either condition (Fig. 5*Bii*), and CMA did not affect the cytotoxic activities against Jurkat cells, irrespective of the culture conditions (data not shown). This finding suggests that perforin or granzyme B does not have a major role in killing Jurkat cells. We evaluated whether TRAIL had a role by adding anti-TRAIL-blocking Ab RIK2 to the medium. RIK2 partially but clearly suppressed the cytotoxic activities against Jurkat cells generated in both conditions without significant differences (Fig. 5*Bii*), although TRAIL expression was slightly higher in the NK cells generated in the D4-Fc plus IL-15 condition (Fig. 5*Cii*). From these observations, we concluded that IL-15 does not influence the killing activity through TRAIL but does enhance the killing activity through perforin/granzyme B. The cytotoxic activity of immature NK cells is TRAIL dependent, while that of mature NK cells is mainly dependent on perforin (29). Therefore, IL-15 might contribute to the maturation of NK cells and confer on them the capacity to exact perforin/granzyme B-medicated cytotoxicity.

Inhibitory effect of anti-Notch1 Ab on Delta4-dependent NK cell development

We prepared mAbs specific for the extracellular domain of Notch1, Notch2, and Notch3 (supplemental Fig. S4*A*). The expression patterns of Notch1, Notch2, and Notch3 in fresh CB mononuclear cells, $CD34^+$ cells, and products during the culture of $CD34^+$ cells are shown in supplemental Fig. S3, *A* and *B*. Notch1 was expressed at higher levels on NK and T cells than on B cells and monocytes. Notch2 was expressed at higher levels on monocytes than on lymphocytes. Notch3 expression was virtually negative on all types of lymphocytes and positive on monocytes. Notch1 and Notch2, but not Notch3, were expressed on $CD34^+$ cells. The $CD34^+$ cell-derived $CD56^+$ NK cells also expressed Notch1 and Notch2, but not Notch3. All three Notch receptors were expressed on cells grown on the control Fc-coated plates (supplemental Fig. S3*B*).

Because $CD34^+$ cells expressed Notch1 and Notch2, but not Notch3 (supplemental Fig. S3*B*), and the established anti-Notch1 Ab, but not anti-Notch2 Ab, blocked binding of the cognate soluble Notch receptor to the ligands (supplemental Fig. S4*B*), we cultured CB $CD34^+$ cells on Delta4-Fc-coated plates in anti-Notch1 Ab-containing medium. Remarkably, the immunophenotype of the cells grown under the presence of anti-Notch1 Ab was almost the same as that of cells grown on control Fc-coated plates, indicating that the effect of Delta4 was completely blocked and NK cell development was shut down by the anti-Notch1 Ab (Fig. 6*A*). Anti-Notch2 Ab did not have such an effect, consistent with the fact that it did not block ligand binding to the cognate receptors (data not shown). CB $CD34^+$ cells cultured with IL-15 on Fc-coated plates in the presence of the anti-Notch1 Ab gave rise to NK cells in a manner indistinguishable from that of cells grown without the Ab (Fig. 6*B*). These results suggest that Notch1 might be a physiologic Notch receptor that mediates Delta4 signaling for NK cell development from CB $CD34^+$ cells and further support the notion that Notch signaling has a role distinct from that of IL-15.

Discussion

In the present study, we demonstrated that functional NK cells developed from CB $CD34^+$ cells when stimulated with the Notch ligand Delta4. Previous reports indicated that NK cells can be derived from in vitro culture of human $CD34^+$ cells prepared from fetal liver, bone marrow, or CB with either IL-2 or IL-15 (30–33), which signal through the shared IL-2/IL-15 receptor β-chain and the common γ-chain. IL-15 has been considered to have a more physiologic role than IL-2 in NK development (30). Notably, IL-15-independent NK cell differentiation has recently been published (6). This culture system, however, has been reported to be stromal cell dependent while the potential molecules and signaling pathways are unknown and, thus, the conclusion whether IL-15 is indispensable is yet to be determined. Notch signaling has been examined in the context of NK cell development as well and appears to affect the very early phase of progenitor development (17–19). In studies of human NK cell development, however, culture systems containing IL-15 and/or a coculture system with the fetal thymus organ or stromal cells are used exclusively. A novel and unexpected finding in the present study was the fact that stimulation of CB $CD34^+$ cells with a soluble Notch ligand, Delta4-Fc, coated onto the plate in the presence of stem cell factor, FL, and IL-7 was sufficient to induce the development of functional NK cells.

Our data do not officially exclude the possibility that endogenous IL-15 is involved in NK cell development in a manner, e.g., that cell-autonomously produced IL-15 activated the signaling by binding to the receptor intracellularly. Given the fact, however, that the exogenous addition of IL-15 resulted in the qualitative rather than quantitative difference in the NK cells developed in the presence of Delta4-Fc, in addition to inefficient blockade by anti-IL-15-neutralizing Ab, IL-15 is likely to be dispensable for human NK cell development in the presence of Delta4-Fc.

The finding that IL-15 is not necessary for human NK cell development in culture contrasts with the absolute necessity of IL-15 signaling for NK development in some mouse phenotypes; mice lacking a gene for IL-15 (3) (34, 35), IL-15 receptor α-chain (36), common β-chain (37), or common γ-chain (38, 39) lack NK cells. This might be due to differences between the in vitro culture conditions and the in vivo environment in which NK cells develop. Another explanation might be a difference between mice and humans, as in the case of IL-7 requirement for T cell development; IL-7 is required for the V-D-J rearrangement of the TCR β-chain gene in humans, whereas it is dispensable in mouse T cell development (40).

Previous studies reported that the effect of Notch signaling in the presence of IL-15 on NK cell development is confined to the very early stages of development. In the present study, we demonstrated that Notch signaling confers CD7 expression competence on cells cultured with or without IL-15 for 1 wk or less, but not for 2 wk, unless also stimulated by Notch. This finding is similar to that in a previous report demonstrating that Notch signaling confers cyCD3 expression competence only on prethymic but not thymic NK cell progenitors or peripheral blood $cyCD3^-$ NK cells (19). We confirmed the Notch signal dependency of cyCD3 expression during NK cell development. Coexpression of CD7 and CD45RA on $CD34^+$ cells might be associated with a restriction toward NK cell development (26, 33). Our data strongly suggest that the vast majority, if not all, of the NK cells derived from $CD34^+$ cells without Notch signaling were generated through $CD7^-$ cells. Therefore, although it is yet to be elucidated whether all of the NK cell progenitors are $CD7^+$ (41), NK cells established in vitro without Notch stimulation might not develop from a physiologic NK progenitor or might skip the physiologic NK/T progenitor stage. Furthermore, our data suggest that the effect of Notch stimulation on CD7 expression is imprinted on cells only if it is administered at the initial stage of the $CD34^+$ cell culture. We, however, failed to prospectively identify the subpopulations in the $CD34^+$ cells that are targets of Delta4 to develop NK-lineage cells. Delta4 stimulation induced NK cell development from both the most immature $CD34^+CD38^-$ and more mature $CD34^+CD38^+$ progenitor populations and both $CD34^+$

CD45RA$^+$ lymphoid progenitors and CD34$^+$CD45RA$^-$ populations (data not shown).

The findings of the present study extend our understanding to more mature stages of NK cell differentiation: the presence of Notch signaling induces generation of functional NK cells in culture conditions that do not generate CD56$^+$ cells without Notch stimulation per se. The precise stages of NK cell development during which Notch signaling determines the progression toward functional NK cells is not known.

In our experiments, even cells cultured with a Notch ligand alone had cytotoxic activity. The level of this activity, however, was weaker than that in NK cells generated by Notch stimulation with IL-15. Indeed, the perforin-mediated cytotoxicity of NK cells generated in the absence of IL-15 was significantly weaker, despite the fact that this is the major pathway of mature NK cells to kill target cells (42). In contrast, the TRAIL-mediated cytotoxicity was almost the same regardless of presence or absence of IL-15. This finding, along with the change in the expression level of CD56, might indicate that IL-15 induces the maturation of CD56^{low} CD161$^+$ immature NK cells generated by Notch stimulation without IL-15. Another difference between the cells cultured with or without IL-15 was the down-regulation of adhesion molecules (CD11a, CD11b, CD62L) on the cell surface. These molecules might be important for homing of the NK cells to the sites at which they function.

To our surprise, cytotoxic activities were not detected in the cell populations generated in the control Fc plus IL-15 condition at either 3 or 6 wk (Fig. 5*B* and data not shown), although these results might be affected by the facts that the frequency of CD56$^+$CD161$^+$ cells was very low at 3 wk and that culture for 6 wk might be too long to evaluate cytotoxic activities while the frequency of CD56$^+$CD161$^+$ cells was much greater. In any case, when clinical application of progenitor-derived NK cells is considered, a Delta4-Fc-coating system would give a significant advantage.

In conclusion, Notch stimulation by Delta4 (or Delta1) was required for initial NK cell differentiation and the development of CD161$^+$CD56^{low} immature NK cells. Among Notch receptors, Notch1 might be essential for physiologic NK cell development, although the involvement of other Notch receptors is yet to be elucidated. IL-15 was not essential for differentiation, but was necessary for maturation. IL-15 might have an indispensable role only in the later part of the NK development. This knowledge might be useful for future approaches toward the ex vivo generation and manipulation of NK cells and their therapeutic application.

Acknowledgments

We thank Y. Mori and E. Nakasone for providing excellent technical assistance.

Disclosures

The authors have no financial conflict of interest.

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