RESEARCH PAPER

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IL-18-based combinatorial adjuvants promote the intranodal production of CCL19 by NK cells and dendritic cells of cancer patients

Jeffrey L Wong[1], Ravikumar Muthuswamy[1], David L Bartlett[1,2], and Pawel Kalinski[1,2,3,*]

[1]Department of Surgery; University of Pittsburgh; Pittsburgh, PA USA;
[2]University of Pittsburgh Cancer Institute; Hillman Cancer Center; University of Pittsburgh; Pittsburgh, PA USA;
[3]Departments of Immunology and Infectious Diseases and Microbiology; University of Pittsburgh; Pittsburgh, PA USA

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Abbreviations: CD40L, CD40 ligand; CFSE, carboxyfluorescein diacetate succinimidyl ester; CTL, cytotoxic T lymphocyte; DC, dendritic cell; GM-CSF, granulocyte macrophage colony-stimulating factor; GzmB, granzyme B; IFN, interferon; IL, interleukin; LN, lymph node; NK, natural killer; PBMC, peripheral blood mononuclear cell; SEB, staphylococcal enterotoxin B; sIFNγR1, soluble IFNγ receptor 1; sTNFR1, soluble TNFα receptor 1; TLS, tertiary lymphoid structures; TNFα, tumor necrosis factor α

The effective accumulation and interaction of mature dendritic cells (DCs) and naïve T cells within lymph nodes (LNs), which are driven by the CCR7-CCL19/CCL21 chemokine axis, are critical for the induction of adaptive T-cell immunity. Human natural killer (NK) cells activated by interleukin (IL)-18 exhibit a unique 'helper' activity in promoting productive DC-T cell interactions, inducing DC maturation and shifting DC-primed T-cell responses toward a T_H1 polarization. Here, we demonstrate that such IL-18-activated 'helper' NK cells uniquely stimulate DCs to produce high levels of CCL19 through tumor necrosis factor α (TNFα) and interferon γ (IFNγ), a process that relies on secondary NK-cell activation by additional inflammatory signals including IFNα, IL-15, IL-12 and IL-2. DCs activated by helper NK cells not only promote the efficient CCR7-mediated recruitment of naïve $CD8^+$ T cells, but also stimulate their expansion and expression of granzyme B. Using an ex vivo explant culture system based on LNs isolated from colorectal cancer patients, we found that CCL19 is upregulated in human tumor-associated lymphoid tissues treated with helper NK cell-stimulating factors. Our findings demonstrate the ability of 2 signal-activated helper NK cells to promote the production of the DC- and naïve/memory T cell-attracting chemokine CCL19 in LNs, and provide a rationale for the therapeutic application of IL-18-containing 'combinatorial adjuvants' to facilitate the induction of antitumor immune responses.

Introduction

The induction of effective T-cell responses requires the productive interaction between antigen-presenting dendritic cells (DCs) and naïve or memory T cells in lymph nodes (LNs). Chemokine (C-C motif) receptor 7 (CCR7) and its main ligands, namely, chemokine (C-C motif) ligand 19 (CCL19) and CCL21, play an essential role in this process, governing the entry of both $CCR7^+$ naïve T cells and activated DCs into LNs,[1,2] their co-localization within the T-cell zones of the LN paracortex,[3] and their efficient dynamic interaction.[4–7] The CCR7-CCL19/CCL21 chemokine axis has likewise been implicated in the optimal recruitment of central memory T cells to LNs,[8,9] facilitating the DC-driven activation of recall responses.

Natural killer (NK) cells have been shown to play key 'helper' roles in directing the DC-mediated priming of adaptive T-cell immunity.[10–12] In particular, NK cells activated by interleukin (IL)-18, a cytokine that is elaborated early in response to tissue damage, infection, or transformation,[13] have been demonstrated to uniquely localize to sites of DC-T cell interaction and provide an important early source of interferon γ (IFNγ) and tumor necrosis factor α (TNFα), promoting DC maturation and polarizing priming toward type 1 helper $CD4^+$ T-cell (T_H1) and $CD8^+$ cytotoxic T lymphocyte (CTL) responses.[14–17] However, it remains unknown whether NK cells can also promote the recruitment of T cells to LNs and their effective interaction with DCs.

Here, we demonstrate that human 2 signal-activated "helper" NK cells, which are uniquely induced upon exposure to IL-18 and secondary pro-inflammatory signals, instruct DCs to secrete high levels of the CCR7 ligand CCL19, driving the efficient DC-mediated recruitment of naïve T cells to LNs as well as subsequent T-cell expansion and acquisition of effector molecules. Moreover, we demonstrate that "IL-18 plus one" adjuvants induce

*Correspondence to: Pawel Kalinski; Email: kalinskip@upmc.edu
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high CCL19 expression levels within the LNs of colorectal cancer patients. These findings have important implications for the prospective clinical application of IL-18-containing combinatorial adjuvants.

Results

2 signal-activated NK cells stimulate DCs to produce high levels of CCL19

We have previously reported the selective ability of IL-18, combined with various secondary stimuli, to promote a unique pathway of human NK cell differentiation. "Helper" NK cells differentiating under these conditions support the DC-mediated priming of T_H1 cells by promoting DC maturation, their expression of co-stimulatory molecules, as well as their secretion of IL-12.[15] Given the critical role of chemokines, particularly CCL19 and CCL21, in directing the interactions between DCs and naïve T cells in lymphoid tissues,[18] we hypothesized that IL-18-driven human helper NK cells may also regulate the capacity of DCs to produce chemotactic factors for naïve T cells, facilitating T-cell priming.

In direct NK cell-DC co-cultures, we observed that the activation of NK cells by either IL-18 or IL-2 (a prototypical NK cell-activating factor)[19] individually had no effect on the levels of CCL19 secreted in the supernatant (**Fig. 1A**, left). In contrast, the combined stimulation of NK cells with IL-18 and IFNα, an immunostimulatory factor produced early in response to viral infection[20] and developing tumors[21] that is known to co-activate cytokine secretion by human NK cells,[22] synergistically enhanced CCL19 production. Such 2-signal-dependent induction of CCL19 secretion persisted even upon harvesting, washing, removal of NK cells, and re-stimulation of the DCs with CD40 ligand (CD40L) (**Fig. 1A**, right). This indicates that the priming of DCs for high CCL19 production achieved under these conditions is stable and may persist even after the initial interaction with NK cells, for instance upon subsequent interaction with CD40L-expressing $CD4^+$ T cells. In contrast, no CCL19 was detected in the supernatant of NK cell cultures exposed to IL-18 and IFNα (**Fig. 2A**). Similarly, only limited levels of CCL19 were found in the supernatant of DC cultures treated with IFNα, either alone or in combination with IL-18 (**Figs. 1A and 2A**). These observations confirm that DCs are the source of CCL19 in this setting and that NK cell-DC interactions are strictly required for the induction of this chemokine. Although other chemokines, including CCL21 and CXCL12, have been reported to function on naïve T cells,[23] we were unable to document the expression of these chemokines by DCs or NK cells under any of the conditions tested (data not shown), in agreement with previous reports.[24–26]

Importantly, the DC-mediated secretion of CCL19 critically depended on the activation of NK cells with IL-18, as a similar phenomenon was not observed when NK cells were exposed to the known activating factors IL-2 and IFNα, either alone or in combination (**Fig. 1A**). Similarly, the activity of IL-18 could not be reproduced using IL-1β, another member of the same cytokine family (**Fig. 1B**). Furthermore, only NK cells activated with IL-18 (but not IL-2) stimulated DCs to produce CCL19 upon exposure to such secondary stimuli as IFNα, IL-15, IL-12, and IL-2 (**Fig. 1C**). Interestingly, a robust production of CCL19 in the course of NK cell-DC interactions could be induced either by the simultaneous application of IL-18 and an additional signal (**Fig. 1A**), or by priming NK cells with IL-18 followed by stimulation with secondary factors, including IL-2 (**Fig. 1C**). In contrast, IL-2-primed NK cells could not trigger the DC-mediated production of CCL19 even upon secondary stimulation with IL-18 (**Fig. 1C**).

Key role of paracrine TNFα and IFNγ in the NK-cell dependent DC-mediated secretion of CCL19

Since the ability of 2 signal-activated NK cells to induce DC maturation has been shown to involve TNFα and IFNγ released by NK cells,[15,22] we tested whether these factors may also stimulate DCs to produce CCL19. Indeed, when NK cells and DCs were co-cultured in the presence of soluble TNFα- and IFNγ-specific decoy receptors, CCL19 levels were significantly decreased (**Fig. 2A**). Likewise, the blockade of TNFα and IFNγ in NK cell-DC co-cultures by specific antibodies significantly reduced the secretion of CCL19 as compared with isotype-matched control antibodies (**Fig. 2B**). These results indicate the key role of TNFα and IFNγ in the NK-driven, DC-mediated secretion of CCL19.

NK cell-activated DCs efficiently recruit naïve T cells and promote their expansion and functional differentiation

Consistent with the significant increase in CCL19 secretion driven by the interaction between IL-18-primed NK cells and DCs, the supernatants from NK cell-DC co-cultures were highly effective at recruiting naïve $CD8^+$ T cells in transwell chemotaxis assays (**Fig. 3A**). Experiments involving the blockade of CCR7 (the CCL19 receptor) with specific antibodies demonstrated that the enhanced migration of naïve $CD8^+$ T cells toward the supernatant of NK cell-DC co-cultures was dependent on CCR7 (**Fig. 3B**). A substantial increase in the number of $CD8^+$ T cells was observed upon culture with NK cell-activated DCs 7 d post T-cell migration (**Fig. 3C**, left). Importantly, such T cells demonstrated a robust proliferative potential as well as elevated expression of the CTL marker granzyme B (**Fig. 3C**, right). These findings indicate that NK cell-activated DCs are capable of efficiently recruiting naïve $CD8^+$ T cells as well as inducing their expansion and activation toward an effector phenotype.

CCL19 is secreted in human tumor-associated lymph nodes in response to NK cell-targeting 2-signal activation

To test the therapeutic potential (as adjuvant interventions) of combinatorial regimens that activate helper NK cells, we investigated the effects of the combined application of IL-18 and IFNα to LN explants from colorectal cancer patients. Indeed, the treatment of patient-derived LNs with IL-18 and IFNα not only promoted the expression of TNFα and IFNγ (**Fig. 4A**), but also resulted in a marked production of CCL19 (**Fig. 4A and B**). These results demonstrate the feasibility of applying IL-18-based, NK cell-activating combinatorial adjuvants to promote T-cell priming in human tumor-associated LNs as well as in peripheral LNs that are targeted by anticancer vaccines.

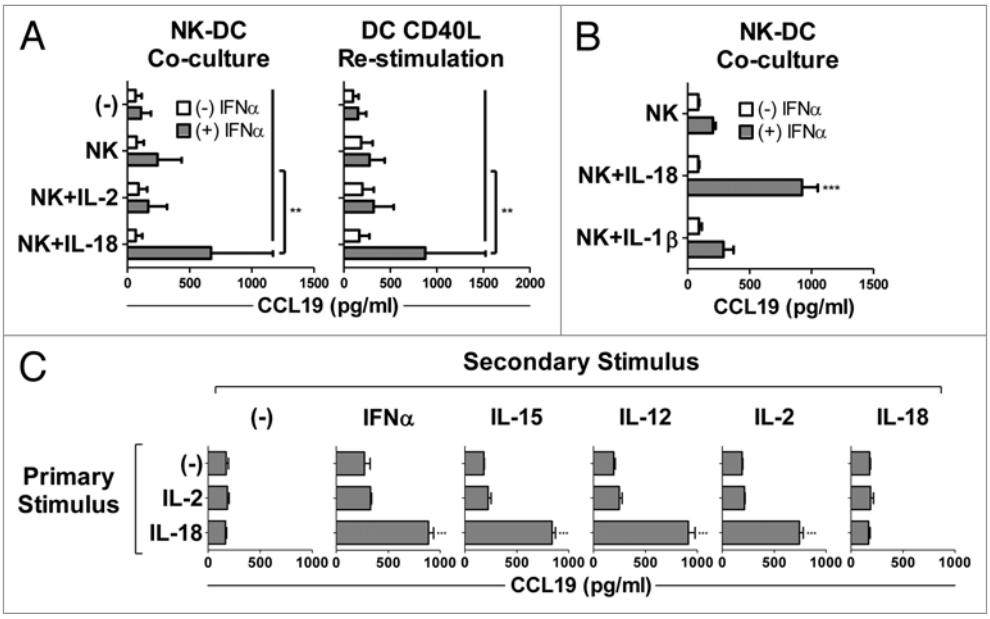

Figure 1. IL-18-activated NK cells stimulate DCs to produce CCL19. (**A**) CCL19 levels in supernatants from DCs cultured for 48 h, alone or together with autologous NK cells (1:2 NK cell:DC ratio), in the presence of interleukin (IL)-2, IL-18 and/or interferon α (IFNα) (left); or for 24 h in the presence of CD40 ligand (CD40L)-expressing cells upon previous co-culture with NK cells (right). Data are reported as means ± SD of samples from 6 independent individuals. (**B**) CCL19 levels in supernatants from DCs cultured for 48 h together with autologous NK cells (1:2 NK cell:DC ratio) in the presence of IL-18, IL-1β, and/or IFNα. Data, which are representative of 1 out of 2 independent experiments yielding similar results, are reported as means ± SD of triplicate cultures. (**C**) NK cells were pre-treated or not for 24 h with IL-2 or IL-18, washed, and re-plated with autologous DCs, alone or in the presence of IFNα, IL-15, IL-12, IL-2, or IL-18. CCL19 levels were analyzed by ELISA 48 h after the administration of the secondary stimulus. Data, which are representative of 1 out of 3 independent experiments yielding similar results, are reported as means ± SD of triplicate cultures. ***$P < 0.001$, **$P < 0.01$, as compared with the indicated samples or all samples when not specified.

Discussion

Our findings unveil a novel link between innate and adaptive immunity, demonstrating the crucial role of helper NK cells in the DC-mediated activation of T cells by promoting DC production of CCL19. DC-derived CCL19 is critical for the recruitment of naïve T cells to the T-cell zones of secondary lymphoid tissues in both mice and humans in vivo,[27,28] as well as for promoting direct interactions between naïve T cells and DCs that are needed for the elicitation of adaptive immune responses.[26] While the production of CCL19 by DCs has previously been shown to be induced "directly" by DC infection[29] or by DC recognition of pathogen-derived molecular motifs,[30] the results presented here demonstrate an alternative "indirect" pathway mediated by NK cells, which specialize in detecting alternative forms of danger, such as oncogenic transformation.[31] At least in part, our findings explain recent data indicating an important role for NK cells in the recruitment of both DCs and naïve T cells to LNs in vivo, a process that is necessary for the optimal induction of protective T-cell immune responses.[32]

NK cells have been previously described to stimulate DCs to express co-stimulatory molecules, produce IL-12, and home to LNs, a series of activities that are regulated in a 2 signal-dependent mechanism driven by IL-18.[17,33] Our current data indicate that a similar 2 signal-dependent mechanism also governs the ability of NK cells to instruct the production of CCL19 by DCs, suggesting that the NK cell-induced migration of DCs to LNs, the recruitment of naïve T cells, and priming responses are coordinately regulated. Our findings also indicate that the NK cell-elicited secretion of CCL19 by DCs persists even after the initial NK cell-DC interactions, suggesting that the NK cell-dependent activation of CCL19 production by DCs and the subsequent recruitment of naïve T cells by DCs for priming do not necessarily need to occur in the same compartment. Nevertheless, numerous reports indicate the ability of murine NK cells to traffic to LNs upon activation in vivo,[11,34,35] a process that in humans may result from the ability of NK cells to acquire CCR7 upon exposure to IL-18,[15] resulting in the coordinate regulation of NK cell, DC and T-cell interactions in secondary lymphoid tissues. Indeed, NK cells and DCs have been demonstrated to co-localize in the T-cell areas of human LNs and to engage in activating interactions.[36]

Given the CCR7-dependent responsiveness to CCL19 shared across NK cells, DCs and T cells, the data presented here suggest the potential for CCL19, as initially elicited by NK cell-DC interactions, to participate in a potent feed-forward loop promoting the recruitment of all 3 cell types, resulting in robust priming responses. Indeed, DCs activated in the presence of IFNγ, a key NK cell-derived factor and an important inducer of CCL19 secretion (as shown in this study), have been demonstrated to reciprocally enhance the responsiveness of NK cells to CCL19,[37] presumably recruiting additional NK cells and engaging a self-amplifying cycle of DC activation. Thus, the NK cell-DC functional collaboration described here suggests the existence

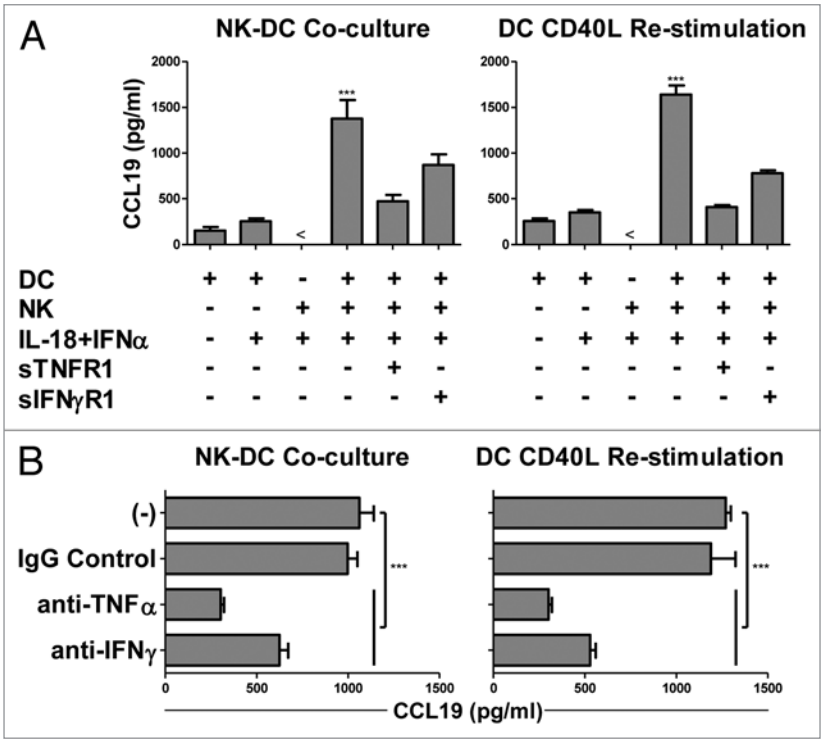

Figure 2. The ability of helper NK cells to stimulate the secretion of CCL19 by DCs depends on TNFα and IFNγ. (**A**) CCL19 levels in supernatants from DCs cultured for 48 h, alone or together with autologous NK cells (1:2 NK cell:DC ratio), in the presence or in the absence of interleukin (IL)-18 plus interferon (IFN)α and soluble tumor necrosis factor α (TNFα) receptor 1 (sTNFR1) or IFNγ receptor 1 (sIFNγR1) decoys (left); or for 24 h in the presence of sTNFR1 and sIFNγR1 decoys and CD40 ligand (CD40L)-expressing cells upon previous co-culture with NK cells (right). Data, which are representative of 1 out of 3 independent experiments yielding similar results, are reported as means ± SD of triplicate cultures. (**B**) CCL19 levels in supernatants from DCs cultured for 48 h together with NK cells activated by IL-18 and IFNα, in the presence of TNFα- or IFNγ-blocking antibodies or isotype-matched control antibodies (left); or for 24 h in the presence of TNFα- or IFNγ-blocking antibodies or isotype-matched control antibodies and CD40 ligand (CD40L)-expressing cells upon previous co-culture with NK cells (right). Data, which are representative of 1 out of 2 independent experiments yielding similar results, are reported as means ± SD of triplicate cultures. ***$P < 0.001$, as compared with the indicated samples or all samples when not specified. < depicts levels that were below the limit of detection of the assay.

of a chemokine-dependent pathway whereby robust immune responses may be elicited upon the detection of relatively weak pathogenic or oncogenic stimuli, and may contribute to the significant protective immunity promoted by NK cell-DC crosstalk in vivo.[38]

The amplification of such a CCL19-promoting NK cell-DC interaction may represent an attractive therapeutic target to stimulate anticancer immune responses. Several studies have demonstrated the beneficial impact of CCL19 in therapeutic tumor models.[39–42] Moreover, CCL19 has been correlated with prolonged survival in cancer patients.[43] Although IL-18 has previously been associated with limited clinical anticancer activity when used as a single agent,[44] our data demonstrate that, in contrast to IL-18 alone, the combination of IL-18 with additional helper NK cell-activating factors can stimulate CCL19 production by DCs in multiple settings, including tumor-associated LNs from colorectal cancer patients. The requirement of 2 distinct signals for such an induction may help explain conflicting reports on the influence of IL-18 on antitumor NK-cell activity,[45–47] with the provision and/or nature of secondary signals likely to determine the outcome of IL-18-mediated NK-cell activation. This indicates the possibility of therapeutically enhancing CCL19-driven T-cell priming in cancer patients using specific "IL-18 plus one" adjuvant regimens (such as IL-18 combined with IFNα) that would target NK cells within neoplastic lesions, tumor-draining LNs, or LNs draining vaccination sites.

Accumulating evidence suggests that CCL19-driven antitumor immune responses developing within neoplastic lesions, especially within tertiary lymphoid structures (TLSs) that host close interactions between mature DCs and naïve T cells, may be critical for the achievement of protective immunity.[48] The presence of such TLSs and other intratumoral tissues that share features with lymphoid organs, such as high endothelial venules, has been shown to correlate with both CCL19 expression levels and favorable clinical outcome in patients affected by non-small-cell lung and breast carcinoma.[49–51] Likewise, in renal cell carcinoma, CCL19 has been shown to localize to tumor regions containing clusters of mature DCs and proliferating $CCR7^+$ T cells,[52] and robust tumor infiltration by $CCR7^+$ T cells has been described to predict prolonged survival among advanced colorectal cancer patients.[53] These findings lend further support to the notion that the activation of intratumoral CCL19 secretion by NK cell-DC interactions might have a robust therapeutic potential.

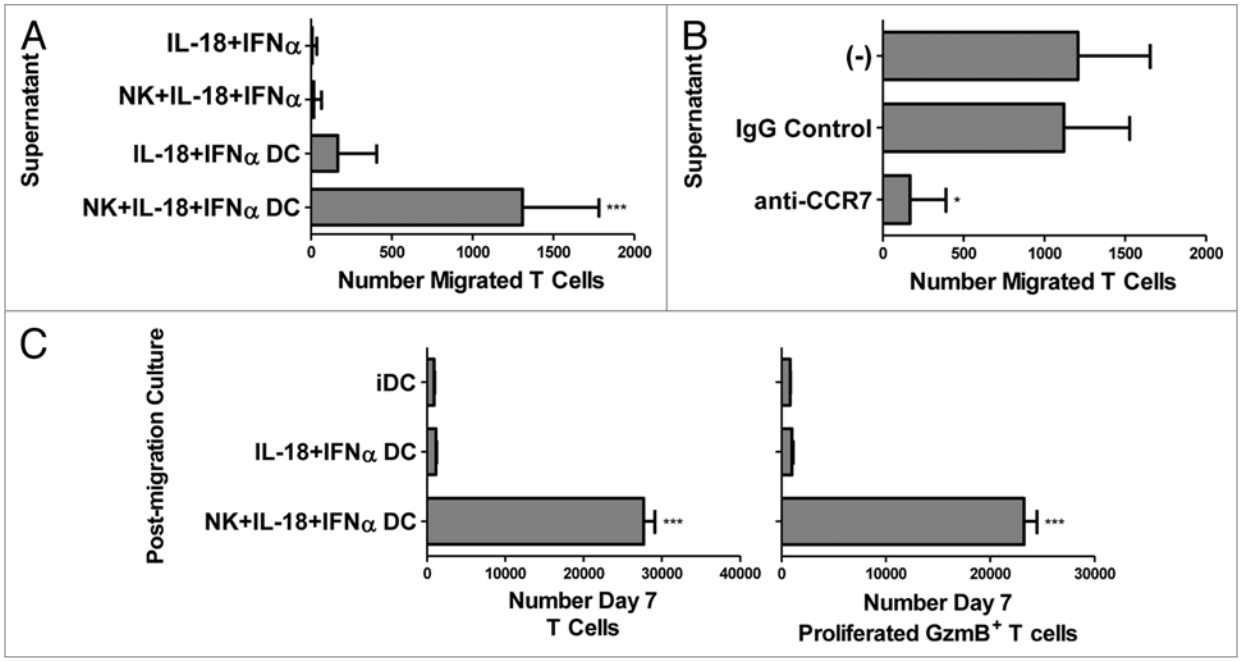

Figure 3. NK cell-activated DCs recruit naïve T cells and promote their expansion and functional differentiation. (**A**) Migration of naïve $CD8^+$ T cells toward supernatants from DCs cultured for 48 h, alone or together with autologous NK cells (1:2 NK cell:DC ratio), in the presence or in the absence of interleukin (IL)-18 plus interferon α (IFNα). Data are reported as means ± SD of samples from 4 independent individuals. (**B**) Migration of naïve $CD8^+$ T cells pretreated or not with an anti-CCR7 antibody toward supernatants from DCs cultured for 48 h together with autologous NK cells activated by IL-18 and IFNα. Data are reported as means ± SD of samples from 4 independent individuals. (**C**) Total number (left) or number of proliferating granzyme B $(GzmB)^+$ $CD8^+$ T cells (right) observed upon the co-culture of migrating T cells with DCs 7 d after migration. Data are reported as means ± SD of triplicate cultures. ***$P < 0.001$, *$P < 0.05$, as compared with all samples.

In summary, our data unveil a novel helper NK cell-driven mechanism promoting T-cell priming by DCs through enhanced DC production of CCL19. Moreover, our findings support the therapeutic application of NK cell-targeting, IL-18-based combinatorial adjuvants to stimulate antitumor immunity in cancer patients.

Materials and Methods

Media and reagents

CellGenix DC medium (CellGenix Technologie Transfer GmbH) was used for the short-term culture of human NK cells and for the generation of DCs. Iscove's Modified Dulbecco's Medium (IMDM) containing 10% fetal bovine serum, 1% L-glutamine and penicillin/streptomycin (all from Gibco, Invitrogen) was used as the standard medium for the outgrowth of T-cell cultures, the culture of human LN explants, and for the maintenance of the CD40L-expressing murine plasmacytoma J558 cells. The following factors were used throughout the study: IL-18 (MBL International); IL-2 (Chiron); IL-1β (Miltenyi Biotech); IFNα-2b (Intron A; Schering-Plough); IL-12 (PeproTech); IL-15 (Sigma-Aldrich); granulocyte macrophage colony-stimulating factor (GM-CSF) and IL-4 (Schering-Plough).

NK-cell and $CD8^+$ T-cell isolation

Peripheral blood from healthy individuals was harvested by venipuncture under protocols approved by the University of Pittsburgh Institutional Review Board, and peripheral blood mononuclear cells (PBMCs) were isolated by density gradient using Lymphocyte Separation Medium (Cellgro Mediatech). NK ($CD56^+CD3^-$) cells and naïve $CD8^+$ T ($CD8^+CD45RA^+CCR7^{high}CD45RO^-CD56^-CD57^-$) cells were further isolated from PBMCs by negative magnetic selection (> 95% pure in both cases) using the EasySep system (StemCell Technologies), according to the manufacturer's instructions.

Generation of DCs

$CD14^+$ monocytes were isolated from PBMCs by positive magnetic selection (Miltenyi Biotech). Immature DCs were generated from monocytes cultured for 6 d in 24-well plates (4×10^5 cells/well) in the presence of 1000 IU/mL GM-CSF and 1000 IU/mL IL-4.

NK cell-mediated activation of DCs

Previously isolated and cryopreserved autologous NK cells were thawed and added (1.5×10^5 cells/well) to DC cultures on day 6 (1:2 NK cell:DC ratio) in the presence of the indicated combination of 200 ng/mL IL-18, 250 IU/mL IL-2, or 25 ng/mL IL-1β, together with 1000 IU/mL IFNα, 5 ng/mL IL-12, and/or 100 ng/mL IL-15. Alternatively, NK cells were pre-treated with IL-18 or IL-2 for 24 h, washed thoroughly, and re-plated with DCs in the presence of IFNα, IL-12, IL-2, IL-15, or IL-18 as a secondary stimulus. When indicated, soluble decoy receptors specific for TNFα (sTNFR1; final concentration = 1 μg/mL; from R&D Systems) and IFNγ (sIFNγR1; final concentration = 10 μg/mL; from R&D Systems), antibodies neutralizing TNFα (clone MAb1; final concentration = 10 μg/mL;

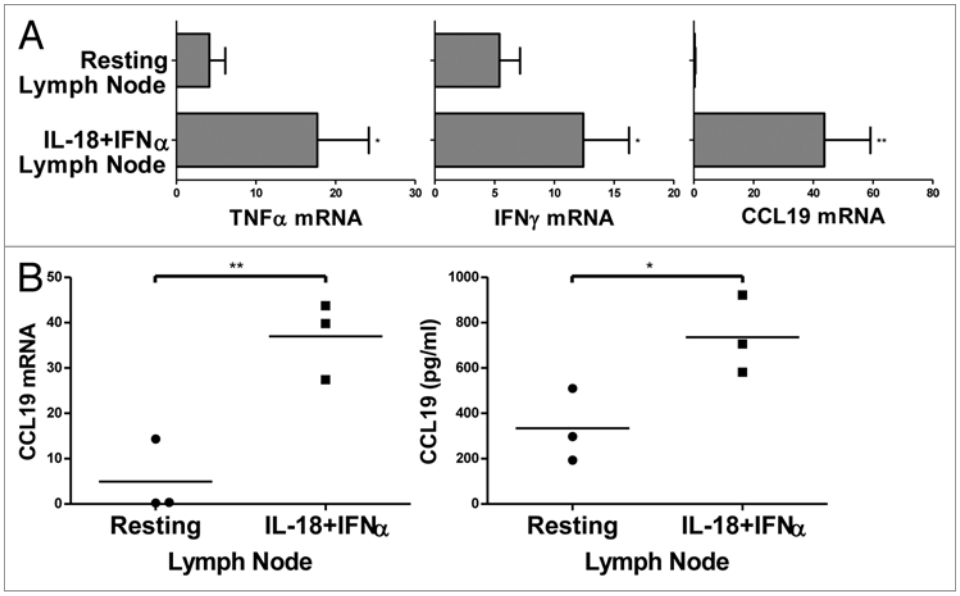

Figure 4. IL-18-based combinatorial adjuvants drive CCL19 production in human tumor-associated lymph nodes. (**A**) Expression of tumor necrosis factor α (TNFα), interferon γ (IFNγ), and CCL19 in lymph nodes from colorectal cancer patients, upon exposure or not to interleukin (IL)-18 plus interferon α (IFNα) for 24 h. Normalized gene expression data, which are representative of 1 out of 3 independent experiments yielding similar results, are reported as means ± SD of triplicate lymph node cultures. (**B**) Expression of the CCL19-coding mRNA in tissues (left) and CCL19 in supernatants (right) of lymph node cultures established with biopsy material from colorectal cancer patients, upon exposure or not to IL-18 plus IFNα for 24 h. Data are reported as means of samples from 3 independent patients. $^{**}P < 0.01$, $^{*}P < 0.05$, as compared with the indicated samples.

from BD Biosciences) or IFNγ (clone B27; final concentration = 10 μg/mL; BD Biosciences), or isotype-matched control antibodies (final concentration = 10 μg/mL; from BD Biosciences) were administered at co-culture initiation. Supernatants were collected 48 h later for the quantification of CCL19 by ELISA (see below). To assess the stability of chemokine production by DCs, NK-DC co-cultures were harvested and washed, $CD56^+$ NK cells were removed by magnetic selection (StemCell Technologies), and DCs were re-plated in 96-well plates (2×10^4 cells/well). To mimic the interaction between DCs and CD40L-expressing $CD4^+$ T cells, DCs were co-cultured with CD40L-expressing J558 cells (a gift from Dr. P. Lane, University of Birmingham, United Kingdom) at 5×10^4 cells/well, which have previously been shown to be equivalent to activated $CD4^+$ T cells and soluble CD40L in this respect.[54] Supernatants were collected 24 h later and CCL19 was quantified by ELISA (see below).

ELISA

Supernatants from cell co-cultures or LN explants (see below) were assayed for CCL19 levels by indirect-sandwich ELISA using specific matched primary and biotinylated-secondary antibody pairs (PeproTech), as previously described.[55]

Chemotaxis

Chemotaxis was evaluated using 24-transwell plates with 5 μm pore-size polycarbonate membranes (Corning), as previously described.[56] The lower chamber was filled with supernatants from NK cell-DC co-cultures, while the upper chamber was loaded with 2×10^5 naïve $CD8^+$ T cells isolated as described above. When indicated, T cells were treated for 30 min with an anti-CCR7 blocking antibody (clone 3D12, final concentration = 20 μg/mL; from BD Biosciences) before the assay to block CCR7-dependent migration. Cells reaching the bottom chambers were harvested after 3 h and fixed in 100 μL of 4% paraformaldehyde, followed by the cytofluorometric assessment of cell number in 60 μL of the fixative reagent. In each condition, specific chemotaxis was calculated as the number of migrating cells upon subtraction of the number of cells migrating toward chambers containing fresh culture medium.

In vitro priming of migrating naïve $CD8^+$ T cells

Naïve $CD8^+$ T cells labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE; from Invitrogen) according to the manufacturer's protocol were allowed to migrate toward DC culture supernatants, as described above. Migrating T cells were pooled from triplicate wells and re-plated with DCs (2×10^4 DCs/well in 96-well plates) that had previously been pulsed with 1 ng/mL staphylococcal enterotoxin B (SEB) for 30 min. 20 IU/mL IL-2 was added to co-cultures on day 5, and T cells were analyzed by flow cytometry on day 7 for cell number, proliferation (as monitored by CFSE dilution), and acquisition of granzyme B expression.

Flow cytometry

Cell surface and intracellular immunostaining analyses were performed using an Accuri C6 Flow Cytometer. NK and T cells were stained with the following dye-conjugated anti-human mouse monoclonal antibodies: CD56-PE-Cy5 (Beckman Coulter), CD3-PE (eBioscience), CCR7-FITC (R&D Systems), granzyme B-PE (Invitrogen), as well as CD16-FITC, CD8-PE-Cy5, CD45RA-FITC, CD45RO-PE, and CD57-FITC (all from BD Biosciences). The corresponding mouse isotype-matched control antibodies IgG1-FITC, IgG2b-FITC, IgG1-PE, IgG2a-PE, and IgG1-PE-Cy5 (BD Biosciences) were used, as appropriate. Before staining, cells were kept for 20 min at 4°C in PBS containing 2%

human serum, 0.5% bovine serum albumin (BSA), 0.1% NaN_3, and 1 μg/mL mouse IgG (Sigma-Aldrich) to block non-specific binding. Cell permeabilization for intracellular staining was performed by placing cells in 0.1% Triton X-100 (Sigma) in PBS for 15 min. Cells were stained for 40 min at 4°C followed by washing in PBS supplemented with 0.5% BSA and 0.1% NaN_3, then fixed and stored in 4% paraformaldehyde until analysis.

Ex vivo culture of human lymph node explants

LNs were obtained from colorectal cancer patients undergoing standard-of-care surgical treatment. All specimens were provided under a protocol approved by the University of Pittsburgh Institutional Review Board (UPCI 02–077) and in accordance with the Helsinki Declaration. Written informed consent was obtained prior to any specimen collection, and the nature as well as possible consequences of the study were explained. Nodal tissues were sectioned using a 4-mm biopsy puncher and placed in IMDM supplemented with 10% fetal bovine serum, 1% L-glutamine and penicillin/streptomycin. When indicated, LN explants were treated 200 ng/mL IL-18 and 1000 IU/mL IFNα. After 24 h of incubation, supernatants were collected for CCL19 quantification by ELISA and tissues were analyzed for mRNA expression (see below).

Quantitative real-time PCR

LN tissues were placed in Lysing Matrix E Tubes (MP Biologicals) containing RLT lysis buffer (Qiagen) and agitated using a FP120 homogenizer (MP Biologicals). Supernatants were collected and total RNA was extracted using the RNeasy kit (Qiagen), according to the manufacturer's protocol. mRNA expression was analyzed using the StepOne Plus System (Applied Biosystems), as previously described,[56] using inventoried primer/probe sets. The expression of TNFα-, IFNγ- and CCL19-coding genes was assessed 24 h after the administration of IL-18 and IFNα. Gene expression was normalized to that of hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) and expressed as fold increase ($2^{-\Delta CT}$), where $\Delta C_T = C_{T\,(\text{target gene})} - C_{T\,(HPRT1)}$.

Statistical analyses

Data were analyzed using unpaired and paired 2-tailed Student t-tests and 1-way and 2-way ANOVA, as appropriate. The threshold for significance was set to P values < 0.05.

Disclosure of Potential Conflicts of Interest

The authors declare that no conflicts of interest exist.

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