CCR5 Usage by CCL5 Induces a Selective Leukocyte Recruitment in Human Skin Xenografts *In Vivo*

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CCR5 is one of the major inflammatory chemokine receptors with potential therapeutical applications in humans. However, the redundancy of chemokines and their receptors, and the species specificity of chemokine receptor antagonists pose challenges to understanding of the role they play in pharmacological situations. To address this question, we used a humanized severe combined immunodeficient mouse model grafted with human skin and autologous leukocytes, and evaluated the effect of a blocking antibody against human CCR5, on CCL5-induced cutaneous leukocyte recruitment *in vivo*. At baseline, CCL5 induced a significant recruitment of T cells mainly of the memory phenotype, of monocytes/macrophages, eosinophils, and IFN-γ^+ but not IL-4$^+$ and IL-5$^+$ cells. *In vivo*, anti-CCR5 antibody was able to almost completely inhibit the recruitment of monocytes/macrophages and T-helper (Th)1-type cells to inhibit partially the attraction of memory T cells, but had no effect on eosinophil infiltration, although all these cell types express other CCL5 binding chemokine receptors than CCR5. These results indicate that the *in vivo* environment regulates target cell specificity of CCL5 leading to differential cell recruitment, suggesting that antagonizing CCR5 receptor may be of therapeutic value in diseases such as acquired immuno deficiency syndrome, where CCL5/CCR5, monocytes, and Th1-type cells play a predominant role.

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INTRODUCTION

The recruitment of effector cells is an important step in the development of an inflammatory response and is orchestrated in part by chemokines, which act through specialized surface receptors. The preferential cell accumulation in tissue suggests that there are specific pathways used for their accumulation *in vivo*. Understanding these mechanisms could aid in developing pharmacological therapies that would block their recruitment. The chemokine regulated on activation, normal T cells expressed and secreted or CCL5 in the new nomenclature mediates the trafficking and homing of T cells and monocytes but also of basophils, eosinophils, natural killer cells, dendritic cells (DCs), and mast cells (Schall, 1991) *in vitro* but also *in vivo* as we have shown in a humanized severe combined immunodeficient (SCID) mouse model (Fahy *et al.*, 2001). CCL5 production which is generated predominantly by CD8$^+$ T cells, epithelial cells, fibroblasts, and platelets is increased in a number of inflammatory disorders and pathologies (Appay and Rowland-Jones, 2001). CCL5 also plays a key role in the immune response to viral infection. It has been reported that CCL5 can mediate enhancement of HIV-specific T-cell cytotoxicity (Hadida *et al.*, 1998), and can suppress the replication of HIV *in vitro* (Cocchi *et al.*, 1995). CCL5 acts by binding to the chemokine receptors CCR1, CCR3, and CCR5. However, the respective role of these different receptors in mediating the response to CCL5 is not well understood. Among them, CCR5 has been particularly studied because of its function of coreceptor for entry of M-tropic strains of HIV into cells (Murphy, 2001). CCR5 is a G-protein-coupled seven-transmembrane receptor that binds macrophage inflammatory protein (MIP)-1α/CCL3, MIP-1β/CCL4, and CCL5. CCR5 is expressed on Th1-type lymphocytes, monocytes/macrophages, basophils, eosinophils, and on DCs when they are in the immature stage (Mueller and Strange, 2004). To evaluate CCR5 usage by CCL5-recruited human cells in tissue *in vivo*, we used a hu-SCID mouse model grafted with autologous human full-thickness skin and peripheral blood mononuclear cells (PBMC) (Tsicopoulos *et al.*, 1998; Fahy

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Abbreviations: DC, dendritic cell; PBMC, peripheral blood mononuclear cell; SCID, severe combined immunodeficient; Th, T-helper

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et al., 2001). The model of SCID mice grafted with skin alone (Juhasz *et al.*, 1993; Yan *et al.*, 1993; Nickoloff *et al.*, 1995; Christofidou-Solomidou *et al.*, 1996, 1997b) or followed by reconstitution with PBMC has been previously extensively used in the field of skin diseases (Christofidou-Solomidou *et al.*, 1997a; Gilhar *et al.*, 1997) and to study different types of T-cell-mediated cutaneous inflammation (Petzelbauer *et al.*, 1996; Tsicopoulos *et al.*, 1998) and graft rejection (Murray *et al.*, 1994; Sultan *et al.*, 1997).

In order to determine the pattern of immune response linked to CCR5 engagement by CCL5, the effects of a neutralizing antibody to human CCR5 on CCL5-induced leukocyte recruitment was investigated. We show that in contrast to the *in vitro* redundancy observed with this receptor–ligand pair, there is a fine specificity of CCR5-mediated cell recruitment by CCL5 *in vivo*.

RESULTS

Neutralizing anti-CCR5 antibody inhibits CCL5-induced human PBMC and murine eosinophil migration *in vitro*

To assess the potency of anti-CCR5 antibody in inhibiting CCL5-induced cell recruitment, *in vitro* chemotaxis assays were performed using human anti-CD3/anti-CD28-activated PBMC and a human CD8 T-cell line incubated with increasing concentrations of anti-CCR5 antibody. The isotype control had no effect on CCL5-induced PBMC migration, whereas anti-CCR5 antibody completely inhibited this migration at a concentration of 3 μg/10^6 cells (Figure 1a).

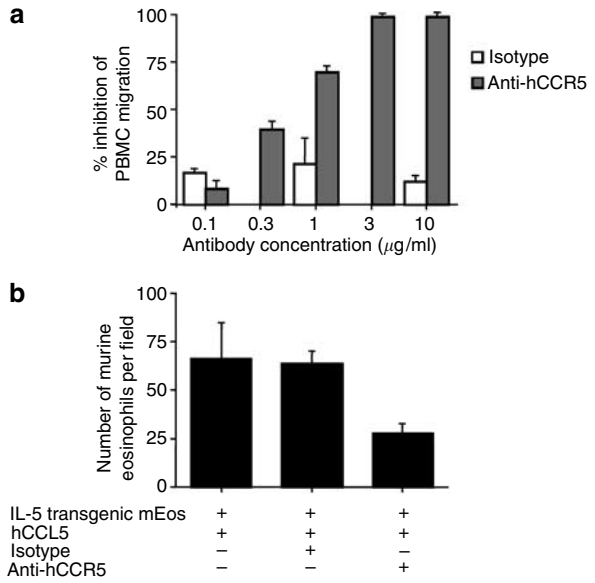

Figure 1. Inhibitory effects of neutralizing anti-CCR5 antibody on human PBMC and murine eosinophil chemotaxis *in vitro*. (**a**) Dose-dependent inhibitory effect of CCR5-blocking antibody on human PBMC chemotaxis. Activated human PBMC were incubated with increasing concentrations of anti-CCR5 or IgG2a antibodies, and chemoattracted by CCL5. Results are expressed as the percentage of inhibition of PBMC migration. **(b)** Functional chemotactic cross-reactivity between anti-human CCR5 and murine eosinophils. Eosinophils from IL-5 transgenic mice were incubated with 10 μg/ml of anti-CCR5 or control IgG2a antibodies and chemoattracted using human CCL5. Results are expressed as means ± SEM number of recruited eosinophils per field ($n = 3$).

The same effect was obtained for the CD8 T-cell line (data not shown).

We have previously shown that in the model of skin xenograft, there are no human eosinophils, whereas eosinophils of murine origin can be attracted into the graft because they react to human chemokines (Fahy *et al.*, 2001; Senechal *et al.*, 2002). Indeed, mice are reconstituted with human PBMC devoided of human eosinophils. Therefore, the cross-reactivity of the anti-human CCR5 antibody with murine eosinophils was evaluated. Anti-CCR5 antibody was able to inhibit partly the chemotaxis of murine eosinophils obtained from IL-5 transgenic mice (anti-CCR5 −57% *versus* control IgG2a −4%), which clearly indicated a functional cross-reactivity between the anti-human CCR5 antibody and the murine CCR5 on eosinophils (Figure 1b).

CCR5 participates in CCL5-induced memory T-cell recruitment *in vivo*

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 following the reconstitution phase. Moreover, the use of two skin grafts per mouse allowed each mouse to be its own control. Previous studies showed that the optimal time point for CCL5-induced maximal recruitment

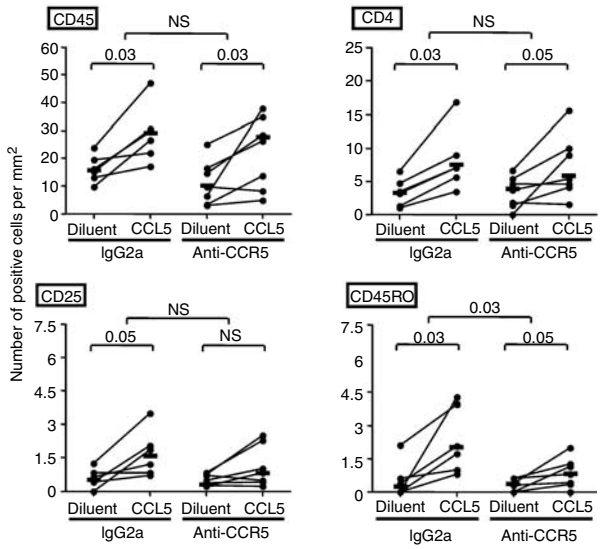

Figure 2. Effect of anti-CCR5 treatment on CCL5-induced skin xenograft lymphocyte infiltration. Infiltration of human $CD45^+$ leukocytes, $CD4^+$ T cells, IL-2R $CD25^+$ T cells, and $CD45RO^+$ memory T cells, following CCL-5 *versus* diluent challenges in 26 skin grafts obtained from normal donors, 24 hours after intravenous antibody injection and intraperitoneal introduction of IgG2a- or anti-CCR5-treated autologous human PBMC. Results are expressed as the number of positive cells per mm^2. Median values are represented by the solid bars. Differences within the four groups were first evaluated by using the Kruskal–Wallis test and were found statistically significant for the four markers. Comparisons within CCL5 and diluent values were performed by using the Wilcoxon test. Comparisons between IgG2a and anti-CCR5-treated groups were performed by using the Mann–Whitney *U*-test after subtraction of the diluent values from CCL5 values. *P*-values as indicated, NS: not significant.

was 24 hours, and the optimal dose 4 μg, in human subjects (Beck *et al.*, 1997). In our hu-SCID mouse model, intradermal injection of CCL5 induced a significant recruitment of total CD45$^+$ leukocytes in both the control and anti-CCR5-treated groups, as compared to the diluent-injected site (Figure 2). There was no difference of recruitment between the two groups. The same pattern was observed for CD4$^+$ T-lymphocytes as well as for CD8$^+$ cells (data not shown). IL-2R CD25$^+$ T cells were recruited by CCL5; however, there was no statistical difference between the anti-CCR5- or isotype-treated groups (Figure 2). For memory CD45RO$^+$ T cells, there was a significant recruitment after CCL5 injection in both groups; however, the absolute number of recruited CD45RO$^+$ cells was significantly lower in the anti-CCR5 than in the control-treated group, showing that anti-CCR5 treatment significantly decreased the number of recruited memory T cells by about 67% (Figure 2).

CCR5 is the main receptor involved in CCL5-induced monocyte/macrophage but not eosinophil recruitment *in vivo*

Compared with the diluent-injected skin graft, CD68$^+$ monocytes/macrophages were attracted by CCL-5 in the isotype-treated group and this recruitment was strongly inhibited following anti-CCR5 treatment (88% inhibition), showing that CCR5 is a major pathway of monocyte/macrophage recruitment by CCL5 (Figure 3). A microphotograph of CD68$^+$ cells after CCL5 injection into human skin xenografts is shown in control- (Figure 5a) and anti-CCR5-treated groups (Figure 5b). In contrast, dermal CD1a$^+$ immature DCs and BB1$^+$ basophils, which have been shown to express CCR5 *in vitro*, were not significantly attracted by CCL5 in our *in vivo* model (Figure 3). Murine eosinophils express CCR1, CCR3, and CCR5 (Oliveira *et al.*, 2002), all able to bind CCL5. In both groups of mice, murine eosinophils were attracted by CCL5, as compared with baseline, whether or not they received anti-CCR5 antibody, suggesting that CCR5 is not a major pathway of eosinophil recruitment by CCL5 *in vivo* (Figure 3).

CCR5 is the primary receptor involved in CCL5-induced IFN-γ^+ cell recruitment *in vivo*

In order to evaluate whether CCR5 has a preferential role in mediating CCL5-induced recruitment of cells characterized by the expression of different cytokines, Th1 and Th2-type cytokine-producing cells were assessed by immunohistochemistry. CCL5 did not induce a significant recruitment of IL-2-, IL-4-, and IL-5-positive cells compared with the diluent-alone, whereas IFN-γ^+ cells were consistently attracted at the site of the chemokine injection (Figure 4). Interestingly, anti-CCR5 treatment strongly reduced this recruitment by about 82% compared to the isotype control ($P = 0.02$). An example of microphotograph showing IFN-γ^+ cells after CCL5 injection into human skin xenografts is shown in control- (Figure 5c) and anti-CCR5-treated groups (Figure 5d). Thus, CCR5 is of major importance for the recruitment of Th1-type cells *in vivo*.

DISCUSSION

Much is known about receptor usage by chemokines *in vitro*, but the effective action of a given chemokine on its receptor *in vivo* is critically dependent upon a number of environmental factors. For example, glycosaminoglycans present on cell surfaces and in the extracellular matrix are involved in chemokine oligomerization and have a critical role in the *in vivo* effect of some chemokines. CCL5 oligomerization is not mandatory for CCR1 and CCR5 activation *in vitro*, although it has been reported to be important for its *in vivo* chemotactic

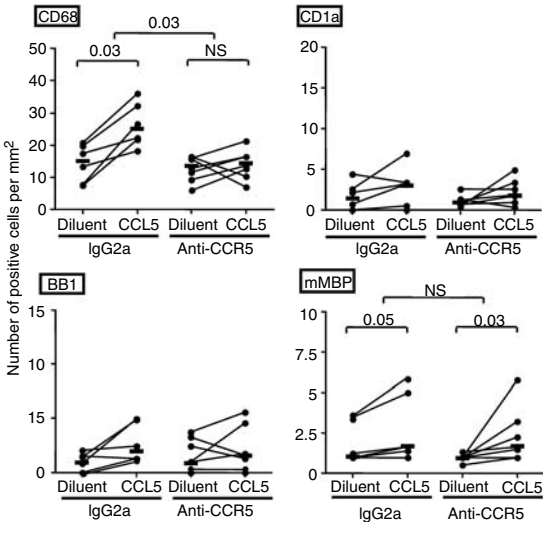

Figure 3. Effect of anti-CCR5 treatment on CCL5-induced skin xenograft leukocyte infiltration. Infiltration of human CD68$^+$ monocytes/macrophages, CD1a$^+$ immature DCs, BB1$^+$ basophils, and murine MBP$^+$ eosinophils, following CCL-5 *versus* diluent challenges as described in Figure 2. Results are expressed as in Figure 2. Kruskal–Wallis test was not significant for CD1a$^+$ and BB1$^+$ cell infiltration.

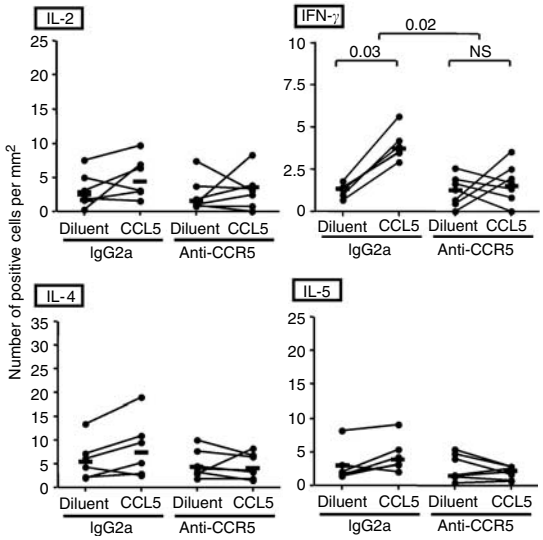

Figure 4. Effect of anti-CCR5 treatment on CCL5-induced skin xenograft cytokine-expressing cell infiltration. Infiltration of human Th1 and Th2 cytokine-expressing cells, following CCL-5 *versus* diluent challenges as described in Figure 2. Results are expressed as in Figure 2. Kruskal–Wallis test was only significant for IFN-γ^+ cell infiltration.

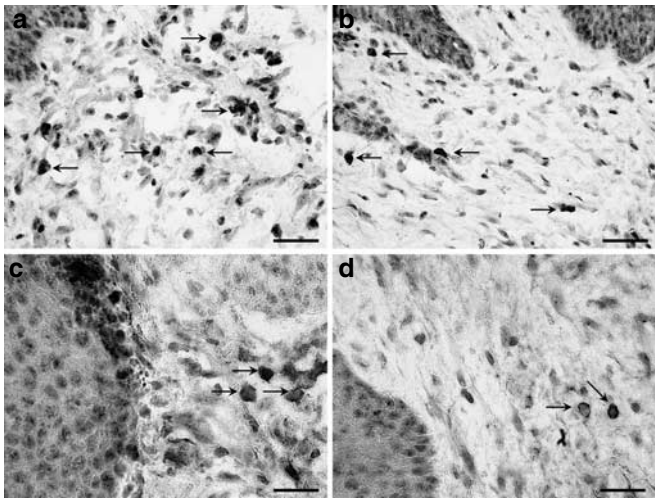

Figure 5. Immunostaining of cryostat sections of human skin grafts. Anti-CD68 staining of CCL5-injected skin grafts in SCID mice treated with **(a)** IgG2a (Bar = 50 μm) or **(b)** anti-CCR5 (Bar = 50 μm) antibody. Anti-IFN-γ staining of CCL5-injected skin grafts in SCID mice treated with **(c)** IgG2a (Bar = 25 μm) or **(d)** anti-CCR5 (Bar = 25 μm) antibody. Immunostaining was performed by using the alkaline phosphatase antialkaline phosphatase technique.

activity (Proudfoot *et al.*, 2003). Moreover, the expression of a given receptor on a cell surface does not mean that it is functional, for example, in a particular cytokine environment, chemokine receptors and in particular CCR5 can act as decoys to eliminate an excess of chemokines, therefore loosing their activation and chemoattractant capacity (D'Amico *et al.*, 2000). Finally, *in vivo*, chemokine receptors exhibit an important plasticity which is the basis for the dynamic migration of the cells bearing those receptors (Lloyd *et al.*, 2000). As more chemokine receptor antagonists appear as potential treatments intended to be used in humans (Proudfoot, 2002), and because of the redundancy of chemokines and their receptors and their species specificities (Liang *et al.*, 2000), it is important to determine *in vivo*, and in a human setting, their spectrum of action. Indeed, targeting only one chemokine receptor might be insufficient to knock down the recruitment of a particular subset of cells given the expression of multiple receptors able to bind the same chemokine. This is particularly true for CCR5, which is coexpressed with CCR1 on monocytes/macrophages and Th1-type cells, and with CCR3 on eosinophils and basophils, all these receptors binding CCL5. We therefore used SCID mice reconstituted with human PBMCs (hu-SCID mice) and grafted with human skin to evaluate CCR5 usage by CCL5-recruited leukocytes. In a previous study, we have used such a model to evaluate the *in vivo* effect of a wide range of chemokines, and in particular of the intradermal injection of CCL5, which induced a cutaneous leukocyte infiltration of human T cells, monocytes/macrophages, basophils, IL-4$^+$ and IFN-γ^+ cells, and murine eosinophils (Fahy *et al.*, 2001). In the present study, in the isotype-treated group, CCL5 induced a recruitment of T cells which consisted mainly of the CD4$^+$ subpopulation, although some CD8$^+$ T cells were also attracted. Among them, CD25$^+$ (either activated or regulatory) and CD45RO$^+$ memory subpopulations were particularly recruited, which is in agreement with the known *in vitro* effect of CCL5 on memory T cells (Schall *et al.*, 1990). As compared with our previous study, more CD68$^+$ monocytes/macrophages and less basophils were recruited by CCL5 in the present work. This may be linked to donor variations, or alternately to the use of the control isotype antibody. The recruitment of murine eosinophils to sites of CCL5 injection is consistent with their known expression of CCR1, CCR3, and CCR5 (Oliveira *et al.*, 2002). We have previously shown that murine eosinophils are attracted by human CCR3 ligands and in particular CCL5 (Fahy *et al.*, 2001), which explains the cross-reactive effect observed in the SCID model here. In terms of cells involved in polarized immune responses, CCL5 can attract both Th2- and Th1-type cells through binding, respectively, to CCR3 and CCR5 (Sallusto *et al.*, 1997; Bonecchi *et al.*, 1998). In the present study, only IFN-γ-producing Th1-type cells were attracted by CCL5 in the isotype-treated group, probably because all the human donors included were non-atopic, and thus there were no Th2-type cells to recruit.

The couple CCL5/CCR5 has been involved in a number of diseases such as acquired immuno deficiency syndrome (He *et al.*, 1997; Blanpain *et al.*, 2002), rheumatoid arthritis (Suzuki *et al.*, 1999), Crohn's disease (Oki *et al.*, 2005), multiple sclerosis (Zang *et al.*, 2000), acute renal allograft rejection (Panzer *et al.*, 2004), hepatitis C virus infection (Hellier *et al.*, 2003), Wegener's granulomatosis (Zhou *et al.*, 2003), and also asthma (Schuh *et al.*, 2002). Because CCR5 antagonists may have therapeutic potential in such human diseases, it is of importance to evaluate the part of CCR5 in the functional effects of CCL5. Studies in mice deficient in CCR5 so far have shown prolongation of graft survival (Luckow *et al.*, 2004; Amano *et al.*, 2005) but only limited defects in cell-mediated immune responses (Field *et al.*, 2003; Algood and Flynn, 2004; Zhong *et al.*, 2004), which might be linked to the appearance of compensatory mechanisms (Chen *et al.*, 2003) that lead to an underestimation of the therapeutic potential of receptor antagonists carriers of the wild-type receptor. To evaluate the effect of CCR5 neutralization on CCL5-induced leukocyte recruitment, we privileged the use of neutralizing antibody, and chose the potent anti-human CCR5 inhibitory antibody 2D7, previously shown to bind to the second extracellular loop of the receptor (Lee *et al.*, 1999). Humanized SCID mice treated with anti-CCR5 antibody did not show modifications of CD45$^+$ leukocyte, CD4$^+$, and CD8$^+$ T-cell recruitment into grafts injected with CCL5. In contrast, there was a partial inhibition of memory T-cell recruitment, showing that CCL5 acts only on a subset of memory T cells through CCR5. Indeed, memory T cells were still significantly attracted *in vivo* by CCL5, suggesting that CCR5 is not the only receptor able to mediate the recruitment of memory T cells. Anti-CCR5 treatment did not inhibit CCL5-induced murine eosinophil recruitment *in vivo*, although eosinophils do express CCR5 (Oliveira *et al.*, 2002), and CCL5 has a higher

affinity for CCR5 than for CCR3 *in vitro* (Blanpain *et al.*, 2001), and although *in vitro* chemotaxis assays showed that the anti-human CCR5 antibody was able to at least partially inhibit CCL5-induced murine eosinophil recruitment. These results suggest that CCR5 usage differs *in vitro* and *in vivo*, and that CCR5 is not a major pathway for eosinophil recruitment by CCL5 *in vivo*. This is consistent with a previous study showing that CCR3 is more likely to be the major pathway of eosinophil recruitment *in vivo*, at least in the human allergic reaction (Senechal *et al.*, 2002) where CCL5 is overexpressed (Ying *et al.*, 1999).

Th1-type cells and monocytes/macrophages express CCR5 (Weber *et al.*, 2000) and CCR1, which both bind CCL5 with high affinity. Therefore, it is of interest that anti-CCR5 treatment dramatically reduced the recruitment of those cells by CCL5. These differential results might be explained by a divergence in CCL5 binding capacity *in vivo* to the different receptors. For example, it has been shown that CD26 dipeptidyl peptidase IV can cleave CCL5 at the amino–terminus, which leads to a truncated form of the molecule, this form showing an altered receptor specificity with reduced activity towards CCR1 but a conserved activity towards CCR5 (Oravecz *et al.*, 1997). Therefore, although there is a strong *in vitro* redundancy of both CCL5 and CCR5 at the cell level, our study shows that cell selectivity can occur *in vivo*.

Taken together, these data suggest that *in vivo* CCL5 selectively uses CCR5 to recruit monocytes/macrophages and Th1-type cells, into human tissue, and that targeting CCR5 may prove therapeutically beneficial in diseases where the protagonists are cell types affected by CCR5 blockade, such as Crohn's disease (Oki *et al.*, 2005), acquired immuno deficiency syndrome (Blanpain *et al.*, 2002), multiple sclerosis (Zang *et al.*, 2000), rheumatoid arthritis (Suzuki *et al.*, 1999), and transplant rejection.

MATERIALS AND METHODS

Reagents

Anti-human CD45, CD25 (IL-2 receptor chain α), and CD4 antibodies were purchased from BD Biosciences (Pont de Claix, France). Anti-human CD8, CD68 (macrophages), CD1a (DC phenotype), CD45RO (memory T-cell phenotype), control IgG, rabbit anti-mouse Ig, and monoclonal alkaline phosphatase antialkaline phosphatase antibodies were from Dako (Glostrup, Denmark). Rabbit antiserum specific for murine major basic protein antibody was produced as described previously (Filley *et al.*, 1981). Monoclonal antibody BB1 was used to stain specifically human basophils (McEuen *et al.*, 1999). Anti-human CCR5 monoclonal antibody (clone 2D7), IgG2a isotype control (clone G155-178), rat anti-human IL-4 (MP4-25D2), IL-5 (JES1-39D10), and IL-2 (MQ1-17H12) were purchased from BD Pharmingen, San Diego, CA. Mouse anti-human IFN-γ (MD2 clone F14) was from HyCult biotechnology (Uden, The Netherlands). Alkaline phosphatase activity was developed using Fast Red/naphtol ASMX tablets (Sigma, St Quentin Fallavier, France). 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 chemokine CCL5 was purchased from Peprotech (Rocky Hill, NJ) and reconstituted with sterile phosphate-buffered saline (PBS). CCL5 was endotoxin-free as assessed by a *Limulus* amebocyte lysate test (Bio Whittaker, Walkersville, MD).

Human donors

Genotype analysis of CCR5 and CCR5Δ32 mutation was performed on a 5 ml blood sample taken before the surgery. Only donors without mutation were allowed to enter the study. Skin from human donors was obtained from truncal operation where 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 Centre Hospitalier Régional et Universitaire ethical committee (no. 96102). All donors signed an informed consent form according to the Declaration of Helsinki Principles.

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 *et al.* (1993). After anesthesia, 6- to 8-week-old mice were prepared for grafting by shaving the hair from a 5-cm^2 area on each side of the lateral abdominal region. Two circular graft beds, approximately 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 $10–15 \times 10^6$ autologous PBMC purified from the donor's blood using a Ficoll–Hypaque (Pharmacia, Uppsala, Sweden) gradient after platelet depletion and resuspended in PBS. PBMCs were incubated 30 minutes before intraperitoneal reconstitution with either 100 μg neutralizing anti-human CCR5 antibody (azide-free, endotoxin level less than 0.01 ng/μl protein) ($n = 7$ mice), or 100 μg irrelevant isotype-matched IgG2a antibody ($n = 6$ mice). In addition, 150 μg of the corresponding antibody was injected intraveneously in each group of mice. CCL5 (4 μg in 50 μl diluent solvent) was 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 BSA 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 13 mice were studied, representing 26 grafts.

Genotype analysis of CCR5 and CCR5Δ32 mutation by PCR

Because the prevalence of the heterozygous Δ32 mutation is 10–15% for Europe, and that individuals with this phenotype exhibit reduced cell surface expression of CCR5 (Berger *et al.*, 1999), we systematically verified the CCR5 genotype of the cell donors. Three 10^6 PBMC were resuspended in TRIzol reagent (Life Technologies, Gaithersburg, MD) and total DNA was extracted according to the manufacturer's procedure and yielded 15 μg genomic DNA. The primers (forward) 5′-TCA AAA AGA AGG TCT TCA TTA CAC C-3′ and (reverse) 5′-AGC CCA GAA GAG AAA ATA AAC AAT C-3′ (Eurogentec, Seraing, Belgium) were used to amplify the CCR5 wild-type and the 32 bp mutation using the following PCR conditions: 100 ng genomic DNA, 5 μl of 10 × buffer, 200 μM dNTPs, 400 nM of each primer, and 2.5 U platinium *Taq* DNA polymerase (Gibco BRL, Cergy Pontoise, France). Amplification was initiated by a 4-minute denaturation step at 94°C, followed by 35 cycles at 94°C for 20 seconds, 58°C for 30 seconds, and 72°C for 1 minute, followed by 10 minutes extension at 72°C using a DNA thermal cycler (Techne, Cyclogene, Duxford, Cambridge, UK). Amplified PCR products were separated by gel electrophoresis in 1.5% agarose gel after Gelstar nucleic acid staining (FMC Bioproducts, Rockland, ME).The CCR5 Δ32 allele yields a product of 209 bp, clearly distinct from the wild-type CCR5 at 241 bp.

Immunohistochemistry

Human skin biopsies were performed at the site of injection marked by Evans blue dye 24 hours after the intradermal injections 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 μm) 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 on acetone/methanol fixed sections using a modified alkaline phosphatase antialkaline phosphatase method as described previously (Tsicopoulos *et al.*, 1994). Briefly, 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 iG antibody and then alkaline phosphatase antialkaline 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 on 4% paraformaldehyde fixed sections by using a modified avidin–biotin complex method, as described previously (Tsicopoulos *et al.*, 2000). Briefly, cryostat 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). Substitution of the primary antibody with an irrelevant antibody of the same species was used as a negative control.

Human T-cell and murine eosinophil chemotaxis assays

Human anti-CD3-/anti-CD28-activated PBMC and a human CD8 T-cell line were resuspended in RPMI medium at a concentration of 10^6 cells/ml for use in a chemotaxis assay. Chemotaxis was performed in 24-well transwell chambers (Costar, VWR International, Leuven, Belgium). Cell suspensions (100 μl) were placed in the upper compartment and 600 μl CCL5 at a concentration of 10 nM in the bottom compartment. Incubation was performed for 1.5 hours at 37°C. Where appropriate, cells were incubated with either neutralizing anti-hCCR5 antibody (2D7), isotype control, or RPMI medium as control. Cells that had migrated through the filter were counted by flow cytometry by counting the events accumulated over 30 seconds.

Murine eosinophils were obtained from IL-5 transgenic mice. After the killing, spleen eosinophils were resuspended at a concentration of 10^6 cells/ml and incubated with medium, control IgG2a or anti-CCR5 antibody at a final concentration of 10 μg/ml. Chemotaxis was induced using human CCL5 at 10^{-7} M. 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 described previously (Fahy *et al.*, 1999). Results were expressed as the mean number of cells migrating to CCL5 after subtraction of the control (medium ± antibody).

Quantification and statistical analysis

Slides were coded and counted in a blinded manner at × 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. Results were expressed as the number of positive cells per mm^2. Statistical analysis was performed using Statview 5 software. Differences within anti-CCR5- and IgG2a-treated groups were first evaluated for the four subgroups using the Kruskal–Wallis test. When statistical significance was observed, differences were subsequently analyzed by using the Wilcoxon test for diluent/CCL5 pairs, and the unpaired Mann–Whitney test for anti-CCR5- and IgG2a-treated groups after subtraction of the diluent value from the chemokine value. Only P-values $\leqslant 0.05$ were considered significant.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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