Role of CCL17 in the Generation of Cutaneous Inflammatory Reactions in Hu-PBMC-SCID Mice Grafted with Human Skin

Jules Gilet1,4, Ying Chang1,2,4, Cécile Chenivesse1,3, Benjamin Legendre1, Han Vorng1, Catherine Duez1, Benoît Wallaert1,3, Henri Porte3, Stéphanie Senechal1 and Anne Tsicopoulos1,3

CCL17 may be of interest in skin inflammation, because it mainly attracts T cells expressing the cutaneous homing receptor and binds the chemokine receptor CCR4, preferentially expressed on Th-2 cells. We evaluated the in vivo effect of CCL17 injection in a humanized mouse model. 125I-CCL17 injection into human skin grafted on severe combined immunodeficient (SCID) mice reconstituted with peripheral blood mononuclear cells resulted in a rapid transportation of CCL17 from the skin to the homolateral lymph nodes, followed 3 hours later by a lymph node infiltration of human memory CD4+ cells and dendritic cells. Intradermal injection of CCL17 resulted 24 hours later in a cutaneous recruitment of human memory CD4+ cells, monocytes, and basophils, but also murine eosinophils. In SCID mice reconstituted with polarized Th-1 or Th-2 cells, intradermal injection of CCL17 resulted in the recruitment of IL-4+ Th-2 cells but not of IFN-γ+ Th-1 cells, whereas CCL17 was able to recruit both subsets in vitro. These results suggest that, in a humanized in vivo model, CCL17 is sufficient per se to induce a lymph node recruitment of memory CD4+ and dendritic cells and a cutaneous recruitment of Th-2-type cells, stressing it as an important actor in the initiation and development of Th-2-associated skin inflammation.

INTRODUCTION

The recruitment of effector cells is an important step in the development of an inflammatory response, and is orchestrated in part by chemokines, a group of potent chemotactic molecules. Chemokines and their receptors are believed to be essential for leukocyte trafficking from the circulation into inflammatory tissues. They constitute a large family of low molecular mass (8–12 kDa) proteins (Baggiolini, 1998; Viola et al., 2006). Conserved cysteine residues approximately spaced are the hallmark of the two major subfamilies of chemokines, namely CXC and CC. Two other chemokine molecules, lymphotactin and fractalkine show distinctive structural characteristics and are, respectively, single members of the C and CX3C subfamilies. Chemokines are involved in a number of inflammatory disorders and in particular in allergy. This disorder involves the recruitment and activation of many inflammatory cells, in particular eosinophils, basophils, and CD4+ lymphocytes, expressing a type 2 cytokine profile (IL-4, IL-5; Robinson et al., 1992). Some chemokine receptors have been shown to be preferentially expressed on these three cell types and may represent a common pathway leading to the development of the allergic reaction. These receptors and their ligands may represent good therapeutical targets in allergic reactions. Among them, CCR4 has been shown to be expressed on type 2 lymphocytes (Sallusto et al., 1998; Imai et al., 1999), and basophils (Power et al., 1995), and one of its ligand, the CC chemokine, CCL17/thymus and activation-regulated chemokine (Imai et al., 1997, 1999), may be of interest in cutaneous allergic inflammatory disorders. CCL17 is produced by activated endothelial cells (Campbell et al., 1999), as well as by keratinocytes in the basal epidermis in atopic dermatitis (Vestergaard et al., 2000). CCL17 is expressed in vitro by keratinocytes stimulated with tumor necrosis factor-α, IFN-γ or IL-1β. Moreover, CCL17 attracts mainly CD4+ T cells, which express the skin homing receptor cutaneous lymphocyte-associated antigen (CLA; Campbell et al., 1999) and binds CCR4, which under physiological conditions is a nonredundant necessary component of skin-specific lymphocyte trafficking (Campbell et al., 2007). Recently, CCL17 expression has been shown to be increased in different skin allergic diseases in humans (Vestergaard et al., 2000; Goebeler et al., 2001; Sebastiani et al., 2002) and to correlate with the clinical activity of atopic dermatitis (Kakinuma et al., 2007).
In Vivo Role of CCL17 in Skin Inflammation

J Gilet

RESULTS

CCL17 accumulates in human skin and in draining lymph nodes after intradermal injection into human skin xenografts

To test whether the chemokine could be transported by afferent lymph in animals, we injected $^{125}$I-CCL17 intradermally into one skin graft, and the diluent into the contralateral skin graft of each mouse. The distribution of $^{125}$I-CCL17 was evaluated in the homolateral and contralateral lymph node (LN) and in different organs 15, 60, and 120 minutes after intradermal injection. A strong signal was detected in the homolateral skin draining LN, as soon as 15 minutes after intradermal injection of CCL17 as compared with the contralateral LN (Figure 1a). The signal in the homolateral skin draining LN was maximal at 60 minutes and remained elevated 120 minutes after injection (Figure 1b and c). A small signal was also observed in the contralateral LN at 60 and 120 minutes showing that some CCL17 may recirculate to the other side. A strong signal was evidenced at all time points in the CCL17-injected skin. Only background levels of $^{125}$I-CCL17 were observed whatever the time point in the spleen, thymus, and liver. A nonsignificant increase appeared in the contralateral kidney at 120 minutes probably reflecting the route of CCL17 clearance. In the peripheral blood, the signal remained low with time, without diffusion in the systemic circulation. These results show that in human skin, intradermally injected CCL17 is distributed to the draining LN.

CCL17 injection into human skin xenografts from humanized SCID mice induces a recruitment of memory T cells in draining peripheral lymph nodes

Given the strong transportation of CCL17 to the superficial draining LN, we examined the potential recruitment of human cells in LN, kinetically after intradermal injection of CCL17 as compared with the contralateral diluent-injected site using immunohistochemistry. Given the small accumulation of CCL17 in the contralateral LN, experiments were performed to check the presence of human cells at baseline after intradermal injection of diluent in the two skin grafts of a given mouse (called diluent/diluent). Human cells mainly T cells and some dendritic cells (DCs) were observed in LN...
draining diluent/diluent-injected skin, showing that there was a spontaneous recirculation of cells following diluent injection in this model (Figure 2a and b). In mice injected with both diluent and CCL17, a significant infiltration of human CD45^+ cells mostly constituted by CD4^+ cells was observed in the LN draining CCL17-injected skin, as compared with the control diluent-injected site, starting at 3 hours after CCL17 injection, until 24 hours (Figure 2a). For the time points before 3 hours and after 24 hours (namely 30 minutes, 1 hour and 30 minutes, and 48 hours after CCL17 injection) no significant cell recruitment was observed as compared with the control. Only memory CD45RO^+ cells (Figure 7a) but not CD45RA^+ cells (data not shown) were differentially recruited to the LN following CCL17 injection. Cells expressing CCR4 were recruited in the LN draining CCL17-injected skin, and were significantly higher than in the LN draining the control site. However, these cells represented only one half of the recruited CD4^+ cells (Figure 2b). In the contralateral LN, the number of recruited T cells at 3 and 6 hours was higher than in the LN from diluent/diluent mice. There was no recruitment of the CD8^+ T-cell subpopulation (data not shown). Concerning DCs, no immature CD1a^+ cell recruitment was observed. For DC-lysosomal-associated membrane protein^+ mature DCs, there was increased recruitment 3 hours after injection in both LN draining CCL17- and diluent-injected sites as compared to the diluent/diluent mice (Figure 2b). Other studies have shown that eosinophils and mast cells are also found in LN (Wang et al., 1998; Shi et al., 2000), however, no mast cell, basophil, eosinophil, and B-cell infiltration was observed in the LN in our study, whatever the time point (data not shown). To check the specificity of CCL17-induced recruitment, experiments were performed with intradermal injections of the same amount of CCL17 mixed with neutralizing

![Figure 2](https://www.jidonline.org/881)

**Figure 2. Kinetics of cell recruitment in draining LN.** Mice were reconstituted i.p. with autologous PBMC and CCL17 or the diluent were immediately injected into human skin xenografts. (a) Kinetics of T-cell recruitment in LN draining CCL17- versus diluent-injected sites, draining diluent/diluent-injected mice, or draining CCL17/anti-CCL17-injected sites. Cryostat sections of the draining LN were immunostained with monoclonal antibodies against CD45^+ leukocytes, CD4^+ T cells, CD45RO^+ memory T cells. Results are expressed as the mean number ± SEM of positive cells per lymph node section for n = 6–8 mice per time point. *P < 0.05, **P < 0.01. (b) Kinetics of CCR4^+ cell and DC recruitment in LN draining CCL17- versus diluent-injected sites, draining diluent/diluent-injected mice, or draining CCL17/anti-CCL17-injected sites. Immature DC were assessed using the CD1a antibody, mature DC using the DC-LAMP antibody. Results are expressed as above.
anti-CCL17 antibody. The number of recruited cells at the different time points was similar to that observed in the diluent/diluent mice showing the specificity of the CCL17-induced recruitment (Figure 2).

CCL17 injection into human skin xenografts from humanized SCID mice induces a recruitment of cutaneous inflammatory cells

The human cellular infiltration following CCL17 injection into skin xenografts obtained from humanized SCID mice was examined using immunohistochemistry. Kinetics and dose-response experiments were performed to determine the optimal time points of action and doses of CCL17. The maximal effect on cell recruitment was observed at a dose of 2.5 μg CCL17, and the optimal time point was found to be 24 hours (data not shown). There was no significant recruitment at 6 hours and the effect was abolished at 48 and 72 hours. These optimal conditions were selected for the rest of the study.

A significant infiltration of human CD45⁺ leukocytes was observed at the site of CCL17 injection as compared with the diluent (Figure 3a). Among the T-lymphocyte population, only the CD4⁺ subpopulation significantly increased after CCL17 injection (Figure 7b and c) whereas the CD8⁺ subpopulation remained stable. Memory and naïve T cells as assessed by the surface markers CD45RO and CD45RA were both recruited following CCL17 injection as compared with the diluent whereas CD25⁺ cells were not recruited. Among the other cell types, small numbers of basophils and CD68⁺ monocytes/macrophages were also recruited after CCL17 injection as compared to the diluent (Figure 3b). No human eosinophils were present in this model, because mice were reconstituted with PBMC only. However, we were able to detect a small recruitment of murine eosinophils (as assessed by the antimurine major basic protein antibody) following CCL17 injection. CCL17 injection had no significant effect on the number of HLA-DR⁺ cells (data not shown).

To check the specificity of CCL17-induced cutaneous cell recruitment, CCL17 was coadministered intradermally with neutralizing anti-CCL17 antibody at a dose able to completely inhibit CCL17 dependent chemotaxis in vitro, and skin biopsies were performed 24 hours later. As observed in LNs, there was no cutaneous leucocyte recruitment 24 hours after this coinjection (data not shown).

Distribution of CLA⁺ and CCR4⁺ cells in circulating blood and skin in humanized SCID mice

The distribution of cells expressing the cutaneous homing receptor CLA, and CCR4 was compared between peripheral blood (by flow cytometry) and skin (by immunohistochemistry). Six hours after reconstitution, 9.3 ± 3.9% (mean ± SEM, n = 4) of blood CD4⁺ cells expressed CLA, and 25.8 ± 2.5% expressed CCR4, which was not very different from the prereconstitution levels (16.9 ± 1.2 CD4⁺CLA⁺ and 19.2 ± 1.7% CD4⁺CCR4⁺). In the skin, 24 hours after reconstitution and CCL17 injection, the mean number of CLA⁺ cells represented 50.6 ± 4.3% of CD4⁺ cells, and the number of CCR4⁺ cells 46.5 ± 2.5% of CD4⁺ cells showing an enrichment compared to the blood (data not shown).

CCL17 preferentially recruits Th-2 cells in human skin xenografts from humanized SCID mice

CCL17 has been shown to preferentially chemoattract Th-2 lymphocytes in vitro (Imai et al., 1999). Less is known about its potential in vivo, in humans. Therefore, we used our humanized model to assess CCL17 capacity to chemoattract human Th-1 or Th-2 cells in vivo. Autologous T cells from the skin donor were polarized in vitro towards Th-1 or Th-2 cells using cytokine cocktails. The generated subsets were well polarized, as assessed by the mean percentage of cytokine secreting cells (Th-1 cells: 73.6 ± 11.2% of IFN-γ⁺ cells and 5.9 ± 2.1% of IL-4⁺ cells; Th-2 cells: 65.8 ± 5.3% of IL-4⁺ cells and 22.9 ± 7.6% of IFN-γ⁺ cells, mean ± SEM of n = 3), both expressed CCR4 although at a higher level on Th-2 cells than on Th-1 cells (Th-2 cells: 53.6 ± 3.7%; Th-1 cells: 37.7 ± 7.5%), and presented a similar degree of apoptosis (Th-2 cells: 26.8 ± 6.3%; Th-1 cells: 31.6 ± 2.3%). A representative example is shown in Figure 4. When SCID mice were reconstituted with Th-1 cells, there was no significant effect of intradermal CCL17 injection on the recruitment of CD4 positive cells, whereas after Th-2 cell reconstitution, a significant infiltration of human CD4 positive cells was observed at the site of CCL17 injection as compared with the diluent (Figure 5a). CCL17 injection induced in Th-2 reconstituted SCID mice, an increase in CCR4⁺ cell recruitment, as compared with the diluent control, whereas no significant effect was observed in Th-1 reconstituted SCID mice (Figure 5a).

To ascertain the cytokine profile of the recruited T helper cells, the production of IFN-γ (type 1 cytokine) and IL-4 (type 2 cytokine) was evaluated by immunohistochemistry in human skin xenografts. In Th-2 reconstituted SCID mice, CCL17 injection induced an increase in IL-4 producing cells (Figure 5d) as compared with the diluent control, whereas no effect was observed for IFN-γ producing cells. In Th-1 reconstituted mice, CCL17 did not recruit either IL-4 or IFN-γ producing cells (Figure 5a).

To understand why Th-1 cells were not recruited to the skin xenografts, LNs draining the injected skin collected at the same time as the skin, that is 24 hours after reconstitution and cutaneous injections, were examined. In Th-2 reconstituted SCID mice, almost no IL-4⁺ CD4⁺ cells were found in the LNs draining CCL17-injected site as compared with the diluent-injected site, whereas in Th-1 reconstituted SCID mice, no difference in the number of IFN-γ⁺ CD4⁺ cells was observed between the LNs draining the diluent and CCL17-injected sites (Figure 5b). This absence of redistribution was not related to a loss of CCR4 expression, as almost all LN Th-1 cells expressed CCR4 (Figure 5b).

Chemoattractant effect of CCL17 in vitro

The above results show that in our in vivo model, cutaneous injection of CCL17 was clearly associated with a human leucocyte infiltration consisting in Th-2 cells, naïve T cells, monocytes, basophils, and also murine eosinophils. This can
result from a direct chemoattractant effect through CCR4 or an indirect effect mediated through other cells present in the skin. CCR4 has been convincingly shown to be expressed on Th-2 memory cells (Imai et al., 1999; Yamamoto et al., 2000; Sebastiani et al., 2002) but its expression on the other cell types cited above remains controversial. To investigate these possibilities, we performed a direct chemotaxis assay with polarized Th-2 and Th-1 cells, naïve T cells, monocytes, basophils, and murine eosinophils. The results showed that in vitro, polarized Th-2 cells were attracted by CCL17 but also Th-1 cells although at a dose 10-fold higher than that necessary for Th-2 cell recruitment. CCL17, in vitro, did not attract CD45RA⁺ naïve T cells (Figure 6a). CCL17 was also able to attract monocytes, murine eosinophils, and to a lesser extent human basophils (Figure 6b).

**DISCUSSION**

In the past years, there has been growing interest in chemokines and their receptors as potential therapeutical targets in different diseases and in particular in allergic
diseases such as atopic dermatitis (Johnson et al., 2005). Mutated antagonists are currently being developed that appear species specific, arguing for the necessity of in vivo humanized animal models. The model of SCID mice grafted with human skin appeared to be particularly suited to CCL17, given its particular effect on the recruitment of CLA\(^+\) cells (Campbell et al., 1999; Vestergaard et al., 2000; Ferenczi et al., 2002) and its increased expression in different skin allergic disorders (Vestergaard et al., 2000; Goebeler et al., 2001; Wenzel et al., 2005).

CCL17 is known to be constitutively expressed in thymus and LN (Lieberam and Forster, 1999), and to be induced in some cells under stimulation (Imai et al., 1999), but it is not known if CCL17 can be transported as a soluble mediator to LN. This way of transport has been previously described for only a few chemokines including CCL21 and CCL2 (Stein et al., 2000; Palframan et al., 2001). Chemokines transported from skin to the draining LN persist for more than 4 hours (Stein et al., 2000) allowing the recruitment of specialized leukocyte subsets. Using \(^{125}\text{I}\)-labeled CCL17, our results demonstrate that in the case of local cutaneous expression of CCL17, this chemokine can be directly transported to superficial draining LN, suggesting that it may participate as a remote control mechanism in the recruitment of cells involved in the initiation of an inflammatory reaction in LNs. In this context, it is of interest that CCL17 has been shown to be released in an IgE-dependent manner by mast cells both in mice and humans (Oliveira and Lukacs, 2001; Wakahara et al., 2001). Mast cells might thus represent an early source.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure4.png}
\caption{Phenotypic characterization of the generated Th-1 and Th-2 subsets. (a) Th-1 and Th-2 subsets were stained with an annexin V/propidium iodide kit and analyzed by flow cytometry. Dot plots show the percentage of apoptotic cells (annexin V\(^+\)/PI\(^-\)) and the percentage of dead cells (annexin V\(^+\)/PI\(^+\)). (b) Th-1 and Th-2 cells were stained using a cytokine secretion assay kit. Histogram plots of IL-4 and IFN-\(\gamma\) secreting cells are overlaid (thick line) on isotype-matched control (thin line). (c) Histogram plots of Th-1 and Th-2 cells expressing CCR4 (thick line) overlaid on isotype-matched control (thin line). One representative experiment out of three is shown.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5.png}
\caption{Cutaneous versus lymph node recruitment of IL-4 and IFN-\(\gamma\) positive cells 24 hours after i.d. CCL17 versus diluent injections in Th-1 or Th-2 reconstituted SCID mice. CD4, CCR4, IL-4, and IFN-\(\gamma\) positive cells were assessed by immunostaining on skin (a) or lymph node (b) cryostat sections. Results are shown as the mean number ± SEM of positive cells per mm\(^2\) for skin, and per lymph node section for lymph nodes, for \(n=6\) mice per group.}
\end{figure}
of CCL17 production very early after an IgE-dependent stimulus. The limited spillover observed after $^{125}$I-labeled CCL17 injection in the contralateral skin draining LNs, led to a small additional recruitment of T cells as compared to the diluent/diluent-injected mice, whereas a more important recruitment of DC was obtained suggesting that the latter may respond to a smaller amount of CCL17 than T cells. CCL17 was sufficient on its own to induce the recruitment of CD4$^+$ memory T cells and DC, but not mast cells and basophils in the draining LN. In the LN draining CCL17-injected skin graft, only part of the recruited CD4$^+$ T-cells expressed the CCL17 receptor CCR4, suggesting either a local desensitization of CCR4 by CCL17, as already described in vivo (de Lavareille et al., 2001), or a lack of sensitivity of the antibody for cells expressing low level of CCR4, or a recruitment through another receptor than CCR4. Controversial data have been published on CCR8 as a potential other receptor for CCL17 (Bernardini et al., 1998; Garlisi et al., 1999).

Kinetics studies showed that memory CD4$^+$ T cells appeared in the skin only 24 hours after intradermal injection of CCL17, at which time these cells had almost entirely disappeared from the LN, suggesting their redistribution from LN to the skin. Intradermal injection of CCL17 into the skin xenografts induced a recruitment of human leukocytes constituted mainly by CD4$^+$ cells, memory and naïve T cells. This is in agreement with previous in vitro studies showing CCL17 chemotactic activity on CD4$^+$ cells (Imai et al., 1996). In contrast, CD8$^+$ cells were not recruited by CCL17 in human skin xenografts conversely to in vitro observations with human CD8$^+$ T-cell lines (Sebastiani et al., 2002). The attraction of memory T cells by CCL17 is well characterized (Yamamoto et al., 2000; Sebastiani et al., 2002), whereas naïve T cells are usually unresponsive to CCL17 (Campbell et al., 1999; Lieberam and Forster, 1999). One surprising result was thus the attraction of naïve T cells in our in vivo model, in contrast to the in vitro data and the known lack of tissue circulation of naïve T cells. One explanation might be that the CD45RA$^+$ cells recruited in the skin are not real naïve T cells but terminally differentiated memory T cells that are CD45RA$^+$ but do not express CCR7 (Saule et al., 2006). Data have suggested that CD4$^+$CD25$^+$ regulatory T cells preferentially expressed the CCR4 and CCR8 receptors and were attracted by CCL17 (Iellem et al., 2001). However, intradermal injection of CCL17 into the human skin xenografts did not induce significant CD25$^+$ cell recruitment, suggesting that additional pathways are required to attract regulatory cells into the skin. Contradictory results have been published in the literature about the presence (Power et al., 1995) or absence (Imai et al., 1997) of CCR4 on monocytes. In our study, CCL17 clearly attracted monocytes both in vitro and in vivo, strongly suggesting the presence of a CCL17 specific receptor on monocytes, such as CCR4.
Human basophils were also attracted by CCL17 in human skin xenografts. Although CCR4 has been cloned from a human basophilic cell line (Power et al., 1995), we evidenced no or little CCR4 expression on human basophils by flow cytometry (data not shown). However, a slight chemoattractant effect was observed in chemotaxis assays, suggesting that this effect might be mediated by another receptor. Such a discrepancy has already been observed for CCL22, another CCR4 ligand, which appears to contribute to eosinophil accumulation without acting through CCR4 (Bochner et al., 1999). The recruitment of murine eosinophils after CCL17 injection into the skin was linked to a cross-reactivity between the human chemokine and murine eosinophils as shown in the chemotaxis assay. This cross-reactivity has also been shown in another study, although the authors were unable to evidence CCR4 expression on murine eosinophils (Borchers et al., 2002).

Although CCR4 is preferentially expressed on Th-2 cells (Bonecchi et al., 1998; D’Ambrosio et al., 1998; Sallusto et al., 1998; Syrbe et al., 1999), under certain conditions of stimulation, CCR4 can also be expressed on Th-1 cells (D’Ambrosio et al., 1998; Andrew et al., 2001). This study shows that in our in vivo model, CCL17 induced the recruitment of Th-2 cells but not Th-1 cells to the skin, which is in contrast with the in vitro chemotaxis assays, showing that both Th-1 and Th-2 cells were attracted by CCL17. It is of note that there was no difference of viability or apoptosis in the generated T helper sub populations, which might have explained a difference of migration of the two subsets. Such responsiveness from polarized Th-1 cells may be explained by the fact that CCR4 expression is induced on Th-1 cells after anti-CD3 stimulation (D’Ambrosio et al., 1998), a condition that we used to polarize the cells. Indeed, CCR4 was expressed on both polarized Th-1 and Th-2 cells, albeit at a lower level on Th-1 cells in vitro. CCR4 expression persisted in vivo on both Th-1 and Th-2 cells present in the LNs, ruling out an absence of Th-1 recruitment related to a loss of CCR4 expression. Because CCL17 could attract Th-1 cells in vitro, through a simple filter, but not in vivo, these results suggest that more complex events take place in vivo, to fine-tune the recruitment of T-cell subpopulations into the skin, through the endothelium layer. Accordingly, a recent study has shown that CCL22, through CCR4, was able to mediate the adhesion of both Th-1 and Th-2 cells on the endothelial adhesion molecule, VCAM-1, whereas CCL17 was only effective on the adhesion of Th-2 cells (D’Ambrosio et al., 2002). In a previous study, we also showed using the same skin grafted SCID model that CCL22 recruited equal numbers of IL-4 and IFN-γ positive cells (Fahy et al., 2001). Therefore, two distinct ligands binding the same receptor can recruit selective T-cell subpopulations, depending on additional costimuli.

Altogether, these data show that an overexpression of CCL17 in the skin leads to its rapid transportation to the draining LN, that allows the recruitment of DC and CCR4+ memory T cells in LN at 3-6 hours, which will be redistributed to the skin at 24 hours. In addition, it suggests a fundamental role for CCL17 both in the initiation of an immune response following antigen encounter with the skin, and in the development of the cutaneous inflammatory reaction.

**MATERIALS AND METHODS**

**Reagents**

Anti-human CD45, CD25 (IL-2 receptor chain α), and CD4 antibodies were purchased from Becton Dickinson (San Jose, CA). Anti-human CD8, CD68 (macrophages), CD1a (DC phenotype), CD45RO (memory T-cell phenotype), HLA-DR were from Dako (Glostrup, Denmark), antimurine major basic protein antibody was an in house rabbit polyclonal antibody, which recognizes specifically murine eosinophil MBP, a kind gift of GJ Gleich (Mayo Clinic, Rochester, MN; Filley et al., 1981) monoclonal antibody BB1 was used to specifically stain human basophils and was a kind gift of AF Walls (Southampton, UK; McEuen et al., 1999). Anti-human DC-lyosomal-associated membrane protein (mature DC phenotype) was from Beckman Coulter (Marseille, France), anti-human CCR4 was from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-human tryptase (mast cell phenotype) was from Chemicon (Temecula, CA). Anti-human CD45RA (naïve T-cell phenotype) and anti-human CD19 (B-cell phenotype) were from Dako, anti-human IL-4 as well as anti-CLA antibodies were from Pharmingen (San Diego, CA), and anti-human IFN-γ antibody was from IbT (Hycult Biotechnology, Uden, the Netherlands). Fast Red (Fast Red/Naphthol ASMX tablets) was from Sigma, St Quentin Fallavier, France. For flow cytometry, anti-human CD4-PECy5, anti-human CCR4-PE, and anti-human CLA-FITC were from BD Biosciences (San Jose, CA).

**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 the same donors was collected on heparin 6 hours after surgery. The protocol was approved by the Centre Hospitalier Régional et Universitaire ethical committee (no. 96,102). 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 Institute Pasteur of Lille in sterilized isolators. Leaky mice (displaying spontaneous IgG production after 6 weeks of age) were discarded. 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-cm² 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 use of two skin grafts per mouse allowed each mouse to be its own control. 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.

**Distribution of intradermally injected ¹²⁵I-CCL17**

To evaluate the fate of intradermal injected CCL17, 61.6 kBq human ¹²⁵I-CCL17 (NE Lifescience Products Inc., Boston, MA) in 50μl phosphate-buffered saline (PBS) was injected intradermally. Mice were killed 15, 60, and 120 minutes after injection. The skin draining and contralateral brachial and inguinal LNs, skin, kidney, mesenteric LN, spleen, liver were removed and mice were bled. Tissues were weighed, homogenized, agitated overnight on a shaker with 2 N NaOH and 0.5% SDS and counted in a γ-counter. The specific radioactivity associated with each organ was expressed per unit wet weight.

**Cell isolation**

**Human PBMC.** PBMCs were prepared from blood collected on heparin. Platelet rich plasma was obtained after centrifugation (120 g, 15 minutes) and discarded. Blood cells were then diluted in RPMI 1640 medium (Life Technologies, Paisley, Scotland; vol/vol) and layered over a Ficoll-Paque gradient (Pharmacia, Uppsala, Sweden). After centrifugation (400 g, 30 minutes), PBMCs were harvested and washed.

**Human CD4⁺ CD45RA⁻ cells.** PBMCs were prepared from blood collected on heparin as described above. Then, human CD45RA⁻ cells were purified by negative selection using CD4⁺ T-cell isolation kit and CD45RO microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer’s recommendations.

**Murine eosinophils.** Murine eosinophils were obtained from transgenic mice expressing murine IL-5 under the control of the human CD2 promoter. Briefly, after killing, spleens of animals were recovered, crushed, resuspended in RPMI and filtered through a nylon filter (Bultex, Sailly, France). Red blood cells were eliminated by hypotonic shocks.

**Human monocytes.** Human monocytes were purified from PBMC by positive selection using CD14 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer’s recommendations.

**Human basophils.** Peripheral blood (50ml) was collected in a heparinized syringe. Ten milliliters of 6% dextran in Hanks’ balanced salt solution was added to be heparinized blood, and erythrocytes were allowed to sediment (during 40 minutes at 37 °C). The buffy layer was then collected and mixed with RPMI-1640 medium (vol/vol) before it was overlaid onto Ficoll-Paque and centrifuged (1,000 g, 30 minutes). Cells at the medium-Ficoll interface (PBMC containing basophils, as well as lymphocytes and monocytes) were collected, washed, and enumerated. Then, human basophils were purified using human basophil isolation kit (Miltenyi Biotec), according to the manufacturer’s recommendations.

**Generation of polarized Th-1/Th-2 cells**

T cells from the skin donor were polarized in vitro towards a Th-1 or a Th-2 cytokine profile as previously described (Asselin et al., 1998; Imai et al., 1999). CD4⁺ CD45RA⁻ were activated with coated anti-CD3 (10 μg ml⁻¹; Pharmingen) in the presence of 2 ng ml⁻¹ IL-12 (R&D Systems, Abington, UK) and 200 ng ml⁻¹ anti-IL-4 (R&D Systems) for induction of Th-1 cells, or 10 ng ml⁻¹ IL-4 (R&D Systems) and 2 μg ml⁻¹ anti-IL-12 (R&D Systems) for induction of Th-2 cells. After 3 days, 10 ng ml⁻¹ IL-2 (Tebu, Le Perray en Yvelines, France) was added to the cultures. At day 10, some cells were restimulated with coated anti-CD3 (2 μg ml⁻¹) and soluble anti-CD28 (2 μg ml⁻¹; Pharmingen) during 5 hours. Polarization was checked by flow cytometry (FACScalibur; BD Biosciences) by assessing IFN-γ and IL-4 secreting cells by using the IFN-γ and IL-4 secretion assay kits (Miltenyi Biotec). Cell apoptosis was determined by flow cytometry using an annexin V/propidium iodide apoptosis assay kit (BD Biosciences). Apoptotic cells stained annexin V⁺ PI⁻ and dead cells stained annexin V⁻ PI⁺. The remaining cells were used to reconstitute the SCID mice and for chemotaxis assays.

**Experimental protocol for lymph node and cutaneous human cell recruitment**

Six weeks after human skin transplantation, antiasialo GM1 (1/20 dilution, Wako, Osaka, Japan) was injected intraperitoneally to neutralize murine NK activity. Twenty-four hours later, SCID mice were reconstituted intraperitoneally with 20 × 10⁶ autologous PBMC purified from the donor’s blood resuspended in PBS, or with 10 × 10⁶ autologous polarized Th-1 or Th-2 cells. 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 allogenic reactions during the reconstitution phase. Recombinant human chemokine CCL17 (PeproTech, Rocky Hill, NJ) was then immediately injected intradermally, 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. In some mice, the two skin grafts were injected with diluent to check the spontaneous cell recirculation following diluent injection. In other experiments, CCL17 was injected concomitantly with a neutralizing anti-CCL17 antibody at a dose of 35 μg per injection (R&D Systems) to check the specificity of the cell recruitment.

For the kinetic study of cell recruitment in peripheral LNs, the latter were recovered 30 minutes, 1 hour, 1 hour and 30 minutes, 3, 6, 24, and 48 hours after 2.5 μg intradermal CCL17 injection. LN were fixed in 4% paraformaldehyde and washed in 15% PBS sucrose before optimum cutting temperature compound embedding, freezing, and storage at –80 °C.

For the cutaneous human cellular recruitment study, human skin biopsies were performed at the site of injection marked by Evans blue dye using a cylindrical sterile punch and cut into two halves. One half was immediately embedded in optimum cutting temperature compound (Labonord, Villeneuve d’Ascq, France), snap-frozen in isopentane precooled in liquid nitrogen, and stored at −80 °C. The other half was fixed in 4% paraformaldehyde, washed in 15% PBS/ sucrose before optimum cutting temperature compound embedding, freezing, and storage at –80 °C. Preliminary dose responses (0.1, 1, 2.5, 5, and 10 μg diluted in 50 μl solvent PBS) and kinetics (6, 24, 48, and 72 hours) were performed to determine the optimal conditions for this study. The best cell recruitment in human skin biopsies was obtained with an intradermal injection of 2.5 μg of CCL17, and at 24 hours.
Distribution of CD4\(^+\) CLA\(^+\) and CD4\(^+\) CCR4\(^+\) cells in the peripheral blood of PBMC-reconstituted SCID mice

To evaluate the proportion of circulating CD4\(^+\) cells expressing the CLA and CCR4, SCID mice were reconstituted intraperitoneally with 20 \(\times\) 10\(^6\) PBMC and bled at different time points after reconstitution. The maximal blood cell recirculation was observed 6 hours after reconstitution. Blood was collected on heparin coated tubes, red cells were lysed using the utilyse kit from Dako. Cells were then double stained with antihuman CD4-PECy5 and antihuman CCR4-PE or antihuman CLA-FITC, and analyzed by flow cytometry.

**Immunohistochemistry**

Cryostat sections (6 \(\mu\)m) of unfixed tissues 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. For all antibodies except antihuman IL-4 antibody, immunohistochemistry was performed using a modified alkaline phosphatase antialkaline phosphatase method as previously described (Tsicopoulos et al., 2004). 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 antimeouse Ig and then monoclonal alkaline phosphatase antialkaline phosphatase antibodies, both from Dako diluted in 20% normal human AB\(^+\) serum. The coloration was developed using Fast Red and sections were counterstained using hematoxylin. Irrelevant primary antibody of the same species was used as negative control. For IL-4, immunohistochemistry was performed by using a modified avidine complex method, as previously described (de Nadai et al., 2006). Briefly, paraformaldehyde-fixed 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-IL-4 antibody in PBS containing 0.1% saponin (Sigma). Sections were treated with the ABC Vectastain Elite kit (Vector) and the color development was performed using diaminobenzidine tetrahydrochloride-nickel-avidine complex method, as previously described (de Nadai et al., 1999).

**Chemotaxis assay**

For all cell types evaluated, CCL17 (at a concentration of 10\(^{-8}\), 10\(^{-7}\), and 10\(^{-6}\)m) as well as control RPMI were used in a 48-well microchemotaxis chamber (Neuro Probe, Cabin John, MD) with 5 \(\mu\)m pore polycarbonate filters (Nucleopore Corp, Pleasanton, CA). Differentiated human Th-1, Th-2, and CD45RA\(^+\) cells were resuspended in RPMI at a concentration of 2 \(\times\) 10\(^6\) cells per ml and incubated 2 hours at 37 °C in 5% CO\(_2\). Cells that had migrated through the filter were counted in the inferior well, and results were expressed as mean number of cells per well. Eosinophils were resuspended at a concentration of 10\(^6\) cells per ml and incubated 45 minutes at 37 °C in 5% CO\(_2\). Monocytes and basophils were resuspended at a concentration of 10\(^5\) cells per ml and incubated 1 hour and 30 minutes at 37 °C in 5% CO\(_2\). Eosinophils, monocytes, and basophils that had migrated through the filter were counted under a microscope at a magnification of x 500. Results were expressed as mean number of cells per field. Each condition was performed in triplicate, and at least four fields were counted for each well. Chemotaxis was distinguished from chemokinesis as previously described (Fahy et al., 1999).

**Statistical analysis**

Slides were encoded and counted in a blinded fashion at \(\times\) 250 magnification using an eyepiece graticule. For skin sections, the upper edge of the grid was placed at the epidermal junction. For each specimen, at least three sections were evaluated, from which three to six fields were counted for immunohistochemistry. Absolute numbers of positive cells were counted per mm\(^2\). For LN, the entire surface of each section was evaluated, and numbers of positive cells were counted per LN section. Statistical analysis was performed using Graph pad software. Results were expressed as mean ± SEM. Comparisons for paired data were performed by using t-test and for kinetics studies by analysis of variance followed by post hoc test for multiple comparisons. Values of \(p\leq 0.05\) were regarded as statistically significant.

**CONFLICT OF INTEREST**

The authors state no conflict of interest.

**ACKNOWLEDGMENTS**

We thank Dr. David Dombrowicz for providing IL-5 transgenic mice. We are thankful to Anne-Sophie Hatzfeld, and Philippe Lassalle for critical review of this work.

**REFERENCES**


**CONFLICT OF INTEREST**

The authors state no conflict of interest.

**ACKNOWLEDGMENTS**

We thank Dr. David Dombrowicz for providing IL-5 transgenic mice. We are thankful to Anne-Sophie Hatzfeld, and Philippe Lassalle for critical review of this work.

**REFERENCES**


Oliveira SH, Lukacs NW (2001) Stem cell factor and IgE-stimulated murine mast cells produce chemokines (CCL2, CCL17, CCL22) and express chemokine receptors. Inflamm Res 50:168–74


www.jidonline.org


