IL-18–induced CD83$^+$CCR7$^+$ NK helper cells

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In addition to their cytotoxic activities, natural killer (NK) cells can have immunoregulatory functions. We describe a distinct "helper" differentiation pathway of human CD56$^+$CD3$^-$ NK cells into CD56$^+$/CD83$^+$/CCR7$^+$/CD25$^+$ cells that display high migratory responsiveness to lymph node (LN)–associated chemokines, high ability to produce interferon-γ upon exposure to dendritic cell (DC)- or T helper (Th) cell–related signals, and pronounced abilities to promote interleukin (IL)-12p70 production in DCs and the development of Th1 responses. This helper pathway of NK cell differentiation, which is not associated with any enhancement of cytolytic activity, is induced by IL-18, but not other NK cell–activating factors. It is blocked by prostaglandin (PG)E_2, a factor that induces a similar CD83$^+$/CCR7$^+$/CD25$^+$ LN-homing phenotype in maturing DCs. The current data demonstrate independent regulation of the "helper" versus "effector" pathways of NK cell differentiation and novel mechanisms of immunoregulation by IL-18 and PGE_2.

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Abbreviations used: Ag, antigen; PG, prostaglandin.

Human CD56$^+$/CD3$^-$ NK cells, representing ~10% of peripheral blood lymphocytes, were identified first based on their unique immediate ability to kill transformed or virally infected cells (1–3). NK cells are recruited rapidly to the sites of virus entry, and are critical for controlling acute viral infections. Individuals who have NK cell deficits also display recurrent viral infections (4), which is suggestive of their impaired ability to develop lasting and effective antigen-specific recall responses. It was demonstrated that NK cells play a major immunoregulatory role for the development of a protective T cell–mediated immunity against intracellular pathogens and cancer (5–8). Such "helper" activity of NK cells is mediated, at least in part, by the functional modulation of DCs. The phenomenon depends on the production of IFN-γ and TNFα by activated NK cells (9–12), and is associated with the enhanced induction of Th1 and CTL responses in human in vitro or mouse in vivo models (12–14). Despite the identification of these two distinct functions of NK cells ("helper" versus "killer"), it remains unclear whether each of these functions can be induced in the same subsets of NK cells and at what stages of their differentiation.

Here we show that in contrast to IL-2—which selectively promotes the cytotoxic activity of NK cells—IL-18 does not enhance the cytolytic activity of NK cells, but induces a distinct "helper" pathway of their differentiation. IL-18, but not other NK cell–activating cytokines, promotes the development of CD56$^+$/CD83$^+$/CCR7$^+$/CD25$^+$ helper NK cells that are characterized by unique expression of several mature DC-associated surface markers, high migratory responsiveness to LN-produced chemokines, and distinctive abilities to support IL-12 production in DCs and to promote Th1 responses of CD4$^+$ T cells.

RESULTS

IL-2 and IL-18 control different aspects of human NK cell activation: LN-homing properties of IL-18–primed NK cells

The entry of multiple cell types to the T cell areas of the LNs depends on the LN-produced CCL19 (MIP-3β/ELC/CKβ-11) and CCL21 (6Ckine/SLC/Exodus2/TCA4) ligands for the chemokine receptor CCR7 (15–17)—and uniquely in mouse—also CXCR3 (18–20). Because recent studies demonstrated the presence of CCR7 expression on a CD56^{bright}/CD16$^-$ subpopulation of human blood NK cells and the presence of NK cells within human LNs (21–24), we analyzed the activation requirements that allow freshly isolated resting peripheral blood NK cells to migrate in response to lymph node–associated chemokines.

We observed that the addition of LPS (but not CD40L) to NK cell–macrophage co-cultures

induced surface expression of CCR7 on CD56$^+$ NK cells (Fig. 1 A). This CCR7 expression was no longer restricted to the CD56^{bright} subset, which is known to express this marker constitutively, but appeared with high intensity on "classic" CD56^{dim} NK cells (Fig. 1 A). Because such co-cultures contained low, but distinct, levels of IL-18—the proinflammatory cytokine that is induced rapidly during acute infections in tissue-residing DCs and macrophages (25) and a known NK cell–activating factor during DC–NK cell interaction (26)—we tested whether recombinant IL-18 also can induce CCR7. As seen in Fig. 1 B, 100 pg of IL-18 induced the expression of CCR7 on highly purified CD56$^+$ (CD3$^-$, CD20$^-$, and MHC II$^-$) NK cells; the concentrations of 10–1,000 ng/ml were optimally effective in different donors.

Freshly isolated CD56$^+$ (CD3$^-$, CD20$^-$, and MHC II$^-$) resting NK cells (Fig. 1 C) showed only marginal migratory activity in response to CCL21, which was consistent with the absence of CCR7 expression on the main population of CD56$^{+(dim)}$/CD16$^+$ NK cell;, only low levels of CCR7 expression were observed selectively on the CD56^{bright}/CD16$^-$ NK cell subset (Fig. 1, A, C, and D) and only a marginal cytotoxic activity was observed against Daudi cells (Fig. 1 F), which is characteristic of resting NK cells.

Although IL-18 and IL-2 promoted a similar level of cluster formation in NK cell cultures (Fig. 1 C), each of

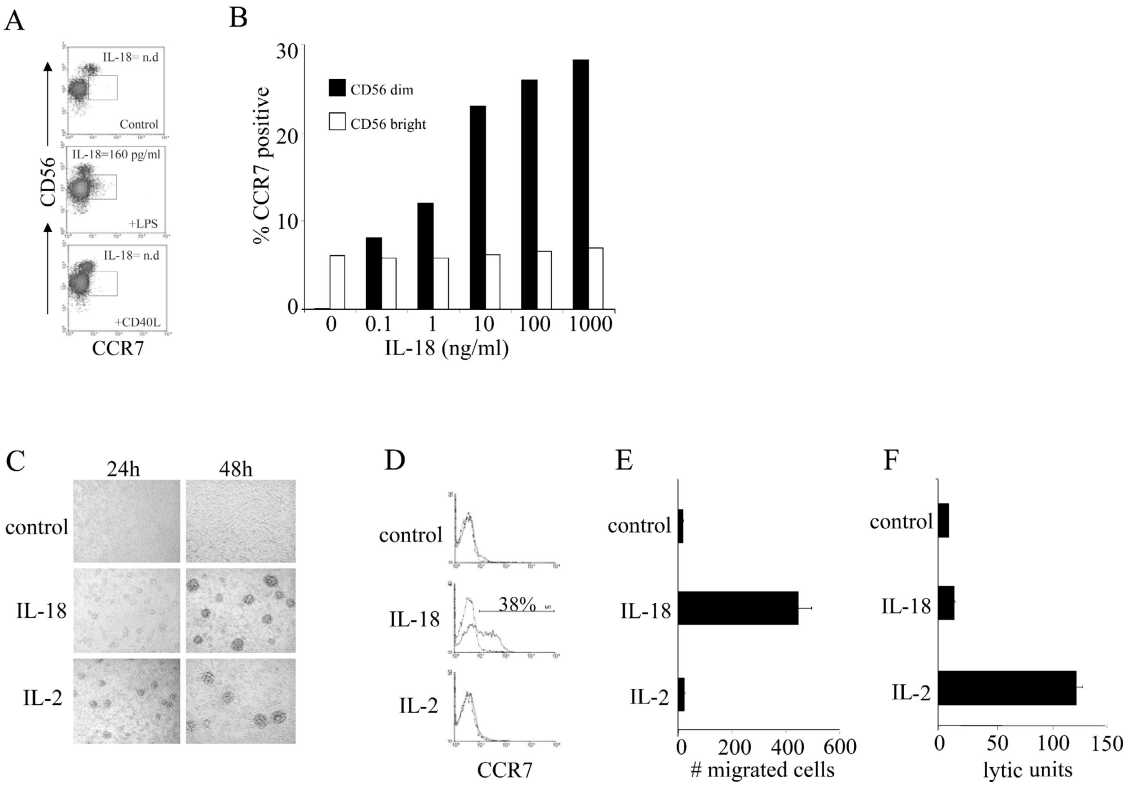

Figure 1. Induction of CCR7-responsiveness in NK cells: IL-18 does not enhance killer activity of NK cells but induces their migratory responsiveness to CCL21. Freshly isolated human NK cells (94% purity, with <1% each of CD3$^+$, CD20$^+$, and HLA-DR$^+$ cells, see Fig. 2) were cultured for 24 or 48 h under serum-free conditions (AIM-V medium) in the presence of rhIL-2 or rhIL-18, before their phenotypic and functional analyses. (A) NK cells were co-cultured with autologous macrophages in the absence or presence of LPS or CD40L for 24 h before harvesting the supernatants and the cells. The expression of CCR7 on NK cells is shown after gating on CD56$^+$CD3$^-$ lymphocytes. n.d., not detected (<20 pg/ml) (B) Freshly-isolated NK cells were cultured for 24 h in the presence of increasing concentrations of IL-18 before the analysis of cell surface phenotype. Data from one of four experiments with blood of different donors that showed the optimum CCR7 induction by 10–1000 ng/ml of IL-18. (C–F) Distinct patterns of NK cell activation by IL-2 or IL-18. All data were obtained in the same experiment and were reproduced in three additional donors. (C) IL-2– or IL-18–dependent activation of NK cells results in formation of cell clusters in 24-h and 48-h cultures. (D) Surface expression of CCR7 induced on NK cells by the exposure to IL-18 (1 μg/ml), but not IL-2 (250 IU/ml), as determined by flow cytometry. (E) Induction of the migratory responsiveness to the LN-associated chemokine CCL21 (6Ckine; 10 ng/ml) in NK cells treated for 48 h with IL-18, but not IL-2. Similar induction of CCL21 responsiveness was observed in 24-h cultures of NK cells that were exposed to IL-18 (but not IL-2). Other modes of NK activation, such as exposure to IL-1β, IFN-α, IL-12, immobilized IgG1, and NK cell sensitive targets (K562 and T2 cells), also were tested and failed to induce expression of CCR7 unless IL-18 was present (not depicted). (F) Cytolytic activity of NK cells against Daudi cells (resistant to killing by resting NK cells) is enhanced by the 48-h exposure to IL-2 but not IL-18, as demonstrated by ^{51}Cr-release assay. Similar, although less pronounced, cytolytic activity against Daudi cells was observed in 24-h IL-2–exposed (but not IL-18–exposed) NK cells. As expected, all three populations of cultured NK cells expressed strong cytolytic activity against K562 cells (not depicted).

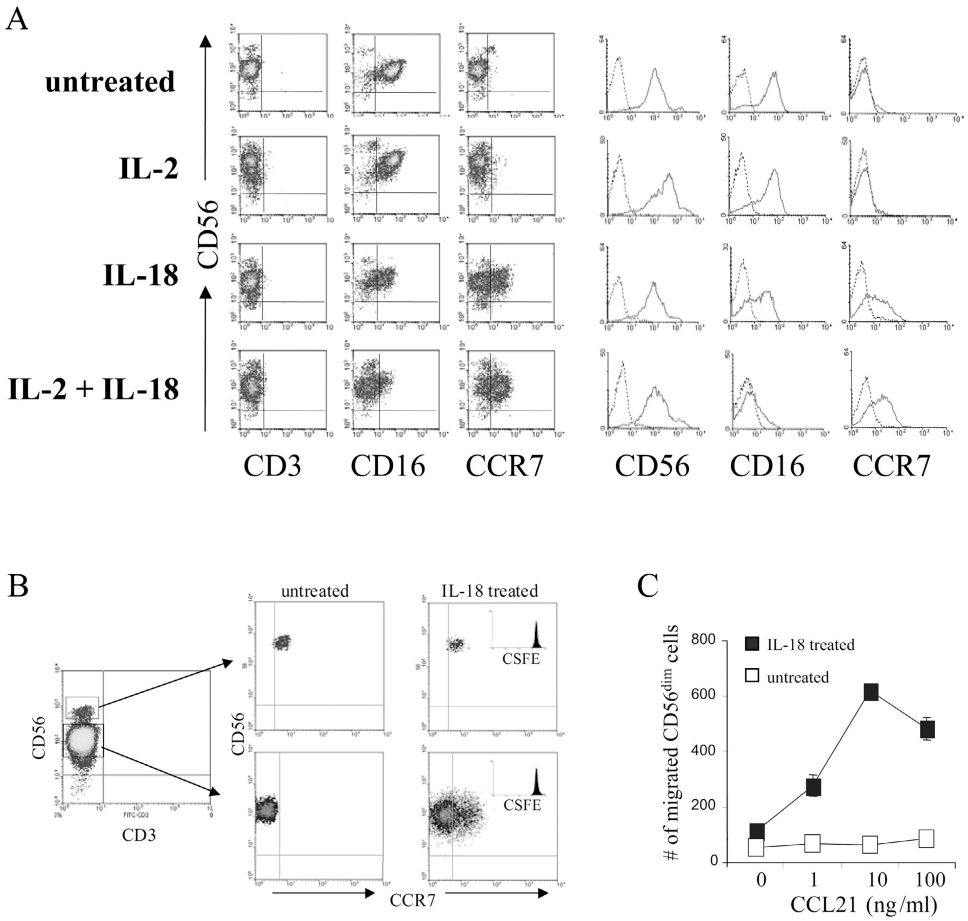

Figure 2. Rapid induction of CCR7 expression and CCL21 responsiveness in IL-18–treated CD56^{dim} NK cells. (A) Phenotypic analysis of 24-h cultured NK cells demonstrating the increase in CCR7 expression and decrease in CD16 expression within the CD56$^+$/CD3$^-$ population of NK cells upon short-term exposure to rhIL-18, but not IL-2. Dot plots and line graphs are shown for visual clarity. Data from one of four experiments that yielded similar results. Similar data also were obtained in 48-h cultures. Although IL-2 alone was completely ineffective in the induction of CCR7$^+$ expression, it supported the IL-18–induced CCR7 expression. Similar ability to support the IL-18–induced CCR7 expression also was observed in the case of IL-12 and IFNα (not depicted). (B) Flow-sorted CD56^{dim} NK cells develop CCR7$^+$ phenotype in 24 h–long IL-18-supplemented cultures. Inset: lack of cell division in IL-18–treated cultures of sorted CD56^{dim} (or CD56^{bright}) NK cells, as demonstrated by the uniform levels of fluorescence of CFSE-labeled NK cells. (C) Flow-sorted CD56^{dim} NK cells develop CCL21 responsiveness in 24 h–long IL-18–supplemented cultures.

these factors induced a different pattern of NK cell activation. Whereas IL-2 selectively enhanced NK cell lytic activity against Daudi cells (Fig. 1 F), without any impact on the ability of NK cells to migrate in response to CCL21 (Fig. 1 E), in sharp contrast, IL-18 selectively induced the migratory responsiveness of NK cells to CCL21, without enhancing their killer activity. In accordance with the crucial role for CCR7 in mediating the migratory responses to CCL21 in the human system (15–17, 19, 20), the above changes were paralleled by a rapid induction of CCR7 on IL-18–treated NK cells. This effect was highly selective for IL-18, because it could not be induced by IL-2 (Fig. 1 D, and Fig. 2), the factor that is highly effective in priming NK cells for enhanced cytotoxic activity (Fig. 1 F), nor by additional NK cell activators tested: IL-1β, -12, and -15, IFN-α, immobilized IgG, K562, T2, or Daudi targets (not depicted).

These data suggested that IL-18 selectively promotes a distinct pathway of NK cell activation that is associated with LN homing, as opposed to the cytotoxic effector pathway of NK cells differentiation that is induced by IL-2.

High CCR7 expression was induced by IL-18 on the main population of CD56$^+$ NK cells (CD56^{dim}) that was exposed to IL-18, and was associated with the reduction of their CD16 expression levels (Fig. 2). In contrast to the optimal induction of cytotoxic activity that required at least 48 h of exposure of NK cells to IL-2, the optimal induction of CCR7 expression was seen after 24 h of exposure to IL-18 (Fig. 2 A). These IL-18–induced changes occurred in the absence of any evidence of cell division in CD56^{dim} or CD56^{bright} NK cells (Fig. 2 B), and could be observed in the isolated population of the flow-sorted CD3$^-$CD56^{dim} (CD16$^+$) NK cells. This indicated that the IL-18–dependent induction of CCR7$^+$ NK

cells reflects the differentiation of the cells belonging to the main population of NK cells, rather than the outgrowth and differentiation of any cells from the minor population of $CD56^{bright}/CD16^{-}$ NK cells or the lineage-negative cells representing 2–10% of the negatively isolated NK cells. Our sorting experiments further demonstrated that the $CCR7^{+}$ NK cells that were derived from $CD56^{dim}$ NK cells can develop into CCL21-responsive cells (Fig. 2 C).

Although IL-2 alone was completely ineffective in inducing CCR7 on resting NK cells, it enhanced the IL-18–induced CCR7 expression (Fig. 2 A). This suggested that the acquisition of $CCR7^{+}$ LN-homing phenotype may be enhanced during recall phase of immune responses, when IL-2–producing $CD4^{+}$ Th cells can interact with DCs in the peripheral tissues. Similar costimulation of the IL-18–dependent induction of CCR7 on resting NK cells also could be

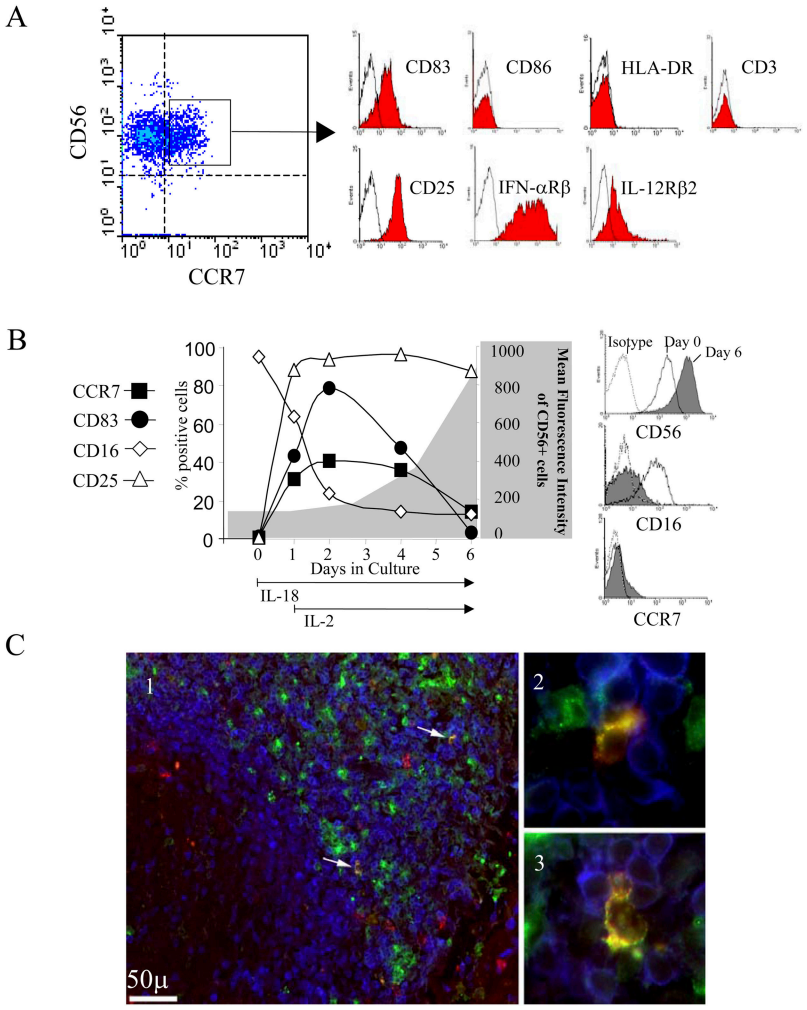

Figure 3. Unique expression of mature DC–associated surface markers and cytokine receptors on IL-18–induced $CD83^{+}/CCR7^{+}/CD25^{+}$ NK cells. (A) Three-color flow cytometry analysis demonstrates coexpression of the mature DC markers CD83, and CD25, on IL-18 induced $CCR7^{+}$ population of $CD56^{+}/CD16^{-}/CD3^{-}/CD86^{-}/DR^{-}$ NK cells. Note the high expression of receptors for IL-2 (CD25), IL-12, and IFN-α on IL-18–induced $CD56^{+}/CCR7^{+}$ NK cells. Only low expression of IFN-αRβ chain, and no detectable CD25 and IL-12Rβ2 were observed on freshly isolated resting NK cells (not depicted). Similar data was obtained in two additional experiments. Open profiles represent the isotype controls. (B) Prolonged culture of IL-18–induced $CCR7^{+}/CD83^{+}$ NK cells in the presence of IL-2 (added 24 h after IL-18) results in an initial enhancement of CCR7 and CD83 expression (up to 40–95% of positive cells in different donors), followed by a progressive reduction in their expression, associated with increased CD56 and loss of CD16 expression. Note the $CD56^{bright}/CD16^{-}/CCR7^{dim}$ phenotype of day 6 cultured NK cells (right). The recovery of viable NK cells was 70%, 69%, 71%, and 85%, on days 1, 2, 4, and 6, respectively, whereas in the absence of IL-2, the cultures showed a rapid decline in cell recovery/viability after 48 h of culture (not depicted). Similar data were obtained in one additional experiment. (C) Three-color florescent microscopy demonstrating the presence of $CD3^{-}$ cells (CD3-positive cells are stained blue) coexpressing CD83 (green) and CD56 (red), within the T cell areas of LNs (arrows point to yellow cells shown in the enlarged photographs). Data from three donors that showed the CD83 expression on 8, 16, and 5% of $CD3^{-}/CD56^{+}$ cells in the T cell areas, respectively. Coexpression of CD83 and CD56 was verified by confocal microscopic analysis (not depicted). Similar cells, although at lower frequency, also were observed in the tonsils of two donors (not depicted).

observed using other NK cell activating factors, IL-12 or IFN-α, in both cases it was strictly dependent on IL-18 as the factor permitting the CCR7 induction (not depicted).

IL-18–induced migratory NK cells express CD83: opposing roles of prostaglandin E_2 in the induction of $CD83^+/CCR7^+/CD25^+$ DCs and $CD83^+/CCR7^+/CD25^+$ NK cells

The induction of CCR7 on IL-18–treated $CD56^+$ NK cells was accompanied by the induction of CD83, another marker of mature DCs. Three-color FACS analysis determined that all of the $CCR7^+$ population of IL-18–activated $CD56^+$ NK cells also coexpressed CD83 (Fig. 3 A). Moreover, IL-18–treated NK cells showed yet another marker of mature DCs (27), CD25 (α chain of the high-affinity IL-2 receptor), which suggests that the IL-18–induced $CD83^+/CCR7^+/CD25^+$ NK cells may home preferentially to the T cell areas of the LNs and may be primed for a distinct pattern of responsiveness to such T cell–produced factors as IL-2. Similarly, the enhanced expression of IL-12Rβ2 and IFN-αRβ on $CD83^+/CCR7^+/CD25^+$ NK cells suggested their enhanced ability to interact with activated myeloid DCs (MDCs) and plasmacytoid DCs (PDCs)—which are known to be sources of IL-12 and IFN-α—the factors that are involved in the induction of Th1-type responses.

Although IL-18 by itself did not support the viability of NK cells (unpublished data), the IL-18–induced $CD83^+/CCR7^+$ NK cells could be maintained in IL-2–supplemented cultures for at least 6 d. However, although a short-term exposure to IL-2 resulted in transient enhancement of CCR7 and CD83 expression, prolonged culture in the presence of IL-2 (to assure the viability of NK cells) led to the eventual disappearance of CD83 expression on $CCR7^+$ NK cells, and a strong down-regulation of their CCR7 expression, in the absence of significant changes in the overall numbers of cultured NK cells (Fig. 3 B). The reduction of CD83 and CCR7 expression was accompanied by the loss of CD16 expression, and the parallel enhancement of the CD56 levels expressed on NK cell surface (Fig. 3 B).

The expression of CD83 on the IL-18–induced $CCR7^+$ NK cells that were responsive to the LN-associated chemokines raises the question of whether the CD83 molecule can be used as a reliable selective marker of mature DCs in the LNs. Triple staining of human LN sections revealed that the CD83 expression also can be observed on the $CD56^+/CD3^-$ cells with NK cell morphology, and frequently juxtaposed to single-positive $CD3^+$ T cells or to single-positive $CD83^+$ cells, in the T cell areas of LNs (Fig. 3 C).

Because the induction of CD83 and CCR7 on maturing DCs (and the development of their responsiveness to CCR7 ligation) are known to be supported by another inflammatory mediator prostaglandin $(PG)E_2$ (28, 29), we tested whether PGE_2 also is involved in the regulation of the migratory ca-

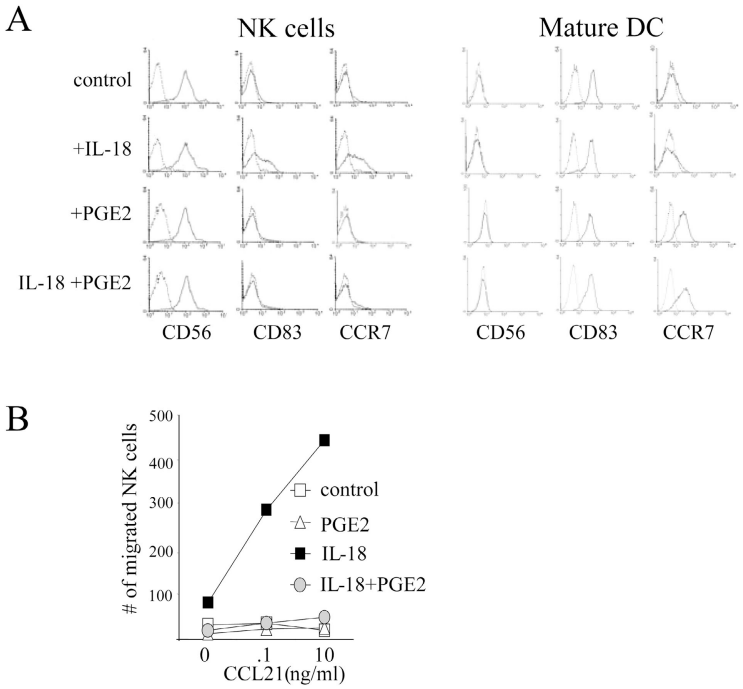

Figure 4. Opposing roles of PGE_2 in the induction of $CD83^+/CCR7^+/CD25^+$ DCs versus NK cells. Freshly isolated NK cells or TNF-α–activated monocyte-derived DCs were treated with IL-18, PGE_2, or their combination. (A) PGE_2 supports the induction of the chemokine receptor CCR7 on maturing DCs, but prevents the induction of CCR7 expression on IL-18–treated NK cells. Although IL-18 does not induce CCR7 expression on mature DCs, it does induce the expression of CCR7 and CD83 on NK cells. (B) PGE_2 prevents the acquisition of the migratory responsiveness to CCL21 in IL-18–primed NK cells. Data from one of three experiments that produced similar results.

pacity of NK cells. In contrast to TNFα-activated CD83$^+$ DCs, which responded to PGE_2 with the enhanced expression of CCR7, NK cells did not increase their CCR7 expression in response to PGE_2 alone or in combination with IL-18 (Fig. 4 A). Instead, PGE_2 completely blocked the IL-18–dependent induction of CCR7 and CD83 on NK cells (Fig. 4 A). As expected, the ability of PGE_2 to block CCR7 expression was accompanied by its ability to abolish the migratory responsiveness of NK cells to CCL21 (Fig. 4 B). Conversely, although IL-18 primed NK cells for CCR7 expression, it could not replace PGE_2 in the induction CCR7 expression on TNFα-activated DCs (Fig. 4 A). These data indicate that the CCL21 responsiveness of DCs and NK cells is regulated by different inflammatory mediators, PGE_2 and IL-18, that display opposing impacts on NK cell activation and acquisition of migratory responsiveness to LN-associated chemokines.

Migratory CD83$^+$/CCR7$^+$/CD25$^+$ NK cells produce IFN-γ upon exposure to Th cell– and DC-related signals: functional stability of the "helper" pathway of NK cell differentiation

Induction of LN-homing receptors and CD25 on the IL-18–treated NK cells prompted us to analyze their responsiveness to IL-2, a product of activated Th cells, and to other factors that are likely to be encountered in the T cell areas of the LNs.

IL-18–pretreated NK cells, generated from flow-sorted CD56^{dim} NK cell population (Fig. 5 A) or from "bulk" CD56$^+$/CD3$^-$ NK cells (Fig. 5, B and C), were washed extensively to remove IL-18 and were exposed for 24 h to

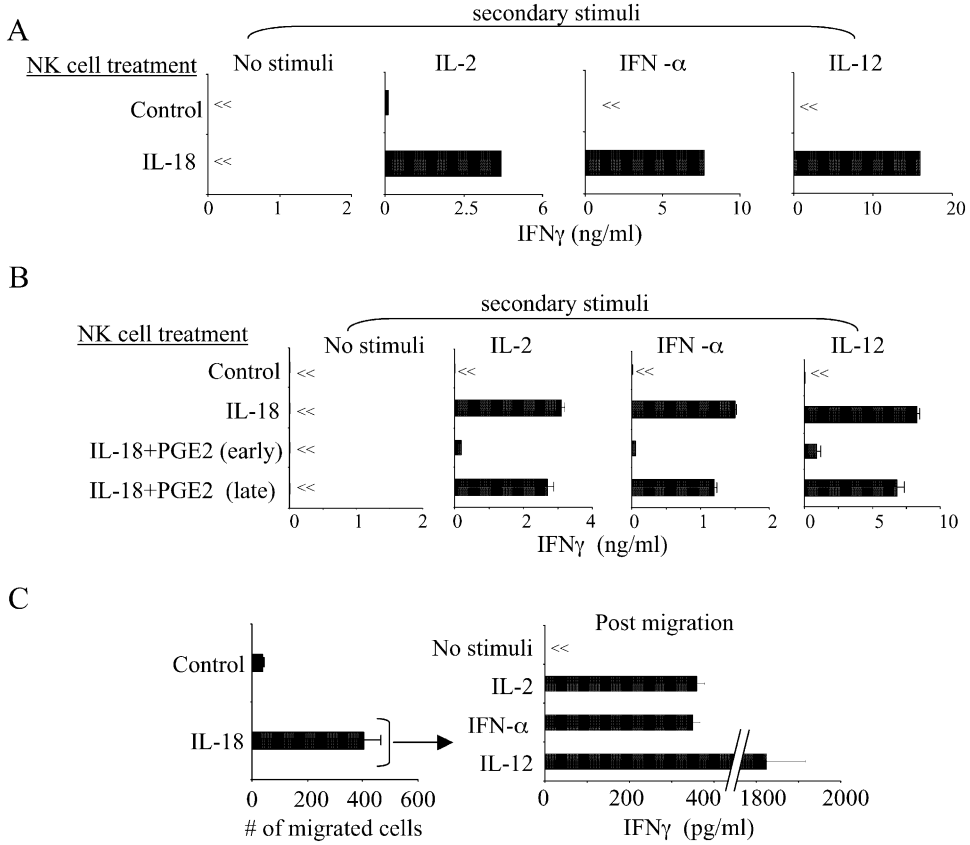

Figure 5. IL-18–induced LN-homing NK cells produce high levels of IFN-γ upon subsequent exposure to MDC-, PDC-, or Th cell–related signals: functional stability of IL-18–primed NK cells. (A) Flow-sorted CD56^{dim} NK cells were treated with IL-18 (1 μg/ml) for 24 h before the subsequent induction of the IFN-γ production with "secondary stimuli." At 24 h, the cells were washed extensively to remove IL-18 and tested for their ability to produce IFN-γ in response to IL-2, IFN-α, or IL-12. (B) Freshly isolated "bulk" CD56$^+$ NK cells were treated with IL-18 (1 μg/ml) for 48 h in the absence or presence of PGE_2. To analyze the functional stability of IL-18–induced NK cells, PGE_2 was added at the beginning of IL-18-priming cultures, or after 24 h, before the subsequent induction of the IFN-γ production in IL-18–primed NK cells. At 48 h, the cells were washed extensively to remove these factors and tested for their ability to produce IFN-γ in response to secondary stimuli: IL-2, IFN-α, or IL-12. Although the addition of PGE_2 at the onset of IL-18 priming inhibits NK cell responsiveness to the secondary stimuli, delayed addition of PGE_2 is completely ineffective. Similar data also were obtained when using IL-15 as a secondary signal (not depicted). (C) IL-18–primed NK cells retain the ability to respond to the secondary IFN-γ–inducing signals, following the CCL21-induced migration. (Left panel) NK cells were cultured in the presence of IL-18 for 48 h, harvested, and assessed for their migratory responsiveness to CCL21 (10 ng/ml) in chemotaxis assay. (Right panel) Following their migration, the CCL21-responsive NK cells were tested for their ability to produce IFN-γ upon the secondary stimulation with IL-2, IFN-α, or IL-12. <<Below the detection limit. Each of the experiments was repeated at least two times.

IL-2, IFN-α, or IL-12, respective products of Th cells, and PDCs and MDCs. Although the IL-18–pretreated NK cells did not produce any detectable levels of IFN-γ spontaneously, their subsequent exposure to IFN-α, IL-12, or IL-2 induced high levels of IFN-γ production. In accordance with previous observations that showed the two-signal requirement for efficient IFN-γ induction in resting NK cells (12), control NK cells were not responsive to any of these individual stimuli (Fig. 5, A–C). In analogy to the CCR7 expression and the responsiveness to LN-associated chemokines, the presence of PGE_2 during the IL-18 priming prevented the acquisition of the ability of NK cells to produce IFN-γ in response to subsequent IFN-α, IL-12, or IL-2 exposure (Fig. 5 B). PGE_2 could prevent the responsiveness of NK cells to these secondary signals only when it was added simultaneously with IL-18 (Fig. 5 B) or before this factor (not depicted). In contrast, the ability of the IL-18–primed NK cells to produce IFN-γ in response to the above LN-associated stimuli could no longer be suppressed by their subsequent exposure to PGE_2; this demonstrated the functional stability of the IL-18–induced NK cell population (Fig. 5 B).

The functional stability of the IL-18–induced $CD83^+$/$CCR7^+$/$CD25^+$ NK cells was underscored further by their ability to respond to the IFN-γ–inducing secondary stimuli after their CCL21-induced migration and subsequent stimulation of the migrated NK cells (Fig. 5 C). These data also demonstrate formally that the CCR7-mediated migratory function and the ability to produce IFN-γ in response to Th cell and PDC- and MDC-associated stimuli are expressed by the same individual IL-18–induced NK cells that codisplay the selectively enhanced LN-homing and IFN-γ–producing functions, without any enhancement of their cytotoxic functions.

IL-18–induced NK "helper" cells support IL-12p70 production and promote DC-mediated induction of Th1 responses, whereas IL-2–primed "cytotoxic" NK cells kill DCs

Because the IL-18–induced $CD83^+$/$CCR7^+$/$CD25^+$ NK cells acquired the migratory responsiveness to secondary lymphoid tissue–expressed chemokines and the ability to produce IFN-γ upon secondary stimulation with LN-associated signals, we tested how such IL-18–primed NK cells would impact mature DCs—the type of DC that is involved in naive Th cell priming—and the development of Th1 and Th2 cells from naive $CD4^+$ T cells.

Although DCs are resistant to lysis by recently activated NK cells (12), prolonged NK cell activation can result in the acquisition of their DC-killing function (11). In accordance with this last possibility, IL-2–activated "killer" NK cells efficiently lysed the LPS-matured DCs (Fig. 6 A). In sharp contrast, IL-18–dependent NK cell activation was not associated with the induction of its DC-killing ability against mature DCs (Fig. 6 A), day 6 immature DCs (not depicted), or tumor cell targets (Fig. 1 E).

In contrast with the IL-2–primed NK cells that effectively eliminated the encountered DCs, the interaction of IL-18–pretreated NK cells with DCs resulted in a low, but distinct, production of IFN-γ. As shown in Fig. 6, in the absence of the Th cell–related signal, CD40L, IL-18–primed NK cells produced only very low levels of IFN-γ upon their interaction with mature DCs. Conversely, mature DCs that were stimulated with CD40L alone produced only low levels of IL-12. In accordance with the requirement for CD40L stimulation and IFN-γ in the effective IL-12p70 induction in DCs (30, 31), and in turn, with the ability of IL-12 to induce IFN-γ production in IL-18–primed NK cells (see Fig. 3 A), the presence of IL-18–induced helper NK cells during the CD40L-dependent DC stimulation resulted in a 10-fold increase in IL-12p70 production, and an even more pronounced (50-fold) enhancement of IFN-γ production (Fig. 6). This ability of IL-18–primed NK cells to support the IL-12 induction in DCs and the ability of CD40L-dependent signals to enhance the efficiency of NK–DC interaction with regard to the DC-dependent induction of IFN-γ in NK cells and the induction of IL-12 production by DCs, indicates the existence of a double-positive feedback that involves the IL-18–primed

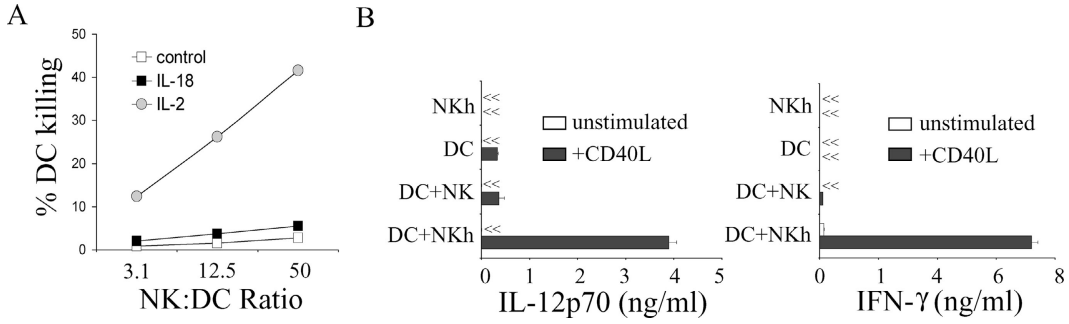

Figure 6. IL-2–primed "effector" NK cells kill DCs, whereas IL-18–primed NK helper cells enhance the ability of DCs to produce IL-12p70. (A) NK cells isolated from peripheral blood were treated with IL-2 or IL-18 for 48 h and used as effectors to test their ability to kill the $CD83^+$ DCs matured by LPS in a 4-h ^{51}Cr-release assay. (B) LPS-matured DCs were stimulated with CD40L-transfected cell line J558 in the absence or presence of NK helper (NKh) cells. Supernatants were collected at 24 h and tested for the presence of IL-12p70 (left panel) and IFN-γ (right panel). Data from one representative experiment of three performed. Similar results also were obtained using soluble rhCD40L instead of J558-CD40L. <<Below the detection limit.

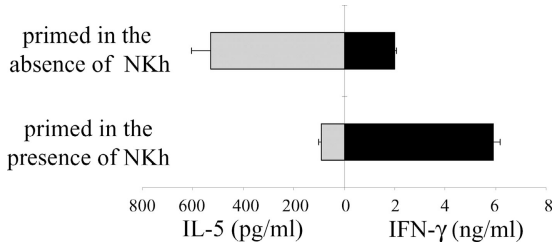

Figure 7. NK helper cells promote the DC-induced development of Th1 cells. In vitro Th cell priming, staphylococcal enterotoxin B–coated LPS-matured DCs were used to stimulate naive $CD4^+$ Th cell proliferation in the absence or presence of γ-irradiated (2,000 rad) IL-18–induced NK helper (NKh) cells. At day 10, the resulting Th cell cultures were restimulated in neutral conditions with activating anti-CD3 and anti-CD28 antibodies. 24-h supernatants were tested for the presence of the respective Th1- and Th2-type cytokines, IFN-γ and IL-5. Similar data were obtained in one additional experiment.

NK cells, DCs, and naive Th cells during the induction of Th1-dominated cell-mediated immunity

In accordance with this possibility, and along with the essential role of IL-12 in the induction and persistence of functional Th1 responses (32), the presence of the IL-18–induced NK "helper" cells during priming of naive $CD4^+$ Th cells promoted their Th1 pathway of differentiation, manifested by their production of enhanced levels of IFN-γ and decreased IL-5 levels upon restimulation (Fig. 7).

DISCUSSION

Our current data demonstrate that in contrast to IL-2 that selectively promotes the cytotoxic "effector" functions of NK cells, but may limit their ability to promote the DC-mediated induction of Th1 responses, IL-18 induces a distinct "helper" pathway of NK cell differentiation. IL-18–induced $CD56^+/CD83^+/CCR7^+/CD25^+$ helper NK cells express several surface markers of mature DCs, show high migratory responses to LN-associated chemokines, and show pronounced abilities to support the IL-12p70 production of DCs and the induction of Th1 responses of $CD4^+$ Th cells.

In analogy to T and B lymphocytes, as well as DCs, whose functions are guided by the conditions of their priming, but which become relatively independent from environmental cues during their mature/memory/effector phase of activity (33), the IL-18–induced "helper" subset of NK cells also displays a striking functional stability. IL-18–primed NK cells modify the pattern of their responsiveness to such cytokines as IL-2, IL-12 and IFN-α, that is not affected by the withdrawal of IL-18, nor by subsequent NK cell migration in response to CCL21, the chemokine that is essential for the entry of immune cells to the T cell areas of LNs (15–17). Moreover, although the ability of NK cells to develop such "helper" function can be prevented effectively at the stage of NK cell priming by PGE_2, an inflammatory mediator with Th2-driving activity that is mediated at the level of Th cells (34–36) and DCs (37), NK cells that have developed the $CD83^+/CCR7^+/CD25^+$ helper status become resistant to inhibition (Fig. 3 B).

The current data suggest that the reciprocal impact of IL-18 and PGE_2 on the development of helper NK cells constitutes an additional mechanism of the respective Th1- versus Th2-promoting activities of these factors. Although IL-18, identified as an IFN-γ–inducing factor (38), is known to support IFN-γ production in multiple cell types, including NK cells (25), the ability of IL-18 to induce the migratory responsiveness of NK cells to CCL21 and to sensitize NK cells to Th cell–, MDC-, and PDC-associated activating signals, may make IL-18 especially powerful in promoting the DC-mediated development of primary Th1 responses. Similarly, although PGE_2 can suppress Th1 responses by directly inhibiting IFN-γ production in Th cells and promoting their alternative Th2 pathway of differentiation (34–36) and by suppressing the Th1-driving impact of IL-12p70 at the levels of IL-12 production (37, 39), by suppressing the responsiveness to this factor (40) and by inducing IL-12 antagonistic p40 homodimer (41), the ability of PGE_2 to interfere with the development of LN-homing helper NK cells is likely to constitute an additional powerful mechanism of the Th2-promoting activity of PGE_2. Such a mechanism is likely to be particularly strong in chronic inflammatory states where continued PGE_2 overproduction (42, 43) may prevent the induction of NK cell helper status, and promote a progressive drift toward Th2 immunity in chronic infections, atopic allergy, or cancer.

IL-18 induced the CCR7 expression selectively on the main population of $CD56^+/CD16^+/CCR7^-$ blood NK cells (Fig. 2), rather than the substantially smaller subset of circulating $CD56^{bright}/CD16^-$ NK cells that express low levels of CCR7 without prior activation (22, 24). This suggested that the NK helper status can be induced in most or all resting NK cells as an alternative to their IL-2-driven "effector" pathway of differentiation. The $CD56^{bright}/CD25^+/CD16^{-/dim}$ phenotype, induced particularly effectively in the additional presence of such T cell–derived factors as IL-2, is similar to the NK cells that are found within secondary lymphoid tissue and peripheral blood (22–24, 44). Because the expression of CD83 disappeared from NK cell surfaces upon their prolonged culture, the phenomenon associated with the strong reduction in CCR7 expression, further enhancement of CD56 expression, and complete loss of CD16 from NK cell surface, it is conceivable that the IL-18–induced $CD56^+/CCR7^+/CD83^+/CD25^+/CD16^{dim}$ NK cells may represent precursors of the $CD56^{bright}/CD16^-$ NK cells that are present in the LNs that subsequently emigrate to peripheral blood and display the ability to produce IFN-γ upon their interaction with activated DCs (22, 24). The presence of CD56 on a small subset of $CD83^+$ cells (classically considered as a reliable marker of mature DCs) in the T cell areas of LNs (Fig. 3 C), and to a lesser extent in the inflamed tonsils (data not shown), supports this last possibility, although low frequency of these cells (5–16% of the $CD56^+$ cells) did

not permit us to isolate and characterize this population with regard to their function. Low frequency of such cells (at least in the absence of acute immune responses that are associated with high IL-18 production), and the possibility of their selective elimination from the sites of chronic inflammation (by such factors such as PGE_2) are in accord with the report from Ferlazzo and colleagues (23), who observed a strongly reduced frequency of NK cells in the chronically inflamed surgically removed tonsils, compared with LNs.

Although we could not observe the expression of CCR7 on >50% of NK cells that were treated with IL-18 alone in any of the experiments, the additional simultaneous or follow-up exposure of the IL-18–treated cells to IL-2 (or to IL-12) induced further elevation of CCR7 expression on all of the NK cells, and was associated with the complete down-regulation of CD16 expression (Fig. 2 A; Fig. 3 B; not depicted). Although the significance of these last observations is not completely clear, they may represent the ability of Th cell– or DC-derived signals to transiently stabilize the IL-18–induced CCR7 expression, and facilitate the effective contribution of NK cells to the Th1 cell priming. After a short period of interaction with DCs and activated T cells, NK cells may down-regulate CCR7 expression and leave the T cell areas in analogy to antigen (Ag)-activated B cells, whose short-term transition from B cell follicles to the T cell areas (to receive T cell helper signals) and back is associated with a transient induction of CCR7 expression (45). This possibility is in accord with our observations that although short-term IL-2 exposure enhances the CCR7 and CD83 expression on IL-18–treated NK cells (Fig. 2 A), both of these markers are down-regulated within the subsequent 72–96 h.

Based on the current data and previous reports from human and mouse systems (9–14), we foresee the following possible mechanisms of the IL-18– and NK cell–mediated support for primary Th1 responses. First, peripheral tissue interaction of NK cells with DCs, in the presence of proinflammatory stimuli (e.g., IL-18 and IFN-α), can result in a stable IFN-γ– and TNFα-mediated polarization of DCs. This instructs DCs to produce high levels of IL-12 during subsequent interaction with LN-based naive Th cells and to induce type-1 responses (12), without the need for tissue-activated NK cells to enter LNs. A second possibility, which is supported by the current data, is that the peripheral activation of NK cells by IL-18 that is produced by pathogen-activated macrophages or immature DCs induces NK cell responsiveness to CCL21 that is produced by lymphatic endothelium. This allows NK cell entry into local lymphatics and their subsequent direct interaction with the CCL21-producing (46) interdigitating DCs in the T cell areas of the draining LNs. In this scenario, NK cell–derived IFN-γ can directly co-stimulate the CD40L-induced IL-12 production in DCs that interact with CD40L-expressing naive Th cells (30, 31) in the T cell areas of LNs, where NK cells mostly can be seen as adjacent to DCs (Bajenoff, M., personal communication).

In addition to the CCR7-dependent immigration of NK cells from pathogen-invaded peripheral tissues, NK cells also may be recruited directly from circulation, in a process that is dependent, at least in the mouse, on CXCR3 (13). It remains unclear whether the CXCR3-mediated pathway allows NK cells to enter the T cell areas, or facilitates their interaction with migrating DCs in the marginal zone. In this last scenario, NK cells could deliver the DC-mediated Th1-inducing signals (13) by way of the stable enhancement of the Th1- and CTL-promoting functions of migrating DCs (12), or receive the additional IL-18–dependent signal in the marginal zone to promote their further CCR7-dependent migration toward the CCL21-producing DCs in the T cell areas.

In the mouse system, although CXCR3 may route NK cells directly to CCL21-producing DCs in the T cell areas of the LNs as a result of the ability of mouse CCL21 to interact with CCR7 and CXCR3 (18), the CCL21–CXCR3 interaction may not be applicable to the human system (19, 20). Therefore, the initial recruitment of human NK cells to the T cell areas and the resulting first wave of NK cell–dependent IFN-γ production are more likely to depend on CCR7, whereas the IFN-γ–inducible chemokines, CXCL-9 (MIG), CXCL-10 (IP-10), and CXCL-11 (I-TAC), are more likely to facilitate later-stage CXCR3-dependent NK cell recruitment and contribute to the stabilization of a Th1-type response pattern. In accordance with this last possibility, although we could not detect the induction of CXCR3 on IL-18–primed NK cells (not depicted), CXCR3 expression can be induced upon prolonged IL-2 exposure of human NK cells (48; not depicted).

In addition to the aforementioned possibilities of the recruitment of NK cells to the LNs under inflammatory conditions, $CD56^{bright}$ NK cells may arise from $CD34^+$ precursors directly in the LNs, as indicated by the ability of $CD34^+$ LN precursors to form $CD56^{bright}$ NK cells in cultures that were supplemented with IL-2 or IL-15 (47).

An intriguing aspect of the current study is the ability of the IL-2–primed NK cells to kill mature DCs (Fig. 6 A). During primary responses, the exposure of NK cells to IL-2 represents a verification of the successful induction of $CD4^+$ T cell responses by Ag-carrying DCs, in which case the NK cell–mediated DC killing may serve a positive role by eliminating the already unnecessary DCs from the LNs. However, at a later stage of immune responses, NK cells that are pre-exposed to memory Th cell–produced IL-2 may facilitate the termination of active immunity by eliminating the subsequently encountered Ag-carrying DCs and preventing the activation of additional T cells. Although the available in vivo reports uniformly demonstrate a proimmunogenic Th1-driving role of NK cells (13, 14), the possible autoregulatory role of IL-2–exposed NK cells in self-limitation of immune responses (analogous to the IL-2–dependent regulatory T cells (49, 50), in chronic inflammatory states, autoimmune diseases, or as a limiting factor during the systemic IL-2 treatment of patients who have cancer, remain to be addressed.

The current data, which show the ability of NK cells to acquire CD83 expression, suggest that this last molecule (CD83) cannot be considered as a selective marker of mature LN-associated DCs and the circulating mature DC in peripheral blood (51–53). They highlight a striking similarity between DCs and NK cells, and between the processes of DC maturation and the development of the helper status in NK cells; in both cases, these lead to the acquisition of a distinct $CCR7^+/CD83^+/CD25^+$ surface phenotype, LN migratory function, and the ability to interact effectively with Th cells and with each other. Both of these processes also are associated with the down-regulation of surface Fcγ receptors: CD16 on IL-18–primed NK cells (current data) and CD32 on maturing DCs (54, 55). At an additional level of similarity, the inhibitory impact of PGE_2 on the priming of NK cells for subsequent production of IFN-γ in response to Th cell–associated IL-2 closely parallels the ability of PGE_2 to suppress the ability of DCs to produce IL-12p70 during the interaction with CD40L-expressing Th cells (55). The responsiveness of both of these cell types to PGE_2 is terminated at their respective "mature" (55) and "helper" (Fig. 5 A) stages of differentiation.

The current data demonstrate that, depending on the conditions of their activation, resting NK cells are able to follow a distinct helper pathway of differentiation, as opposed to the "killer" pathway of differentiation. They also highlight the existence of an additional level of the antagonistic activity of IL-18 versus PGE_2 in the regulation of the Th1/Th2 patterns of immune responses that is mediated by the reciprocal impact of these cytokines on NK cell differentiation. During acute infections—typically associated with the induction of inflammatory-type immunity—production of factors (e.g., IL-18, IFN-α, TNFα) is likely to promote the simultaneous accumulation of NK cells and DCs in the LNs and their interaction, which supports the development of type-1 responses. Such interaction is likely to be suppressed during chronic phase of immunity, when PGE_2 that is overexpressed at the sites of chronic inflammation (42, 43) selectively promotes the LN migratory activity of DCs (28, 29), and prevents NK cells from developing their LN-homing ability and helper activity. These contribute to the progressive Th1 to Th2 shift that is associated with chronic infections and cancer (56–59).

MATERIALS AND METHODS

Culture media, reagents, and cell lines. Serum-free AIM-V medium (GIBCO BRL and Invitrogen) was used for short-term culturing of isolated human NK cells. Cell lines and human DCs were cultured in IMDM containing L-glutamine supplemented with 1% penicillin/streptomycin and 10% FBS, all of which were purchased from GIBCO BRL. The following cytokines and reagents were used to culture and activate isolated NK cells, to obtain immature DCs, to activate and induce maturation of DCs, or to culture and activate $CD4^+$ Th cells: IL-18 (Glaxco-SmithKline); IL-2 (Chiron Corp); IFN-α (Intron A- IFN-α-2b; Schering-Plough); *rhu*IL-12 (Genetics Institute) and soluble trimeric *rh*CD40L (sCD40LT) (gifts from Immunex, Seattle, WA), and purchased from Alexis; *rhu* GM-CSF and IL-4 (Schering-Plough); rhuTNF-α and rhuIL-1β (both from Strathmann Biotech Gmbh); and PGE_2 (Sigma-Aldrich). Anti–human CD3 and CD28 stimulating mAb's were purchased from CLB. The CD40L-transfected J558 plasmacytoma cell line was a gift from P. Lane (University of Birmingham, Birmingham, England,UK). The Daudi cell line (susceptible to activated NK cells, but not resting NK cells) and K562 cells (susceptible to freshly isolated NK cells) were gifts from T. Whiteside (University of Pittsburgh, Pittsburgh, PA).

Cell isolation. Mononuclear cells from peripheral blood of healthy donors were isolated by density gradient separation using Lymphocyte Separation Medium (Cellgro Mediatech). Resting NK cells ($CD56^+$, $CD3^-$, $CD20^-$, $HLA\text{-}DR^-$) and naive ($CD45RA^+/RO^-$) $CD4^+$ T cells were isolated by negative magnetic selection using the StemSep system (StemCell Technologies Inc.). Isolated populations had >94% purity as determined by flow cytometry; most of the contaminating cells were negative for common lineage markers. Contamination with $CD3^+$, $CD20^+$, and $HLA\text{-}DR^+$ cells was each <1%. When indicated, $CD3^-CD56^{dim}/CD16^+$ and $CD3^-/CD56^{bright}/CD16^-$ NK cell subsets were flow-sorted using MoFlo high-speed cell sorter (DakoCytomation), after labeling with the appropriate antibodies.

Flow cytometry. Two- and three-color cell surface immunostaining analysis was performed using a Beckman Coulter Epics XL Flow Cytometer. FITC- and PE-labeled anti–human CD3, CD45RO, CD45RA, CD16, CD25, and the corresponding isotype (mouse–IgG1) control mAb's were purchased from BD Biosciences. FITC- and PE-labeled anti–human CD83 (IgG2b- clone HB15a), CD86 (IgG2b), PC5-labeled anti–human CD56 (IgG1), and the corresponding isotype (FITC- and PE-mouse IgG2b and PC5-mouse IgG1) control mAb's were purchased from Coulter Immunotech. The FITC-labeled anti–human CCR7 (IgG2a- clone #150503) mAb was purchased from R&D Systems; the corresponding control isotype (mouse IgG2a) was purchased from Coulter Immunotech. Rabbit polyclonal antibody against human IFN-αR chain 2 (β-chain) was obtained from Research Diagnostics, Inc. Th antibody against human IL-12Rβ2 chain was a gift from S.F. Wolf (Genetics Institute, Andover, MA). Before all stainings, the cells were treated for 15 min in PBS (GIBCO BRL) containing 10% human serum (Atlanta Biologicals) to block nonspecific Fc binding sites. Immunostaining of cells was performed in PBS buffer, containing 2% human serum, and 0.1% NaN_3. Samples were fixed and stored in a 1% paraformaldehyde solution until cell analysis. For the analysis of NK cell division, the cells were stained with 5 μM CFSE (Invitrogen) for 10 min, before being washed three times and cultured.

NK cell culture. Isolated NK cells were cultured in serum free AIM-V medium (addition of serum resulted in the inhibition of the IL-18–dependent development of $CD83^+/CCR7^+$ NK cells) in 48-well plates (Falcon, Becton Dickinson) at a concentration of 10^6 cells/m in the absence or presence of IL-18 (100 pg/ml–1 μg/ml). The duration of the short-term cultures of differentially activated NK cells ranged between 24 h and 48 h. When indicated, NK cells also were exposed to IL-2 (250 IU/ml), IFN-α (1,000 IU/ml), IL-12 (1 ng/ml), and PGE_2 (10^{-6} M), either alone, in combination with IL-18 (1 μg/ml), or after IL-18 pretreatment. Alternatively, NK cells were co-cultured for 24 h with 6-d GM-CSF–generated (plastic adherent) macrophages (2×10^5 cells/ml) in the presence of LPS (250 ng/ml) or CD40L (1 μg/ml).

Cytokine production by NK cells. Cultured NK cells were washed extensively to remove excess cytokines, placed in fresh AIM-V medium, and plated in 96-well round bottom plates at 10^5 cells/200 μl/well. The untreated control and IL-18–treated NK cells were tested for their ability to produce IFN-γ spontaneously or in response to subsequent exposure to IL-2 (250 IU/ml), IFN-α (1,000 IU/ml), or IL-12 (1 ng/ml). IFN-γ levels in 24-h supernatant were measured by ELISA.

Cytolytic activity of NK cells. Cytolytic activity was determined by performing standard 4-h ^{51}Cr-release assays. The results were calculated and recorded as lytic units or percent target killing as described (12).

Three-color in situ analysis of human LN material. All human material was obtained under protocols approved by our Institutional Review Board. Sections (7-μm) of noninvolved LNs from patients who underwent tumor resection were washed in PBS with 4% BSA and labeled with mouse anti–human CD56-PC5 (Immunotech), followed by Cy3-conjugated Fab goat anti–mouse mAb (Jackson ImmunoResearch Laboratories) and overnight blocking with unconjugated Fab goat anti–mouse mAb (Jackson ImmunoResearch Laboratories). Tissue was incubated with mouse anti–human CD83 FITC (Immunotech) and rabbit anti–human CD3 mAb (DakoCytomation) followed by a secondary Alexa 488–labeled goat anti–mouse (Invitrogen) and goat anti–rabbit Cy5 (Jackson ImmunoResearch Laboratories). The sections were analyzed using Olympus BX51 fluorescent microscope (Olympus America Inc.) and Magnafire software (Optoronix).

Generation of human DCs. Human monocytes were isolated from peripheral blood of healthy donors using a two-step process. Mononuclear cells were isolated by density gradient separation using Lymphocyte Separation Medium (Cellgro Mediatech). Monocytes were isolated from mononuclear cell fraction using a Percoll (Sigma-Aldrich) density separation technique, followed by plastic adherence, as described (36). To obtain immature "day 6 DCs" (53), monocytes were cultured for 6 d in 24-well plates (Falcon, Becton Dickinson) at 5×10^5 cells per well in IMDM supplemented with L-glutamine (1%), penicillin/streptomycin (1%), 10% FBS, *rhu* GM-CSF, and IL-4 (both 1,000 IU/ml). The typical cultures yielded 2–3 $\times 10^5$ per well of $CD14^-$, $CD86^+$, $CD83^-$ "immature DCs."

DC maturation. The following DC maturation factors were used (as described in the individual experiments) and added to day 6 DC cultures at these concentrations: TNFα (50 ng/ml), IL-1β (25 ng/ml), PGE_2 (10^{-6} M), and LPS (250 ng/ml), as described (41, 55). In all cases, after 48 h, the $CD83^+$ DCs were harvested and analyzed for their expression of maturation-associated surface markers, co-culture with NK cells, and their ability to produce IL-12p70 in response to CD40L stimulation.

Determination of the IL-12p70-producing capacity of DCs. To determine the IL-12p70–producing capacity of DCs, the cells were harvested, washed, and plated in flat bottom 96-well plates at 2×10^4 cells/well and stimulated with soluble trimeric CD40L protein (1 μg/ml), or with CD40L-transfected J558 cells (5×10^4) in the presence or absence of differentially activated NK cells (5×10^4). Supernatants were collected after 24 h and tested for the presence of IL-12p70 by ELISA.

Cytokine measurements. Concentrations of IL-12p70, IFN-γ, and IL-5 were determined by specific ELISAs, performed with matched antibody pairs, standards, and reagents from Endogen. IL-18 concentrations were measured by ELISA using matched antibody pairs and standards from Medical and Biological Laboratories Co.

Chemotaxis. Chemokine-induced cell migration was measured using the 96-well 5-μm pore ChemoTx disposable chemotaxis system (Neuro Probe). Various concentrations of CCL21 (6C-kine; Biosource International) in AIM-V medium were added to the appropriate wells and below the membrane. NK cells (3×10^4 in 30 μl AIM-V medium) were placed on the membrane surface and incubated for 90 min at 37°C. The cells remaining on the topside of the membrane were removed by gentle washing with cold PBS and wiping, followed by removal of the membrane. The migrated cells were viewed at the bottom of the wells after gentle centrifugation (2 min at 100 *g*) of the assay plates. Using an inverted microscope, cells were examined at 10×, and cell counts within four nonoverlapping random areas (1 mm^2) were recorded. Results are expressed as the mean sum of the four areas ± SEM for duplicate wells. To determine the IFN-γ–producing capacity of the CCL21-responsive migratory NK cells, IL-2 (250 IU/ml), IL-12 (1 ng/ml), or IFN-α (1,000 IU/ml) was added directly to wells containing the migrated NK cells; 24-h supernatants were collected and analyzed by ELISA as described earlier.

Differential priming of Th cells. LPS-matured DCs were harvested and pulsed with staphylococcal enterotoxin B (1 ng/ml) for 1 h (30, 37). The SEB-coated DCs were washed and placed into culture (2×10^4 cells/well) with naive ($CD45RA^+/RO^-$) $CD4^+$ Th cells (10^5 cells/well) in the presence or absence of autologous irradiated (2,000 rad) IL-18–treated NK cells. On day 4, rhIL-2 (50 IU/ml) was added to the cultures. At day 10, the expanded $CD4^+$ Th cells were washed and counted, plated in 96-well plates (10^5 cells/well), and stimulated with CD3 (1 μg/ml) and CD28 (2 μg/ml) mAb's (30, 35, 37). The supernatants from restimulated $CD4^+$ Th cells were collected after 24 h and analyzed by ELISA for the presence of IL-5 and IFN-γ.

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