



Role of lymphotoxin- α in cigarette smoke-induced inflammation and lymphoid neogenesis

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ABSTRACT: In chronic obstructive pulmonary disease (COPD), chronic inflammation is accompanied by peribronchial lymphoid aggregates. Lymphotoxin (LT)- α , crucial in secondary lymphoid organogenesis, may be involved in lymphoid neogenesis.

We examined cigarette smoke (CS)-induced pulmonary lymphoid neogenesis and inflammation *in vivo* in LT α knockout (LT $\alpha^{-/-}$) and wild-type (WT) mice and studied the expression of lymphoid chemokines by lung fibroblasts *in vitro*.

T-cell numbers (in bronchoalveolar lavage fluid (BALF) and lungs) and lymphoid aggregate numbers were significantly higher in air-exposed LT $\alpha^{-/-}$ mice than in WT animals, and increased upon chronic CS exposure in both genotypes. In contrast, local immunoglobulin A responses upon chronic CS exposure were attenuated in LT $\alpha^{-/-}$ mice. CXC chemokine ligand (CXCL) 13 and CC chemokine ligand (CCL) 19 mRNA in total lung and CXCL13 protein level in BALF increased upon CS exposure in WT, but not in LT $\alpha^{-/-}$ mice. *In vitro* lymphotoxin- β receptor (LT β R) stimulation induced CXCL13 and CCL19 mRNA in WT lung fibroblasts. Furthermore, *in vitro* exposure to CS extract upregulated CXCL13 mRNA expression in WT, but not in LT β R $^{-/-}$ lung fibroblasts.

In this murine model of COPD, CS induces pulmonary expression of lymphoid chemokines CXCL13 and CCL19 in a LT α –LT β R-dependent fashion. However, LT α is not required for CS-induced pulmonary lymphocyte accumulation and neogenesis of lymphoid aggregates.

KEYWORDS: Chronic obstructive pulmonary disease, cigarette smoking, cytokines and chemokines, lymphotoxin- α , mouse models

Chronic obstructive pulmonary disease (COPD) is characterised by a slowly progressive airflow limitation that is poorly reversible [1]. COPD is one of the leading causes of death with cigarette smoking as the main risk factor [2]. The molecular and cellular mechanisms responsible for the development of COPD are poorly understood. For that reason, a murine smoke model of COPD was developed, showing a pulmonary pathology comparable to the disease in COPD patients [3], including inflammation and remodelling of the small airways as well as destruction of the lung parenchyma and emphysema.

Recently, BRACKE *et al.* [4], as well as others [5], reported the appearance of lymphoid aggregates in murine lung tissue upon chronic cigarette smoke (CS) exposure. Similar lymphoid follicles with clearly delineated T- and B-cell areas can be seen in lung sections of patients with severe COPD [6, 7]. The underlying mechanisms and

functional properties of lymphoid follicle formation in the lung remain to be elucidated.

Lymphotoxin (LT)- α , essential in organogenesis of secondary lymphoid organs, may induce the development of tertiary lymphoid tissue. LT α knockout (LT $\alpha^{-/-}$) mice have no lymph nodes or Peyer's patches and aberrant splenic architecture [8, 9], while site-specific LT α expression in transgenic animals causes local inflammation with a cellular composition and organisation similar to those of lymph nodes [10].

LT α exists as a soluble homotrimer (LT α_3) or as a membrane-bound heterotrimer with lymphotoxin- β (LT $\alpha_1\beta_2$). LT α_3 , produced by activated T-cells and early B-cells, is functionally redundant with tumour necrosis factor (TNF)- α [11]. LT $\alpha_1\beta_2$ on activated T- and B-lymphocytes and natural killer (NK) cells binds exclusively to the TNF receptor (TNFR)-like LT β receptor (LT β R), on stromal and myeloid lineage cells [12]. The LT α –LT β R pathway is crucial in lymphoid

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organ development and triggers the expression of lymphoid chemokines, such as CXC chemokine ligand (CXCL) 12, CXCL13, CC chemokine ligand (CCL) 19 and CCL21 [13, 14]. Furthermore, the $LT\alpha\beta$ – $LT\beta R$ pathway has been implicated in the thymic emigration of ($V\alpha 14$) invariant NK T (iNKT) cells [15].

To reveal the *in vivo* role of $LT\alpha$ in CS-induced inflammation, pulmonary emphysema, lymphoid neogenesis and mucosal immunoglobulin (Ig) A production, we subjected wild-type (WT) and $LT\alpha^{-/-}$ mice to subacute (4 weeks) or chronic (24 weeks) CS exposure. Moreover, we studied *in vitro* the expression of lymphoid chemokines by lung fibroblasts under baseline conditions and upon stimulation with agonistic $LT\beta R$ antibody (Ab) and/or cigarette smoke extract (CSE).

MATERIALS AND METHODS

Animals

Homozygous male C57Bl/6 $LT\alpha^{-/-}$ ($LT\alpha^{tm1Dch}$) mice and C57Bl/6 WT mice (8 weeks old) were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) [8]. The local ethics committee for animal experimentation of the faculty of Medicine and Health Sciences (Ghent University Hospital, Ghent, Belgium) approved all *in vivo* manipulations.

Smoke exposure

Mice (n=8 per group) were exposed to CS, as described previously [16]. Briefly, groups of eight mice were exposed whole body to the tobacco smoke of five cigarettes (Reference Cigarette 2R4F without filter; University of Kentucky, Lexington, KY, USA), four times a day with 30 min smoke-free intervals, 5 days a week for 4 weeks (subacute exposure) or 24 weeks (chronic exposure). During the exposure an optimal smoke:air ratio of 1:6 was obtained. The control groups were exposed to air. Carboxyhaemoglobin in the serum of smoke-exposed mice reached a nontoxic level of $8.3 \pm 1.4\%$ (compared with $1.0 \pm 0.2\%$ in air-exposed mice, n=7 for both groups), similar to carboxyhaemoglobin blood concentrations of human smokers [17].

Bronchoalveolar lavage

24 h after the last exposure, mice were euthanised with an overdose of pentobarbital and bronchoalveolar lavage fluid (BALF) was collected, as described previously [16]. A total cell count was performed in a Bürker chamber, and differential cell counts (on ≥ 400 cells) were performed on cytocentrifuge preparations using standard morphologic criteria after May–Grünwald–Giemsa staining. Flow cytometric analysis of BAL cells was performed to enumerate dendritic cells (DCs) and $CD4^+$ and $CD8^+$ T-lymphocytes.

Preparation of lung single-cell suspensions

After rinsing of pulmonary and systemic circulation, the left lung was used for histology, and the right lung for the preparation of a single-cell suspension, as detailed previously [16]. Cell counting was performed with a Z2 Beckman Coulter particle counter (Beckman Coulter, Ghent, Belgium).

Labelling of BAL cells and single-cell suspensions for flow cytometry

The following monoclonal Abs (mAbs) were used to identify mouse DC populations: anti- $CD11c$ -allophycocyanin (APC; HL3) and anti-I-Ab-phycoerythrin (PE; AF6-120.1). We discriminated

between macrophages and myeloid DCs using the methodology described by VERMAELEN and PAUWELS [18]. The following mAbs were used to stain mouse T-cell subpopulations: anti- $CD4$ -fluorescein isothiocyanate (FITC; GK1.5), anti- $CD8$ -FITC (53-6.7), anti- $CD3$ -APC (145-2C11) and anti- $CD69$ -PE (H1.2F3). Using anti- $CD19$ -PE (1D3) and anti- $CD11c$, B-lymphocytes were identified as the $CD11c$ -low and $CD19$ -positive population. All mAbs were obtained from BD Pharmingen (San Diego, CA, USA). iNKT cells were stained with anti- $CD3$ and PE-conjugated $CD1d$ tetramer loaded with α -galactosylceramide (α GalCer) [19]. In a last step before analysis, cells were incubated with 7-aminoactinomycin D (or viaprobe; BD Pharmingen) to check cell viability. Flow cytometry data acquisition was performed on a FACScalibur™ running CellQuest™ software (BD Biosciences, San Jose, CA, USA). FlowJo software was used for data analysis (TreeStar Inc., Ashland, OR, USA).

Histology

The left lung was fixated by intratracheal infusion of fixative (4% paraformaldehyde), as described previously [16]. The lung lobe was embedded in paraffin and cut into 3 μ m transversal sections. Photomicrographs were captured using a Zeiss KS400 image analyser platform (KS400, Zeiss, Oberkochen, Germany).

Quantification of pulmonary emphysema

To evaluate pulmonary emphysema, we determined the enlargement of the alveolar spaces by measuring the mean linear intercept (L_m), as described previously [4, 16]. Using image analysis software (ImageJ 1.34s; National Institutes of Health, Bethesda, MD, USA) a $100 \times 100 \mu$ m grid was placed over images of haematoxylin and eosin-stained lung sections, acquired and scored in a blinded fashion. The total length of each line of the grid divided by the number of alveolar intercepts gives the average distance between alveolated surfaces, the L_m .

Morphometric quantification of lymphoid infiltrates

To evaluate the presence of lymphoid infiltrates in lung tissues, sections obtained from formalin-fixed, paraffin-embedded lung lobes were subjected to an immunohistological $CD3/B220$ double-staining as described previously [4]. Infiltrates in the proximity of airways and blood vessels were counted. Dense accumulations of ≥ 50 cells were defined as lymphoid aggregates. Counts were normalised for the number of bronchovascular bundles per lung section.

Immunohistochemistry for CXCL13

Paraffin sections were incubated with primary anti-CXCL13 Ab (R&D Systems, Minneapolis, MN, USA), followed by biotinylated rabbit anti-goat-Ab from the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA). After incubation with streptavidin-horseradish peroxidase, slides were coloured with diaminobenzidine (Dako, Carpinteria, CA, USA) and counterstained with Mayer's haematoxylin (Sigma-Aldrich, St Louis, MO, USA).

ELISA

Commercially available ELISA kits were used to determine CXCL13 and CCL21 protein levels in BALF (R&D Systems) as well as IgM (ZeptoMetrix, Buffalo, NY, USA) and IgA (Alpha

Diagnostic International, San Antonio, TX, USA) titers in serum and BALF. Secretory-IgA (S-IgA) was measured with a sandwich ELISA, developed in the laboratory of co-author C. Pilette. BALF samples were assayed using a polyclonal goat Ab to rat secretory component (SC), cross-reactive with mouse SC, as capture antibody and biotinylated anti-mouse IgA (Sigma-Aldrich) as detection antibody. S-IgA data were expressed as corrected optical density, in the absence of a murine standard.

Reverse transcriptase PCR analysis

Total lung RNA was extracted with the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA from cultured cells was extracted with the ChargeSwitch Total RNA Cell Kit (Invitrogen Corp, Carlsbad, CA, USA). Expression of CXCL13, CCL19, CCL20, CXCR5 and CC chemokine receptor (CCR) 7 mRNA, relative to hypoxanthine guanine phosphoribosyltransferase mRNA, was analysed with the Assays-on-Demand Gene Expression Products (Applied Biosystems, Foster City, CA, USA). RT-PCR was performed on a LightCycler 480 Instrument (Roche Diagnostics, Basel, Switzerland) with murine leukemia virus reverse transcriptase (Applied Biosystems) under previously described conditions [4].

Lung fibroblast culture

Lungs from a WT and LT β R^{-/-} mouse (C57Bl/6) [20] were digested, as described previously, into single-cell suspensions. Cells were seeded in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, L-glutamine and penicillin/streptomycin (all from Gibco BRL; Invitrogen Corp) and incubated in a humidified 37°C incubator with 5% CO₂. 24 h after plating, nonadherent cells were removed by medium change. Cells were passaged at subconfluency. At passage 5, fibroblastic phenotype was checked on Lab-Tek chamber slides (Nalge Nunc, Rochester, NY, USA). In addition to positive staining of the cells for vimentin with monoclonal anti-vimentin Ab (clone VIM 13.2, Sigma-Aldrich), fibroblast morphology was confirmed by inverted phase-contrast microscopy.

Stimulation of cultured lung fibroblasts

At passage 6, cells were plated onto 24-well plates at a density of 4×10^4 cells·well⁻¹ and grown to confluency. Cells were stimulated for 48 h with 1 mL culture medium, isotype control (2 µg·mL⁻¹), agonistic LT β R Ab (2 µg·mL⁻¹), 5% CSE, or the combination of agonistic LT β R Ab with CSE. 100% CSE was prepared as described previously [21]. Agonistic LT β R Ab consisted of a 9:1 mix of 4H8 and 3C8 (C.F. Ware, La Jolla Institute, San Diego, CA, USA). Isotype control consisted of a 9:1 mix of rat IgG_{2a} (A110-2) and rat IgG₁ (R3-34; BD Pharmingen).

Statistical analysis

Reported values are expressed as mean $\pm$ SEM. Statistical analysis was performed with Sigma Stat software (SPSS 15.0 Inc., Chicago, IL, USA) using nonparametric tests (Kruskal–Wallis and the Mann–Whitney U-test). A p-value $p \leq 0.05$ was considered significant.

RESULTS

Modulation of CS-induced inflammation in BALF of LT α ^{-/-} mice

Subacute and chronic CS exposure increased the number of total cells, alveolar macrophages, DCs, neutrophils and lymphocytes

in the BALF of both WT and LT α ^{-/-} mice, compared with air-exposed animals (fig. 1). Neutrophilic inflammation was significantly attenuated in LT α ^{-/-} mice at both time points (fig. 1d). In contrast, baseline levels of BAL lymphocytes, more specifically both CD4⁺ and CD8⁺ T-cells, were significantly higher in air-exposed LT α ^{-/-} mice than in WT mice, and increased further upon CS exposure (fig. 1e and f).

CS-induced pulmonary inflammation and emphysema

The CS-induced increase in lung macrophages and DCs was comparable between WT and LT α ^{-/-} mice (fig. 2a and b). Baseline B- and T-cell numbers were higher in the lungs of air-exposed LT α ^{-/-} mice *versus* WT mice. Contrary to the B-cell numbers, T-cell numbers increased strongly upon chronic CS exposure in the lung compartment of both WT and LT α ^{-/-} mice (fig. 2c and d).

In both WT and LT α ^{-/-} mice, chronic CS exposure induced significantly higher numbers of activated CD4⁺CD69⁺ and CD8⁺CD69⁺ T-cells. Yet again, both cell types were significantly increased in air-exposed LT α ^{-/-} mice in comparison with WT mice (fig. 2e and f). These steady state differences in lymphocyte numbers, between air-exposed WT and LT α ^{-/-} mice, were confirmed in the short-term experiment (data not shown).

The Lm increased significantly upon chronic CS exposure in WT mice (air 34.55 ± 0.35 *versus* smoke 37.35 ± 0.71 µm; 8.1% increase; $p=0.037$) as in LT α ^{-/-} mice (air 33.71 ± 0.50 *versus* smoke 36.71 ± 0.82 µm; 8.9% increase; $p=0.009$). There was no significant difference in Lm between CS-exposed WT and LT α ^{-/-} mice ($p=0.505$).

Increase of iNKT cells in the lung upon subacute CS exposure is LT α -dependent

iNKT cell numbers were determined through high specificity binding to CD1d-tetramer loaded with α GalCer [19] (fig. 3e). Subacute CS exposure caused a significant increase of iNKT cells in WT but not in LT α ^{-/-} mice (fig. 3a and b). In contrast, chronic CS exposure did not change iNKT cell numbers in the WT and knockout mice, but LT α ^{-/-} mice had lower percentages of iNKT cells compared with WT mice (fig. 3c and d).

LT α is not required for lymphoid neogenesis upon chronic CS exposure

Lymphoid aggregates were scarce in lung sections of air-exposed WT mice (fig. 4a and e), whereas the lungs of air-exposed LT α ^{-/-} mice were strongly infiltrated with lymphocytes (fig. 4c and 5). Chronic (24 weeks) CS exposure significantly increased the number of peribronchial and perivascular lymphoid aggregates in the lungs of both WT and LT α ^{-/-} mice (fig. 4). Lymphoid aggregates were absent in the lungs of WT mice after subacute (4 weeks) exposure to air or CS (data not shown). In contrast, lungs of air-exposed LT α ^{-/-} mice already showed a high degree of lymphocyte infiltration, but short-term CS exposure did not induce additional aggregates (aggregates/bronchovascular bundles: air-exposed LT α ^{-/-} mice: 0.31 ± 0.059 , CS-exposed LT α ^{-/-} mice: 0.28 ± 0.054 ; mean $\pm$ SEM, $n=5$ animals·group⁻¹).

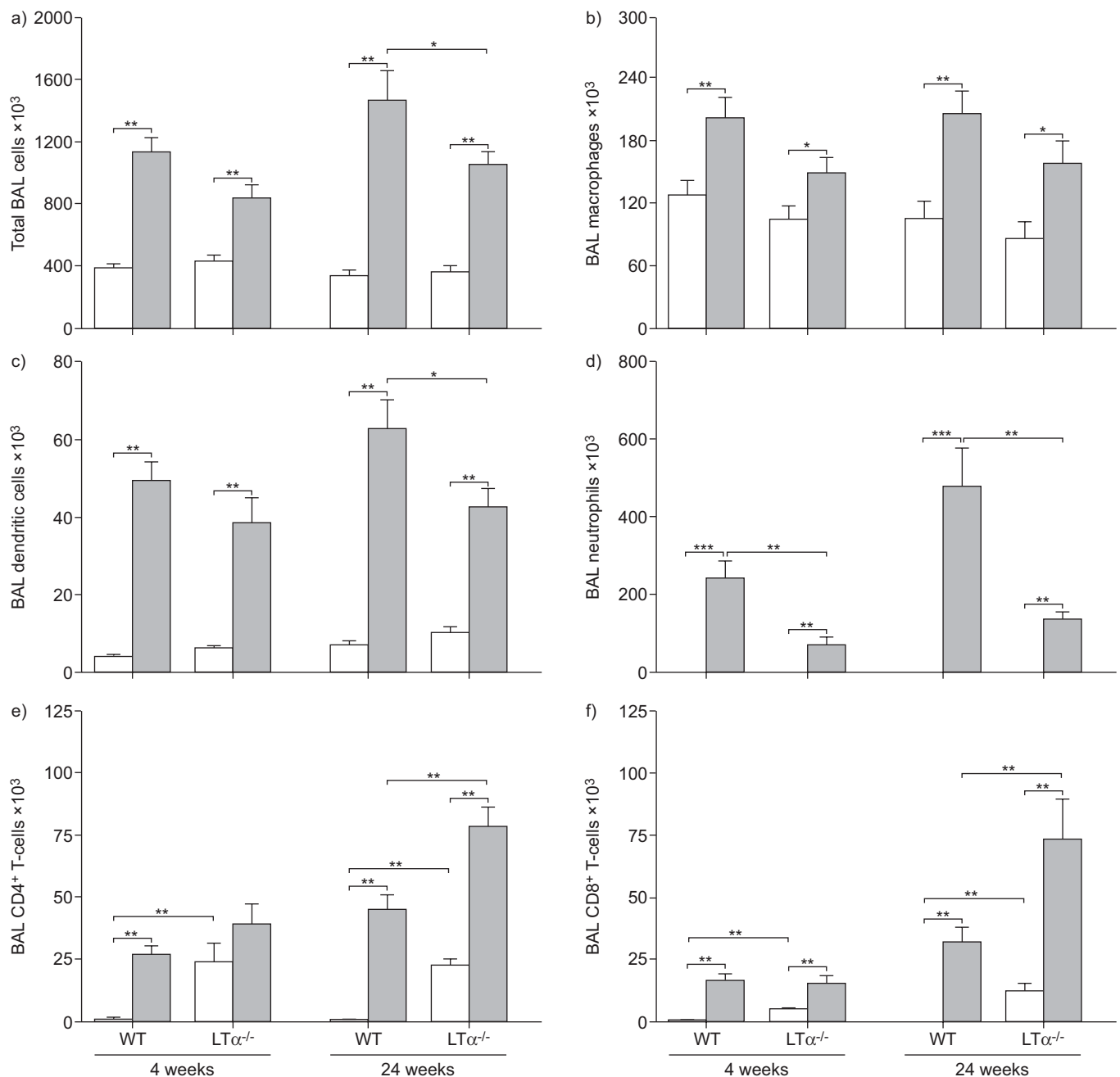


FIGURE 1. Total bronchoalveolar (BAL) cells and cell differentiation in BAL fluid of wild-type (WT) and lymphotoxin- α knockout (LT $\alpha^{-/-}$) mice after subacute (4 weeks) or chronic (24 weeks) exposure to air (□) or cigarette smoke (■). a) Total BAL cells, b) macrophages, c) dendritic cells, d) neutrophils, e) CD4⁺ T-cells and f) CD8⁺ T-cells (a and d were calculated on cytopins; b, c, e and f were enumerated by flow cytometry). Results are expressed as mean $\pm$ SEM. n=8 animals/group¹. *: p<0.05; **: p<0.01; ***: p<0.001.

Local and systemic IgA response upon chronic CS exposure is attenuated in LT $\alpha^{-/-}$ mice

Serum and BALF IgA levels were significantly diminished in LT $\alpha^{-/-}$ mice compared with WT animals, both at baseline in air-exposed mice and upon CS exposure for 24 weeks (fig. 5). In serum, the relative increase in IgA levels upon chronic CS exposure was larger in the LT $\alpha^{-/-}$ mice compared with WT (fig. 5a), whereas in BALF, the opposite was observed (fig. 5b). Chronic CS exposure increased S-IgA levels in BALF of WT

mice, while BALF S-IgA levels in LT $\alpha^{-/-}$ mice were significantly lower, both in air- and CS-exposed animals (fig. 5c), indicating impairment of S-IgA responses. In contrast, IgM levels were higher in serum and BALF of LT $\alpha^{-/-}$ mice, both at baseline and upon CS exposure (fig. 5d and e).

LT α affects chemokine expression upon CS exposure

mRNA expression of CXCL13 and CCL19 was significantly increased in the total lung after CS exposure in WT animals,

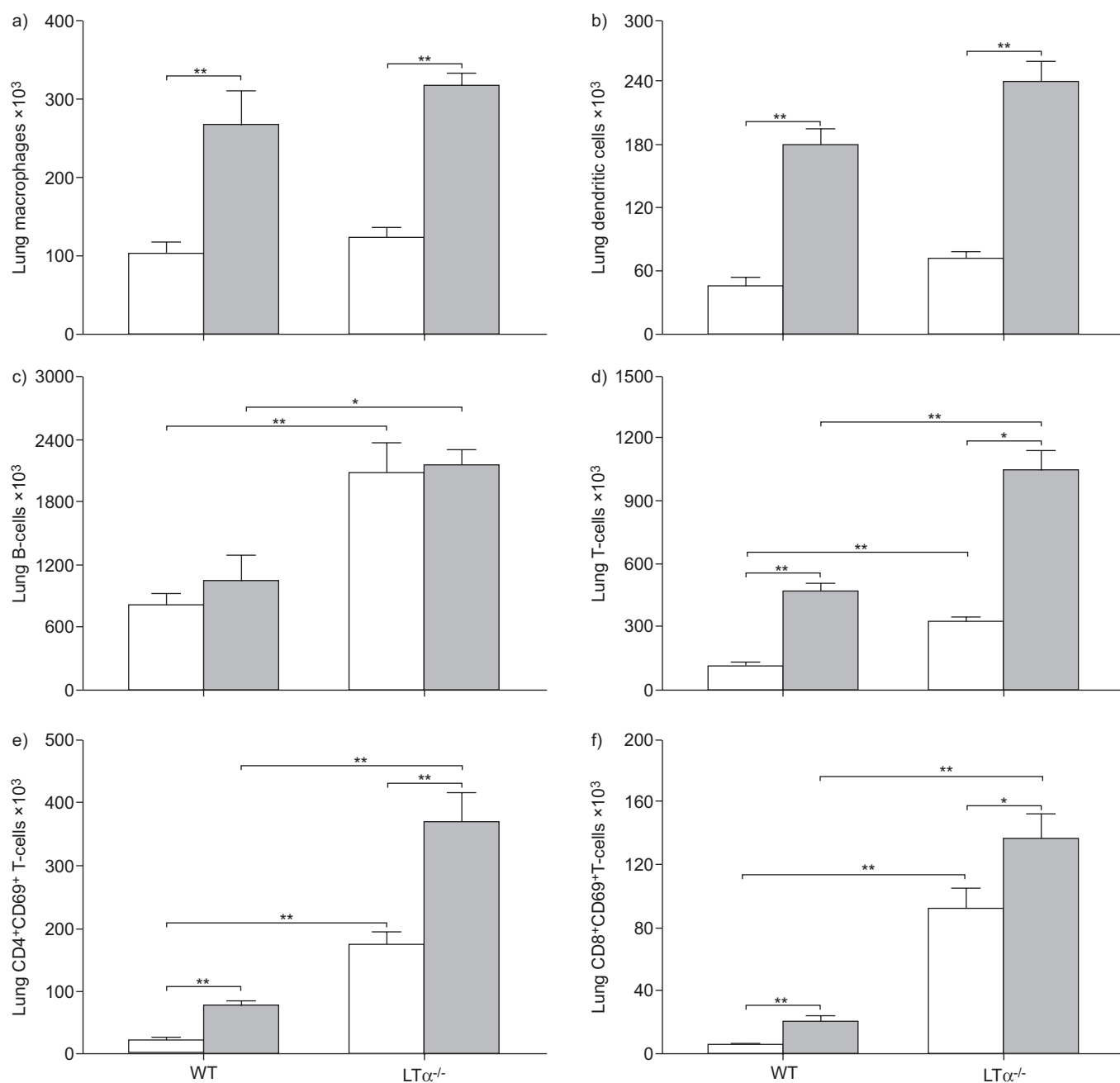


FIGURE 2. Cell differentiation in the lungs of wild-type (WT) and lymphotoxin- α knockout (LT $\alpha^{-/-}$) mice after chronic (24 weeks) exposure to air (□) or cigarette smoke (■). a) Macrophages, b) dendritic cells, c) B-cells, d) T-cells, e) activated CD4⁺ T-cells and f) activated CD8⁺ T-cells (all cell types were enumerated by flow cytometry). Results are expressed as mean $\pm$ SEM. n=8 animals/group⁻¹. *: p<0.05; **: p<0.01.

whereas this increase could not be seen in LT $\alpha^{-/-}$ mice (fig. 6a and c). mRNA levels of CCL20/macrophage inflammatory protein (MIP)-3 α , an attractant for immature DCs and lymphocytes, were increased in lungs of WT mice upon CS exposure (fig. 6d). Baseline MIP-3 α expression was higher in LT $\alpha^{-/-}$ mice compared with WT mice, with a strong tendency to increase after chronic CS exposure (p=0.076).

Total lung mRNA levels of CXCR5 and CCR7, the receptors for CXCL13 and CCL19, respectively, were higher at baseline in LT $\alpha^{-/-}$ mice (CXCR5: WT mice in air 0.28 ± 0.045 versus smoke 0.36 ± 0.065 ; LT $\alpha^{-/-}$ mice in air 2.09 ± 0.52 versus smoke 1.74 ± 0.26 ;

CCR7: WT mice in air 0.39 ± 0.038 versus smoke 0.73 ± 0.086 ; LT $\alpha^{-/-}$ mice in air 1.29 ± 0.19 versus smoke 1.33 ± 0.16). CCR7 mRNA expression increased upon subacute CS exposure in WT, but not in LT $\alpha^{-/-}$ mice.

CXCL13 protein levels in BALF were upregulated upon subacute and chronic CS exposure, again only in WT mice (fig. 6b). Immunohistochemistry in WT mice revealed CXCL13 expression in CS-induced lymphoid aggregates (fig. 6e and f). CS exposure did not affect protein levels of total CCL21 in BALF of both WT and LT $\alpha^{-/-}$ mice (data not shown).

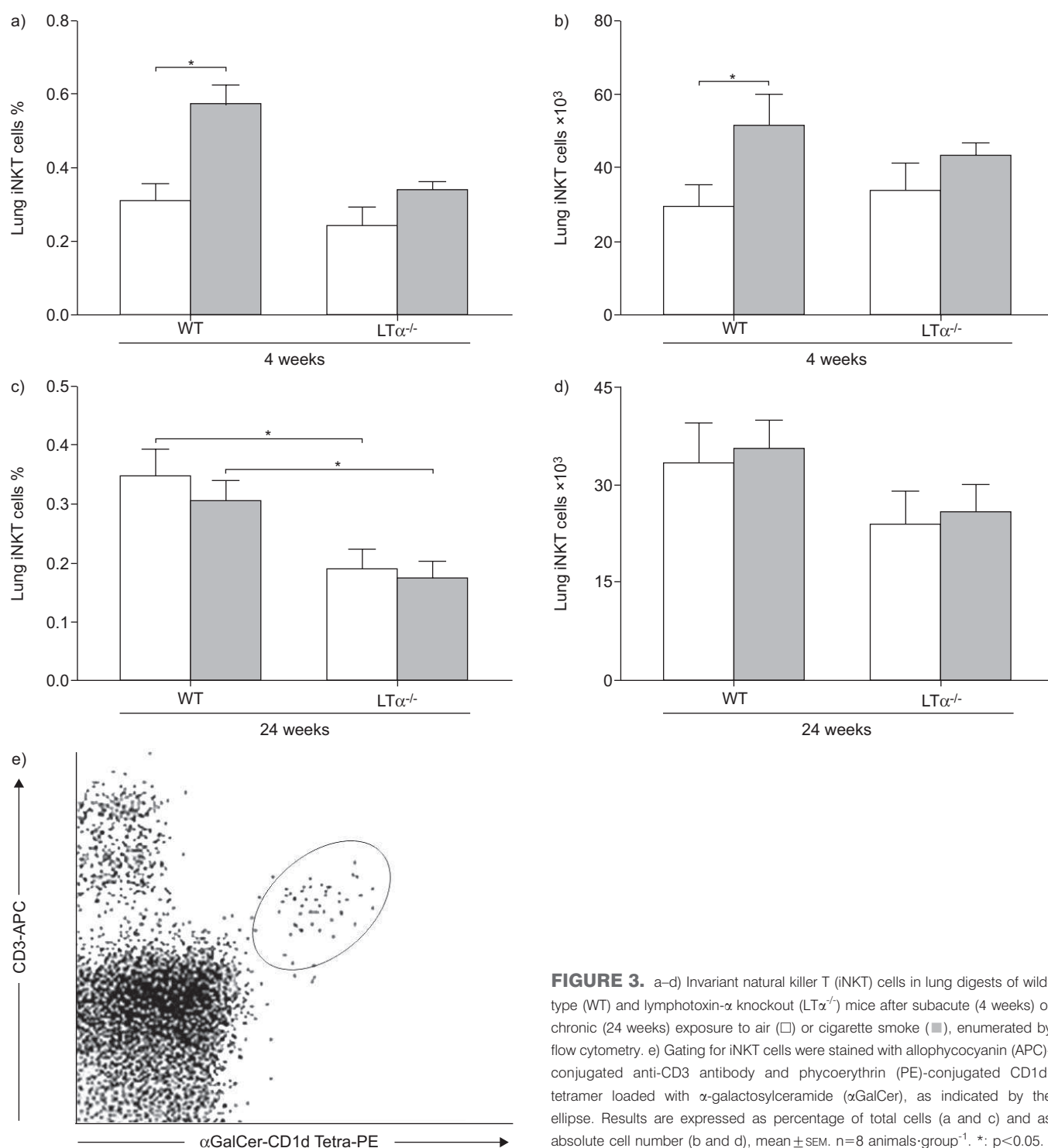


FIGURE 3. a–d) Invariant natural killer T (iNKT) cells in lung digests of wild-type (WT) and lymphotoxin- α knockout (LT $\alpha^{-/-}$) mice after subacute (4 weeks) or chronic (24 weeks) exposure to air ($\square$) or cigarette smoke ($\blacksquare$), enumerated by flow cytometry. e) Gating for iNKT cells were stained with allophycocyanin (APC)-conjugated anti-CD3 antibody and phycoerythrin (PE)-conjugated CD1d-tetramer loaded with α -galactosylceramide (α GalCer), as indicated by the ellipse. Results are expressed as percentage of total cells (a and c) and as absolute cell number (b and d), mean $\pm$ SEM. n=8 animals/group $^{-1}$. *: p<0.05.

***In vitro* expression of CXCL13 and CCL19 in lung fibroblasts is LT β R mediated**

To reveal the mechanism by which CXCL13 and CCL19 are induced in the lung, we stimulated cultured WT and LT β R $^{-/-}$ lung fibroblasts with agonistic LT β R Ab and/or CSE.

In WT lung fibroblasts, *in vitro* stimulation with agonistic LT β R Ab or CSE resulted in a 60-fold and five-fold increase in

expression of CXCL13 mRNA, respectively. Furthermore, the combination of LT β R Ab with CSE induced a 100-fold upregulation in CXCL13 mRNA levels (fig. 7a). A similar CCL19 mRNA response was seen upon stimulation with LT β R Ab or LT β R Ab with CSE, but there was no significant effect of CSE exposure alone (fig. 7c). The different stimulations did not affect CXCL13 and CCL19 mRNA expression in LT β R $^{-/-}$ lung fibroblasts (fig. 7b and d), however, baseline mRNA levels of

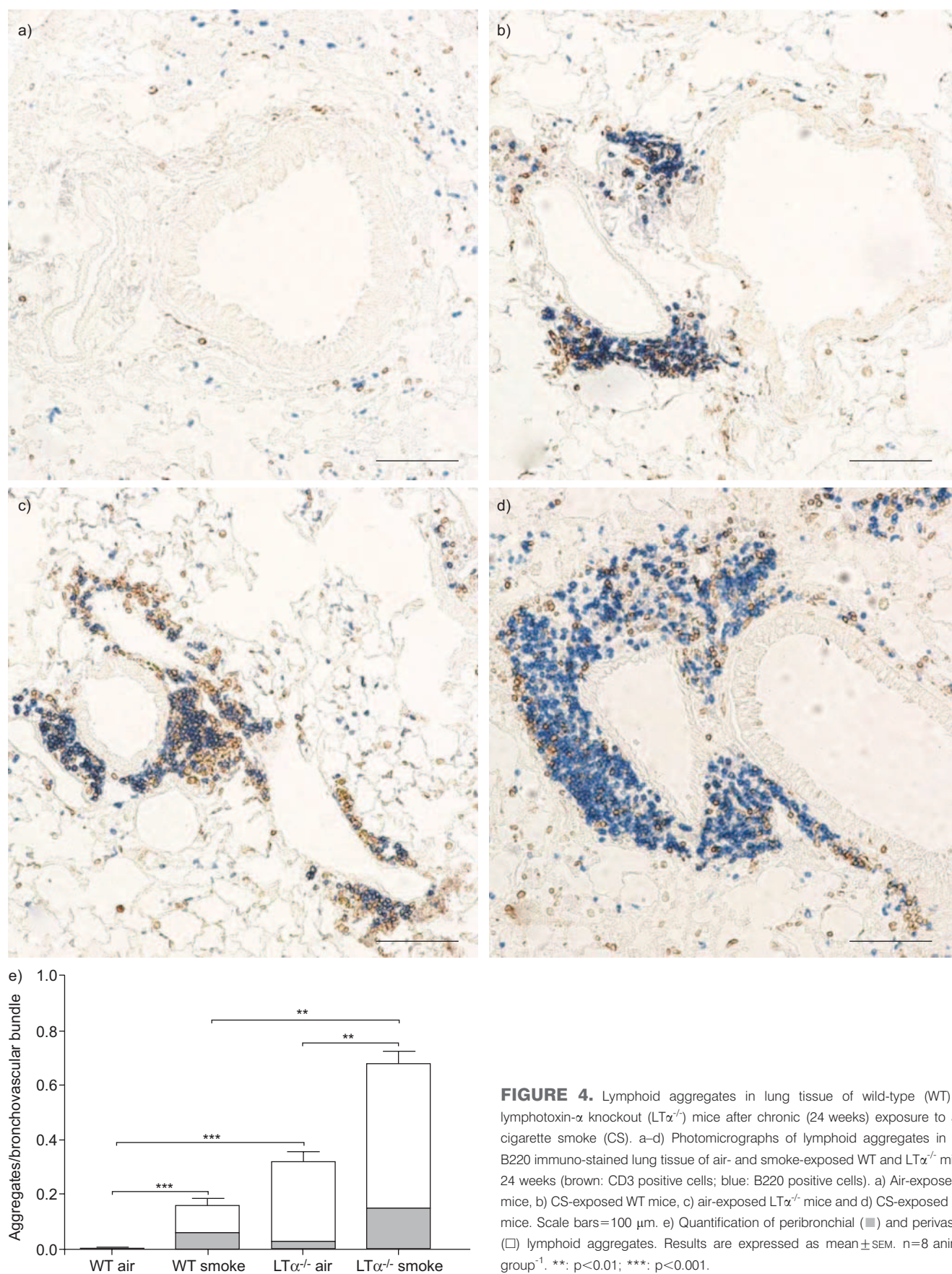


FIGURE 4. Lymphoid aggregates in lung tissue of wild-type (WT) and lymphotoxin- α knockout ($LT\alpha^{-/-}$) mice after chronic (24 weeks) exposure to air or cigarette smoke (CS). a–d) Photomicrographs of lymphoid aggregates in CD3/B220 immuno-stained lung tissue of air- and smoke-exposed WT and $LT\alpha^{-/-}$ mice at 24 weeks (brown: CD3 positive cells; blue: B220 positive cells). a) Air-exposed WT mice, b) CS-exposed WT mice, c) air-exposed $LT\alpha^{-/-}$ mice and d) CS-exposed $LT\alpha^{-/-}$ mice. Scale bars=100 μ m. e) Quantification of peribronchial (■) and perivascular (□) lymphoid aggregates. Results are expressed as mean $\pm$ SEM. n=8 animals/group⁻¹. **, p<0.01; ***, p<0.001.

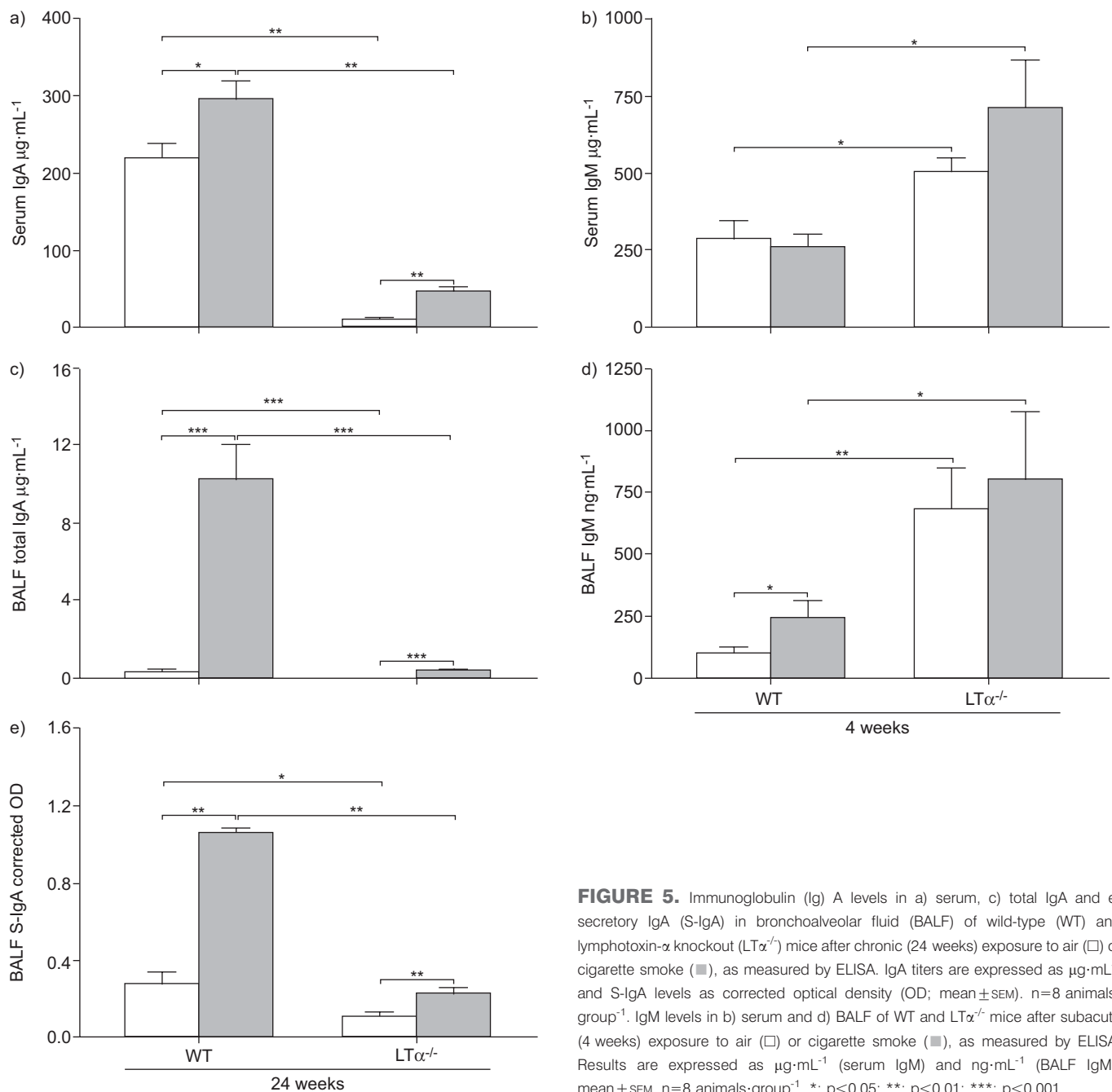


FIGURE 5. Immunoglobulin (Ig) A levels in a) serum, c) total IgA and e) secretory IgA (S-IgA) in bronchoalveolar fluid (BALF) of wild-type (WT) and lymphotoxin- α knockout ($\text{LT}\alpha^{-/-}$) mice after chronic (24 weeks) exposure to air (□) or cigarette smoke (■), as measured by ELISA. IgA titers are expressed as $\mu\text{g}\cdot\text{mL}^{-1}$ and S-IgA levels as corrected optical density (OD; mean $\pm$ SEM). $n=8$ animals·group $^{-1}$. IgM levels in b) serum and d) BALF of WT and $\text{LT}\alpha^{-/-}$ mice after subacute (4 weeks) exposure to air (□) or cigarette smoke (■), as measured by ELISA. Results are expressed as $\mu\text{g}\cdot\text{mL}^{-1}$ (serum IgM) and $\text{ng}\cdot\text{mL}^{-1}$ (BALF IgM), mean $\pm$ SEM. $n=8$ animals·group $^{-1}$. *: $p<0.05$; **: $p<0.01$; ***: $p<0.001$.

both CXCL13 and CCL19 were higher compared with WT lung fibroblasts (data not shown).

DISCUSSION

Using *in vivo* studies with WT and $\text{LT}\alpha^{-/-}$ mice, we have demonstrated for the first time that CS-induced pulmonary lymphocyte accumulation and lymphoid neogenesis do not require $\text{LT}\alpha$. Moreover, $\text{LT}\alpha^{-/-}$ mice are not protected against the development of CS-induced emphysema. In contrast, $\text{LT}\alpha$ is required for CS-induced upregulation, but not for baseline expression, of the lymphoid chemokines CXCL13 and CCL19. We have shown *in vitro* that the upregulation of CXCL13 and CCL19 mRNA levels in lung fibroblasts is $\text{LT}\beta\text{R}$ mediated.

Furthermore, CSE itself is sufficient to induce CXCL13 expression in cultured lung fibroblasts *via* $\text{LT}\beta\text{R}$.

In accordance with previous reports [9], the lungs of air-exposed $\text{LT}\alpha^{-/-}$ mice were strongly infiltrated with B- and T-cells. Moreover, we found high steady state numbers of CD4^{+} and CD8^{+} T-cells in BALF of air-exposed $\text{LT}\alpha^{-/-}$ mice compared with WT mice, in line with earlier research [22]. Extensive serology proved all animals to be free of a wide range of viruses and bacteria. Most likely, the lack of lymph nodes in $\text{LT}\alpha^{-/-}$ mice causes an accumulation of lymphocytes in organs such as the lungs and the liver, as described previously [9].

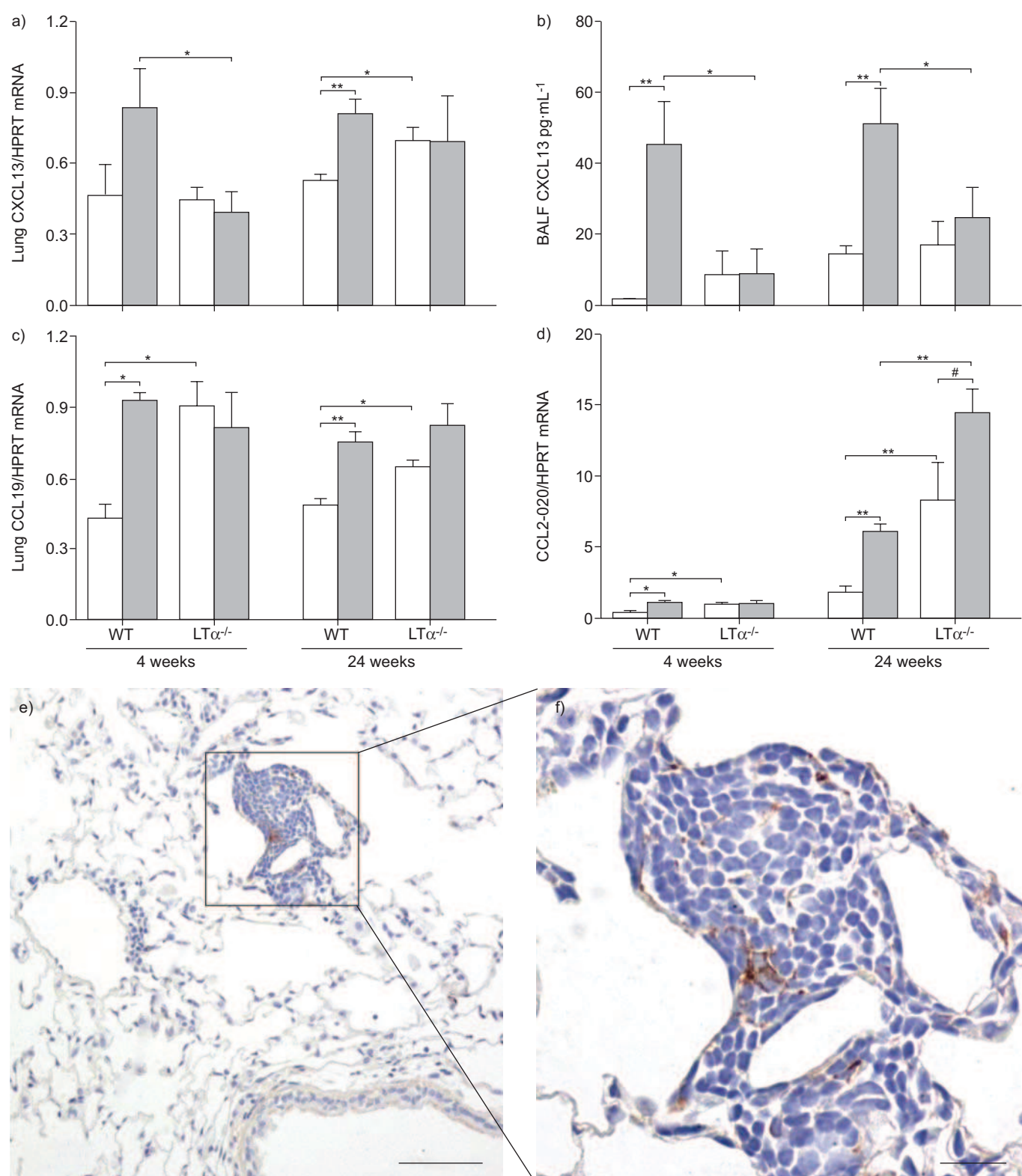


FIGURE 6. Measurement of chemokines in bronchoalveolar fluid (BALF) and lung tissue. mRNA levels of a) CXC chemokine ligand (CXCL) 13, c) CC chemokine ligand (CCL)19 and d) CCL20 in total lung tissue of wild-type (WT) and lymphotoxin- α knockout (LT $\alpha^{-/-}$) mice after subacute (4 weeks) or chronic (24 weeks) exposure to air ($\square$) or cigarette smoke ($\blacksquare$), as measured by RT-PCR. Results are expressed as ratio with hypoxanthine guanine phosphoribosyltransferase (HPRT) mRNA (mean $\pm$ SEM). n=5 animals·group⁻¹. b) Protein levels of CXCL13 in BALF of WT and LT $\alpha^{-/-}$ mice after subacute (4 weeks) or chronic (24 weeks) exposure to air ($\square$) or cigarette smoke ($\blacksquare$), as measured by ELISA. Results are expressed as pg·mL⁻¹ (mean $\pm$ SEM). n=8 animals·group⁻¹. e and f) Photomicrographs of a CXCL13-positive lymphoid aggregate in a smoke-exposed WT animal at 24 weeks. e) Scale bar=100 μ m; f) scale bar=20 μ m. *: p<0.05; **: p<0.01; #: p=0.076.

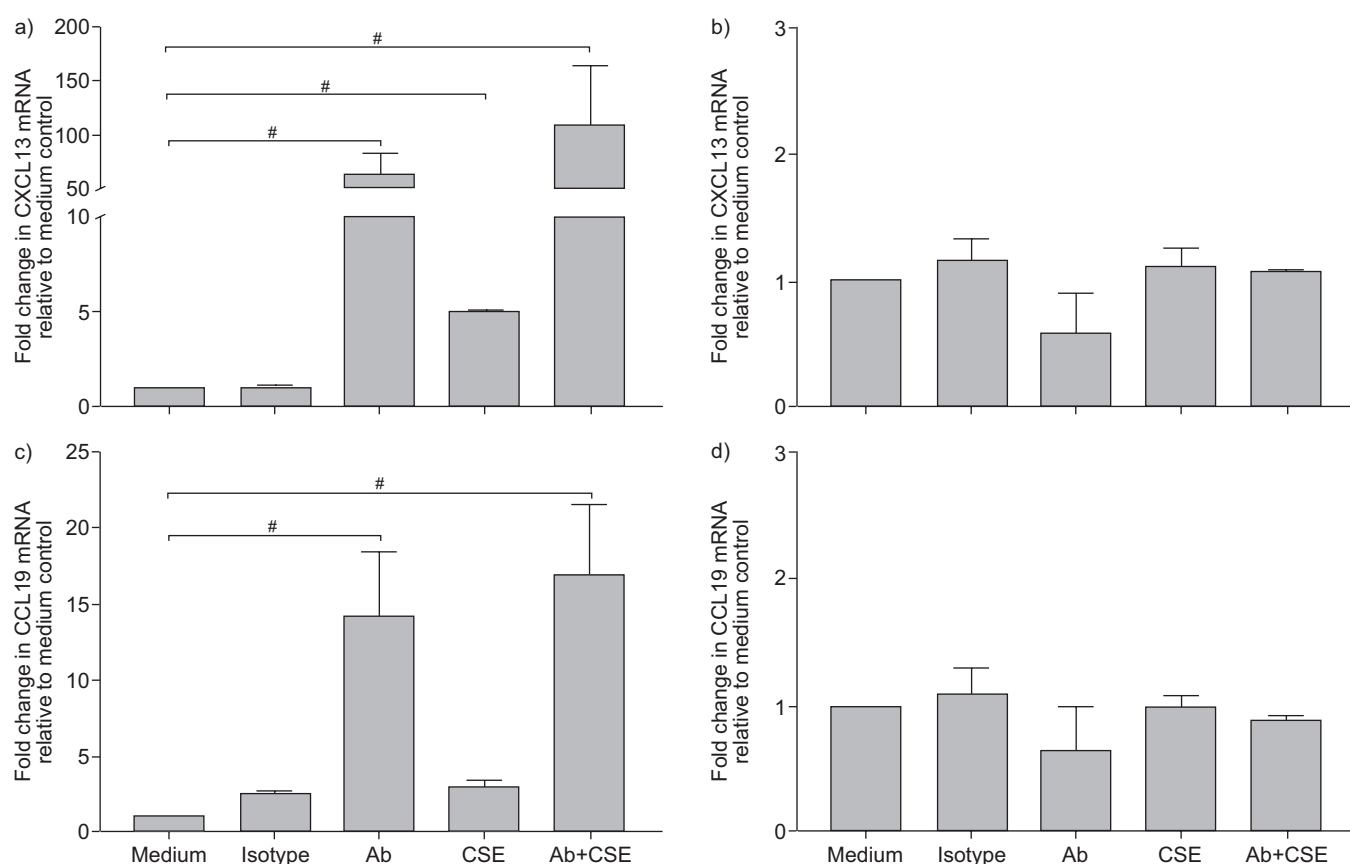


FIGURE 7. Expression of lymphoid chemokines CXC chemokine ligand (CXCL) 13 and CC chemokine ligand (CCL) 19 by lung fibroblasts, in response to lymphotoxin- β receptor (LT β R) stimulation *in vitro*. a–d) Cultured lung fibroblasts were exposed for 48 h to Dulbecco's modified Eagle medium, isotype control, agonistic LT β R antibody (Ab), cigarette smoke extract (CSE) or the combination of LT β R Ab with CSE (Ab+CSE). mRNA levels of CXCL13 (a and b) and CCL19 (c and d) in wild-type (WT; a and c) and LT β R^{-/-} fibroblasts (b and d), as measured by RT-PCR. Results are expressed as ratio with hypoxanthine guanine phosphoribosyltransferase mRNA; mean of three experiments $\pm$ SEM. #: $p \leq 0.05$.

Lymphoid chemokines are critical for neogenesis and organisation of lymphoid tissue [23, 24]. The lymphoid chemokines CXCL13 and CCL21 are greatly downregulated in the spleen, but not in the lungs of LT α ^{-/-} mice [13, 25, 26], possibly causing a reversed chemokine gradient and consequent homing of lymphocytes to the lung. We found baseline mRNA levels of CXCL13, CCL19 and CCL20 to be elevated in lungs of air-exposed LT α ^{-/-} mice compared with WT mice, which further explains increased recruitment of B- and T-cells to the lungs.

In WT mice, CS exposure increases the expression of CXCL13 and CCL19, a B-cell and T-cell attractant, respectively. Furthermore, the presence of CXCL13 in pulmonary lymphoid aggregates of smoke-exposed WT mice indicates a role for this chemokine in ongoing organisation. Whereas LT α is not required for the baseline expression of CXCL13 and CCL19, it is essential for the CS-induced upregulation of these lymphoid chemokines. In secondary lymphoid tissue organogenesis, a positive feedback loop mechanism has been described: CXCL13 induces LT α ₁ β ₂ expression on attracted B-cells through CXCR5 and subsequent stimulation of LT β R on stromal cells, which in turn upregulates CXCL13 production [27, 28]. Indeed, we found cultured lung fibroblasts to be

responsive to LT β R stimulation through enhanced CXCL13 and CCL19 expression. More importantly, CSE induced *in vitro* CXCL13 expression in WT, but not in LT β R^{-/-} lung fibroblasts. In line with a previous report on LT β expression in stromal cells [29], we detected a discreet expression of LT β in fibroblasts, which was upregulated by CSE, only in WT fibroblasts (data not shown). CSE may thus contain or induce LT β R ligands, implicating the LT β R pathway in CS-induced CXCL13 expression in the lung. However, we can not exclude a general nonresponsiveness of LT β R^{-/-} lung fibroblasts towards the different stimuli. In figure 8, we propose a mechanism for lymphoid chemokine expression in the lung upon CS exposure.

Interestingly, CXCR5 and CCR7, the receptors for CXCL13 and CCL19, respectively, appear to be important for lymphoid neogenesis in chronic inflammatory autoimmune disease [30]. In contrast, the inability of LT α ^{-/-} mice to generate a CXCL13- and CCL19-response does not prevent the development of pulmonary lymphoid aggregates upon chronic CS exposure, suggesting other chemokines are involved. We demonstrated that CCL20, a chemokine that attracts immature DCs and lymphocytes, was higher in lungs of air-exposed LT α ^{-/-} mice

compared with WT mice, and increased further upon chronic CS exposure. CCL20 is thus possibly involved in both steady state and CS-induced lymphocyte recruitment and lymphoid aggregate formation in $LT\alpha^{-/-}$ mice.

In WT mice, chronic CS exposure induces lymphoid neogenesis with germinal centre formation [5]; the latter is known to be $LT\alpha$ dependent [31]. Accordingly, baseline and CS-induced IgA levels in BALF and serum were significantly attenuated in $LT\alpha^{-/-}$ mice compared with WT animals, whereas $LT\alpha^{-/-}$ mice also showed higher baseline IgM titers in BALF and serum, consistent with a putative Ig isotype switching deficiency. WT mice generated a strong IgA response in BALF upon chronic CS exposure. Similarly, IgA appears to be increased in bronchial secretions of chronic bronchitis patients [32, 33]. However, when IgA levels are corrected for albumin, to exclude diffusion effects, there is in fact a decrease of indices of local active transport of IgA across the epithelium [32, 33], which could relate to reduced expression of epithelial polymeric immunoglobulin receptor/transmembrane secretory component in COPD airways [34]. In CS-exposed WT mice, IgA transport towards the airway lumen appeared intact, whereas the strongly reduced S-IgA concentrations in BALF of $LT\alpha^{-/-}$ animals could relate to decreased local IgA synthesis (general defect in class switching to IgA, which could also underlie reduced serum IgA levels) and/or to decreased trans-epithelial transport. Altogether, our B-cell data could indicate impairment in class switch recombination mechanisms in $LT\alpha^{-/-}$ mice, as previously suggested by others [35].

Currently, there is considerable controversy on the role of iNKT cells in airway disease [36, 37], a cell population that is profoundly diminished in the periphery of $LT\alpha$ deficient mice [15]. Therefore, iNKT cell frequencies were monitored during the course of CS exposure. iNKT cell percentages were significantly reduced in lungs of $LT\alpha^{-/-}$ mice in the chronic experiment. Moreover, subacute CS-exposure increased pulmonary iNKT cell numbers in a $LT\alpha$ -dependent fashion. This is

in line with the modulating role of iNKT cells between innate and adaptive immunity, since at this time point (4 weeks), immune responses shift from innate to adaptive in this experimental model of COPD [16]. When the adaptive immune system has kicked in, iNKT cell numbers are no longer different between air and CS exposed lungs of WT mice. Corresponding with data from COPD patients [37], our findings do not indicate a role for iNKT cells in the chronic disease model.

In COPD patients, lymphoid aggregates have been correlated with disease severity [6, 7]. Hypothesising that the pathogenesis of COPD has an autoimmune component, pulmonary lymphoid aggregates could add to the perpetuating nature of COPD and enhance the production of auto-antibodies in the germinal centres. Conversely, ectopic lymphoid tissue could very well have a local protective role against infections [7]. Although $LT\alpha$ polymorphisms are not directly associated with COPD [38, 39], potential indirect effects on inflammatory mediators should not be excluded.

In conclusion, we have demonstrated that CS-induced pulmonary lymphoid neogenesis does not require $LT\alpha$. CS induces the pulmonary expression of lymphoid chemokines CXCL13 and CCL19 in a $LT\alpha\beta$ - $LT\beta$ R-dependent fashion. This murine model of COPD will be useful to further characterise the developing mechanisms and function of CS-induced lymphoid neogenesis.

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STATEMENT OF INTEREST

None declared.

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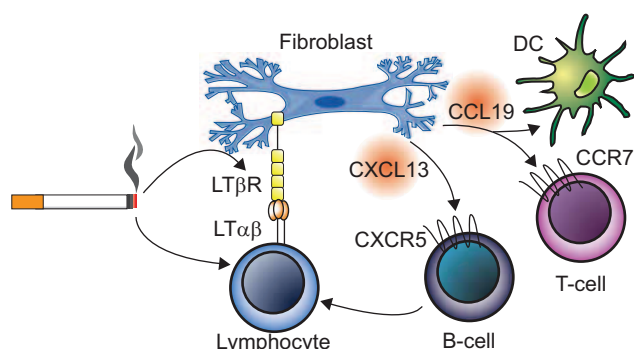


FIGURE 8. Proposed mechanism for lymphoid chemokine expression in the lung upon cigarette smoke (CS) exposure. CS induces pulmonary inflammation and subsequent expression of lymphotoxin (LT)- $\alpha\beta$ on lymphocytes, but may also directly stimulate the $LT\beta$ receptor ($LT\beta$ R) on stromal cells, such as lung fibroblasts. $LT\beta$ R stimulation leads to the production of CC chemokine ligand (CCL) 19 and CXC chemokine ligand (CXCL) 13 which attract dendrite cells (DCs) and T- and B-cells, respectively. Finally, CXCL13 may induce $LT\alpha\beta$ on B-cells, creating a positive feedback loop mechanism. CXCR: CXC chemokine receptor; CCR: CC chemokine receptor.

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