

CX3CR1 is required for airway inflammation by promoting T helper cell survival and maintenance in inflamed lung

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Allergic asthma is a T helper type 2 (T_H2)-dominated disease of the lung. In people with asthma, a fraction of CD4⁺ T cells express the CX3CL1 receptor, CX3CR1, and CX3CL1 expression is increased in airway smooth muscle, lung endothelium and epithelium upon allergen challenge. Here we found that untreated CX3CR1-deficient mice or wild-type (WT) mice treated with CX3CR1-blocking reagents show reduced lung disease upon allergen sensitization and challenge. Transfer of WT CD4⁺ T cells into CX3CR1-deficient mice restored the cardinal features of asthma, and CX3CR1-blocking reagents prevented airway inflammation in CX3CR1-deficient recipients injected with WT T_H2 cells. We found that CX3CR1 signaling promoted T_H2 survival in the inflamed lungs, and injection of B cell leukemia/lymphoma-2 protein (BCL-2)-transduced CX3CR1-deficient T_H2 cells into CX3CR1-deficient mice restored asthma. CX3CR1-induced survival was also observed for T_H1 cells upon airway inflammation but not under homeostatic conditions or upon peripheral inflammation. Therefore, CX3CR1 and CX3CL1 may represent attractive therapeutic targets in asthma.

Allergic asthma is a chronic inflammatory lung disease characterized by airway hyper-responsiveness (AHR), excessive mucus production, lung eosinophilia and airway remodeling¹. Although T_H2 cells have a major role in this disease², the mechanisms underlying their recruitment, reactivation and survival in airways remain unknown.

The chemokine CX3CL1 binds only to and is the unique ligand of CX3CR1, making this ligand-receptor pair an attractive therapeutic target. In humans, CX3CL1 is constitutively expressed by intestinal epithelial cells, synovial cells, macrophages and dendritic cells (DCs)^{3,4}. CX3CL1 is expressed by endothelial cells upon stimulation by inflammatory cytokines and is upregulated in Crohn's disease⁵, rheumatoid arthritis, atherosclerosis⁶ and systemic lupus erythematosus⁷. In both humans and mice, CX3CR1 is expressed by monocytes, macrophages, mucosal DCs, natural killer cells, CD8⁺ T cells and a subset of effector-memory CD4⁺ T cells⁸⁻¹¹. In humans, most CX3CR1-expressing lymphocytes are T_H1 cells^{12,13}. Because CX3CL1 is upregulated upon inflammation, it has been proposed to contribute to inflammatory diseases by promoting the transmigration of CX3CR1-expressing cells to inflamed tissues.

CX3CR1- and CX3CL1-deficient mice do not show gross abnormalities. WT and CX3CL1-deficient mice have similar lymph node cellular composition, and CX3CR1-deficient DCs are not impaired in migration or differentiation in response to microbial antigens

or contact sensitizers¹⁰. However, both CX3CR1-deficient and CX3CL1-deficient mice show reduced atherosclerosis when crossed to mice lacking apolipoprotein E¹⁴. In addition, a CX3CL1 antagonist delays the initiation of lupus nephritis in the lupus-prone MRL *lpr/lpr* strain¹⁵, and antibody to CX3CL1 improves experimental autoimmune myositis in SJL/J mice¹⁶ and collagen-induced arthritis in DBA mice⁶. In individuals with atopic dermatitis, CX3CL1 was expressed by skin lesion endothelial cells, and serum CX3CL1 concentrations were associated with disease severity¹⁷. In individuals with asthma, a fraction of memory-effector CD4⁺ T cells expressed CX3CR1, and CX3CL1 expression was increased in airway smooth muscle, lung epithelium and bronchoalveolar lavage fluids (BALF) upon allergen challenge^{18,19}. Furthermore, two CX3CR1 single nucleotide polymorphisms were associated with asthma and atopy in French-Canadian populations²⁰ and German children²¹. In this study, we seek to investigate the role of CX3CR1 and CX3CL1 in the development of allergic airway inflammation in mice.

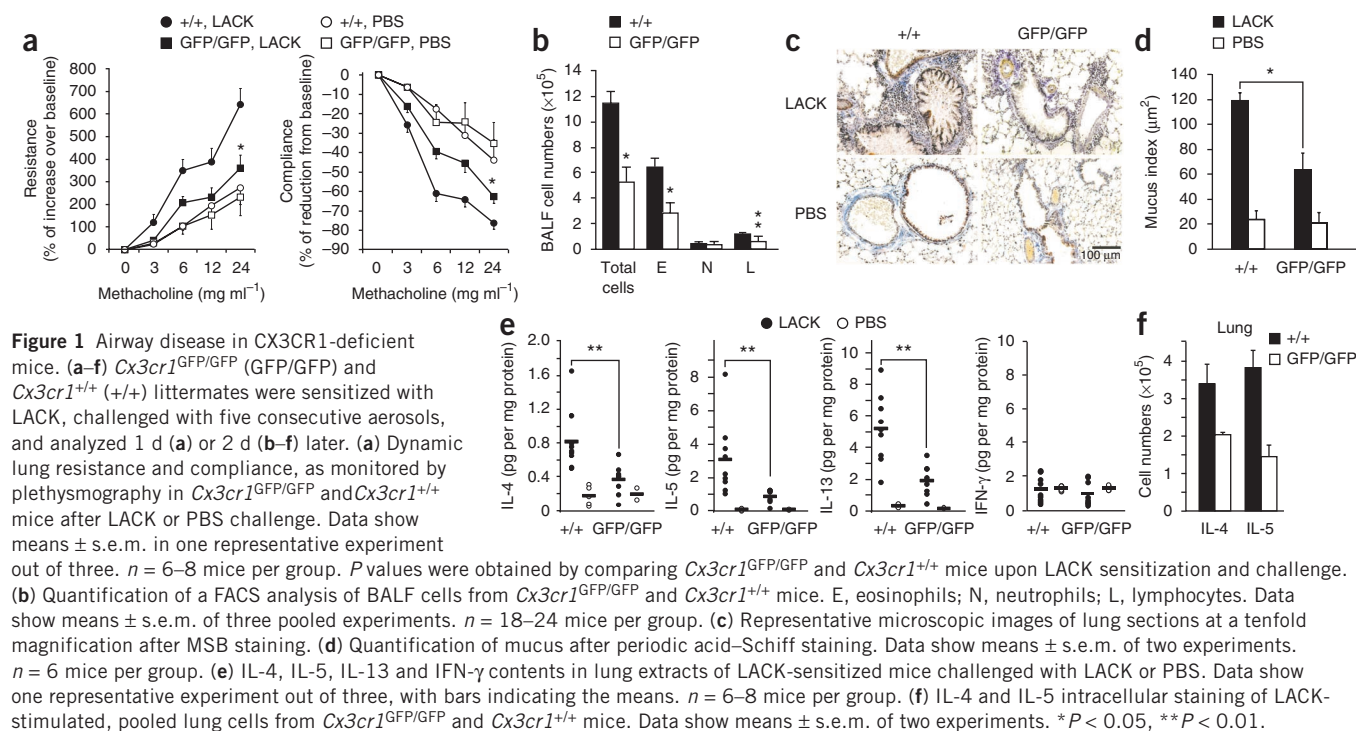
RESULTS

CX3CR1 is required for allergic airway inflammation in mice

To investigate the role of CX3CR1 and CX3CL1 in allergic airway inflammation, we used *Cx3cr1*^{GFP/GFP} mice in which the two alleles of the *Cx3cr1* gene were replaced by the *EGFP* reporter gene¹⁰. We sensitized CX3CR1-deficient *Cx3cr1*^{GFP/GFP} and their

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Received 30 July; accepted 4 October; published online 31 October 2010; doi:10.1038/nm.2253



CX3CR1-proficient *Cx3cr1*^{+/+} littermates and challenged them with the *Leishmania* homolog of receptors for activated C kinase (LACK) antigen. Compared to *Cx3cr1*^{+/+} mice, *Cx3cr1*^{GFP/GFP} mice showed reduced AHR (Fig. 1a), fewer total cells and eosinophils in BALF (Fig. 1b), reduced lung infiltrates (Fig. 1c) and decreased mucus secretion (Fig. 1d). The lungs of *Cx3cr1*^{GFP/GFP} mice contained lower T_H2 cytokine amounts than those of *Cx3cr1*^{+/+} mice (Fig. 1e). Interferon- γ (IFN- γ) amounts remained low in both groups (Fig. 1e). Interleukin-4 (IL-4)- and IL-5-secreting CD4⁺ T cell numbers in the lung were lower in *Cx3cr1*^{GFP/GFP} mice than in *Cx3cr1*^{+/+} mice (Fig. 1f). We obtained similar results upon ovalbumin (OVA) (Supplementary Fig. 1), ragweed (Supplementary Fig. 2) and house dust mite (HDM) (Supplementary Fig. 3) sensitization and challenge. Of note, *Cx3cr1*^{+/GFP} and *Cx3cr1*^{+/+} mice developed similar airway inflammation (Supplementary Fig. 4).

To confirm the role of CX3CR1 in airway inflammation, we treated WT mice with a CX3CR1-blocking antibody or the CX3CL1 antagonist FKN-AT¹⁵ (Fig. 2a). We sensitized mice with LACK, treated them with CX3CR1-blocking antibody or FKN-AT or left them untreated, and then exposed them to LACK aerosols. Compared to controls, prophylactic treatment with either FKN-AT or antibody to CX3CR1 decreased LACK-induced AHR (Fig. 2b,c), airway inflammation and eosinophilia (Fig. 2d) and impaired IL-4, IL-5 and IL-13 secretion by lung CD4⁺ T cells (Fig. 2e). FKN-AT was also protective when given as a curative treatment (Fig. 2b,d,e). Altogether, these data show that CX3CR1 and CX3CL1 are required for optimal disease development.

CX3CR1 expression by CD4⁺ T cells is required for asthma

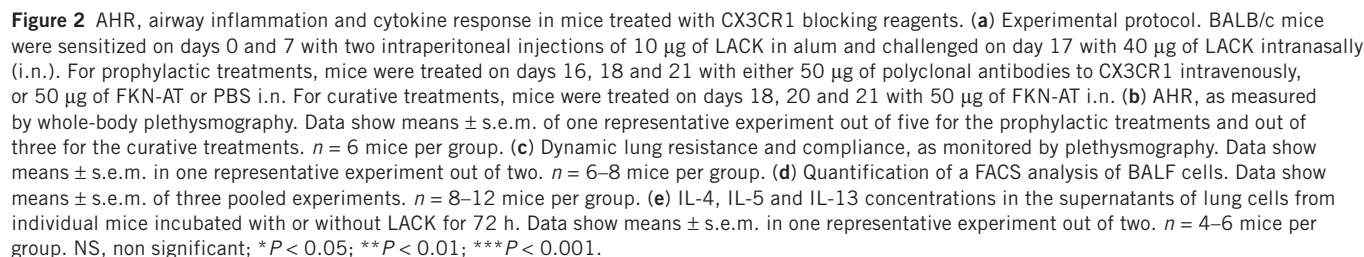
We next investigated whether *Cx3cr1*^{GFP/GFP} mice were impaired in their CD4⁺ T cell response. Mediastinal lymph node cells from LACK-immunized WT and *Cx3cr1*^{GFP/GFP} mice secreted similar IL-4, IL-5, IL-10, IL-13 and IFN- γ amounts, suggesting that CD4⁺ T cell responses were not impaired in *Cx3cr1*^{GFP/GFP} mice (Supplementary Fig. 5).

We next compared the ability of CD4⁺ T cells from CX3CR1-deficient and CX3CR1-proficient mice to restore airway inflammation when injected into CX3CR1-deficient mice. In contrast to *Cx3cr1*^{GFP/GFP} cells, WT CD4⁺ T cells restored AHR and eosinophilia in *Cx3cr1*^{GFP/GFP} mice (Fig. 3a,b). To confirm this result, we injected *Cx3cr1*^{GFP/GFP} mice with CX3CR1-proficient LACK-specific T_H2 cells, treated with FKN-AT or left untreated and exposed to LACK. Both AHR and eosinophilia were reduced in FKN-AT-treated mice compared to controls (Fig. 3c,d). Both the frequency of donor cells and T_H2 cytokine secretion were reduced upon treatment with FKN-AT (Fig. 3e,f). Therefore, CX3CR1 expression by T_H2 cells is required for the optimal development of airway disease.

T_H2 cells express CX3CR1 in the airways

We monitored CX3CR1 expression in *Cx3cr1*^{+/GFP} mice, using GFP as a surrogate marker for CX3CR1 expression^{9,10}. As previously reported¹⁰, the frequency of GFP⁺CD4⁺ T cells in naive *Cx3cr1*^{+/GFP} mice was lower than 0.5% in blood, spleen, mesenteric lymph node (MLN), lung and BALF (Fig. 4a). This frequency slightly increased in all compartments after LACK sensitization and increased even further after challenge in lung and BALF. We obtained similar data in OVA-sensitized and challenged *Cx3cr1*^{+/GFP} mice (Supplementary Fig. 6). Quantitative PCR confirmed that BALF and lung CD4⁺ T cells expressed higher levels of *Cx3cr1* mRNA than CD4⁺ T cells from spleen and MLN in LACK sensitized and challenged WT mice (Fig. 4b).

We further analyzed airway-infiltrating T cells for expression of the T_H2-specific marker T1/ST2. In lung and BALF, 18% and 25% of CD4⁺ T cells were T1/ST2⁺ (Fig. 4c). Furthermore, 20% and 27% of lung and BALF T1/ST2⁺ cells were GFP⁺ (Fig. 4c), indicating that 20–30% of T_H2 cells in inflamed airways expressed CX3CR1. Likewise, lung GFP⁺ cells were enriched in T_H2 cytokine-secreting cells, as indicated by both intracellular cytokine staining and supernatant analysis after restimulation of purified GFP⁺ cells (Fig. 4d). Indeed, the percentage of IL-5- and IL-13-producing CD4⁺ T cells



The key role of CX3CR1 in airway inflammation and the presence of CX3CR1⁺CD4⁺ T cells in the airways could reflect the preferential migration of CX3CR1⁺CD4⁺ cells to the airways, the ability of the lung tissue to induce or maintain CX3CR1 expression by T_H2 cells, a

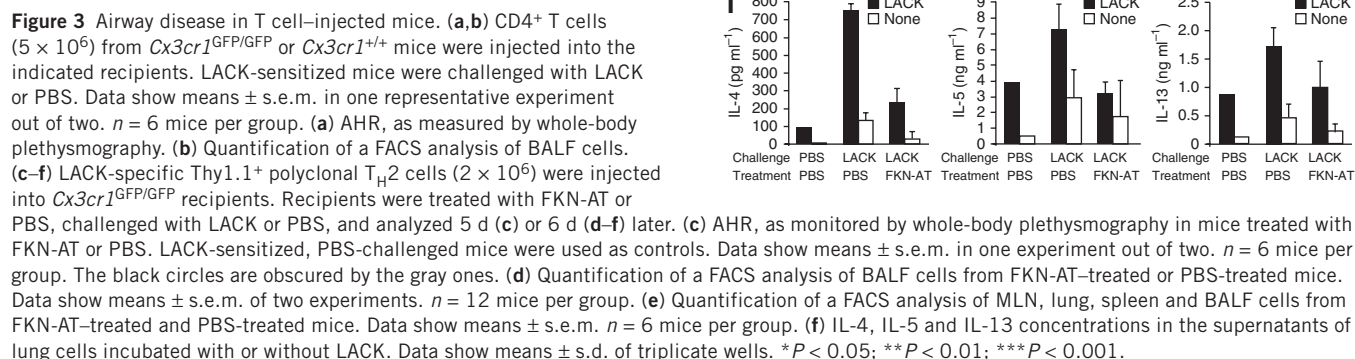
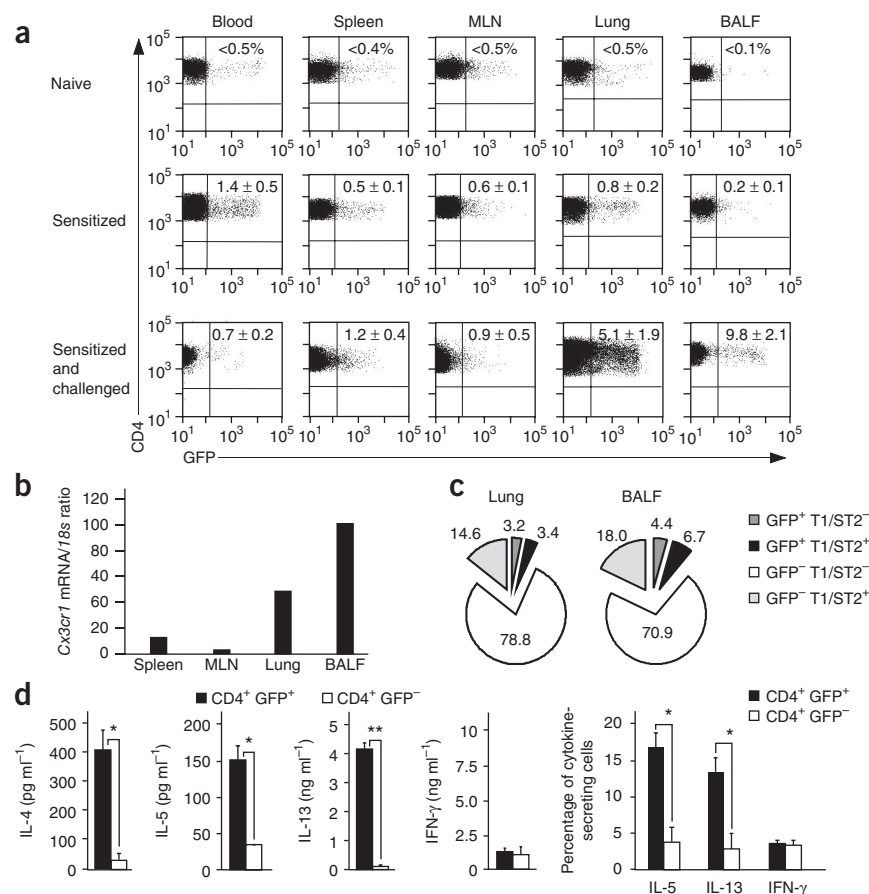


Figure 4 Number and phenotype of CX3CR1-expressing CD4⁺ T cells. (a–d) *Cx3cr1*^{+/GFP} (a,c,d) and WT (b) mice were sensitized and challenged with LACK and killed 1 d after the last aerosol. (a) GFP expression, after gating on live CD3⁺CD4⁺ cells, in the indicated organs in which blood cells were removed by cardiac perfusion with PBS. Data show representative FACS profiles. Numbers indicate the mean frequency $\pm$ s.e.m. of GFP⁺ cells among CD4⁺ T cells. $n = 12$ mice per group pooled from three experiments. (b) *Cx3cr1* mRNA levels, as measured by quantitative PCR, in CD4⁺ T cells purified from spleen, MLN, lung and BAL. Data are expressed as *Cx3cr1* / 18s rRNA ratio. (c) FACS analysis of T1/ST2 expression. Data show mean frequencies after gating on live CD3⁺CD4⁺ cells. $n = 6$ mice per group. (d) T cell cytokine secretion assessed by ELISA and intracellular staining. Top, 2×10^4 sorted GFP⁺ or GFP[−] cells were incubated with LACK and 5×10^5 T cell-depleted spleen cells for 72 h. Supernatants were analyzed for IL-4, IL-5, IL-13 and IFN- γ contents. Data show means $\pm$ s.d. of three experiments. Bottom, quantification of intracellular staining of sorted GFP⁺ or GFP[−] cells. Data show means $\pm$ s.e.m. of three experiments. * $P < 0.05$; ** $P < 0.01$.



selective advantage conferred by CX3CR1 on the maintenance of T_H2 cells or a combination of the above mechanisms. To address these issues, we generated *Thy1^{a/b} Cx3cr1^{+/GFP}* and *Thy1^{a/a} Cx3cr1^{GFP/GFP}* WT15 T cell receptor (TCR)-transgenic mice that respectively expressed the Thy1.1 and Thy1.2 antigens or the Thy1.1 antigen only and differentiated their CD4⁺ T cells *ex vivo* into T_H2 cells. Both populations were GFP[−] after differentiation and secreted similar amounts of cytokines, indicating that CX3CR1-deficient T_H2 cells had no intrinsic defect in cytokine secretion (Supplementary Fig. 8a–c). In addition, CX3CR1-deficient and CX3CR1-proficient T_H2 cells showed similar FACS profiles upon staining with antibodies to CD69, CD25, CD44 and CD62 ligand (Supplementary Fig. 8d). We injected equal numbers of CX3CR1-proficient and CX3CR1-deficient T_H2 cells into *Thy1^{b/b}* WT mice that we then exposed to LACK. Before antigen exposure, CX3CR1-deficient and CX3CR1-proficient donor cells were present at the same low frequency in all tissues, suggesting that the homing of CX3CR1-deficient T cells to lymphoid and non-lymphoid organs in steady-state conditions was not altered (Fig. 5a and Supplementary Fig. 9). The frequency of both CX3CR1-deficient and CX3CR1-proficient donor cells rapidly increased in lung but not in mediastinal lymph node after the first aerosol challenge. After the third aerosol challenge, both populations were detectable in BALF, and their frequency further increased in lung and MLN, but not in spleen (Fig. 5a and Supplementary Fig. 9). Furthermore, although both T_H2 populations were found at the same low frequency in lung, BALF, peripheral lymph node (PLN) and spleen, CX3CR1-proficient T_H2 cells outnumbered CX3CR1-deficient T_H2 cells in MLN. After the fifth aerosol challenge, CX3CR1-proficient T_H2 cells outnumbered CX3CR1-deficient T_H2 cells in BALF, lung and MLN but not in PLN or spleen. Therefore, CX3CR1-proficient T_H2 cells have a selective advantage compared to CX3CR1-deficient T_H2 cells, resulting in their preferential expansion or maintenance in the MLN and the inflamed lung.

Before and after the first aerosol, both CX3CR1-deficient and CX3CR1-proficient T_H2 cells remained GFP[−] (Fig. 5b). After the third aerosol, both populations expressed GFP in BALF, lung, MLN and spleen (Fig. 5b). GFP expression in lung and BALF remained high after the fourth and fifth aerosols but decreased in both MLN and spleen to background level (Fig. 5b). To elucidate how CX3CR1 expression is regulated, we injected equal numbers of both CX3CR1-proficient and CX3CR1-deficient LACK-specific T_H2 cells into OVA-sensitized and challenged mice and into OVA-sensitized recipients that were exposed to OVA and LACK. As expected, all recipients showed airway inflammation, and CX3CR1-proficient cells outnumbered CX3CR1-deficient cells in lung and BALF of mice exposed to both OVA and LACK (Fig. 5c). In contrast, we found both populations at a similar frequency in the inflamed lung and BALF of mice challenged with OVA only (Fig. 5c). Therefore, CX3CR1-proficient and CX3CR1-deficient LACK-specific T_H2 cells were equally capable of migrating to the inflamed lung, suggesting that T_H2 cells were recruited to the airways through a CX3CR1-independent mechanism. Whereas both CX3CR1-proficient and CX3CR1-deficient T_H2 cells in BALF and lung expressed GFP in mice exposed to OVA and LACK, both populations remained GFP[−] in mice exposed to OVA only (Fig. 5d). Therefore, inflammation was not sufficient to induce infiltrating T_H2 cells to express CX3CR1 but CX3CR1 upregulation required TCR engagement.

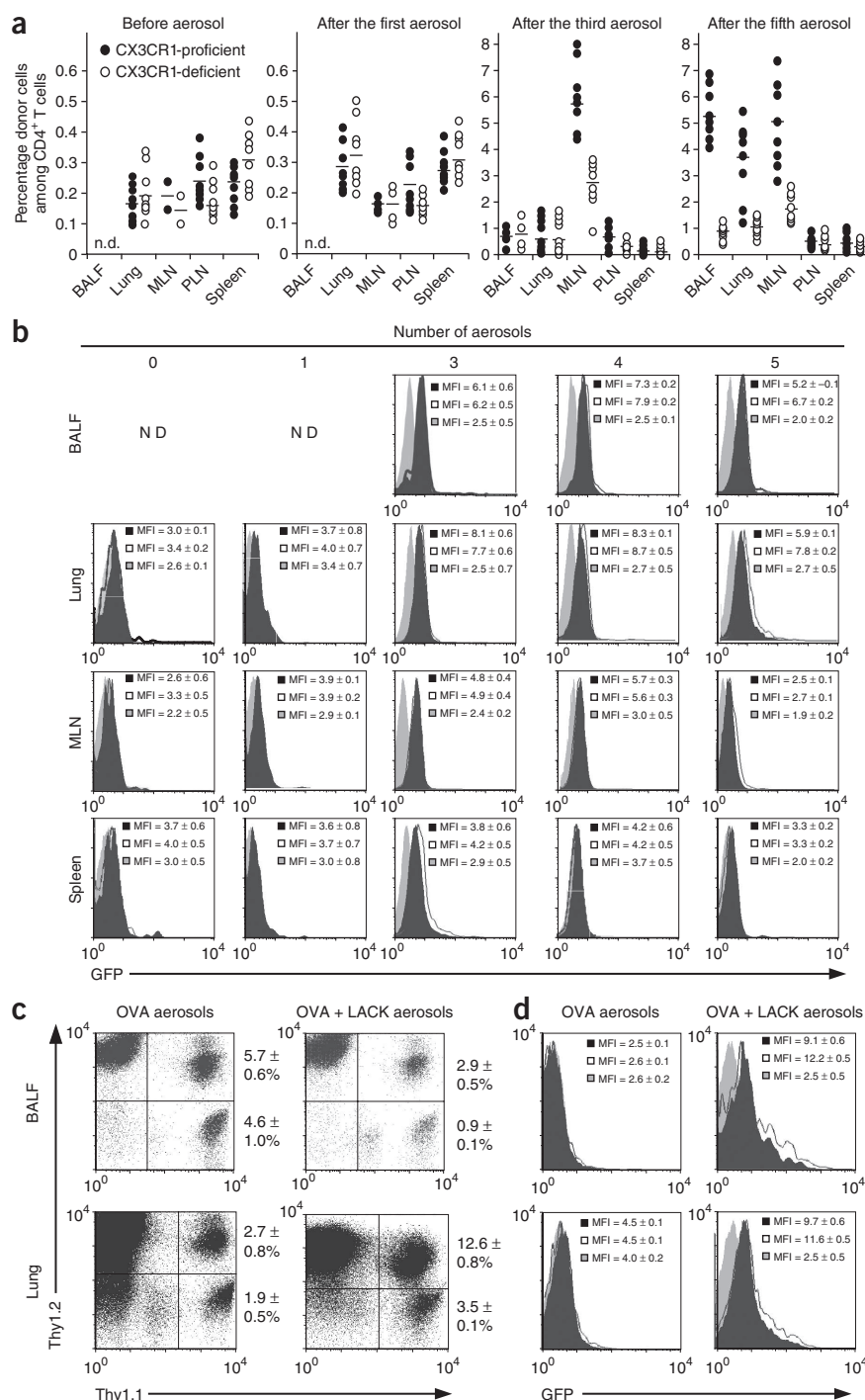
CX3CR1 provides a survival signal to lung T_H2 cells

To compare the ability of CX3CR1-deficient and CX3CR1-proficient LACK-specific T_H2 cells to proliferate *in vivo*, we injected equal numbers of these cells into recipients that were then exposed to LACK and

Figure 5 Frequency and phenotype of injected T_H2 cells. **(a–d)** $Thy1.1^{+}Thy1.2^{-}Cx3cr1^{GFP/+}$ and $Thy1.1^{+}Thy1.2^{-}Cx3cr1^{+/GFP}$ TCR-transgenic T_H2 cells were co-injected into naive **(a,b)** or OVA-sensitized **(c,d)** BALB/c mice (1.5×10^6 cells of each per mouse). Recipients were exposed to LACK aerosols, and cells were analyzed by FACS. **(a)** The frequency of $Cx3cr1^{GFP/+}$ and $Cx3cr1^{+/GFP}$ donor cells in individual mice in two experiments. $n = 8$ mice per group, except for MLN before aerosol and BALF after the third aerosol, for which each dot represents pools of four and two mice, respectively. **(b)** Representative profiles after gating on CX3CR1-proficient (black histogram) and CX3CR1-deficient (empty histogram) donor or endogenous $CD4^{+}$ T cells (gray histogram). Numbers indicate mean fluorescence intensities (MFI) $\pm$ s.e.m. of eight mice from two experiments. **(c,d)** Mice were exposed to five aerosols of OVA only or LACK and OVA, and lung and BALF cells were analyzed by FACS 1 d later. **(c)** Representative FACS profiles after gating on live $CD4^{+}$ T cells. Numbers indicate mean frequencies $\pm$ s.e.m. of donor cells in one representative experiment out of two. $n = 4$ mice per group. **(d)** GFP expression by donor and endogenous $CD4^{+}$ T cells in OVA-sensitized, challenged mice. Data show representative FACS profiles after gating on CX3CR1-proficient (black histogram), CX3CR1-deficient (empty histogram) and endogenous $CD4^{+}$ T cells (gray histogram). Numbers indicate mean fluorescence intensities $\pm$ s.e.m. of eight mice from two experiments.

treated with BrdU. Both populations proliferated similarly (**Supplementary Fig. 10a**). We next monitored the death of T_H2 donor cells *in vivo*. In lung and MLN, the frequencies of both TUNEL⁺ and 7-aminoactinomycin D (7-AAD)⁺ cells were higher among CX3CR1-deficient cells than among CX3CR1-proficient cells (**Fig. 6a** and **Supplementary Fig. 10b**). In contrast, we saw no difference in the PLN. To confirm these data, we monitored the death of endogenous $GFP^{+}CD4^{+}$ T cells in sensitized and challenged mice. Whereas $26.8 \pm 0.8\%$ of GFP^{+} cells were 7-AAD⁺ in $Cx3cr1^{+/GFP}$ mice, this frequency increased to $42.6 \pm 1.1\%$ in $Cx3cr1^{GFP/+}$ mice (**Supplementary Fig. 11**). Therefore, CX3CR1-deficient T helper cells were more susceptible to death than CX3CR1-proficient T helper cells.

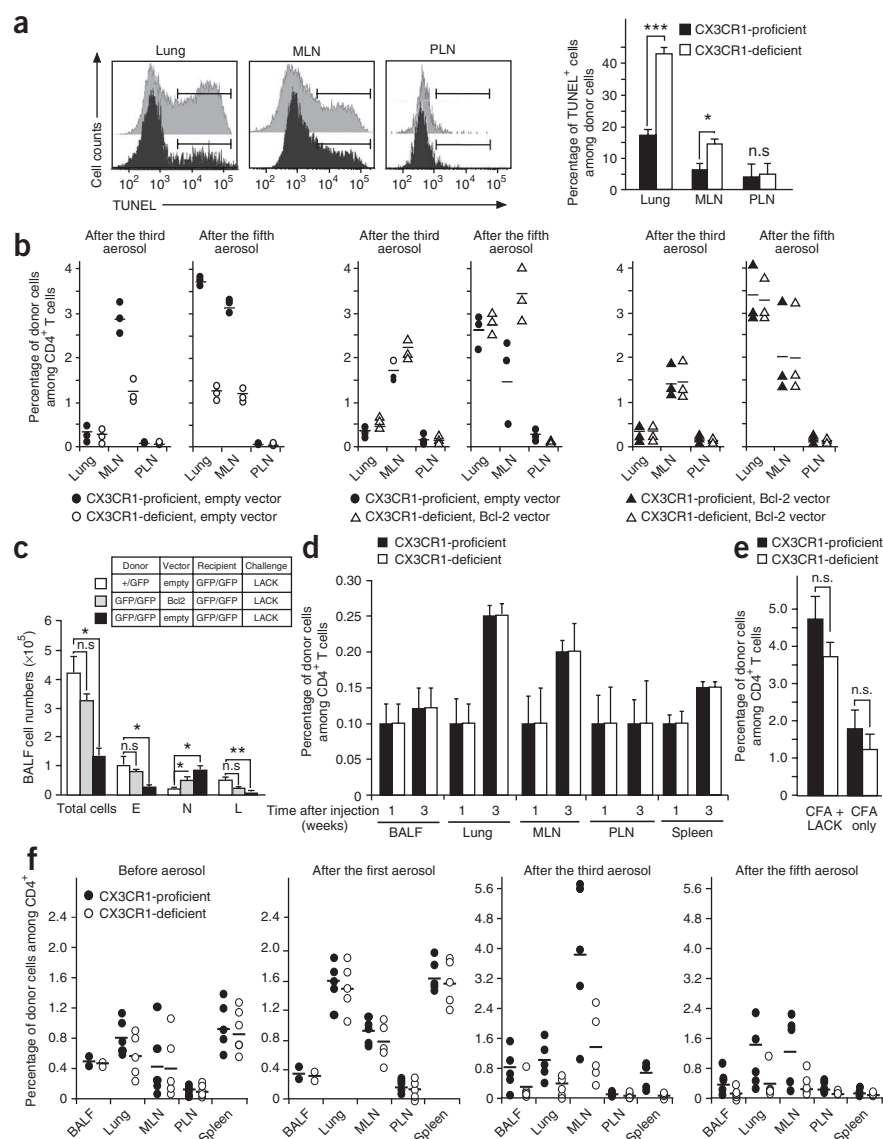
We next monitored the death of CX3CR1-deficient and CX3CR1-proficient T_H2 cells that had been induced to express GFP upon incubation with lung cells. We incubated sorted GFP^{-} and GFP^{+} cells without serum to induce cell death in the presence or absence of CX3CL1. Most GFP^{+} and GFP^{-} CX3CR1-deficient cells as well as most GFP^{-} CX3CR1-proficient cells died, as indicated by the high frequency of 7-AAD⁺ cells, irrespective of the presence of CX3CL1 (**Supplementary Fig. 12**). In contrast, the frequency of GFP^{+} CX3CR1-proficient cells that died upon serum deprivation decreased from 72% to 26% upon addition of CX3CL1 (**Supplementary Fig. 12**). Therefore, CX3CR1 provided a survival signal to T_H2 cells. The



frequency of 7-AAD⁺ cells was also decreased by the addition of either CXCL12 or CCL22, but this effect was similar in all populations irrespective of CX3CR1 expression (**Supplementary Fig. 12**).

We next investigated whether the increased sensitivity to apoptosis of CX3CR1-deficient T_H2 cells accounted for their rapid disappearance from the lungs in asthmatic mice. We infected CX3CR1-deficient and CX3CR1-proficient LACK-specific T_H2 cells with a retroviral vector carrying the genes encoding antiapoptotic BCL-2 and GFP (BCL-2 vector) or only GFP (empty vector). We sorted GFP^{+} infected cells. This was possible because neither CX3CR1-deficient nor CX3CR1-proficient T_H2 cells expressed

Figure 6 T cell proliferation and apoptosis. (a–f) Equal numbers of LACK-specific *Cx3cr1*^{GFP/GFP} and *Cx3cr1*^{+/GFP} TCR-transgenic T_H2 (a–e) or T_H1 (f) cells were injected into naive mice. (a) Apoptosis of transferred cells, as assessed by TUNEL. Recipients were challenged with LACK aerosols and analyzed 24 h after the 3rd aerosol. Data show representative FACS profiles after gating on CX3CR1-proficient or CX3CR1-deficient cells. Data show mean frequencies $\pm$ s.e.m. of TUNEL⁺ cells among CX3CR1-proficient (black histograms) and CX3CR1-deficient (gray histograms) cells. One representative experiment out of two is shown. $n = 6$ mice per group. (b) Frequencies of infected T cells following aerosol challenges. CX3CR1-proficient and CX3CR1-deficient (T_H2 cells) were infected with a BCL-2–encoding or a control retroviral vector. Equal numbers of transduced cells were injected into naive recipients. Data show donor cell frequency in individual mice, with bars indicating the mean in one representative experiment out of two. (c) Quantification of a FACS analysis of BALF cells 1 d after *Cx3cr1*^{GFP/GFP} recipients were injected with transduced T_H2 cells and exposed to LACK aerosols. Data show means $\pm$ s.e.m. of two pooled experiments. $n = 6$ mice per group. (d) Quantification of a FACS analysis of donor cells 1 or 3 weeks after injection. Data show mean frequency $\pm$ s.e.m. of CX3CR1-proficient and CX3CR1-deficient donor cells in a representative experiment out of two. $n = 6$ mice per group. (e) Quantification of a FACS analysis of lymph node cells 4 d after the mice were immunized with LACK in CFA or CFA only 1 d after cell injection. Data show mean frequency $\pm$ s.e.m. of CX3CR1-proficient and CX3CR1-deficient donor cells in a representative experiment out of two. $n = 3$ mice per group. (f) Quantification of a FACS analysis of CD4⁺ donor T_H1 cells from mice exposed to LACK aerosols. Data show the frequency of CX3CR1-proficient or CX3CR1-deficient donor cells in individual mice in one representative experiment out of two. $n = 5$, except for BALF before and after the first aerosol, for which each dot represents a pool of three mice. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.



GFP after *in vitro* differentiation. Infected CX3CR1-deficient and CX3CR1-proficient LACK-specific cells were either incubated without serum or co-injected into CX3CR1-deficient mice that were then challenged with LACK. As expected, CX3CR1-deficient cells were partially rescued from serum deprivation–induced apoptosis when infected with the BCL-2–transducing retroviral vector but not with the empty vector (Supplementary Fig. 13). Upon injection into recipients, and in agreement with data obtained with uninfected cells, CX3CR1-proficient cells infected with the empty vector outnumbered CX3CR1-deficient cells infected with the same virus in MLN after the third aerosol and in both lung and MLN after the fifth aerosol (Fig. 6b). In contrast, BCL-2–transduced CX3CR1-deficient cells were present at the same frequency as CX3CR1-proficient cells in MLN after the third aerosol and in lung after the fifth aerosol (Fig. 6b). BCL-2–transduced CX3CR1-deficient cells even outnumbered CX3CR1-proficient cells in MLN after the fifth aerosol (Fig. 6b). Therefore, BCL-2 rescued CX3CR1-deficient T_H2 cells from apoptosis *in vivo*. Together, our data demonstrate that CX3CR1-deficient T_H2

cells show an increased sensitivity to apoptosis, therefore accounting for their disappearance from the airways of allergen-sensitized, challenged mice.

To further investigate how the increased sensitivity to death of CX3CR1-deficient T_H2 cells affects allergic airway disease, we injected CX3CR1-deficient LACK-specific T_H2 cells infected with the BCL-2–encoding vector or the empty vector into *Cx3cr1*^{GFP/GFP} mice that were next exposed to LACK aerosols. As controls, *Cx3cr1*^{GFP/GFP} mice received CX3CR1-proficient T_H2 cells infected with the empty vector (Fig. 6c). When infected with the empty vector, CX3CR1-proficient T_H2 cells induced a more severe airway inflammation in *Cx3cr1*^{GFP/GFP} mice than did CX3CR1-deficient T_H2 cells (Fig. 6c). In contrast, *Cx3cr1*^{GFP/GFP} mice injected with BCL-2–transduced CX3CR1-deficient T_H2 cells developed similar airway inflammation as those injected with CX3CR1-proficient T_H2 cells (Fig. 6c). Taken together, our results show that CX3CR1 provides a survival signal to T_H2 cells that is required for airway disease.

CX3CR1-independent maintenance in steady-state and inflamed PLN

We next investigated whether CX3CR1 increased T_H2 cell survival in general or only in the inflamed airways. We injected equal numbers of CX3CR1-deficient and CX3CR1-proficient T_H2 cells into naive mice and monitored the frequency of each population 1 and 3 weeks later. The survival rate of these two populations was similar, suggesting that CX3CR1 is not involved in T_H2 cell maintenance under homeostatic conditions (Fig. 6d). Mice were also co-injected with CX3CR1-deficient and CX3CR1-proficient T_H2 cells and further immunized subcutaneously with LACK in CFA or CFA only. Although both populations were detected in inflamed lymph node, mice immunized with LACK in CFA had more donor cells in lymph node than those immunized with CFA only (Fig. 6e). In contrast to what we had observed in inflamed airways, CX3CR1-deficient and CX3CR1-proficient donor T_H2 cells were present at the same frequency in inflamed LN in both groups. Therefore, while CX3CR1 provided a survival signal to T_H2 cells in inflamed airways, it did not increase T_H2 cell survival in inflamed popliteal lymph node.

Previous studies have shown that T_H1 cells induce airway inflammation but not eosinophilia upon injection into recipients and aerosolized antigen challenge²². We used this model to investigate whether CX3CR1 provided a survival signal to T_H1 cells, as it did for T_H2 cells. We differentiated CX3CR1-proficient and CX3CR1-deficient LACK-specific T cells into T_H1 cells. Both populations remained GFP⁺ and showed similar FACS profiles after intracellular cytokine staining and staining with antibodies to CD62 ligand, CD44, CD25 and CD69 (Supplementary Fig. 14a–c). We then injected equal numbers of these cells into recipients that we next exposed to LACK. Before antigen exposure, we found CX3CR1-deficient and CX3CR1-proficient donor T_H1 cells at the same low frequencies in all tissues (Fig. 6f). After the first aerosol, the frequency of both populations rapidly increased in lung, MLN and spleen but not in PLN (Fig. 6f). After the third aerosol, the frequency of both populations further increased in MLN but not in other organs (Fig. 6f). Furthermore, although we found the two populations at the same low frequency in PLN, CX3CR1-proficient T_H1 cells outnumbered CX3CR1-deficient T_H1 cells in MLN, lung, BALF and spleen (Fig. 6f). After the fifth aerosol, the frequency of both populations decreased in lung, with CX3CR1-proficient T_H1 cells outnumbering CX3CR1-deficient cells T_H1 in BALF, lung and MLN (Fig. 6f). Further experiments showed that CX3CR1-deficient and CX3CR1-proficient T_H1 cells proliferated similarly *in vivo* and that CX3CR1 protected CX3CR1-proficient T_H1 cells from serum deprivation-induced cell death *in vitro* (Supplementary Fig. 14d,e). Together, our results show that CX3CR1 confers a survival advantage on both T_H1 and T_H2 cells in inflamed airways but not under homeostatic conditions or in inflamed PLN.

DISCUSSION

The role of chemokine receptors has previously been investigated in animal models of asthma. For example, targeted deletion of the genes encoding CCR3 or CCR4 in mice did not inhibit asthma^{23,24}, and neutralization of CCR4 in guinea pig did not prevent cell recruitment to the lung²⁵. In apparent contrast, neutralization of the CCR4 ligand CCL17 decreased AHR and T_H2 cytokine amounts²⁶, and CCR4 was shown to be required for T_H2 entry into the lung in mice²⁷. CCR8 was dispensable in two studies but not in others^{27–30}. CXCR4 was required for airway inflammation, but it was not clear whether its expression was required on T cells or other cells^{31,32}. These studies suggested that chemokine receptors could play a part in airway inflammation, but it was not clear on which cell types they are required.

Here we have shown that CX3CR1-deficient mice show reduced AHR, eosinophilia, mucus secretion and T_H2 cytokine levels compared to their littermates in whom neither or only one of the two *Cx3cr1* alleles was disrupted. This was shown in mice sensitized and challenged with LACK, OVA, HDM or ragweed. In addition, blocking CX3CR1–CX3CL1 interactions with either a CX3CR1-specific antibody or a CX3CL1 antagonist prevented airway inflammation in WT mice. CD4⁺ T cells purified from WT mice, but not from CX3CR1-deficient mice, restored lung inflammation when injected into CX3CR1-deficient mice, demonstrating that CX3CR1 expression by T_H2 cells is essential for disease. Furthermore, treatment with FKN-AT inhibited disease in CX3CR1-deficient mice injected with CX3CR1-proficient T_H2 cells.

Although in initial studies chemokines were shown to induce chemotaxis *in vitro*, they also have a role in angiogenesis, tissue repair, tumor progression, hematopoiesis, antimicrobial mechanisms and pain control^{33–41}. In addition to its role in chemotaxis and adhesion⁸, CX3CL1 acts as a survival factor for microglial cells and monocytes^{42,43}. Here we have shown that CX3CR1 is dispensable for T cell migration to the lung but required for T cell maintenance and survival in inflamed airways. In contrast, T cell survival under homeostatic conditions or in inflamed PLN was CX3CR1 independent. It remains to be determined which factors induce T helper cells to express CX3CR1 *in vivo* and the survival signals that are induced by the binding of CX3CL1 to CX3CR1.

A fraction of memory-effector peripheral CD4⁺ T cells express CX3CR1 in humans^{13,18}. In one study, human CX3CR1⁺CD4⁺ T cells expressed CCR5, suggesting that they were T_H1 cells¹³. Likewise, human polarized T_H1 cells expressed a high level of *Cx3cr1* mRNA, whereas polarized T_H2 cells did not¹². In mice in our study, we found that neither T_H1 nor T_H2 *in vitro*-differentiated cells expressed CX3CR1 unless incubated with lung cells or injected into sensitized mice exposed to aerosols of their cognate antigen. Therefore, CX3CR1 expression was inducible in T helper cells. This also explains why CX3CR1 is not required for T helper cell survival under homeostatic conditions and T helper cell recruitment to inflamed lymph nodes. Of note, three promoter regions control the human CX3CR1 gene, leading to the transcription of several mature mRNAs⁴⁴. This highly complex regulation may allow versatile and tightly regulated CX3CR1 expression in different cell types. IL-2 induces CX3CR1 on both human peripheral blood mononuclear cells and mouse splenocytes, and transforming growth factor- β 1, IL-10 and IFN- γ upregulate CX3CR1 on rat microglial and monocytic cells^{45,46}. It is unknown whether any of these cytokines induce T helper cells to express CX3CR1 in airways.

Although asthma is a paradigm for T_H2 -driven inflammation, concomitant T_H1 activation has been observed in humans^{47–49}, and the presence of T_H1 cells in sputum correlated with both the loss of asthma control and exacerbations⁵⁰. Therefore, our results showing that CX3CR1 and CX3CL1 are required for the survival of both T_H1 and T_H2 cells in inflamed airways makes these molecules interesting therapeutic targets. In addition, the fact that CX3CL1 antagonist was protective when administered intranasally is particularly promising, because corticosteroids and β 2-agonist bronchodilators that constitute the standard therapy of asthma are given as inhaled drugs. Furthermore, although inhaled corticosteroids are very effective, they have side effects, and 10% of patients with asthma do not respond to this treatment⁵¹. More specific therapeutic agents such as antibodies to IL-5 or IgE have been tested in clinical trials but the results have been disappointing⁵². As there is an urgent need for better therapy, blocking CX3CR1 signaling may represent a promising alternative therapy.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/naturemedicine/>.

Note: Supplementary information is available on the Nature Medicine website.

ACKNOWLEDGMENTS

We thank N. Guy, V. Thieffin and A. Barbot for animal care, J. Cazareth and F. Larbret for cell sorting and S. Kirschnek (Institute for Medical Microbiology, Immunology and Hygiene, Technical University Munich) for providing the MIG-mBcl-2 and MIGR1 vectors. IFN- γ -specific antibody R4-6A2 and IL-4-specific antibody 11B11 were kind gifts from DNAX Research Institute. *Cx3cr1*^{GFP/GFP} mice were a gift from S. Jung (Weizmann Institute of Science, Immunology Department). This research was supported by the Fondation pour la Recherche Médicale, the Agence Nationale de la Recherche, the French Society of Allergy, the French Pneumologie Society, the Genavie foundation and the Pays de la Loire and Provence Alpes Cote d'Azur regional councils.

AUTHOR CONTRIBUTIONS

C.M. and V.B. conducted most of the experiments and contributed to data analysis. A.K., S.F. and D.D. measured lung resistance and compliance and performed lung histology. V.M. provided technical help. D.L., M.L., Y.L. and A.M. performed experiments with HDM. E.H. and R.C. contributed to early experiments aimed at monitoring chemokine receptor expression in lung T cells. N.G. and V.J. wrote the manuscript. V.J. conceived of and directed the project.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. BALB/c ByJ mice were purchased from The Centre d'Élevage Janvier and housed under specific pathogen-free conditions. *Cx3cr1^{+/GFP}* mice were generated by mating *Cx3cr1^{GFP/GFP}* mice (a gift from S. Jung) with BALB/c ByJ mice for 12 generations. *Cx3cr1^{GFP/GFP}*, *Cx3cr1^{+/GFP}* and *Cx3cr1^{+/+}* mice were generated by intercrossing *Cx3cr1^{+/GFP}* mice. *Cx3cr1^{GFP/GFP}* and *Cx3cr1^{+/GFP}* Thy1.1^{+/−} WT15 TCR-transgenic mice were generated by crossing *Cx3cr1^{GFP/GFP}* WT15 TCR transgenic mice to *Thy1^{u/a}* congenic BALB/c ByJ mice⁵³. All experiments were approved by the Regional Ethical Committee for Animal Experiments of the Côte d'Azur.

Asthma induction. For LACK and OVA-induced asthma, mice were sensitized by two intraperitoneal injections of 10 µg of LACK or OVA (Sigma) in 2 mg of aluminum hydroxide (Pierce) at day 0 and day 7. From day 17 to day 21, mice were exposed to LACK (1.5 mg ml^{−1}) or OVA (10 mg ml^{−1}) aerosols for 30 min using an ultrasonic nebulizer (AeroNeb, Emka Technologies). Mice were analyzed for AHR on day 22 and killed on day 23. Alternatively, mice were challenged i.n. with 40 µg of LACK on day 17 and assessed for AHR 5 d later. For HDM-induced asthma, mice were sensitized on days 0, 7, 14 and 21 by topical administration of 500 µg HDM (Stallergenes) in 20 µl DMSO onto the ears and challenged on day 27 with 250 µg HDM i.n. The protocol for ragweed-induced asthma was as previously described⁵⁴. LACK was produced and detoxified with Endotrap columns (Profos)⁵⁵. Endotoxin levels, assessed by LAL assay (Pierce), were below 5 ng per mg of protein.

mRNA levels. Total RNA was isolated with the RNeasy 96 kit (Qiagen). cDNA was prepared and analyzed by real-time PCR with the ABI Prism 7700 sequence detection system and TaqMan primers and probes (Perkin Elmer). 18s rRNA PCR amplification was performed and used for data normalization.

Fractalkine antagonist. The CX3CL1 chemokine domain was amplified by PCR using the American Type Culture Collection clone MGC-5859 as a template and the primers 5'-AAGCTAGCCATATGACGAAATGGCGAAATCATGTGCG-3' and 5'-AAGTCGACTTACACCATCATCATCATACCATTTTGTAGTGAGGGC-3'. The PCR fragment was cloned into the pET3a plasmid, transfected into the BL21(DE3) pLysS *Escherichia coli* strain (Promega), produced and detoxified using an Endotrap column as previously described⁵⁵. Endotoxin levels assessed by LAL assay were below 5 ng per mg of protein.

Airway hyper-reactivity. Enhanced pause was measured by whole-body plethysmography (Emka Technologies)⁵⁶. Dynamic lung resistance and compliance were measured using a flexiVent (SCIREQ)⁵⁷.

Histology. Lungs were treated as previously described⁵⁸, and 4-µm sections were stained with Martius scarlet blue or periodic acid–Schiff⁵⁹.

Bronchoalveolar lavage fluid and lung cell analysis. Cardiac perfusion with PBS was performed to remove blood cells from the collected organs. In some experiments, cells were stained with antibodies to Thy1.1 (Ox7 clone, BD Biosciences), Thy1.2 (53-2.1 clone, BD Biosciences) and CD4 (RM4-5 or GK1.5 clones, BD Biosciences) to discriminate the two populations of donor cells and endogenous CD4⁺ T cells.

Cell purification. Spleen CD4⁺ T cells were enriched by negative selection, stained with antibody to CD4 and αGalCer-loaded CD1d tetramers (US National Institutes of Health) and sorted by FACS. Lung CD4⁺ T cells from 20

Cx3cr1^{+/GFP} mice were enriched by positive selection with CD4-specific beads (Miltenyi) and sorted by FACS after staining with antibodies to CD4 (RM4-5, BD Biosciences) and CD3 (145-2C11, BD Biosciences). For preparing T_H1 and T_H2 cells, CD4⁺ T cells from TCR-transgenic mice were purified by negative selection, and 1.5 × 10⁶ cells were incubated for 3 d with 7.5 × 10⁵ T cell-depleted splenocytes in complete RPMI with 50 nM LACK_{156–173} peptide and 10 ng ml^{−1} recombinant IL-4 (R&D Systems) and 10 µg ml^{−1} antibody to IFN-γ (R4-6A2, DNAX Research Institute) for T_H2 cells or 10 ng ml^{−1} recombinant IL-12 (R&D Systems) and 10 µg ml^{−1} antibody to IL-4 (11B11, DNAX Research Institute) for T_H1 cells. T_H1 and T_H2 cells were purified by negative selection, rested for 3 d with T cell-depleted splenocytes and purified by positive selection with CD4-specific beads (Miltenyi). To assess cytokine secretion, 5 × 10⁵ T cells were incubated with 1 × 10⁵ T cell-depleted splenocytes and 10 nM LACK_{156–173} in complete RPMI in a 96-well plate.

Terminal deoxynucleotide transferase dUTP nick end labeling assay. Cells were stained with antibodies to Thy1.1, Thy1.2 and CD4 and treated according to BD Biosciences instructions.

Retroviral transduction. The cDNA encoding human BCL-2 was cloned into the mouse bicistronic retroviral expression vector MIGR. T_H2 cells were incubated with viral supernatant containing 5 µg ml^{−1} Polybrene (Sigma) and spun at 650g for 8 h at 32 °C. Viral supernatant was replaced with fresh medium. GFP-expressing T_H2 cells were sorted 3 d later.

Cytokine assays. Lungs were either homogenized in HBSS for protein extract or digested with collagenase I and DNase I (Roche) for 30 min at 37 °C for cell preparation. 3 × 10⁶ cells were cultured in 48-well plates for 72 h in complete RPMI with or without 100 µg ml^{−1} of LACK. Cytokine levels were measured by ELISA or cytometric bead array. Alternatively, 3 × 10⁶ lung cells were incubated with 100 µg ml^{−1} LACK and 1 µg ml^{−1} antibody to CD28 (BD Biosciences) for 6 h and brefeldin A (Sigma, 5 µg ml^{−1}) during the last 4 h. Cells were stained with antibody to CD4 (BD Biosciences), fixed, permeabilized, stained with cytokine antibodies and analyzed by FACS. Otherwise, cells were incubated for 6 h with 10 ng ml^{−1} PMA (Sigma), 1 ng ml^{−1} ionomycin (Sigma) and 5 µg ml^{−1} brefeldin A (BD Biosciences), permeabilized with Cytofix/Cytoperm buffer (BD Biosciences), stained with cytokine antibodies and analyzed by FACS.

Statistical analyses. Analysis of variance was used for plethysmography measurements. Otherwise, pair comparisons were performed by unpaired two-tailed Student's *t* test. Significance levels were set at a *P* value of 0.05.

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