Natural Killer Cells Produce T Cell–Recruiting Chemokines in Response to Antibody-Coated Tumor Cells

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Abstract
In the current report, we have examined the ability of natural killer (NK) cells to produce T cell–recruiting chemokines following dual stimulation with interleukin (IL)-2 or IL-12 and human breast cancer cells coated with an antitumor antibody (trastuzumab). NK cells stimulated in this manner secreted an array of T cell–recruiting chemotactic factors, including IL-8, macrophage-derived chemokine, macrophage inflammatory protein 1α (MIP-1α), monocyte chemoattractant protein 1, and regulated on activation, normal T-cell expressed and secreted (RANTES), whereas stimulation of NK cells with either agent alone had minimal effect. Furthermore, these factors were functional for T-cell chemotaxis as culture supernatants derived from costimulated NK cells induced migration of both naive and activated T cells in an in vitro chemotaxis assay. T-cell migration was significantly reduced when neutralizing antibodies to IL-8, MIP-1α, or RANTES were added to culture supernatants before their use in the chemotaxis assay. In addition, coadministration of trastuzumab-coated tumor cells and IL-12 to mice led to enhanced serum MIP-1α. As a clinical correlate, we examined the chemokine content of serum samples from breast cancer patients enrolled on a phase I trial of trastuzumab and IL-12, and found elevated levels of IL-8, RANTES, IFN-γ-inducible protein 10, monokine induced by IFN-γ, and MIP-1α, specifically in those patients that experienced a clinical benefit. Sera from these patients exhibited the ability to direct T-cell migration in a chemotaxis assay, and neutralization of chemokines abrogated this effect. These data are the first to show chemokine production by NK cells, specifically in response to stimulation with antibody-coated tumor cells, and suggest a potential role for NK cell–derived chemokines in patients receiving therapeutic monoclonal antibodies. (Cancer Res 2006; 66(1): 517-26)

Introduction
Natural killer (NK) cells are bone marrow–derived, large granular lymphocytes that participate in the innate immune response to virally infected and malignant cells (1). In addition to their ability to recognize and eliminate these altered cells by cytotoxic mechanisms (2), activated NK cells are a potent source of immune modulatory cytokines that directly aid in the clearance of pathogens and also indirectly augment a developing adaptive immune response (3). NK cell–derived IFN-γ, for example, can directly affect the growth and immune recognition of virally infected cells (4) as well as promote the differentiation of naïve CD4+ T cells into Th1-type helper effector cells (5). Thus, NK cells are able to promote an immune as well as a specific immune response to intracellular pathogens and malignant cells via the secretion of specific cytokines.

The proper recruitment of immune cells to sites of inflammation is critical to the initiation and effector phases of an effective immune response. After presentation of antigens to T lymphocytes, delivery of costimulatory signals, and expansion, effector T cells must receive proper homing signals to be directed to their sites of action. Throughout this process, chemotactic cytokines, or chemokines, play a pivotal role in orchestrating the adaptive immune response. It is not surprising that NK cells, in keeping with their unique role in bridging innate and adaptive immunity, are potent sources of chemokines. In fact, even resting NK cells have been shown to express mRNA for the chemokines macrophage inflammatory protein 1α (MIP-1α), monocyte chemoattractant protein (MCP)-2, and activation-induced, T cell–derived, and chemokine-related (ATAC), although the cells produce very little protein in the absence of stimulatory signals (6, 7). Following activation, however, NK cells have been shown to dramatically up-regulate their expression of chemokine transcripts and to secrete several factors with the capacity to recruit T cells, B cells, neutrophils, and other activated NK cells (8–10). It has been shown that NK cells produce chemokines after engagement of CD43 (11) or following activation of their NK receptors during antigen presentation (12). Interleukin (IL)-2- or IL-12-activated NK cells have been reported to secrete low amounts of several T cell–recruiting chemokines, including MIP-1β, MIP-1β, IL-8, macrophage-derived chemokine (MDC), and regulated on activation, normal T-cell expressed and secreted (RANTES; refs. 14, 15). MIP-1γ secretion has also been reported from NK cells following stimulation with immunoglobulin E (16) and cross-linking of CD16 (6). These studies support a role for NK cells in the initiation of a specific immune response by facilitating T-cell recruitment. Despite this evidence, little is known about NK cell chemokine production in response to antibody-coated targets.

We have previously shown that NK cells costimulated with antibody-coated tumor cells and the immune stimulatory cytokines IL-2 or IL-12 secrete large quantities of immune modulatory cytokines, including IFN-γ, tumor necrosis factor–α, and granulocyte macrophage-colony stimulating factor (17, 18). In the current report, we have examined the chemokine response of NK cells in response to this stimulus. We show that costimulated NK cells secrete a broad array of T cell–attracting chemokines, including MIP-1α, MCP-1, RANTES, IL-8, and MDC. Furthermore, these

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chemokines have potent T cell–recruiting activity as NK cell–derived culture supernatants induced significant migration of both naïve and activated T cells in an in vitro chemotaxis assay. In addition, mice receiving IL-12 and HER2-positive tumor cells coated with an anti-HER2 monoclonal antibody (mAb; trastuzumab) exhibited enhanced systemic levels of the chemokine MIP-1α as compared with mice receiving either agent alone. As a clinical correlate, we detected increased circulating levels of NK cell–derived chemokines within a select group of patients who exhibited a clinical response or significant stabilization of disease during therapy with an antitumor antibody and IL-12. Taken together, these data suggest a potential role for NK cell–derived chemokines in the immune response to antibody-coated targets.

Materials and Methods

Cytokines and antibodies. Recombinant human IL-12 was kindly provided by Genetics Institute, Inc. (Cambridge, MA). Recombinant human IL-2 was obtained from Hoffman-LaRoche (Nutley, NJ). All cytokines were reconstituted in 1× PBS containing 0.1% bovine serum albumin. Trastuzumab, a humanized anti-HER2 mAb, was provided by Genentech Corp. (San Francisco, CA). Polyclonal human immunoglobulin G (IgG) was purchased from Sigma-Aldrich (St. Louis, MO).

Cell lines. The SKBR3 (HER2-overexpressing) and MDA-468 (HER2-negative) human breast adenocarcinoma lines were obtained from the American Type Culture Collection (Manassas, VA). Cell lines were propagated in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum (FBS), 300 μg/mL of gl-tumegline, 100 units/mL of penicillin, 100 μg/mL of streptomycin, 0.25 μg/mL of amphotericin B, and 0.06 mg/mL of anti-PPLO agent (all from Life Technologies, Inc., Rockville, MD).

Isolation of human NK cells. NK cells were isolated directly from fresh peripheral blood leukopacks (American Red Cross, Columbus, OH) by 30-minute incubation with RosetteSep cocktail (Stem Cell Technologies, Vancouver, BC) before Ficoll Hypaque (Sigma) density gradient centrifugation. Isolated CD56− NK cells were >96% pure by fluorescence-activated cell sorting analysis. Human NK cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated pooled human AB serum (HAB; C-Six Diagnostics, Germantown, WI), 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of amphotericin B (10% HAB medium).

In vitro chemokinesis. Tumor cells were grown to confluence overnight and then treated with medium alone, trastuzumab alone, or cytokine alone. Control conditions consisted of NK cells plus tumor cells treated with medium alone, trastuzumab alone, or cytokine alone. Culture supernatants were harvested after 72 hours and analyzed by ELISA.

Chemokines and antibodies. In vitro chemotaxis experiments, wells of a 96-well flat-bottomed plate were coated with 100 μg/mL of human IgG in cold PBS overnight at 4°C, washed with cold PBS, and then plated with human NK cells (2 × 10^6 per well) and 10 ng/mL of IL-2 or IL-12 as previously described (18). At the indicated time points, cell-free culture supernatants were harvested and analyzed for levels of various chemokines [MIP-1α, MIP-1β, RANTES, MCP-1, IL-8, MDC, monokine induced by IFN-γ (MIG), IFN-γ inducible protein 10 (IP-10), and growth-related oncogene α (GRO-α)] using commercially available ELISA kits (Endogen, Inc., Woburn, MA). For the in vitro coculture assay, wells of a 96-well flat-bottomed culture plate were seeded with either the HER2-overexpressing human breast cancer cell line SKBR3 or the HER2-negative human breast adenocarcinoma MDA-468 at a density of 5 × 10^5 tumor cells per well. Tumor cells were grown to confluence overnight and then treated with 100 μg/mL of trastuzumab for 1 hour at 37°C as previously described (18). After washing off unbound trastuzumab, purified NK cells were then added at 2 × 10^6 per well in 200 μL of 0.5% HAB medium containing 10 ng/mL of IL-12 or IL-2. Control conditions consisted of NK cells plus tumor cells treated with medium alone, trastuzumab alone, or cytokine alone. Culture supernatants were harvested after 72 hours and analyzed by ELISA. The lower detection limit for all ELISAs was <20 pg/mL. All results shown are the mean of triplicate wells ± SE.

Measurement of T-cell chemotaxis. Activated T cells were generated by sequential culture of peripheral blood-derived T cells from a normal donor for 2 days with 1 μg/mL phytohemagglutinin (PHA; Sigma-Aldrich) and then for 3 days with 500 pmol/L huIL-2 in RPMI 1640 complete medium supplemented with 10% FBS (19). Transwell inserts for the migration experiments (Corning, Somerset, NJ) were prepared by coating with 0.01% gelatin at 37°C overnight, followed by 3 μg of human fibronectin (Life Technologies, Grand Island, NY) at 37°C for 3 hours to mimic endothelial and extracellular matrix components as previously described (19). Migration experiments were done by placing 2 × 10^6 purified naïve or activated T cells in 100 μL of 10% HAB medium in the upper chambers of precoated 5-μm-pore Transwell inserts. Inserts were then placed into wells of a 24-well plate. NK cell–derived culture supernatants (400 μL) were placed in the lower chambers of the wells. In some experiments, 400 μL of uniplet dBH patient serum were used as the chemokine source. Plain medium or medium supplemented with 1 μg/mL MIG served as negative and positive controls, respectively. The plates were then incubated for 3 hours at 37°C with 5% CO2, followed by a 10-minute incubation at 4°C to loosen any cells adhering to the undersides of the insert membranes (20). The fluid in the lower chambers was then collected separately and migrated cells were counted using trypsin blue exclusion. Some of the cells were analyzed for CD4 and CD8 expression by flow cytometry to assess the CD4/CD8 ratio of migrated T cells. In some experiments, NK cell culture supernatants or patient serum samples were incubated with 10 μg/mL of neutralizing antibodies to MIP-1α (R&D Systems, Minneapolis, MN), RANTES (American Research Products, Belmont, MA), or IL-8 (BD Pharmingen, San Diego, CA) or with equivalent amounts of isotype-matched control IgG (Sigma) for 1 hour at 4°C before use in chemotaxis assays.

In vivo chemotaxis. A BALB/c murine colon carcinoma line overexpressing human HER2/neu, CT-26(HER2/neu), was obtained from Dr. P. Kaumaya (Ohio State University Biochemistry Program, The Ohio State University, Columbus, OH). CT-26(HER2/neu) cells were incubated at 4°C in PBS (cell density, 1 × 10^6/mL) with either Herceptin or normal human IgG (both at 1 mg/mL) for 45 minutes. Cells were then washed twice in sterile PBS, resuspended in PBS, and injected i.p. into female BALB/c mice, ages 5 to 7 weeks (The Jackson Laboratory, Bar Harbor, ME). Mice also received a separate i.p. injection of 1 μg murine IL-12. Control groups (n = 5) received injections of IgG-treated tumor alone, Herceptin-coated tumor alone, or IgG-treated tumor and IL-12. Serum was harvested at 24 hours postinjection and analyzed for MIP-1α levels by ELISA (R&D Systems).

Patient tissue and serum samples. Fifteen patients received treatment on an National Cancer Institute (NCI)–sponsored phase I trial of IL-12 with trastuzumab for patients with HER2-overexpressing malignancies (T99-0032). All studies were conducted in accordance with the rules and regulations of The Ohio State University Institutional Review Board (OSU-9968). Patients with nonhematologic malignancies that expressed HER2 were eligible for enrollment. Consecutive patients received a loading dose of 4 mg/kg trastuzumab iv., followed 1 week later by a maintenance dose of 2 mg/kg. On the third week and every week thereafter, patients received 2 mg/kg trastuzumab on day 1 of the weekly cycle followed by s.c. IL-12 on days 2 and 5. The starting dose of IL-12 was 30 ng/kg. Patients received this therapy for 14 weeks, at which time they were restaged with appropriate imaging modalities. Successive cohorts of three patients received increasing doses of IL-12 (30, 100, 300, and 500 ng/kg s.c.). However, neither the dose of trastuzumab nor IL-12 was escalated within a given patient. Patients with a clinical response or stable disease at week 14 were allowed to continue therapy for a total of 54 weeks or until disease progression. Patient characteristics and response criteria have been previously described. Peripheral blood was obtained via phlebotomy and serum was collected per standard protocol (21). Surgical specimens were obtained after acquisition of patient informed consent through the Cooperative Human Tissue Network facility at the Ohio State University (Columbus, OH). Following biopsy, tumors were fixed in formalin, embedded in paraffin, sectioned into 5-μm slices using a standard microtome, and attached to a lysis-coated slide. When possible, these specimens were selected to include tumor and adjacent nonmalignant tissue.

Immunohistochemistry and in situ reverse transcription-PCR. Sections were deparaffinized in xylene (twice, 10 minutes each at room temperature) and rehydrated by stepwise washes at decreasing ethanol/H2O ratios. Endogenous peroxidase activity was blocked with diH2O containing 3% hydrogen peroxide for 5 minutes, followed by repeated rinses in diH2O.
Antigen retrieval was achieved in DAKO target retrieval solution (DAKO S1699) by heating slides in a steamer at 94°C for 30 minutes and cooling at room temperature for 15 minutes. After rinsing in dH₂O, slides were incubated for 60 minutes with primary antibodies specific for CD4+ T cells, CD8+ T cells, and granzyme b (BD Transduction Laboratories, San Diego, CA). Detection was achieved with the Isab+ system and 3,3'-diaminobenzidine chromogen (DAKO, Carpinteria, CA). Some tumor sections were tested for CD56 mRNA expression using in situ reverse transcription-PCR (RT-PCR) with CD56-specific primers (F-\text{TTGTTTTTCCTGGGAACCTGC, R'-CCCAGACTCGACGTTTTGTA}). Covariates were applied following counterstaining with Mayer's hematoxylin and dehydration. Tissue specimens were processed in a single batch to ensure a valid comparison between samples. Several high-power fields per tumor section were analyzed using a Nikon Eclipse E400 microscope (Tokyo, Japan). Care was taken to eliminate compromised areas that could bias the analysis, such as artifacts generated during processing or staining.

Statistics. All statistical analyses were done using the Student’s t test with $P < 0.05$ considered significant.

Results

Human NK cells stimulated with immobilized antibody in the presence of immune stimulatory cytokines secrete high levels of T cell–attracting chemokines. The ability of NK cell–activating cytokines such as IL-2 and IL-12 to enhance NK cell chemokine production in the presence of immobilized antibody was evaluated. NK cells produced moderate amounts of the chemokines IL-8, MIP-1α, and RANTES, but not MDC, in response to IL-12 or IL-2 alone (Fig. 1A). IgG alone induced NK cell production of IL-8 only. Both IL-2 and IL-12 significantly enhanced the production of IL-8, MIP-1α, MIG, and IP-10 from NK cells costimulated with immobilized IgG (Fig. 1A and data not shown).

In addition, NK cells costimulated with IgG and IL-2 produced increased quantities of MDC whereas NK cells costimulated with IgG and IL-12 produced large quantities of RANTES. Secretion of MIP-1β, MCP-1, 6-cysteine chemokine (6ckine), MIP-3β, or GRO-α was not observed under any of the conditions tested (data not shown). Cross-linking of FcγRIIIa with an anti-CD16 mAb (clone 3g8, Mederex, Annandale, NJ) in the presence of IL-2 or IL-12 resulted in levels of chemokine secretion that were comparable to that obtained via costimulation with immobilized IgG and cytokine (data not shown).

To extend these observations in the context of trastuzumab plus cytokine combination therapy, we employed an in vitro assay to better reflect the tumor microenvironment (18). HER2-overexpressing (SKBR3) and HER2-negative (MDA-468) cell lines were grown to confluence in flat-bottomed wells and coated with trastuzumab. Each well was then supplemented with purified human NK cells in

![Chemokine Production by FcR and Cytokine Costimulation](www.aacrjournals.org)

**Figure 1.** Human NK cells stimulated with immobilized antibody in the presence of immune-stimulatory cytokines secrete high levels of T cell–attracting chemokines. A, human NK cells were cultured on wells precoated with human IgG. Recombinant human IL-2 or recombinant human IL-12 were added at a concentration of 10 ng/mL. Control wells consisted of NK cells cultured with medium alone (Medium), immobilized human IgG alone (IgG), or cytokine alone (IL-2 or IL-12; Cytokine). Culture supernatants were harvested after 72 hours and analyzed for the indicated chemokines by ELISA.

B, human NK cells were cocultured with a trastuzumab-coated HER2-overexpressing human breast cancer cell line (SKBR3) in the presence of IL-2 or IL-12 (10 ng/mL). Control wells consisted of NK cells cocultured with untreated tumor cells (Medium), trastuzumab-treated tumor cells alone (Tras), or cytokine alone (IL-2 or IL-12; Cytokine). NK cells cultured in the presence of tumor cells that had been treated with control IgG produced negligible amounts of chemokines (data not shown). Supernatants were harvested at 72 hours and analyzed for chemokine content. *, $P < 0.05$, versus all conditions shown.
media containing IL-2 or IL-12. Trastuzumab-coated HER2-overexpressing tumor cells served as a potent costimulus for NK cell production of the chemokines IL-8, MIP-1α, MDC, MCP-1, MIG, and IP-10 in the presence of IL-2 or IL-12 (Fig. 1B and data not shown). NK cell production of RANTES was also observed in response to stimulation with IL-12 or IL-2 alone; however, the amount of RANTES secreted did not increase on costimulation with trastuzumab (data not shown). Chemokine production was minimal in response to a HER2-negative cell line regardless of the presence of cytokine (data not shown). Secretion of the chemokines 6ckine, MIP-3β, and GRO-α was not observed under any conditions tested (data not shown).

Supernatants from NK cells costimulated with antibody-coated tumor plus cytokine induce chemotaxis of naïve and activated T cells. Having shown that NK cells costimulated with antibody-coated breast cancer cells and IL-2 or IL-12 produce large quantities of several T cell–attracting chemokines, we next wanted to determine the capacity of these NK cell–derived factors to induce T-cell migration. Peripheral blood T cells were isolated via magnetic separation and were either left untreated (i.e., naïve) or activated with PHA and IL-2 (see Materials and Methods). The naïve and activated phenotypes of these T cells were confirmed via examination of activation marker expression (CD25 and CD69) and IFN-γ secretion on T-cell receptor restimulation (ref. 23; Fig. 2A). T cells were then placed in the upper chamber of Transwell inserts (prepared as described in Materials and Methods). The lower chambers contained cell culture supernatants from the various conditions of the costimulation assays that were described in Fig. 1. Medium supplemented with MIG, a chemotactic factor known to induce T-cell migration (24), served as a positive control for T-cell chemotaxis. Naïve T cells exhibited slight migration (10% T-cell migration) in response to supernatants from NK cells stimulated with trastuzumab-coated tumor alone (Fig. 2B). Interestingly, this migration was slightly inhibited in response to supernatants derived from NK cells that had been costimulated with antibody and IL-2. In contrast, culture supernatants from NK cells costimulated with trastuzumab-coated tumor and IL-12 induced significant levels of chemotaxis (∼36% of naïve T cells migrated) as compared with supernatants derived from single stimulation conditions. Similar results were obtained with activated T cells although activated T cells were able to respond with greater migration across all conditions examined (Fig. 2C). CD4/CD8 ratios of donor T cells were within the reference range (0.5-2.3) and did not change on T-cell activation. Interestingly, for each individual donor, the CD4/CD8 ratio of the migrated T cells was similar to that of the starting T-cell population (not shown), suggesting that each T-cell subset within the generated populations expressed a similar capacity to respond to secreted chemokines. Taken together, these results suggested that factors derived from NK cells stimulated by antibody-coated tumor cells in the presence of activating cytokines such as IL-2 or IL-12 were capable of recruiting naïve and activated T cells.

Figure 2. Supernatants from NK cells costimulated with immobilized antibody plus cytokine induce chemotaxis of naïve and activated T cells. A, phenotype of naïve and activated peripheral blood-derived T cells with regard to surface expression of the activation markers CD69 and CD25 (middle and bottom flow cytometric dot plots), as well as IFN-γ secretion in response to CD3 stimulation. Naïve (B) and activated (C) T cells were assayed for chemotaxis in response to culture supernatants derived from NK cells costimulated with trastuzumab-coated tumor plus IL-2 or IL-12. Supernatants from control-stimulated NK cells were also used. *, P < 0.01, versus all conditions shown.
Neutralization of chemokines reduces T-cell migration in response to NK cell supernatants. To confirm the role of NK cell–derived chemokines in driving T-cell chemotaxis, supernatants derived from IL-12 and trastuzumab costimulated NK cells were incubated with neutralizing concentrations of antibodies to MIP-1α, RANTES, and/or IL-8 before use in the chemotaxis assays. Neutralization of either MIP-1α or RANTES had a minimal effect on the migration of naïve T cells whereas neutralization of IL-8 reduced migration by ~50%. Neutralization of all three chemokines reduced naïve T-cell migration to ~25% of the original level (Fig. 3A). Neutralization of either MIP-1α or RANTES also had a slight (~10%) effect on the migration of activated T cells whereas neutralization of IL-8 reduced migration of activated T cells by ~60%. As with naïve T cells, neutralization of all three chemokines had a dramatic effect on the migration of activated T cells, reducing chemotaxis to ~20% of the original level (Fig. 3B).

IL-12 enhances in vivo chemokine production in response to Herceptin-coated tumor cells. We next wanted to determine whether administration of mAb-coated tumor cells and IL-12 could lead to enhanced chemokine levels in vivo. An in vivo costimulation experiment was done in which IL-12 and trastuzumab-coated, human HER2-overexpressing murine tumor cells were delivered to naïve mice via the i.p. route. Serum was harvested at 24 hours and evaluated for MIP-1α content. No MIP-1α was detected in the serum of mice that had received control IgG-coated tumor cells or trastuzumab-coated tumor cells alone. Mice receiving IL-12 and control IgG–treated tumor cells produced modest amounts of MIP-1α (111 ± 29 pg/mL) whereas mice receiving IL-12 and trastuzumab-coated tumor cells had significantly elevated serum levels of this chemokine (402 ± 42 pg/mL; P < 0.005). These results show that coadministration of IL-12 and trastuzumab-coated tumor cells can lead to elevated chemokine levels in vivo.

Examination of chemokine levels in patients enrolled in a phase 1 clinical trial of trastuzumab plus IL-12 combination therapy. We next examined whether patients with HER2-overexpressing malignancies undergoing treatment with trastuzumab and IL-12 were able to exhibit a similar NK cell chemokine response in the peripheral blood (see ref. 21 for treatment schema). Serum had been obtained before the administration of trastuzumab or IL-12 on days 1, 2, and 5 of each weekly cycle. Serum samples from each patient from all cycles of therapy were analyzed for the presence of the chemotactic factors IP-10, MIG, IL-8, MDC, MIP-1α, RANTES, and MCP-1 (those up-regulated in the in vitro models). Pretreatment (baseline) sera from all patients examined had measurable levels of each of these factors that were at or slightly above levels found within healthy adults (n = 10 normals examined; Fig. 4A and B, dashed line). Levels of serum MDC and MCP-1 did not significantly change during the course of treatment for any of the patients examined (data not shown). In contrast, levels of IL-8 (Fig. 4A) and RANTES (Fig. 4B), as well as IP-10, MIG, and MIP-1α (20), increased throughout the course of treatment only in those patients who exhibited a complete clinical response or prolonged stabilization of disease (i.e., lasting ≥12 months). Whereas all patients on trial (n = 15) experienced an early increase in serum IL-8 and MIP-1α following the initial dose of IL-12 in cycle 3, only the three patients experiencing clinical benefit continued to produce significant quantities of these chemokines following subsequent doses of IL-12 (Fig. 4A and data not shown). Serum IL-8 and MIP-1α levels for these three patients continued to increase throughout the duration of the trial; in contrast, serum levels of these chemokines remained at baseline levels in the nonresponding patients (Fig. 4A and data not shown). Serum levels of RANTES did not follow the same pattern in terms of regulation by IL-12 during each individual cycle but did increase over the course of therapy, specifically in patients who exhibited a clinical benefit (Fig. 4B and data not shown). Indeed, an examination of chemokine levels in the last cycle of therapy revealed that patients with progressive disease had levels of IL-8, MIP-1α, and RANTES that were equal to or lower than baseline values. In contrast, in the last cycle of therapy, the patients that experienced clinical benefit (E, M, and Q) exhibited 7.0-fold, 3.9-fold, and 4.6-fold increases in serum levels of IL-8, MIP-1α, and RANTES, respectively, as compared with each patient’s baseline value (data not shown; P < 0.01).

Sera from patients experiencing a clinical benefit on a phase 1 trial of trastuzumab and IL-12 induce chemotaxis of naïve and activated T cells. We next replicated the chemotaxis assay described above using serum samples from patients undergoing therapy with trastuzumab and IL-12 as the chemokine source. For each patient, three serum samples were analyzed: the baseline sample, the sample procured at day 1 of cycle 4 (when all patients displayed an increase in serum IL-8 and MIP-1α), and the sample obtained at day 5 of cycle 5 (when only clinical benefit patients exhibited an increase in these factors). Sera from two normal donors were also examined for comparison. Sera from both
groups of patients (clinical benefit and progressive disease) exhibited an enhanced ability to induce chemotaxis of naïve T cells at day 1 of cycle 4 (just after IL-12 was added to the treatment regimen) as compared with each patient’s respective baseline sample. However, the serum from patients E, M, and Q (clinical benefit) induced chemotaxis at a later time point (day 5 of cycle 5) whereas the serum from patients H, J, and P (progressive disease) did not have this capacity at the same time point (Fig. 5A and B). A similar pattern of migration was observed for activated T cells, for which the level of chemotaxis was proportionately higher across all samples tested (Fig. 5C and D). These results suggested that therapy with trastuzumab and IL-12 induced a functional program of chemokine secretion within a subset of patients that responded favorably to the treatment. To determine whether the chemokines found to be up-regulated in the sera of responding patients were responsible for the observed T-cell migration, we preincubated serum from patient Q (obtained at day 5 of cycle 5) with neutralizing antibodies to IL-8, MIP-1α, and RANTES before use in the chemotaxis assay. Treatment of the serum in this fashion reduced the migration of T cells to ~10% of the original value, confirming a vital role for these chemokines in directing T-cell migration (Fig. 5E).

Tumor-infiltrating lymphocytes in a patient who exhibited a complete response to trastuzumab plus IL-12 therapy consist mainly of cytotoxic CD8+ T cells. To determine the effect of secreted chemokines on the infiltration of tumor deposits by effector T cells, we examined tumor samples obtained from patients enrolled on NCI trial T99-0032. Figure 6 shows representative immunohistochemical staining of lymph nodes from a patient with metastatic breast cancer obtained before (A-D) or during (E-H) therapy with trastuzumab and IL-12. This patient later exhibited a complete clinical response to therapy. The H&E stains (Fig. 6A and E) revealed extensive replacement of the lymph node with poorly differentiated adenocarcinoma. Focal infiltration of the tumor by lymphocytes was observed both before and during therapy. Immunostaining of the samples revealed an overall increase in the number of tumor-infiltrating T cells during the course of therapy as compared with the pretreatment sample. The majority of the tumor infiltrate was of the CD8+ phenotype (Fig. 6B and F) whereas very few CD4+ T cells were observed at either time point (data not shown). In addition, the CD8+ T cells in the pretreatment sample did not seem to be cytotoxic as no staining for granzyme b was observed (Fig. 6C) whereas the CD8+ T cells in the sample obtained during therapy showed marked granzyme positivity (Fig. 6G), suggesting a cytotoxic phenotype. In situ RT-PCR with primers specific for CD56 revealed rare infiltrating NK cells within the posttreatment sample but not within the pretreatment sample (Fig. 6D and H). These observations suggested that administration of trastuzumab and IL-12 had led to T-cell infiltration of a nodal tumor deposit, possibly in response to NK cell–derived factors.

**Figure 4.** Serum IL-8 and RANTES levels increased during trastuzumab plus IL-12 therapy specifically in patients experiencing a clinical benefit. Serum samples from all cycles of therapy from all patients on trial were analyzed for the presence of the chemotactic factors IL-8, MIP-1α, and RANTES at three time points within each weekly treatment cycle (days 1, 2, and 5) to evaluate the effects of the addition of IL-12 to the trastuzumab treatment regimen. Results for IL-8 (A) and RANTES (B) from patient E (complete response) compared with patient J (representative progressive disease patient) for the first seven cycles of therapy. Trends similar to those of patient E were observed for patients M and Q (significant stabilization of disease). Similar results were obtained in later cycles (data not shown). Dashed line, average level of the chemokine within normal sera (mean of 10 different normal donors). No significant increases or correlations with response were observed for levels of serum MDC or MCP-1 in these patients (not shown). *, P < 0.001, versus the level of chemokine at the previous time point.
Discussion

The current study examined the ability of primary human NK cells to produce chemokines following FcR engagement and the enhancement of this response by immune stimulatory cytokines such as IL-2 or IL-12. NK cells produced modest amounts of chemokines in response to different types of antibody-coated targets, including immobilized IgG- and mAb-coated human breast cancer cells. Costimulation of NK cells with IL-2 or IL-12 resulted in enhanced production of several of these chemokines, including IL-8, MIP-1α, MIP-1β, MDC, MCP-1, and RANTES. Culture supernatants from costimulated NK cells were also able to direct the migration of naïve and activated T cells. This migration was reduced when culture supernatants were preincubated with neutralizing antibodies to IL-8, RANTES, and/or MIP-1α before use in the chemotaxis assays. In addition, enhanced levels of NK cell–derived chemokines with the ability to direct T-cell chemotaxis were also detected within the sera of a subset of patients experiencing clinical benefit on a phase I trial of trastuzumab and IL-12.

Although NK cell chemokine secretion in response to activation with IL-2 or IL-12 is well documented (14, 15), less is known about the NK cell chemokine response to Fc receptor stimulation. Oliva et al. (6) have shown that cross-linking of CD16 on NK cells in the presence of IL-2 resulted in enhanced secretion of RANTES, MIP-1α, and MIP-1β. In contrast to the present report, however, IL-12 did not enhance the production of MIP-1α from CD16 cross-linked NK cells. This discrepancy could be due to the fact that these studies used NK cells derived from HIV+ donors and employed CD16 cross-linking as the FcR stimulus rather than antibody-coated targets. The current report extends these findings to Fc receptor stimulation via tumor-bound antibody. To date, little has been reported about the ability of NK cell–derived factors to recruit T cells although Somersalo et al. (10) have shown that supernatants from IL-2-activated NK cells could induce the chemotaxis of CD4+ and CD8+ T cells through fibronectin-coated filters in vitro and identified IL-8 as one of the factors responsible for this activity. The present report has identified MIP-1α and RANTES as additional factors that are secreted in response to FcR/cytokine receptor costimulation and documented their role in mediating T-cell chemotaxis. The fact that the presence of tumor-infiltrating T cells has been correlated with decreased incidence of recurrence and increased overall survival in patients with ovarian, breast, prostate, esophageal, and colorectal carcinomas highlights the clinical importance of T-cell infiltration of the tumor (25–29). Our finding that NK cells costimulated with antibody-coated tumor cells and

Figure 5. Chemotactic capacity of patient serum samples. Naïve (A and B) and activated (C and D) T cells were assayed for their chemotactic response to serum samples obtained from patients undergoing therapy with trastuzumab and IL-12. Sera were analyzed from all patients experiencing clinical benefit (E, M, and Q) and from three representative patients with progressive disease (H, J, and P). Samples analyzed were those obtained before any treatment (baseline) and those obtained on day 1 of cycle 4 and day 5 of cycle 5 of therapy. Samples from all patients on trial had been observed to contain elevated levels of IL-8 and MIP-1α at day 1 of cycle 4 whereas only samples obtained from clinical benefit patients contained elevated levels of these chemokines at day 5 of cycle 5 (see Fig. 4). Medium supplemented with MIG served as a positive control. Normal sera from two healthy donors are shown for comparison. E, migration of activated T cells was measured in response to patient serum that had been preincubated with neutralizing concentrations of antibodies to IL-8, RANTES, and MIP-1α or with isotype-matched control IgG. The serum sample was obtained from a responding patient (patient Q) on day 5 of cycle 5 of therapy and had been previously observed to contain high levels of IL-8, RANTES, and MIP-1α and to direct significant migration of naïve and activated T cells (see A and C). *, P < 0.05, versus the level of chemotaxis of the baseline sample for that patient.
IL-2 or IL-12 can recruit naïve and activated T cells suggests an additional mechanism through which T-cell infiltration of the tumor can be achieved.

NK cell chemokine secretion has been shown within several infectious disease models. The ability of NK cells to secrete a broad array of chemokines was shown by Dorner et al. (30) who showed by flow cytometry that at the single-cell level, MIP-1α/β, RANTES, and ATAC were all cosecreted with IFN-γ by individual activated NK cells in a murine model of listeriosis. The authors proposed that these cytokines acted together as a functional unit to recruit CD8+ T cells and mediate the transition to an antigen-specific phase of immune defense. The same group later reported a similar effect within a murine model of cytomegalovirus (13). NK cell secretion of the IFN-γ-induced chemokines MIG and IP-10 has been shown to facilitate the migration of T cells into the liver in a murine model of adenovirus infection (31). Enhanced circulating levels of MIG and IP-10 have also been detected in human patients with meliodosis, a Gram-negative bacterial infection (32). The secretion of these and other chemokines by human NK cells costimulated with antibody and IL-2 or IL-12 may therefore reflect a normal physiologic process through which NK cells promote an adaptive immune response during the course of an infection.

A limited number of studies have examined the role of chemokines within the context of experimental tumor models. Increased numbers of tumor-infiltrating lymphocytes were observed when mice bearing 4T1 adenocarcinomas were treated with IL-12. Enhanced levels of mRNA for the chemokines MIG, IP-10, RANTES, and MCP-2 were also detected within tumors following IL-12 administration (33). In addition, TS/A tumors engineered to secrete murine IL-12 showed strong NK cell and CD8+ T-cell infiltration, as well as expression of MIG, IP-10, RANTES, MCP-1,
and IL-8, although the cellular source of these chemokines was not identified (34). In human cancer patients, enhanced chemokine expression at the mRNA and protein levels has been detected in circulating peripheral blood mononuclear cells following administration of IL-12 to patients with melanoma or renal cell carcinoma (35–37). In each of these reports, significant induction of the IFN-γ-regulated, antiangiogenic chemokines IP-10 and MIG was found on IL-12 administration, supporting the role of IL-12 in the initiation of a chemokine response. Less is known about the NK cell chemokine response during the administration of therapeutic mAbs. Examination of serum chemokines in breast cancer patients on a clinical trial of an antitumor antibody (trastuzumab) in combination with IL-12 revealed enhanced circulating chemotactic factors in patients who experienced a clinical benefit from the treatment regimen. Of 15 patients, 1 had a complete response and 2 had stabilization of disease lasting ≥12 months. Specifically, levels of IL-8, MIG, IP-10, MIP-1α, and RANTES were up-regulated in these three patients during the course of therapy following the addition of IL-12 to the treatment regimen. In our previous work, we have shown that NK cells were responsible for the production of IFN-γ in patients that received trastuzumab and IL-12 and experienced a clinical benefit (21). Whereas the unavailability of cryopreserved peripheral blood mononuclear cell samples precludes a similar analysis of the cellular source of chemokines in these same patients, we postulate that the NK cell compartment is the likely source of these factors. Recently, several antibody-chemokine fusion proteins have been generated by recombinant technology and tested in vitro as well as in vivo models (38, 39). For example, an anti-HER2 IgG3 antibody fused with the chemokine RANTES (RANTES.Her2.IgG3) bound HER2-overexpressing cell lines and elicited trans-endothelial migration of peripheral blood T cells (38, 39). Although these chimeric proteins have been effective in recruiting T cells in vitro, the ultimate success of these agents will depend on their ability to simultaneously bind HER2 and recruit the appropriate number of cells to tumor sites. Our clinical data suggest that a NK cell chemokine response to antibody-coated targets can also be generated by systemic administration of costimulatory cytokines, such as IL-12, and illustrate a functional role for these NK cell–derived factors in patients undergoing immune therapies for cancer.

In summary, the current study shows that NK cells secrete a broad array of potent chemotactic factors in response to costimulation with antibody-coated tumor cells plus IL-2 or IL-12. These chemokines had the functional capability to induce the migration of naïve or activated T cells. In addition, elevated circulating levels of IL-8, MIP-1α, and RANTES were observed in patients who benefited from a phase I clinical trial of trastuzumab plus IL-12, and these factors were able to direct the migration of naïve and activated T cells. Although these results should be interpreted with some caution due to the small size of the patient group, we believe they provide evidence that NK cells can be induced to secrete a panel of chemotactic factors on costimulation via CD16 and specific cytokine receptors.

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