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The Notch Signaling Pathway Controls Short-Lived Effector CD8+ T Cell Differentiation but Is Dispensable for Memory Generation

Mélissa Mathieu,*†,1 Frédéric Duval,*†,1 Jean-François Daudelin,* and Nathalie Labrecque*‡,‡

Following an infection, naive CD8+ T cells expand and differentiate into two main populations of effectors: short-lived effector cells (SLECs) and memory precursor effector cells (MPECs). There is limited understanding of the molecular mechanism and cellular processes governing this cell fate. Notch is a key regulator of cell fate decision relevant in many immunological pathways. In this study, we add to the role of Notch in cell fate decision and demonstrate that the Notch signaling pathway controls the MPEC/SLEC differentiation choice following both Listeria infection and dendritic cell immunization of mice. Although fewer SLECs were generated, Notch deficiency did not alter the rate of memory CD8+ T cell generation. Moreover, we reveal that the Notch signaling pathway plays a context-dependent role for optimal cytokine production by effector CD8+ T cells. Together, our results unravel critical functions for the Notch signaling pathway during effector CD8+ T cell differentiation. The Journal of Immunology, 2015, 194: 5654–5662.

Upon Ag encounter, CD8+ T lymphocytes become activated, proliferate, and differentiate into effector T (Te) cells able to kill target cells and secrete cytokines. Following pathogen clearance, only ~5–10% of Te cells will survive and further differentiate into long-lived memory T (Tm) lymphocytes. This generation of Tm cells is essential for protection against reinfection.

The differentiation of naive CD8+ T cells into Te and Tm cells requires the proper integration of several signals provided by the APC and the environment in which the immune response occurs. At the peak of the CD8+ T cell response, different subsets of effectors can be identified using CD127 and KLRG1 expression (1, 2). The short-lived effector cells (SLECs; CD127highKLRG1high) are destined to die during the contraction phase of the CD8+ T cell response, whereas the memory precursor effector cells (MPECs; CD127highKLRG1low) will further differentiate into long-lived Tm cells. The fate of effectors is mainly dictated by the inflammatory cytokine signals that they perceived during the immune response. High amount of IL-12 favors their differentiation into SLECs by increasing the expression level of the transcription factor T-bet (1).

Furthermore, SLECs differentiate from effectors that have maintained the expression of the IL-2Rα chain (CD25) (3), which allows for the induction of the expression of the transcriptional repressor Blimp-1 (3, 4), a key molecule controlling SLEC differentiation (5, 6). Although the cytokine milieu plays a very important role during the differentiation of effectors, it is still possible that other receptor–ligand interaction provided by APCs may also play a crucial role during the differentiation of naive CD8+ T cells into Te and Tm cells.

The Notch signaling pathway is an evolutionary conserved pathway that is well known for its role during various differentiation events. In vertebrates, this pathway is composed of four receptors (Notch1–4) and five ligands (Jagged-1, -2, and Delta-like-1, -3, -4). The interaction of Notch with its ligands leads to its proteolytic cleavage releasing the Notch intracellular domain (NICD) (7). The NICD translocates to the nucleus, where it associates with RBP-J and allows assembly of a transcriptional activator complex (NICD, MAML1–3, and MED-8 mediator transcription activation complex) that induces transcription of target genes (7). HES family members are common targets of Notch signaling in many tissues, whereas other target genes are tissue specific. Furthermore, a broad number of genes can be regulated by Notch, as shown using genome-wide methodologies (8–11).

In the immune system, the Notch signaling pathway has been shown to control the development of multiple cell types, including T cells, marginal zone B cells, innate lymphoid cells, and dendritic cells (DCs) (12). Apart from playing crucial roles for the development of immune cells, the Notch signaling pathway also regulates T cell response. Indeed, T cells express Notch receptors, whereas APCs can express the ligands (12). In CD4+ T cells, Notch signaling regulates the differentiation and response of various Th cell subsets (12). Indeed, the induction of different Notch ligands by danger signals on DCs controls the Th1 and Th2 differentiation choice (13). This initial observation has now been expanded to the differentiation of several Th cell subsets (12). Specifically, the Notch signaling pathway was shown to directly regulate the transcription of Thx21 (encoding for T-bet) and to increase IFN-γ.

Abbreviations used in this article: DC, dendritic cell; EEC, early effector cell; GVHD, graft-versus-host disease; Lm-OVA, Listeria monocytogenes expressing ovalbumin; MPEC, memory precursor effector cell; NICD, Notch intracellular domain; SLEC, short-lived effector cell; Te, effector T; Tm, memory T.

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production in Th1 cells (14, 15). The Notch pathway is also a direct regulator of Gata3 transcription during Th2 differentiation (16, 17).

More recently, the Notch signaling pathway was also shown to regulate Th9, Th17, and T regulatory differentiation (18–20). The mechanism by which the Notch signaling pathway orchestrates the various Th differentiation program was recently identified by the group of Pear (21). It appears that Notch does not dictate the Th differentiation choice, but rather it potentiates the cytokine signal received by CD4+ T cells during differentiation. Thus, by itself, the Notch signaling pathway cannot initiate the differentiation of CD4+ T cells toward one subset or another, but it acts as an amplifier of Th cell differentiation.

Much less is known about the involvement of the Notch signaling pathway during CD8+ T cell response, and most of the experiments addressing its function have been performed in vitro. For instance, Notch signaling was shown to directly regulate, via binding of the NICD to their promoter, the transcription of many effector molecules, such as perforin, granzyme B, and IFN-γ, in mouse CD8+ T cells (22–24). Similarly, the Notch signaling pathway controls the production of IFN-γ by human CD8+ T cells (25). Furthermore, the transcription factor Eomes was defined as a transcriptional target of the Notch signaling pathway in in vitro activated CD8+ T cells (23).

In vivo, Notch2 expression by CD8+ T cells is essential for the efficient killing of target cells (24), for the control of Trypanosoma cruzi infection (24), and for potent antitumor activity (26). Moreover, inhibition of the Notch signaling pathway using either genetic deletion of Rhbpj or expression of a dominant-negative form of MAML protected mice from graft-versus-host disease (GVHD) (27, 28). The protective effect of Notch inhibition was not a consequence of decreased alloreactive CD8+ T cell expansion, but was most likely the result of defective IFN-γ production (27, 28).

However, in this system, Eomes expression was increased, whereas T-bet expression was preserved (27, 28). Very interestingly, inhibition of the Notch signaling pathway during an active immune response. In this study, we reported that Notch signaling influences the formation of SLECs following Listeria infection or DC vaccination. These two experimental systems allow us to evaluate the influence of an inflammatory response on the role of the Notch signaling pathway during an active immune response. In this study, we report that Notch signaling influences the formation of SLECs following Listeria infection and DC vaccination, whereas it is dispensable for memory CD8+ T cell differentiation. The reduced generation of SLECs in absence of Notch signaling correlates with defective CD25 expression by effectors. The Notch signaling pathway was also required for maximal IFN-γ production by effectors after Listeria infection and DC vaccination. However, Notch signaling is selectively required for the production of IL-2 and TNF-α following DC vaccination, revealing a context-dependent role for Notch during CD8+ T cell response. Furthermore, IFN-γ production was more severely decreased by Notch deficiency following DC vaccination than infection. This more severe reduction in IFN-γ production leads to impaired control of Listeria growth following rechallenge. Our results highlight a crucial role for the Notch signaling pathway for the differentiation of functional CD8+ T cells.

**Materials and Methods**

**Mice**

B6.SJL (C57BL/6 × B6.SJL-F1), and OT-1 Rag-1−/− deficient (29) mice were bred at the Maisonneuve-Rosemont Hospital Research Center facility. Notch1fl/fl (B6.129.S-Notch1tm2J/J) and Notch2fl/fl (B6.129S-Notch2tm2Grid/J) (31), and E8I-cre (C57BL/6-Tg(Cd8a-cre)Itan/J) (24) mice were purchased from The Jackson Laboratory. Notch1fl/fl were backcrossed to the C57BL/6 background and bred to Notch1fl/fl and E8I-Cre to obtain E8I-Cre+/− Notch1fl/fl Notch1fl/fl mice (Notch1/2-deficient mice, Δα), and their Notch1fl/fl Notch2fl/fl littermates (Notch1/2-sufficient mice, fl/fl). E8I-Cre+/− Notch1fl/fl Notch2fl/fl mice were crossed to OT-1 Rag-1−/− mice to generate Notch-sufficient and -deficient OT-1 Rag-1−/− mice. Mice were housed at the Maisonneuve-Rosemont Hospital Research Center Facility, in a pathogen-free environment, and treated in accordance with the Canadian Council on Animal Care guidelines.

**L. monocytogenes infection, DC immunization, and analysis of T cell response**

*L. monocytogenes* expressing ovalbumin (Lm-OVA) was grown, as previously described (32). A sublethal dose of 2 × 108 CFUs was injected i.v. for analysis of primary T cell response, whereas a lethal dose of 2 × 109 CFUs was used for challenge experiments. Bone marrow–derived DCs were generated, as previously described (33), matured with LPS (1 µg/ml), and loaded overnight with the OVA325-358 peptide (SINFEKL, 2 µg/ml, Mid-west Biotech). A total of 1.25 × 107 DCs was injected i.v. for vaccination. In some experiments, 15,000 U rIL-2 (Novartis) was injected i.p. twice daily from the day of vaccination until day 7, as described previously (4). The primary endogenous CD8+ T cell response was analyzed in the spleen with either Kb-OVA tetramer staining or intracellular cytokine staining following a short in vitro stimulation with the OVA peptide. In some experiments, 105 Notch-sufficient or -deficient OT-1 CD8+ T cells were adoptively transferred in B6.SJL recipient mice before *Listeria* infection or DC vaccination.

**Hematopoietic chimeras**

(C57BL/6 × B6.SJL-F1) (CD45.1+CD45.2+) mice were lethally irradiated (12 Gy) and reconstituted with a 50:50 mixture of 5 × 106 bone marrow cells from B6.SJL (CD45.1+) mice and E8I-Cre+/− Notch1fl/fl Notch2fl/fl mice (Notch1/2-deficient mice, Δα, CD45.2+). Chimeric mice were used at 8 wk postreconstitution.

**Abs, flow cytometry, and cell sorting**

Anti-CD44 (IMT), anti-CD8 (53-6-7), anti-CD25 (PC61), anti-Ly6C (HK1.4), anti-IL-2 (JE55-5H4), and anti-TNF-α MP6-XT22 Abs were from BioLegend; anti–IFN-γ (XM11.2) Abs was from Life Technologies; anti-CD127 (A7R34), anti-KLRG1 (2F1), anti-T-bet (4B10), and anti-Eomes (Dan1img) Abs were from eBioscience. Anti-Ki67 (B56) was purchased from BD Biosciences. Cell surface staining, intracellular staining, tetramer staining, and OVA peptide loading on Kβ MHC were performed, as previously described (33–35). Cell death was measured using 7-amino-actinomycin D (BD Biosciences) and annexin V (BioLegend) staining, as previously described (4).

**Naïve CD8+ T cells (CD8+CD44+), total OVA-specific CD8+ effector cells (CD8+CD44hi/Tet-OVA), OVA-specific CD8+ SLECs (CD8+CD44hi/Tet-OVA/CD127 KLRG1+), and OVA-specific CD8+ Tm cells (CD8+CD44hi/Tet-OVA+)** were sorted with a BD FACSAria III.

**Quantitative real-time PCR**

Quantification of Prdm1, Eomes, and Th2f1 mRNAs from sorted OVA-specific CD8+ T cells was performed, as previously described (4, 35). Briefly, total RNA was isolated using Trizol (Life Technologies) and reverse transcribed into cDNA using SuperScript II with oligo(dT) primers (Life Technologies). Real-time PCR was performed in triplicates using Power SYBR Green (Life Technologies) on an Applied Biosystems 7500 Real-Time PCR System. Sequences of primers used were as follows: Prdm1, 5'-ACA-CACAGGGAGAAGCCCATAGA-3' and 5'-TGCGAGTGGTTGTCC-3'; Eomes, 5'-GGTACCCCAAGGATCCTCAAC-3' and 5'-CAAG GTTCGTGTGATTT-3'; Th2f1, 5'-AGGGGACACTGATATCACAGA-3' and 5'-AGGGGGTCCTCCAAACATG-3'.

**PCR for measurement of the deletion of the floxed Notch1 and Notch2 genes**

Genomic DNA was extracted from sorted naïve CD8+ T cells, OVA-specific CD8+ effectors, OVA-specific CD8+ SLECs, and OVA-specific Tm cells. PCR were performed as described by Zheng et al. (36).
Results

Notch signaling controls SLEC differentiation

It was shown by us and others that CD8^+ T cells upregulate Notch1 and Notch2 expression following TCR stimulation (22, 24, 37, 38). However, Notch3 and Notch4 mRNA were not increased (24, 39) (data not shown); thus, the deletion of Notch1 and Notch2 genes should completely abrogate Notch signaling in CD8^+ T cells. To elucidate the role of the Notch signaling pathway during in vivo CD8^+ T cell response, Notch1 and Notch2 expression was specifically abrogated in mature CD8^+ T cells using Notch1^fl/fl Notch2^fl/fl-E8I-Cre^+/− mice (referred as Notch1/2-deficient mice or Δ/Δ through the manuscript). These mice express the Cre recombinase under the CD8α enhancer, allowing for Notch1 and Notch2 deletion only in mature CD8^+ T cells (24). The efficient and specific deletion of the Notch1 and Notch2 genes in mature CD8^+ T cells was assessed by flow cytometry. Indeed, Notch1 and Notch2 cell surface expression could not be detected on CD8^+ T cells from Notch-deficient mice following TCR stimulation (data not shown). Moreover, mice with deletion of the Notch1 and Notch2 genes in CD8^+ T cells did not show any difference in peripheral CD8^+ T cell populations (naive, effector, and memory) nor in the expression of various cell surface markers (TCR, CD3, CD44, CD62L, CD127, CD25, CD5, and TCRVβ usage) in the steady state when compared with their wild-type littermates (data not shown).

To delineate the role of the Notch signaling pathway in CD8^+ T cell response, Notch1/2-sufficient and -deficient mice were infected with a sublethal dose of the intracellular bacteria Lm-OVA, allowing us to follow the response of OVA-specific CD8^+ T cells in an inflammatory environment. The effector response was assessed in the spleen 7 d postinfection using Kb-OVA tetramer staining. As shown in Fig. 1A and 1B, Notch deficiency in CD8^+ T cells promoted a better expansion of OVA-specific CD8^+ T cells compared with their wild-type counterpart. The phenotypic characterization of the effectors generated revealed a crucial role for Notch signaling for their proper differentiation into SLECs (Fig. 1C, 1D). OVA-specific CD8^+ T cells lacking expression of Notch1/2 show a 2-fold reduction in the proportion of SLECs. Although proportionally less SLECs were generated by NotchΔ/Δ CD8^+ T cells, this did not lead to a direct reciprocal increase in MPECs (Fig. 1C, 1D). However, an increase in early effector cells (EECs; KLRG1low and CD127low), the precursors of both SLECs and MPECs (40), is also observed in NotchΔ/Δ mice (Fig. 1C, 1D). These results suggest that Notch deficiency is most important for the EEC to SLEC transition.

In our model, Cre-mediated inactivation of the Notch1 and Notch2 genes should only occur in mature peripheral CD8^+ T cells in favor of a cell-autonomous role for Notch signaling in CD8^+ T cells. However, it is possible that the Cre transgene is expressed by other cells, which may then influence the CD8^+ T cell response. To exclude that possibility, we generated mixed bone marrow chimeras by grafting in the same recipient a 1:1 ratio of wild-type (CD45.1^+ and NotchΔ/Δ (CD45.2^−) bone marrow cells. Following infection with Lm-OVA, SLEC generation was selectively impaired in OVA-specific Te cells originating from NotchΔ/Δ hematopoietic cells (Supplemental Fig. 1). These results confirm that Notch signaling acts in a cell-autonomous manner on CD8^+ Te cell differentiation.

To confirm the role of the Notch signaling pathway during CD8^+ T cell response, we immunized mice with LPS-matured DCs pulsed with the OVA peptide (DC-OVA). In contrast to Lm-Ova, the CD8^+ T cell response is primed in a low inflammation setting, and, as a result, fewer SLECs are generated, whereas memory T cell differentiation is accelerated (1, 4, 33, 41, 42). Although CD8^+ T cell expansion was similar in absence or presence of Notch signaling (Fig. 2A, 2B), SLEC generation was almost abolished following DC vaccination in NotchΔ/Δ mice (Fig. 2C, 2D). Therefore, the Notch signaling pathway plays a very important role during CD8^+ Te cell terminal differentiation irrespective of the inflammation level. The defect in SLEC generation in NotchΔ/Δ mice could be the consequence of an increased death rate of SLECs, a reduced proliferation of SLECs, or an inability of EECs to differentiate into SLECs. To understand better how Notch signaling affects SLEC differentiation, we evaluated the death rate of the different effector populations using annexin V staining. We did not observe any significant difference in the percentage of annexin V^+ cells at day 7 postinfection or postvaccination (Supplemental Fig. 2A). We also did not observe any difference in the rate of effector proliferation as measured using Ki67 staining (Supplemental Fig. 2B). Altogether, these results suggest that the decreased generation of SLECs in absence of Notch1/2 is not the consequence of an increased death rate or reduced proliferation of SLECs, but rather from a defective differentiation of EECs into SLECs.

Although SLEC generation was severely affected following both Lm infection and DC vaccination, few cells with a SLEC phenotype were still present in NotchΔ/Δ mice. To address whether these cells were derived from naive T cells that did not properly delete the Notch1 or Notch2 genes, we evaluated whether the residual SLEC population was enriched for cells that have not deleted the floxed
As there is evidence that the Notch signaling pathway controls CD25 expression in immature thymocytes (44) and activated CD8+ T cells (45), we evaluated whether Notch deficiency affects CD25 expression by CD8+ T cells. The expression level of CD25 is maximal a few days before and becomes undetectable at the peak of the CD8+ T cell response (3). Therefore, to analyze CD25 expression at day 4–5 postimmunization, we adoptively transferred 10^7 OVA-specific OT-I T cells lacking (OT-I/Notch^fl/fl) or not (OT-I/Notch^h/h) Notch1 and Notch2 expression in naive B6.SJL congenic recipients. These mice were then infected with Lm-OVA or vaccinated with DC-OVA. As shown in Fig. 3B, OT-I/Notch^h/h CD8+ effectors failed to properly express CD25 when compared with OT-I/Notch^fl/fl CD8+ T cells. Furthermore, both the percentage of cells expressing CD25 and the amount expressed by the positive cells are severely reduced in absence of Notch signaling (Fig. 3B). These results suggest that, in our two distinct immunization protocols, Notch signaling controls CD25 expression to promote SLEC generation.

As Notch^h/h Ag-specific CD8+ T cells probably received less IL-2 signals, we tested whether twice daily administration of rIL-2 during the expansion phase of the response to DC-OVA vaccination could restore SLEC generation in absence of Notch signaling. As shown in Supplemental Fig. 4, in vivo administration of rIL-2 was not sufficient to promote the generation of SLEC in Notch^h/h mice, whereas it enhances SLEC generation in Notch^fl/fl mice.

One of the mechanisms proposed to explain how the maintenance of CD25 expression on effectors promotes SLEC differentiation is via the upregulation of Blimp-1 (encoded by the gene Prdm1) expression (3, 4, 43). Therefore, we evaluated whether Notch^h/h effectors express lower level of Prdm1 using quantitative RT-PCR. As shown in Fig. 3C, Prdm1 expression was not statistically different between Notch^fl/fl and Notch^h/h effectors. These results suggest that Notch influences SLEC generation via another mechanism than regulation of the expression of the key transcription factors (T-bet and Blimp-1) known to control this differentiation process.

Notch controls optimal IFN-γ and IL-2 production by CD8+ Te cells

Our results clearly indicate that the Notch signaling pathway affects effector differentiation; we then tested whether the functionality of CD8+ Te cells was also impaired in absence of Notch1/2. Upon Listeria infection, we found a similar frequency of CD8+ Te cells producing IFN-γ in Notch^h/h and Notch^fl/fl mice following a short in vitro stimulation with the OVA peptide (Fig. 4A, 4C). However, the amount of IFN-γ produced by cells was reduced in Notch1/2-deficient effectors when compared with Notch-sufficient effectors (Fig. 4A, 4C). Notably, a more drastic effect on IFN-γ production was observed following DC vaccination. Very few Notch-deficient CD8+ Te cells produced IFN-γ, and the amount produced by each cell was also severely decreased (Fig. 4B, 4D). Furthermore, the proportion of effectors producing IL-2 and TNF-α was only affected by Notch deficiency following DC vaccination (Fig. 4A–D). These results suggest a differential role for the Notch signaling pathway in the regulation of cytokine production by CD8+ effectors depending on the context of the CD8+ T cell response.

We next undertook to explain the variation in IFN-γ production. The two transcription factors, T-bet and Eomes, have been shown to be important to regulate IFN-γ expression in CD8+ T cells (46, 47). Indeed, in absence of both transcription factors, effector CD8+ T cells lose their ability to produce IFN-γ, whereas single deficiency did not alter their ability to produce IFN-γ (46, 47). Interestingly, both Tbet21 and Eomes are potential Notch target genes (14, 15, 23, 24). However, a decrease in the expression level of T-bet cannot explain the more severe reduction of IFN-γ production...
by Notch^ΔΔ effectors generated following DC vaccination (Fig. 3A). Therefore, we evaluated whether Eomes expression was affected by Notch deficiency. As shown in Fig. 5, Eomes expression was lower in effectors that were deficient for the expression of Notch1/2. Eomes expression was similarly reduced following both DC vaccination and *Listeria* infection (Fig. 5), suggesting that this reduction is not responsible for the more defective production of IFN-γ by Notch^ΔΔ effectors following DC vaccination.

To evaluate whether the reduction in IFN-γ production by Notch^ΔΔ CD8^+ effectors generated following DC vaccination had a functional impact, we challenged the mice with a lethal dose of *Lm*-OVA. In this system, efficient elimination of the bacteria will depend on the ability of OVA-specific CD8^+ Te cells to produce IFN-γ (48, 49). As expected, OVA-specific CD8^+ T cells lacking or not Notch1/2 expression generated following *Listeria* infection were very efficient at controlling a lethal challenge with Lm-OVA (Fig. 4E). However, Notch^ΔΔ CD8^+ effectors generated with DC vaccination were inefficient at controlling bacterial growth (Fig. 4F), whereas their wild-type counterparts were very effective.

*Notch signaling is not required for the generation of CD8^+ Tm cells*

In the absence of Notch signaling, OVA-specific CD8^+ Te cells successfully differentiate into MPECs. However, these effectors express less Eomes (Fig. 5), which might affect their differentiation into long-lived CD8^+ Tm cells. We then evaluated whether these MPECs efficiently give rise to Tm cells. At day 45 postinfection with *Lm*-OVA or postvaccination with DCs, OVA-specific CD8^+ Tm cells were generated regardless of the expression of Notch1 and Notch2 receptors (Fig. 6A, 6C). However, Notch1/2 deficiency leads to an increased yield (% Tm cells/% of Te cells) of OVA-specific CD8^+ Tm cells following DC vaccination, but not following *Listeria* infection (Fig. 6A, 6C). Furthermore, the CD8^+ Tm cells generated in Notch^ΔΔ mice are not derived from the survival of cells that did not delete the *Notch1* and *Notch2* genes (Supplemental Fig. 3). These results indicate that the Notch signaling pathway is not required for CD8^+ Tm cell generation, and that in some circumstances it may impede CD8^+ Tm cell differentiation.

Although OVA-specific CD8^+ Tm cells were generated in Notch^ΔΔ mice, it was important to evaluate whether Notch deficiency had altered their functionality. To address that, we evaluated their capacity to produce cytokines following a short in vitro restimulation with the OVA peptide. No difference in IFN-γ production was observed in Notch^ΔΔ and Notch^fl/fl OVA-specific CD8^+ Tm cells that were generated following Lm-OVA infection (Fig. 6B). However, a lower frequency of OVA-specific CD8^+ Tm cells produces IFN-γ in Notch^ΔΔ mice compared with wild-type mice following DC immunization (Fig. 6D). Moreover, the amount of IFN-γ produced on a per cell basis upon DC vaccination is significantly lower in OVA-specific CD8^+ Tm cells lacking expression of the Notch1 and Notch2 receptors (Fig. 6D). Thus, in absence of Notch1/2, the ability to produce IFN-γ is significantly impaired following DC vaccination.

**Discussion**

Our results demonstrate that the Notch signaling pathway plays specific and crucial roles during the response of CD8^+ T cells to (day 7 or 8 postinfection/vaccination) was evaluated by quantitative RT-PCR. The results from two independent experiments are shown (one at day 7 and one at day 8). *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.
infection and vaccination. Furthermore, our results uncover a context-dependent role for the Notch signaling during these immune responses.

In this study, we report that the Notch signaling pathway is very important for the proper generation of SLECs during the response of CD8+ T cells to both infection and vaccination. One mechanism by which Notch signaling could have impacted SLEC differentiation is via its ability to regulate T-bet expression. Indeed, Tbx21 has been reported to be a direct transcriptional target of Notch in CD4+ T cells (14, 15). In CD8+ T cells, inhibition of Notch signaling following in vitro stimulation of CD8+ T cells leads to reduced expression of T-bet, but, unfortunately, the direct binding of the NICD to the Tbx21 gene was not evaluated in this study (23). In our in vivo experimental models, we did not observe any significant difference in T-bet protein expression level between Notch-deficient and wild-type effectors. Our results are in agreement with other studies in which no effect of the Notch signaling pathway on T-bet expression in vivo experimental models, we did not observe any significant difference in T-bet protein expression level between Notch-deficient and wild-type effectors. Our results are in agreement with other studies in which no effect of the Notch signaling pathway on T-bet expression

![Diagram of CD8+ Te cells generated following DC vaccination](http://www.jimmunol.org/)

**FIGURE 4.** Ag-specific Notch-deficient CD8+ Te cells generated following DC vaccination have a more severe impairment in functionality than the one obtained following Listeria infection. Notch-sufficient (fl/fl) and -deficient mice (Δ/Δ) were infected with Lm-OVA (A, C, and E) or immunized with DCs (B, D, and F). Cytokine production was assessed on day 7 postinfection or postvaccination following a short in vitro restimulation with the OVA peptide. (A and B) Representative dot plots demonstrating the frequency of IFN-γ or IL-2–producing CD8+ Te cells are shown. The percentage of CD8+ Te cells producing IFN-γ or IL-2 and their mean fluorescence intensity (MFI) are indicated on the respective dot plot. (C and D) Graphs represent the proportion of cells producing the cytokine (% cytokine+/% Tet-OVA+, left panel) and MFI (right panel) of IFN-γ (top panels) or IL-2 (middle panels)– or TNF-α (bottom panels)–producing effectors. (E and F) Bacterial burden in spleen and liver at day 3 postchallenge with a lethal dose of Lm-OVA of mice that were infected 7 d before with Lm-OVA (E) or vaccinated with DC-OVA (F). Data are representative of two (E) or three independent experiments with ≥2 mice per group. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

**FIGURE 5.** Notch-deficient CD8+ Te cells express decreased level of Eomes. Notch-sufficient (fl/fl) and -deficient (Δ/Δ) mice were infected with Lm-OVA (top panels) or immunized with DCs (bottom panels), as in Figs. 1 and 2. The expression of Eomes was assessed by flow cytometry 7 d postinfection or postimmunization. The representative overlay histogram shows Eomes expression by OVA-specific CD8+ Te cells (TetOVA+CD44hi), whereas the lower number is the MFI for the isotype control staining. The graphs show compilation of MFI of Eomes expression (middle) or the relative mRNA expression level of Eomes by OVA-specific Te cells. Flow cytometry data are representative of three (Lm infection) or four (DC immunization) independent experiments with ≥2 mice per group, whereas the quantitative RT-PCR data are from two independent experiments with three mice per group. *p ≤ 0.05, **p ≤ 0.01.
CD25 expression and as such receive less IL-2 signaling. This observation is in agreement with the recent publication of Backer et al. (50), who has also observed reduced expression of CD25 by Notch-deficient Ag-specific CD8\(^+\) Te cells. Therefore, Notch signaling influences SLEC generation by regulating CD25 expression. Further studies are required to determine whether Cd25 is a direct Notch target gene in CD8\(^+\) T cells, a likely possibility because it has been reported that the NICD can be recruited to the Cd25 promoter in double-negative thymocytes (44) and to the Cd25 enhancer in leukemic T cells (10).

The maintenance of CD25 expression on a subset of effector CD8\(^+\) T cells is required for the generation of SLECs, and it was proposed, by us and others, that this was necessary to allow for the proper upregulation of Blimp-1 (3, 4), a key transcriptional repressor controlling SLEC differentiation (5, 6). Unexpectedly, in our models, the defective expression of CD25 by Notch\(^{ΔΔ}\) effectors did not prevent the upregulation of Prdm1 transcription. Furthermore, rIL-2 supplementation during the expansion phase of the CD8\(^+\) T cell response to DC-OVA vaccination did not rescue SLEC generation, suggesting that Notch affects SLEC differentiation independently of Blimp-1. A possible mechanism by which Notch regulation of CD25 expression could affect SLEC differentiation is via modulation of T cell metabolism, as IL-2 signaling is known to regulate the activity of the Akt/mTOR pathway (52–55). This possibility is in agreement with the very recent observation of Backer et al. (50), who have shown reduced activation of the Akt-mTOR pathway in Notch-deficient CD8\(^+\) Te cells.

It is intriguing that we observe a severe SLEC differentiation defect even if Notch\(^{ΔΔ}\) effectors were able to upregulate the expression of T-bet and Blimp-1. This suggests that either Notch signaling acts in concert with T-bet and/or Blimp-1 during SLEC differentiation or that it regulates another pathway (for example metabolism) controlling SLEC differentiation. Further studies are required to better define how the Notch signaling pathway influences SLEC differentiation.

Unlike what would have been expected from in vitro studies (22, 23, 37), ablation of Notch1 and Notch2 receptor expression did not decrease the in vivo proliferation of CD8\(^+\) T cells. This difference might be the result of unspecific effect of the Notch inhibitors that were used for the in vitro studies. Furthermore, CD8\(^+\) T cell in vivo proliferation was not affected by Notch deficiency during the alloresponse that occurs during GVHD (27, 28). In our models, following in vivo response to an infection with Listeria, but not following DC vaccination, CD8\(^+\) T cell expansion was enhanced in absence of Notch1/2 expression by CD8\(^+\) T cells. This suggests that Notch activation during an immune response to a pathogen that induced high level of inflammation is deleterious to T cell expansion. Further studies are required to understand the distinct effect of the Notch signaling pathway during different in vivo CD8\(^+\) T cell responses.

Our results also highlight that the Notch signaling pathway controls the ability of CD8\(^+\) Te cells to produce cytokines in a context-dependent manner. Its seems that when the CD8\(^+\) T cell response is primed in the context of a pathogen (Listeria infection), the absence of Notch signaling on cytokine production by CD8\(^+\) T cells is less important than in a low inflammatory context (DC vaccination). Indeed, IL-2 and TNF-\(\alpha\) production by Ag-specific CD8\(^+\) Te cells is only affected following DC vaccination in absence of Notch1 and Notch2. This illustrates a context-dependent role for the Notch signaling pathway and is reminiscent of what has been observed during GVHD (27, 28), a nonpathogen-driven immune response. When inflammation is low, it is possible that there is less induction of the key transcription factors controlling the production of IL-2 and TNF-\(\alpha\), which then renders the optimal production of these cytokines dependent on Notch signaling. Therefore, the NICD may directly bind to the promoter/enhancer of the \(\text{Il}2\) and \(\text{Tnfa}\) genes. Alternatively, Notch signaling may act in a noncanonical manner (without binding of the NICD to RBPI) to regulate the production of these cytokines. Indeed, the NICD has been reported to interact with signaling components of the TCR (56–58), and thus, it is possible that Notch deficiency affects TCR signaling, which in turn influences cytokine production. Further studies are required to decipher the mechanism by which Notch activation controls the production of IL-2 and TNF-\(\alpha\) in CD8\(^+\) Te cells.

The production of IFN-\(\gamma\) was affected following both Listeria infection and DC vaccination, although the effect was more severe after DC vaccination. This suggests that priming of CD8\(^+\) T cells during infection is able to induce some transcription of the Ifng gene in absence of the NICD, whereas binding of the NICD to the Ifng promoter is required following DC vaccination, which occurs in a low inflammatory context. It was shown that the two transcription factors, T-bet and Eomes, act together to regulate IFN-\(\gamma\) production in effector CD8\(^+\) T cells; when both are absent, CD8\(^+\) T cells lose
their ability to produce IFN-γ (47). Although we have observed that Notch-deficient CD8+ T cells express lower amount of Eomes, this decrease cannot explain the selective defect of IFN-γ that we observed following DC vaccination because it occurs following both Listeria infection and DC vaccination. However, following DC vaccination, less T-bet is induced than following infection. Thus, it is possible that it is the combined decrease of Eomes and T-bet expression observed in NotchΔΔΔΔ cells that regulates the transcription of the Ifng gene in DC vaccination. In contrast, it was shown by others that the Ifng gene is a direct target gene of the Notch signaling pathway (14, 21). Therefore, when the inflammation level is high, such as following an infection, it is possible that a different set or quantity of transcription factors is induced, and that this can compensate for the lack of NICD binding to the Ifng promoter. Alternatively, the difference in the duration of antigenic presentation that occurs following DC vaccination versus Listeria infection may affect the expression of key transcription factors controlling the transcription of the Ifng gene, making Notch signaling interesting to induce maximal production of IFN-γ by CD8+ T cells. Interestingly, the defective ability of NotchΔΔΔΔ CD8+ T cells to produce IFN-γ was maintained at the memory stage, suggesting that the absence of binding of the NICD to the Ifng gene has a permanent impact on the ability of the Ag-specific CD8+ T cells to produce this cytokine.

The severe reduction of IFN-γ and TNF-α production by NotchΔΔΔΔ CD8+ T cells generated following DC vaccination has a functional consequence because these effectors were not able to control a challenge with a lethal dose of Lm-OVA. The fact that the CD8+ T cells generated following Listeria infection are able to control the same challenge, although they also produce less IFN-γ, might be explained by their maintenance of normal TNF-α production, a cytokine that has been shown to be important for Listeria elimination (49, 59).

Our results also demonstrate that Notch signaling is dispensable for the generation of CD8+ Tm cells. However, in the DC vaccination model, the yield of Ag-specific CD8+ Tm cells was higher in absence of the Notch1 and Notch2 receptors. One possible explanation could be that the reduced production of IFN-γ by NotchΔΔΔΔ CD8+ T cells allows for a better survival of Ag-specific T cells during the contraction phase of the T cell response. Indeed, it was previously shown that IFN-γ contributes to the contraction phase of the CD8+ T cell response (60, 61).

In summary, the Notch signaling pathway influences various aspects of the CD8+ T cell response. Interestingly, some of the effects of the Notch signaling pathway are dependent on the context in which the CD8+ T cell response occurs. Further identification of the Notch target genes during different types of CD8+ T cell response should shed light on the mechanism by which the Notch signaling pathway influences the differentiation of Ag-specific CD8+ T cells during different immune responses.

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Disclosures

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