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

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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, whereas 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 affected neither 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 experiments established CX_3CR1 as a key regulator of $CD4^+$ T cell retention in inflamed skin, indicating a new function for this chemokine receptor. Therefore, although CX_3CR1 and CX_3CL1 act through distinct mechanisms in different pathologies, our results further indicate their interest as promising therapeutic targets in allergic diseases.

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Abbreviations used: 7-AAD, 7-aminoactinomycin D; AD, atopic dermatitis; AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage fluid; LACK, *Leishmania major*–activated C kinase.

Atopic dermatitis (AD) is a common, chronic inflammatory dermatosis that frequently occurs 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,b). 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); allergic asthma or allergic rhinitis are more exclusively Th2-dominated diseases.

S. Fleury and A. Lazzari contributed equally to this paper.

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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, and -26) bind to CCR3 and attract eosinophils, and CCL26 appears to be particularly involved in AD (Kagami et al., 2003; Owczarek et al., 2010). CCL27 together with CCR10 and CCR4 expression ensures T cell skin domiciliation (Reiss et al., 2001; Homey et al., 2002). More recently, CCR8 and CCL8 have been elegantly demonstrated to direct Th2 cell recruitment into allergen-inflamed skin and draining LNs 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 (Mionnet et al., 2010; Julia, 2012). In AD patients, CX_3CL1 is up-regulated 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, although CX_3CR1 mRNA expression is consistently up-regulated 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, 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 AD 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), with that from their proficient ($^{+/+}$) WT counterparts. Neither strain exhibited an inflammatory phenotype in the absence of LACK sensitization (Fig. 1 A). Compared with 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. 1 B) associated with more pronounced hyperkeratosis, spongiosis, and dermal cellular infiltrates, including mast cells, eosinophils, MHC-II^+, and $CD4^+$ T cells (Fig. 1 C), as well as increased skin and inguinal LN expression of inflammatory and Th1- and Th2-associated cytokines, chemokines, and chemokine receptors (not depicted). In sharp contrast, $CX_3CR1^{gfp/gfp}$ mice did not develop a skin inflammatory response upon LACK sensitization (Fig. 1, A and 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. 1 C). Furthermore, expression of Th1 and inflammatory response genes was also significantly decreased (not depicted). Humoral response was also altered in $CX_3CR1^{gfp/gfp}$ compared with $CX_3CR1^{+/+}$ 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. 1 D).

As in the human pathology, epicutaneous sensitization also induced lung inflammation and airway hyperresponsiveness (AHR) after a single antigenic airway challenge. Airway resistance upon LACK sensitization was significantly lower in $CX_3CR1^{gfp/gfp}$ compared with $CX_3CR1^{+/+}$ animals (Fig. 1 E). Furthermore, cellular inflammatory infiltrates in the bronchoalveolar lavage 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 with BALF from $CX_3CR1^{+/+}$ mice (Fig. 1 F).

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. 2 A). Both administration schedules fully inhibited antigen-induced epidermal thickening (Fig. 2 B), as well as dermal mast cell, eosinophil, and $CD4^+$ T cell infiltration (Fig. 2 C). Upon LACK aerosol challenge, AHR and inflammatory cell infiltration in the airways were also significantly decreased upon both prophylactic and therapeutic treatments (Fig. 2, C and D). Collectively, these results confirm the key role of CX_3CR1–CX_3CL1 in AD in nongenetically 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 naive T cell proliferation but regulates both Th1- and Th2-induced skin inflammation

As CX_3CR1 is expressed by various myeloid cells, such as blood monocytes, DC progenitors, plasmacytoid DCs, 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 into 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

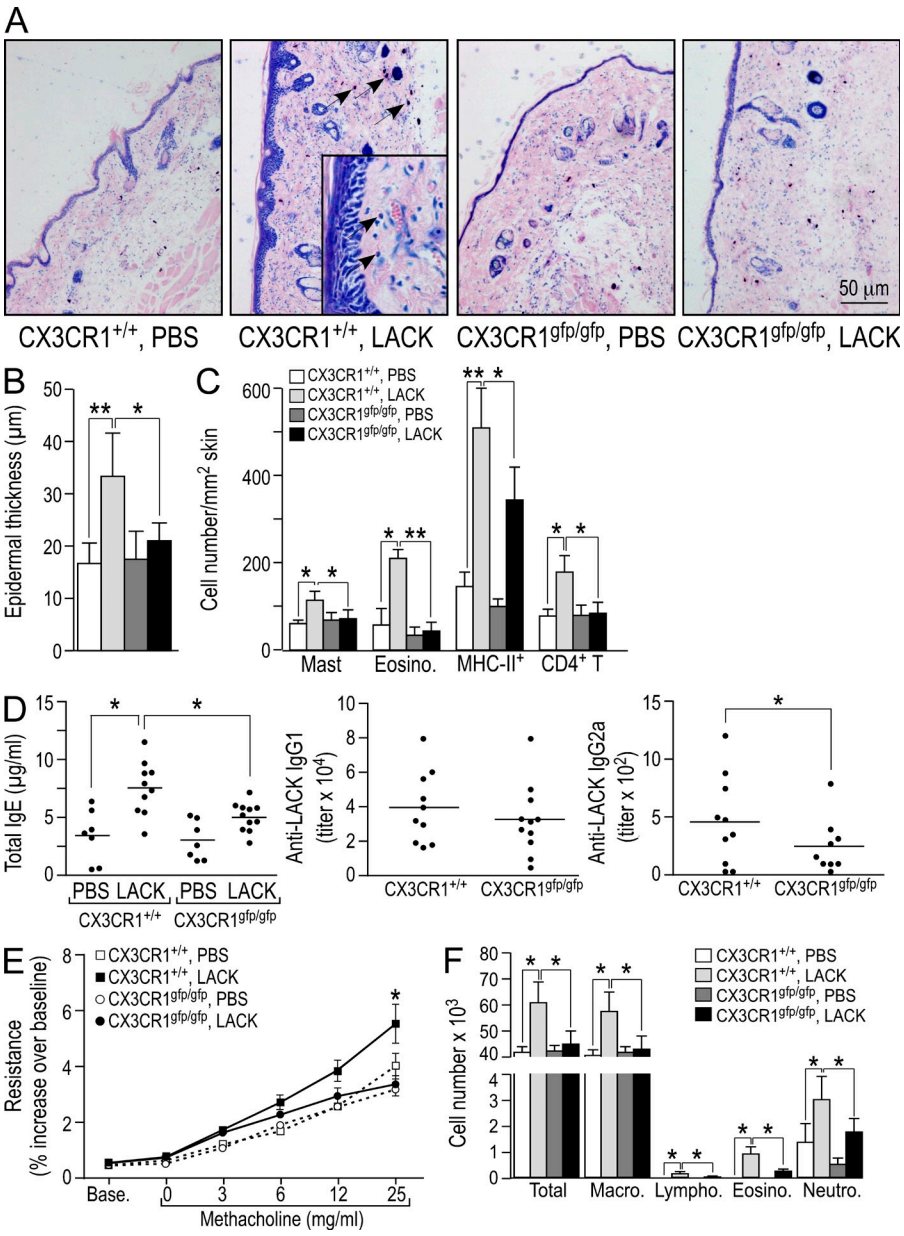

Figure 1. Absence of AD and attenuation of associated humoral and lung inflammatory response in CX_3CR1-deficient mice. AD was induced on abdominal skin in $CX_3CR1^{+/+}$ and $CX_3CR1^{gfp/gfp}$ mice by epicutaneous LACK sensitization for three 1-wk periods, with a 2-wk interval between applications. 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 BALF was collected and analyzed on cytospin preparations. Skin samples were collected at the site of sensitization. (A) May-Grünwald Giemsa staining of skin sections. 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) Ig concentrations in serum. Total IgE (left), LACK-specific IgG_1 (middle), and LACK-specific IgG_{2a} (right) concentrations are shown. Horizontal bars indicate mean. (E) AHR to increasing methacholine concentrations. Resistance was evaluated by invasive plethysmography. (F) Lung inflammatory response: total number of cells, macrophages, neutrophils, lymphocytes, and eosinophils in BALF. Data are expressed as mean ± SEM (n = 6–10 animals per group). One out of two independent experiments is shown for each panel. *, $P < 0.05$; ** $P < 0.01$.

comparable in both $CX_3CR1^{+/gfp}$ and $CX_3CR1^{gfp/gfp}$ mice (Fig. 3 A), 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 coinjected 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. 3 B). Altogether these results suggest that CX_3CR1 deficiency does not alter naive T cell proliferation.

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

As both Th2 and Th1 cells are associated with the acute and chronic phases of AD, respectively (Spergel et al., 1999), we investigated whether CX_3CR1 expression was required by Th1 or 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. Although both CX_3CR1-proficient Th1 and Th2 cells alone were able to induce a four- to fivefold epidermal thickening upon a single round of epicutaneous antigen exposure, CX_3CR1-deficient Th2 cells only induced a less than twofold increase in epidermal thickness, and injection of CX_3CR1-deficient Th1 cells

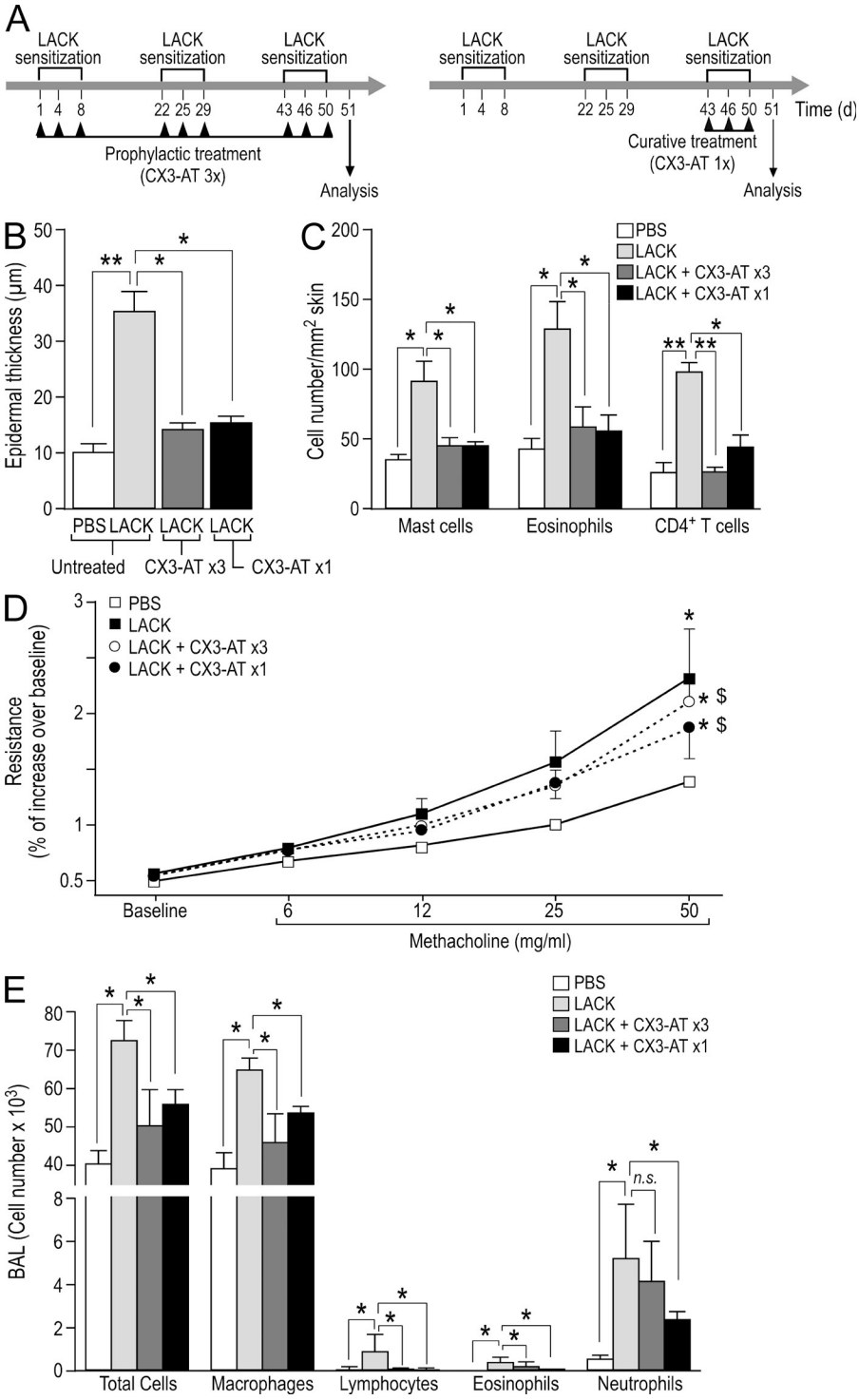

Figure 2. Abrogation of AD features in WT mice treated with CX_3-AT. (A) Timeline of CX_3-AT administration during AD induction. (B) Epidermal thickness. (C) Eosinophil, mast cell, MHC II$^+$, and CD4$^+$ T cell numbers in dermis. (D) AHR to increasing methacholine concentrations. Resistance was evaluated by invasive plethysmography. (E) Lung inflammatory response: total number of cells, macrophages, neutrophils, lymphocytes, and eosinophils in BALF. Data are expressed as mean ± SEM (n = 6–10 animals per group). One out of two 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: $, $P < 0.05$.

barely led to epidermal thickening compared with PBS-treated animals (Fig. 3 C). 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. 3 D). 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. 3 D). Collectively, 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

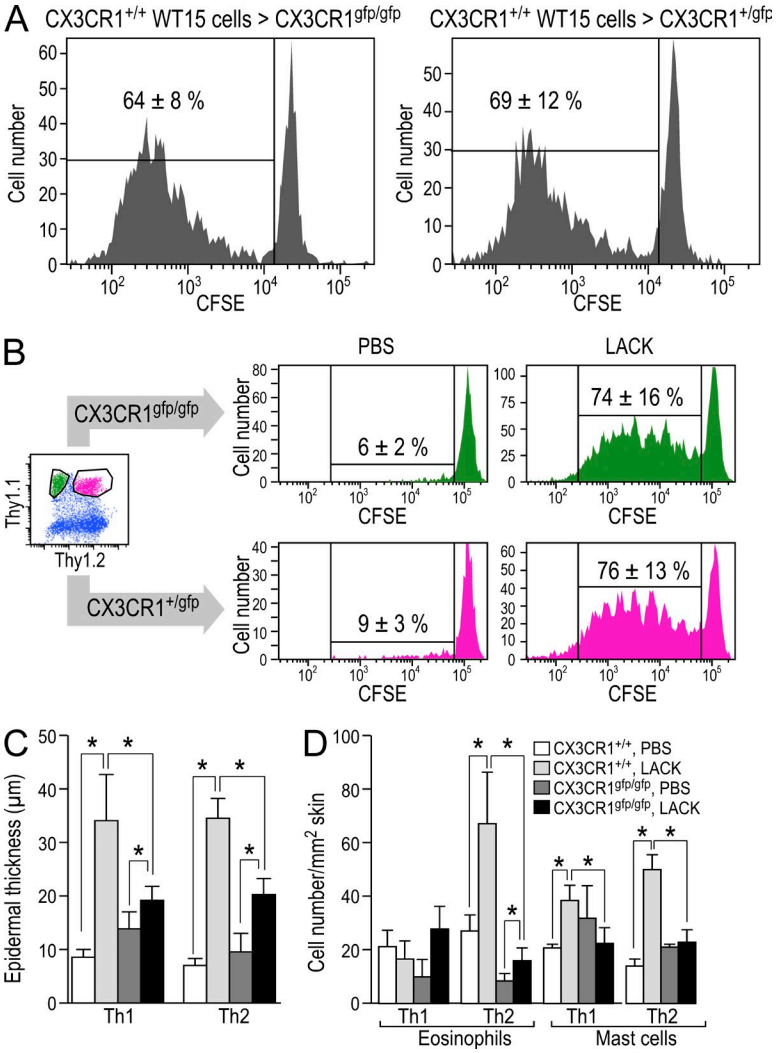

Figure 3. CX_3CR1 expression regulates both Th1- and Th2-induced skin inflammation. (A) CSFE-labeled $CX_3CR1^{+/+}$ WT15 Thy1.1$^{+/+}$ CD4$^+$ T cells were injected i.v. into $CX_3CR1^{gfp/gfp}$ or $CX_3CR1^{+/gfp}$ mice 1 d before epicutaneous sensitization with LACK. Donor cells were analyzed by flow cytometry 5 d later in inguinal LNs 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 coinjected i.v. into WT Thy1.1$^{-/-}$ Thy1.2$^{+/+}$ mice 1 d before LACK or PBS epicutaneous sensitization. Donor cells were analyzed by flow cytometry 4 d later in inguinal LNs 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). (C and D) WT 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 two independent experiments is shown for each panel. *, $P < 0.05$.

both CX_3CR1-proficient and -deficient, LACK-specific Th1 and Th2 cells upon coinjection into WT mice that were exposed to LACK and further fed with BrdU. 3 d after antigen exposure, although Th1 and Th2 donor cells of both genotypes had not yet incorporated BrdU (not depicted), CX_3CR1-proficient and -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. 4 A). 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 d after antigen exposure. In sharp contrast, frequencies of CX_3CR1-proficient donor cells outnumbered CX_3CR1-deficient cells on day 7 (Fig. 4 B). However, donor cells of both genotypes proliferated at the same rate (Fig. 4 C). Therefore, as observed for naive 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 (Jung et al., 2000; Geissmann et al., 2003). Upon differentiation, antigen-specific effector cells remained GFP$^-$ as previously described (Mionnet et al., 2010). At day 3 after initial sensitization, <0.3% of antigen-specific effector cells expressed CX_3CR1 (i.e., GFP) in draining LNs, and around 4% of these cells expressed CX_3CR1 (i.e., GFP) in skin (Fig. 4 D). At day 7, although 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. 4 D). 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

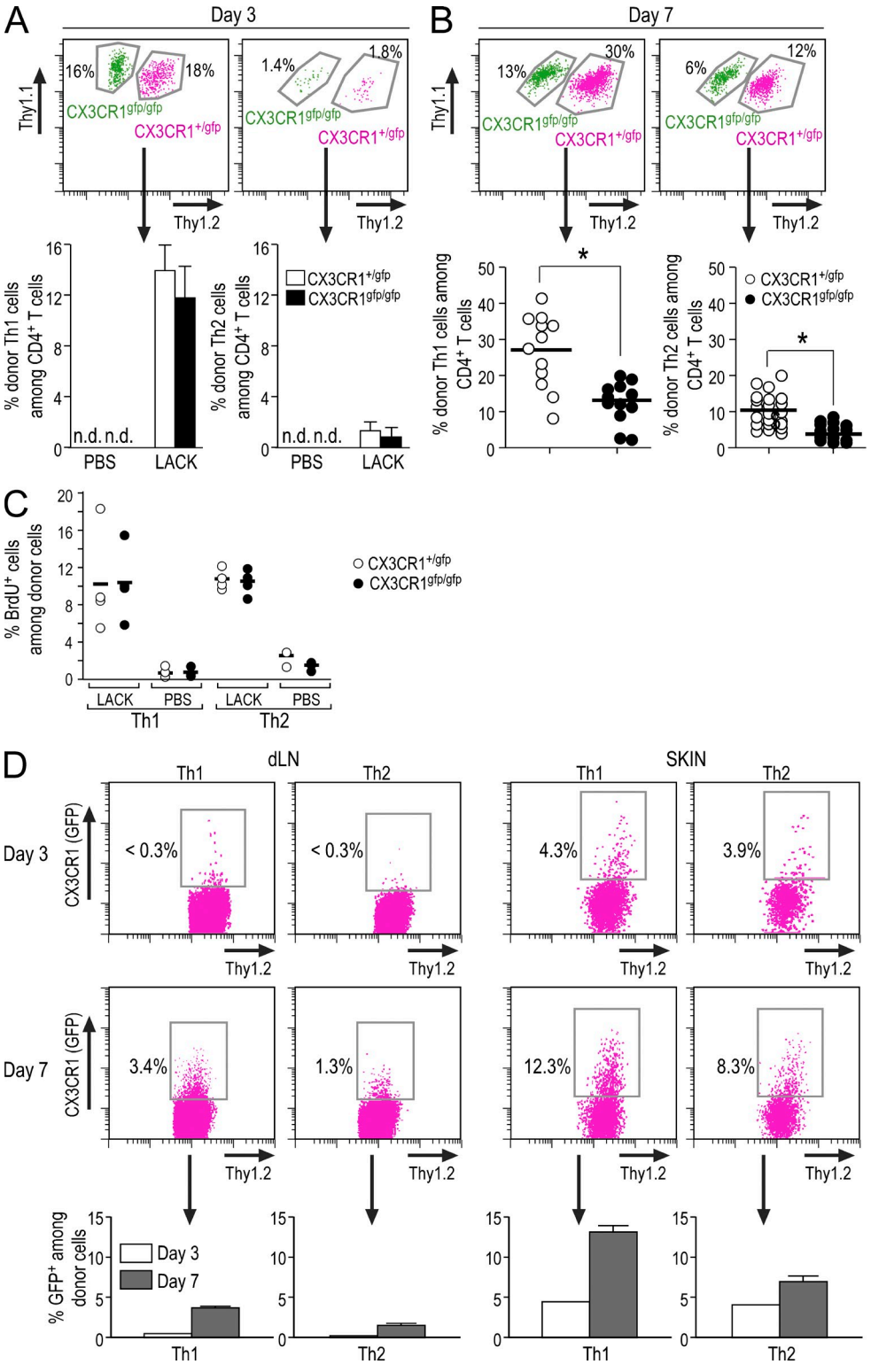

Figure 4. CX_3CR1 provides a selective advantage to effector CD4⁺ T cells. Equal numbers of LACK-specific $CX_3CR1^{gfp/gfp}$ and $CX_3CR1^{+/gfp}$ Th2 or Th1 cells were coinjected into WT mice at day 0. Recipients were sensitized with LACK or PBS at days 1 and 4 and analyzed at day 3 (A, C, and D) or 7 (B and D). (A) Donor cells were analyzed in skin by flow cytometry. Data show representative flow cytometry profiles (top). Data show mean frequencies ± SEM of donor Th1 (bottom left) or Th2 (bottom right) 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 (top). Data show donor cell frequency of Th1 (bottom left) or Th2 (bottom right) in individual mice with horizontal bars indicating the mean from three experiments ($n = 16$ mice per group). *, $P < 0.05$. (C) 18 h before sacrifice, recipient

this was also the case in inflamed skin. WT mice were coinjected with CX_3CR1-proficient and -deficient, antigen-specific Th1 or Th2 donor cells and 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-aminoactinomycin D^+ (7-AAD^+) $CX_3CR1^{+/gfp}$ and $CX_3CR1^{gfp/gfp}$ donor cells were found in both skin and draining LNs (Fig. 5 A and not depicted), suggesting that CX_3CR1 is not involved in T cell survival. However, as Th2 donor cells were difficult to monitor in skin because of 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 antiapoptotic BCL-2 (an empty vector was used as control) and transferred into WT recipients that were sensitized by LACK. Although overexpression of BCL-2 led to a decrease and, respectively, an increase in the recovery of transduced cells in the skin and draining LNs, 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. 5 B).

We next compared the migration of CX_3CR1-proficient and -deficient effector T cells into the inflamed skin and draining LNs of WT mice with established AD. Recipient mice were submitted to two rounds of epicutaneous sensitization before the injection of CX_3CR1-proficient and -deficient Th1 or Th2 cells the day before the third and last antigen application. 3 d later, although BrdU incorporation was very low, frequencies of donor cells of both genotypes were similar (Fig. 5 C), 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 coinjected with CX_3CR1-proficient and -deficient, LACK-specific effector T cells, challenged for 4 d with LACK, and further challenged with the antigen while being topically treated with CX_3-AT for 3 d. Upon topical CX_3-AT treatment, frequency of skin CX_3CR1-proficient T cells decreased to the level of coinjected CX_3CR1-deficient cells, whereas their frequency increased in draining LNs (Fig. 5 D), 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 d 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. 5, E–G). Finally, to further confirm that the effect of CX_3CR1 in AD was caused by its expression on T cells, we adoptively transferred CX_3CR1-proficient or -deficient naive T cells into CX_3CR1-deficient animals and induced AD. After 7 wk, animals reconstituted with CX_3CR1-proficient T cells developed a pathology (epidermal thickening, mast cell and eosinophil infiltration) that was comparable with that of CX_3CR1-proficient animals. Reconstitution with CX_3CR1-deficient T cells did not induce symptoms of AD (Fig. 5, H–J). Furthermore, topical application of CX_3-AT during the last 3 d 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 a similar effect as on (nonreconstituted) CX_3CR1-proficient animals (Fig. 5, H–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

To assess the human relevance of our observations, we investigated whether CX_3CR1^+ $CD4^+$ 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 cells from AD patients. In addition, no significant differences were found between healthy individuals and AD and psoriasis patients in agreement with previously published work (Fig. 6 A; Echigo et al., 2004). About 7% of skin-isolated $CD4^+$ T cells expressed CX_3CR1, in keeping with our findings in the murine model of AD. As CX_3CR1^- cells, CX_3CR1^+ $CD4^+$ T cells display a very heterogeneous cytokine profile, with the Th1 and Th2 subsets being the most represented as compared with Th17 (Fig. 6 B). Collectively, 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 caused by 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_3-AT 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. 3 A and not

mice were injected with BrdU, and donor cells were analyzed by flow cytometry after staining with anti-BrdU, anti-Thy1.1, anti-Thy1.2, anti-CD4, and anti-CD45 antibodies. Data show cell frequency of donor Th1 or Th2 cells in individual mice with horizontal bars indicating the mean in LACK-sensitized (n = 4 mice) and PBS-sensitized mice (n = 2–3 mice). One representative experiment out of two is shown. (D) Donor cells were analyzed in draining LNs (dLN) and skin by flow cytometry for GFP expression at days 3 and 7. Data show representative flow cytometry profiles after aggregating files from individual mice (top; n = 6 mice per group). One experiment out of three is shown. Histograms show mean frequencies ± SEM of GFP^+ cells among Th1 (left) or Th2 (right) donor cells (n = 12 mice per group at day 3 and n = 18 mice at day 7).

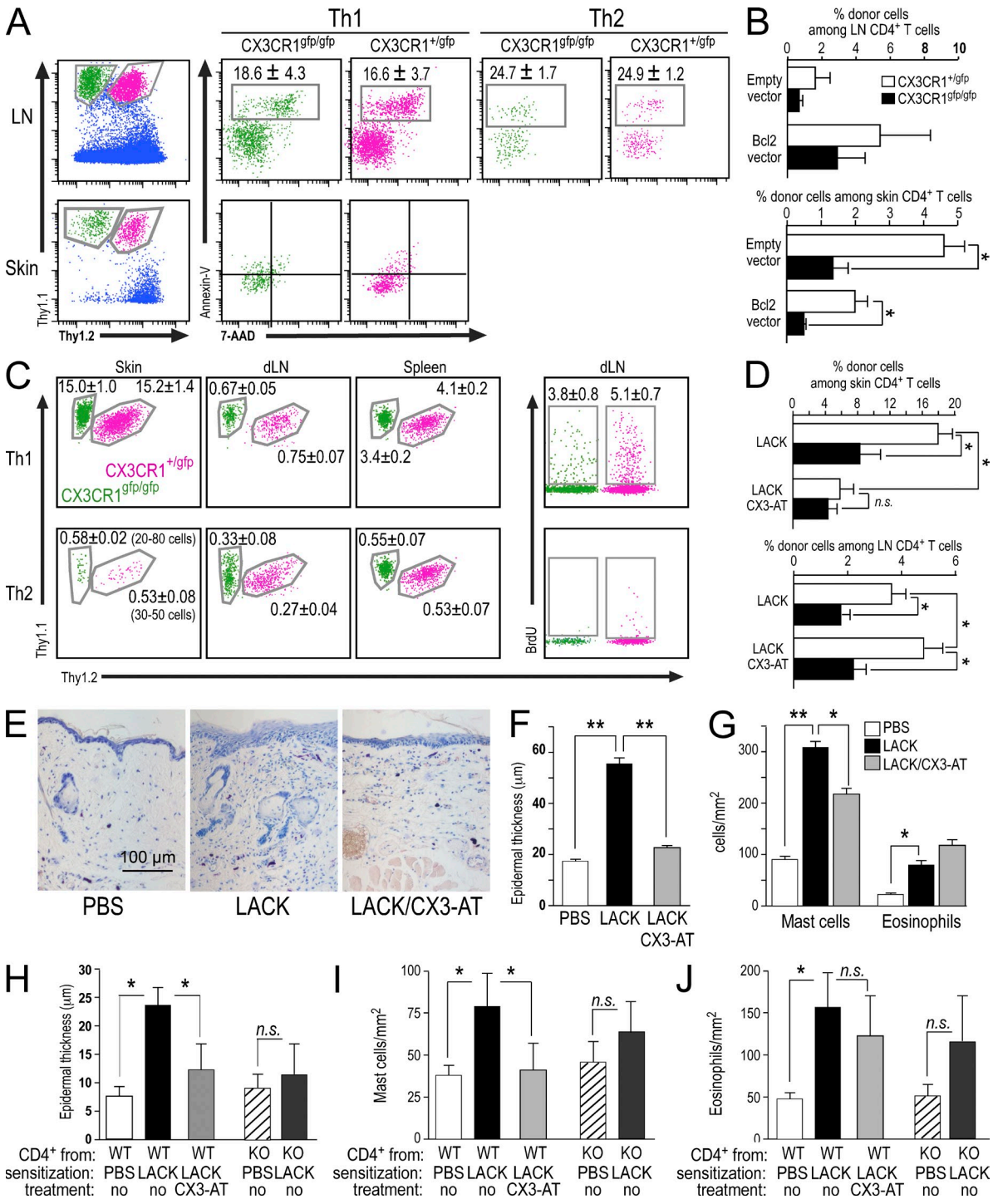

Figure 5. CX_3CR1 is required for T cell retention in chronically inflamed skin. (A) Equal numbers of LACK-specific $CX_3CR1^{gfp/gfp}$ and $CX_3CR1^{+/gfp}$ Th2 or Th1 cells were coinjected into WT recipient mice at day 0. Recipients were sensitized with LACK at days 1 and 4 and analyzed at days 3 and 7. Donor cells were analyzed by flow cytometry 3 d later in inguinal draining LNs (dLN) after staining with antibodies against CD45, CD4, Thy1.1, Thy1.2, annexin-V, and 7-AAD. Data show mean frequencies of annexin-V+ cells among donor cells ± SEM. (B) $CX_3CR1^{+/gfp}$ and $CX_3CR1^{gfp/gfp}$ Th2 cells were infected with a Bcl2 or an empty retroviral vector. Equal numbers of transduced cells were coinjected into recipients that were further sensitized with one round of LACK sensitization. Data show the mean of donor cell frequency in skin (bottom) and inguinal draining LN (top) of individual mice in one representative experiment out of two (n = 5 mice per group). *, $P < 0.05$. (C) AD was induced in WT mice as described in the legend of Fig. 1, and equal numbers of LACK-specific $CX_3CR1^{gfp/gfp}$ and $CX_3CR1^{+/gfp}$ Th1 (top) or Th2 (bottom) cells were coinjected 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 (top) or Th2 (bottom) cells among the CD4+ T cell population. One representative experiment out of two is shown (n = 12 mice per group). (right) 18 h

depicted). 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, proliferation, early or late migration, and survival were not affected by CX_3CR1 deficiency on $CD4^+$ effector T cells, whereas blocking CX_3CR1–CX_3CL1 interactions in situ prevented WT allergen-specific T cell accumulation in skin and induced their accumulation in draining LNs (Fig. 5 D). Interestingly, upon such treatment, although 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 which might suggest that residence in 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 DCs promote 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 ensure lymphocyte circulation or recirculation 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 toward the skin and not by facilitating their retention in the inflammatory sites. Indeed, Islam et al. (2011) demonstrated that CCL8 is involved in skin homing of CCR8-expressing Th2 cells in the same animal model of AD. Likewise, Homey et al. (2002) 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. Although 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 study demonstrating such a role in vivo. However, it remains to be determined which cell types interact with $CD4^+$ T cells to retain them within the skin and why/how the CX_3CR1 pathway plays a different role in the skin compared with 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. Although in skin CX_3CL1 is expressed in epithelial cells and endothelium, as well as in the lesional skin of AD patients, in asthmatic lungs it is also highly up-regulated 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 (unpublished data).

The particular importance of CX_3CR1 in the development of AD, compared with 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.

CX_3CR1 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; unpublished data). Likewise, an earlier study has 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_3-AT further demonstrates that the CX_3CR1–CX_3CL1 axis represents a new promising therapeutic target in allergic inflammatory diseases like allergic asthma and AD.

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

before sacrifice, recipient mice were injected with BrdU and donor cells were analyzed by flow cytometry after staining with anti-BrdU, anti-Thy1.1, anti-Thy1.2, anti-CD4, or anti-CD45 antibodies. Data show representative cytometry profiles, and numbers indicate the mean (n = 12 mice). One representative experiment out of two is shown. (D) Equal numbers of LACK-specific $CX_3CR1^{gfp/gfp}$ and $CX_3CR1^{+/gfp}$ effector cells were coinjected into WT recipient mice at day 0. Recipients were sensitized with LACK at days 1 and 4. At day 4, some recipient mice topically received LACK together with CX_3-AT. 3 d later, the frequencies of donor cells were analyzed in skin and draining LNs 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 CX_3-AT (50 µg/mouse) through the patch together with LACK as indicated in the figure. (E) May-Grünwald Giemsa staining of skin sections. (F) Epidermal thickness. (G) Mast cell and eosinophil numbers. Data for 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 naive WT or $CX_3CR1^{gfp/gfp}$ (KO) $CD4^+$ T cells 24 h before AD induction. During the last round of sensitization, mice also received CX_3-AT (50 µg/mouse) through the patch together with LACK as indicated in the figure. (H) Epidermal thickness. (I and J) Mast cell and eosinophil numbers. Data for H–J show mean of cell numbers in one representative experiment (n = 5 mice per group). *, $P < 0.05$. Error bars indicate SEM.

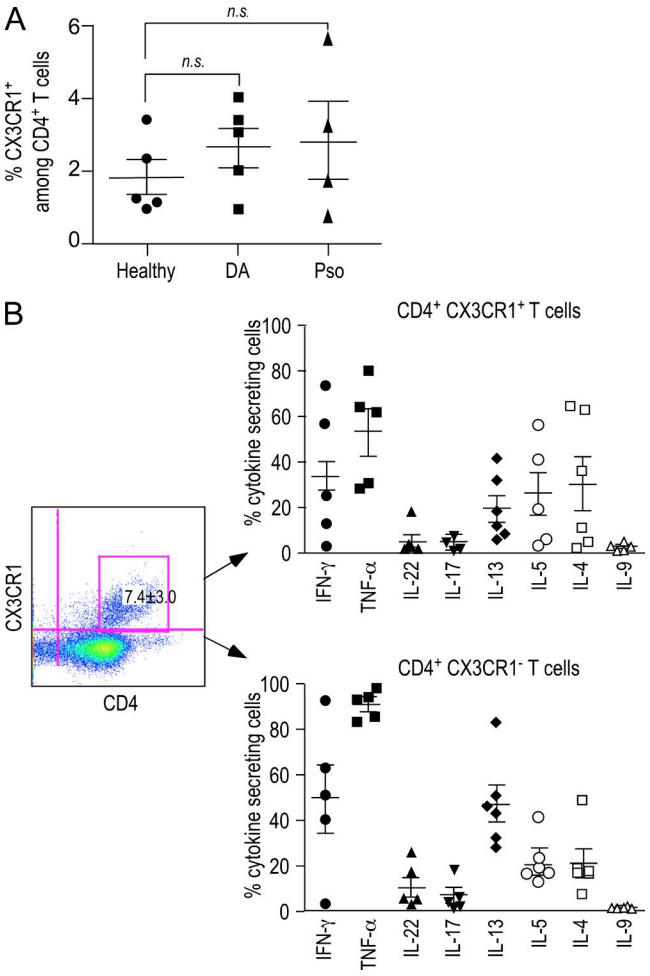

Figure 6. CX_3CR1 is expressed by skin-infiltrating CD4+ T cells in AD patients. (A) CX_3CR1 expression by circulating CD4+ T cells. PBMCs from patients with AD or from healthy donors were analyzed by flow cytometry for CX_3CR1 expression. Data show frequency of CX_3CR1^+ cells among CD4+ T cells in individual donors. (B) Cells from skin biopsies were characterized by surface and intracellular staining by flow cytometry. Left panel shows a representative FACS profile. Numbers indicate the mean ± SEM of the frequency of CX_3CR1^+ among CD4+ T cells from $n = 6$ patients. Right panels show frequencies of cytokine-secreting CX_3CR1^+ and CX_3CR1^- CD4+ T cells for each patient. Error bars indicate SEM.

BALB/c ByJ background (12 backcrosses) as previously described (Mionnet et al., 2010). 8–12-wk-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 AD. As previously described, AD was induced by epicutaneous sensitization (Staumont-Sallé et al., 2008) with LACK antigen (Spergel et al., 1998). In brief, patches soaked with 25 µl of 0.2% LACK solution in PBS or with vehicle were applied on abdominal skin 24 h after shaving and were left on for three 1-wk periods (with patch renewal at midweek), with a 2-wk interval between applications. At the time of the last patch removal (day 49), animals were challenged for 20 min by means of aerosol nebulization with LACK (0.2% in PBS) by using an ultrasonic nebulizer (Syst'am), and serum was collected. On the next day, 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. BALF was collected and analyzed on cytospin preparations after RAL 555 staining. Skin and inguinal LNs 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 (Thermo Fisher Scientific) were below 5 ng/mg of protein.

Treatment with a CX_3CL1 antagonist (CX_3-AT) in a model of AD. CX_3-AT blocking reagent was prepared as described previously (Mionnet et al., 2010) and administered by weekly i.p. injections (50 µg/mouse) either during the three 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 d 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-Sallé et al., 2008). In brief, samples were fixed in ImmunoHistoFix (Interstiles) and embedded in ImmunoHistoWax (Interstiles) at 37°C. 5-µm sections were stained with May-Grünwald Giemsa for measurement of epidermal thickness and eosinophil and mast cells counts (Staumont-Sallé et al., 2008) by using a microscope with Arcturus XT software. Epidermal thickness was determined at 250-fold magnification; a mean 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), and cryopreserved sections were stained with anti-CD4 mAb (clone RM4-5, rat IgG2a; BD) as previously described (Angeli et al., 2004). For each section, 10 random fields were examined at 400×.

Ig concentrations. Ig (IgE, IgG_1, and IgG_{2a}) concentrations in serum were measured by ELISA as previously described (Staumont-Sallé et al., 2008). Twofold serial dilutions were prepared for each serum (starting dilution 1:25 for IgE, 1:5,000 for IgG_1, and 1:1,000 for IgG2a). Antibody titers were calculated as the dilution corresponding to twice the mean absorbance value obtained for nonsensitized mouse sera.

Murine cell purification and in vivo assays. For the antigen-presenting capacity assay, splenic CD4+ T cells were purified from $CX_3CR1^{+/+}$ WT15 $Thy1.1^{+/+}$ $Rag\text{-}2^{-/-}$ TCR transgenic mice, encoding a LACK-specific TCR, by positive selection using anti-CD4 beads (MACS, purity >95%; Miltenyi Biotec), stained with CFSE (Sigma-Aldrich), and further injected (2×10^6 cells) via the lateral tail vein into $CX_3CR1^{gfp/gfp}$ and $CX_3CR1^{+/gfp}$ recipient mice 1 d before epicutaneous LACK sensitization. Proliferation of donor cells in the inguinal LN was assessed by flow cytometry 5 d 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 coinjected (2×10^6 of each population) into $Thy1.1^{-/-}$ $CX_3CR1^{+/+}$ WT mice 1 d before epicutaneous LACK sensitization. Proliferation was assessed in the inguinal LN 4 d later by flow cytometry.

For preparation of Th1 and Th2 cells, CD4+ T cells from TCR transgenic mice were purified by negative selection and 1.5×10^6 cells were incubated for 3 d with 0.75×10^6 T cell–depleted splenocytes in complete RPMI with 50 nM $LACK_{156–173}$ peptide and (a) 10 ng/ml r-IL-4 and 10 µg/ml antibody to IFN-γ (R4-6A2) for Th2 cells or (b) 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 (2×10^6 cells per mouse) by i.v. 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 coinjected in $Thy1.1^{-/-}$ $Thy1.2^{+/+}$ WT mice (1.5×10^6 of each genotype per mouse) 1 d before LACK epicutaneous sensitization. When indicated, recipient mice treated as described above were injected i.p. with 200 µg BrdU (BD) for the last 18 h. BrdU incorporation of CX_3CR1-proficient and -deficient Th1 and Th2 donor cells was studied by flow cytometry in inguinal LNs. Survival of Th1 and Th2 donor cells was analyzed by annexin-V and 7-AAD staining in both skin and inguinal LNs. In reconstitution experiments, 5×10^6 naive $CX_3CR1^{gfp/gfp}$ or $CX_3CR1^{+/gfp}$ $CD4^+$ T cells were adoptively transferred into $CX_3CR1^{gfp/gfp}$ mice 24 h before 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). PBMCs 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-Aldrich), and 20 U IL-2/ml (Novartis). After 10–13 d, 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) according to the manufacturer's instructions. The following fluorochrome-conjugated antibodies were used: anti–IFN-γ–V450 (B27), anti-TNF–Alexa Fluor 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), anti-IL4-PE (3010.211; BD), anti-IL22-APC (142928; R&D Systems), and CX_3CR1-FITC (2A9-1; BioLegend).

Retroviral transduction. The human Bcl2 cDNA was cloned into the mouse bicistronic retroviral expression vector MIGR. Th2-differentiated cells were incubated with viral supernatant containing 5 µg/ml Polybrene (Sigma-Aldrich) and spun at 32°C for 8 h, as described previously (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 the Student's *t* test, except for plethysmographic data, for which ANOVA for repeated measures was used. GraphPad and STATview softwares were used, respectively. Results are expressed as means ± SEM. A p-value < 0.05 was considered significant.

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