Chemokine-Induced Cutaneous Inflammatory Cell Infiltration in a Model of Hu-PBMC-SCID Mice Grafted with Human Skin

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Recently, certain chemokines and chemokine receptors have been preferentially associated with the selective recruitment *in vitro* of type 1 T cells, such as IP-10 and its receptor CXCR3, or type 2 T cells such as monocyte-derived chemokine (MDC) and eotaxin and their receptors CCR4 and CCR3. Very few models have provided confirmation of these findings *in vivo*. Taking advantage of the humanized SCID mouse model grafted with autologous human skin, the ability of the chemokines IP-10, MDC, eotaxin, and RANTES to stimulate cell recruitment was investigated. Intradermal IP-10 injection resulted in an influx of CD4⁺ T lymphocytes but also surprisingly in the recruitment of dendritic cells. MDC recruited mainly CD8⁺ T lymphocytes, and had little effect on eosinophils. As predicted, eotaxin was a potent inducer of eosinophil and basophil migration, also recruiting CD4⁺ T cells. RANTES, a ubiquitous chemokine associated with both type 1 and type 2 profiles, was able to recruit all cell types. CXCR3-positive cells were preferentially recruited by IP-10, whereas CCR3- and CCR4-positive cells were predominantly found after injection of eotaxin and MDC. Thus, in a human environment *in vivo*, some chemokines have the ability to recruit cells expressing chemokine receptors preferentially expressed on type 1 or type 2 cells. Further investigations revealed that MDC and eotaxin induced the recruitment of type 2, but not type 1, cytokine-producing cells. RANTES, on the other hand, induced the migration of both type 1 and type 2 cytokine-secreting cells, whereas IP-10 did not induce the recruitment of either subtype. These studies provide detailed information on the properties of MDC, eotaxin, IP-10, and RANTES as chemotactic molecules in skin *in vivo*. The use of the humanized SCID mouse model grafted with human skin is validated as a useful model

for the evaluation of chemokine function in the inflammatory reaction, and suggests that therapeutic targeting of certain chemokines might be of interest in diseases associated preferentially with a type 1 or type 2 profile. (*Am J Pathol* 2001, 158:1053–1063)

The recruitment of effector cells is an important step in the development of an inflammatory response, and it is orchestrated in part by a group of chemotactic cytokines named chemokines.¹ During the last few years, much has been learned from *in vitro* studies of chemokine function. The list of chemokines described is growing rapidly and the concept is emerging that these mediators are involved not only in leukocyte migration but also in hematopoiesis and leukocyte activation.²

Extending findings from *in vitro* to more complex *in vivo* systems has posed a challenge. Studies with tissue biopsies and samples of biological fluid, (eg, bronchoalveolar lavages from human patients) have yielded some interesting data and have indicated differential expression of various chemokines in given pathologies such as asthma and arthritis.^{3,4} However, the downstream processes of cellular recruitment cannot be investigated extensively in such models.

The use of animal models has advanced understanding of chemokine function *in vivo*, but the observations made may not reflect accurately the situation in humans. Ethical considerations have restricted the scope of investigations in man, although few studies have been conducted. Intradermal injection of RANTES (CCL5) into the skin of allergic and nonallergic patients has been found to result in significant eosinophil recruitment in both groups, although it occurred more rapidly in the group of allergic patients.⁵ Intradermal administration in human volunteers of MIP-1 α (CCL3), a chemokine active on several target cells, including eosinophils, monocytes, and T and B lymphocytes, has been noted to induce not only the accumulation of monocytes, lymphocytes, and

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eosinophils (as would have been expected on the basis of studies *in vitro*), but also the recruitment of neutrophils.⁶ Such observations call for a fuller evaluation of chemokine function *in vivo*.

A Hu-SCID mouse model grafted with autologous human full thickness skin and peripheral blood mononuclear cells (PBMCs)⁷ presents a new approach for the study of chemokine-induced human cell recruitment into tissue *in vivo*. We have investigated the effects of four chemokines: the CC-chemokines eotaxin (CCL11), regulated on activation, normal T cells expressed and secreted (RANTES), and monocyte-derived chemokine (MDC) (CCL22), and the CXC-chemokine interferon- γ -inducible protein (IP-10) (CXCL10). These have been selected as they have been reported to be involved in cutaneous T-cell-mediated inflammatory reactions such as the tuberculin-induced delayed-type hypersensitivity^{8,9} and the cutaneous late-phase reaction.¹⁰ We have also sought to study the pattern of expression of the receptors for these chemokines. Eotaxin and MDC are ligands for the CCR3 and CCR4 receptors, respectively, whereas IP-10 binds the CXCR3 receptor.¹¹ RANTES acts mainly via CCR3 and CCR5, but also via CCR1 and CCR4 receptors. CCR3 is expressed on Th2 cells,¹² eosinophils, and basophils,¹³ whereas CCR4 is found on Th2 cells¹² and basophils,¹⁴ suggesting the involvement of these receptors in the late-phase reaction. CXCR3 and CCR5 are found mainly on Th1 cells,¹² suggesting their involvement in delayed-type hypersensitivity. To evaluate the nature of cell recruitment, we have injected these cytokines into human skin grafted onto SCID mice reconstituted with human mononuclear cells.

Materials and Methods

Reagents

Anti-human CD45, CD25 (interleukin-2 receptor chain α) and CD4 antibodies were purchased from Becton Dickinson (San Jose, CA). Anti-human CD8, CD68 (macrophages), CD1a dendritic cell (DC) phenotype, CD45RO (memory T cells phenotype), HLA-DR, control IgG, rabbit anti-mouse Ig, and monoclonal alkaline phosphatase anti-alkaline phosphatase antibodies were from DAKO (Glostrup, Denmark). Rabbit antiserum specific for murine major basic protein (mMBP) antibody was produced as previously described.¹⁵ EG2, which recognizes the activated form of human eosinophil cationic protein, was obtained from Pharmacia (Uppsala, Sweden). Monoclonal antibody BB1 was used to specifically stain human basophils.¹⁶ Goat polyclonal anti-human CCR4 and CXCR3 were purchased from Tebu (Santa Cruz Biotechnology, Santa Cruz, CA). Rabbit anti-goat IgG biotin conjugate antibody and ExtrAvidin alkaline phosphatase conjugate were from Sigma Chemical Co. (St. Louis, MO). Anti-human CCR3 monoclonal antibody (7B11), from Leukosite Inc., was obtained through the AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Disease, National Institutes of Health.^{17,18} Rat anti-human interleu-

kin (IL)-4 (MP4-25D2), IL-5 (JES1-39D10), and IL-2 (MQ1-17H12) were purchased from PharMingen (San Diego, CA), and mouse anti-human interferon (IFN)- γ (MD2 clone F14) from HyCult Biotechnology (Uden, The Netherlands). Mouse anti-PCNA (proliferating cell nuclear antigen) was purchased from DAKO (Trappes, France). AP activity was developed using Fast Red/naphthol ASMX tablets (Sigma Chemical Co.). Anti-human antibodies displayed no cross-reactivity with murine structures, as verified by immunohistochemistry on cryostat sections from biopsies performed at the border between human and murine tissue. Recombinant human chemokines eotaxin, RANTES, MDC, and IP-10 were all purchased from Preprotech (Rocky Hill, NJ) and reconstituted with sterile phosphate-buffered saline (PBS). All recombinant reagents were endotoxin-free as assessed by a *Limulus* amoebocyte lysate test (BioWhittaker, Walkersville, MD).

Human Donors

Skin from human donors was obtained from truncal operation in which skin was discarded. Skin was kept in sterile normal saline with added penicillin and streptomycin and transplanted onto SCID mice within 2 hours after harvesting. Blood from donors was collected on heparin 6 weeks after surgery. The protocol was approved by the Center Hospitalier Régional et Universitaire ethical committee (no. 96-102). All donors signed an informed consent form.

Animals

Inbred mice with severe combined immunodeficiency (CB-17 SCID mice) were obtained from breeding pairs originally provided by M. Lieberman (Stanford University, Stanford, CA) maintained at the Institut Pasteur de Lille in sterilized isolators. Leaky mice (displaying spontaneous IgG production after 6 weeks of age) were discarded. The mice were housed under pathogen-free conditions. Animals were handled according to the ethical principles of animal experimentation established by the European Center of Tufts University.

Skin Grafting

Skin grafting was performed as described by Yan and colleagues.¹⁹ After anesthesia, 6- to 8-week-old mice were prepared for grafting by shaving the hair from a 5-cm² area on each side of the lateral abdominal region. Two circular graft beds, ~1.5 cm diameter, were prepared by removing shaved murine skin. Full-thickness human skin grafts of the same size were placed onto wound beds. The transplants were held in place using 6/0 silk suture material and covered with an adhesive wound dressing and then with a standard bandage. Dressing material and sutures were removed 10 days after transplantation.

Experimental Protocol

Six weeks after human skin transplantation, anti-asialo GM1 (1/20 dilution; Wako, Osaka, Japan) was injected intraperitoneally. Twenty-four hours later, SCID mice were reconstituted intraperitoneally with 10 to 15×10^6 autologous peripheral blood mononuclear cells (PBMCs) purified from the donor's blood using a Ficoll-Hypaque (Pharmacia) gradient after platelet depletion and resuspended in PBS. Chemokines (1 to $10 \mu\text{g}$ in $50 \mu\text{l}$ of diluent solvent) were then injected intradermally immediately, with 5% Evans blue dye (Sigma) to mark the site of injection. The contralateral graft of each mouse was injected with diluent containing an equivalent amount of bovine serum albumin and 5% Evans blue dye to serve as control. Mice with spontaneously activated grafts as evidenced by leukocyte infiltration in the diluent-injected site were discarded. A total of four different donors were included in this study with a total of 35 mice studied, representing 68 grafts.

Immunohistochemistry and Statistical Evaluation

Human skin biopsies were performed at the site of injection marked by Evans blue dye after 6, 24, 48, or 72 hours using a cylindrical sterile punch and cut into two halves. One half was immediately embedded in OCT compound (Labonord, Villeneuve d'Ascq, France), snap-frozen in isopentane precooled in liquid nitrogen, and stored at -80°C . Cryostat sections ($6 \mu\text{m}$) were cut, air-dried, fixed in a mixture of 60% acetone and 40% methanol, dried, wrapped in aluminum foil, and stored at -20°C for immunohistochemistry. The other half was fixed in 4% paraformaldehyde and washed in 15% PBS/sucrose before OCT embedment, freezing, and storage as described above. For all antibodies except antibodies against IL-4, IL-5, and IL-2, immunohistochemistry was performed using a modified alkaline-phosphatase anti-alkaline phosphatase method as previously described.²⁰ Briefly, acetone/methanol-fixed cryostat sections were incubated with the primary antibody for 1 hour, washed in Tris-buffered saline, and successively incubated 30 minutes with rabbit anti-mouse and then alkaline-phosphatase anti-alkaline phosphatase diluted in 20% normal human AB⁺ serum. The color was developed using Fast Red and sections were counterstained with hematoxylin. Irrelevant primary antibody of the same species was used as negative control. For IL-4, IL-5, and IL-2, immunohistochemistry was performed by using a modified avidin-biotin complex method, as previously described.²¹ Briefly, sections were incubated in 0.3% Triton X-100 for 20 minutes and with PBS containing 1% hydrogen peroxide. Endogenous biotin was quenched by using a Vector Laboratory kit (Peterborough, UK). Sections were preincubated with rabbit serum and incubated overnight with the anti-cytokine antibodies in PBS containing 0.1% saponin (Sigma). Sections were treated with the ABC Vectastain Elite kit (Vector) and the color developed by using diaminobenzidine tetrahydrochloride-nickel (Vector).²² Substitution of the primary antibody with an irrele-

vant antibody of the same species was used as a negative control.

Slides were coded and counted in a blinded manner at $\times 250$ magnification using an eyepiece graticule. At least three sections from each biopsy specimen were cut at different levels and stained with each antibody. Absolute numbers of positive cells were counted along the epithelial edge, on a surface of 1-mm wide and 0.4-mm deep for both chemokine-injected and diluent control samples. To allow comparison between the results obtained for different chemokines, the values counted for each contralateral control were subtracted from the respective values counted for the chemokine-injected graft. Results were expressed as mean $\pm$ SEM. In the diluent control samples, there were low numbers of DCs, basophils, memory T cells, and murine eosinophils (range, 0–5 cells/mm²), and normal levels of macrophages, CD4⁺, and CD8⁺ T cells (range, 5–15 cells/mm²).

Murine Eosinophil Chemotaxis Assay

Murine eosinophils were obtained from transgenic mice expressing murine IL-5 under the control of the human CD2 promoter.²³ Briefly, after sacrifice, the spleens of the animals were recovered, crushed, resuspended in RPMI, and filtered through a nylon filter (Bultex, Saily, France). Red blood cells were eliminated by hypotonic shocks. Eosinophils (30 to 40%) were resuspended at a concentration of 10^6 cells/ml for use in a chemotaxis assay. RANTES, MDC, and eotaxin (at a concentration of 10^{-8} mol/L, 10^{-7} mol/L, and 10^{-6} mol/L) as well as control RPMI were used in a 48-well micro chemotaxis chamber (NeuroProbe, Cabin John, MD) with 5- μm pore polycarbonate filters (Nucleopore Corp., Pleasanton, CA) during 45 minutes at 37°C in 5% CO₂. Eosinophils that had migrated through the filter were counted under a microscope at a magnification of 500-fold. Each condition was performed in triplicate, and at least four fields were counted for each well. Chemotaxis was distinguished from chemokinesis as previously described.²⁴

Results

The use of mononuclear cells and skin specimens obtained from the same human donor for each group of mice was of importance in this SCID mouse model, because it avoided any allogenicity during the reconstitution phase. Moreover, the use of two skin grafts per mouse allowed each mouse to be its own control. It is of note that no consistent swelling was observed after either diluent or chemokine injections, but most of the chemokine-injected sites displayed a discrete erythema whereas control grafts did not. To allow comparison between the results obtained for different chemokines, the values counted for each contralateral control were subtracted from the respective values counted for the chemokine-injected graft.

Figure 1. Chemokines MDC, eotaxin, RANTES, and IP-10 induce local recruitment of human CD45-positive cells. The chemokines or the diluents alone were injected into one of the two human skin grafts of each mouse just after reconstitution with PBMCs. Skin biopsies were performed 24 hours later for eotaxin, RANTES, and IP-10 injection, and 48 hours later for MDC injection. CD45-positive cells were detected by immunohistochemistry. Results are shown as the mean number (±SE) of positive cells in chemokine-injected skin sites after subtraction of the numbers in the corresponding contralateral diluent-injected control grafts ($n = 4$ for all chemokines).

Total Leukocyte Recruitment

The CD45 surface marker for leukocytes was used to determine the most suitable time points for the biopsies. The period of maximal recruitment was 24 hours for eotaxin, RANTES, and IP-10, and 48 hours for MDC. Initial dose-response experiments (with a range of concentrations from 1 to 10 μg) allowed the selection of a general 5- μg dose as optimal for total leukocyte recruitment for MDC, eotaxin, and IP-10. The dose used for RANTES injections was 4 μg , which has been shown to be optimal in a previous study in human patients.⁵

As shown in Figure 1, all four chemokines were able to recruit human leukocytes (CD45⁺ cells) at the site of injection. The most potent effect was observed for RANTES and IP-10 (corresponding to a 61 and 109% increase, respectively, when compared with the contralateral skin grafts injected with diluent), followed by that for MDC (see Figure 5, a and b) and eotaxin (41 and 51%, respectively).

Recruitment of T Cell Subpopulations and Expression of Markers for Cell Activation

In contrast with findings with the other chemokines, the optimal time point for cell recruitment in response to MDC was 48 hours. A representative experiment is shown in Figure 2A. Results are shown after subtraction of the values in the diluent control at each of the different time points (6 hours, 24 hours, 48 hours, and 72 hours).

The CD4⁺ and CD8⁺ T cell subpopulations as well as the CD45RO⁺ memory T cells were evaluated. MDC in-

Figure 2. **A:** Kinetics of T-lymphocyte recruitment by MDC. MDC (5 μg) was injected into human skin immediately after reconstitution with PBMCs. Each time point represents a different mouse. Tissue sections were stained immunohistochemically with monoclonal antibodies against the CD4 and CD8 T-cell subpopulations, and the CD45RO memory T cell marker. Results are shown as the mean number of positive cells in chemokine-injected skin sites after subtraction of the respective contralateral solvent-injected control grafts. **B:** Characterization of the T cell infiltrate. Chemokines MDC, eotaxin, RANTES, and IP-10 were injected in the human skin as described in Material and Methods. Results are expressed as mean (±SE) as in Figure 1 ($n = 4$ for all chemokines). **C:** Characterization of lymphocyte and leukocyte activation. CD25 (the α -chain subunit of the IL-2 receptor) and HLA-DR-positive cells were counted on tissue cryostat sections, and results are expressed as mean (±SE) as in Figure 1 ($n = 4$ for all chemokines).

duced the recruitment of all three cell types, although the effect was predominantly on CD8⁺ cells (see Figure 5d). Eotaxin had almost no effect on the recruitment of T cells, except for a modest increase in CD4⁺ cell numbers. RANTES, and IP-10 were potent stimuli for T cell accumulation, and acted preferentially on the CD4⁺ subpopulation and on memory cells (Figure 2B).

Eotaxin, RANTES, and IP-10 recruited few, if any, activated T lymphocytes (CD25⁺ cells) or activated leukocytes in general (HLA-DR⁺ cells). MDC, however, was very effective in inducing recruitment of activated cells, both T lymphocytes and other leukocytes (Figure 2C).

Macrophage and DC Recruitment

There was no effect of MDC and eotaxin on monocyte (CD68⁺) recruitment, whereas RANTES, and IP-10 to a lesser extent, attracted this cell type (Figure 3A).

Figure 3. Recruitment of effector cells after chemokine injection. **A:** Recruitment of DCs (CD1a marker) and of monocytes/macrophages (CD68 marker). **B:** Recruitment of human basophils, assessed by the BB1 antibody, and of murine eosinophils, assessed by the anti-mouse major basic protein antibody. Cryostat sections of the human skin biopsies were stained with the different antibodies by using immunohistochemistry. Results are shown as the mean number ($\pm$ SE) of positive cells in chemokine-injected skin sites after subtraction of the numbers in the corresponding contralateral solvent-injected control grafts ($n = 4$ for all chemokines).

For Langerhans cells, CD1a⁺ cells were counted both in the epidermis and the dermis. Results of the epidermal counts are shown in Figure 3A. In eotaxin and MDC-injected skin there was almost no additional recruitment of CD1a⁺ cells. On the other hand, in RANTES and IP-10-injected sites, additional CD1a⁺ cells were observed in the epidermis (see Figure 5c). For the dermal counts, few cells were visualized, and only after RANTES and IP-10 injection (data not shown).

Basophil and Eosinophil Recruitment

The recently developed BB1 antibody allowed the specific staining of human basophils on cryostat sections. As shown in Figure 3B, MDC and IP-10 had no effect on human basophil recruitment, but RANTES and eotaxin induced to a limited extent the migration of this cell type toward the sites of injection (see Figure 5e).

Because we used mononuclear cells to reconstitute SCID mice, human basophils but very few human eosinophils were present in mice. However, precursors of eosinophils might be present. We used the EG2 marker to evaluate the presence of eosinophils in the skin grafts. EG2 staining gave no evidence of activated human eosinophils recruitment after chemokine injections (data not shown). However, using a specific antibody recognizing murine eosinophils (anti-mMBP), we were able to detect

Figure 4. Chemotaxis of murine eosinophils under a range of human chemokine concentrations. Recombinant human chemokines eotaxin, RANTES, and MDC were used at 10^{-8} , 10^{-7} , and 10^{-6} molar in a chemotaxis assay using a Boyden chamber. RPMI was used as solvent control. Murine eosinophils that had migrated through the filter were counted after Giemsa staining. One experiment that was representative of three is illustrated.

these cells in the skin grafts. Anti-mMBP staining showed no differential recruitment of murine eosinophils after IP-10 injection, and little recruitment after MDC injection (corresponding to a +40% increase when compared with the relevant diluent control), but a strong response was seen in the recruitment of positive cells after eotaxin (see Figure 5f) or RANTES injection as compared with diluent controls (+265% and +148%, respectively) (Figure 3B).

Murine Eosinophil Chemotaxis Assay

The findings suggest either that the human chemokines had a direct chemoattractant effect on murine eosinophils linked to interspecies cross-reactivity, or an indirect effect mediated through other cells present in the skin. To investigate these possibilities, we performed a direct *in vitro* chemotaxis assay with murine eosinophils, using various concentrations of human chemokines known to have chemoattractant properties for human eosinophils (namely eotaxin, RANTES, and MDC). The results demonstrated that human MDC and RANTES were fully able to induce migration of murine eosinophils, as well as human eotaxin, which displayed a very strong and dose-dependent effect (Figure 4).

Recruitment of CCR3-, CCR4-, and CXCR3-Expressing Cells

Monoclonal antibody against CCR3 (a receptor for eotaxin and RANTES) and polyclonal antibodies against CCR4 (receptor for MDC and RANTES) and CXCR3 (receptor for IP-10) were used to evaluate their membrane expression using immunohistochemistry.

As seen in Figure 6, the injection of MDC in the human skin resulted in a moderate increase in the number of CCR4⁺ cells recruited to the site of injection, together with a very slight increase in CCR3⁺ cells. Eotaxin induced an increase in CCR3⁺ cells as compared with the control, with no effect on CCR4- and CXCR3-positive cells. RANTES had a highly significant effect on the recruitment of CCR3⁺ cells (Figure 5g), with a moderate effect on CCR4⁺ cells. IP-10 injection favored the recruit-

Figure 5. Microphotographs of immunohistochemistry on cryostat skin sections. **a** and **b**: Human skin graft 24 hours after diluent (**a**) or MDC (**b**) injection, immunostained with anti-CD45 antibody. **c**: Anti-CD1a staining, in an IP-10-injected site. Positive cells appear in the epidermis. **d**: Anti-CD8 staining in a MDC-injected site. **e**: Human basophils in a cryostat section of a human skin graft injected with RANTES, stained with BB1 monoclonal antibody. **f**: Murine eosinophils, stained with an anti-murine major basic protein antibody, in a cryostat section of a human skin graft injected with eotaxin. **g** and **h**: Human chemokine receptors CCR3 (**g**) and CXCR3 (**h**) staining, in a RANTES-injected and a IP-10-injected site, respectively. **Inset**: Corresponds to a deeper field of a similar section showing CCR3-positive cells with a dendritic morphology. **Arrow** points out CXCR3-positive cells. Immunostaining was performed by using the alkaline phosphatase anti-alkaline phosphatase technique. Original magnifications, $\times 250$ (**a-h**) and $\times 400$ (**inset**).

Figure 6. CCR3, CCR4, and CXCR3 expression after injection of chemokines. The expression of the chemokine receptors CCR3, CCR4, and CXCR3 was assessed on cryostat sections of human skin after injection of MDC, eotaxin, RANTES, or IP-10. Results are shown as the mean number ($\pm$ SE) of positive cells in chemokine-injected skin sites after subtraction of the numbers in the corresponding contralateral solvent-injected control grafts ($n = 4$ for all chemokines).

ment of CXCR3⁺ cells (Figure 5h), with no effect on the other two receptors studied.

Recruitment of Type 1 and Type 2 Cytokine-Expressing Cells

The production of type 1 cytokines (IFN- γ and IL-2) and type 2 cytokines (IL-4 and IL-5) was evaluated by immunohistochemistry to characterize better the profile of recruited cells after chemokine injection. As shown in Figure 7, MDC and more dramatically, eotaxin both stimulated the recruitment of IL-5-producing cells. RANTES injection caused an increase in both type-1 and type-2 cytokine-producing cells, and in particular of IL-4 and IFN- γ -expressing cells. IP-10 injection had no effect on the recruitment of type 1 or type 2 cytokine-producing cells, as compared with their respective diluent controls.

Discussion

The ability of chemokines to stimulate cell recruitment *in vivo* has been relatively little explored. The results of studies *in vitro* and recently the accumulation of information on the nature of chemokine receptors has lead to speculation as to the cell populations that would be recruited in response to given chemokines,²⁵ but this has not been validated experimentally. SCID mice reconstituted with human PBMCs (hu-SCID mice) have been used to evaluate the effects of various chemokines on the migration of human leukocytes *in vivo*,^{26,27} but in these models the murine peripheral lymphoid tissue or skin have been the target organs. The grafting of human skin on to these mice has allowed us to improve further the reliability of the model. Similar models have proved useful in studies of skin diseases such as psoriasis,^{28–30} cutaneous lymphoma,³¹ cytotoxic dermatitis,³² and toxin- or tuberculin-induced delayed-type hypersensitivity.^{7,33}

Figure 7. Cytokine profiles of recruited cells after injection of chemokines. The expression of type 1 cytokines (IL-2 and IFN- γ) and type 2 cytokines (IL-4 and IL-5) was assessed by immunostaining on cryostat sections of human skin after injection of MDC, eotaxin, RANTES, or IP-10. Results are shown as the mean number ($\pm$ SE) of positive cells in chemokine-injected skin sites after subtraction of the numbers in the corresponding diluent-injected control grafts ($n = 4$ for all chemokines).

Recently, using an allogeneic model, Kunstfeld and colleagues³⁴ evaluated the human T cell response to various chemokines reported to be chemoattractants for T cells *in vitro*. They observed relatively little accumulation of human leukocytes after intradermal injection of recombinant human (rh) RANTES, rh SDF-1, or rh IP-10, but injection of rh MIP-1 α or rh MCP-1 was found to be a potent stimulus for the recruitment of human cells. In that study, however, only the migration of T-cell populations was investigated, and allogenicity may have been a confounding factor as the SCID mice were reconstituted with human PBMCs and grafted with human skin from a different donor.

In the present studies, we took advantage of the hu-SCID-mouse model grafted with human skin and used autologous mononuclear cells from the same donor to evaluate ability of chemokines to stimulate the recruitment of different cell types *in vivo*. As the chemokines were injected into the grafts immediately after reconstitution, we were able to avoid bias because of possible

depletion or anergy of the human leukocytes that generally occur 3 weeks after cell reconstitution.³⁵

T-helper lymphocytes, which have key roles in orchestration of the immune response have been classified into two subsets depending on their cytokine production: T-helper type 1 lymphocytes (Th1) that produce mainly IFN- γ , and type 2 (Th2) that produce mainly IL-4 and IL-5. More recently, it has been demonstrated that Th1 and Th2 cells also express different chemokine receptors on their surface, suggesting that this may provide a means for selective recruitment of these cells.³⁶ Th1 cells express preferentially CCR5 and CXCR3, whereas Th2 cells have CCR3, CCR4, and CCR8 on their surface.¹² IP-10 is a major ligand for CXCR3, suggesting its involvement in type 1 cell recruitment, whereas eotaxin and MDC bind preferentially CCR3 and CCR4, respectively, suggesting an activity on type 2 cells. RANTES, which binds several of these receptors, would seem to be involved in Th1 and Th2 recruitment.

The potent T lymphocyte recruitment we observed after injection of IP-10 is consistent with the expression of its receptor (CXCR3) on certain T cell subsets. Interestingly, IP-10 injection also induced a marked recruitment of CXCR3-positive cells in the skin, with no effect on the CCR3- and CCR4-positive cells. The number of CXCR3⁺ cells was similar to the number of CD4⁺ cells recruited by IP-10, suggesting that there is a preferential recruitment of T cells through this receptor. IP-10 injections triggered no selective recruitment of IL-4- or IL-5-producing cells as might be expected, and surprisingly failed to induce the migration of IFN- γ - and/or IL-2-producing cells. This may be explained in part by the immunohistochemical method used being less sensitive for the detection of lymphocyte subsets than for cells displaying a larger cytoplasmic compartment.³⁷ In the present study lymphocytes were the predominant cell population recruited to the site of IP-10 injections. In pathological conditions involving IP-10, such as delayed type hypersensitivity, lymphocytes have been found to be the main cellular source of IFN- γ production.

IP-10 also induced the recruitment of DCs to the epidermis. Previously we have noted the presence of human DCs in SCID mice reconstituted with PBMCs.³⁸ These cells have been found mainly in the lungs but also in other organs. In the model of the hu-SCID mouse grafted with human skin, DCs were also present and might presumably originate from precursors and mature circulating cells of the donor's blood, or they may have been present already in the skin transplant and may have entered the circulation after its vascularization. The local proliferation of pre-existing DCs is unlikely. Indeed, immunostaining of proliferating cells using a specific antibody (PCNA)³⁹ gave similar results for both the chemokine-injected and the diluent-injected skin grafts (data not shown). DCs have not hitherto been reported to respond to IP-10, and these cells are not known to express CXCR3. It is possible that T-cell populations recruited by IP-10 could release other chemotactic mediators, which may in turn recruit DCs and other cell types. IP-10 has been shown to be strongly expressed in cutaneous delayed-type hypersensitivity,⁸ a condition that may be mediated by Th1

lymphocytes.⁴⁰ The recruitment of CXCR3-positive cells after an isolated IP-10 injection suggests that IP-10 may play a key role in the pathophysiology of delayed-type hypersensitivity reactions.

The results with RANTES in the SCID model are in accord with the findings of a previous investigation of its effects after injection into the skin of human volunteers,⁵ and we deliberately used the same dose. Beside the recruitment of T lymphocytes and eosinophils reported in the study in human patients, we found that there was recruitment of other effector cells such as monocytes, DCs, and basophils. The T-cell recruitment consisted almost exclusively of the CD4⁺ T-cell subpopulation and CD45RO⁺ memory cells. CD68⁺ monocytes and CD1a⁺ DCs also migrated toward the site of injection, but to a lesser extent than T cells. This is not a surprising result in itself, because monocytes and macrophages are known to express CCR5 and other receptors for RANTES.⁴¹ The recruitment of eosinophils and basophils is also consistent with reports that they express CCR3 and CCR4, both of which bind RANTES. CCR3 seemed to be an important receptor mediating RANTES-induced cell accumulation, as illustrated by the marked increase in numbers of CCR3-positive cells. Surprisingly, the number of CCR3⁺ cells recruited exceeded the number of lymphocytes and basophils recruited. This might be because of other cell types expressing CCR3 such as DCs and mast cells, as has been observed by immunohistochemistry.⁴² On the other hand, CCR4, another receptor for RANTES, seemed to be much less involved. The cells recruited after injection of RANTES appeared to express both Th1 and Th2 cytokines. This is consistent with the known association of this chemokine with various pathologies. Indeed, the involvement of RANTES has been described in several immunological conditions, including tuberculin-induced delayed-type hypersensitivity (a Th1-mediated reaction) and allergen-induced late-phase reaction (a Th2-mediated reaction).^{9,10} The close similarity between the findings in humans and in the SCID model (in both dose response and time course) and the strong responses induced by RANTES are in accord with a report that a RANTES antagonist can strongly inhibit allergic reactions *in vivo*.⁴³

MDC was highly effective at inducing the migration of T CD8⁺ lymphocytes. To date, MDC has been shown *in vitro* to act mainly on monocytes, activated T cells, and eosinophils.^{44–46} Recently, MDC has been shown to be expressed by both DCs and T lymphocytes in skin biopsies from patients with atopic dermatitis.⁴⁷ In the present studies we found that MDC had a preferential effect on CD8⁺ T lymphocytes when injected in the human skin, although it was also able to stimulate the ingress of CD4⁺ and CD45RO⁺ subsets to some extent. Interestingly, Campbell and colleagues⁴⁸ have demonstrated that not only CD4⁺ but also CD8⁺ T lymphocytes expressing CCR4 can respond *in vitro* to MDC. CCR4 is expressed specifically on type 2 and not type 1 CD8⁺ lymphocytes that are not activated.⁴⁹ This suggests that the CD8⁺ cells recruited after MDC injection in the human skin might be type 2 CD8⁺ lymphocytes. It is noteworthy that injection of MDC was less potent as a stimulus for the

recruitment of CCR4-positive cells than for T cells. This could be accounted for by the existence of another receptor for MDC, which has been suggested on the basis of studies *in vitro*.^{46,50} Alternatively, the recruitment of T lymphocytes could be mediated by indirect actions of MDC.

MDC had no effect in the recruitment of type 1 cytokine-expressing cells, but induced the selective recruitment of IL-5-producing cells. This selective effect was not observed for the other major type 2 cytokine (IL-4), but our results are in accord with previous studies that have demonstrated the association of this chemokine with the development of type 2 diseases such as atopic dermatitis.⁴⁷ The effects of MDC that have been described on eosinophil chemokines *in vitro*⁴⁶ were only partially confirmed in our *in vivo* model. Whereas MDC and eotaxin were reported to be of similar potency as a stimulus for human eosinophil chemotaxis, we found that eotaxin was far more potent than MDC in inducing the accumulation of murine eosinophils. Although murine eosinophils can respond to both MDC and eotaxin, one cannot exclude the possibility that the differences in potency could be accounted for the human eotaxin being more similar functionally to its murine counterpart than is the case for MDC.

The effects of eotaxin on the recruitment of eosinophils in the SCID model is of interest when considering what has been described previously both *in vitro* and *in vivo*. Intradermal injection of human eotaxin in monkeys has been reported to result in eosinophil accumulation at the injection site.⁵¹ There has been just one preliminary study involving intradermal injection of eotaxin in human skin, and this seemed to have only a limited effect on eosinophil recruitment.⁵² The local accumulation of polymorphonuclear cells was observed after injection into healthy volunteers, but no eosinophils were present. One hypereosinophilic patient was also found to display a polymorphonuclear cell-rich infiltrate, but with 25% eosinophils. The dose of eotaxin used in that study was low (50 to 100 ng), presumably on account of ethical considerations. Knowing the deleterious effects of this cell type when overstimulated and massively recruited in the skin as in atopic dermatitis disease, regulation of this cell type represents a major target for therapeutic research. As was the case for eosinophils, eotaxin was the most potent chemoattractant for basophils. Both of these cell types have very high expression of CCR3.¹³ T-lymphocyte migration was observed to a smaller extent after eotaxin injection. CCR3 is present on Th2 lymphocytes, but only a very small proportion of the peripheral blood T cells express this receptor (ranging from 0 to 2%).³⁶ This could explain the consistent but limited degree of T-cell recruitment after eotaxin injection in the SCID model. Eotaxin induced a marked recruitment of IL-5-producing cells. Although we did not perform double-staining experiments, in view of the morphology and localization (data not shown), it is possible that these IL-5-producing cells correspond to mMBP⁺ murine eosinophils that were recruited after injection of eotaxin.

In seeking to interpret our findings, and those of the few other studies that have involved injection of chemo-

kines into the skin of human volunteers, an important question remains. While our model involved the injection of just one chemokine, the recruitment of various cell types implies a series of events including adhesion to endothelial cells, transendothelial migration, and chemotactic movement along a concentration gradient for each chemokine. These steps require sequential expression of more than a single molecule by the cells responsible for triggering cell recruitment. This can be considered both as one of the limitations as well as one of the advantages of our model. When a cell population is recruited, we can reasonably imagine that the chemokine injected in the skin is responsible for the induction of various other signals and mediators necessary for effective cell migration. Our model will be useful in exploring further the mechanisms *in vivo* whereby the migration of immune cells may be controlled in the skin. Moreover, the hu-SCID-mouse model grafted with human skin may represent a valuable first step in evaluating of the capacity of therapeutic agents to inhibit cell migration *in vivo* in response to various chemoattractants.

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