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MHC Recognition in Thymic Development: Distinct, Parallel Pathways for Survival and Lineage Commitment

David Chang, Patricia Valdez, Thomas Ho, and Ellen Robey

The molecular events triggered by MHC recognition and how they lead to the emergence of mature CD4 and CD8 lineage thymocytes are not yet understood. To address these questions, we have examined what signals are necessary to drive the development of CD8 lineage thymocytes in TCRα− mice in which TCR/MHC engagement cannot occur. We find that the combination of constitutive Notch activity and constitutive Bcl-2 expression are necessary and sufficient to allow the appearance of mature CD8 lineage thymocytes in TCRα− mice. In addition, Notch activity alone in TCRα− mice can induce the up-regulation of HES1, suggesting that thymocytes are competent to respond to Notch signaling in the absence of MHC recognition. These data indicate that survival and lineage commitment represent distinct, parallel pathways that occur as a consequence of MHC recognition, both of which are necessary for the development of mature CD8 lineage T cells.

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**Flow cytometry**

Thymus and lymph nodes (cervical, axillary, brachial, and mesenteric) were teased apart in cold M199 medium (Life Technologies, Grand Island, NY) supplemented with 2% FBS, and the cells were filtered through nylon mesh. A total of 10^6 cells were incubated with 10 μl Ab on ice for 20 min. Cells were then washed twice with staining buffer containing 1% HBSS (Fisher, Pittsburgh, PA), 0.2% sodium azide, and 0.2% bovine albumin (Sigma, St. Louis, MO). Data (50,000 events) were collected and analyzed using an Epics XL-MCL flow cytometer (Coulter, Hialeah, FL). Dead cells were excluded on the basis of forward and side scatter. Dot plot images were produced with the aid of WinMDI version 2.1.2 by Joseph Trotter (Scripps Research Institute, La Jolla, CA). Abs used were FITC-labeled were produced with the aid of WinMDI version 2.1.2 by Joseph Trotter (Scripps Research Institute, La Jolla, CA). FITC-labeled anti-CD8 (53-6.7; PharMingen, San Diego, CA), FITC-labeled goat anti-mouse (Mr. Big, San Francisco, CA), PE-labeled anti-CD4 (H129;1; Life Technologies), PE-labeled anti-TCR (H57-597; PharMin- gen), PE-labeled CD4 (Becton Dickinson, Mountain View, CA), FITC- labeled anti-αβTCR (PharMingen), anti-heat stable Ag (HSA) (11.d cell culture supernatant), R613-labeled anti-Rat Ig (Life Technologies), and rat γ-globulin (Calbiochem, San Diego, CA), PE-labeled anti-γδ TCR (PharMingen), FITC-labeled anti-CD8β (PharMingen), and FITC-labeled anti-KK (PharMingen).

**Functional assays**

Thymocytes (~10^7) were stained with Abs against CD4 and CD8 and populations were isolated by FACS. Mature CD4^+CD8^- and CD4^-CD8^+ populations were preenriched before sorting by treating thymocytes with HSA and complement. Sorted double positive (DP) and CD4 single positive (SP) thymocytes were >98% pure. Sorted CD8 SP thymocytes were 80-97% pure. The contaminating population was primarily DP thymocytes and there was <0.5% contamination with CD4 SP or double negative (DN) thymocytes. Sorted thymocytes (20,000/well) were cultured in round-bottom 96-well plates for 24 h in the presence of PMA (5 ng/ml) and ionomycin (A23187) (125 ng/ml). Culture supernatants were assayed for IL-2 using an ELISA kit (OptEIA; PharMingen) according to the manufacturer’s instructions. For measurement of thymocyte survival, cell suspensions of total thymocytes were cultured in RPMI containing 10% FCS for 37°C at initial cell concentrations of either 10^6/ml or 2 × 10^7/ml. Cell viability was measured after various times in culture using trypan blue exclusion.

**Northern blot analyses**

For preparation of thymocyte RNA, thymuses were teased in media and cell suspensions were filtered through nylon mesh. Cell suspensions prepared in this manner consist of >99% thymocytes and are free of stromal cells. RNA was isolated using Tripure Isolation Reagent (Boehringer Mannheim, Indianapolis, IN). For Northern blot analysis, 20 μg of total RNA was used (10). An equal amount of RNA was loaded in each lane based on ethidium bromide staining of the 18S and 28S ribosomal RNAs. The 1-kb EcoRI/DdeI fragment from rat HES1 cDNA was used as a probe for HES1 (kindly provided by R. Kageyama, Kyoto University, Kyoto, Japan). The 350-bp EcoRI/HindIII fragment from the EcoRI/EcoRI fragment of the E47 cDNA was used as a probe for E2A (kindly provided by C. Murre, University of California, San Diego, CA).

**Results**

Previous studies indicate that Notch activity can permit CD8 T cell development in the absence of class I MHC, but not in the absence of both class I and class II MHC (7). This requirement for MHC may reflect the need for a survival signal, independent of Notch signaling, to allow CD8 lineage T cells to emerge. Alternatively, MHC recognition might turn on components of the Notch signaling pathway, and thus render thymocytes competent to respond to activated Notch. If MHC recognition provides a survival signal independent of Notch signaling, it might be possible to supply this survival signal using a constitutive Bcl-2 transgene. Bcl-2 is an inhibitor of programmed cell death, and previous studies have shown that constitutive expression of Bcl-2 inhibits apoptosis in thymocytes (9, 11, 12). We therefore crossed both a constitutive Bcl-2 transgene (11) and an activated Notch transgene onto a TCRα mutant background (8) and examined thymic development in the offspring of this cross.

As shown in Fig. 1, A and B, wild-type mice contain a small population of CD4^-CD8^- thymocytes, the majority of which express low levels of HSA, indicating that they are mature CD8 lineage thymocytes. Mice lacking the αβTCR, due to a targeted mutation of the TCRα gene (8), display a block in the development of mature CD4 and CD8 lineage cells, and this is reflected in the reduced number of CD4^-CD8^-HSA^low thymocytes in these mice (Fig. 1, A and B). The presence of either a constitutive Bcl-2 transgene, or an activated Notch transgene alone, in TCRα mutant mice leads to only a small increase in the number of CD4^-CD8^-HSA^low thymocytes (Fig. 1, A and B). This is consistent with previous studies showing that neither the Bcl-2 transgene nor the activated Notch transgene are sufficient to permit the development of CD8 lineage thymocytes in the absence of both class I and class II MHC (9, 13).

Strikingly, the combination of activated Notch and constitutive Bcl-2 leads to the appearance of a large population of thymocytes that are CD4^-CD8^-HSA^low. Similar to the CD4^-CD8^- thymocytes from wild-type mice, the CD4^-CD8^- population from TCRα^-NotchIC, Bcl-2 transgenic mice lacks expression of γδTCR, but expresses high levels of class I MHC (Fig. 1C). In addition, the CD4^-CD8^- population from TCRα^-NotchIC, Bcl-2 transgenic mice expresses CD8β, a characteristic of conventional CD8 lineage thymocytes. The levels of both CD8α and CD8β are somewhat lower on CD4^-CD8^- cells from TCRα^-NotchIC, Bcl-2 transgenic mice, compared with CD4^-CD8^- population from wild-type mice, perhaps a result of inappropriately high Notch activity in these cells. Together, these analyses indicate that the combination of Notch activity and a survival signal are necessary and sufficient to permit the development of phenotypically mature CD8 lineage thymocytes in the absence of positive selection.

The phenotype of CD8^-CD4^-HSA^low thymocytes in NotchIC, Bcl-2 transgenic, TCRα mutant mice suggests that they represent mature CD8 lineage T cells. To confirm this, we examined the ability of these cells to produce IL-2. We isolated CD4^-CD8^-HSA^low (CD8 SP), CD4^-CD8^-HSA^low (CD8 DP), or CD4^-CD8^- DP thymocytes from wild-type mice and from NotchIC, Bcl-2 transgenic, TCRα mutant mice, cultured them in the presence of PMA and ionomycin, and measured IL-2 in the culture supernatants. As shown in Fig. 2, CD8^-CD4^- thymocytes from NotchIC, Bcl-2 transgenic TCRα mutant mice produce significant levels of IL-2, indicating that they are functionally, as well as phenotypically, mature. Interestingly, despite the evidence for functional maturity of CD8 cells in the thymus, CD8 T cells fail to accumulate in the lymph nodes of NotchIC, Bcl-2 transgenic, TCRα mutant mice (data not shown). The failure of these phenotypically mature CD8 thymocytes to migrate and survive in the periphery may be a result of the absence of αβTCR expression on these cells and the known requirement for TCR-MHC interactions for the maintenance of peripheral T cells (14). An alternative explanation is that the NotchIC and Bcl-2 transgenes induce only a subset of the properties of normal mature CD8 T cells that allow them to accumulate in the periphery.

The appearance of CD8 lineage thymocytes in Bcl-2 and NotchIC transgenic, TCRα mutant mice implies that the downstream events of Notch signaling can occur in the absence of MHC recognition. If this is the case, it might be possible to see indications of Notch signaling in thymocytes in the absence of the αβTCR. We have previously shown that expression of activated Notch in thymocytes leads to the up-regulation of HES1 (15), a gene that encodes a basic helix-loop-helix transcription factor that is related to components of the Notch signaling pathway in Dro-
We find that up-regulation of HES1 mRNA also occurs in thymocytes from TCRα mutant, NotchIC transgenic mice (Fig. 3). This indicates that activated Notch can turn on the Notch signaling pathway in the absence of TCR-MHC engagement, although it is not sufficient to drive the development of CD8 lineage T cells.
In most systems, Bcl-2 acts to promote cell survival, whereas Notch affects cell fate decisions. There are reports that Notch activity can affect thymocyte survival in some experimental settings (18, 19). However, it seems unlikely that NotchIC is exerting its CD8-promoting effects via a survival signal, because Notch and Bcl-2 activity are jointly required to drive CD8 cell development in the absence of positive selection, and because Bcl-2 alone is known to deliver a potent survival signal. To explore this issue further, we directly compared the NotchIC transgene and the Bcl-2 transgene for their ability to promote thymocyte survival. Thymocytes from both normal and NotchIC transgenic mice die within a few days when placed in culture without thymic stromal cells (Fig. 4). In contrast, thymocytes from Bcl-2 transgenic mice show significantly enhanced survival under the same culture conditions (Fig. 4, and Ref. 20). We also note that the turnover rate of CD4⁺CD8⁻ thymocytes, as measured by the rate of 5-bromo-2'-deoxyuridine incorporation, is identical in NotchIC transgenic and wild-type mice (7). In contrast, the turnover of CD4⁺CD8⁻ cells from Bcl-2 transgenic mice is dramatically delayed compared with wild type (Ref. 21, and data not shown). These data indicate that, unlike Bcl-2, the NotchIC transgene does not provide a general survival signal to thymocytes. Together, the data are most consistent with the interpretation that Notch and Bcl-2 act in distinct ways to promote the CD8 fate: with Bcl-2 promoting survival, and NotchIC promoting the CD8 lineage choice.

Discussion

The generation of mature CD4 or CD8 lineage thymocytes from CD4⁺CD8⁻ precursors is driven by recognition of MHC proteins by TCR expressed by thymocytes, but the nature of the signaling pathways that contribute to this process is poorly understood. Here, we show that the combination of a constitutive Notch signal and Bcl-2 up-regulation is necessary and sufficient to drive the development of phenotypically and functionally mature CD8⁺ thymocytes in the absence of TCR-MHC engagement. We also provide evidence that the Notch signaling pathway can be activated in the absence of TCR-MHC engagement, but that this is not sufficient to allow the emergence of mature CD8⁺ thymocytes in the absence of a survival signal. Given the well-established roles for Bcl-2 in survival and for Notch in lineage commitment, these data support a model (Fig. 5) in which TCR-MHC engagement during positive selection of thymocytes activates two distinct signaling pathways, one which regulates survival and one which regulates lineage commitment. The survival signal is required for the emergence of both CD4 and CD8 lineage T cells, but does not control lineage choice. The lineage commitment signal differentially modulates Notch signaling in response to class I or class II MHC recognition. Thus, class I MHC recognition (Fig. 5A) would both up-regulate Notch signaling and provide a survival signal, leading to the development of CD8 lineage T cells. Class II MHC recognition would both decrease Notch signaling and provide a survival signal, thus leading to the development of CD4 lineage T cells (Fig. 5B). In the absence of MHC recognition (Fig. 5C), CD8 cells can be generated by providing both a lineage commitment signal in the form of an activated Notch transgene, and a survival signal in the form of a Bcl-2 transgene.

Although our data show that Notch and Bcl-2 transgenes can provide survival and lineage commitment signals in the absence of positive selection, they leave open the question of which molecules provide these signals during normal positive selection. There is evidence that CD4⁺CD8⁻ thymocytes up-regulate Bcl-2 in response to positive selection signals (9, 22, 23), suggesting that...
Bcl-2 may contribute to a survival signal generated during positive selection. However, positive selection occurs normally in mice lacking Bcl-2 (24, 25), indicating that other anti-apoptotic molecules may also contribute to this process. In the case of the lineage commitment signal, the effects of an activated form of Notch on the CD4 vs CD8 lineage choice implicate the Notch signaling pathway; however, it is not yet clear which endogenous proteins are normally involved. A conditional disruption of the Notch1 gene produces a very early block in thymic development (26), precluding an examination of the effect of the mutation on the CD4 vs CD8 lineage choice. The effect of blocking Notch1 function in a thymic organ culture system provides evidence that Notch1 is involved in the developmental progression of CD8 lineage, but not CD4 lineage, thymocytes (27). However, Notch1, Notch2, and Notch3 are expressed by thymocytes (28), suggesting the possibility that multiple Notch homologues may contribute to the CD4 vs CD8 lineage choice. With regard to proteins acting downstream of Notch, we have identified one possible candidate, the basic helix-loop-helix transcription factor, HES-1. The observation that HES-1 is up-regulated in response to Notch activity in thymocytes (15), together with the fact that HES-1 related genes function downstream of Notch in other systems (16, 17), suggests that HES-1 up-regulation by Notch may contribute to the CD4 vs CD8 lineage choice.

Recently, Deftos et al. (19, 29) have put forth a very different view of the role of Notch in which Notch provides survival signals that promote the development of both CD4 and CD8 cell development. Their view is based in part on the observation that Notch activity can induce Bcl-2 expression in a thymocyte cell line and inhibit glucocorticoid-mediated cell death in thymocytes, suggesting the possibility that the effect of activated Notch on the CD4 vs CD8 lineage choice is an indirect consequence of disregulating Bcl-2 expression. The data presented here, showing that activated Notch and Bcl-2 perform distinct and separable functions during positive selection, argue strongly against this view and are most compatible with the notion that Notch exerts its effect directly on lineage commitment. Interestingly, whereas the Bcl-2 transgene is not sufficient to allow the positive selection of CD8 T cells in MHC or TCRα-deficient mice, it can allow CD8 cell development in class I MHC-deficient mice (9). Although the explanation for this is not yet clear, one possibility is that constitutive Bcl-2 expression leads to disregulation of Notch, causing thymocytes that recognize class II MHC to receive an inappropriately high Notch signal and directing them to the CD8 lineage.

FIGURE 5. Distinct pathways for survival and lineage commitment. According to this model, one consequence of class I MHC recognition (A) would be a survival signal, perhaps supplied in part by up-regulation of Bcl-2 (9, 22, 23). In addition, class I MHC recognition would lead to increased Notch signaling that would promote the CD8 cell fate, in part by causing up-regulation of HES1 (15). The combination of the survival signal and increased Notch signaling would lead to the appearance of mature CD8 lineage T cells. Class II MHC recognition (B) would also produce a survival signal, but would lead to decreased Notch signaling. The combination of a survival signal and decreased Notch signaling would lead to the appearance of mature CD4 lineage cells. In the absence of MHC recognition (C) the combination of constitutive Bcl-2 expression and constitutive Notch activity could mimic class I MHC recognition and lead to the appearance of mature CD8 lineage cells. Italics are used to denote steps, which may contribute to, but are not absolutely required for, the indicated pathway. See text for discussion.
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