CX_3CL1 (Fractalkine) and its receptor CX_3CR1 regulate atopic dermatitis by controlling effector T cell retention in inflamed skin

Delphine Staumont-Sallé [1,2,3,4,5], Sébastien Fleury [1,2,3,4*], Anne Lazzari [6,7,*], Olivier Molendi-Coste [1,2,3,4], Nicolas Hornez [3,5], Céline Lavogiez [3,5], Akira Kanda [1,2,3,4], Julien Wartelle[1,2,3,4], Anissa Fries [6,7], Davide Pennino[8], Cyrille Mionnet [6,7], Janne Prawitt [1,2,3,4], Emmanuel Bouchaert [1,2,3,4], Emmanuel Delaporte [5], Nicolas Glaichenhaus [6,7], Bart Staels [1,2,3,4], Valérie Julia [6,7], and David Dombrowicz [1,2,3,4].

[1] Inserm U1011 ; [2] Institut Pasteur de Lille ; [3] Université Lille 2. Lille, France. [4] European Genomic Institute of Diabetes. Lille, France.

[5] Department of Dermatology, Claude Huriez Hospital. Lille, France.

[6] CNRS UMR7275 - [7] Université Nice - Sophia Antipolis, France

[8] ZAUM-Center of Allergy and Environment, Technische Universität and Helmholtz Center Munich, Munich, Germany

[*] SF and AL contributed equally to this work

Corresponding authors:

David Dombrowicz, Inserm U1011, Institut Pasteur de Lille. 1, rue Prof. Calmette BP245. 59019 Lille Cedex. France. E-mail : david.dombrowicz@pasteur-lille.fr.

Phone: +33 3 20 87 79 67 Fax: +33 3 20 87 78 88.

Valérie Julia, CNRS UMR7275, Université Nice-Sophia Antipolis. 660 route des Lucioles. 06560 Valbonne. France. E-mail: vjulia@ipmc.cnrs.fr

Phone: +33 4 93 95 77 82 Fax: +33 4 93 95 77 08.

Sentence caption: Fractalkine interactions with its receptor, CX3CR1, regulate CD4+ T cell retention in atopic dermatitis and offer a potential therapeutic target in allergic disease.

Source of funding: This study was in part funded by grants from the Agence Nationale de la Recherche (ANR) ANR-06-MIME-008, ANR-10-LABX-46, from the AVIESAN Institut Thématique Multiorganisme (ITMO) Immunologie and from the Fondation pour la Recherche Médicale (FRM).

The authors declare no financial conflict of interest.

Abbreviations used:

AD: Atopic dermatitis

AHR: Airway hyperresponsiveness

BAL: Bronchoalveolar lavage

LACK: Leishmania homolog of receptors for activated C kinase

WT: wild-type

ABSTRACT

Atopic dermatitis (AD) is a chronic allergic dermatosis characterized by epidermal thickening and dermal inflammatory infiltrates with a dominant Th2 profile during the acute phase, while a Th1 profile is characteristic of the chronic stage. Among chemokines and chemokine receptors associated with inflammation, increased levels of CX_3CL1 (fractalkine) and its unique receptor, CX_3CR1, have been observed in human AD. We have thus investigated their role and mechanism of action in experimental models of AD and psoriasis. AD pathology and immune responses, but not psoriasis, were profoundly decreased in CX_3CR1-deficient mice and upon blocking CX_3CL1/CX_3CR1 interactions in wild type mice. CX_3CR1 deficiency neither affected antigen presentation nor T cell proliferation *in vivo* upon skin sensitization, but CX_3CR1 expression by both Th2 and Th1 cells was required to induce AD. Surprisingly, unlike in allergic asthma, where CX_3CL1 and CX_3CR1 regulate the pathology by controlling effector $CD4^+$ T cell survival within inflamed tissues, adoptive transfer studies established CX3CR1 as a key regulator of $CD4^+$ T cell retention in inflamed skin, indicating a new function for this chemokine receptor. Therefore, while CX_3CR1 and CX_3CL1 act through distinct mechanisms in different pathologies, our results further evidence their interest as promising therapeutic targets in allergic diseases.

INTRODUCTION

Atopic dermatitis (AD) is a common, chronic inflammatory dermatosis frequently occuring in individuals with a personal or family history of atopic diseases. AD pathophysiology is complex and results from skin barrier dysfunction and a dysregulated immune response, influenced by genetic and environmental factors (Guttman-Yassky et al., 2011a; Guttman-Yassky et al., 2011b). Indeed, most patients with AD have increased serum IgE levels, with specific IgE directed against allergens or microbial proteins such as *Staphylococcus aureus* (Leung et al., 2004). Lesions in AD are characterized by increased epidermal thickness and a dermal inflammatory cell infiltrate, consisting of mast cells, eosinophils and T lymphocytes. In acute AD lesions, a preferential recruitment of Th2 cells occurs, whereas in the chronic lesions a Th1 profile is predominant (Grewe et al., 1998), while allergic asthma or allergic rhinitis are more exclusively Th2-dominated diseases.

Chemokines and their receptors play a key role in leukocyte recruitment to inflamed skin (Schall and Proudfoot, 2011). Eotaxins 1, 2 and 3 (CCL11, 24, 26) bind to CCR3 and attract eosinophils, and CCL26 appears to be particularly involved in AD (Kagami et al., 2003; Owczarek et al., 2011). CCL27 together with CCR10 and CCR4 expression insure T cell skin domiciliation (Homey et al., 2002; Reiss et al., 2001). More recently, CCR8 and CCL8 have been elegantly demonstrated to direct Th2 cell recruitment into allergen-inflamed skin and draining lymph nodes in a murine model of AD (Islam et al., 2011).

Besides chemoattraction, chemokine - chemokine receptor interactions also regulate other functions. Indeed, we have recently demonstrated that CX_3CR1, the receptor for CX_3CL1 (fractalkine -CX_3-), identified also as a receptor for CCL26 (Nakayama et al., 2010) in humans, controls the development of allergic asthma by providing a survival signal to the $CD4^+$ effector T lymphocytes in the inflammatory airways (Julia, 2012; Mionnet et al., 2010). In AD patients, CX_3CL1 is upregulated in both endothelial cells and skin lesions and serum

CX_3CL1 levels are positively associated with disease severity (Echigo et al., 2004). Another study reported that, while CX_3CR1 mRNA expression is consistently upregulated in AD skin, CX_3CL1 mRNA levels are only increased in some patients with a significant correlation to the disease severity (Nakayama et al., 2010), a result likely to explain the earlier failure to detect CX_3CL1 in skin lesions (Fraticelli et al., 2001). Furthermore, two CX_3CR1 single nucleotide polymorphisms have been associated with asthma and atopy in French-Canadian populations (Tremblay et al., 2006) and German children (Depner et al., 2007).

Thus, in order to functionally delineate the role of CX_3CL1/CX_3CR1 in AD, we used a mouse model of epicutaneous sensitization, by a protein antigen in the absence of adjuvant, faithfully mimicking features of human AD. Unexpectedly, we found that CX_3CL1/CX_3CR1 controlled atopic dermatitis to an even greater extent than allergic asthma through a new and distinct mechanism.

RESULTS

Upon skin sensitization, CX_3CR1-deficient mice develop neither AD nor subsequent lung inflammation.

To assess the contribution of CX_3CR1 to AD development, we used a previously described model of AD based on repeated epicutaneous sensitizations (Spergel et al., 1998) with *Leishmania major activated C Kinase* (LACK) as antigen and compared the response of CX_3CR1-deficient ($^{gfp/gfp}$) mice, in which the CX_3CR1 gene has been replaced by gfp (Jung et al., 2000), to that from their proficient ($^{+/+}$) WT counterparts. Neither strain exhibited an inflammatory phenotype in the absence of LACK sensitization **(Fig. 1A)**. Compared to vehicle (i.e. PBS)-sensitized $CX_3CR1^{+/+}$ mice, LACK-sensitized $CX_3CR1^{+/+}$ mice exhibited a significant skin inflammatory response, characterized by a 50% increase in epidermal thickening **(Fig. 1B)** associated with more pronounced hyperkeratosis, spongiosis and dermal cellular infiltrates including mast cells, eosinophils, MHC-II^+ and $CD4^+$ T cells **(Fig. 1C)** as well as increased skin and inguinal lymph node (LN) expression of inflammatory and Th1- and Th2-associated cytokines, chemokines and chemokine receptors **(not shown)**. In sharp contrast, $CX_3CR1^{gfp/gfp}$ mice did not develop a skin inflammatory response upon LACK sensitization **(Fig. 1A-B)**. Compared with PBS-sensitized $CX_3CR1^{gfp/gfp}$ mice, only MHC-II^+ cell numbers were increased, but to a lesser extent than in LACK-sensitized $CX_3CR1^{+/+}$ animals **(Fig. 1C)**. Furthermore, expression of Th1- and inflammatory response genes was also significantly decreased **(not shown)**.

Humoral response was also altered in $CX_3CR1^{gfp/gfp}$ compared to $CX_3CR1^{+/+}$ mice, with decreased total Th2-associated IgE concentrations (but not IgG_1 titers), as well as decreased Th1-associated antigen-specific IgG_{2a} titers **(Fig. 1D).**

As in the human pathology, epicutaneous sensitization also induced lung inflammation and airway hyperreactivity (AHR) following a single antigenic airway challenge. Airway resistance upon LACK-sensitization was significantly lower in $CX_3CR1^{gfp/gfp}$ compared to $CX_3CR1^{+/+}$ animals **(Fig. 1E)**. Furthermore, cellular inflammatory infiltrates in the broncho-alveolar fluid (BALF) of LACK-sensitized $CX_3CR1^{gfp/gfp}$ mice were decreased by 32% for macrophages, 70% for lymphocytes and eosinophils, and 40% for neutrophils compared to BALF from $CX_3CR1^{+/+}$ mice **(Fig. 1F)**.

A CX_3CL1 antagonist strongly reduces features of AD.

To further confirm the key role of CX_3CR1 in AD development, we next investigated whether inhibition of CX_3CL1/CX_3CR1 interactions would inhibit the pathology in WT animals. We investigated the efficacy of a CX_3CR1 antagonist (CX_3-AT), whose potency was already validated in an allergic asthma model (Mionnet et al., 2010), using prophylactic or therapeutic administration protocols (**Fig. 2A**). Both administration schedules fully inhibited antigen-induced epidermal thickening (**Fig. 2B**), as well as dermal mast cell, eosinophil and $CD4^+$ T cell infiltration **(Fig. 2C)**. Upon LACK aerosol challenge, AHR and inflammatory cell infiltration in the airways were also significantly decreased upon both prophylactic and therapeutic treatments **(Fig. 2C-D)**. Taken together, these results confirm the key role of CX_3CR1/CX_3CL1 in AD in non-genetically manipulated mice and further demonstrate that pharmacological inhibition of CX_3CL1/CX_3CR1 interactions abrogate skin and lung inflammation.

CX_3CR1 is neither required for antigen presentation nor for naïve T cell proliferation but regulates both Th1- and Th2- induced skin inflammation

As CX_3CR1 is expressed by various myeloïd cells, such as blood monocytes, dendritic cell

(DC) progenitors, plasmacytoid DC, and macrophages (Bar-On et al., 2010; Kim et al., 2011; Zhang et al., 2012), we next assessed whether antigen presentation was affected in the absence of CX_3CR1. To this aim, antigen-specific $CD4^+$ T cells from WT15 TCR transgenic mice (Wang et al., 2001), were labeled with CSFE and injected to both $CX_3CR1^{+/gfp}$ and $CX_3CR1^{gfp/gfp}$ mice that were further sensitized via epicutaneous LACK administration. Frequencies of divided antigen-specific $CD4^+$ T cells in the draining LN were comparable in both $CX_3CR1^{+/gfp}$ and $CX_3CR1^{gfp/gfp}$ mice **(Fig. 3A)**, suggesting that, upon epicutaneous sensitization, CX_3CR1 deficiency does not affect antigen presentation. We next investigated whether CX_3CR1 deficiency affected T cell proliferation induced upon epicutaneous antigen sensitization. To address this issue, we generated $Thy1^{+/-}$ $CX_3CR1^{+/gfp}$ and $Thy1^{+/+}$ $CX_3CR1^{gfp/gfp}$ LACK-specific TCR transgenic mice that respectively expressed the Thy1.1 and Thy1.2 antigens or the Thy1.1 antigen only. $CD4^+$ T cells from both genotypes were prepared, stained with CSFE and co-injected into WT $Thy1.1^{-/-}$ $Thy1.2^{+/+}$ mice. Upon epicutaneous sensitization with LACK, frequencies of dividing $CX_3CR1^{+/gfp}$ and $CX_3CR1^{gfp/gfp}$ WT15 cells were comparable **(Fig. 3B)**. Altogether these results suggest that CX_3CR1 deficiency does not alter naïve T cell proliferation.

CX_3CR1 expression regulates both Th1- and Th2- induced skin inflammation.

As both Th2 and Th1 cells are associated to the acute and chronic phases of AD, respectively (Spergel et al., 1999), we investigated whether CX_3CR1 expression was required by Th1, Th2 cells or both to induce skin inflammation. LACK-specific $CD4^+$ T cells were prepared from either $Thy1^{+/-}$ $CX_3CR1^{+/gfp}$ or $Thy1^{+/+}$ $CX_3CR1^{gfp/gfp}$ WT15 mice, differentiated *in vitro* into Th1 or Th2 cells and injected into WT mice that were further sensitized to LACK. While both CX_3CR1-proficient Th1 and Th2 cells alone were able to induce a 4 to 5-fold epidermal thickening upon a single round of epicutaneous antigen exposure, CX_3CR1-deficient Th2

cells only induced a less than 2- fold increase in epidermal thickness and injection of CX_3CR1-deficient Th1 cells barely led to epidermal thickening compared to PBS-treated animals **(Fig. 3C)**. Likewise, dermal mast cell infiltration was also induced by both CX_3CR1-proficient Th1 and Th2 cells, but not upon injection of CX_3CR1-deficient effector T cells **(Fig. 3D)**. Eosinophil infiltration was only induced upon transfer of CX_3CR1-proficient Th2 cells and strongly reduced when CX_3CR1-deficient Th2 cells were injected **(Fig. 3D)**. Taken together, these results suggest that CX_3CR1 expression by Th2- and Th1-cells regulates the key features of AD.

CX_3CR1 expression confers a selective advantage to skin-infiltrating T cells.

To decipher the mechanisms accounting for the role of CX_3CR1 expression by T helper cells in skin inflammation, we monitored the recruitment and proliferative capacities of both CX_3CR1-proficient and CX_3CR1-deficient LACK-specific Th1 and Th2 cells upon co-injection into WT mice that were exposed to LACK and further fed with BrdU. Three days following antigen exposure, while Th1 and Th2 donor cells of both genotypes had not yet incorporated BrdU (data not shown), CX_3CR1-proficient and CX_3CR1-deficient LACK-specific donor cells were detected at the same frequency in skin, suggesting that early migration of effector T cells into the skin did not require CX_3CR1 **(Fig. 4A)**. It is worth noting that antigen-induced recruitment of LACK-specific Th1 cells was more pronounced than recruitment of Th2 cells as early as 3 days after antigen exposure. In sharp contrast, frequencies of CX_3CR1-proficient donor cells outnumbered CX_3CR1-deficient cells on day 7 **(Fig. 4B)**. However, donor cells of both genotypes proliferated at the same rate (**Fig. 4C**). Therefore, as observed for naïve T cells, CX_3CR1 deficiency neither affects the early recruitment, nor the proliferation of Th1- and Th2- effector cells.

We next monitored CX_3CR1 expression using GFP as a surrogate marker for CX_3CR1

expression (Geissmann et al., 2003; Jung et al., 2000). Upon differentiation, antigen-specific effector cells remained GFP^- as previously described (Mionnet et al., 2010). At day 3 after initial sensitization, less than 0.3% of antigen-specific effector cells expressed CX_3CR1 (i.e. GFP) in draining LN, and around 4% of these cells expressed CX_3CR1 (i.e. GFP) in skin (**Fig. 4D**). At day 7, while the frequencies of CX_3CR1-expressing Th1 and Th2 cells in the draining LN slightly increased to 3.6 and 1.4 %, respectively, their frequencies in skin steadily increased to 12.3 and 8.3 %, respectively (**Fig. 4D).** These latter results suggest that CX_3CR1 expression is likely to be induced early in skin.

CX_3CR1-deficiency impairs effector T cell retention into inflamed skin.

The higher frequency of CX_3CR1-proficient $CD4^+$ effector cells in skin could be explained by several hypotheses: the preferential late recruitment of CX_3CR1^+ effector T cells into the skin, their prolonged survival, or their selective advantage for residence in the inflamed skin. As CX_3CR1 is involved in effector T cell survival in allergic lung inflammation (Mionnet et al., 2010), and as the role of CX_3CR1/CX_3CL1 in microglial cell (Meucci et al., 1998) and monocyte survival (Landsman et al., 2009) has also been reported, we next assessed whether this was also the case in inflamed skin. WT mice were co-injected with CX_3CR1-proficient and -deficient antigen-specific Th1- or Th2- donor cells, sensitized by epicutaneous antigen administration and the frequencies of apoptotic and/or necrotic donor cells were measured. Similar frequencies of annexin-V^+ and/or 7-AAD^+ $CX_3CR1^{+/gfp}$ and $CX_3CR1^{gfp/gfp}$ donor cells were found in both skin and draining LN **(Fig. 5A and not shown)**, suggesting that CX_3CR1 is not involved in T cell survival. However, as Th2 donor cells were difficult to monitor in skin due to their low frequencies, additional experiments were performed to confirm these data. $CX_3CR1^{+/gfp}$ and $CX_3CR1^{gfp/gfp}$ antigen-specific Th2 cells were transduced with a retroviral construct leading to the expression of anti-apoptotic BCL-2 (an empty vector was

used as control) and transferred into WT recipients that were sensitized by LACK. While overexpression of BCL-2 led to a decrease, and respectively an increase in the recovery of transduced cells in the skin and draining LN, it did not affect the ratio between CX_3CR1-proficient and -deficient Th2 cells within these tissues, ruling out a role of CX_3CR1 in T cell survival **(Fig. 5B)**.

We next compared the migration of CX_3CR1-proficient and -deficient effector T cells into the inflamed skin and draining LN of WT mice with established AD. Recipient mice were submitted to two rounds of epicutaneous sensitization prior to the injection of CX_3CR1-proficient and -deficient Th1 or Th2 cells the day before the third and last antigen application. Three days later, while BrdU incorporation was very low, frequencies of donor cells of both genotypes were similar (**Fig. 5C**), demonstrating that CX_3CR1 is not required for the late migration of effector T cells into the inflamed skin.

To investigate whether CX_3CR1 was required for T cell retention into the inflamed skin, mice were co-injected with CX_3CR1-proficient and -deficient LACK-specific effector T cells, challenged for 4 days with LACK, and further challenged with the antigen while topically treated with CX_3-AT for 3 days. Upon topical CX_3-AT treatment, frequency of skin CX_3CR1-proficient T cells decreased to the level of co-injected CX_3CR1-deficient cells, while their frequency increased in draining LN (**Fig. 5D**), demonstrating that blocking CX_3CR1-CX_3CL1 interactions *in situ* prevents CX_3CR1^+ T cell retention.

Next, to further corroborate these findings with endogenously generated T cells, WT mice were treated topically with CX_3-AT for the last 3 days of the third and last round of epicutaneous sensitization. CX_3-AT strongly decreased epidermal thickening, mildly inhibited mast cell infiltration and did not significantly affect eosinophil infiltration (**Fig. 5E-G**). Finally, to further confirm that the effect of CX_3CR1 in AD was due to its expression on T cells, we adoptively transferred CX_3CR1-proficient or -deficient naïve T cells into CX_3CR1-

deficient animals and induced AD. After 7 weeks, animals reconstituted with CX_3CR1-proficient T cells developed a pathology (epidermal thickening, mast cell and eosinophil infiltration) that was comparable to that of CX_3CR1-proficient animals. Reconstitution with CX_3CR1-deficient T cells did not induce symptoms of AD (**Fig. 5H-J**). Furthermore, topical application of CX_3-AT during the last 3 days of sensitization, to CX_3CR1-deficient mice reconstituted with CX_3CR1-proficient T cells, the only cells expressing CX_3CR1 in this experimental setting, exerted similar effect than on (non-reconstituted) CX_3CR1-proficientanimals (**Fig. 5H-J**).

Altogether, these data demonstrate that blocking interactions between CX_3CR1 and CX_3CL1 *in situ* prevents disease symptoms by impairing effector T cell retention in inflamed skin.

Human Th2 - and Th1- CX_3CR1^+ cells infiltrate AD skin lesions.

In order to assess the human relevance of our observations, we investigated whether $CX3CR1^+$ $CD4^+$ T cells could be detected in AD patients. In agreement with the absence of CX_3CR1 expression on circulating mouse $CD4^+$ T cells, even upon antigenic sensitization, in human, we detected a very low expression on circulating $CD4^+$ T from AD patients. In addition, no significant differences were found between healthy individuals, AD and psoriasis patients ~~patients~~ in agreement with previously published work (Echigo et al., 2004) **(Fig. 6A).** About 7% of skin isolated $CD4^+$ T cells expressed CX_3CR1, in keeping with our findings in the murine model of AD. As $CX3CR1^-$ cells, CX_3CR1^+ $CD4^+$ T cells display a very heterogeneous cytokine profile with the Th1 and Th2 subsets being the most represented as compared to Th17 (**Fig. 6B, C**). Taken together, these data confirm the presence and functional properties of CX_3CR1 cells in human.

DISCUSSION

We have demonstrated that in an AD model based on epicutaneous antigen sensitization, CX_3CR1 deficiency prevented skin inflammatory response and severely reduced the pulmonary symptoms as well as humoral responses. This defect was solely due to CX_3CR1 expression by $CD4^+$ T cells as demonstrated by reconstitution experiments in which transfer of WT $CD4^+$ T cells in CX_3CR1-deficient mice restored skin disease that could be further blocked upon CX_3CR1 antagonist treatment. Paradoxically for an allergic disease and unlike asthma, the most pronounced inhibitory effects were observed on the Th1-associated rather than Th2-associated response (Fig. 3A and not shown). These findings further underline the major role exerted by CX_3CR1 on Th1 cells, prominently associated to the chronic phase of the disease, which adds to its key effect on Th2 cells in allergic asthma (Mionnet et al., 2010). CX_3CL1 and expression of its unique receptor, CX_3CR1 on T cells play a crucial role in the development of AD by retaining effector T cells in the inflamed skin. Indeed, neither proliferation, early or late migration, nor survival were affected by CX_3CR1 deficiency on $CD4^+$ effector T cells, while blocking CX_3CR1/CX_3CL1 interactions *in situ* prevented WT allergen-specific T cell accumulation in skin and induced their accumulation in draining LN (Fig. 5D). Interestingly, upon such treatment, while allergen-specific T cells were more abundant in the periphery, this later also prevented the development of pulmonary symptoms observed upon intranasal antigen challenge. A phenomenon that might suggest that residence into inflammatory skin leads to further T cell education and subsequent preferential access to the lungs. This hypothesis fits with our unpublished observations in which T-cell containing lung inflammatory foci develop even in the absence of the single terminal airway antigen challenge in AD mice. Such "reprogramming" for distal mucosal homing is reminiscent of previously reported reprogramming from gut to skin homing (Oyoshi et al., 2011). Furthermore, it was recently shown that lung DC promotes T cell lung homing through

induction of CCR4, which is also a skin-homing receptor (Mikhak et al., 2013). These findings illustrate the mechanisms at play to insure lymphocyte (re)circulation between distinct sites within the common mucosal immune system (Lazarus et al., 2003).

Chemokines known so far to be involved in skin diseases contribute to the pathology by inducing migration of inflammatory cells towards the skin and not by facilitating their retention in the inflammatory sites. Indeed, Islam *et al* demonstrated that CCL8 is involved in skin homing of CCR8-expressing Th2 cells in the same animal model of AD (Islam et al., 2011). Likewise, Homey *et al* showed that intradermal CCL27 injection attracted lymphocytes *in vivo* and, conversely, neutralization of interactions between CCL27 and its receptor CCR10 impaired lymphocyte recruitment to the skin leading to the suppression of DNFB (dinitrofluorobenzene)-induced skin inflammation in a murine model of contact hypersensitivity (Homey et al., 2002). While the membrane form of CX_3CL1 had been previously shown to mediate adhesion of CX_3CR1-expressing leukocytes *in vitro*, to our knowledge this is the first report demonstrating such a role *in vivo*. It remains, however to be determined which cell types interact with $CD4^+$ T cells to retain them within the skin and why/how the CX3CR1 pathway plays a different role in the skin compared to the lung. To this latter point, we can hypothesize that CX_3CL1-expressing cells required for the delivery of CX_3CR1 signaling are different in skin and lung. While in skin CX_3CL1 is expressed in epithelial cells, endothelium as well as in lesional skin of AD patients, in asthmatic lungs it is also highly upregulated within airway smooth muscle (El-Shazly et al., 2006). Indeed, we have also found in our LACK-induced asthma model that infiltrating antigen-specific $CD4^+$ T cells can be localized within the smooth muscle cells (our unpublished data).

The particular importance of CX_3CR1 in the development of AD, compared to a "pure" Th2 pathology such as allergic asthma, probably results from its combined action on both Th1 and Th2 arms of the disease. Such a major effect could be evidenced in a model of mild-

inflammation induced by epicutaneous sensitization in the absence of adjuvant and might not have been found by using models of highly polarized Th2 inflammation obtained upon immunization with adjuvant.

CX3CR1 does not appear involved in every skin disease as CX_3CR1-deficient and -proficient mice displayed similar epidermal thickening and inflammation in a well established experimental model mimicking human psoriasis by repeated applications of Imiquimod (a TLR7/8 agonist) (van der Fits et al., 2009) (not shown). Likewise, earlier studies had demonstrated that CX_3CR1-deficient and -proficient mice also exhibited similar responses in a model of contact hypersensitivity/DTH to oxazolone (Jung et al., 2000). This reinforces the high potential of CX_3CR1 and CX_3CL1 as therapeutic targets in allergic diseases.

In conclusion, the identification of a new mode of action for a chemokine and the full inhibition of AD by a CX_3CR1 antagonist further demonstrates that the CX_3CR1/CX_3CL1 axis represents a new promising therapeutic target in allergic inflammatory diseases like allergic asthma and atopic dermatitis.

MATERIALS AND METHODS

Animals

Littermate $CX_3CR1^{gfp/gfp}$, $CX_3CR1^{+/gfp}$, $CX_3CR1^{gfp/gfp}$ and $CX_3CR1^{+/gfp}$ $Thy1.1^{+/-}$ WT15 TCR transgenic mice were generated in a Balb/c ByJ background (12 backcrosses) as previously described (Mionnet et al., 2010). Eight- to 12-week-old female mice were used for all experiments. Animals were housed within the specific pathogen-free facility from the Institut Pasteur de Lille or from the Institut de Pharmacologie Moléculaire et Cellulaire in Sophia-Antipolis. Experiments were performed after approval by the Ethics Committee for Animal Experimentation from Lille and Nice.

Experimental atopic dermatitis

As previously described, AD was induced by epicutaneous sensitization (Staumont-Salle et al., 2008) with LACK antigen. Briefly, patches soaked with 25 µL of 0.2% LACK solution in PBS or with vehicle were applied on abdominal skin 24 hours after shaving and were left on for three 1-week periods (with patch renewal at midweek), with a 2-week interval between applications. At the time of the last patch removal (day 49), animals were challenged for 20 minutes by means of aerosol nebulization with LACK (0.2% in PBS) by using an ultrasonic nebulizer (Systam, Villeneuve sur Lot, France) and serum was collected. On the next day, airway hyperresponsiveness (AHR) to increasing concentrations of methacholine was measured by means of invasive plethysmography using a FlexiVent and expressed by dynamic lung resistance (SCIREQ) (Kanda et al., 2009). Animals were sacrificed by cervical dislocation. Bronchoalveolar lavage fluid (BALF) was collected and analyzed on cytospin preparations after RAL 555 staining. Skin and inguinal lymph nodes were collected for histological analyses and real-time PCR after RNA extraction. LACK was produced and

detoxified using Endotrap columns (Profos). Endotoxin levels assessed by LAL assay (Pierce) were below 5 ng/mg of protein.

Treatment with a CX3CL1 antagonist (CX3-AT) in a model of AD

CX3-AT blocking reagent was prepared as described (Mionnet et al., 2010) and administered by weekly intraperitoneal injections (50 µg/mouse) either during the 3 sensitization periods (prophylactic protocol) or only during the last week of sensitization (therapeutic protocol) and animals were analyzed as described above. Alternatively, CX_3-AT was applied topically (50 µg/mouse) by patch together with the antigen during the last 3 days of a single round of sensitization or during the third and last week-long round of sensitization.

Histology and immunohistochemistry

Tissue biopsy specimens were processed as previously described (Staumont-Salle et al., 2008). Briefly, samples were fixed in ImmunoHistoFix (Interstiles, Brussels, Belgium) and embedded in ImmunoHistoWax (Interstiles) at 37°C. Five µm sections were stained with May Grünwald Giemsa for measurement of epidermal thickness and eosinophil and mast cells counts (Staumont-Salle et al., 2008) by using a microscope with Arcturus XT software. Epidermal thickness was determined at 250-fold magnification; an average of 10 measures was calculated for each sample. Eosinophils and mast cells were enumerated by examining 20 random fields at 400-fold magnification; cell frequency was converted to cells per square millimeter and results were expressed as mean ± SD. For immunohistochemical analysis, sections embedded in ImmunoHistoWax were immunostained with anti-I-A^d/I-E^d (MHC II) mAb (clone M5/114, rat IgG2b, BD Biosciences) and cryopreserved sections were stained with anti-CD4 mAb (clone RM4-5, rat IgG2a, BD Biosciences) as previously described (Angeli et al., 2004). For each section, 10 random fields were examined at x400.

Immunoglobulin concentrations

Immunoglobulin (IgE, IgG_1 and IgG_{2a}) concentrations in serum were measured as previously described by ELISA (Staumont-Salle et al., 2008). Two-fold serial dilutions were prepared for each serum (starting dilution 1:25 for IgE, 1:5000 for IgG_1, 1:1000 for IgG2a). Antibody titers were calculated as the dilution corresponding to twice the mean absorbance value obtained for non-sensitized mouse sera.

Murine cell purification and *in vivo* assays

For the antigen-presenting capacity assay, splenic $CD4^+$ T cells were purified from $CX3CR1^{+/+}$ WT15 $Thy1.1^{+/+}$ $Rag\text{-}2^{-/-}$ TCR transgenic mice, encoding a LACK specific TCR, by positive selection using anti-CD4 beads (MACS, Miltenyi) (purity >95%), stained with CFSE (Sigma) and further injected (2 X 10^6 cells) via the lateral tail vein into $CX3CR1^{gfp/gfp}$ and $CX3CR1^{+/gfp}$ recipient mice, 1 day before epicutaneous LACK sensitization. Proliferation of donor cells in the inguinal LN was assessed by flow cytometry 5 days later.

For analysis of $CX_3CR1^{+/+}$ and $CX_3CR1^{gfp/gfp}$ T cell proliferation, splenic $CD4^+$ T cells were purified from $CX_3CR1^{gfp/gfp}$ $Thy1.1^{+/+}$ and $CX_3CR1^{+/gfp}$ $Thy1.1^{+/-}$ WT15 donor mice, stained with CFSE, and co-injected (2 X 10^6 of each population) into $Thy1.1^{-/-}$ $CX_3CR1^{+/+}$ wild-type mice, one day before epicutaneous LACK sensitization. Proliferation was assessed in the inguinal LN 4 days later by flow cytometry.

For preparation of Th1 and Th2 cells, $CD4^+$ T cells from TCR transgenic mice were purified by negative selection and 1.5 X 10^6 cells were incubated for 3 days with 0.75 X 10^6 T cell-depleted splenocytes in complete RPMI with 50 nM $LACK_{156\text{-}173}$ peptide and (i) 10 ng/ml r-IL-4 and 10 µg /ml antibody to IFNγ (R4-6A2) for Th2 cells or (ii) 10 ng/ml r-IL-12 and 10 µg /ml antibody to IL-4 (11B11) for Th1 cells. In some experiments, $Thy1.1^{-/-}$ $Thy1.2^{+/+}$ WT

mice received either $CX_3CR1^{gfp/gfp}$ or $CX_3CR1^{+/gfp}$ LACK-specific Th1 or Th2 cells (2 X 10^6 cells per mouse) by intravenous injection and then underwent one single round of LACK epicutaneous sensitization. Animals were sacrificed at day 7 and skin samples were collected for histological analysis of AD characteristics as previously described. In other experiments, the same number of both $CX_3CR1^{gfp/gfp}$ and $CX_3CR1^{+/gfp}$ LACK-specific Th1 or $CX_3CR1^{gfp/gfp}$ and $CX_3CR1^{+/gfp}$ LACK-specific Th2 cells were co-injected in $Thy1.1^{-/-}$ $Thy1.2^{+/+}$ WT mice mice (1.5 X 10^6 of each genotype per mouse), one day before LACK epicutaneous sensitization. When indicated, recipient mice treated as described above, were injected i.p. with BrdU (BD Biosciences) (200 μg) for the last 18 hrs. BrdU incorporation of CX_3CR1 -proficient and -deficient Th1 and Th2 donor cells was studied by flow cytometry in inguinal LN. Survival of Th1 and Th2 donor cells was analyzed by annexin-V and 7-aminoactinomycin D (7-AAD) staining in both skin and inguinal LN.

In reconstitution experiments, 5 X 10^6 naïve $CX_3CR1^{gfp/gfp}$ or $CX_3CR1^{+/gfp}$ $CD4^+$ T cells were adoptively transferred into $CX_3CR1^{gfp/gfp}$ mice 24h prior to induction of AD.

Human T cells

PBMC and skin samples were obtained from patients with AD from the Department of Dermatology or from healthy donors from the Department of Plastic Surgery. This study was approved by the Ethical Committee from the University Hospital of Lille. All subjects provided written informed consent. Patients with AD were selected according to the Hanifin and Rajka criteria (Hanifin and Rajka, 1980). PBMC were obtained after elimination of granulocytes on a Ficoll gradient. Punch skin biopsies (diameter 5 mm) were cultured in complete RPMI 1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin (all Invitrogen), 5% human serum (Sigma) and 20U IL-2/ml (Novartis). After 10-13 days, emigrating cells were collected and characterized by surface and intracellular staining by flow

cytometry. Surface and intracellular cytokine staining was performed using the Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's instructions. The following fluorochrome-conjugated antibodies were used: ant-IFN-γ-V450 (B27), anti-TNF-Alexa flluor 700 (Mab11), anti-IL-4-PerCP-Cy5.5 (8D4-8), anti-IL17A-PE (N49-653), anti-IL-5-APC (TRFK5), anti-IL-9 PerCP-Cy5.5 (MH9A3), anti-CD4-APC-Cy7 (RPA-T4), anti-IL-13-Horizon-V450 (JES10-5A2) (all BD Bioscience), anti-IL4-PE (3010.211), (BD FastImmune), anti-IL22-APC (142928) (R&D), CX3CR1-FITC (2A9-1) Biolegend.

Retroviral transduction

The human Bcl2 cDNA was cloned into the mouse bi-cistronic retroviral expression vector MIGR. Th2 differentiated cells were incubated with viral supernatant containing 5 µg/ml Polybrene (Sigma) and spun at 32°C for 8 h as described (Mionnet et al., 2010). Viral supernatant was replaced with fresh medium. GFP-expressing T cells were sorted 3 d later.

Statistical analysis

Statistical significance was determined by Student T test, except for plethysmographic data, for which ANOVA for repeated measures was used. Graphpad and STATview softwares were used, respectively. Results were expressed as means ± SEM. A P value <0.05 was considered significant.

Acknowledgement

The authors wish to thank Drs S. Eyrich (ZAUM and Technische Universität, München, Germany) and L. Cifuentes (Department of Dermatology, Technische Universität, München, Germany) for their help.

REFERENCES

Angeli, V., D. Staumont, A.S. Charbonnier, H. Hammad, P. Gosset, M. Pichavant, B.N. Lambrecht, M. Capron, D. Dombrowicz, and F. Trottein. 2004. Activation of the D prostanoid receptor 1 regulates immune and skin allergic responses. *J Immunol* 172:3822-3829.

Bar-On, L., T. Birnberg, K.L. Lewis, B.T. Edelson, D. Bruder, K. Hildner, J. Buer, K.M. Murphy, B. Reizis, and S. Jung. 2010. CX3CR1+ CD8alpha+ dendritic cells are a steady-state population related to plasmacytoid dendritic cells. *Proc Natl Acad Sci U S A* 107:14745-14750.

Depner, M., M.S. Kormann, N. Klopp, T. Illig, C. Vogelberg, S.K. Weiland, E. von Mutius, C. Combadiere, and M. Kabesch. 2007. CX3CR1 polymorphisms are associated with atopy but not asthma in German children. *Int Arch Allergy Immunol* 144:91-94.

Echigo, T., M. Hasegawa, Y. Shimada, K. Takehara, and S. Sato. 2004. Expression of fractalkine and its receptor, CX3CR1, in atopic dermatitis: possible contribution to skin inflammation. *J Allergy Clin Immunol* 113:940-948.

El-Shazly, A., P. Berger, P.O. Girodet, O. Ousova, M. Fayon, J.M. Vernejoux, R. Marthan, and J.M. Tunon-de-Lara. 2006. Fraktalkine produced by airway smooth muscle cells contributes to mast cell recruitment in asthma. *J Immunol* 176:1860-1868.

Fraticelli, P., M. Sironi, G. Bianchi, D. D'Ambrosio, C. Albanesi, A. Stoppacciaro, M. Chieppa, P. Allavena, L. Ruco, G. Girolomoni, F. Sinigaglia, A. Vecchi, and A. Mantovani. 2001. Fractalkine (CX3CL1) as an amplification circuit of polarized Th1 responses. *J Clin Invest* 107:1173-1181.

Geissmann, F., P. Revy, N. Brousse, Y. Lepelletier, C. Folli, A. Durandy, P. Chambon, and M. Dy. 2003. Retinoids regulate survival and antigen presentation by immature dendritic cells. *J Exp Med* 198:623-634.

Grewe, M., C.A. Bruijnzeel-Koomen, E. Schopf, T. Thepen, A.G. Langeveld-Wildschut, T. Ruzicka, and J. Krutmann. 1998. A role for Th1 and Th2 cells in the immunopathogenesis of atopic dermatitis. *Immunol Today* 19:359-361.

Guttman-Yassky, E., K.E. Nograles, and J.G. Krueger. 2011a. Contrasting pathogenesis of atopic dermatitis and psoriasis--part I: clinical and pathologic concepts. *J Allergy Clin Immunol* 127:1110-1118.

Guttman-Yassky, E., K.E. Nograles, and J.G. Krueger. 2011b. Contrasting pathogenesis of atopic dermatitis and psoriasis--part II: immune cell subsets and therapeutic concepts. *J Allergy Clin Immunol* 127:1420-1432.

Hanifin, J.M., and G. Rajka. 1980. Diagnostic features of atopic dermatitis. *Acta Derm Venerol* Suppl 92:44-47.

Homey, B., H. Alenius, A. Muller, H. Soto, E.P. Bowman, W. Yuan, L. McEvoy, A.I. Lauerma, T. Assmann, E. Bunemann, M. Lehto, H. Wolff, D. Yen, H. Marxhausen, W. To, J. Sedgwick, T. Ruzicka, P. Lehmann, and A. Zlotnik. 2002. CCL27-CCR10 interactions regulate T cell-mediated skin inflammation. *Nat Med* 8:157-165.

Islam, S.A., D.S. Chang, R.A. Colvin, M.H. Byrne, M.L. McCully, B. Moser, S.A. Lira, I.F. Charo, and A.D. Luster. 2011. Mouse CCL8, a CCR8 agonist, promotes atopic dermatitis by recruiting IL-5+ T(H)2 cells. *Nat Immunol* 12:167-177.

Julia, V. 2012. CX3CL1 in allergic diseases: not just a chemotactic molecule. *Allergy* 67:1106-1110.

Jung, S., J. Aliberti, P. Graemmel, M.J. Sunshine, G.W. Kreutzberg, A. Sher, and D.R. Littman. 2000. Analysis of fractalkine receptor CX(3)CR1 function by targeted

deletion and green fluorescent protein reporter gene insertion. *Mol Cell Biol* 20:4106-4114.

Kagami, S., T. Kakinuma, H. Saeki, Y. Tsunemi, H. Fujita, K. Nakamura, T. Takekoshi, M. Kishimoto, H. Mitsui, H. Torii, M. Komine, A. Asahina, and K. Tamaki. 2003. Significant elevation of serum levels of eotaxin-3/CCL26, but not of eotaxin-2/CCL24, in patients with atopic dermatitis: serum eotaxin-3/CCL26 levels reflect the disease activity of atopic dermatitis. *Clin Exp Immunol* 134:309-313.

Kanda, A., V. Driss, N. Hornez, M. Abdallah, T. Roumier, G. Abboud, F. Legrand, D. Staumont-Salle, S. Queant, J. Bertout, S. Fleury, P. Remy, J.P. Papin, V. Julia, M. Capron, and D. Dombrowicz. 2009. Eosinophil-derived IFN-gamma induces airway hyperresponsiveness and lung inflammation in the absence of lymphocytes. *J Allergy Clin Immunol* 124:573-582, 582 e571-579.

Kim, K.W., A. Vallon-Eberhard, E. Zigmond, J. Farache, E. Shezen, G. Shakhar, A. Ludwig, S.A. Lira, and S. Jung. 2011. In vivo structure/function and expression analysis of the CX3C chemokine fractalkine. *Blood* 118:e156-167.

Landsman, L., L. Bar-On, A. Zernecke, K.W. Kim, R. Krauthgamer, E. Shagdarsuren, S.A. Lira, I.L. Weissman, C. Weber, and S. Jung. 2009. CX3CR1 is required for monocyte homeostasis and atherogenesis by promoting cell survival. *Blood* 113:963-972.

Lazarus, N.H., E.J. Kunkel, B. Johnston, E. Wilson, K.R. Youngman, and E.C. Butcher. 2003. A common mucosal chemokine (mucosae-associated epithelial chemokine/CCL28) selectively attracts IgA plasmablasts. *J Immunol* 170:3799-3805.

Leung, D.Y., M. Boguniewicz, M.D. Howell, I. Nomura, and Q.A. Hamid. 2004. New insights into atopic dermatitis. *J Clin Invest* 113:651-657.

Livak, K.J., and T.D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25:402-408.

Meucci, O., A. Fatatis, A.A. Simen, T.J. Bushell, P.W. Gray, and R.J. Miller. 1998. Chemokines regulate hippocampal neuronal signaling and gp120 neurotoxicity. *Proc Natl Acad Sci U S A* 95:14500-14505.

Mikhak, Z., J.P. Strassner, and A.D. Luster. 2013. Lung dendritic cells imprint T cell lung homing and promote lung immunity through the chemokine receptor CCR4. *J Exp Med* 210:1855-1869.

Mionnet, C., V. Buatois, A. Kanda, V. Milcent, S. Fleury, D. Lair, M. Langelot, Y. Lacoeuille, E. Hessel, R. Coffman, A. Magnan, D. Dombrowicz, N. Glaichenhaus, and V. Julia. 2010. CX3CR1 is required for airway inflammation by promoting T helper cell survival and maintenance in inflamed lung. *Nat Med* 16:1305-1312.

Nakayama, T., Y. Watanabe, N. Oiso, T. Higuchi, A. Shigeta, N. Mizuguchi, F. Katou, K. Hashimoto, A. Kawada, and O. Yoshie. 2010. Eotaxin-3/CC chemokine ligand 26 is a functional ligand for CX3CR1. *J Immunol* 185:6472-6479.

Owczarek, W., M. Paplinska, T. Targowski, K. Jahnz-Rozyk, E. Paluchowska, A. Kucharczyk, and B. Kasztalewicz. 2011. Analysis of eotaxin 1/CCL11, eotaxin 2/CCL24 and eotaxin 3/CCL26 expression in lesional and non-lesional skin of patients with atopic dermatitis. *Cytokine* 50:181-185.

Oyoshi, M.K., A. Elkhal, J.E. Scott, M.A. Wurbel, J.L. Hornick, J.J. Campbell, and R.S. Geha. 2011. Epicutaneous challenge of orally immunized mice redirects antigen-specific gut-homing T cells to the skin. *J Clin Invest* 121:2210-2220.

Reiss, Y., A.E. Proudfoot, C.A. Power, J.J. Campbell, and E.C. Butcher. 2001. CC chemokine receptor (CCR)4 and the CCR10 ligand cutaneous T cell-attracting chemokine (CTACK) in lymphocyte trafficking to inflamed skin. *J Exp Med* 194:1541-1547.

Schall, T.J., and A.E. Proudfoot. 2011. Overcoming hurdles in developing successful drugs targeting chemokine receptors. *Nat Rev Immunol* 11:355-363.

Spergel, J.M., E. Mizoguchi, J.P. Brewer, T.R. Martin, A.K. Bhan, and R.S. Geha. 1998. Epicutaneous sensitization with protein antigen induces localized allergic dermatitis and hyperresponsiveness to methacholine after single exposure to aerosolized antigen in mice. *J Clin Invest* 101:1614-1622.

Spergel, J.M., E. Mizoguchi, H. Oettgen, A.K. Bhan, and R.S. Geha. 1999. Roles of TH1 and TH2 cytokines in a murine model of allergic dermatitis. *J Clin Invest* 103:1103-1111.

Staumont-Salle, D., G. Abboud, C. Brenuchon, A. Kanda, T. Roumier, C. Lavogiez, S. Fleury, P. Remy, J.P. Papin, J. Bertrand-Michel, F. Terce, B. Staels, E. Delaporte, M. Capron, and D. Dombrowicz. 2008. Peroxisome proliferator-activated receptor alpha regulates skin inflammation and humoral response in atopic dermatitis. *J Allergy Clin Immunol* 121:962-968 e966.

Tremblay, K., M. Lemire, V. Provost, T. Pastinen, Y. Renaud, A.J. Sandford, M. Laviolette, T.J. Hudson, and C. Laprise. 2006. Association study between the CX3CR1 gene and asthma. *Genes Immun* 7:632-639.

van der Fits, L., S. Mourits, J.S. Voerman, M. Kant, L. Boon, J.D. Laman, F. Cornelissen, A.M. Mus, E. Florencia, E.P. Prens, and E. Lubberts. 2009. Imiquimod-induced psoriasis-like skin inflammation in mice is mediated via the IL-23/IL-17 axis. *J Immunol* 182:5836-5845.

Wang, Q., L. Malherbe, D. Zhang, K. Zingler, N. Glaichenhaus, and N. Killeen. 2001. CD4 promotes breadth in the TCR repertoire. *J Immunol* 167:4311-4320.

Zhang, X., S. Yu, K. Hoffmann, K. Yu, and R. Forster. 2012. Neonatal lymph node stromal cells drive myelodendritic lineage cells into a distinct population of CX3CR1+CD11b+F4/80+ regulatory macrophages in mice. *Blood* 119:3975-3986.

FIGURE LEGENDS

Figure 1. Absence of atopic dermatitis and attenuation of associated humoral and lung inflammatory response in CX_3CR1-deficient mice. AD was induced on abdominal skin in $CX_3CR1^{+/+}$ and $CX_3CR1^{gfp/gfp}$ mice by epicutaneous LACK sensitization for three 1-week periods, with a 2-week interval between applications. At day 49, sera were collected and animals were challenged by LACK nebulization. At day 50, AHR to increasing concentrations of methacholine was measured by invasive plethysmography. Then, mice were sacrificed and BAL fluid was collected and analyzed on cytospin preparations. Skin samples were collected at the site of sensitization. **A**. May Grünwald Giemsa staining of skin sections (original magnification X10 for the main panels and X63 for the inset). Black arrows indicate mast cells and, in the inset, eosinophils. **B.** Epidermal thickness. **C**. Eosinophil, mast cell, MHC II^+ and $CD4^+$ T cell numbers in dermis. **D.** Immunoglobulin concentrations in serum. Total IgE (upper left panel), -LACK-specific IgG_1 (bottom left panel) and LACK-specific IgG_{2a} (bottom right panel) concentrations. **E**. Airway hyperreactivity to increasing methacholine concentrations. Resistance was evaluated by invasive plethysmography. **F**. Lung inflammatory response: total number of cells, macrophages, neutrophils, lymphocytes and eosinophils in BAL fluid. Data are expressed as mean ± SEM (*n*=6-10 animals per group). One out of two independent experiments is shown for each panel. * $P < 0.05$; ** $p< 0.01$.

Figure 2. Abrogation of AD features in wild-type mice treated with CX3-AT. A. Timeline of CX3-AT administration during AD induction. **B.** Epidermal thickness. **C**. Eosinophil, mast cell, MHC II^+ and $CD4^+$ T cell numbers in dermis. **D**. Airway hyperreactivity to increasing methacholine concentrations. Resistance was evaluated by invasive plethysmography. **E**.

Lung inflammatory response: total number of cells, macrophages, neutrophils, lymphocytes and eosinophils in BAL fluid. Data are expressed as mean ± SEM (*n*=6-10 animals per group). One out of 2 independent experiments is shown for each panel. Statistically different from PBS-treated mice * $P < 0.05$ and ** $p< 0.01$; $ statistically different from vehicle-treated mice ($P < 0.05$).

Figure 3. CX_3CR1 expression regulates both Th1- and Th2- induced skin inflammation.

A. CSFE-labeled CX3CR1$^{+/+}$ WT15 Thy1.1$^{+/+}$ CD4^{+} T cells were injected i.v. into $CX_3CR1^{gfp/gfp}$ or $CX_3CR1^{+/gfp}$ mice one day before epicutaneous sensitization with LACK. Donor cells were analyzed by flow cytometry 5 days later in inguinal LN after gating onto CD4^{+} Thy1.1^{+} cells. Data show one representative flow cytometry profile of CFSE staining with numbers indicating frequencies of dividing donor cells ± SEM (*n*=6). **B.** CSFE-labeled $CX_3CR1^{+/gfp}$ Thy1.1$^{+/-}$ and $CX_3CR1^{gfp/gfp}$ Thy1.1$^{+/+}$ WT15 CD4^{+} T cells were co-injected i.v. into WT Thy1.1$^{-/-}$ Thy1.2$^{+/+}$ mice one day before LACK or PBS epicutaneous sensitization. Donor cells were analyzed by flow cytometry 4 days later in inguinal LN after gating CD4^{+} Thy1.1^{+} cells. Data show a representative flow cytometry profile of CFSE staining with numbers indicating dividing $CX_3CR1^{+/+}$ and $CX_3CR1^{gfp/gfp}$ donor cells (*n*=5 mice in each group). Wild-type mice were injected at day -1 with LACK-specific $CX_3CR1^{gfp/gfp}$ or $CX_3CR1^{+/gfp}$ Th1 or Th2 cells, sensitized for one single week with LACK or PBS at day 0 and further analyzed at day 7. **C**. Epidermal thickness. **D.** Eosinophil and mast cell numbers in dermis at the site of sensitization. Data are expressed as mean ± SEM (*n*=6-10 animals per group). One out of 2 independent experiments is shown for each panel. * $P < 0.05$.

Figure 4. CX_3CR1 provides a selective advantage to effector CD4^{+} T cells. Equal numbers of LACK-specific $CX_3CR1^{gfp/gfp}$ (black histograms or symbols) and $CX_3CR1^{+/gfp}$ (white

histograms or symbols) Th2 or Th1 cells were co-injected into WT mice at day 0. Recipients were sensitized with LACK or PBS at day 1 and day 4 and analyzed at day 3 (A,C,D) or day 7 (B,D). **A**. Donor cells were analyzed in skin by flow cytometry. Data show representative flow cytometry profiles (upper panels). Data show mean frequencies ± SEM of donor Th1 (bottom left panel) or Th2 (bottom right panel) cells among the $CD4^+$ T cell population. One representative experiment out of two is shown. (*n*=6 mice per group). **B.** Donor cells were analyzed in skin by flow cytometry (upper panels). Data show donor cell frequency of Th1 (bottom left panel) or Th2 (bottom right panel) in individual mice with bars indicating the mean from three experiments (*n*=16 mice per group). * $P < 0.05$. **C**. 18 h before sacrifice, recipient mice were injected with BrdU, and donor cells were analyzed by flow cytometry after staining with anti-BrdU, anti-Thy1.1, -Thy1.2, -CD4, and -CD45 antibodies. Data show cell frequency of donor Th1 or Th2 cells in individual mice with bars indicating the mean in LACK-sensitized (*n*=4 mice) and PBS-sensitized mice (*n*=2-3 mice). (One representative experiment out of two). **D**. Donor cells were analyzed in draining LN and skin by flow cytometry for GFP expression at days 3 (white bars) and 7 (grey bars). Data show representative flow cytometry profiles after aggregating files from individual mice (upper panels) (*n*=6 mice per group). One experiment out of three is shown. Histograms show mean frequencies ± SEM of GFP^+ cells among Th1 (left panels) or Th2 (right panels) donor cells (*n*=12 mice per group at day 3 and *n*=18 mice at day 7).

Figure 5. CX3CR1 is required for T cell retention in chronically inflamed skin.

A. Equal numbers of LACK-specific $CX_3CR1^{gfp/gfp}$ (black histograms) and $CX_3CR1^{+/gfp}$ (white histograms) Th2 or Th1 cells were co-injected into WT recipient mice at day 0. Recipients were sensitized with LACK at day 1 and day 4 and analyzed at day 3 and day 7. Donor cells were analyzed by flow cytometry 3 days later in inguinal draining LN after

staining with antibodies against CD45, CD4, Thy1.1, Thy1.2, and annexin-V and 7-AAD. Data show mean frequencies of annexin-V^+ cells among donor cells ± SEM. **B**. $CX_3CR1^{+/gfp}$ (white histograms) and $CX_3CR1^{gfp/gfp}$ (black histograms) Th2 cells were infected with a Bcl2 or an empty retroviral vector. Equal numbers of transduced cells were co-injected into recipients that were further sensitized with one round of LACK sensitization. Data show the mean of donor cell frequency in skin (left panel) and inguinal draining LN (right panel) of individual mice in one representative experiment out of two (*n*=5 mice per group). **C.** AD was induced in WT mice as described in the legend of Fig.1 and equal numbers of LACK-specific $CX_3CR1^{gfp/gfp}$ (green population) and $CX_3CR1^{+/gfp}$ (pink population) Th1 (upper panels) or Th2 (lower panels) cells were co-injected at day 41. Donor cells were analyzed in skin, draining LN and spleen by flow cytometry. Data show representative flow cytometry profiles. Numbers indicate frequencies ± SEM of donor Th1 (upper panels) or Th2 (bottom panels) cells among the $CD4^+$ T cell population. One representative experiment out of two is shown (*n*=12 mice per group). 18 h before sacrifice, recipient mice were injected with BrdU and donor cells were analyzed by flow cytometry after staining with anti-BrdU, -Thy1.1, -Thy1.2, -CD4 or -CD45 antibodies. Data show representative cytometry profiles and numbers indicate the mean (*n*=12 mice) (One representative experiment out of two). **D.** Equal numbers of LACK-specific $CX_3CR1^{gfp/gfp}$ (black histograms) and $CX_3CR1^{+/gfp}$ (white histograms) effector cells were co-injected into WT recipient mice at day 0. Recipients were sensitized with LACK at day 1 and day 4. At day 4, some recipient mice topically received LACK together with CX3-AT. Three days later, the frequencies of donor cells were analyzed in skin and draining LN by flow cytometry. Data show mean of donor cell frequency of individual mice in one representative experiment out of two (*n*=5 mice per group). * $P < 0.05$). **E-G.** AD was induced as described in the legend of Fig.1. During the last round of sensitization, some mice also received CX3-AT (50 μg/mouse) through the patch together with LACK as indicated on

the figure. **E**. May Grünwald Giemsa staining of skin sections (original magnification X10). **F.** Epidermal thickness. **G**. Mast cell and eosinophil numbers. Data for panels E-G show mean of cell numbers in one representative experiment out of two (*n*=6 mice per group). * P < 0.05. **H-J** $CX_3CR1^{gfp/gfp}$ mice were injected with polyclonal naïve WT or $CX_3CR1^{gfp/gfp}$ (KO) $CD4^+$ T cells 24h prior to AD induction. During the last round of sensitization, mice also received CX3-AT (50 µg/mouse) through the patch together with LACK as indicated on the figure. **H.** Epidermal thickness. **I-J**. Mast cell and eosinophil numbers. Data for panels H-J show mean of cell numbers in one representative experiment (*n*=5 mice per group). * P < 0.05; n.s. Not significant.

Figure 6: **CX3CR1 is expressed by skin-infiltrating $CD4^+$ T cells in AD patients.**

A. CX3CR1 expression by circulating $CD4^+$ T cells. PBMC were from patients with AD or from healthy donors were analyzed by flow cytometry for CX3CR1 expression. Data show frequency of $CX3CR1^+$ cells among $CD4^+$ T cells in individual donor. n.s. Not significant. B. Cells from skin biopsies were characterized by surface and intracellular staining by flow cytometry. Left panel show a representative FACS profile. Numbers indicate the mean ± SEM of the frequency of $CX3CR1^+$ among $CD4^+$ T cells from n=6 patients. Right panels show frequencies of cytokine-secreting $CX3CR1^+$ and $CX3CR1^-$ $CD4^+$ T cells for each patient.