program and suppressed the MPC-specific program. Expression of Notch was induced on naive CD8+ T cells by inflammatory mediators and interleukin 2 (IL-2) via pathways dependent on the metabolic checkpoint kinase mTOR and the transcription factor T-bet. These pathways were subsequently amplified downstream of Notch, creating a positive feedback loop. Notch thus functions as a central hub where information from different sources converges to match effector T cell differentiation to the demands of an infection.

The adaptive immune system must simultaneously curb the acute threat of microbial infections and generate immunological memory. Adaptive immunity to intracellular pathogens depends largely on CD8+ T cells. Protective CD8+ T cell responses require the proliferation of naive antigen-specific cells and their differentiation into cytolytic and cytokine-producing effector cells1. Different types of effector cells are generated that are responsible for acute protection or the generation of memory1,2. At one extreme is a population of terminally differentiated KLRG1−CD127− effector cells, the ‘terminal effector cells’ (TECs), which are mostly short-lived1-2. The ability to differentiate into long-lived memory cells is found predominantly in a KLRG1−CD127− population, collectively referred to as ‘memory precursor cells’ (MPCs)3. Beyond their ability to survive, there are considerable differences between these populations. TECs have higher expression of effector molecules than do MPCs3 and favor migration to nonlymphoid tissues and splenic red pulp. In contrast, MPCs preferentially home to lymph nodes and splenic white pulp4. Both lineages express distinct transcriptional programs. The transcription factors Blimp1 and T-bet are critical regulators of the TEC gene-expression program2,3,5, whereas the transcription factors eomesodermin, Tcf-1, Foxo1, STAT3 and Id3 control various aspects of MPC biology6–12.

TECs and MPCs can both derive from a single naive CD8+ T cell, but there is large variation in the proportion of TECs and MPCs among descendant effector populations derived from individual naive precursors13. Cells committed to either lineage can be identified among KLRG1−CD127− early effector cells within 3 d of infection9,14,15. An important question is how differentiating CD8+ T cells ‘choose’ among effector cell fates. This ‘choice’ may be affected by asymmetric segregation of fate-determining factors16. It stands to reason that the generation of TECs is proportional to the severity of the infection17; a greater infectious load requires the generation of more fully armed effector cells without an increase in the demand for memory cells. Such proportionality would require instructive signals that relay information about the severity of infection. Candidates for this include inflammatory cytokines, such as interleukin 12 (IL-12) and type I interferons, help by CD4+ T cells, and strong signaling via the receptor for IL-2, all of which promote the generation of TECs2,14,15,18,19. Given the potentially harmful consequences of the erroneous generation of highly cytotoxic TECs, it makes intuitive sense that their generation requires ‘licensing’ by multiple signals.

The cell-surface receptor Notch is a conserved regulator of binary cell-fate ‘decisions’20. Notch responds to membrane-bound ligands of the Delta-like (DLL) and Jagged families. The Notch intracellular domain (NICD) acts as a transcriptional activator after its ligand-induced cleavage and translocation to the nucleus20. All four receptors of the mammalian Notch family activate a pathway that...
RESULTS

Induction of Notch expression by TEC-promoting signals

To study how the degree of viral infection affects effector CD8+ T cell differentiation, we infected mice intranasally with various concentrations of the influenza virus strain A/HKx31 (called simply ‘HKx31’ here). We identified influenza virus–specific CD8+ T cells at the peak of the response (day 10; data not shown) through the use of H-2Db tetramers loaded with the immunodominant peptide of influenza virus nucleoprotein (amino acids 366–374) (H-2Dβ–NP)22. Increasing viral loads across a 100-fold range resulted in a great elevation in the number of TECs, whereas the number of MPCs remained constant (Fig. 1a). This result suggested the existence of signals that might couple the severity of the infectious threat to generation of TECs.

To determine whether Notch might be involved in that process, we investigated whether expression of the Notch family of receptors on CD8+ T cells was regulated by signals known to promote TEC differentiation. Important among those signals are inflammatory mediators produced by antigen-presenting cells (APCs). We incubated naive CD8+ T cells with dendritic cells (DCs) and added the RNA analog R-848, a mimic of RNA viruses. These conditions indeed induced surface expression of Notch1 on the naive CD8+ T cells within 24 h (Fig. 1b). Expression of Notch2 was induced only marginally (Fig. 1b). The addition of R-848 to naive CD8+ T cells without DCs did not elevate the expression of Notch1, but the addition of supernatants of R-848-treated DCs did (Supplementary Fig. 1a,b). This induction required the presence of the Toll-like receptor adaptor MyD88 in DCs but not in T cells (Supplementary Fig. 1b,c). Lipopolysaccharide similarly induced the expression of Notch1 on naive CD8+ T cells

Figure 1 TEC-promoting signals induce Notch expression on CD8+ T cells. (a) Quantification of KLRG1+CD127− (top) and KLRG1−CD127+ (bottom) H-2Db−NP+CD8+ T cells in mouse spleens 10 d after infection with various doses of HKx31 (horizontal axis; half-maximal tissue culture infectious dose (TCID50)). (b) Expression of Notch1 (top) and Notch2 (bottom) on CD8+ T cells cultured for 16 h with bone marrow–derived DCs in the presence (+ R-848) or absence (−R-848) of R-848, and on naive CD8+ T cells (Naive). MFI, mean fluorescence intensity. (c) Expression of Notch1 (top) and Notch2 (bottom) on OT-I CD8+ T cells activated overnight by bone marrow–derived DCs and DC supernatants (DC sup); results are presented relative to those of the control gene Actb (which encodes β-actin). (d) Expression of Notch1 (top) and Notch2 (bottom) on OT-I CD8+ T cells activated overnight by bone marrow–derived DCs and OVA protein in the presence or absence of R-848 (key); results were calculated by subtraction of those obtained for staining with an isotype-matched control antibody (ΔMFI). (e) Notch1 expression on CD8+ T cells stimulated overnight with anti-CD3 and supernatants of untreated DCs (DC sup) or supernatants of R-848-treated DCs (R-848 DC sup); results are presented relative to those of the control gene Actb (which encodes β-actin). (f) Notch1 expression on wild-type CD8+ T cells (WT) and CD8+ T cells deficient in the receptor for type I interferons (IFNAR1-KO), left unstimulated (US) or stimulated with supernatants as in c. (g) Notch1 expression on CD8+ T cells left unstimulated or stimulated with supernatants as in c in the presence (+) or absence (−) of rapamycin. (h) Notch1 expression on wild-type or Tbx21−/− (T-bet-KO) CD8+ T cells stimulated in the presence of anti-CD3 and various concentrations of IL-2 (horizontal axis). NS, not significant; *P < 0.05, **P < 0.01 and ***P < 0.001 (two-tailed t-test (b,c,g) or one-way analysis of variance (ANOVA) with Bonferroni corrections (b,h)). Data are representative of two experiments (a,f,h,i), more than five experiments (b) or three experiments (d,e,g) mean and s.e.m. of three mice per group (a), technical triplicates (b,h), technical duplicates (d-f), three to six technical replicates per group (g) or technical duplicates and quadruplicates (i)) or are from two experiments, each with technical duplicates (c; mean and s.e.m.).
via a MyD88-dependent pathway in DCs (Supplementary Fig. 1b). Thus, the activation of Toll-like receptors stimulate DCs to produce soluble factors that in turn induced surface expression of the Notch family of receptors on naive CD8+ T cells. Such soluble factors also elevated the expression of Rbpj mRNA (Fig. 1c), which suggested general enabling of the Notch pathway.

The T cell antigen receptor (TCR)-mediated activation of naive OT-I CD8+ T cells by DCs presenting an ovalbumin (OVA) peptide of amino acids 257–264 resulted in modest induction of the expression of Notch1 and Notch2 (Fig. 1d). Induction of each of these receptors was markedly enhanced by addition of R-848 (Fig. 1d). This was not due to improved antigen presentation, as supernatants of R-848-treated DCs also enhanced Notch1 expression on CD8+ T cells activated with antibody to CD3 (anti-CD3) (Fig. 1e).

To identify the soluble mediators responsible for the induction of Notch, we focused on type I interferons. These cytokines are produced by APCs upon recognition of viral nucleic acids and promote the differentiation of TECs15,19,28. CD8+ T cells lacking expression of IFNAR1, the receptor for type I interferons, failed to elevate Notch1 expression in response to supernatants of R-848-treated DCs (Fig. 1f). Thus, CD8+ T cells responded to type I interferons by elevating their surface expression of Notch1.

We further investigated whether signaling pathways known to regulate the differentiation of TECs might control Notch expression. The rapamycin-sensitive complex TORC1 is required for the differentiation of TECs29–31 and is activated by signaling via the receptor for type I interferons32. Treatment with rapamycin almost completely abrogated the induction of Notch1 expression on CD8+ T cells by supernatants of R-848-treated DCs (Fig. 1g). The differentiation of TECs also depends on T-bet2. Expression of T-bet was induced in naive CD8+ T cells by supernatants of R-848-treated DCs (data not shown) and was partially responsible for the induction of Notch1 expression, as surface expression of Notch1 was lower in T-bet-deficient (Tbx21−/−) T cells than in T-bet-sufficient (wild-type) cells (Fig. 1h). Finally, strong signaling via the receptor for IL-2 enhances TEC differentiation4,41,42, and IL-2 also acted in synergy with stimulation of the TCR to elevate expression of Notch1 (Fig. 1i).

Consistent with a role in driving TEC differentiation, Notch abundance was also transiently elevated in vivo on early KLGl− effector cells compared with its abundance on KLRG1− effector cells (Supplementary Fig. 2). Thus, promoters of TEC differentiation also induced the receptivity of the Notch pathway in CD8+ T cells. The convergence of all these signals on Notch expression suggested that perhaps Notch has a role in guiding the ‘decision’ between TEC differentiation and MPC differentiation.

**Induction of Notch ligands on APCs by viral infection**

Two major populations of APCs in lungs are CD11c+MHCIIInt alveolar macrophages and CD11c+MHCIIhi migratory DCs (mDCs), characterized by intermediate size and large size, respectively33,34 (Fig. 2a and Supplementary Fig. 3a). These mDCs carry influenza virus antigen to the mediastinal lymph nodes and are responsible for priming naive CD8+ T cells through direct presentation34,35. Both mDCs and alveolar macrophages from the lungs from uninfected mice had undetectable expression of DLL4 and Jagged2 and low expression of DLL1 (Fig. 2a). However, Notch ligands were almost completely undetectable on other populations of MHCII+ cells present in lungs, even after infection with influenza virus (Supplementary Fig. 3c). Therefore, infection with influenza virus induced the expression of Notch ligands on the APCs that primed naive CD8+ T cells, positioning these ligands for a potential role in the differentiation of virus-specific CD8+ T cells.

**Notch control of the acquisition of effector function**

The results presented above showed that the Notch signaling module was assembled on both sides of the T cell–APC interface under conditions favoring the differentiation of TECs. To investigate whether Notch controls differentiation of these cells, we used mice lacking expression of both Notch1 and Notch2 in the T cell lineage. Notch1 and Notch2 were both expressed in CD8+ T cells (Fig. 1), whereas expression of Notch3 and Notch4 is undetectable26. We therefore generated mice lacking expression of both Notch1 and Notch2 by crossing mice expressing a transgene encoding Cre recombinase from the T cell–specific Cd4 promoter (Cd4-Cre) with mice homozygous for loxP-flanked alleles of Notch1 (Notch1fl/fl) and Notch2 (Notch2fl/fl). The resultant Notch1fl/flNotch2fl/flCd4-Cre mice (called ‘Notch1-2 KO’ mice here) lacked expression of both Notch1 and Notch2 in mature CD4+ T cells and CD8+ T cells (data not shown). Thymic T cell development is not overtly affected in these mice36.

After infecting mice with HKx31, we found similar numbers of CD8+ T cells that bound H-2Dβ–NP in blood, spleen or lungs of wild-type and Notch1-2 KO mice at the peak of the response (Fig. 3a–c). However, the proportion of influenza virus–specific CD8+ T cells was
consistently higher in mediastinal lymph nodes from Notch1-2-KO mice than in those from wild-type mice (Fig. 3d). Also, Notch1-2-KO CD8+ T cells ‘preferentially’ localized to sites in the spleen not accessible to antibodies injected into the bloodstream (Fig. 3e), which suggested localization to the white pulp. Although the proportion of cells that produced IL-2 in response to the NP peptide was similar among either wild-type CD8+ effector T cells or Notch1-2-KO CD8+ effector T cells, production of IFN-γ and tumor-necrosis factor was reduced in the latter (Fig. 3f). Production of the cytolytic effector molecule granzyme B was almost completely undetectable in Notch1-2-KO CD8+ T cells, and the abundance of mRNA encoding granzyme B or perforin was reduced in these cells (Fig. 3g). Low expression of these molecules ‘translated’ into reduced cytolytic effector function in an in vivo cytology assay. When we loaded splenocytes with NP peptide and labeled them with a low dose of the cytotoxic dye CFSE (CFSElo cells) and then injected them into wild-type mice that had been infected with HKx31 10 d earlier, nearly all of these cells were rapidly killed (as shown by the absence of CFSElo cells; CFSEhi splenocytes not loaded with peptide and injected together with the peptide-loaded CFSElo splenocytes were not killed). In contrast, the killing of that population of cells was less effective in Notch1-2-KO mice treated similarly (Fig. 3h). As expected from such defects in effector function, viral clearance and weight recovery were compromised in Notch1-2-KO mice infected with the aggressive A/PR/8/34 strain of influenza virus (Fig. 3i). Although Notch1-2-KO mice also lacked expression of Notch in CD4+ T cells, the defective viral clearance in these mice was not caused by the ineffective provision of help to B cells, as titers of neutralizing antibodies to influenza virus strain A/PR/8/34 were similar in wild-type mice and Notch1-2-KO mice (Fig. 3k). Thus, under these experimental conditions, Notch was required for the generation of fully functional CD8+ effector T cells.

Control of TEC differentiation by Notch

High expression of effector molecules and residence outside lymph nodes and splenic white pulp are characteristics of TECs. In contrast, MPCS ‘preferentially’ localize inside such locations and tend to produce IL-2 exclusively3,4,37. Our results were therefore consistent with a defect in the TEC compartment and a greater abundance of MPCs.
Notch is required for TEC differentiation. (a) Flow cytometry of KLRG1 and CD127 on splenic H-2D<sup>b</sup>–NP<sup>+</sup>CD8<sup>+</sup> T cells obtained from wild-type and Notch1-2-KO mice (n = 3 per group) 10 d after infection with Hkx31 (top), and frequency of KLRG1<sup>+</sup>CD127<sup>+</sup> TECs (bottom left) and KLRG1<sup>−</sup>CD127<sup>−</sup> MPCs (bottom right) among CD8<sup>+</sup> T cells from the mice above. Numbers in quadrants (top) indicate percent KLRG1<sup>−</sup>CD127<sup>−</sup> cells (top left) or KLRG1<sup>−</sup>CD127<sup>−</sup> cells (bottom right). (b) Time course of the appearance (frequency) of CD127<sup>−</sup>KLRG1<sup>−</sup> TECs among H-2D<sup>b</sup>–NP<sup>+</sup>CD8<sup>+</sup> T cells in the blood of mice as in a (n = 3–20 per data point). (c) Frequency of KLRG1<sup>−</sup>CD127<sup>−</sup> TECs in the blood of wild-type, Notch1-2-KO, Notch1-deficient (Notch1-KO) or Notch2-deficient (Notch2-KO) mice (n ≥ 6 mice per group) (e) or wild-type or RBPJ-deficient (RBPJ-KO) mice (n = 3 per group) (d) at 10 d after infection as in a. (e) Flow cytometry of KLRG1 and CD127.1 on H-2D<sup>b</sup>–NP<sup>−</sup>CD8<sup>+</sup> T cells from chimeras reconstituted with wild-type (CD45.1<sup>+</sup>) plus wild-type (CD45.1<sup>+</sup>) bone marrow (WT-WT), wild-type (CD45.1<sup>+</sup>) bone marrow Notch1-2-KO (CD45.2<sup>+</sup>) bone marrow (KO-KO), or Notch1-2-KO (CD45.2<sup>+</sup>) plus Notch1-2-KO (CD45.2<sup>+</sup>) bone marrow (KO-KO), then infected as in a and assessed 10 d later. (f) Frequency of KLRG1<sup>+</sup> H-2D<sup>b</sup>–NP<sup>−</sup>CD8<sup>+</sup> T cells in chimeras as in e (n ≥ 8 mice per group). (g) Frequency of KLRG1<sup>−</sup>Notch2 KO OT-I (CD45.2<sup>+</sup>) T cells in wild-type (CD45.1<sup>+</sup>) mice (n = 3 per group) 5 d after infection with OVA-influenza and transfer of Notch1<sup>−/−</sup>Notch2<sup>−/−</sup> OT-I CD8<sup>+</sup> T cells transduced to express empty vector control retrovirus (EV) or retrovirus expressing human Cre (hCre), presented as transduced (TD) and untransduced (UT) cells from the same culture. (h) Frequency of KLRG1<sup>−</sup>Notch1-2-KO OT-I (CD45.2<sup>+</sup>) T cells in wild-type (CD45.1<sup>+</sup>) mice (n = 2 per group) 5 d after infection with OVA-influenza and transfer of Notch1-2-KO OT-I cells transduced to express empty vector control or NICD1-expressing retrovirus (presented as presented in g). NS, not significant; *P < 0.05, **P < 0.01 and ***P < 0.001 (two-tailed t-test (a,d,g,h) or one-way ANOVA with Bonferroni corrections (c,f)). Data are representative of more than ten experiments (a), two experiments (d) or three experiments (e,g,h) or are from three experiments (b,c,f). mean and s.e.m. in a–d,f–h.

In Notch1-2-KO mice, indeed, almost no (influenza virus–specific) KLRG1<sup>−</sup>CD127<sup>−</sup> TEC T cells were detectable in Notch1-2-KO mice at the peak of the response to HKx31, whereas the proportion of (influenza virus–specific) KLRG1<sup>−</sup>CD127<sup>−</sup> MPC T cells was greater than that in wild-type mice treated the same way (Fig. 4a). TECs were absent from Notch1-2-KO mice at all time points examined after infection with HKx31 (Fig. 4b), which demonstrated that their absence did not reflect altered response kinetics. We observed a partial loss of the population of TECs in mice lacking expression of only Notch1 or Notch2 (Fig. 4c) or mice heterozygous for deletion of both Notch1 and Notch2 (Supplementary Fig. 4). These results showed that Notch1 and Notch2 acted redundantly and that the presence of TECs was sensitive to the ‘dose’ of Notch1 and/or Notch2. Furthermore, TECs were almost completely absent when the canonical Notch effector RBP-J was deleted in T cells (Fig. 4d and Supplementary Fig. 5). Dependence of Notch on HKx31, as no TECs were detectable after infection with influenza virus strain WSN-OVA (called ‘OVA-influenza’ here), which expresses the H-2K<sup>b</sup>-restricted OVA peptide of amino acids 257–264 (Supplementary Fig. 7). These data showed that Notch controlled TEC development in a CD8<sup>+</sup> T cell–intrinsic manner and independently of the influenza virus strain and TCR specificity.

As developmental defects could potentially have indirectly perturbed TEC differentiation in Notch1-2-KO mice, we inactivated the expression of Notch1 and Notch2 in mature CD8<sup>+</sup> T cells. For this, we retrovirally introduced Cre into mature Notch1<sup>−/−</sup>Notch2<sup>−/−</sup> OT-I CD8<sup>+</sup> T cells and transferred those cells into mice infected with OVA-influenza (infection was required for full population expansion in vivo; data not shown). Expression of Cre abrogated the development of KLRG1<sup>+</sup> cells (Fig. 4g). In contrast, the introduction of NICD1 (the constitutively active intracellular domain of Notch1) restored the ability of Notch1-2-KO OT-I T cells to generate KLRG1<sup>+</sup> cells (Fig. 4h). We concluded that Notch1 and Notch2 acted directly in mature CD8<sup>+</sup> T cells to drive the development of cells that phenotypically resembled TECs.

Control of the TEC gene-expression signature by Notch

To determine whether Notch was required for adoption of the full TEC identity, we examined the global gene-expression profiles of Notch1-2-KO and wild-type CD8<sup>+</sup> effector cells by whole-transcriptome RNA sequencing. For this, we sorted CD8<sup>+</sup> T cells that bound the H-2D<sup>b</sup>–NP tetramer by flow cytometry from mice infected 10 d earlier with HKx31. This population consisted of well over 99% pure effector cells, as indicated by their CD44<sup>+</sup> phenotype (data not shown)

We used the c7 gene set of the Molecular Signatures Database (http://www.broadinstitute.org/gsea/msigdb/) for gene-set--enrichment
analysis of immunological signatures to obtain an unbiased view of the types of processes affected by Notch deficiency. Consistent with a role for Notch in the control of TEC differentiation, nine of the top ten gene sets with the most significant enrichment pertained to the differentiation of CD8+ effector T cells in general (yellow, Supplementary Data Set 1) and the differentiation of TECs and MPCs in both viral and bacterial models in particular (red, Supplementary Data Set 1).

To more specifically determine whether the TEC program is controlled by Notch, we generated molecular definitions of TECs and MPCs by whole-transcriptome sequencing of sorted KLRG1−CD127+ and KLRG1+CD127− H-2Db–NP–specific CD8+ T cell populations from wild-type mice. We found 132 genes that were specifically expressed in TECs (‘TEC specific’), whereas another 250 genes were ‘preferentially’ expressed in MPCs (‘MPC specific’) (Supplementary Data Set 2). Assignment of those identities to the genes with different expression in wild-type H-2Db–NP–specific CD8+ T cells than in their Notch1-2-KO counterparts showed that a large proportion of Notch-dependent genes (that is, genes with higher expression in wild-type cells) were TEC specific (Fig. 5a). Indeed, more than 40% of the TEC-specific transcriptome had lower expression in Notch1-2-KO H-2Db–NP–specific CD8+ T cells than in their wild-type counterparts (Fig. 5b). Conversely, less than 2% of the MPC-specific genes had lower expression in Notch1-2-KO H-2Db–NP–specific CD8+ T cells than in their wild-type counterparts (Fig. 5b).

**Figure 5** Notch regulates the TEC transcriptome. (a) High-throughput RNA sequencing analysis of splenic H-2Db–NP+ CD8+ T cells from wild-type and Notch1-2-KO mice 10 d after infection with HKx31, presented as mean expression versus difference in expression (log2 fold) in Notch1-2-KO cells versus wild-type cells (KO/WT), of ‘undedicated’ genes (without specific expression in TECs or MPCs; gray), TEC-specific genes (green) and MPC-specific genes (magenta) (Supplementary Data Set 2) with significantly different expression (DE) in Notch1-2-KO cells versus wild-type cells (adjusted P value, <0.05) or without significantly different expression (Not DE). (b) Frequency of TEC-specific or MPC-specific genes with significantly lower expression (Reduced) or higher expression (Induced) in Notch1-2-KO H-2Db–NP+CD8+ T cells than in their wild-type counterparts (adjusted P value, <0.05). (c) Gene-set–enrichment analysis map of genes expressed differently (false-discovery rate, <0.5) in Notch1-2-KO versus wild-type cells, with analysis restricted to genes encoding transcription factors (pathway and regulation), with a difference in expression of at least twofold for these comparisons (e–i, genes encoding CD44 and Sell included for completeness). Data are from three experiments, with results pooled from three mice per genotype in each.
expression in Notch1-2-KO CD8+ effector T cells than in their wild-type counterparts (Fig. 5b). Instead, the expression of a large proportion (36%) of MPC-specific genes was higher in Notch1-2-KO CD8+ effector T cells than in their wild-type counterparts (Fig. 5a,b). These results suggested that Notch promoted the TEC fate and may have inhibited the formation of MPCs.

As Notch controlled the expression of many, but not all, TEC signature genes (Fig. 5b), we investigated whether Notch selectively controls specific functional categories. For this, we made a side-by-side analysis of the gene ontology terms (biological pathways from the database of the Gene Ontology Consortium) that showed enrichment in the comparison of the wild-type and Notch1-2-KO transcriptomes and those that showed enrichment in the comparison of the MPC and TEC transcriptomes. We focused on the major functional categories that define the difference between MPCs and TECs, including those associated with effector function, migration, viability, activation and differentiation (Fig. 5c). The nearly complete overlap of enrichment for gene ontology terms revealed representation of Notch–dependent genes in all these categories (Fig. 5c). Thus, Notch did not control individual aspects of TEC differentiation but exerted broad control throughout the TEC gene-expression program. That conclusion was further supported by comparison of the expression patterns of specific genes encoding transcription factors (involved in the differentiation of effector cells), chemokine receptors, adhesion molecules, cytotoxic effector molecules or killer lectin-like receptors. Expression of TEC-specific genes in all these categories was lower in Notch1-2-KO effector cells than in their wild-type counterparts (Fig. 5d–i and Supplementary Data Set 3). Although some genes were either unaffected or affected only weakly, no TEC gene had significantly higher expression in Notch1-2-KO effector cells than in their wild-type counterparts (Fig. 5d–i). Reciprocally, expression of MPC genes was generally higher in Notch1-2-KO effector cells than in their wild-type counterparts or was not different, but it was never lower in Notch1-2-KO effector cells than in their wild-type counterparts (Fig. 5d–i). Together these results identified Notch as a major regulator of the ‘decision’ between TEC differentiation and MPC differentiation.

Notch feeds back onto TEC-promoting pathways

Early TECs are characterized by high expression of CD25 (the α-chain of the receptor for IL-2 (IL-2Rα)), and signaling via IL-2R promotes the differentiation of TECs13,18. CD25 (IL-2Rα) expression is induced on CD8+ T cells by constitutively active Notch38, which suggests that this component of IL-2R is a downstream target of Notch. Indeed, expression of mRNA encoding CD25 (IL-2Rα) was lower in Notch1-2-KO effector CD8+ T cells than in their wild-type counterparts (Fig. 6a), as was CD25 (IL-2Rα) expression on Notch1-2-KO OT-1 CD8+ T cells at early stages of the response to OVA-influenza (Fig. 6b), when differentiation takes place.

The Akt-mTOR pathway, an important regulator of TEC differentiation39,40, is activated by signaling via IL-2R and functions downstream of Notch in thymocytes and T cell leukemia41,42. We therefore investigated whether Notch controls the activity of this pathway during CD8+ T cell responses. Activation of the kinase Akt requires phosphorylation of Thr308, which is the substrate site for the kinase PKD1, and this modification positively correlates with high CD25 (IL-2Rα) expression in early CD8+ effector T cells in vivo39,40. Phosphorylation of Akt at Thr308 was detectable in OT-I effector cells isolated from mice 5 d after infection with OVA-influenza (Fig. 6c).
In contrast, in Notch1-2-KO OT-I T cells, phosphorylation of this residue was diminished to the background amount found in unactivated CD4+CD8+ T cells (Fig. 6c). We obtained similar results for Akt-mediated phosphorylation of the metabolic checkpoint kinase mTOR (at Ser2448) and of Foxo1-Foxo3 (at Thr24 and Thr32), which were both diminished to background amounts in Notch1-2-KO OT-I cells (Fig. 6c and Supplementary Fig. 8a). Of note, expression of constitutively active Akt41 in Notch1-2-KO OT-I T cells was sufficient to restore the generation of a KLRG1+ population (Fig. 6d). These results supported the conclusion that the Akt-mTOR pathway functions downstream of Notch in the differentiation of TECs.

T-bet is an important transcriptional regulator of TEC development2 (Fig. 6e). Tbx21 is a direct target of Notch in CD4+ T cells24,25 and, correspondingly, the abundance of Tbx21 mRNA was also lower in Notch1-2-KO effector CD8+ T cells than in their wild-type counterparts (Fig. 6f). Genetic deficiency in T-bet largely abrogated the ability of retrovirally expressed NICD1 to induce the development of KLRG1+ effector CD8+ T cells (Fig. 6g), whereas ectopic expression of T-bet restored the differentiation of such cells from Notch1-2-KO CD8+ T cells (Fig. 6h). These results suggested that T-bet acts downstream of Notch to drive the differentiation of TECs.

In conclusion, expression of CD25 (IL-2Rα) and T-bet, as well as activity of Akt and mTOR, all depended on Notch. These factors also acted upstream of Notch by inducing its expression on naive CD8+ T cells (Fig. 1). Therefore, our results have shown that Notch functions as a central hub in a network that integrates signal input from different sources (inflammation, IL-2 and Notch ligands) to induce the generation of TECs (Supplementary Fig. 8b).

DISCUSSION

How activated CD8+ T cells ‘decide’ between committing to the memory effector cell lineage or the terminal effector cell lineage is a conceptual question with implications for vaccine design. The requirement for balancing effective rejection of invading microorganisms and the risk of immunopathology is involved in this. Given their destructive potential, the generation of TECs must be regulated by signals that shape the response to the threat posed by the infection. Indeed, inflammatory signals promote the development of TECs2,15,17,19. We have now shown that Notch bridges signaling by innate pattern recognition receptors and inflammation to TEC development. Type I interferons induce expression of receptors of the Notch family and RBPJ on or in CD8+ T cells, while Toll-like receptors and inflammatory cytokines induce expression of Notch ligands on APCs13,44. Thus, microbial and inflammatory stimuli activate both sides of the T cell–APC interface to assemble the Notch signaling module. Receptors of the Notch family and their ligands never reach very high abundance on the cell surface. However, we note that such abundance is often actively kept low, for example, by internalization45,46, a property that may promote ‘dose sensitivity’ of the pathway47. TEC differentiation was indeed sensitive to the Notch ‘dose’, as shown in mice that lacked Notch1 or Notch2 individually or mice heterozygous for the absence of both. Notably, combined induction of receptors, ligands and the downstream mediator RBPJ probably results in cumulative amplification of the signal ‘amplitude’.

Our results showed that Notch, IL-2R, mTOR and T-bet together formed a positive feedback module that integrated signals from various sources. These signals might signify the detection of living microorganisms (type I interferons), direct interaction of the APC with those microorganisms (Notch ligands) and perhaps help by CD4+ T cells (IL-2). Such a system that requires the integration of multiple signals is presumably less prone to spuriously develop the potentially destructive TECs than one that depends on an individual signal. Together these factors (and possibly others) could serve as a ‘licensing code’ that allows full differentiation of TECs only when justified by the severity of the infection. The finding that delivery of all these signals together sets up a positive feedback loop suggests the existence of a mechanism to ensure maximal separation between the TEC fate and MPC fate.

Notch and Akt promote the survival of various cell types48,49, but this does not appear to explain their roles in effector CD8+ T cells. Activation of Akt in fact reduces the survival of CD8+ effector T cells in vivo, presumably by promoting the differentiation of the short-lived TEC fate39,50. Likewise, our results were not consistent with a major role for Notch in the survival of TECs. First, Notch deficiency did not affect the magnitude of the influenza virus–specific CD8+ T cell response, inconsistent with the loss of a major population. Second, if Notch deficiency compromised TEC survival, loss of the entire TEC signature in Notch1-2-KO mice would be expected. Instead, we found that approximately half of the TEC-specific gene-expression signature was unaffected by deficiency in Notch1 and Notch2. Furthermore, there were considerable differences in the degree to which different TEC-specific genes were affected. These data suggested that Notch controls TEC differentiation by regulating a large proportion of the TEC-specific gene-expression program. In this program, some genes relied more heavily on Notch than did others. However, in the absence of Notch, the program as a whole collapsed, which resulted in a severe differentiation defect. Genes that encode molecules with dedicated roles in effector CD8+ T cells have been identified as targets of Notch. These include the genes encoding granzyme B, perforin, IFN-γ and T-bet22,24–28. Furthermore, Notch controlled the expression of a critical component of IL-2R as well as the amplitude of signaling via Akt and mTOR during effector CD8+ T cell differentiation. All of these components promote the differentiation of effector CD8+ T cells and regulate additional transcription factors involved in this process, including STAT5, Foxo and HIF-1 proteins39,40,51–53. Thus, in addition to directly regulating genes encoding key effector molecules, Notch mobilizes additional signaling pathways and thereby coordinates an extensive differentiation program. Still, the finding that approximately half of the TEC-specific transcriptome was unaffected by the absence of Notch showed that the TEC differentiation program is modular. It seems likely that the other module(s) are controlled by additional signals activated by inflammation.

Loss of part of the TEC signature in Notch1-2-KO mice was mirrored by higher expression of roughly a third of the MPC-specific transcriptome. This probably did not reflect a relative increase in the number of NPCs due to the absence of TECs, as that would result in higher expression of the entire MPC transcriptome. The simplest interpretation of these data, therefore, is that apart from inducing a TEC-specific gene-expression program, Notch represses part of the MPC program. This program might further be suppressed by additional TEC-promoting signals and/or might require the provision of MPC-promoting signals, such as proteins of the Wnt family and activators of STAT3, including IL-10 and IL-21 (refs. 6,11,12). Nonetheless, our results do suggest that avoiding (strong) Notch signaling is a condition that allows differentiation into the MPC lineage. How and where this condition exists during an infection should be addressed by future research.

METHODS

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

Accession codes. ArrayExpress, E-MTAB-2999.
Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
R.A.B. designed, performed and analyzed experiments and wrote the manuscript; C.H.L., R.G., A.K., B.J.L., C.X.D., Y.S.D., S.E.V.T., R.v.B. and A.T.B. performed and analyzed experiments; A.M.W. and A.H.C.v.K. analyzed data from high-throughput RNA sequencing; G.F., S.M.K., J.M.B. and K.v.G. designed and analyzed experiments; and D.A. supervised the study, designed experiments and wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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ONLINE METHODS

Infection with influenza virus. Mice. All mice were on a C57BL/6 background. Notch1<sup>fl/fl</sup>Notch2<sup>fl/fl</sup>CD4-Cre mice or Rbpj<sup>fl/fl</sup>CD4-Cre mice were used<sup>41,43</sup>. Cre-negative littermates were used in all experiments. Transgenes encoding the OT-1 TCR (003831) or P14 TCR (004694), as well as mice lacking MyD88 (009088), IFNAR1 (032045), Tbx21 (004648) and the recombinase component RAG-1 (002216), are all available from Jackson Laboratories. Mice were bred and housed in specific pathogen-free conditions at the Animal Centers of the Academic Medical Center (Amsterdam, the Netherlands), Mount Sinai School of Medicine and Yale University School of Medicine. Mice (both male and female) were between 8 and 16 weeks of age at the start of the experiment. During infection experiments, wild-type and Notch1-2 KO mice were housed together to avoid ‘cage bias’. No intentional method for randomization was used. No formal method for blinding was used, except for determination of viral loads and hemagglutination assay, for which the operator did not know mouse genotype. Chimeras were generated by intravenous injection of 5 × 10<sup>6</sup> to 10 × 10<sup>6</sup> donor bone marrow cells (wild-type and Notch1-2 KO cells at a ratio of 1:1) into lethally irradiated RAG-1-deficient mice. Wild-type and Notch1-2 KO cells of donor origin were identified with the congenic markers CD45.1 and CD45.2. The chimeras were used at 12 weeks after engraftment. All mice were used in accordance of institutional and national animal experimentation guidelines. All procedures were approved by the local Animal Ethics Committees.

Media, reagents and monoclonal antibodies. The culture medium (‘IMDMc’) was Iscove’s modified Dulbecco’s medium (Lonza) supplemented with 10% heat-inactivated FCS (Lonza), 200 U/ml penicillin, 20 µg/ml streptomycin ( Gibco), GlutaMAX ( Gibco) and 50 µM β-mercaptoethanol (Invitrogen). All directly conjugated monoclonal antibodies used for flow cytometry were from eBioscience, unless stated otherwise: anti-CD3 (145-2C11), anti-CD4 (GK1.5), anti-CD8α (Ly-2; 53-6.7), anti-CD8β (Ly-3; eBio341), anti-CD25 (anti-IL-2Rα; 7D4), anti-CD28 (37.51; anti-CD41 (IM7), anti-CD45.1 (A20; BD Biosciences), anti-CD45.2 (104), anti-CD62L (MEL-14), anti-CD69 (H1.2F3), anti-CD127 (anti-IL7Rα; A7R34), anti-DLL1 (HMD1-5), anti-DLL4 (HMD4-1), anti-granzyme B (GB-11; Sanquin PeliCluster), anti-IL-2 (JS56- SH4), anti-IFN-γ (XMG1.2), anti-Jagged1 (HMJ1-29), anti-Jagged2 (HJM2-1), anti-KLRG-1 (2F1), anti-Notch1 (HMN1-12; Biolegend), anti-Notch2 (HMN2-35; Biolegend), antibody to tumor-necrosis factor (MP6-XT22), antibody to Akt phosphorylated at Thr308 (14098), antibody to mTOR phosphorylated at Ser2448 (5336), antibody to FcXo1 and FcXo3 phosphorylated at Thr24 and Thr25 (25995) and isotype-matched control antibody (39005; Cell Signaling Technology).

Infection with influenza virus. Mice were infected intranasally with 100–200 TCD<sub>50</sub> of the H3N2 influenza A virus strain HKx31 (ref. 27), influenza virus strain A/WSN/33, influenza virus strain A/WSN/33–OV<sub>A</sub> (‘ova’influenza)<sup>14</sup>, influenza virus strain A/PR/8/83 (H1N1) or the recombinant influenza virus strain A/PR/8/34 expressing epitope of amino acids 33–41 of the lymphocytic choriomeningitis virus glycoprotein<sup>55</sup>. Stocks and viral titers were obtained by infection of MDCK (Mardin-Darby canine kidney) cells or LLC-MK2 (rhesus monkey kidney) cells as described<sup>56</sup>.

At various time intervals, blood samples were drawn from the tail vein or mice were killed and organs were collected for quantification of influenza virus–specific CD8<sup>+</sup> T cells. Influenza virus–specific CD8<sup>+</sup> T cells were quantified with anti-CD8<sup>+</sup> (53-6.7; eBioscience) and phycoerythrin– or allophycocyanin-conjugated tautomers of H-2D<sup>B</sup> containing influenza A virus–derived NP (amino acids 366–374 (ASNNENMETM)) in the presence of 10 µg/ml brefeldin A (Sigma) to prevent cytokine release. Cells were stained for 30 min at 4 °C with the relevant fluorochrome-conjugated monoclonal antibodies (identified above) in PBS containing 0.5% BSA and 0.02% NaN<sub>3</sub>. For intracellular staining, cells were fixed and permeabilized with Cytofix/Cytoperm (BD Biosciences). For phosphorylation–specific flow cytometry, cells were fixed and permeabilized with Phosflow lysis and Phosflow PermWash I reagents according to the manufacturer’s recommendations (BD Biosciences) and were subsequently stained for 1 h at 4 °C with phosphorylation-specific antibodies (identified above). Where required, cells were washed and then were additionally incubated for 40 min with secondary antibody (Alexa Fluor 488–conjugated goat antibody to rabbit immunoglobulin G (heavy and light chains) (A11008 (lot 65E1-1); Molecular Probes)). Data acquisition and analysis was done on a FACScanto (Becton Dickinson) and with FlowJo software.

For the isolation of H-2D<sup>B</sup>–NP tetramer–positive CD8<sup>+</sup> T cells from influenza virus–infected mice, single-cell suspensions of spleens were stained with influenza virus–specific tautomers and antibodies to the relevant markers (identified above). Cells were sorted with a FACSAria cell sorter (BD Biosciences).

For the discrimination of circulating T cells from T cells in spleen white pulp, intravascular CD8<sup>+</sup> T cells were stained by intravenous injection of 1 µg phycoerythrin–anti-CD8β (eBio341) 8 min before mice were killed. Organs were processed as described above, and CD8<sup>+</sup> T cells were stained in vitro with a different, noncompeting anti-CD8α (53-6.7) along with antibodies to other surface markers (identified above).

Retroviral transduction and adoptive transfer of CD8<sup>+</sup> T cells. Virus was produced in PlatE retroviral packaging cells as described<sup>57</sup>. Total splenocytes from CD45.2<sup>+</sup> wild-type or Notch1-2 KO OT-I mice were incubated with 1 nM OVA peptide (amino acids 257–264), and the next day cells were ‘spin-infected’
(700g for 90 min at 37 °C) with viral supernatant (with 8 µg/ml polybrene), followed by incubation for 5 h at 37 °C. The medium was replaced, and the next day live T cells were isolated by density centrifugation (Lymphoprep; Axis-shield PoC), and 7.5 × 10⁶ to 5 × 10⁷ cells were transferred into CD45.1 mice given ‘timed’ infection with OVA-influenza. Donor OT-I T cells were detected 5–10 d after transfer as CD45.2 CD8+ and Thy-1.1+ or GFP+ (triple-positive) cells. Retroviral vectors MSCV-Thy1.1, NICD1-MSCV-Thy1.1, pMIY and myrAkt-pMIY were described previously.

**In vivo cytotoxicity assay.** Target spleen cells from C57BL/6 mice were pulsed for 35 min with 1 µg/ml influenza virus–derived NP peptide (peptide amino acids 366–374) and were subsequently labeled for 15 min at 37 °C with 0.2 µM CFSE (carboxyfluorescein diacetate succinimidyl ester; Invitrogen) (CFSE55, specific target cells) or were not pulsed with peptide and were labeled with 2 µM CFSE (CFSE65; non-specific target cells). The two target populations were mixed in equal numbers, and 5 × 10⁶ cells were transferred intravenously into mice that had been infected with influenza A virus strain HKx31 10 d before or into untreated control mice. Mice were killed 4 h later and the ratio of peptide-loaded target cells to ‘empty’ target cells was quantified by flow cytometry.

**Gene-expression profiling.** CD8+ T cells that bound H-2D b–NP (NP peptide amino acids 366–374) were isolated by flow cytometry from spleens of influenza virus–infected mice. Total RNA was extracted with TRIzol reagent according to the manufacturer’s protocol (Invitrogen). For deep-sequencing analysis, total RNA was further purified by nucleosipin RNAII columns (Macherey-Nagel) and RNA was amplified with the Superscript RNA amplification system (Invitrogen) and labeled with the ULS system (Kreatech) with indocarbocyanine or indodicarbocyanine dye (Amersham). Sequences were obtained by pooling of ten samples in one lane on a HiSeq2000 machine. Between 17 × 10⁶ and 27 × 10⁶ ‘reads’ were obtained per sample.

‘Read’ mapping (with TopHat software) and determination of differently expressed genes (with the DESeq software package of the R project for statistical computing) was done as described54. The ‘reads’ were mapped against the mouse reference genome (build mm9 of the National Center for Biotechnology Information assembly of the mouse genome) with TopHat software (version 1.4.0), which allows spanning of exon-exon junctions. The TopHat software was supplied with a known set of gene models (National Center for Biotechnology Information build 37, version 64). To obtain ‘per sample gene counts’, the script HTSeq-count was used. This tool generates ‘gene counts’ for each gene present in the GTF (gene-transfer format) file provided. Genes with no counts in any sample were removed from the data set. The R package DESeq was used for statistical analysis. Genes with different expression in TEC versus MPC samples or in wild-type versus mutant samples were identified. DESeq assumes that ‘gene counts’ can be modeled by a negative binomial distribution. For sample normalization, the ‘size factors’ were determined from the count data. The empirical dispersion was determined by the ‘pooled’ method, which uses samples from all conditions with replicates for estimation of a single pooled dispersion value. Subsequently, a parametric fit determined the dispersion-mean relationship for the expression values. By setting the DESeq argument ‘sharingMode’ to ‘maximum’, DESeq selected the dispersion estimate (empirical or fitted value) that is most conservative in calculating the p values. Finally, P values and P values corrected by the false-discovery rate were calculated.

To highlight biological processes over-represented in the set of genes with different expression, we used the Bioconductor package GOseq59, which was developed for the analysis of data from high-throughput RNA sequencing. First, we selected all genes with a false-discovery rate of <0.05 from the TEC-MPC and wild-type–mutant comparisons. Subsequently, the gene sets of the gene-ontology (GO) term ‘Biological Processes’ were used to determine over-represented processes. In addition we used the C7 gene set of the Molecular Signatures Database (a collection of annotated gene sets). Gene set C7 comprises immunologic signatures composed of gene sets that represent cell types, states and perturbations in the immune system. The signatures were generated by manual curation of published microarray studies of human and mouse immunology. This gene set was generated as part of the Human Immunology Project Consortium. An in-house R script was developed to convert the C7 gene set into a format that could be used by GoSeq.

To visualize the results for the GoSeq analysis based on the GO biological processes, we used the Cytoscape Enrichment Map plug-in60. We first constructed a gene matrix transposed file containing the GO terms and corresponding gene identifiers from the Ensembl project of genome databases. GO terms associated with more than 250 genes were removed because these represent too general terms that are difficult to interpret. In addition, very small gene sets (<15 genes) were removed because these are more likely to become significant by chance alone. Subsequently, from the results of the GoSeq analysis, we selected biological processes related to chemokines, cytokines, cytotoxicity, differentiation, adhesion, migration, apoptosis, activation and proliferation. Selected GO terms with a false-discovery rate of <0.05 were visualized as an enrichment map.

**Quantitative RT-PCR.** cDNA was made with oligo(dT) and random hexamers through the use of the First Strand cDNA synthesis kit (Fermentas). Quantitative PCR was done with SYBR Green (Bio-Rad) and a C1000 Thermal Cycler (Bio-Rad). Relative concentrations were determined by normalizing to the gene encoding β-actin (Actb) with Bio-Rad CFX Manager software. Melt curves ensured amplification of a single product. Sequences of primers used were as follows: Actb, 5′-GAAGTCCTCACCCTCCCAA-3′ and 5′-GGC ATGGACGGACCA-3′; Rp51, 5′-CGGTCTGCTTATCAACTTTCC-3′; TdT, 5′-ATTCAAGTCGAGATGGCTA-3′; and Tbx21, 5′-CAACAACCC TTGGGCAAG-3′ and 5′-TCCCCCAACGAGTTGACGT-3′.

**Statistical analysis.** A standard Student’s t-test (unpaired, two-tailed) was applied with GraphPad Prism software. For comparison of three or more groups, one-way ANOVA with Bonferroni correction was used. P values of <0.05 were considered statistically significant.