Leptin Modulates Innate and Adaptive Immune Cell Recruitment after Cigarette Smoke Exposure in Mice


*J Immunol* 2010; 184:7169-7177; Prepublished online 19 May 2010;
doi: 10.4049/jimmunol.0900963
http://www.jimmunol.org/content/184/12/7169

**References**
This article cites 57 articles, 25 of which you can access for free at:
http://www.jimmunol.org/content/184/12/7169.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Leptin Modulates Innate and Adaptive Immune Cell Recruitment after Cigarette Smoke Exposure in Mice

Juanita H. J. Vernooy,*§† Ken R. Bracke,*† Nadja S. A. Pauwels,*‡ Lennart Zabeau,§ Robert Jan van Suylen,§ Jan Tavernier,§ Guy F. Joos,‡ Emiel F. M. Wouters,*§† and Guy G. Brusselle‡

Leptin, a pleiotropic type I cytokine, was recently demonstrated to be expressed by resident lung cells in chronic obstructive pulmonary disease patients and asymptomatic smokers. To elucidate the functional role of leptin in the onset of chronic obstructive pulmonary disease, we tested leptin-deficient ob/ob mice (C57BL/6), leptin receptor-deficient db/db mice (C57BKS), and littermates in a model of cigarette smoke (CS)-induced pulmonary inflammation. Wild-type (WT) C57BL/6 mice were exposed for 4 or 24 wk to control air or CS. Pulmonary leptin expression was analyzed by immunohistochemistry and real-time PCR. Pulmonary inflammation upon 4 wk CS exposure was evaluated in bronchoalveolar lavage fluid (BALF) and lung tissue of WT, ob/ob, and db/db mice. Immunohistochemical analysis revealed leptin expression in bronchial epithelial cells, pneumocytes, alveolar macrophages, and bronchial/vascular smooth muscle cells. The 4 and 24 wk CS exposure increased leptin expression in bronchial epithelial cells and pneumocytes versus air-exposed WT mice (p < 0.05). The 4 wk CS exposure resulted in increased accumulation of neutrophils, dendritic cells, macrophages, and lymphocytes in BALF and lung tissue of WT, ob/ob, and db/db mice. CS-exposed ob/ob and db/db mice showed in general higher numbers of neutrophils and lower numbers of CD4+, CD8+, and dendritic cells versus CS-exposed WT mice. Consistently, CXCL1 levels were enhanced in BALF of CS-exposed ob/ob and db/db mice versus WT mice (p < 0.05). Exogenous leptin administration completely restored the skewed inflammatory profile in ob/ob mice. These data reveal an important role of leptin in modulating innate and adaptive immunity after CS inhalation in mice. The Journal of Immunology, 2010, 184: 7169–7177.

Chronic obstructive pulmonary disease (COPD) is one of the leading causes of mortality and morbidity worldwide, and its prevalence is expected to increase in the next decades (1). The disease is characterized by the development of airflow limitation, which is slowly progressive and poorly reversible. The airflow limitation is due to a combination of chronic obstructive bronchiolitis, destruction of lung parenchyma (emphysema), and structural alterations of the small airways (2). Cigarette smoking is the major risk factor for the development of COPD, and it has been shown that chronic exposure to cigarette smoke (CS) leads to an abnormal inflammatory response of the lungs. Different kinds of inflammatory cells have been described to participate in this chronic pulmonary inflammation in COPD, including neutrophils, macrophages, and T lymphocytes (3). In addition, a recent study showed a significant increase in dendritic cell (DC) numbers in small airways of COPD patients, which correlated with disease severity (4). The infiltrating inflammatory cells are capable of releasing inflammatory mediators and proteases, which are believed to play a role in the progressive lung destruction in COPD (3).

Several cytokines and chemokines are assumed to regulate the activation, recruitment, and survival of these inflammatory cells into the airways and pulmonary tissue of COPD patients, such as TNF-α, CCL2, and CXCL8 (5). A novel candidate to regulate pulmonary immune function in COPD is leptin (6), a 16-kDa nonglycosylated peptide encoded by the ob gene. Leptin was originally described as an adipocyte-derived hormone involved in regulating the balance between food intake and energy expenditure by activating its functional receptor (Ob-Rb) (7) in the hypothalamus (8). Recent studies reported that leptin secretion not only by adipocytes but also by various epithelial cell types, including bronchial epithelial cells (BECs) (6, 9) and type II pneumocytes (6). The structure of leptin and its receptor classify leptin as a type I cytokine. The almost universal distribution of leptin receptors in the lung (9) suggests that leptin might play an important role in the function of both innate and adaptive immunity (10), making it an ideal candidate for a role in chronic inflammatory airway diseases such as COPD. On neutrophils and macrophages, leptin stimulates chemotaxis (11) and enhances functional capacities, such as oxidative burst (11), phagocytosis (12), and cytokine...
secretion (13, 14). In addition, leptin exerts proliferative (15) and antiapoptotic effects (16) on T lymphocytes and promotes Th1 cell differentiation (17). Furthermore, leptin enhances host response to inflammation and infection by stimulating tissue repair via its mitogenic and angiogenic properties on epithelium and endothelium (18, 19).

Normal lung tissue displays particularly high levels of Ob-Rb (20, 21), and specific leptin-binding sites have been identified in BECs and pneumocytes (22–24). Recent observations that leptin is actually present in induced sputum (25), proximal airway biopsies (9), and peripheral lung tissue (6) of COPD patients designate the lung as a peripheral site of action for leptin. In addition, we showed that exposure of cultured (primary) epithelial cells to CS concentrate, cytokines, or glucocorticoids resulted in increased expression of both leptin and Ob-Rb (6). In light of the immunomodulating role of leptin, leptin may regulate pulmonary immune function in chronic inflammatory airway diseases, such as COPD. To elucidate the functional role of leptin in the onset of COPD, we tested leptin-deficient mice (ob/ob), leptin receptor-deficient mice (db/db), and their littermates in a model of CS-induced pulmonary inflammation (26). We hypothesized that CS exposure would enhance pulmonary leptin expression and therefore analyzed pulmonary leptin in wild-type (WT) mice after subacute (4 wk) or chronic (24 wk) exposure to CS. In addition, we hypothesized that ob/ob mice and db/db mice would exhibit a disturbed inflammatory response to CS exposure that could be corrected by the administration of leptin in vivo. To this end, we quantified the inflammatory responses in both bronchoalveolar lavage fluid (BALF) and lung tissue in ob/ob, db/db, and WT mice after 4 wk CS exposure. In addition, we investigated whether leptin replacement therapy could restore the altered inflammatory profile in ob/ob mice.

Materials and Methods

Animals

Eight-week-old male leptin-deficient ob/ob mice on a C57BL/6 background (B6.V-Lepob), leptin receptor-deficient db/db mice on a C57BKS background (BKS.Cg-M/m+/-Lepob), and WT control mice (C57BL/6 and C57BKS background) were purchased from Taconic Europe (Ry, Denmark). Mice were maintained with standard rodent chow and had free access to water. All of the in vivo manipulations were approved by the local Ethics Committee for animal experimentation of the Faculty of Medicine and Health Sciences (University of Ghent, Ghent, Belgium).

Smoke exposure

Mice (n = 8 per group) were exposed whole body to CS as described previously (26, 27). Briefly, groups of eight mice were exposed to the tobacco smoke of five cigarettes (Reference Cigarette 2R4F without filter, University of Kentucky, Lexington, KY) four times a day with 30 min smoke-free intervals, 5 d a week for 4 or 24 wk. An optimal smoke-to-air ratio of 1:6 was obtained. The control groups were exposed to air. Carboxyhemoglobin concentrations were determined in serum of smoke-exposed mice reached a nontoxic level of 8.3 ± 1.4% in air-exposed mice (n = 7 for both groups), which is similar to carboxyhemoglobin blood concentrations of human smokers (28). Total body weight was recorded every week. Mice were killed 24 h after the last smoke exposure with an overdose of pentobarbital (Sanofi, Libourne, France), and blood was sampled from the orbital sinus. After insertion of a tracheal cannula, lungs were prepared for BAL. Following BAL, the pulmonary and systemic circulation was rinsed. The left lung was used for histology; the right lung was used for isolation of RNA and for preparation of lung single-cell suspensions.

Exogenous leptin administration

To replace leptin in ob/ob mice, C57BL/6 WT mice and ob/ob mice (n = 8 per group) were exposed to CS or air for 4 wk. An l.p. injection of recombinant murine leptin (45 μg per mouse per day) was administered daily, following CS or air exposure. Recombinant murine leptin, cloned in the PET11a vector, was expressed in Escherichia coli, solubilized in 7 M urea, and refolded. Protein was purified to homogeneity by anion-exchange chromatography on a HiTrap ANX FF column (GE Healthcare Life Sciences, Diegem, Belgium) followed by gel filtration on a HiLoad Superdex 75 column (GE Healthcare). Leptin concentrations were determined by absorbance at 280 nm. Endotoxins were removed using polymyxin B-agarose (Sigma-Aldrich, St. Louis, MO), and final levels were <0.1 endotoxin units per milligram of protein as measured with the Limulus amebocyte lysate method (Lonza, Basel, Switzerland). This leptin replacement protocol prevented body weight gain in ob/ob mice. Mice were killed 24 h following the final exposure, and leptin injection and lungs were harvested for BAL.

Immunohistochemistry

The left lung was fixed by gentle infusion of 4% paraformaldehyde through the tracheal cannula (26). After excision, the lung was immersed in fresh fixative for 2 h and embedded in paraffin. Histological sections (3 μm) were cut and processed for immunohistochemical staining for leptin according to Löffler et al. (29) with slight modifications. Immunoreactive epitopes of leptin were exposed by treatment with 0.05% Pronase E in 0.5 M Tris/HCl (pH 7.6), and nonspecific binding was blocked with 5% BSA in TBS. Leptin was detected using a rabbit polyclonal Ab against human leptin (SC-842, Santa Cruz Biotechnology, Santa Cruz, CA), which cross-reacts with mouse leptin. After application of biotin-conjugated goat anti-rabbit IgG Ab (E-0431, DakoCytomation, Glostrup, Denmark) and alkaline phosphatase–labeled avidin–biotin complex (ABC-AP, K-0376, DakoCytomation), enzymatic reactivity was visualized using Blue Substrate Kit III (SK-5300, Vector Laboratories, Burlingame, CA). Sections were counterstained with VectorFast Red and mounted. Negative controls for nonspecific binding by 1) omitting the primary detecting Ab or 2) preincubating the primary Ab with recombinant leptin (R&D Systems, Minneapolis, MN) revealed no signal. The slides were observed using a light microscope (Leica DMRB, Leica Microsystems, Rijswijk, The Netherlands) at ×20 magnification in a blinded fashion. Immunostaining for leptin was quantified in BECs and pneumocytes. For BECs, at least 500 cells were counted by one independent observer unaware of the experimental conditions; data are expressed as percentage leptin-positive BECs of total counted BEC. For pneumocytes, 5 to 10 fields of lung parenchyma were randomly selected for each section, and leptin-positive pneumocytes were scored semiquantitatively on an arbitrary five-point scale by two independent observers unaware of the experimental conditions. This five-point scale was defined as: 1, no or hardly any staining; 2, weak staining; 3, moderate staining; 4, strong staining; 5, very intense staining. The data represent the mean of the two observers; the coefficient variation between observers was <10%.

Quantitative real-time PCR

RNA was isolated from lung tissue stored at −80°C in RNAlater RNA Stabilization Reagent (Qiagen, Benelux, Venlo, The Netherlands) using the RNeasy Mini kit (Qiagen) including protease K and DNase I treatment. Total RNA (0.5 μg) was reverse-transcribed into cDNA using the Reverse Transcription-1st Strand Synthesis kit and oligo(dT) primers (Abgene, Epsom, U.K.). Primer pairs used in this study were designed with Primer Express 2.0 software (Applied Biosystems, Foster City, CA) and are listed in Table I. Real-time PCR reactions were performed in duplicate using diluted cDNA template (dilution 1:5 for leptin and 1:25 for reference genes), 0.6 pmol each primer (Table I), and IQTM SYBR Green Superscript 1 dye (Bio-Rad, Hercules, CA) in a total volume of 20 μl. A standard curve derived from the serial dilutions of a mixture of all of the samples was included on each plate. Quantitation and real-time detection of the PCR products was followed on an iCycler IQ Real-Time PCR Detection System (Bio-Rad) with the following cycling conditions: 3 min at 95°C for Platinum Taq activation and 40 cycles for the melting (15 s, 95°C) and annealing/extension (45 s, 60°C) steps. These conditions generate specific PCR products of the desired length, which was verified by digestion with an ethidium bromide-stained 2% agarose gel and by nucleotide sequencing. Data were processed using the standard curve-based method. Expression of leptin was corrected by a normalization factor that was calculated based on expression of the three reference genes (hprt1, pfkl, and rpl13a) using the geNorm applet (http://medgen.ugent.be/~vdesomp/genorm/) according to the guidelines and theoretical framework previously described (30).

BAL

The tracheal cannula was used to instill three times 300 μl HBSS, free of ionized calcium and magnesium and supplemented with 1% BSA, followed by three times 1 ml HBSS supplemented with 0.6 mM EDTA. The six lavage fractions were recovered by gentle manual aspiration, pooled, and centrifuged. The cell pellet was washed twice and finally resuspended
in 200 μl buffer (PBS supplemented with 1% BSA, 5 mM EDTA, and 1% sodium azide). A total cell count was performed in a Bürcker chamber, and the differential cell counts (on at least 400 cells) were performed on cytospun preparations using standard morphologic criteria after May–Grünewald-Giemsa staining. Flow cytometric analysis of BAL cells was performed to enumerate DCs and CD4+ and CD8+ T cells.

**Preparation of lung single-cell suspensions**

Lung single-cell suspension was prepared as detailed previously (31). Briefly, the lung was thoroughly minced, digested, subjected to BREC lysis, passed through a 50-μm cell strainer, and kept on ice until labeling. Cell counting was performed with a Z2 Beckman Coulter particle counter (Beckman Coulter, Ghent, Belgium).

**Labeling of BAL cells and lung single-cell suspensions for flow cytometry**

Cells were preincubated with FcR-blocking Ab (anti-CD16/CD32, clone 2.4G2) to reduce nonspecific binding. mAbs used to identify mouse DC populations were: allophycocyanin-conjugated anti-CD11c (clone HL3) and PE-conjugated anti-IAβ (AF6-120.1). We discriminated between macrophages and DCs using the methodology described by Vermaelen et al. (32). After gating on the CD11c-bright population, two peaks of autofluorescence can be distinguished. Macrophages are identified as the CD11c-bright, high autofluorescent population and do not express MHC class II. DCs are identified as CD11c-bright, low autofluorescent cells, which strongly express MHC class II. DCs enumerated by these criteria correspond with myeloid DCs. The following Abs were used to stain mouse T cell subpopulations: FITC-conjugated anti-CD4 (L3T4), FITC-conjugated anti-CD8 (Ly-2), and allophycocyanin-conjugated anti-CD3 (145-2C11) mAbs. The additional marker used for activation was PE-conjugated anti-CD69 (H1.2F3). The additional marker used for activation was PE-conjugated anti-CD69 (H1.2F3). PE-conjugated anti-GR1 (Ly-6G) and allophycocyanin-conjugated anti-CD11c Abs were used to characterize pulmonary granulocytes. All of the mAbs were obtained from BD Pharmingen (San Diego, CA). As a last step before analysis, cells were incubated with 7-aminoactinomycin D (Via-Probe, BD Pharmingen) for dead cell exclusion. Cells were preincubated with FcR-blocking Ab (anti-CD16/CD32, clone 2.4G2) to reduce nonspecific binding. mAbs used to identify mouse DC populations were: allophycocyanin-conjugated anti-CD11c (clone HL3) and PE-conjugated anti-IAβ (AF6-120.1). We discriminated between macrophages and DCs using the methodology described by Vermaelen et al. (32). After gating on the CD11c-bright population, two peaks of autofluorescence can be distinguished. Macrophages are identified as the CD11c-bright, high autofluorescent population and do not express MHC class II. DCs are identified as CD11c-bright, low autofluorescent cells, which strongly express MHC class II. DCs enumerated by these criteria correspond with myeloid DCs. The following Abs were used to stain mouse T cell subpopulations: FITC-conjugated anti-CD4 (L3T4), FITC-conjugated anti-CD8 (Ly-2), and allophycocyanin-conjugated anti-CD3 (145-2C11) mAbs. The additional marker used for activation was PE-conjugated anti-CD69 (H1.2F3). The additional marker used for activation was PE-conjugated anti-CD69 (H1.2F3). PE-conjugated anti-GR1 (Ly-6G) and allophycocyanin-conjugated anti-CD11c Abs were used to characterize pulmonary granulocytes. All of the mAbs were obtained from BD Pharmingen (San Diego, CA). As a last step before analysis, cells were incubated with 7-aminoactinomycin D (Via-Probe, BD Pharmingen) for dead cell exclusion. All of the labeling reactions were performed on ice in FACS EDTA buffer.

Flow cytometry data acquisition was performed on a FACSCalibur flow cytometer running CellQuest software (BD Biosciences, Erembodegem, Belgium). FlowJo software (Tree Star, Ashland, OR) was used for data analysis.

**ELISA**

After 4 wk of CS-exposure, protein levels of CXCL1 (KC) and CCL2 (MCP-1) in BALF and leptin protein levels in serum were determined using commercially available ELISA kits (R&D Systems).

**Statistical analysis**

Results are presented as mean ± SEM. Statistical analysis was performed using SPSS, version 13.0 for Windows (SPSS, Chicago, IL) using non-parametric tests (Kruskall-Wallis; Mann-Whitney U). A p value <0.05 denotes the presence of a significant statistical difference.

**Results**

Increase in leptin-expressing BECs and pneumocytes upon 4 and 24 wk CS exposure

Immunohistochemistry for leptin protein in lung tissue of air-exposed C57BL/6 WT mice revealed leptin expression in BECs, pneumocytes, alveolar macrophages, and smooth muscle cells surrounding the airways and pulmonary vessel walls (Fig. 1A, 1C). The 4 wk (Fig. 1B) and 24 wk (Fig. 1D) exposure to CS resulted in a profound increase in leptin expression in BECs and pneumocytes. Fig. 1E shows quantification of leptin-expressing BECs, and the percentage of leptin-positive BECs increased 2- to 3-fold in CS-exposed versus air-exposed WT mice. In addition, upon both 4 and 24 wk CS exposure, there was a significant increase in leptin-expressing pneumocytes, determined semiquantitatively on a five-point scale (Fig. 1F). Furthermore, quantitative real-time PCR analyses on RNA isolated from total lung tissue using the primers listed in Table I revealed a 64% increase in normalized leptin mRNA expression levels in 4 wk CS-exposed versus air-exposed WT mice (data not shown). On the basis of these results, leptin-deficient ob/ob mice and their littermates were subjected to the 4 wk CS exposure protocol to determine the role of leptin in pulmonary inflammation induced by CS.
Altered pulmonary inflammatory cell recruitment after 4 wk CS inhalation in ob/ob mice

Body weights of ob/ob mice were significantly higher as compared with those of their littermates and showed a slight increase in the 4 wk period (Fig. 2A). In all of the groups, 4 wk CS exposure did not affect body weight. Fig. 2B shows serum leptin concentrations as measured by ELISA. The 4 wk CS exposure decreased serum leptin levels in WT mice on a C57BL/6 background. As expected, circulating leptin levels were undetectable in leptin-deficient mice.

The inflammatory profile in the lung was monitored in BALF as well as in single-cell suspensions of total lung. The baseline inflammatory profile in BALF (Fig. 3) was identical between leptin-deficient mice and their littermates except for the level of DCs, which was significantly lower in air-exposed ob/ob mice (Fig. 3D). The 4 wk CS exposure increased the absolute numbers of neutrophils, DCs, and CD4+ and CD8+ T lymphocytes in the BALF as compared with those in air-exposed animals, whereas a trend of increase was observed for the absolute numbers of total cells and alveolar macrophages. CS exposure in leptin-deficient mice tended to increase the neutrophil numbers in BALF as compared with those in air-exposed animals, whereas the numbers of all of the cell types examined, which reached significance in leptin-deficient mice for total cell counts and DCs and in WT mice for total cell counts, neutrophils, interstitial macrophages, DCs, and CD4+ T lymphocytes. In line with the data obtained in BALF, 4 wk CS-exposed ob/ob mice showed significantly higher numbers of neutrophils. In addition, the counts of interstitial macrophages and CD8+ T lymphocytes were lower in CS-exposed ob/ob mice versus those in WT mice, but differences did not reach the level of significance. In addition, the fraction of activated CD4+ and CD8+ cells was measured by double-labeling with an anti-CD69 Ab. Again, exposure to the smoking protocol increased the numbers of both CD4+CD69+ and CD8+CD69+ T cells as well as the percentage active (CD69+) over total CD4+ and CD8+ cells. However, no differences were observed between leptin-deficient mice and their littermates except for the fact that the percentage activated CD8+ cells was significantly decreased in leptin-deficient mice (Fig. 3D-F).

In lung single-cell suspensions (Fig. 4), baseline levels of neutrophils were significantly higher in air-exposed ob/ob mice versus those in their littermates. The 4 wk CS exposure increased the numbers of all of the cell types examined, which reached significance in leptin-deficient mice for total cell counts and DCs and in WT mice for total cell counts, neutrophils, interstitial macrophages, DCs, and CD4+ T lymphocytes. In line with the data obtained in BALF, 4 wk CS-exposed ob/ob mice showed significantly higher numbers of neutrophils. In addition, the counts of interstitial macrophages and CD8+ T lymphocytes were lower in CS-exposed ob/ob mice versus those in WT mice, but differences did not reach the level of significance. In addition, the fraction of activated CD4+ and CD8+ cells was measured by double-labeling with an anti-CD69 Ab. Again, exposure to the smoking protocol increased the numbers of both CD4+CD69+ and CD8+CD69+ T cells as well as the percentage active (CD69+) over total CD4+ and CD8+ cells. However, no differences were observed between leptin-deficient mice and their littermates except for the fact that the percentage activated CD8+ cells was significantly decreased in leptin-deficient mice (Fig. 3D-F).

![FIGURE 2](http://www.jimmunol.org/) Body weight and plasma leptin concentration of (CS-exposed) C57BL/6 WT and ob/ob mice. C57BL/6 WT mice (circles) and ob/ob mice (squares) were exposed to air (open) or CS (closed) for 4 wk. A and B, Body weight (A) and plasma leptin concentration (B) (detected by ELISA) were monitored after 1, 2, 3, and 4 wk of exposure. Results are expressed as mean ± SEM. n = 8 animals per group; *p < 0.05.

![FIGURE 3](http://www.jimmunol.org/) Total BAL cells and cell differentiation in BALF of (CS-exposed) C57BL/6 WT and ob/ob mice. C57BL/6 WT mice and ob/ob mice were exposed to air or CS for 4 wk. A, Total cell counts. B, Neutrophils were calculated on cytospins. C–F, Macrophages (C), DCs (D), CD4+ T lymphocytes (E), and CD8+ T lymphocytes (F) were enumerated by FACS analysis. Results are expressed as mean ± SEM. n = 8 animals per group; *p < 0.05.
increased in CS-exposed ob/ob mice (10%) versus that in WT mice (6%; \( p = 0.029 \)).

The CS-induced pulmonary inflammatory profile is comparable between db/db and ob/ob mice

Leptin-resistant db/db mice, lacking the functional B isoform of the leptin receptor, were exposed to the same 4 wk CS protocol to confirm the results obtained in leptin-deficient mice. Fig. 5A shows that body weights of db/db mice were significantly higher than those of their littermates, again slightly increasing in the 4 wk period. Similar to ob/ob mice, CS exposure did not affect body weight in all of the groups. Leptin-resistant mice showed, however, hyperleptinemia, and CS exposure did not affect the serum leptin levels in these mice on a C57BKS background (Fig. 5B).

BALF and single-cell suspensions of total lung were examined to determine the inflammatory profile in lung. BALF levels of CD4+ and CD8+ T lymphocytes were 24-fold and 19-fold lower, respectively, in air-exposed WT mice on a C57BKS (Fig. 6) versus those on a C57BL/6 background (Fig. 3), indicating interstrain differences in the baseline inflammatory profile. The 4 wk CS exposure increased the absolute numbers of total cells, neutrophils, alveolar macrophages, DCs, and CD4+ and CD8+ T lymphocytes in the BALF of leptin receptor-deficient mice as well as littermates (except for total cells counts) as compared with those of air-exposed animals. In line with the BALF data in ob/ob mice, CS exposure in db/db mice resulted in significantly increased numbers of neutrophils (Fig. 6B) and decreased counts of DCs (Fig. 6D) in BALF as compared with those in WT animals.

FACS analysis of single-cell suspensions of total lung showed accumulation of neutrophils in air-exposed db/db mice, whereas counts for DCs and CD4+ and CD8+ T cells at baseline were lower as compared with those of air-exposed WT mice (Fig. 7). CS exposure induced a general pulmonary inflammation in the db/db strain, which reached significance for DCs and the two lymphocyte populations. Comparison between CS-exposed db/db mice and littermates confirmed the cellular profile observed in ob/ob mice: lack of correct leptin signaling via the Ob-Rb receptor results in increased neutrophil recruitment and decreased influx of DCs and lymphocytes into the lung interstitium. Within the lymphocyte population, the CS-induced accumulation of both CD4+ and CD8+ T cells was attenuated in leptin-resistent mice. Again, absolute numbers of total cells (Fig. 7A) and alveolar macrophages (Fig. 7C) after CS exposure were not affected in db/db mice. Determining the fraction of activated CD4+ and CD8+ cells revealed that exposure to the smoking protocol increased the numbers of both CD4+CD69+ and CD8+CD69+ cells in WT and db/db mice. In addition, an increase was observed in air-exposed db/db mice versus their littermates when calculating the percentage CD69+ over total CD4+ cells (WT air 10% and db/db air 15%; \( p = 0.022 \)), which further significantly increased after CS exposure (WT CS 16% and db/db CS 23%; \( p = 0.01 \)). The percentage CD69+ over total CD8+ cells was not different among the four groups (range 7–10%).

**Chemokine levels**

To obtain a mechanistic explanation for the observed changes in inflammatory cell influx in ob/ob and db/db mice after CS
exposure, we measured the protein levels in BALF of the neutrophil chemoattractant CXCL1 and the chemokine CCL2 involved in recruiting monocytes and DCs. ELISA analyses showed that baseline CXCL1 and CCL2 BALF levels in leptin-deficient and leptin receptor-deficient mice were comparable. The 4 wk exposure to CS significantly increased the protein levels of CXCL1 in WT as well as ob/ob and db/db mice (Fig. 8A), and BALF levels were further increased in both ob/ob and db/db mice as compared with those in their littermates. Unexpectedly, CCL2 showed the same pattern as CXCL1 (Fig. 8B).

Restoration of altered pulmonary inflammatory profile in CS-exposed ob/ob mice with exogenous leptin administration

We next determined whether the observed alterations in the pulmonary inflammatory profile in leptin-deficient ob/ob mice exposed to 4 wk CS could be restored with exogenous leptin administration. Air-exposed WT and ob/ob mice treated with leptin did not gain weight in the 4 wk period, and body weights were, respectively, 10 and 15% lower (p < 0.05) as compared with those of untreated WT and ob/ob mice (Fig. 9). Moreover, CS-exposed ob/ob mice even lost weight after exogenous leptin administration, and their body weights were 25% lower (p < 0.05) as compared with those of untreated CS-exposed ob/ob mice.

The effect of exogenous leptin administration on the inflammatory profile in the lung was monitored in BALF. Fig. 10 shows that the baseline cellular inflammatory profile, including DCs, was similar between WT and ob/ob mice treated with leptin. In addition, the observed trends in differences in the CS-induced cellular inflammatory profile between WT and ob/ob mice (Fig. 3) could be fully restored by exogenous leptin administration (Fig. 10).

Discussion

Our studies are the first to show that CS exposure induces leptin accumulation in murine lung, specifically in BECs and pneumocytes. Mice deficient in leptin signaling (e.g., ob/ob and db/db mice) show a disbalance in innate and adaptive immune cells recruited to the lung at baseline and after CS exposure, which could be restored after exogenous leptin administration in ob/ob mice. Together, these findings suggest that leptin receptor activation is important in balancing the smoke-induced inflammatory response in the lung.

Adipocytes located in various fat depots are a major, but not the only, source of leptin. Cells other than adipocytes, including gastric mucosa, bone marrow, skeletal muscle, and various epithelia, have also been shown to produce leptin in certain circumstances (7). Using immunohistochemistry, we show for the first time leptin localization in lung tissue of mice, revealing leptin expression in BECs, pneumocytes, alveolar macrophages, and bronchial and vascular smooth muscle cells. The findings of the current study are corroborated by our clinical data that showed that BECs, pneumocytes, and macrophages are a rich source of leptin in human peripheral lung (6). Moreover, the current murine study and our previous human study examining lung tissue and (primary) cell cultures together convincingly demonstrate that smoke is a trigger of the inflammatory response in the lung.
for pulmonary leptin expression, specifically in BECs and pneumocytes. An extra argument for local leptin production is the increased leptin mRNA expression in total lung of CS-exposed animals. We did, however, not quantify leptin protein levels in BALF or lung homogenates, which would have strengthened our results that resident lung cells are a rich source of leptin in the pulmonary compartment. Our suggestion that leptin is produced by epithelial cells during homeostasis and pulmonary inflammation may imply that lung epithelial cells are a source of leptin detected in BALF in animal models of infectious diseases (33–36) and hyperoxia-induced acute lung injury (24) in addition to systemic leptin leaking into the lung interstitium and alveolar spaces together with other plasma proteins. Future studies are necessary to address the involvement of pulmonary leptin in respiratory (patho-)physiology.

Recent studies demonstrate that leptin influences both innate and adaptive immunity, modulating the activation and function of different cell types. This fits with our data showing that leptin-deficient and leptin receptor-deficient mice showed in general an increased recruitment of neutrophils but a decreased recruitment of DCs and CD4+ and CD8+ cells to the lung in response to CS exposure. In addition, we could demonstrate that the trends in differences in the CS-induced cellular inflammatory profile between WT and ob/ob mice were fully restored by exogenous leptin administration. The latter experiment indicates that the skewed inflammatory response in CS-exposed ob/ob and db/db mice is a direct consequence of the deficient leptin pathway and is not secondary to the several endocrine and metabolic abnormalities of these mutant mice. Consistently, the BALF levels of the neutrophil chemoattractant CXCL1 (functional homologue of human CXCL8) were significantly enhanced in CS-exposed ob/ob and db/db mice versus those in WT mice. An unexpected observation was the increased BALF concentration of the chemokine CCL2 in CS-exposed ob/ob and db/db mice versus that in WT mice. This is not in line with the decreased DC numbers or unaltered numbers of monocytes/macrophages, for which we have no explanation at this point. Baseline levels of neutrophils were found to be significantly higher in air-exposed ob/ob mice versus those in littermates, which is in line with a previous report from Faggioni et al. (37) demonstrating that peripheral blood neutrophil counts in leptin-deficient mice were twice that of WT animals (37). They also showed that exogenous leptin administration normalized peripheral blood neutrophil counts in ob/ob mice, which indicates that leptin affects peripheral neutrophil counts (37). Because excessive recruitment and consequently activation of neutrophils results in overall lung injury due to the release of reactive oxygen species and proteases, controlled recruitment of neutrophils is very important.
Previous murine studies investigating the role of leptin in the pulmonary compartment during pneumococcal pneumonia (38), experimental tuberculosis (35), and ozone-induced airway hyperresponsiveness (39, 40) suggested that the presence of leptin is likely required for effective balancing of the neutrophilic response in the pulmonary compartment induced by various triggers. One mechanism by which leptin may modulate trafficking of neutrophils in response to these triggers including CS is the induction of neutrophilic chemokines, such as CXCL1, by resident lung cells. In addition, leptin itself has been described to be a chemotactant for neutrophils (11, 41). On the contrary, leptin is known to reduce expression of neutrophilic chemotaxants and inhibits neutrophil migration in response to classical chemotaxants (41, 42). Furthermore, leptin regulates functional capacities of neutrophils (11, 12) and can protect neutrophils from apoptosis (43). Further investigations are required to unravel the role of leptin in neutrophil recruitment to the lungs in response to CS exposure.

DCs are a cellular population widely acknowledged as a master for induction and regulation of immune responses. The airways and lungs contain a rich network of DCs, localized near the epithelial surface (44). Consequently, DCs are ideally localized to initiate an inflammatory reaction in response to inhaled CS. Our current data show that CS-induced recruitment of DCs and T lymphocytes (CD4+ and CD8+) to the lung tissue is impaired in ob/ob and db/db mice. This may be explained by recent studies showing that leptin promotes differentiation, survival, and immunostimulatory functions of DCs, resulting in stronger heterologous T cell responses. In addition, Maia et al. (45) showed that circulating steady-state DC numbers are decreased in ob/ob mice. Also, in db/db mice (prediabetic stage) (46), it was shown that the total number of DCs generated from bone marrow cultures is lower than that in WT controls due to increased apoptosis. These studies also pointed at impaired DC function in ob/ob and db/db mice. Moreover, leptin is also known to act on lymphocytes by suppressing Fas/CD95-mediated apoptosis, hereby promoting survival. This is supported by our current data, showing a decrease in T cells in ob/ob and db/db mice after CS inhalation. In this model, leptin signaling does not affect activation of T cells, because the numbers of activated (CD69+) CD4+ and CD8+ cells are not different between ob/ob or db/db mice and their littermates, and in the db/db study, the percentage of CD69+CD4+ cells over total CD4+ cells is even increased. Together, these data point at a regulatory role for leptin in DC function necessary for an adequate immune response.

Recent studies from Chen et al. (47–50) investigated the relationship among smoking, neuropeptide Y, and weight loss and demonstrated that short-term (4 d) (47) and long-term (5, 7, and 12 wk) (48–50) CS exposure led to a significant reduction in body weight and systemic leptin concentration. This is partly in line with our current and previous data, showing that 24 wk—but not 4 wk—CS exposure induces significant weight loss (51). The difference between our findings and the studies by Chen et al. (47–50) are most likely due to interstrain differences. These investigators used BALB/c mice, whereas in the current study C57BL/6 and C57BKS mice were used. The latter two are closely related but also showed interstrain differences regarding circulating leptin levels, with significantly reduced systemic leptin concentrations in C57BL/6 mice but not in C57BKS mice after CS exposure. We speculate that exposure to CS may decrease the abdominal and gonadal fat pad mass in a strain-dependent manner, resulting in decreased circulating leptin levels, but additional studies need to confirm this potential extrapulmonary aspect of CS exposure.

The reports thus far on body weight changes in ob/ob mice after leptin replacement therapy are inconclusive. Numerous treatment protocols are being used, varying in the dose of leptin per day (30–300 μg per animal per day), once or twice daily injections, treatment period (2 d to 6 wk), and origin (and thus biological activity) of leptin (12, 52–56), eventually resulting in a change in body weight of +3% to −25%. In our hands, leptin replacement therapy with a relatively low dose (45 μg) of murine leptin (E. coli) once daily for 4 wk maintained the initial body weight in C57BL/6 WT mice and air-exposed ob/ob mice, which is in line with several previous reports (52, 54–56). An interesting finding in this respect is that leptin-treated ob/ob mice exposed to CS did lose up to 10% of their body weight, resulting in a comparable body weight loss induced by CS exposure in C57BL/6 mice and leptin-treated ob/ob mice.

Specific leptin-binding sites have been identified in BECs and pneumocytes (22–24). An interesting observation was recently published by Hansel et al. (57). They showed that upon 4 mo of smoke exposure in AKR/J mice, there was a reduction of Ob-R staining in the airspace and airway wall. If this will eventually result in a reduced leptin is difficult to say, because the Ab was directed against the extracellular part of the Ob-R, hereby detecting all of the leptin receptor isoforms. However, Bellmeyer et al. (24) showed that intratracheal leptin administration, which is likely to expose lung epithelial cells to relatively higher levels of leptin, caused acute lung injury in WT mice but not in db/db mice, whereas systemic leptin administration failed to modulate acute lung injury (58). We therefore propose that epithelial cells play a central role in the response to leptin in the pulmonary compartment. To validate the role of pulmonary leptin in CS-related disease, it is necessary to develop transgenic mice that lack functional leptin signaling in the pulmonary compartment. These new transgenic mice will also overcome the complex phenotype of ob/ob and db/db mice, being obesity, hyperglycemia, overproduction of glucocorticosteroids, thymic atrophy, and lowered peripheral blood leukocyte counts that hampered our current investigations on the immunological role of leptin in CS-related disease.

In summary, this report shows for the first time immunohisto-localization of leptin in murine lung tissue and provides novel insights into the role of leptin in the regulation of innate and adaptive immune responses upon CS exposure. These results support the hypothesis that leptin is a key molecule in balancing innate and adaptive immune responses and provide a rationale for future studies exploring the role of leptin in lung epithelial cells. Detailed studies are currently in progress to determine functional target genes of leptin signaling in lung epithelial cells and to elucidate the chemotactic action of leptin in lung, which may add to understanding the role of lung epithelial cells in the pathogenesis of smoking-related pulmonary diseases, such as COPD.

Acknowledgments
The authors thank Greet Barbier, Eliaene Castraque, Indra De Borle, Philippe De Gryze, Kaileen De Saedeleer, Anouck Goethals, Marie-Rose Mouton, Ann Neessen, Christelle Sauwaert, and Evelyn Spruyt for technical assistance.

Disclosures
The authors have no financial conflicts of interest.

References