Neutrophil and Eosinophil Granulocytes as Key Players in a Mouse Model of Chemical-Induced Asthma

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Received July 27, 2012; accepted October 16, 2012

Disocyanates are an important cause of chemical-induced occupational asthma. This type of immunologically mediated asthma is often characterized by a predominant granulocytic inflammation in the airways, rather than an infiltration by lymphocytes. We sought to determine the contribution of granulocytes in the outcome of chemical-induced asthma using general and specific leukocyte depletion strategies in an established mouse model of isocyanate asthma. On days 1 and 8, BALB/c mice received dermal applications with toluene-2,4-diisocyanate (TDI) or vehicle (acetone olive oil), followed by two ip injections of cyclophosphamide (CP, days 11 and 13), or one iv injection of antigranulocyte receptor 1 (aGR1, day 13) monoclonal antibody (mAb), or two iv injections of Ly6G-specific mAb (1A8, days 13 and 14). On day 15, the mice were challenged (oropharyngeal administration) with TDI or vehicle. The next day, we assessed methacholine airway hyperreactivity (AHR); bronchoalveolar lavage differential cell count; histopathology and total serum IgE; and auricular lymphocyte subpopulations and release of interleukin (IL)-2, IL-4, IL-10, IL-13, and gamma interferon by these lymphocytes. CP depleted all leukocyte types and completely prevented AHR and airway inflammation. aGR1 depleted granulocytes and CD8+ lymphocytes, which resulted in a partial prevention in AHR but no decrease in airway inflammation. Depletion of Ly6G-positive granulocytes, i.e., both neutrophils and eosinophils, prevented AHR and lung epithelial damage and significantly reduced airway inflammation. Injection of aGR1 or 1A8 led to significantly changed cytokine release patterns in TDI-treated mice. Granulocytes, both neutrophils and eosinophils, are key cellular players in this model of chemical-induced asthma.

Key Words: chemical-induced asthma; neutrophil depletion; TDI; cyclophosphamide; aGR1; 1A8.

Asthma is a chronic disorder of the airways characterized by reversible airway obstruction, nonspecific airway hyperreactivity (AHR), and airway inflammation (Lemanske and Busse, 2010). Many types of cells are involved in the pathophysiology of asthma. The contribution of mast cells, lymphocytes, and eosinophils in the induction and effector phase has been well established (Monteseirin, 2009). Although airway eosinophilic inflammation is an important feature of allergic asthma, increasing evidence exists that neutrophils also play an important role in allergic processes, especially asthma. Thus, increased neutrophil levels have been found in patients with acute severe asthma, whereas the contribution of neutrophils in mild asthma is still controversial (Ordonez et al., 2000; Profita et al., 2003; Taha et al., 2001). Furthermore, it has become apparent that certain phenotypes of asthma are characterized by an influx of neutrophils in the airways, as assessed by analysis of induced sputum or bronchoalveolar lavage (BAL) fluid (Cundall et al., 2003). This is especially the case with some forms of occupational asthma (OA) (Lemiere et al., 2002). Thus, airway neutrophilia has been reported in patients with isocyanate-induced OA, as well as in experimental animal models (De Vooght et al., 2009; Matheson et al., 2005; Park et al., 1999). Neutrophils play an important role in the immune system, acting as a first line of defense against viral, bacterial, and fungal infections. Besides their role in fighting infections, it is now well established that neutrophils can release various mediators that have effects on the Airways of asthmatic individuals (Monteseirin, 2009).

We have developed and validated a mouse model of chemical-induced asthma using toluene-2,4-diisocyanate (TDI), one of the most common causes of OA (De Vooght et al., 2010a,b; Vanoirbeek et al., 2006, 2008). In this model, dermal sensitization with TDI followed by a single airway challenge with TDI leads, among other effects, to airway inflammation that is mainly characterized by neutrophils (De Vooght et al., 2009; Vanoirbeek et al., 2004). Because we did not know whether these neutrophils are only bystanders or play an active role, we...
set out to unravel their role in the effector phase of the disease by depleting neutrophils in vivo using three different strategies. A first method was to deplete neutrophils using cyclophosphamide (CP), an agent that suppresses myelopoiesis (Daley et al., 2008). However, CP depletes not only neutrophils but also other leukocyte types. Therefore, we also used two types of antibodies against granulocytes, namely antigranulocyte receptor 1 (aGR1) that recognizes mouse neutrophils (Ly6G) and a subset of macrophages (Ly6C) and the more neutrophil-specific anti-Ly6G (1A8) (Daley et al., 2008; Li et al., 2010).

MATERIALS AND METHODS

Reagents. TDI (98% pure, CAS 584-84-9) (MW: 174.15), acetyl-β-methylcholine (methacholine), acetone, and CP were obtained from Sigma-Aldrich (Bornem, Belgium). Isoflurane (Foren) was obtained from Abbott Laboratories (S.A. Abbott N.V., Ottignies, Belgium) and pentobarbital (Nembutal) from Sanofi Santé Animale (CEVA, Brussels, Belgium). aGR1 antibody (aGR1, mouse IgG1, clone RB6.8C5) was obtained from the Immunobiology Section, Department of Microbiology and Immunology, KU Leuven, Belgium, and anti-Ly6G (1A8, mouse IgG2a) was obtained from BioXCell (West Lebanon, NH) (Li et al., 2010). For dermal applications, the vehicle (acetone olive oil [AOO] (AOO) consisted of 2 volumes of acetone (A) and 3 volumes of olive oil (Selection de Almazara, Carbonell, Madrid, Spain). For the oropharyngeal aspiration, the vehicle (AOO) consisted of a mixture of 1 volume of acetone and 4 volumes of olive oil. Concentrations of TDI are given as percent (vol/vol) in AOO. CP, aGR1, and 1A8 were dissolved in saline (0.9% NaCl), Hanks balanced salt solution (HBSS) (Invitrogen, Merelbeke, Belgium), and PBS− (Invitrogen), respectively.

Animals. Male BALB/c Olahsd mice (6 weeks old, ± 20 g) were obtained from Harlan (Horst, The Netherlands). The mice were housed in a conventional animal house with 12-h dark/light cycles. They were housed in filter top cages and received lightly acidified water and pelleted food (Trouw Nutrition, Ghent, Belgium) ad libitum. All experimental procedures were approved by the local Ethical Committee for Animal Experiments.

Groups of animals and treatment protocol. On days 1 and 8, the animals received a dermal application (20 µl) of 0.3% TDI or vehicle (AOO, 2.3%) on the dorsum of both ears. On day 15, the mice received, under light isoflurane anesthesia, an oropharyngeal aspiration (20 µl) of saline (0.9% NaCl), Hanks balanced salt solution (HBSS) (Invitrogen, Merelbeke, Belgium), and PBS− (Invitrogen), respectively.

Lymph node cells analysis. Retro-auricular lymph nodes were obtained from the same mice and were processed for each mouse separately. The lymph nodes were kept on ice in RPMI-1640 (Invitrogen) and cell suspensions were obtained by pressing the lymph nodes through a cell strainer (100 µm) (BD Biosciences). Flow cytometry (FACSArray, BD Biosciences) was performed according to the manufacturer’s instructions. Lower detection limits were 0.2, 0.3, 9.6, 13.6, 2.4, and 0.5 pg/ml, respectively.

AHR. AHR was measured 24 h after the oropharyngeal challenge, using a forced oscillation technique (FlexiVent, SCIREQ, Montreal, Canada). As previously described, mice were anesthetized with pentobarbital (70 mg/kg body weight) and airway resistance (R) to increasing concentrations of methacholine (0–10 mg/ml) was measured using a “snapshot” protocol. For each mouse, R was plotted against methacholine concentration (0–10 mg/ml) and the area under the curve (AUC) was calculated (Vanoirbeek et al., 2010).

Pulmonary inflammation (BAL). After the functional airway measurements (i.e., 1 day after challenge), mice were deeply anesthetized by an ip injection of pentobarbital (90 mg/kg body weight). Blood was first sampled from the retro-orbital plexus, and the mice were then killed by section of the abdominal vessels. Serum samples, obtained after centrifugation (14,000 × g, 10 min) of whole blood, were stored at −80°C until analysis. The lungs were lavaged, in situ, three times with 0.7 ml sterile saline (0.9% NaCl), and the recovered fluid was pooled. Cells were counted using a Bürker hemocytometer (total cells) and the BAL fluid was centrifuged (1000 × g, 10 min). The supernatant was frozen (−80°C) until further analyses. For differential cell counts, 250 µl of the resuspended cells (100,000 cells/ml) was spun (300 × g, 6 min) (Cytoospin 3, Shandon, TechGen, Zellik, Belgium) onto microscope slides, air-dried, and stained (Diff-Quik method, Medical Diagnostics, Düdingen, Germany). For each sample, 200 cells were counted for the number of macrophages, eosinophils, neutrophils, and lymphocytes.

Levels of monocyte chemotactic protein 1 (MCP-1), tumor necrosis factor-alpha (TNF-α) (Invitrogen, Nivelles, Belgium), matrix metalloproteinase-9 (MMP-9) (R&D Systems, Abingdon, UK), and interleukin (IL)-17 (R&D Systems) were measured in homogenized lung tissue with standard ELISA, according to the manufacturer’s instructions. Lower limits of detection were 9, 3.09, and 2 pg/ml, respectively.

After taking BAL fluid, the lungs were instilled with 4% formaldehyde until full inflation of all lobes, as judged visually. Evaluation of lung injury on slides stained by H&E, anti-F480 (macrophages/monocytes), or anti-Ly6G (neutrophils) was performed by an experienced pathologist who was blinded to the treatment given to the mice. Inflammation (i.e., infiltration of inflammatory cells) and epithelial damage (i.e., disruption of the epithelial barrier in the airways) were scored on semiquantitative scale of 0–3.

Total serum IgE. The OptEIA Mouse IgE set from Pharmingen (BD Biosciences) was used to measure total serum IgE (diluted 1/70). Measurements were performed according to the manufacturer’s instructions.

Data analysis. A normal distribution of the data was assessed by the D’Agostino & Pearson omnibus normality test. All data are presented as means and SD, except for the AUC data of the AHR, which is shown as individual data.
and group means. All data were analyzed using one-way ANOVA test followed by a Dunnett’s multiple comparison test (Graphpad Prism 5.01, Graphpad Software Inc., San Diego, CA). A level of $p < 0.05$ (two-tailed) was considered significant.

**RESULTS**

**Effect of Leukocyte Depletion on Body Weight**

The treatment with two CP injections, as well as the single aGR1 injection caused a significant decrease of the body weight of both AOO- and TDI-treated mice, but weight loss did not occur after two 1A8 injections. Control injections did not alter the body weight (Supplementary fig. 1).

**Effect of Leukocyte Depletion on Airway Hyperreactivity**

Figure 2 shows the average dose-response curves of the airway resistance (R) upon methacholine provocation, 24 h after the challenge with TDI or vehicle, separately for the depletion experiments using CP (Fig. 2A), aGR1 (Fig. 2B), and 1A8 (Fig. 2C). Figure 2D combines all these data and presents the areas under the R versus methacholine concentration curve (AUC) for each mouse, and the group means. In all three series of experiments, TDI sensitization followed by TDI challenge gave, as expected, a pronounced increase in airway reactivity to methacholine in nondepleted animals (TDI/Con/TDI), and the depleting agents did not by themselves affect the response to methacholine in AOO-treated control mice. Treatment with

![FIG. 1. Treatment protocols for granulocyte depletion. On days 1 and 8, the animals received a dermal application (20 µl) of 0.3% TDI or vehicle (AOO, 2:3) on the dorsum of both ears. On day 15, the mice received an oropharyngeal aspiration (20 µl) of 0.01% TDI (challenge) or vehicle (AOO, 1:4). Mice were sacrificed 24 h after the challenge. In a first neutrophil-depletion protocol, the mice received two ip injections (100 µl) of saline (control) or CP on day 11 (250 mg/kg CP) and day 13 (150 mg/kg CP). In a second neutrophil-depletion protocol, the mice were iv injected in the tail vein (100 µl) with HBSS- (control) or the neutrophil-depleting aGR1 antibody (500 µg per mouse) on day 13. In a third neutrophil-depleting protocol, the mice were ip injected (250 µl) with anti-Ly6G (1A8) (1000 µg per mouse) or PBS- (control) on days 13 and 14.](https://academic.oup.com/toxsci/article-abstract/131/2/406/1642267)
FIG. 2. Effect of granulocyte depletion on AHR 24 h after challenge with TDI or vehicle (AOO). (A)–(C) AHR to increasing concentrations of methacholine after granulocyte depletion with CP (A), aGR1 antibody (B), and anti-Ly6G (1A8) (C). (D) AUC of the airway resistance (R) for each individual mouse and the group means. The first abbreviation identifies the agent (AOO or TDI) used for dermal application on days 1 and 8 (sensitization), the second abbreviation identifies the granulocyte depletion agent (CP, aGR1, 1A8, or control), and the third abbreviation identifies the agent (AOO or TDI) used for the airway challenge on day 15. Injections with saline, HBSS−, and PBS− will always be referred to as control injections. (A)–(C) Data are presented as means; (D) individual values and group means are depicted, n = 7–11 per group. **p < 0.01 and ***p < 0.001.
CP completely abolished the AHR induced by TDI sensitization and challenge (Figs. 2A and 2D, left). Treatment with 1A8 had a similar effect (Figs. 2C and 2D, right), whereas treatment with aGr1 reduced AHR only partially (Figs. 2B and 2D, middle).

**Effect of Leukocyte Depletion on Pulmonary Inflammation**

Figure 3 shows the total numbers of macrophages, neutrophils, and eosinophils in the BAL fluid, 24h after the challenge. In the TDI-treated groups, receiving a sham injection (TDI/Con/TDI, TDI/Con/1A8, and TDI/Con/1A8), a predominantly neutrophilic inflammation was found, along with a small but significant influx of eosinophils, as previously found (De Vooght et al., 2009). In contrast, after each of the three depletion treatments neither neutrophils, nor eosinophils could be found in the BAL fluid of TDI-treated mice.

Figures 4A and 4B show the lung tissue concentrations of MCP-1 and MMP-9, respectively. CP injections significantly increased the level of MCP-1 (Fig. 4A) in both the vehicle-treated group (AOO/CP/AOO) and the TDI-treated group (TDI/CP/1A8). aGr1 and 1A8 injections did not alter the levels of MCP-1 in the lungs of control or TDI-treated mice. On the other hand, MMP-9 levels were significantly decreased in CP, aGr1, and 1A8 injected control as well as TDI-treated mice. No differences were found in levels of lung tissue TNF-α and IL-17 (data not shown).

Figure 5 shows the semiquantitative scoring of the lung inflammation (presence of alveolar macrophages and neutrophils) (Fig. 5A) and epithelial damage (Fig. 5B) on histological slides of the different groups (Fig. 5C). The lungs of TDI-treated mice who received a sham injection (TDI/Con/1A8) showed pronounced airway inflammation (macrophages/monocytes and neutrophils) and epithelial shedding. The TDI-treated and CP injected mice (TDI/CP/1A8) did not show any histological alterations, whereas the TDI-treated aGr1 (TDI/aGr1/1A8) or 1A8 (TDI/1A8/1A8) injected mice showed signs of mild airway inflammation by monocytes. Furthermore, TDI-treated aGr1 mice also showed some epithelial shedding, whereas TDI-treated 1A8 mice did not.

**Effect of Leukocyte Depletion on Lymph Node Cells and Mediators**

Figure 6A shows the total cell counts of the auricular lymph nodes, 24h after the challenge. TDI-treated mice, that received a sham injection, showed a significant increase in total number of lymphocytes. CP injections led to an almost complete disappearance of the lymphocytes in the auricular lymph nodes (Fig. 6A), thus rendering it impossible to determine lymphocyte subpopulations and cytokine release profiles in CP-treated mice. In contrast, aGr1 or 1A8 injection did not alter the total number of lymphocytes in the auricular lymph nodes (Fig. 6A). aGr1 injection did not alter the CD3+CD4+ T helper cells (Fig. 6B), the CD4+CD25+ activated/regulatory T cells (Fig. 6C), and the CD19+ B cell (Fig. 6E) subpopulations, but aGr1 injection did lead to an almost complete vanishing of the CD3+CD8+ cytotoxic T-cell subpopulation (Fig. 6D). Injection with 1A8 did not at all alter the lymph node subpopulations composition.

Figure 7 shows the cytokine release profiles from cultured auricular lymphocytes. All TDI-treated mice showed increased levels of IL-4, IL-10, IL-13, and IFN-γ and a decreased level...
of IL-2 (Figs. 7A–E), compared with the vehicle treated mice. Injection of aGR1 in TDI-treated mice led to a decreased level of IL-13 (Fig. 7E) and IFN-γ (Fig 7D), whereas IL-2 was increased again (Fig. 7B) compared with TDI-treated and sham-injected mice. In TDI-treated 1A8 injected mice, IL-2 (Fig 7B), IL-4 (Fig. 7A), and IL-13 (Fig. 7E) were significantly decreased, compared with TDI-treated, control injected mice (TDI/Con/TDI).

**DISCUSSION**

We investigated the role of leukocytes on AHR and airway inflammation in an established mouse model of chemical-induced asthma (De Vooght et al., 2009). We depleted leukocyte types before airway challenge using three different depleting
FIG. 5. Effect of granulocyte depletion on histological analysis of the lung 24 h after challenge with TDI or vehicle (AOO). The lungs were collected, fixed, stained with H&E, anti-F4/80, or anti-Ly6G, and scored. Semiquantitative scores ranging from 0 to 3 were given for airway inflammation (A) and epithelial damage (B). (C) A large airway of the different treatment groups; a ×400 magnification with an insert of a ×100 magnification is shown in each treatment group. Experimental groups are identical to Figure 2. Data are presented as means ± SD, n = 7–11 per group. *p < 0.05 and **p < 0.01.
agents. All agents caused a complete neutrophil and eosinophil depletion in the lungs, but they also affected other cell types and cytokine production profiles. Granulocyte depletion clearly influenced AHR, suggesting a key causative role for granulocytes in our mouse model of chemical-induced asthma.

Neutrophils are polymorphonuclear leukocytes produced by hematopoiesis in the bone marrow. In human adults, neutrophils make up 40–75% of circulating leukocytes, which is much more than the percentage of eosinophils (1–6%) (Opdenakker et al., 1998). Neutrophils, normally found in the blood circulation, are the first cells that migrate toward the site of inflammation during an innate immune response to tissue damage and/or infection (Sanz and Kubes, 2012). Neutrophil migration involves the successive steps of tethering, rolling, and adhesion to finally migrate between (paracellular route) or directly through (transcellular route) endothelial cells to distinct sites of inflammation along gradients of chemoattractants (Borregaard, 2010). Although airway eosinophilic inflammation is an important feature of asthma, certain subtypes of OA (De Vooght et al., 2009; Matheson et al., 2005; Park et al., 1999) and severe asthma (Ordonez et al., 2000; Profita et al., 2003; Taha et al., 2001) are characterized by a predominant neutrophilic inflammation.

The use of genetically modified animals lacking one or more immune cell types and the depletion of specific immune effector cells have extensively contributed to the understanding of several immune-related disorders (Daley et al., 2008). Because of the lack of neutrophil knockout mice, researchers have relied on the use of chemical agents or antibodies to deplete neutrophils in animal models. To our knowledge, we are the first to investigate the functional role of granulocytes in a mouse model of OA and, by depleting these cells, to demonstrate their pivotal role in the disease, both in the initiation phase (auricular lymph node alterations in T- and B-lymphocyte activation) and in the effector phase (AHR, lung inflammation, and epithelial damage).

CP was initially developed and is still used as an anticancer agent (Emadi et al., 2009). Moreover, it is also a potent immunosuppressive agent and has been used by many research groups to deplete neutrophils in mice and other animal models (Gordeuk et al., 1988; Saoulidis et al., 2011; Xu et al., 2009). CP, however, has depleting effects not only on neutrophils,
FIG. 6. Effect of granulocyte depletion on lymphocyte subpopulations in the auricular lymph nodes 24 h after challenge with TDI or vehicle (AOO). The total amount of lymphocytes (A) for the different treatment groups was assessed. Different subpopulations after aGR1 or 1A8 treatment: (B) CD3⁺CD4⁺ (T helper lymphocytes), (C) CD3⁺CD4⁺CD25⁺ (activated/T regulatory lymphocytes), (D) CD3⁺CD8⁺ (T cytotoxic lymphocytes), and (E) CD19⁺ (B lymphocytes). Experimental groups are the same as in Figure 2. Data are presented as means ± SD, n = 6–11. *p < 0.05 and ***p < 0.001.
FIG. 7. Effect of granulocyte depletion on cytokine levels in auricular lymphocytes supernatant 24h after challenge with TDI or vehicle (AOO). Auricular lymphocytes were cultured and levels of IL-4 (A), IL-2 (B), IL-10 (C), IFN-γ (D), and IL-13 (E) were measured in the lymph node cell supernatant. Experimental groups are the same as in Figure 2. Data are presented as means ± SD, n = 6–11. *p < 0.05, **p < 0.01, ***p < 0.001.
but also on leukocyte types including lymphocytes and blood monocytes (Zuluaga et al., 2006). In our study, CP treatment led to a complete depletion of neutrophils, eosinophils, and lymphocytes, whereas macrophages were not affected. The AHR induced in our model was completely abolished in CP-treated mice. However, this does not constitute proof that neutrophils were involved, because we previously found no AHR response in TDI-treated severe combined immunodeficiency disease mice (Tarkowski et al., 2007). These mice lack lymphocytes, similar to CP-treated mice, and they did not show AHR upon methacholine provocation. In addition, Matheson et al. (2001) demonstrated that athymic TDI-treated mice did not show AHR, indicating the importance of lymphocytes in the development of AHR. Overall, due to its nonspecific character, CP is not an ideal tool to study the role of neutrophils in animal models. We therefore tested two more specific neutrophil-depleting agents—aGR1 and 1A8.

aGR1 monoclonal antibody (mAb) targets the granulocyte surface marker Ly6G and binds to Ly6C antigen, which is expressed on CD8+ T cells, monocytes/macrophages and dendritic cells (Daley et al., 2008; Wojtasiak et al., 2010). Because 1A8 only binds Ly6G, 1A8 allows for a more specific neutrophil depletion without the unintended effect of binding to cells expressing Ly6C (Daley et al., 2008; Wojtasiak et al., 2010). In our study, both antibodies induced complete granulocyte depletion. In addition, the few eosinophils present in the BAL of the TDI-treated group were depleted after injection with the different agents as well, whereas no differences were found in the number of macrophages. From the literature, aGR1 also depletes monocytes, but evidence is lacking about the (direct) depleting effects of this compound on mature macrophages (Tate et al., 2009). Tate et al. (2009) found that the expression of Ly6C was low or absent on lung macrophages of wild-type animals, whereas expression was upregulated following influenza virus infection.

Surprisingly, AHR was completely abolished after 1A8 treatment, whereas only a partial decrease was observed after aGR1 injection. Considering that 1A8 is a more specific neutrophil-depleting antibody than aGR1, we had expected at least the same or a smaller decrease in AHR after 1A8 treatment. Matheson et al. (2005) already showed reductions in AHR, cytokine expression, serum antibodies, airway inflammation and histopathological lesions in CD8+ knockout mice, sensitized and challenged with TDI, compared with normal mice. Our depletion experiments with aGR1 (also recognizing CD8+ T cells) are in line with these findings. However, our data with 1A8 demonstrate clearly the pivotal role of myeloid cells. A critical role of CD8+ cells has also been demonstrated in mouse models of ovalbumin-induced asthma, with the disappearance of AHR upon depletion of CD8+ cells (Hamelmann et al., 1996). Thus, in view of the importance of CD8+ cells in asthma, aGR1 treatment causing depletion of both neutrophils and CD8+ cells does not allow us to define the specific role of neutrophils.

Possible explanations why some mice still exhibited AHR after aGR1 treatment while all mice did not react after 1A8 treatment can be found in the histological analysis of the lungs. The critical feature appeared to consist in the presence of epithelial damage, because epithelial damage was not present after 1A8 treatment, while aGR1 had no effect on the epithelial damage seen in asthmatic mice. Epithelial damage causes a less tight barrier between the airway smooth muscle cells and the airway lumen filled with inflammatory cells and cytokines. Thus, we can speculate that after aGR1 treatment the airway smooth muscle cells are probably more accessible to inflammatory cytokines and the muscarinic receptor agonist methacholine, used to provoke smooth muscle contraction. In addition, histologically assessed airway inflammation was still more pronounced after aGR1 treatment than after...
1A8 treatment. It is also known that aGR1 clone RB6.8C5 depletes myeloid-derived suppressor cells which can explain the inflammation that is still present in the lung tissue (Mauti et al., 2011).

CD4+ T lymphocytes and its cytokines IL-4 and IL-13 are known as critical participants in the pathophysiology of asthma (Venkayya et al., 2002; Wynn, 2003). More specifically, animal models reveal an essential role for IL-13 in the induction of AHR. Thus, administration of recombinant IL-13 to nonimmunized BALB/c mice is sufficient to induce an asthmatic phenotype including airway inflammation and AHR (Grunig et al., 1998; Wills-Karp et al., 1998). Similar results were seen in genetically engineered mice overexpressing IL-13 (Zhu et al., 1999). Furthermore, AHR failed to develop in ovalbumin-sensitized and challenged mice after selective blockade of IL-13 (Grunig et al., 1998; Wills-Karp et al., 1998). All these studies suggest that IL-13 is necessary and sufficient to induce AHR. Accordingly, we have observed decreases in IL-13 concentration secreted by auricular lymph nodes, along with decreases in AHR after antibody treatment. At this point, we do not know whether the observed decreases of IL-13 and some other cytokines are a direct (side) effect of antibody treatment or an indirect effect of granulocyte depletion. However, we did not find published information on neutralizing effects of the used antibodies on cytokines, suggesting that the observed decrease of IL-13 is an indirect consequence of granulocyte depletion.

Overall, our results suggest an essential role for granulocytes in the development of AHR in our mouse model of chemical-induced asthma. In contrast, Park et al. (2004) demonstrated that neutrophil depletion by aGR1 antibody did not affect the development of AHR after O3-induced lung inflammation. Likewise, AHR to methacholine remained unchanged after neutrophil depletion in a mouse model of LPS-induced airway disease (Savov et al., 2002). Although the latter mouse models are associated with some classic features of asthma including airway inflammation, AHR, and airway obstruction, substantial cellular and immunological differences are present compared with our mouse model of chemical-induced asthma. Recently, Mizutani et al. (2012) demonstrated that depletion of neutrophils by the aGR1 mAb greatly suppressed AHR in OVA-sensitized mice, which is similar to our present findings in a completely different mouse model of asthma.

Although it is known that the ligation of the mAbs to Ly6G activates the complement cascade and that the activation of these complement factors can result in AHR, such phenomenon does not seem to apply here, because we observed a suppression of AHR after treatment with these mAbs (Abbitt et al., 2009).

In conclusion, we have demonstrated that granulocytes play a key role in our mouse model of chemical-induced asthma and more specifically on AHR.

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

**FUNDING**

Interuniversity Attraction Pole Program, Belgian State, Belgian Science Policy P7/30; “Fonds voor Wetenschappelijk Onderzoek Vlaanderen” (FWO), FWO G.0547.08. V.D.V. and J.A.J.V. are postdoctoral fellows of the FWO.

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