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Evidence for NK Cell Subsets Based on Chemokine Receptor Expression[1]

Robert D. Berahovich,[2]* Nu L. Lai,* Zheng Wei,* Lewis L. Lanier,† and Thomas J. Schall*

To help understand the role of chemokines in NK cell trafficking, we determined the chemokine receptor profiles of three different human NK cell lines and freshly isolated primary human NK cells. The cell lines overlapped in their chemokine receptor profiles: CXCR3 and CXCR4 were expressed by all three lines, whereas CCR1, CCR4, CCR6, CCR7, and CX3CR1 were expressed by only one or two of the lines, and no other chemokine receptors were detected. Freshly isolated primary NK cells were found to express CXCR1, CXCR3, and CXCR4, and to contain subsets expressing CCR1, CCR4, CCR5, CCR6, CCR7, CCR9, CXCR5, and CXCR6. With the exception of CCR4, these chemokine receptors were expressed at higher percentages by CD56^{bright} NK cells than by CD56^{dim} NK cells. In particular, CCR7 was expressed by almost all CD56^{bright} NK cells but was not detected on CD56^{dim} NK cells. CCR9 and CXCR6 have not been described previously on primary NK cells. These results indicate that within both the CD56^{bright} and CD56^{dim} NK cell populations, subsets with the capacity for differential trafficking programs exist, which likely influence their functions in innate and adaptive immunity. ***The Journal of Immunology,*** **2006, 177: 7833–7840.**

Natural killer cells are lymphocytes that participate in both innate and adaptive immune responses to malignant tumors, intracellular pathogens, and foreign cells. NK cells circulate in peripheral blood, but rapidly move to sites of immune reactions in peripheral tissues, due to gradients of chemokines and lysolipids produced in adjacent capillaries (1–3). At these sites, NK cells recognize target cells via ligation of a variety of activating receptors. Once activated, NK cells lyse the offending cells and release cytokines, including IFN-γ, which recruit other cell types and modulate subsequent T cell responses.

In human blood, two types of NK cells are found, based on expression of CD56 and CD16: CD56^{dim}CD16^{+} cells, which account for ~90% of the NK cells, and CD56^{bright}CD16^{-} cells, which comprise the remaining ~10% of the NK cells. CD56^{dim} CD16^{+} cells are the more cytotoxic population, whereas CD56^{bright} CD16^{-} cells express the high-affinity IL-2R CD25 and thereby proliferate to a greater extent after exposure to IL-2 and typically produce greater amounts of cytokines (3). CD56^{bright}CD16^{-} cells are also found at low frequencies in secondary lymphoid organs, e.g., lymph node and tonsil, and respond vigorously to locally produced IL-2 (4). CD56^{bright}CD16^{-} cells have also been found in inflammatory lesions obtained from a wide variety of diseases (5).

At present, the relationship between the two populations of NK cells is controversial. Evidence exists that CD56^{bright}CD16^{-} cells are direct precursors of CD56^{dim}CD16^{+} cells; that CD56^{bright}CD16^{-} cells are direct descendents of CD56^{dim}CD16^{+} cells; or that CD56^{bright}CD16^{-} cells are a separate lineage of NK cells than CD56^{dim}CD16^{+} cells. For example, CD56^{bright}CD16^{-} cells isolated from lymph node up-regulated perforin and become cytotoxic after 1 wk of culture in medium containing IL-2 (6). In contrast, upon entry of CD56^{dim}CD16^{+} NK cells into peripheral tissues, CD56 was up-regulated (3). It is likely that peripheral blood CD56^{bright}CD16^{-} NK cells are a heterogeneous population, potentially containing at least three different subsets: 1) immature cells coming from the bone marrow, 2) mature cells activated in the lymph nodes, and 3) CD56^{dim}CD16^{+}-derived cells returning to the bloodstream after activation in peripheral tissues. In addition, a subset of CD56^{bright}CD16^{-} NK cells was found to be activated (e.g., HLA-DR^{+}CD45RO^{+}) (7).

There are also inconsistencies in the literature regarding the chemokine receptors that regulate NK cell trafficking (3). Many laboratories analyzed IL-2-activated NK cells, sometimes with contaminating T cells, and most laboratories did not separate the CD56^{bright}CD16^{-} and CD56^{dim}CD16^{+} NK cells. In the two most complete analyses of fresh NK cells (8, 9), discrepancies were observed with regard to no less than five chemokine receptors. Therefore, we sought to re-evaluate the chemokine receptors on freshly isolated NK cells by using flow cytometry and chemotaxis assays. Moreover, we used these methods to determine the chemokine receptor profile on three commonly used NK cell lines: NKL, KHYG-1, and NK-92. The NKL cell line was isolated from peripheral blood of a patient with CD56^{+}CD16^{+} large granular lymphocyte leukemia (10). The KHYG-1 cell line was isolated from peripheral blood of a patient with an aggressive NK leukemia (11). The NK-92 cell line was isolated from peripheral blood of a non-Hodgkin's lymphoma patient containing large granular lymphocytes detected in the bone marrow (12). The NK lines were used, in part, because they might be derived from rare NK subsets and, as such, might express chemokine receptors not expressed on a substantial fraction of peripheral blood NK cells. In this study, we present a comprehensive analysis of chemokine receptor expression and function on these three cell lines, and a more selective analysis of freshly isolated human NK cells.

*ChemoCentryx, Mountain View, CA 94043; and †Department of Microbiology and Immunology and the Cancer Research Institute, University of California San Francisco, San Francisco, CA 94143

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[2] Address correspondence and reprint requests to Dr. Robert Berahovich, ChemoCentryx, 850 Maude Avenue, Mountain View, CA 94043. E-mail address: rberahovich@chemocentryx.com

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Materials and Methods

Cells

Human buffy coats were purchased from the Stanford Blood Center. PBMC were prepared by centrifugation of the buffy coats over Ficoll-Hypaque (Sigma-Aldrich) for 45 min, then collecting and rinsing the cells at the interface with PBS. NK cells used in chemotaxis assays were prepared by negative depletion of the PBMC, using a commercial kit (Miltenyi Biotec) and an AutoMACS instrument (Miltenyi Biotec). NKL cells were a gift from M. Robertson (University of Indianapolis, IN) and cultured in RPMI 1640 (Mediatech), 10% FBS (HyClone), 10 mM HEPES (Mediatech), 1 mM sodium pyruvate (Mediatech), 1× nonessential amino acids (Mediatech), 0.1 mM 2-ME (Sigma-Aldrich), and 200 U/ml IL-2 (R&D Systems). KHYG-1 cells were purchased from the Health Science Research Resources Bank and cultured in RPMI 1640, 10% FBS, and 100 U/ml IL-2. NK-92 cells were purchased from the American Type Culture Collection and cultured in Alpha MEM without ribonucleosides and deoxyribonucleosides (Mediatech), 12.5% horse serum (HyClone), 12.5% FBS, 2 mM L-glutamine (Mediatech), 1.5 g/L sodium bicarbonate (Mediatech), 0.2 mM inositol (Sigma-Aldrich), 0.02 mM folic acid (Sigma-Aldrich), 0.1 mM 2-ME, and 100 U/ml IL-2 (R&D Systems).

Flow cytometry

PBMC were exposed to human IgG at 50 μg/ml in buffer (PBS containing 5% FBS) on ice for 15 min to reduce nonspecific binding in subsequent steps. Cells were rinsed with buffer and exposed to anti-chemokine receptor Abs or isotype-matched control Abs (all purchased from R&D Systems) at 10 μg/ml in buffer on ice for 30 min. Cells were rinsed with buffer and exposed to PE-conjugated $F(ab')_2$ of anti-mouse IgG Ab (Beckman Coulter) on ice for 20 min. Cells were rinsed with buffer and exposed to 5% normal mouse serum (Sigma-Aldrich) in buffer on ice for 15 min and rinsed again. Cells were finally exposed to a mixture of CyChrome-conjugated anti-CD16, Alexa 647-conjugated anti-CD56, and FITC-conjugated anti-CD3, -CD14, and -CD19 Abs (BD Biosciences) in buffer on ice for 20 min. After rinsing with buffer, cells were analyzed on a FACScan (BD Biosciences). NK cell lines were analyzed using the chemokine receptor Abs and PE-conjugated anti-mouse IgG Ab described above.

Chemotaxis assay

NK cell lines and enriched NK cells were resuspended in HBSS containing 0.1% BSA. Chemotaxis assays were performed in 96-well ChemoTx microplates (Neuroprobe) as follows. Chemokines (Table I) were added to the lower wells (final volume 29 μl), and 20 μl of cell suspension (5×10^6 cells/ml) was added to the polycarbonate filter (5-μm pore size). After incubation at 37°C in a humidified environment for 2 h, the filters were removed. Cells that migrated into the lower chamber were quantified by using the CyQuant cell proliferation assay kit (Molecular Probes) and were analyzed with a Tecan fluorometer (excitation at 480 nm, emission at 530 nm). Data were analyzed and plotted in arbitrary units of fluorescence using Prism (GraphPad Software).

Table I. *Chemokines used in this study*

Receptor	Ligand	Common Name
CCR1	CCL15	Leukotactin
	CCL23	CKβ8Δ24
CCR1,5	CCL3	MIP-1α
	CCL5	RANTES
CCR2	CCL2	MCP-1
CCR3	CCL11	Eotaxin
CCR4	CCL22	MDC
	CCL17	TARC
CCR5	CCL4	MIP-1β
CCR6	CCL20	MIP-3α
CCR7	CCL19	MIP-3β
	CCL21	SLC
CCR8	CCL1	I-309
CCR9	CCL25	TECK
CCR10	CCL27	CTACK
CXCR1,2	CXCL8	IL-8
CXCR2	CXCL2	Groβ
CXCR3	CXCL11	I-TAC
	CXCL10	IP10
CXCR4	CXCL12	SDF-1α
CXCR5	CXCL13	BLC
CXCR6	CXCL16	CXCL16
CX3CR1	CX3CL1	Fractalkine

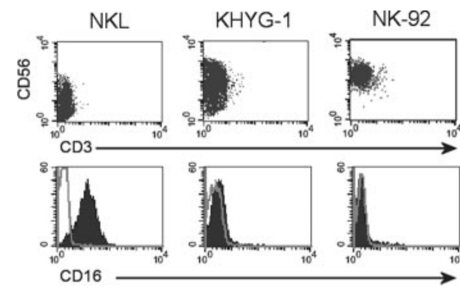

FIGURE 1. Expression of CD56 and CD16 on NK cell lines. NKL, KHYG-1, and NK-92 cells were analyzed by flow cytometry for surface expression of CD3, CD16, and CD56. *Top*, Expression of CD3 vs CD56. *Bottom*, Staining by anti-CD16 Ab (filled histogram) vs isotype-matched control Ab (open histogram).

Results

Determination of chemokine receptor expression on NK cell lines

Flow cytometric analysis of the NKL, KHYG-1, and NK-92 cell lines with Abs specific for CD3, CD56, and CD16 indicated that the lines were uniformly CD3 negative, but varied in CD56 and CD16 expression: NKL cells were $CD56^{-/dim}CD16^{+}$, KHYG-1 cells were $CD56^{+}CD16^{-}$, and NK-92 cells were $CD56^{bright}CD16^{-}$ (Fig. 1). These results are in accordance with the previously published data (10–12).

Each cell line was analyzed for chemokine receptor expression by two methods: flow cytometric staining with Abs specific for chemokine receptors and chemotaxis assays with ligands specific for chemokine receptors. NKL cells were found by flow cytometry to express CCR4, CCR7, CXCR3, and CXCR4; in contrast, CCR1, CCR2, CCR5, CCR6, CCR8, CCR9, CXCR1, CXCR2, CXCR5, and CXCR6 were not detected (Fig. 2*A*). In chemotaxis assays analyzing responsiveness to three concentrations of chemokines, ligands specific for CCR4 (CCL22/MDC), CCR7 (CCL19/MIP-3β), and CXCR4 (CXCL12/SDF-1α) induced migration of the NKL cells, whereas ligands specific for CCR1 and CCR5 (CCL3/MIP-1α), CCR2 (CCL2/MCP-1), CCR3 (CCL11/eotaxin), CCR5 (CCL4/MIP-1β), CCR6 (CCL20/MIP-3α), CCR8 (CCL1/I-309), CCR9 (CCL25/TECK), CXCR1 and CXCR2 (CXCL8/IL-8), CXCR3 (CXCL11/I-TAC), CXCR5 (CXCL13/BCL), and CXCR6 (CXCL16) failed to induce migration of the NKL cells (Fig. 2*B*). In chemotaxis assays using a wider range of chemokine concentrations, CCL22/MDC recruited NKL cells with an EC_{50} of 5 pM, CCL19/MIP-3β exhibited an EC_{50} of 3 nM, and CXCL12/SDF-1α exhibited an EC_{50} of 4 pM (Fig. 2*C*). In addition, a ligand specific for CX3CR1 (the chemokine domain of CX3CL1/fractalkine, herein termed "FK-CK") recruited NKL cells with an EC_{50} of 50 nM. Ligands for CCR8, CCR9, CCR10, CXCR3, and CXCR6 failed to recruit the NKL cells (Fig. 2*C*).

The KHYG-1 cell line was analyzed in a similar fashion. By flow cytometry, the cells were positive only for CCR6, CXCR3, and—to a very low extent—CXCR4 (Fig. 3*A*). In chemotaxis assays using three concentrations of ligand, only CCL20/MIP-3α, CXCL11/I-TAC, and CXCL12/SDF-1α induced migration of the KHYG-1 cells (Fig. 3*B*). In chemotaxis assays using a broader range of ligand concentrations, CCL20/MIP-3α recruited the cells with an EC_{50} of 120 pM, CXCL11/I-TAC exhibited an EC_{50} of 110 pM, and CXCL12/SDF-1α exhibited an EC_{50} of 2 nM

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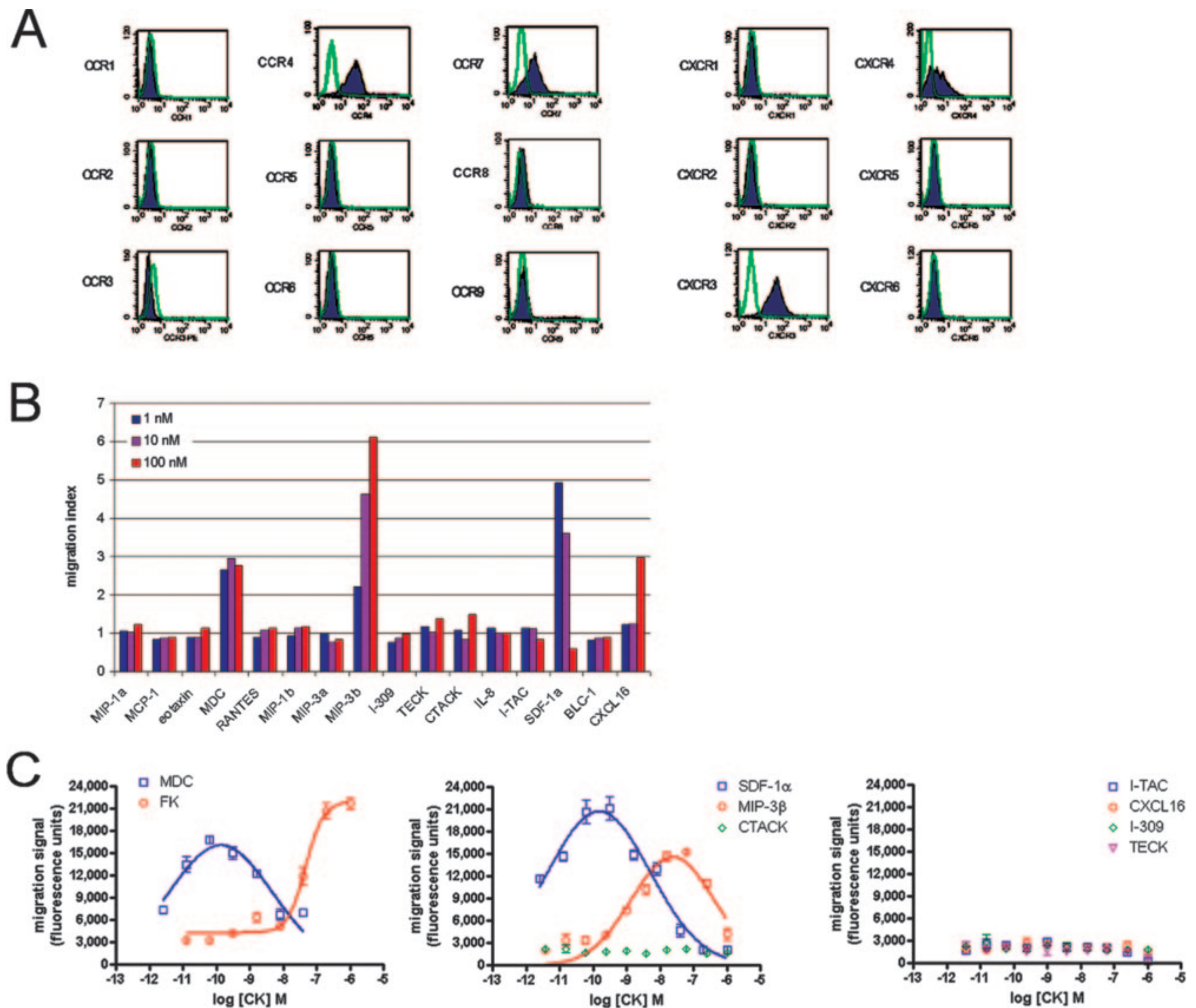

FIGURE 2. Chemokine receptor expression on the NKL cell line. *A*, Flow cytometric analysis. NKL cells were stained with Abs specific for human chemokine receptors (filled histograms) vs isotype-matched control Abs (open histograms). *B*, Chemotaxis assay. NKL cells were tested for migration to chemokines at three concentrations (1, 10, and 100 nM), and then the cells in the bottom chamber were quantified by DNA content. Data were plotted as the ratio of migration induced by chemokine to the migration induced by buffer. *C*, Full-dose chemotaxis assay. NKL cells were tested for migration to chemokines at multiple doses, ranging from 3 pM to 1 μM, and then the migrated cells were quantified by DNA content. Data were plotted as the extent of migration (in arbitrary units of fluorescence) vs chemokine concentration, and curve-fitting was performed by nonlinear regression.

(Fig. 3*c*); ligands for CCR3 (CCL11/eotaxin), CCR7 (CCL21/SLC), and CX3CR1 (FK-CK) failed to recruit the KHYG-1 cells (Fig. 3*C*).

The NK-92 cell line was positive only for CXCR3, although a very slight staining with the CCR1 and CXCR4 Abs was usually observed (Fig. 4*A*). In chemotaxis assays using three concentrations of ligand, ligands for CXCR3 (CXCL11/I-TAC) and CXCR4 (CXCL12/SDF-1α) induced migration of the cells; in addition, migration of the cells toward ligands for CCR1 and CCR5 (CCL5/RANTES) and CCR7 (CCL19/MIP-3β) was observed, but only at the highest concentrations (Fig. 4*B*). In full-dose chemotaxis assays, CXCL11/I-TAC recruited the NK-92 cells with an EC_{50} of 6 pM and CXCL12/SDF-1α exhibited an EC_{50} of 3 nM, whereas CCL19/MIP-3β recruited a small number of cells with an EC_{50} of 12 nM (Fig. 4*C*). Ligands for CCR4 (CCL22/MDC), CCR5 (CCL4/MIP-1β), and CCR10 (CCL27/CTACK) failed to induce migration, whereas the ligand for CX3CR1 recruited a small number of cells at the two highest doses (Fig. 4*C*). CCL3/MIP-1α, a CCR1 ligand, did not induce migration of the NK-92 cells; however, N-terminally truncated CCL23/CKβ8, a more potent CCR1 ligand, recruited the cells with an EC_{50} of 410 pM (Fig. 4*C*). Table II summarizes the data obtained from the flow cytometric and chemotaxis assays for these three NK cell lines.

Determination of chemokine receptor expression on primary NK cells

Because the published studies are discrepant with regard to chemokine receptor expression on primary peripheral blood NK cells, we sought to determine which chemokine receptors are expressed by these cells. Toward this end, we enriched freshly isolated human NK cells from the peripheral blood of two healthy human donors and performed chemotaxis assays in vitro. Enriched NK cells (95% $CD56^+CD3^-$) were exposed to increasing concentrations of ligands for CCR4 (CCL22/MDC), CCR5 (CCL4/MIP-1β), CCR6 (CCL20/MIP-3α), CCR7 (CCL21/SLC), CXCR1,2 (CXCL8/IL-8), CXCR3 (CXCL11/I-TAC), and CXCR4 (CXCL12/SDF-1α) (Fig. 5). The α chemokines CXCL8/IL-8, CXCL11/I-TAC, and CXCL12/SDF-1α induced migration of both donors' cells: CXCL8/IL-8 exhibited EC_{50}s of 250 and 70 pM for donor 1 and donor 2, respectively,

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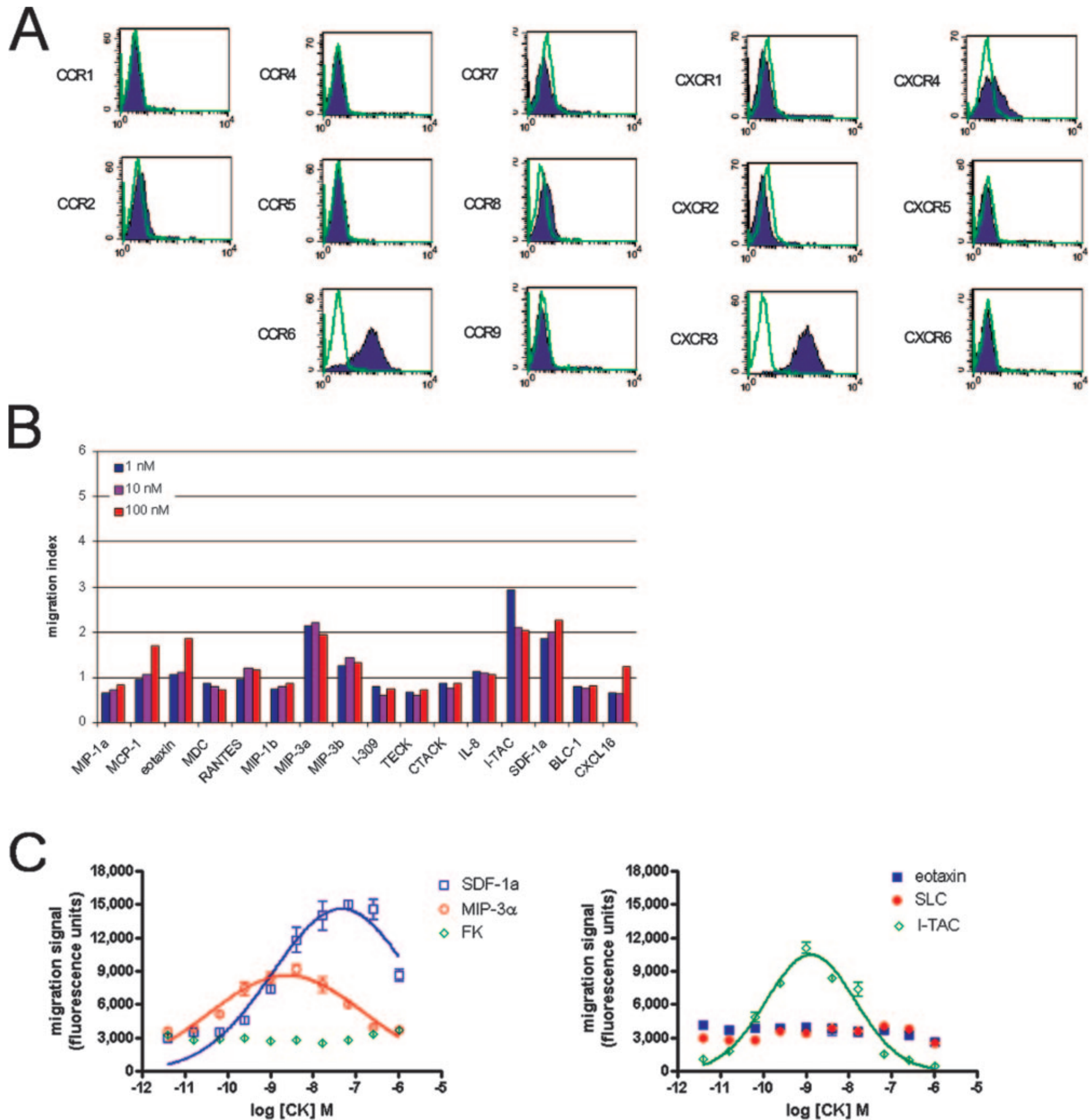

FIGURE 3. Chemokine receptor expression on the KHYG-1 cell line. *A*, Flow cytometric analysis. KHYG-1 cells were stained with Abs specific for human chemokine receptors (filled histograms) vs isotype-matched control Abs (open histograms). *B*, Chemotaxis assay. KHYG-1 cells were tested for migration to chemokines at three concentrations (1, 10, and 100 nM), and then the cells in the bottom chamber were quantified by DNA content. Data were plotted as the ratio of migration induced by chemokine to the migration induced by buffer. *C*, Full-dose chemotaxis assay. KHYG-1 cells were tested for migration to chemokines at multiple doses, ranging from 3 pM to 1 μM, and then the migrated cells were quantified by DNA content. Data were plotted as the extent of migration (in arbitrary units of fluorescence) vs chemokine concentration, and curve-fitting was performed by nonlinear regression.

whereas CXCL11/I-TAC exhibited EC_{50}s of 1.6 and 0.8 nM and CXCL12/SDF-1α exhibited EC_{50}s of 7.1 and 3.5 nM. None of the β chemokines induced migration. A chemotaxis assay was also performed on pooled IL-2-activated NK cells from two more donors; migration was induced by CXCL8/IL-8, CXCL11/I-TAC, and CXCL12/SDF-1α but not by CCL22/MDC, CCL20/MIP-3α, or ligands for CCR9 (CCL25/TECK), CXCR6 (CXCL16), or CK3CR1 (FK-CK) (data not shown).

To determine whether chemokine receptors are expressed on minor subpopulations of primary NK cells, flow cytometric analysis was performed on PBMC from 11 donors, using Abs specific for a number of chemokine receptors in combination with Abs specific for CD3, CD14, CD16, CD19, and CD56. For both $CD56^{bright}$ and $CD56^{dim}$ NK populations, data were plotted in chemokine receptor vs CD16 dot plots to reveal potentially rare subsets (data from a representative donor are depicted in Fig. 6). CCR1, CCR4, CCR5, CCR6, CCR7, and CXCR6 were detected on some but not all NK cells in each of the 11 donors, with all but CCR4 preferentially expressed in the $CD56^{bright}$ population (Table III). The proinflammatory chemokine receptors CCR1 and CCR5 were detected on approximately one-third of $CD56^{bright}$ cells, although that amount is likely an underestimate due to the exclusion

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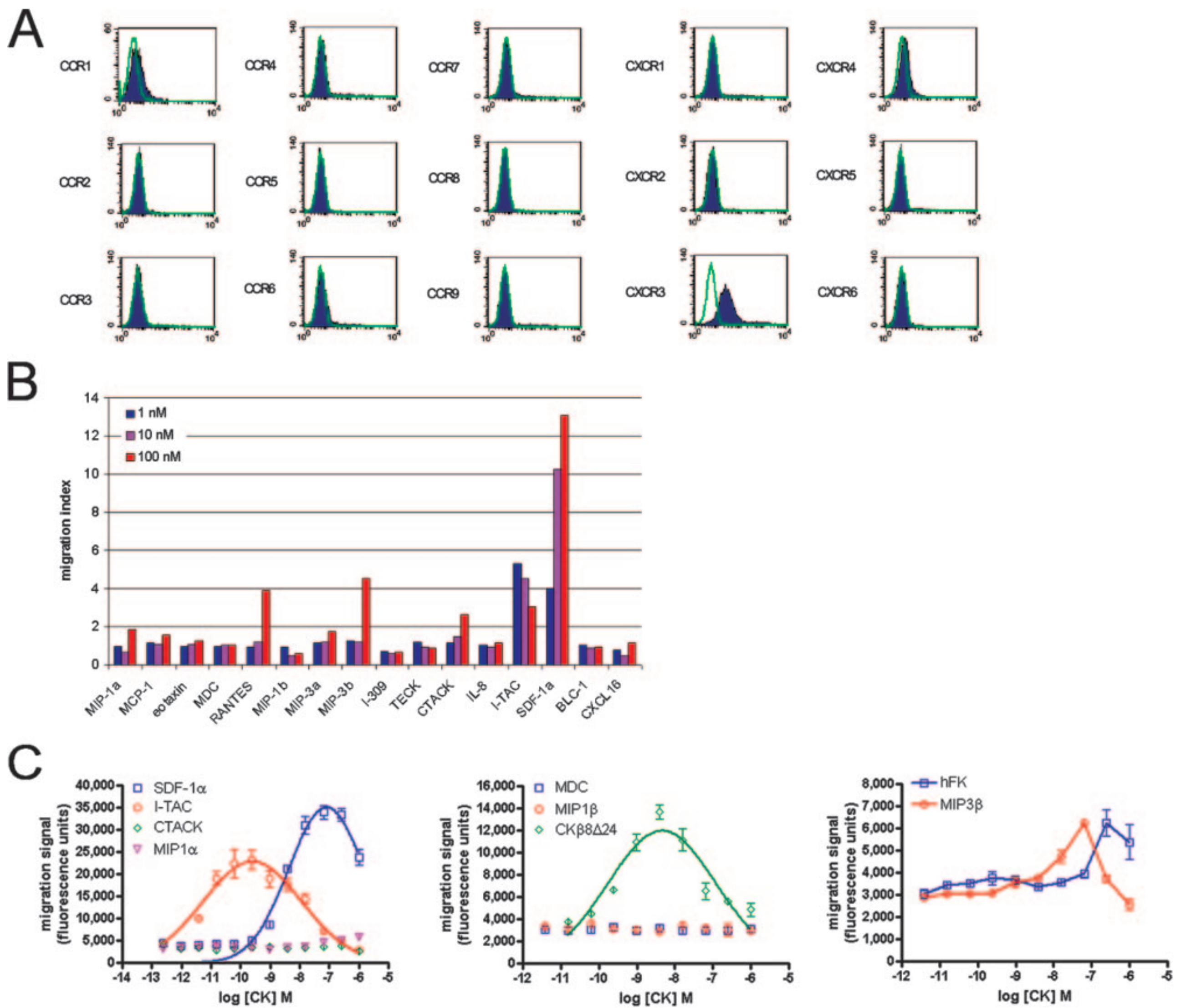

FIGURE 4. Chemokine receptor expression on the NK-92 cell line. *A*, Flow cytometric analysis. NK-92 cells were stained with Abs specific for human chemokine receptors (filled histograms) vs isotype-matched control Abs (open histograms). *B*, Chemotaxis assay. NK-92 cells were tested for migration to chemokines at three concentrations (1, 10, and 100 nM), and then the cells in the bottom chamber were quantified by DNA content. Data were plotted as the ratio of migration induced by chemokine to the migration induced by buffer. *C*, Full-dose chemotaxis assay. NK-92 cells were tested for migration to chemokines at multiple doses, ranging from 3 pM to 1 μM, and then the migrated cells were quantified by DNA content. Data were plotted as the extent of migration (in arbitrary units of fluorescence) vs chemokine concentration, and curve-fitting was performed by nonlinear regression.

of CCR1^{dim} and CCR5^{dim} cells during quantitation. CCR1 and CCR5 were also detected on ~10% of CD56^{dim} NK cells. CCR7, a receptor critical for entry of leukocytes into secondary lymphoid organs, was detected on at least 60% of CD56^{bright} cells, but <5% of CD56^{dim} NK cells. CCR4, CCR6, and CXCR6, chemokine receptors important for targeting leukocytes to specific tissues, were detected on ~4, 9, and 24% (respectively) of CD56^{bright} NK cells and on ~7, 3, and 4% of CD56^{dim} NK cells.

In contrast, CCR9 and CXCR5 were detected on NK cells from only a few donors (Table III). CCR9, a receptor important for homing of leukocytes to the intestines and possibly lung, was detected on a small subset of both CD56^{bright} and CD56^{dim} NK cells from one donor (the one depicted in Fig. 6) and on a small subset of CD56^{dim} NK cells from three other donors (data not shown). CXCR5, a receptor important for homing of leukocytes to lymphoid follicles, was detected on a small subset of both CD56^{bright} and CD56^{dim} NK cells from two donors (one depicted in Fig. 7) and on CD56^{dim} NK cells from a third donor (data not shown). Within the positive donors, CCR9 and CXCR5 were detected on 2–3% of CD56^{bright} NK cells and on only 0.2–0.8% of CD56^{dim} NK cells.

Discussion

In this study, we have determined that peripheral blood NK cells are heterogeneous in their expression of certain chemokine receptors, and thereby have the capacity for differential trafficking within the body. Using flow cytometry, we identified subsets of peripheral blood NK cells expressing the chemokine receptors CCR1, CCR4, CCR5, CCR6, CCR7, CCR9, CXCR5, and CXCR6. (At present, we do not know whether these chemokine receptors are on the same NK cell or are on different NK cells.) The chemokine receptors were detected on both CD56^{bright} and CD56^{dim} NK cells, though each receptor, except CCR4, was expressed preferentially on the CD56^{bright} NK population. CCR1, CCR4, CCR5, CCR6, and CXCR6 were detected on all 11 donors analyzed,

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Table II. *Analysis of chemokine receptor expression on NK cell lines by migration assay and FACS*

	NKL Migration	FACS	KHYG-1 Migration	FACS	NK-92 Migration	Flow Cytometry
CD56		−/dim		+		Bright
CD16		+		−		−
CCR1	−	−	−	−	+ (CKβ8; EC_{50} 410 pM)	+
CCR2	−	−	−	−	−	−
CCR3	−	−	−	ND	−	−
CCR4	+ (MDC; EC_{50} 5 pM)	+	−	−	−	−
CCR5	−	−	−	−	−	−
CCR6	−	−	+ (MIP-3α; EC_{50} 120 pM)	+	−	−
CCR7	+ (MIP-3β; EC_{50} 3 nM)	+	−	−	+/− (MIP-3β; EC_{50} 12 nM)	−
CCR8	−	−	−	−	−	−
CCR9	−	−	−	−	−	−
CCR10	−	ND	−	ND	−	ND
CXCR1	−	−	−	−	−	−
CXCR2	−	−	−	−	−	−
CXCR3	−	+	+ (I-TAC; EC_{50} 110 pM)	+	+ (I-TAC; EC_{50} 6 pM)	+
CXCR4	+ (SDF-1α; EC_{50} 4 pM)	+	+ (SDF-1α; EC_{50} 2 nM)	+	+ (SDF-1α; EC_{50} 3 nM)	+
CXCR5	−	−	−	−	−	−
CXCR6	−	−	−	−	−	−
CX3CR1	+ (FK-CK; EC_{50} 50 nM)	ND	−	ND	+/− (FK-CK; EC_{50} > 150 nM)	ND

whereas CCR9 and CXCR5 were detected on a subset of the donors. The size of the NK subsets expressing chemokine receptor varied from <1% (e.g., CCR9 and CXCR5 on CD56^{dim} NK cells) to the vast majority (e.g., CCR7 on CD56^{bright} NK cells).

In addition, we have described the complete chemokine receptor profiles of three commonly studied NK cell lines: NKL (10), KHYG-1 (11), and NK-92 (12). The cell lines each expressed CXCR3 and CXCR4, but varied in expression of other chemokine receptors. NKL, which was CD56$^{-/dim}$CD16^{+}, expressed CCR4, CCR7, and CX3CR1. KHYG-1 was CD56^{+}CD16^{-} and expressed CCR6, whereas NK-92 was CD56^{bright}CD16^{-} and expressed CCR1. The expression of CCR7 on NKL but not KHYG-1 appears contradictory, because we detected CCR7 on a much higher frequency of CD56^{bright}CD16^{-} primary NK cells than CD56^{dim}CD16^{+} cells. However, the primary tumor from which KHYG-1 developed was originally described as CD16^{+}, with CD16 disappearing during culture (11). In addition, we did detect CCR7 on a small percentage of CD56^{dim}CD16^{+} primary NK cells; the NKL line might have been derived from one of these rare cells. Results from chemotaxis assays correlated well with flow cytometric data, with the exception that CXCR3 on NKL was nonfunctional and CCR1 on NK-92 was functional only for CCL5/RANTES and CCL23/CKβ8Δ24, but not CCL3/MIP-1α. The presence of CCR1, CCR4, CCR6, and CCR7 variably on NK cell lines supports the primary NK cell flow cytometric data, indicating that these receptors are expressed variably on primary NK cells.

Enriched primary NK cells migrated to ligands for CXCR1, CXCR3, and CXCR4, as reported previously (8). Because many of the other chemokine receptors were present on very minor subsets of the CD56^{dim} NK cell population or were expressed on the rare CD56^{right} NK cell population, we were unable to detect migration of these cells in chemotaxis assays, which are not sufficiently sensitive to detect the migration of very rare cell types within a heterogeneous population. We attempted to measure chemokine receptor signaling in enriched primary NK cell preparations, using a calcium mobilization assay, but never detected robust signaling with any chemokine.

Our data obtained using primary NK cells have a few discrepancies with the two prior surveys of chemokine receptor expression by human NK cells, and the two prior studies were themselves very discrepant. Campbell et al. (8) performed flow cytometric and chemotaxis assays on freshly isolated CD56^{dim}CD16^{+} and CD56^{bright}CD16^{-} cells separately; the former cells were found to be positive only for CXCR1–4 and CX3CR1, whereas the CD56^{bright}CD16^{-} subset was found to be positive only for CCR5, CCR7, CXCR3, CXCR4, and, to a small extent, CX3CR1. Unlike in our study, Campbell et al. (8) did not detect CCR4, CCR6, CCR9, CXCR5, or CXCR6 on NK cells. Inngjerdingen et al. (9) performed both assays on total freshly isolated NK cells, with no distinction between the CD56^{dim}CD16^{+} and CD56^{bright}CD16^{-} subpopulations. By flow cytometric analysis, these NK cells were found to be positive for CXCR4, CCR7, and, to a lesser extent, CXCR3 and CCR4. By chemotaxis assays, the freshly isolated NK cells migrated to ligands for CCR2, CCR4, CCR6, CCR7, CXCR3, and CXCR4; CCR9 and CXCR6 were not evaluated. The reasons for the discrepancies in chemokine receptor detection between our study, the Campbell et al. (8)

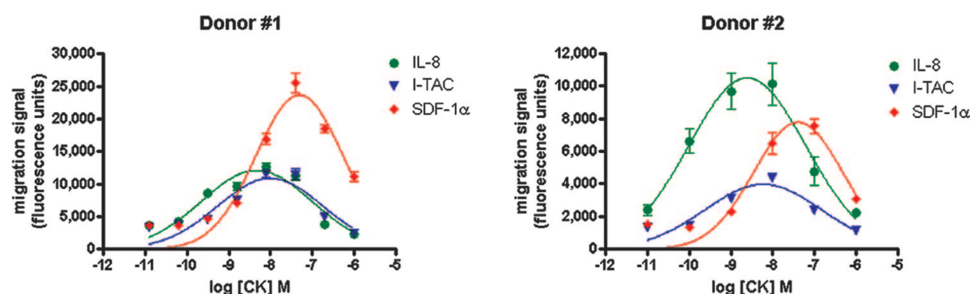

FIGURE 5. Chemotactic analysis of fresh NK cells. Freshly isolated NK cells from two donors were tested for migration to chemokines at multiple doses, ranging from 3 pM to 1 μM, and then the migrated cells were quantified by DNA content. Data were plotted as the extent of migration (in arbitrary units of fluorescence) vs chemokine concentration, and curve-fitting was performed by nonlinear regression.

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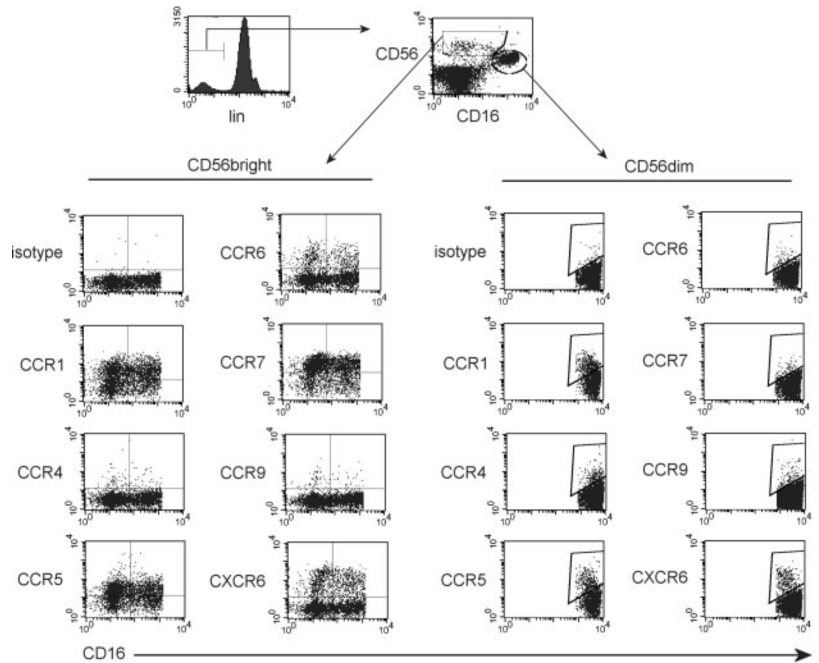

FIGURE 6. Chemokine receptor expression on NK cells in freshly prepared human PBMC. Lineage (CD3, CD14, CD19)-negative cells (histogram, *top left*) were analyzed for CD56 vs CD16 expression (dot plot, *top right*). $CD56^{bright}$ cells and $CD56^{dim}$ cells were gated and each analyzed for chemokine receptor vs CD16. $CD56^{bright}$ cells were observed to include $CD16^{-}$ and $CD16^{dim}$ cells. Chemokine receptor-positive cells were quantitated by quadrant analysis ($CD56^{bright}$ NK cells) or by gating ($CD56^{dim}$ NK cells).

study, and the Inngjerdingen et al. (9) study are not known, but may relate to differences in cell isolation techniques and/or the Abs used for detection of the chemokine receptors. Importantly, both of the prior studies analyzed histograms displaying the flow cytometric data to evaluate receptor expression; however, histograms are not sensitive for the detection of very minor subpopulations. Instead, our analysis used two-parameter dot plot displays of the flow cytometry data, in which every cell analyzed is visualized—a more sensitive technique for detecting and displaying very rare cell populations.

Our observation that NK cell subsets express CCR1, CCR4, CCR5, CCR6, CCR7, CCR9, CXCR5, and CXCR6 suggests that peripheral blood NK cells, primarily the $CD56^{bright}$ cells, may vary in their abilities to migrate to different tissues. CCR1 and CCR5 are chemokine receptors that have been implicated in response to inflammation, suggesting that subsets of $CD56^{bright}$ and $CD56^{dim}$ NK cells have the capacity to migrate to sites of inflammation. CCR7 is critical for entry of leukocytes into secondary lymphoid organs, such as lymph nodes; our results support the hypothesis that CCR7-positive $CD56^{bright}$ NK cells are immunoregulatory cells with roles inside secondary lymphoid organs, whereas CCR7-negative $CD56^{dim}$ NK cells are cytotoxic effectors maintained in the peripheral blood. CCR4 is expressed on cutaneous T cells and is thought to be necessary for homing to skin, whereas CCR9 is expressed on mucosal T cells and is thought to be necessary for homing to the intestine and perhaps lung (13). CCR6 has been implicated in homing of leukocytes to skin (i.e., Langerhans cells), intestine (14), and lung (15); CXCR6 has been implicated in homing of T cells to rheumatoid arthritic joints (16) and liver (17), and CXCR5 is critical for migration of T cells into B cell follicles in lymphoid organs (18) and lymphoid aggregates in nonlymphoid organs (19–21). It remains to be seen whether NK cells expressing these chemokine receptors are enriched at the receptors' target tissues. However, the presence of these chemokine receptors on minor subsets of NK cells in peripheral blood may provide the mechanism whereby subsets of NK cells migrate to these tissues during immune responses to infectious agents and cancer.

Table III. *Chemokine receptor expression in primary NK cell populations*

	No. of Donors[a]	Average[b]	SD
CCR1			
$CD56^{bright}$	9/9	33.8	19.0
$CD56^{dim}$	9/9	11.6	8.9
CCR4			
$CD56^{bright}$	11/11	3.6	2.0
$CD56^{dim}$	11/11	6.8	5.0
CCR5			
$CD56^{bright}$	9/9	31.3	22.0
$CD56^{dim}$	9/9	10.4	9.0
CCR6			
$CD56^{bright}$	11/11	9.3	3.8
$CD56^{dim}$	11/11	2.6	2.8
CCR7			
$CD56^{bright}$	11/11	59.7	20.0
$CD56^{dim}$	11/11	3.3	2.4
CCR9			
$CD56^{bright}$	1/11	2.1	
$CD56^{dim}$	4/11	0.4	0.1
CXCR5			
$CD56^{bright}$	2/11	2.7	0.1
$CD56^{dim}$	3/11	0.5	0.2
CXCR6			
$CD56^{bright}$	11/11	24.0	18.0
$CD56^{dim}$	11/11	3.7	3.4

[a] Number of donors expressing the chemokine receptor/number of donors analyzed.
[b] Percentage of cell population expressing the receptor.

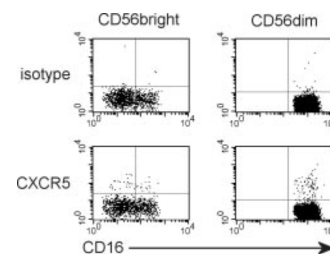

FIGURE 7. CXCR5 expression on NK cells in freshly prepared human PBMC. $CD56^{bright}$ and $CD56^{dim}$ NK cells, identified as indicated in the Fig. 5 legend, were analyzed for CXCR5 vs CD16 expression. $CXCR5^{+}$ cells were quantitated by quadrant analysis.

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Disclosures

The authors have no financial conflict of interest.

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