Inflammatory signatures for eosinophilic vs. neutrophilic allergic pulmonary inflammation reveal critical regulatory checkpoints

Pieter Bogaert,1,2* Thomas Naessens,1* Stefaan De Koker,1 Benoit Hennuy,3 Jonathan Hacha,4 Muriel Smet,1 Didier Cataldo,4 Emmanuel Di Valentin,5 Jacques Pette,5 Kurt G. Tournoy,6 and Johan Grooten1

1Laboratory of Molecular Immunology, Department of Biomedical Molecular Biology, Ghent University, and 2Department for Molecular Biomedical Research, VIB, Ghent; 3Transcriptomic Unit, 4Laboratory of Biology of Tumors and Development, and 5Laboratory of Fundamental Virology and Immunology, GIGA Research, University of Liège, Liège; and 6Department of Respiratory Medicine, Ghent University Hospital, Ghent, Belgium

Submitted 21 June 2010; accepted in final form 14 February 2011

Bogaert P, Naessens T, De Koker S, Hennuy B, Hacha J, Smet M, Cataldo D, Di Valentin E, Pette J, Tournoy KG, Grooten J. Inflammatory signatures for eosinophilic vs. neutrophilic allergic pulmonary inflammation reveal critical regulatory checkpoints. Am J Physiol Lung Cell Mol Physiol 300: L679–L690, 2011. First published February 18, 2011; doi:10.1152/ajplung.00202.2010.—Contrary to the T-helper (Th)-2 bias and eosinophil-dominated bronchial inflammation encountered in most asthmatic subjects, other patients may exhibit neutrophil-predominant asthma subphenotypes, along with Th-1 and Th-17 cells. However, the etiology of many neutrophil-dominated asthma subphenotypes remains ill-understood, in part due to a lack of appropriate experimental models. To better understand the distinct immune-pathological features of eosinophilic vs. neutrophilic asthma types, we developed an ovalbumin (OVA)-based mouse model of neutrophil-dominated allergic pulmonary inflammation. Consequently, we probed for particular inflammatory signatures and checkpoints underlying the immune pathology in this new model, as well as in a conventional, eosinophil-dominated asthma model. Briefly, mice were OVA sensitized using either aluminum hydroxide (alum) or complete Freund’s adjuvant, followed by OVA aerosol challenge. T-cell, granulocyte, and inflammatory mediator profiles were determined, along with alveolar macrophage genomewide transcriptome profiling. In contrast to the Th-2-dominated phenotype provoked by alum, OVA/complete Freund’s adjuvants adjuvant-based sensitization, followed by allergen challenge, elicited a pulmonary inflammation that was poorly controlled by dexamethasone, and in which Th-1 and Th-17 cells additionally participated. Analysis of the overall pulmonary and alveolar macrophage inflammatory mediator profiles revealed remarkable similarities between both models. Nevertheless, we observed pronounced differences in the IL-12/IFN-γ axis and its control by IL-18 and IL-18 binding protein, but also in macrophage arachidonic acid metabolism and expression of T-cell instructive ligands. These differential signatures, superimposed onto a generic inflammatory signature, denote distinctive inflammatory checkpoints potentially involved in orchestrating neutrophil-dominated asthma.

neutrophil-predominant asthma; allergic inflammation; alveolar macrophage; transcriptome; mouse models

* P. Bogaert and T. Naessens contributed equally to this work.

http://www.ajplung.org 1040-0605/11 Copyright © 2011 the American Physiological Society L679
addition, we exploited this new model of neutrophilic pulmonary inflammation, as well as a conventional model of eosinophilic allergic pulmonary inflammation, to seek, by comparative analysis, different inflammatory mediators and cell populations in different models of pulmonary inflammation. To this end, we developed a new model of neutrophilic pulmonary inflammation, to seek, by comparison with a conventional model of eosinophilic allergic pulmonary inflammation, to identify novel mediators and cell populations in different models of pulmonary inflammation.

**METHODS**

**Mouse models and collection of samples.** Female, 6- to 8-wk-old C57BL/6 mice (Janvier, Le Genest St-Isle, France) were housed under specific pathogen-free conditions. Mice were individually housed in standard cages with food and water ad libitum except specific feeding regimens. Mice were used 1 mo after weaning. The mouse experiments were performed at the Laboratory Animal Centre of University of Brussels. The animal experiments were approved by the Institutional Ethics Committee of University of Brussels, according to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health, 1996).

**Lung CD4 T cells.** All mice (6 mice/group) were used. T cells were isolated from pooled lungs, minced, and digested with collagenase (Sigma-Aldrich) and DNase I, and cytokines and chemokines in the culture supernatant were measured using ELISA. For RT-quantitative PCR analysis, cDNA was prepared from 1 μg total RNA using oligo(dT), RNase inhibitor, and Reverse Transcription kit (Invitrogen). PCR reactions were performed with the SybrGreen-I qPCR Core kit (Invitrogen). The PCR primer pair sequences are provided in the supplemental information. The PCR primer pair sequences are provided in the supplemental information.

**Flow cytometric analysis.** For the detection of changes in cell surface expression, cells were cultured for another 4 h, upon which time they were harvested for flow cytometric analysis, as described above. Cells were first stained for surface markers (CD45, I-Ab, F4/80, CD4), then fixed and permeabilized, and subsequently stained for intracellular cytokines (IL-17, IL-4, IFN-γ).

**Measurement of inflammatory mediators in BAL fluid.** Inflammatory mediators were measured in the first 0.5 ml fraction of the collected BAL fluid. Cytokines and chemokines were measured using the Bioplex suspension array system (Bio-Rad), according to the manufacturer’s instructions, using recombinant cytokine standards (Bio-Rad). Matrix metalloproteinase (MMP)-9 and MMP-12 levels were determined by SDS-PAGE Western blot analysis, using specific monoclonal antibodies (R&D Systems) and peroxidase-conjugated rabbit-anti-mouse IgG (Dako, Denmark) for detection. Active MMP-9 was analyzed by zymography on a SDS-10% polyacrylamide gel + 1 mg/ml gelatin, detected as a lyses 95-kDa band. Culture medium from HT1080 cells served as internal standard. MMP-12 enzymatic activity was analyzed using the EnzoLyte 490 kit (Anaspec, San Jose, CA), optimized to detect MMP-12 activity in biological samples using a fluorescence quenched substrate (EDANS/DabcylPlus FRET peptide). MMP-12 purified enzyme used as positive control was purchased from Sigma (St. Louis, MO). Complement C3a levels were determined by enzyme-linked immunosorbent assay, using anti-C3a clone I87-1162 (BD Pharmingen) for capture and biotinylated I87-419 clone (BD Pharmingen) for detection. Purified mouse C3a protein (BD Pharmingen) was used as standard.

**RT-quantitative PCR analysis.** Total RNA was prepared using the Aurum total RNA mini kit (Bio-Rad, Hercules, CA). RNA quality was determined spectrophotometrically. For RT-quantitative PCR (qPCR), cDNA was prepared from 1 μg total RNA using oligo(dT), RNase inhibitor, and Superscript II reverse transcriptase (Invitrogen). PCR reactions were performed with the SybrGreen-I qPCR Core kit (Eurogentec, Liège, Belgium) and an iCycler (Bio-Rad) instrument. Data were normalized for the expression levels of housekeeping genes (ribosomal protein L13a and TATA box binding protein). Amplification specificity was confirmed by evaluation of the melting curves. n-Fold differences between samples were calculated using the 2^−ΔΔCt method. The PCR primer pair sequences (Invitrogen) are provided in the supplemental information.

**Transcriptome analysis.** RNA integrity was confirmed with the automated electrophoresis Experion System using the RNA StdSens Analysis Kit (Bio-Rad). Total RNA (4 μg), labeled using the GeneChip Expression 3′ Amplification One-Cycle Target Labeling kit (Affymetrix, Santa Clara, CA), was hybridized to Genechip Mouse Genome 430A 2.0 (Affymetrix), according to the manufacturer’s instructions. Arrays were scanned with an Affymetrix/Hewlett-Packard GeneChip Scanner 3000 7G (Palo Alto, CA). Data were generated with the MAS 5.0 algorithm included in Genechip Operating Software. The naive condition was set as baseline for pairwise comparison with OVA-induced experimental conditions. The probe sets were filtered on signal log ratio (greater than 1 for upregulated and less than −1 for downregulated transcripts) and on P value associated with the change status (<0.001 for upregulated probe sets, >0.999 for downregulated probe sets). The complete microarray data set can be consulted in the EBI Array Express Database (http://www.ebi.ac.uk/arrayexpress, accession number E-MEXP-2500).

**Statistical analysis.** Statistical significance on numbers of cells, cell composition, and mediator levels was determined with the Mann-Whitney U-test (2 categorical variables) or Kruskal-Wallis test with post hoc Bonferroni correction (>2 categorical variables). Significant P values were ranked as P < 0.05 (*), P < 0.01 (**), and P < 0.001 (***). Data are represented as means ± SE or ± SD (as indicated in Figs. 1–5).
RESULTS

Immunization with CFA predisposes mice to develop a neutrophilic asthmalike pulmonary inflammation that is poorly controlled by glucocorticoids. To verify to what extent Th-1/Th-17-biased sensitization supports the development of neutrophilic asthmalike inflammation, C57BL/6 mice were systemically immunized against the model antigen OVA in the presence of CFA. Sensitization promoting an eosinophilic asthmalike immunopathology was generated by immunization against OVA in the presence of the Th-2-skewing adjuvant alum. Thereafter, mice from both groups were challenged with nebulized OVA for 2 consecutive days. This then resulted in a significant cell infiltrate in the BAL fluid compared with naive mice (Fig. 1A).

Flow cytometric analysis of cell surface markers (Fig. 1B) and histological analysis of hematoxylin-eosin-stained lung tissue sections (Fig. 1C) revealed profound differences in the cellular infiltration between OVA/CFA- and OVA/alum-sensitized mice. As expected, the BAL fluid from the well-established OVA/alum model contained mainly eosinophils (±70%), AM/DC (±15%), lymphocytes (±10%), and fewer neutrophils (<10%). Histological analysis showed infiltration of eosinophils and monocytic cells almost exclusively around the peribronchiolar and perivascular areas. Contrarily, in the OVA/CFA-sensitized group, we found a substantial infiltration into the bronchoalveolar space of neutrophils, AM/DC, and lymphocytes (each ±30%), and to a lesser extent eosinophils (±10%) (Fig. 1B). Analysis of lung sections showed a strong infiltration of lymphocytes, monocyctic cells, and neutrophils around peribronchiolar and perivascular areas, although also a patchy interstitial infiltrate was observed (Fig. 1C).

To investigate the responsiveness to anti-inflammatory glucocorticoid treatment, mice from both OVA/alum and OVA/CFA immunized groups received ip injections of Dex.
OVA inhalation, and BAL cellular infiltration was examined after two OVA challenges (Fig. 2, Supplemental Fig. S2A). In the OVA/alum model, pretreatment with either 1 mg/kg or 2.5 mg/kg Dex showed a potent reduction in infiltrated eosinophils, neutrophils, recruited (CD11b<sup>+</sup>) AM, DC, and CD4 T cells. In the OVA/CFA model, however, 1 mg/kg Dex had no effect on cellular inflammation, while 2.5 mg/kg Dex reduced the number of DC and CD4 T cells and to a lesser extent of eosinophils, but had no effect on the neutrophil cell count. Thus, using either alum or CFA as adjuvants predisposes mice to develop divergent types of pulmonary inflammation that are in line with many inflammatory aspects of, respectively, human eosinophilic and neutrophilic asthma types.

**Lung CD4 T-cell phenotypes.** We characterized the local Th phenotype generated in the OVA/CFA and OVA/alum groups by performing RT-qPCR on magnetically purified lung tissue CD4 T cells (Fig. 3A) and by flow cytometric phenotyping of interstitial lung lymphocytes after in vitro culture with CD3<sup>-</sup>/CD3<sup>+</sup> (Fig. 3B, Supplemental Fig. S2B). mRNA expression of the Th-1 transcription factor, T-bet, was induced solely in the OVA/CFA model, while its Th-2 counterpart, GATA-3, was slightly induced in OVA/alum and OVA/CFA mice. mRNA expression of the Th-17 transcription factor, retinoid-related L682 IMMUNE MEDIATORS IN MODELS OF AIRWAY ALLERGY

Fig. 2. Characterization of BAL immune cell populations in OVA/alum (A) and OVA/CFA (B) mice, treated with 0, 1, or 2.5 mg/kg of dexamethasone (Dex) (intrapertitoneal) 3 h before OVA challenge (n = 7/group). Average BAL cellular composition ± SE is shown, determined by flow cytometric analysis of eosinophils (CD3<sup>+</sup>, I-Ab<sup>-</sup>, CD11c<sup>-</sup>, CD11b<sup>+</sup>, CCR3<sup>+</sup>, SSC<sup>-</sup>), neutrophils (CD3<sup>+</sup>, I-Ab, CD11c<sup>-</sup>, CD11b<sup>+</sup>, CCR3<sup>-</sup>, SSC<sup>-</sup>), resident AM (CD3<sup>-</sup>, I-Ab<sup>-</sup>, CD11b<sup>-</sup>, CD11c<sup>-</sup>, autofluo<sup>+</sup>), recruited AM (CD3<sup>-</sup>, I-Ab<sup>-</sup>, CD11b<sup>-</sup>, CD11c<sup>-</sup>, autofluo<sup>+</sup>), DC (CD3<sup>-</sup>, I-Ab<sup>+</sup>, CD11c<sup>+</sup>, autofluo<sup>+</sup>), and CD4 T cells (CD3<sup>-</sup>, CD4<sup>+</sup>, CD11c<sup>-</sup>). *P < 0.05, **P < 0.01, ***P < 0.001. Gating strategy is provided in Supplemental Fig. S2A.

Fig. 3. Characterization of local pulmonary T-cell populations in OVA/CFA, OVA/alum, and naive mice. A: relative mRNA expression levels ± SD of T-helper (Th)-1 (T-bet), Th-2 (Gata3), and Th-17 (Rorgt) associated transcription factors in purified pulmonary CD4 T cells, pooled from 5 (OVA/CFA, OVA/alum) or 10 (naive) mice/group. B: average levels ± SD of pulmonary Th populations in lung leukocytes (pooled from 6 mice/group) after in vitro α-CD3 reactivation, determined by flow cytometric analysis of Th-1 (CD45<sup>+</sup>, CD4<sup>+</sup>, I-Ab/F4/80<sup>+</sup>, IL-17<sup>-</sup>, IFN-γ<sup>-</sup>), Th-2 (CD45<sup>+</sup>, CD4<sup>+</sup>, I-Ab/F4/80<sup>+</sup>, IL-4<sup>+</sup>), and Th-17 (CD45<sup>+</sup>, CD4<sup>+</sup>, I-Ab/F4/80<sup>+</sup>, IL-17<sup>-</sup>). Gating strategy is provided in Supplemental Fig. S2B. C: average BAL fluid levels of T-cell-associated cytokines ± SE (n = 4/group). *P < 0.05, **P < 0.01, ***P < 0.001.

AJP-Lung Cell Mol Physiol • VOL 300 • MAY 2011 • www.ajplung.org
orphan receptor-γt, was also induced in both models, albeit more prominently in OVA/CFA mice. Quantification of in vitro reactivated lung tissue CD4+ T cells confirmed this differential Th phenotype: larger IFN-γ+ IL-17+ Th-1 and IL-17+ Th-17 CD4+ T-cell populations in the lung leukocytes from OVA/CFA compared with OVA/alum, and similar levels in both models of IL-4+ Th-2 CD4+ T-cell population.

We also quantified the absolute levels of T-cell-derived or -associated cytokines in the BAL fluid by a multiplexed antibody assay (Fig. 3C). Reflecting the higher numbers of BAL T cells, IL-2 levels were increased in the OVA/CFA group. A robust increase in IFN-γ levels, and significantly higher presence of IL-12p40 in OVA/CFA mice compared with OVA/alum mice, further confirmed the Th-1 component in the OVA/CFA model. Importantly also, the levels of Th-2 cytokines (IL-4, IL-13, and IL-5) were increased in OVA/CFA, with only IL-5 levels being significantly higher in the OVA/alum group. The dramatically increased IL-17 levels in the OVA/CFA group then confirmed the presence of Th-17 cells in this model. Finally, IL-10 levels remained unchanged in either group.

**Innate inflammatory mediator signatures.** We verified to what extent the differential T-cell polarization in OVA/CFA vs. OVA/alum conditions reflected at the local inflammatory mediator level. First, we performed RT-qPCR on total lung mRNA (Fig. 4A). Reflecting the cellular environment, mRNA expression of Th-1 chemoattractant (CXCL10) was induced solely in the OVA/CFA condition, whereas the induction of Th-2- and eosinophil-recruiting chemokine (CCL11) was observed only in the OVA/alum group. However, expression of two functionally similar chemokines, Th-1 chemoattractant CXCL9 and Th-2/eosinophil-recruiting CCL9, was induced in...
both conditions and only showed quantitative differences between both groups.

Next, BAL fluid levels of proinflammatory cytokines (IL-1β, IL-6 and TNF-α) and chemokines (CXCL1/KC, CCL2/monocyte chemoattractant protein-1, CCL3/macrophage inflammatory protein-1α, CCL4/macrophage inflammatory protein-1β, and CCL5/regulated on activation normal T-expressed and presumably secreted) were analyzed by a multiplexed antibody assay (Fig. 4B). This revealed merely quantitative differences in inflammatory mediator expression between both groups. Of the cytokines and chemokines analyzed, only TNF-α was not induced, whereas the levels of IL-1β, IL-6, and monocyte/lymphocyte-attracting chemokines CCL2, CCL3, CCL4, and CCL5 were elevated in both groups. Surprisingly also, the levels of the neutrophil chemotactant, CXCL1, were elevated in both groups to a similar extent. Yet IL-1β, IL-6, CCL4, and CCL5 levels were more prominent in the OVA/CFA mice.

Next to cytokines and chemokines, we also analyzed the expression levels of additional inflammatory mediators. The MMPs, MMP-9 and MMP-12, are thought to play detrimental roles in asthma. In line herewith, we observed increased levels of total or processed MMP-9 and MMP-12 levels in OVA/alum mice, but also in the OVA/CFA model (Fig. 4C). Finally, as complement C3a has also been implicated in acute allergic airway inflammation, we examined C3a levels in the BAL fluid by enzyme-linked immunosorbent assay (Fig. 4D). Again, C3a levels were elevated in both models, but more prominently so in OVA/CFA mice. These results show that the innate inflammatory mediator signature only partially reflects the differential cellular inflammation in the models, with the OVA/CFA model showing more biomarkers (IL-1β, IL-6, C3a) associated with neutrophil-predominant asthma.

Genomewide transcriptome profiling of AM. AM are long-lived cells residing in the bronchoalveolar cavities that are among the first cells to encounter inhaled particles and initiate immune responses and are a common denominator in the BAL immune cell composition of both eosinophil- and neutrophil-dominated asthma. To identify (anti-)inflammatory signatures differentially induced by the inflammatory microenvironment in AM from neutrophil-dominated vs. eosinophil-dominated pulmonary inflammation, we performed genomewide transcriptome profiling of flow cytometric purified CD11chigh autofluorescencehigh AM. As shown in Fig. 5, both OVA/CFA and OVA/alum groups exhibited robust changes in the AM transcriptome with over 2,800 genes differentially expressed in each condition compared with naive AM, a large majority of which was upregulated. Strikingly, ~60% of those genes had a concurrent expression pattern, defined as greater than twofold up- or downregulated in both conditions. Functional clustering of the commonly upregulated gene set using online “Database for Annotation, Visualization and Integrated Discovery” bioinformatics resources (19) and the “Kyoto Encyclopedia of Genes and Genomes” and “Protein Information Resource” databases identified mainly inflammation-related biological themes, such as cytokine-cytokine receptor interaction, chemotaxis, myosin heavy chain (MHC) II-mediated antigen presentation, JAK-STAT signaling, and toll-like receptor signaling. Other gene clusters (type 1 diabetes mellitus, cell adhesion molecules) also comprised genes involved in inflammatory signaling and transendothelial migration of inflammatory cells.

Fig. 5. Identification of common and condition-specific over- and underrepresented biological themes in the AM transcriptome by genomewide transcriptome profiling of pooled AM, isolated from 10 (OVA/CFA, OVA/alum) or 15 (naive) mice/group. Transcripts, more than twofold differentially expressed in OVA/CFA and/or OVA/alum vs. naive condition, were withheld for unbiased in silico functional clustering analysis. Shown are n-fold enrichment of genes within each biological theme and its P value [Expression Analysis Systematic Explorer (EASE) score]. EASE < 1.10^-4 was considered significantly over- or underrepresented. MHC, myosin heavy chain.
As apparent from Fig. 4, genes induced exclusively in the OVA/CFA condition displayed an overrepresentation of immune effector functions, which was absent in genes induced exclusively in the OVA/alum group. In the downregulated gene sets, no inflammation-related biological themes were found (Fig. 5).

**Generic and condition-specific immune effector gene signatures in AM.** The above clustering analysis of differentially expressed AM genes does not take into account quantitative differences in gene expression levels. Therefore, we selected for further analysis an immune effector gene set consisting of chemokines, cytokines, growth factors, genes involved in arachidonic acid metabolism, and genes involved in antigen processing and presentation. Genes having an expression pattern not more than twofold different between OVA/CFA and OVA/alum vs. naive were assigned to the generic signature. Genes showing twofold to fivefold differences in expression between the respective inflammatory conditions vs. naive were assigned to the condition-biased signature. Finally, genes over fivefold differentially expressed between the inflammatory models were assigned to the condition-specific signature. The expression levels of selected genes were further tested by RT-qPCR for confirmation in an independent repeat experiment. The results of this refined transcriptome analysis are shown in Fig. 6 and discussed in the ensuing paragraphs.

**Inflammatory mediator differentials.** The majority of differentially expressed (vs. naive) chemo- and cytokines and growth factors showed mRNA levels that were identical or similar in OVA/CFA and OVA/alum conditions (Fig. 6, A and B), indicating that AM are dedicated to establishing a general proinflammatory environment and recruiting other leukocytes in both conditions. Importantly, a much smaller condition-specific gene set supplemented this generic gene signature. In line with our previous results, we found elevated expression of CXCL9, CXCL10, and CCL5 to be part of the OVA/CFA-specific gene signature (Fig. 6A). Conversely, the Th-2-/eosinophil-attracting chemokines CCL22 and CCL24, but also the neutrophil/monocyte-attracting CXCL7, were part of the OVA/...
alum-specific gene signature. Moreover, we found evidence for immune-modulatory functions of AM. Thus, whereas expression of IL-12b, promoting Th-1 function, was generically increased, IL-18 expression, a potent IFN-γ-inducing factor acting synergistically with IL-12, was repressed (Fig. 6B). Repression of IL-18 activity may be even more pronounced in the OVA/CFA condition, as suggested by the OVA/CFA-specific expression of IL-18BP, a soluble IL-18 scavenger. IL18-BP expression may be induced by an endogenous IFN-γ regulated feedback loop, present only in the OVA/CFA condition (Fig. 6B).

Interestingly, we observed a condition-specific dichotomy in the expression of arachidonic acid-metabolizing enzymes (Fig. 6C). Arachidonic acid can be metabolized into pro- or anti-inflammatory eicosanoids. Expression of the proinflammatory leukotriene-synthesizing enzymes, Alox5, Lta4h, and Ltc4s, was generically repressed. Instead, it appears that the main eicosanoids produced in OVA/CFA-AM are anti-inflammatory prostaglandin(PG) E2 and I2 through Ptgs2, encoding COX-2. Conversely, in OVA/alum-AM, expression of Ptgs1, encoding COX-1, is induced along with a concurrent strong induction of Alox15, suggesting a shift toward anti-inflammatory lipoxin (LX) synthesis.

**Antigen processing and presentation differentials.** Transcriptome analysis suggested that AM gained increased capacities to interact with T cells and present antigen (Fig. 6D). mRNA expression of CD40, which activates T cells through binding of CD40 ligand, and of ICAM-1, which ligates T cells through lymphocyte function-associated antigen-1, were generically induced. Increased input of MHC II molecules into antigen-processing endosomes is suggested by induced mRNA expression of MHC II transcription factor (C2ta), CD74 invariant chain (li), I-A α-and β-chains (H2-Aa, H2-Ab1), and I-E α-chain (H2-Eb1). Although part of the generic gene signature, these genes were invariable higher expressed in the OVA/CFA than in the OVA/alum condition. The OVA/CFA-induced expression of peptide editors (H2-Dmb1, H2-Dmb2) and their inhibitor (H2-Oa) are then indicative of a broader range of peptides presented in this condition. Notably, although expression of the MHC I common light chain (H2-M3), Qa-1, and Qa-2 genes was biased toward the OVA/CFA condition. Combined, these data indicate that AM-mediated antigen presentation to CD4 and certainly to CD8 T cells may be more effective in OVA/CFA-AM than in OVA/alum-AM. The stimulatory vs. inhibitory outcome of antigen presentation depends on accompanying costimulatory signals. Costimulatory inducible costimulator ligand mRNA expression was repressed in both conditions, whereas CD86 (but not CD80) expression was increased. Yet, also costimulatory Pdcd11g2 (encoding for PD-L2) expression was increased in both conditions and further supplemented specifically in the OVA/CFA condition with elevated levels of coinhibitory CD274 mRNA (encoding for PD-L1).

**Expression of costimulatory molecules on AM and DC.** To confirm these findings at the protein level, we performed flow cytometric analysis of selected surface markers on CD11c<sup>high</sup> AM, as well as CD11c<sup>high</sup> AM, as well as CD11c<sup>low</sup> DC (Fig. 7, A and B). In agreement with the transcriptome analysis, we observed moderately elevated surface levels of MHC II on OVA/alum-AM and strongly elevated levels on OVA/CFA-AM (Fig. 7C). Furthermore, a fraction of these MHC II<sup>high</sup> AM also was CD4<sup>high</sup>. Analysis of costimulatory CD80 and CD86, as well as coinhibitory PD-L1 and PD-L2, confirmed the findings made at the transcriptome level: in the OVA/CFA condition, a larger fraction of AM were MHC II<sup>high</sup>CD86<sup>high</sup> and only few CD8<sup>high</sup>AM were observed (Fig. 7, D and E). Both conditions also showed elevated levels of the coinhibitory PD-Ls, with PD-L1 elevated mainly in OVA/CFA-AM and PD-L2 mainly in OVA/alum-AM (Fig. 7, F and G). Furthermore, in OVA/CFA-AM, expression of PD-L1 coincided with MHC II expression, indicating that, especially in this model, AM exert a T-cell restraining function.

Analysis of alveolar DC showed, as expected, a majority of the cells to be MHC II<sup>high</sup>CD40<sup>high</sup> in both OVA/CFA and OVA/alum conditions (Fig. 7C). Costimulatory CD86 expression was more pronounced in DC than in AM, especially in the OVA/CFA model (Fig. 7E). Nonetheless, we also observed elevated levels of coinhibitory PD-L1 and PD-L2, coinciding with elevated MHC II levels (Fig. 7, F and G).

**DISCUSSION**

In this study, we addressed the issue of whether mouse models of neutrophil-dominated allergic pulmonary and bronchial inflammation could be developed suitable to effectively study the cellular and molecular pathways underlying such conditions. In humans, neutrophil-predominant asthma forms display some undeniable parallels with HP: neutrophilic infiltration; presence of Th-1, Th-17, and Tc cells; and aleukiosis (1, 5, 10, 13–18, 21, 25, 28). A classical protocol to predispose mice for HP consists of immunization against the actinomycete *Sacccharopolyspora rectivertigula* in the presence of CFA as Th-1/Th-17-inducing immunogenic cofactor (41). Conversely, mice become predisposed to develop features of eosinophil-predominant asthma by systemic sensitization with OVA in the presence of the Th-2-skewing adjuvant alum (14). We here combined these protocols by sensitizing mice against OVA in the presence of CFA. Following OVA inhalation, the resulting pulmonary inflammation featured several cellular and molecular characteristics of neutrophil-predominant asthma: infiltration of lymphocytes, neutrophils, and macrophages, and polarization of local CD4 T cells toward Th-1 and Th-17, similar to clinical findings (1, 5, 9, 10, 13–15, 28, 42). Importantly, we also observed eosinophilic infiltration and evidence of a Th-2-cell component.

In general, asthmatic subjects with neutrophil- and Th-17-dominated inflammation tend to display an increased severity of disease, and both murine and clinical data suggest a relation with steroid resistance (1, 32, 42). Indeed, we also found that, whereas Dex treatment dampened bronchoalveolar cellular infiltration, as well as lung Th-2 numbers (not shown) in OVA/alum mice, little to no effect on bronchoalveolar infiltration or lung Th-1, Th-2, and Th-17 numbers (not shown) was seen in OVA/CFA mice. From these features, we conclude that this model is clinically relevant and appears suitable to study commumal aspects of the immunopathophysiology of the heterogeneous neutrophilic asthma condition. A previous study (32), in which OVA-induced airway inflammation was elicited by adoptively transferred Th-2 or Th-17, showed that Th-17...
could critically mediate steroid-resistant airway inflammation, as well as airway hyperresponsiveness (not assessed in this study) in asthma. This hypothesis is strengthened by the steroid resistance of inflammation in our OVA/CFA model, in which the inflammatory features of neutrophilic and Th-17-dominated asthma may be better represented by the additional Th-1 and Th-2 components. Furthermore, extrapolating to clinical asthma, our results also indicate that eosinophil- vs. neutrophil-dominated or steroid-sensitive vs. -resistant asthma types may diverge already at the stage of allergic sensitization, dictated by the nature of the immune-modulating danger signaling elicited during sensitization.

Comparative analyses of the two models revealed, as expected, a strong parallel between chemokine expression and cellular composition of the inflammatory response. In the OVA/CFA model, Th-1 chemoattractants were prominently expressed, in contrast with the OVA/alum model, where Th-2-/eosinophil-recruiting chemokines predominated. However, protein levels of inflammatory mediators in BAL fluid also showed some remarkable resemblances between the models.
Thus IL-1β, IL-6, IL-12p40, CXCL-1, α-chemokines, and C3a levels were elevated in both conditions. This observation, along with the notion that there is redundancy in the chemokine and cytokine system (29, 30, 38), could indicate that cytokine measurements in BAL fluid as such may not be adequate to distinguish between the different pathologies. It is, nonetheless, interesting to point out the significantly higher levels of C3a, IL-1β, IL-6, and especially IFN-γ in the OVA/CFA mice, since these mediators have been shown to be associated with severe asthma attacks (2, 23, 28, 36, 44). Moreover, IL-17 and MMP-9 expression have been correlated with asthma severity (3, 9, 12, 31). Although IL-17 was markedly increased in the OVA/CFA model, we found no differences in total and processed MMP-9 in BAL fluid. Also, MMP-12 levels and proteolytic activity were strongly increased, in line with a previous report showing a role for MMP-12 in a model of cockroach-induced, neutrophil-dominated asthma (48). However, again we found no differences between the models. Thus, although the two experimental models clearly represent different pathologies in terms of cellular inflammation, glucocorticoid sensitivity, and Th2-cell phenotypes, the inflammatory microenvironment displays a remarkable number of common denominators.

A common constituent in the pulmonary immune cell composition of both eosinophil- and neutrophil-predominant asthma are AM. These long-lived cells are presumed to play pivotal roles in the orchestration of local inflammation by producing secreted inflammatory mediators and modulating T-cell responses through their antigen-presenting capacities. AM display a remarkable plasticity and can be excellent sensors of the inflammatory microenvironment. Because of these features, their activation state may reflect distinct inflammatory signatures involved in (counter) acting regulatory checkpoints in neutrophil- or eosinophil-dominated allergic disease. Yet only fragmented and often contradicting information is available on the precise functions of AM in asthma (24, 39). We, therefore, performed a genomewide transcriptome analysis on these cells. A striking first observation was the identification of an important generic inflammatory transcriptional program comprising multiple proinflammatory cytokines, chemokines, and growth factors, in line with the common proinflammatory environment we also observed in the BAL fluid. Genes specifically associated with neutrophilic or eosinophilic inflammatory signatures could mostly be linked with differences in BAL cell composition (Th1-attracting CXCL9 and CXCL10 in neutrophil- vs. Th2-/eosinophil-recruiting CCL22 and CCL24 in eosinophil-predominant inflammation) or with the earlier mentioned notion that the inflammatory setting in the neutrophil-dominated condition possesses similarities with severe asthma attacks in clinical disease (higher expression of IFN-γ and IL-6).

Importantly, we found indications that AM may also exert subtle immune-modulatory functions. First, we observed a dramatic increase in the mRNA expression of the IL-18 scavenger, IL-18BP, in the neutrophilic severe asthmalike model. Along with a generic downregulation of IL-18, a cytokine that strongly synergizes with IL-12 and the induction of IL-12b mRNA, this result indicates that, in the neutrophil-predominant asthma model, IL-12-driven IFN-γ production by Th1 cells is tightly kept in control. Possibly, an endogenous IFN-γ-regulated feedback loop (11) underlies this regulatory mechanism. It is tempting to speculate that loss of control at the level of the IL-18 down/IL-18BP up balance by genetic or environmental factors, resulting in increased IL-18 activity, may contribute to a shift from eosinophil- to neutrophil-predominant asthma and that neutrophil-predominant asthma may lead to exacerbation of pathology and lung damage. A second (anti-)inflammatory checkpoint is suggested by the strongly polarized mRNA levels of AM arachidonic acid-metabolizing key enzymes: in eosinophilic allergic inflammation, a pronounced upregulation of Alox15 and Ptgls is observed, as opposed to the increased expression of Ptg2 in neutrophil-dominated inflammation. A shift toward LX production through Alox15 in eosinophilic asthma has been associated with anti-inflammatory activity, as specifically LXA4 was shown to have beneficial effects in mouse models (4, 7, 27). Conversely, in AM from the neutrophil-dominated inflammation model, a shift toward chiefly prostanoid production is suggested by the increased expression of Ptg2, COX-2 (encoded by Ptg2) has a higher affinity for arachidonic acid than COX-1 (encoded by Ptgs1) (34) and preferentially synthesizes PGE3 and PGD3 (8). The role of these two prostanoids in clinical asthma subphenotypes is elusive (26), but, in the allergically inflamed lung, PGE3 and PGD3 are considered as anti-inflammatory agents (35, 46). Thus it appears that there exists a dichotomy in the AM arachidonic acid metabolism by which these cells attempt to control different types of inflammation. Clearly, more studies are required to confirm whether the differential Ptgs2 vs. Ptgs1/Alox15 pathways in AM could be a discriminative feature of neutrophil vs. eosinophil asthma types, respectively.

Finally, transcriptome and flow cytometric analyses also provided clues as to how AM may directly modulate local T-cell functions in an antigen-dependent manner. Especially in the neutrophil-predominant condition, an increased capability to exert MHC I- and MHC II-mediated antigen presentation is apparent; increased expression of genes involved in T-cell communication (Cd40, antigen processing (B2m, Tap1, Tapbp, C2ta, H2-Dmb1, H2-Dmb2, H2-Oa), and presentation (H2-M3, Qa-1, Qa-2, li, H2-Aa, H2-Ab1, H2-Eb1) supports this. However, the outcome of antigen recognition by local T cells may be attenuation rather than activation. Thus we observed a dominant expression of coinhibitory (Cd274 and Pdcd1l2) over costimulatory (Cd80, Cd86, and Icosl) ligands. The gene products of Cd274 and Pdcd1l2, respectively, PD-L1 and PD-L2, have been reported to counteract established Th1- and Th2-mediated immune responses and to induce immunological tolerance (22, 40). Flow cytometric analysis of AM further supported this proposition. In the neutrophil-predominant condition, MHC II surface levels were clearly upregulated, indicating enhanced antigen presentation capacities. Likewise, coinhibitory PD-L1 levels were strongly increased in the total AM population. In contrast, AM from the eosinophilic condition showed only little upregulation of MHC II. As expected, DC showed all characteristics of potent APC, strongly increased surface levels of CD40, MHC II, and CD86, in either model, but again in the neutrophilic model a bias toward T-cell attenuation is indicated by the selective increase of PD-L1.

In conclusion, in this study, we established a CFA-based mouse model mimicking the inflammatory and glucocorticoid-resistant component of neutrophil-predominant asthma. Moreover, our results demonstrate the crucial role of immune potentiating factors present during allergic sensitization in
determining the nature of the ensuing bronchial allergic response. Furthermore, we identified distinctive endogenous regulatory checkpoints superimposed onto a generic inflammatory environment potentially controlling the observed phenotype. These findings underscore the clinical relevance of comparative studies using the here documented OVA/CAF-based model of neutrophilic allergic inflammation as a counterpart for the conventional OVA/alum-based model of eosinophilic allergic inflammation. Mechanistic studies addressing the identified anti-inflammatory balances, tight control of IL-12/IL-18 activity, prostanoids-biased arachidonic acid metabolism, and local T-cell attenuation, may lead to new insights and innovative therapeutic approaches to treat neutrophil-dominated asthma types.

ACKNOWLEDGMENTS

We thank Seppe Vander Beken for technical assistance and Chris Van Hove for help with the manuscript.

REFERENCES