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# Transient Neutrophil Infiltration After Allergen Challenge Is Dependent on Specific Antibodies and Fc $\gamma$ RIII Receptors<sup>1</sup>

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Following allergen challenge of sensitized mice, neutrophils are the first inflammatory cells found in bronchoalveolar lavage (BAL) fluid. To determine the underlying mechanism for their accumulation, mice were sensitized to OVA on days 0 and 14, and received, on day 28, a single intranasal challenge (s.i.n.) with either OVA or ragweed. Eight hours after the s.i.n., BAL fluid was obtained. BALB/c mice sensitized and challenged with OVA showed significantly higher total cell counts and numbers of neutrophils in BAL fluid compared to the OVA-sensitized and ragweed-challenged or nonsensitized mice. Levels of neutrophil chemokines in BAL fluid supernatants were markedly elevated in the sensitized and OVA-challenged mice; Fc $\epsilon$ RI-deficient mice showed comparable numbers of neutrophils and neutrophil chemokines in BAL fluid after s.i.n. But in sensitized mice lacking the Fc common  $\gamma$ -chain and B cell-deficient mice, the number of neutrophils and levels of neutrophil chemokines in BAL fluid were significantly lower. Further, mice lacking the Fc $\gamma$ RIII did not develop this early neutrophil influx. Neutrophil infiltration could be induced in naive mice following intranasal instillation of allergen combined with allergen-specific IgG1. In addition, macrophages from sensitized mice were stimulated with allergen and activated to produce neutrophil chemokines. These results demonstrate that neutrophil influx after allergen challenge requires prior sensitization, is allergen-specific, is mediated through Fc $\gamma$ RIII, and is dependent on the presence of Ab. *The Journal of Immunology*, 2003, 170: 4301–4309.

**A**sthma is a complex syndrome, characterized by obstruction, hyperresponsiveness, and inflammation of the airways. Inflammation in asthma is characterized by an accumulation of eosinophils, lymphocytes, and mast cells in the bronchial wall and lumen (1). Neutrophils also accumulate in the airways in patients with allergic and nonallergic asthma (2, 3). Increased numbers of neutrophils have been found in patients with more severe disease (4), in nocturnal asthma (5, 6), in steroid-dependent asthma (7, 8) and during asthma exacerbation (9). After allergen challenge of patients with allergic asthma, neutrophils are the first inflammatory cells to accumulate within the airways (10, 11) and neutrophil numbers in bronchoalveolar lavage (BAL)<sup>3</sup> fluid of patients with allergic asthma after allergen challenge have been calculated to be about 90 times higher than healthy controls (12). However, the factors regulating this transient neutrophil influx are not well-defined.

Murine models for allergic airway inflammation and airway hyperresponsiveness (AHR) are used to identify mechanisms of airway inflammation. In one such model, an increase in numbers of neutrophils has been compared to the kinetics of airway inflammation and AHR (13). In this model, mice sensitized to OVA, received a single intranasal OVA challenge. Interestingly, the initial inflammatory response (8 h after challenge) within the airways was almost exclusively neutrophilic. This increased number of neutrophils was transient (24 h), followed by a later influx of eosinophils and lymphocytes and development of AHR (13). Increased levels of the CXC chemokines macrophage inflammatory protein (MIP)-2 and cytokine-inducible neutrophil chemoattractant (KC) were found in BAL fluid (13) and they appear to be important chemoattractants for neutrophils in this early phase of the response (14).

In the present study, we have further characterized this early and transient influx of neutrophils into the lung following challenge of sensitized mice and examined the underlying mechanism for this influx. We demonstrate that neutrophil influx requires prior sensitization, is dependent on allergen-specific Ab, and is mediated through Fc $\gamma$ RIII.

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<sup>3</sup> Abbreviations used in this paper: BAL, bronchoalveolar lavage; AHR, airway hyperresponsiveness; MIP, macrophage inflammatory protein; KC, cytokine-inducible neutrophil chemoattractant; s.i.n., single intranasal challenge; EU, ELISA units; AM, alveolar macrophage.

## Material and Methods

### Animals

Female BALB/c, C57BL/6, and B6/129 mice were obtained from The Jackson Laboratory (Bar Harbor, ME), and mice with a disruption of the  $\alpha$  subunit of the high affinity IgE receptor (Fc $\epsilon$ RI<sup>-/-</sup>) (BALB/c background) were obtained from D. Dombrowicz and J. Kinet (Beth Israel Hospital, Boston, MA). Mice with a disruption of the J<sub>H</sub> gene (J<sub>H</sub><sup>-/-</sup>) (C57BL/6 background) (15) were kindly provided by Dr. L. Wysocki (National Jewish Medical and Research Center, Denver, CO). All mice, including those lacking the FcR common  $\gamma$ -chain (FcR $\gamma$ <sup>-/-</sup>) (C57BL/6 background) (16) and Fc $\gamma$ RIII (Fc $\gamma$ RIII<sup>-/-</sup>) (B6/129 background) (17) were maintained in the animal facility.

All animals were maintained on an OVA-free diet. All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of the National Jewish Medical and Research Center.

### Sensitization and airway challenge

Mice were sensitized using a previously described protocol (13). Briefly, 8- to 12-wk-old mice were sensitized by i.p. injection of either 20  $\mu$ g of ragweed (Greer Laboratories, Lenoir, NC) or 20  $\mu$ g of OVA (Grade V; Sigma-Aldrich, St. Louis, MO) suspended in 2.25 mg of aluminum hydroxide (AlumImject; Pierce, Rockford, IL) in a total volume of 100  $\mu$ l on days 0 and 14. Mice received a single intranasal challenge (s.i.n.) with ragweed (50  $\mu$ l, 2 mg/ml in normal saline) or with OVA (50  $\mu$ l, 2 mg/ml in normal saline) on day 28. For kinetic studies BAL fluid was obtained at different time points (0.25, 0.5, 1, 2, 4, 8, 12, 24, 48 h) after the challenge. In all other experiments BAL fluid was obtained 8 h after the allergen challenge.

In separate experiments nonsensitized mice received s.i.n. with either OVA (50  $\mu$ l, 2 mg/ml in normal saline), mouse IgG (50  $\mu$ l, 10  $\mu$ g/ml; Sigma-Aldrich) preincubated with 100  $\mu$ g of OVA, mouse monoclonal anti-OVA IgG1 (50  $\mu$ l, 10  $\mu$ g/ml; Sigma-Aldrich) and OVA-specific IgG1 (50  $\mu$ l, 10  $\mu$ g/ml; Sigma-Aldrich) preincubated with 100  $\mu$ g of OVA. Preincubation was performed for 30 min at 4°C. Eight hours after the single intranasal application, BAL fluid was obtained.

### Determination of cell numbers and cytokine levels in BAL

Lungs were lavaged via the tracheal cannula with HBSS (1 ml). Total cell numbers were determined by counting of cells using a Coulter Counter (Beckman-Coulter, Hialeah, FL). Differential cell counts were made from cytospin preparations (Cytospin 2; Shandon, Runcorn, Cheshire, U.K.), stained with Leukostat (Fisher Diagnostics, Pittsburgh, PA). Cells were identified as macrophages, eosinophils, neutrophils, and lymphocytes by standard hematological procedures and at least 200 cells were counted under immersion oil. BAL supernatants were collected and kept frozen at -80°C until assayed. To assess levels of intracellular cytokines, lungs were lavaged three times with 1 ml of HBSS. Cell number was determined using a Coulter Counter (Beckman-Coulter). Samples were then centrifuged (1500 rpm for 10 min) and the cell pellet was isolated. Cells were lysed and cell lysates frozen at -80°C until assayed.

The levels of cytokines secreted into the supernatants of BAL fluid samples and in the cell lysates were determined by ELISA. KC, MIP-2, and TNF- $\alpha$  (all from R&D Systems, Minneapolis, MN) were measured following the manufacturer's instructions (18). The limits of detection were <2 pg/ml for KC and <5 pg/ml for MIP-2 and TNF- $\alpha$ .

### Measurement of serum OVA-specific Ab and total IgE

Total IgE levels and OVA-specific IgE, IgG1, and IgG2a Ab levels in the serum were measured by ELISA as previously described (19). Briefly, Immulon-2 plates were coated with 5  $\mu$ g/ml OVA. After addition of serum samples, a biotinylated anti-IgE Ab (02122D; BD PharMingen, San Diego, CA) was used as the detecting Ab, and the reaction was amplified with avidin-HRP (Sigma-Aldrich). To detect IgG1 and IgG2a, alkaline phosphatase-labeled Abs (02003 E and 02013 E; BD PharMingen) were used. The OVA-specific Ab titers of samples were related to an internal pooled standard derived from sera of OVA-sensitized and -challenged BALB/c mice, which was arbitrarily assigned to be 500 ELISA units (EU). The total IgE level was calculated by comparison with a known mouse IgE standard (55 3481; BD PharMingen). The limit of detection was 100 pg/ml for total IgE.

### Assessment of neutrophil chemokine production by alveolar macrophages in vitro

BALB/c mice, 8–12 wk of age, were sensitized to OVA using the protocol described above. Fourteen days later (day 28) nonsensitized and sensitized mice were sacrificed and BAL fluid was obtained. At this time point, 96% of the cells in BAL fluid are alveolar macrophages. Cells were resuspended in culture medium (RPMI 1640 with 10% FCS and 10  $\mu$ g/ml polymyxin B supplemented with penicillin and streptomycin) and plated at 10<sup>5</sup> cells (250  $\mu$ l) per well on a 24-well plate. Cells were stimulated with 100  $\mu$ g OVA or ragweed in a final volume of 500  $\mu$ l. After 6 h of incubation at 37°C at 5% CO<sub>2</sub>, supernatants were collected and the levels of KC, MIP-2, and TNF- $\alpha$  secreted into the supernatant were determined by ELISA as described above.

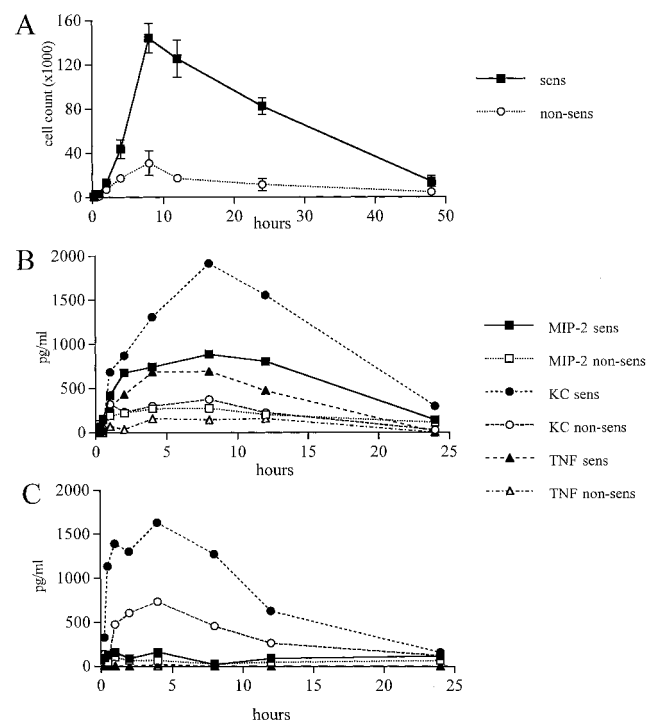
### Statistical analysis

Values of all measurements are expressed as the mean and SEM. ANOVA was used to determine the levels of difference between all groups. Comparisons for all pairs were performed by the Tukey-Kramer honest significant difference (HSD) test. Statistical significance was assumed for  $p < 0.05$ .

## Results

### Kinetics of early and transient neutrophil inflammation and neutrophil chemokine levels in BAL fluid and blood

Following a single intranasal OVA challenge, BAL fluid was obtained in the different groups of BALB/c mice at different time points (0.25, 0.5, 1, 2, 4, 8, 12, 24, and 48 h after the challenge). Nonsensitized and sensitized but not challenged mice had low neutrophil numbers (1.2  $\times 10^3$ ) in BAL fluid. Following allergen challenge, nonsensitized mice developed a small increase in neutrophil numbers at 2, 4, 8, and 24 h compared to unchallenged mice (Fig. 1A). In contrast, sensitized mice showed a significant ( $p < 0.05$ ) increase in neutrophil numbers from 2 h after the allergen challenge, peaking at 8 h (Fig. 1A). Neutrophil numbers were significantly ( $p < 0.05$ ) higher in sensitized and challenged animals compared to the nonsensitized but challenged mice at 2, 4, 8, 12, and 24 h. At 48 h there was no significant difference in neutrophil numbers between the groups (Fig. 1A). C57BL/6 mice showed similar kinetics of neutrophil influx following allergen challenge (Table I).



**FIGURE 1.** Kinetics of neutrophil infiltrate in BAL fluid and MIP-2, KC, and TNF- $\alpha$  levels in BAL fluid and serum following single intranasal OVA challenge. Neutrophil numbers in BAL fluid (A) were determined in mice receiving OVA challenge alone (non-sens) ( $n = 8$  for each time point) and OVA-sensitized and -challenged mice (sens) ( $n = 8$  for each time point) at 0.25, 0.5, 1, 2, 4, 8, 12, 24, and 48 h following the airway challenge. Means  $\pm$  SEM are given. Levels of MIP-2, KC, and TNF- $\alpha$  in BAL fluid (B) and serum (C) were determined by ELISA as described in *Materials and Methods* at 0.25, 0.5, 1, 2, 4, 8, 12, and 24 h following the airway challenge in mice receiving OVA challenge alone (MIP-2 non-sens, KC non-sens, TNF- $\alpha$  non-sens) ( $n = 8$  for each time point) and OVA-sensitized and -challenged mice (MIP-2 sens, KC sens, TNF- $\alpha$  sens) ( $n = 8$  for each time point). Mean values are given.

Table I. Neutrophil count and BAL levels of MIP-2, KC, and TNF- $\alpha$  following allergen challenge in sensitized C57BL/6 mice<sup>a</sup>

	Time (h)					
	1	2	4	8	12	24
Neutrophil count ( $\times 10^3$ )	0.2 $\pm$ 0.2	4.8 $\pm$ 1.8	25.7 $\pm$ 7.6	118.1 $\pm$ 28.7	85.7 $\pm$ 14.6	42.2 $\pm$ 6.1
MIP-2 (pg/ml)	40.2 $\pm$ 6.0	107.8 $\pm$ 6.5	184.3 $\pm$ 12	379.6 $\pm$ 64.3	276 $\pm$ 41.3	16.2 $\pm$ 5.8
KC (pg/ml)	92 $\pm$ 39.5	178.8 $\pm$ 12.2	349.3 $\pm$ 79.2	887.3 $\pm$ 98.1	342.3 $\pm$ 31.5	37 $\pm$ 25
TNF- $\alpha$ (pg/ml)	50.3 $\pm$ 26.5	94.2 $\pm$ 14.5	192.1 $\pm$ 41.2	317.1 $\pm$ 91.5	159.9 $\pm$ 33.1	0 $\pm$ 0

<sup>a</sup> Mice were sensitized and challenged as described in *Materials and Methods*. BAL fluid was obtained at different time points after the challenge. Mean values  $\pm$  SEM are given.

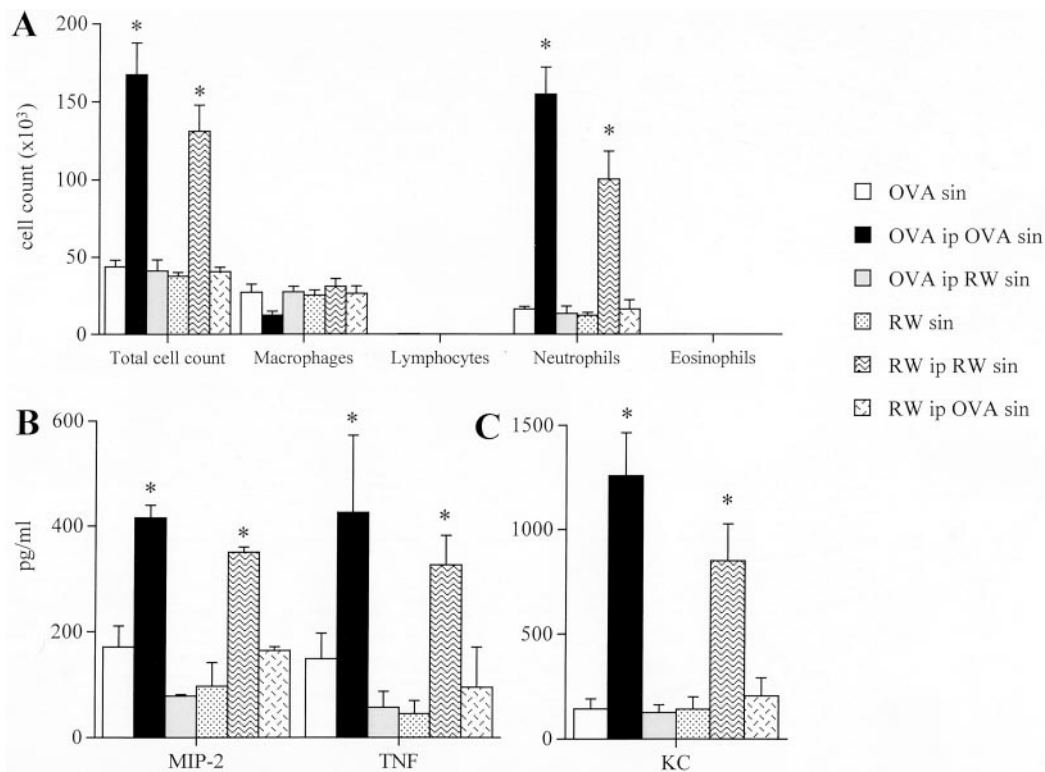
The chemokines MIP-2 and KC serve as chemoattractants for neutrophils and were measured in BAL fluid and blood. In the BAL fluid of sensitized mice there was a rapid increase of MIP-2, KC, and TNF- $\alpha$  levels starting 1 h after challenge and peaking at 8 h (Fig. 1B). At 24 h the levels of all three cytokines returned to baseline. Nonsensitized mice receiving s.i.n. showed a small increase in MIP-2, KC, and TNF- $\alpha$ , which was significantly ( $p < 0.05$ ) lower compared to the sensitized mice at 1, 2, 4, 8, and 12 h after the challenge. C57BL/6 mice showed similar kinetics for neutrophil chemokine levels in BAL fluid following allergen challenge (Table I).

In both sensitized and nonsensitized mice, no significant changes in serum levels of MIP-2 and TNF were found after s.i.n.; at most of the time points, levels were not detectable by ELISA (Fig. 1C). However, KC levels were significantly increased in serum at 30 min, reaching a maximum at 4 h and declined to baseline values by 24 h after the challenge (Fig. 1C).

#### Influx of neutrophils and release of neutrophil chemokines is allergen-specific

To assess whether this early and transient neutrophil influx is allergen-specific, mice were sensitized to OVA or ragweed and subsequently challenged with either OVA or ragweed. BAL fluid was collected 8 h after challenge and analyzed for neutrophil numbers and chemokine levels. Mice sensitized and challenged with the same allergen demonstrated significantly ( $p < 0.001$ ) higher numbers of neutrophils in BAL fluid compared to nonsensitized and challenged mice (Fig. 2A). Such increases were not observed in the BAL fluid of mice challenged with the noncorresponding allergen (Fig. 2A).

Levels of MIP-2, TNF- $\alpha$  (Fig. 2B), and KC (Fig. 2C) in BAL fluid supernates were significantly increased in sensitized mice that were challenged with the corresponding allergen, compared to sensitized mice challenged with the noncorresponding allergen and to nonsensitized mice (Fig. 2, B and C).



**FIGURE 2.** Cellular infiltrate and levels of MIP-2, KC, and TNF- $\alpha$  in BAL fluid 8 h following single intranasal allergen challenge. BAL fluid cellular composition (A) and BAL fluid levels of MIP-2, TNF- $\alpha$  (B), and KC (C) were determined as described in *Materials and Methods*. Groups consisted of mice receiving OVA challenge alone (OVA s.i.n.), OVA-sensitized and -challenged mice (OVA i.p. OVA s.i.n.), OVA-sensitized and ragweed-challenged mice (OVA i.p. RW s.i.n.), ragweed alone challenged mice (RW s.i.n.), ragweed-sensitized and -challenged mice (RW i.p. RW s.i.n.), and ragweed-sensitized and OVA-challenged mice (RW i.p. OVA s.i.n.) ( $n = 8$  for each group). Means  $\pm$  SEM are given. \*,  $p < 0.05$  compared to OVA s.i.n., OVA i.p. RW s.i.n., RW s.i.n., and RW i.p. OVA s.i.n.

Table II. Levels of serum Igs in sensitized and nonsensitized mice<sup>a</sup>

	BALB/c Nonsensitized	BALB/c Sensitized	Fc $\epsilon$ R <sup>-/-</sup> Sensitized	C57BL/6 Nonsensitized	C57BL/6 Sensitized	Fc $\gamma$ R <sup>-/-</sup> Sensitized	J <sub>H</sub> <sup>-/-</sup> Sensitized
Total IgE (ng/ml)	84.2 ± 8.1	176.3 ± 39.2	152.9 ± 45.2	20.3 ± 2.1	45.7 ± 4.5	56.3 ± 8.8	<0.1
OVA-specific IgE (EU/ml)	<10	853.4 ± 156.5	632.8 ± 56.5	<10	154.9 ± 40.7	186.7 ± 38.2	<10
OVA-specific IgG1 (EU/ml)	<10	737.1 ± 163.6	689.7 ± 202.5	<10	171.6 ± 25.3	140.6 ± 40.6	<10
OVA-specific IgG2a (EU/ml)	<10	70.6 ± 19.5	91.5 ± 37.1	<10	81.2 ± 48.3	64.2 ± 31.1	<10

<sup>a</sup> Mice were sensitized and challenged as described in *Materials and Methods*. Serum levels of Igs were assessed 8 h after the challenge. Mean values ± SEM are given; Fc $\epsilon$ R<sup>-/-</sup>: mice deficient of the high affinity IgE receptor; Fc $\gamma$ R<sup>-/-</sup>: mice deficient in the FcR common  $\gamma$ -chain; J<sub>H</sub><sup>-/-</sup>: mice with a disruption of the J<sub>H</sub> gene.

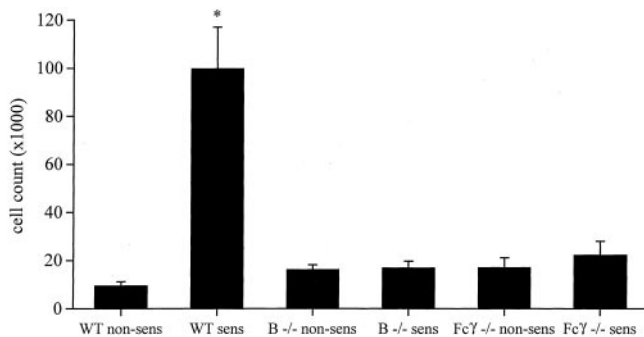
### B cell-deficient mice do not develop early neutrophil inflammation

Given the Ag specificity, we then determined whether allergen-specific Abs are required for this response. J<sub>H</sub><sup>-/-</sup> mice, devoid of serum Igs and B cells (15), were sensitized to OVA and then received s.i.n. No Igs were detected in the serum of sensitized J<sub>H</sub><sup>-/-</sup> mice compared to sensitized C57BL/6 wild-type mice, which demonstrated significantly ( $p < 0.05$ ) higher levels of total IgE, OVA-specific IgE, IgG1 and IgG2a compared to the nonsensitized wild-type mice (Table II). Similar to BALB/c mice, sensitized and challenged C57BL/6 wild-type mice showed significantly ( $p < 0.001$ ) higher numbers of neutrophils in the BAL fluid 8 h after allergen challenge (Fig. 3) compared to nonsensitized controls. In contrast, sensitized and challenged B cell-deficient mice showed no increase in neutrophil numbers (Fig. 3).

Levels of MIP-2, KC, and TNF- $\alpha$  (mean ± SEM) were not increased in BAL fluid in sensitized and challenged J<sub>H</sub><sup>-/-</sup> mice (MIP-2: 106 ± 21.3 pg/ml, KC: 108.4 ± 32.1 pg/ml, and TNF- $\alpha$ : 16.2 ± 6.9 pg/ml, respectively) compared to challenged only J<sub>H</sub><sup>-/-</sup> mice (77.6 ± 21.3, 152.7 ± 87.6, and 10.3 ± 5.2 pg/ml, respectively). In contrast, sensitized and challenged wild-type mice developed significantly ( $p < 0.01$ ) increased levels of all three cytokines following allergen challenge (419.1 ± 43.4, 826.1 ± 58.7, and 315.6 ± 29.6 pg/ml, respectively).

### Early neutrophil influx is dependent on Fc $\gamma$ RIII

As the neutrophil response was dependent on Abs, we next determined whether Ig receptors were playing a role. We first investi-



**FIGURE 3.** Neutrophil numbers in BAL fluid 8 h after a single intranasal OVA challenge. BAL fluid neutrophil numbers were determined in challenged-alone B cell-deficient mice (B<sup>-/-</sup> non-sens) ( $n = 13$ ), sensitized and challenged B cell-deficient mice (B<sup>-/-</sup> sens) ( $n = 19$ ), challenged alone mice lacking the Fc common  $\gamma$ -chain (Fc $\gamma$ <sup>-/-</sup> non-sens) ( $n = 8$ ), sensitized and challenged mice lacking the Fc common  $\gamma$ -chain (Fc $\gamma$ <sup>-/-</sup> sens) ( $n = 9$ ), challenged alone respective wild-type mice (WT non-sens) ( $n = 8$ ), and sensitized and challenged wild-type mice (WT sens) ( $n = 8$ ). Mean ± SEM are given. \*,  $p < 0.001$  compared to all other groups.

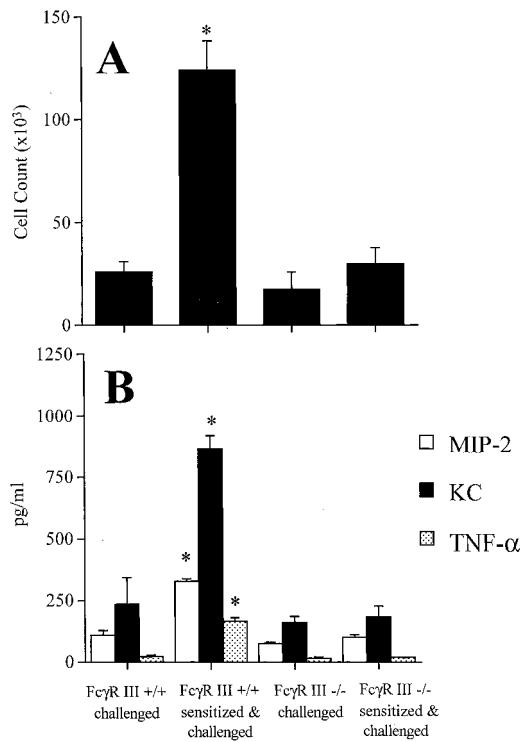
gated mice lacking the Fc common  $\gamma$ -chain (Fc $\gamma$ R<sup>-/-</sup>) which fail to express functional Fc $\gamma$ RI, Fc $\gamma$ RIII, and Fc $\epsilon$ RI. Sensitized and nonsensitized Fc $\gamma$ R<sup>-/-</sup> and wild-type (C57BL/6) mice were challenged. Ig levels in the serum were not statistically different between sensitized wild-type and sensitized Fc $\gamma$ R<sup>-/-</sup> animals (Table II). Following allergen challenge, sensitized wild-type mice showed an increase in neutrophil numbers in BAL fluid (Fig. 3) compared to nonsensitized control mice. In contrast, the number of neutrophils in BAL fluid of sensitized Fc $\gamma$ R<sup>-/-</sup> mice was not statistically different from nonsensitized animals (Fig. 3). In addition, the levels of MIP-2, KC, and TNF- $\alpha$  (mean ± SEM) in BAL fluid supernates were significantly ( $p < 0.01$ ) lower in sensitized (226 ± 53.8, 442.1 ± 175.3, and 79.4 ± 40 pg/ml, respectively) and nonsensitized (196 ± 72.1, 388.5 ± 112.8, and 52.8 ± 26.1 pg/ml, respectively) Fc $\gamma$ R<sup>-/-</sup> mice compared to sensitized wild-type mice (552.6 ± 36, 1133.7 ± 84.9, and 212.4 ± 46.2 pg/ml, respectively).

Similarly, sensitized Fc $\gamma$ RIII<sup>-/-</sup> mice showed low neutrophil counts following allergen challenge (Fig. 4A) which were not different from nonsensitized but challenged Fc $\gamma$ RIII<sup>-/-</sup> mice and significantly ( $p < 0.001$ ) lower when compared to the respective sensitized and challenged wild-type animals (Fig. 4A). In addition, sensitized and challenged Fc $\gamma$ RIII<sup>-/-</sup> mice showed low levels of KC, MIP-2, and TNF- $\alpha$  in BAL fluid, not statistically different from challenged only Fc $\gamma$ RIII<sup>-/-</sup> mice, but significantly ( $p < 0.05$ ) lower compared to sensitized and challenged wild-type animals (Fig. 4B). Serum Ig levels were similar in sensitized Fc $\gamma$ RIII<sup>-/-</sup> mice (mean ± SEM; total IgE: 37.7 ± 5.9 pg/ml, OVA-specific IgE: 79.8 ± 20.9 EU/ml; OVA-specific IgG1: 141.6 ± 39.4 EU/ml; OVA-specific IgG2a 56.5 ± 23.3 EU/ml) and sensitized wild-type mice (36.8 ± 7.2 pg/ml; 76.2 ± 18.9 EU/ml; 166.2 ± 41.4 EU/ml; 45.4 ± 27.7 EU/ml, respectively).

To determine whether there was a role for Fc $\epsilon$ RI in mediating this neutrophil response, mice with disruption of the  $\alpha$  subunit of the Fc $\epsilon$ RI (Fc $\epsilon$ RI<sup>-/-</sup>) were sensitized and challenged. Ig levels in serum were not different between sensitized Fc $\epsilon$ RI<sup>-/-</sup> mice and sensitized wild-type BALB/c controls (Table II). Eight hours after the OVA challenge, sensitized Fc $\epsilon$ RI<sup>-/-</sup> mice showed significant ( $p < 0.001$ ) increases in BAL neutrophil numbers (Fig. 5) and levels of MIP-2, KC, and TNF- $\alpha$  (mean ± SEM) (428.2 ± 36, 1286 ± 84.9, 356.7 ± 46.2 pg/ml, respectively) similar to those measured in sensitized wild-type mice (415.2 ± 53.8, 1258 ± 175.3, 425.1 ± 40 pg/ml, respectively).

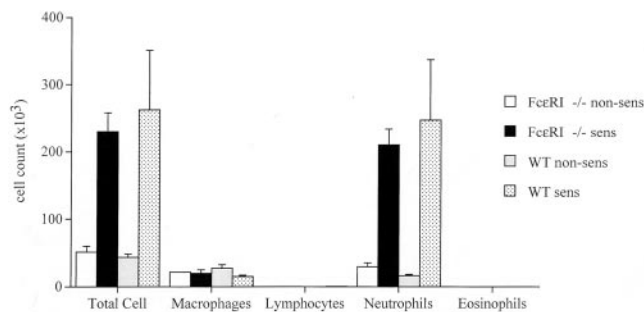
### OVA-specific IgG1 can induce neutrophil inflammation in nonsensitized mice

To determine whether OVA can mediate neutrophil inflammation through an interaction with OVA-specific IgG1, nonsensitized

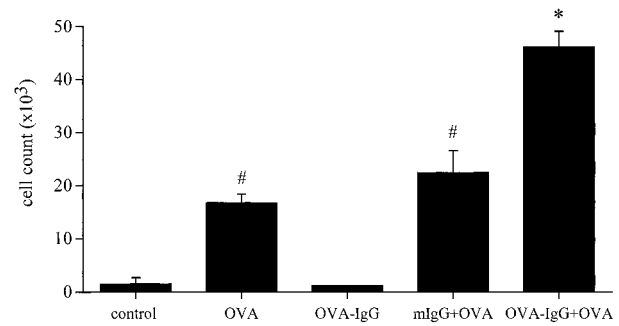


**FIGURE 4.** FcγRIII-deficient mice do not develop early neutrophil infiltration. BAL fluid neutrophil numbers (A) and BAL fluid levels of MIP-2, KC, and TNF-α (B) were determined 8 h following allergen challenge in challenged alone FcγRIII-deficient mice (FcγRIII<sup>-/-</sup> challenged), sensitized and challenged FcγRIII<sup>-/-</sup>-deficient mice (FcγRIII<sup>-/-</sup> sensitized & challenged), challenged alone respective wild-type mice (FcγRIII<sup>+/+</sup> challenged), and sensitized and challenged wild-type mice (FcγRIII<sup>+/+</sup> sensitized & challenged) (*n* = 4 in all groups). Mean ± SEM are given. \*, *p* < 0.05 compared to all other groups.

BALB/c mice received either OVA alone (OVA), OVA preincubated with nonspecific mouse IgG1 (OVA + mIgG), OVA-specific mouse IgG1 (OVA-IgG1) alone, or OVA preincubated with OVA-specific mouse IgG1 (OVA + OVA-IgG1) as described in *Materials and Methods*. Eight hours after s.i.n., BAL fluid was analyzed. Mice receiving OVA or OVA + mIgG showed an increase in the numbers of neutrophils in BAL fluid compared to controls. Mice receiving OVA-IgG1 alone showed no increase compared to sensitized but nonchallenged mice (Fig. 6). However,



**FIGURE 5.** Cellular count in BAL fluid 8 h after single intranasal OVA challenge. BAL fluid cell count numbers were determined in challenged FcεRI-deficient mice (FcεRI<sup>-/-</sup> non-sens) (*n* = 7), sensitized and challenged FcεRI deficient mice (FcεRI<sup>-/-</sup> sens) (*n* = 8), challenged alone wild-type controls (WT non-sens) (*n* = 8), and sensitized and challenged WT control (WT sens) (*n* = 8). Mean ± SEM are given. \*, *p* < 0.001 compared to FcεRI<sup>-/-</sup> non-sens and WT non-sens.



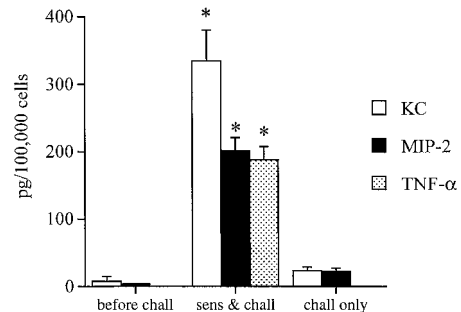
**FIGURE 6.** Neutrophil numbers in BAL fluid 8 h after s.i.n. Mice were either sensitized and not challenged (control) (*n* = 4), or nonsensitized and received either with OVA alone (OVA) (*n* = 8), OVA-specific IgG alone (OVA-IgG) (*n* = 6), unspecific mouse IgG preincubated with OVA (mIgG + OVA) (*n* = 8), OVA-specific IgG preincubated with OVA (OVA-IgG + OVA) (*n* = 14). Mean ± SEM are given. #, *p* < 0.01 compared to OVA-IgG and control; \*, *p* < 0.01 to all other groups.

mice receiving complexes of OVA + OVA-IgG1 showed significantly higher neutrophil numbers in BAL fluid compared to all other groups (Fig. 6).

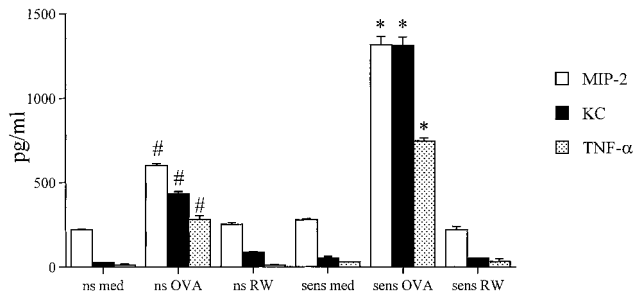
*Alveolar macrophages from sensitized mice release neutrophil chemokines when stimulated with allergen in vitro*

Alveolar macrophages are a major source of neutrophil chemoattractants. To identify whether in this model macrophages are a source of neutrophil chemokines, lungs of sensitized and nonsensitized BALB/c mice were lavaged before the challenge and 1 h following the allergen challenge. The levels of intracellular cytokines were assessed as described in *Materials and Methods*. At this time point, total cell counts and differentials were similar in all groups, about 95% of the cells being macrophages. Cell lysates prepared from nonchallenged mice showed low levels of intracellular KC, MIP-2, and TNF-α, similar to challenged only mice (Fig. 7). In contrast, sensitized and challenged mice demonstrated a significant (*p* < 0.001) increase in intracellular content of MIP-2, KC, and TNF-α (Fig. 7).

To further confirm the in vivo results were due to lung macrophage activation, we examined the response of alveolar macrophages to allergen challenge in vitro. Alveolar macrophages were obtained by lavage from OVA-sensitized (but nonchallenged) BALB/c mice, as they constitute over 95% of total BAL fluid cells. Sensitized mice developed significantly (*p* < 0.01) higher serum



**FIGURE 7.** Levels of MIP-2, KC, and TNF-α in cell lysates from alveolar macrophages (AM). AM were isolated either before airway challenge (before chall) (*n* = 8) or 1 h following single intranasal OVA challenge from either OVA-sensitized (sens & chall) (*n* = 16) or nonsensitized (chall only) (*n* = 12) mice and then lysed. MIP-2, KC, and TNF-α concentrations were analyzed by ELISA in cell lysates and normalized for cell number. Mean ± SEM are given; \*, *p* < 0.001 to all other groups.



**FIGURE 8.** Levels of MIP-2, KC, and TNF- $\alpha$  in supernatants from cultured AM. AM were isolated from either OVA-sensitized (sens) or non-sensitized (ns) mice from BAL fluid and then stimulated with either medium alone (med) OVA (OVA) or ragweed (RW). Six hours after stimulation the supernatant was collected and analyzed by ELISA. AM from mice sensitized to OVA showed an increase in MIP-2, KC, and TNF- $\alpha$  levels when stimulated with OVA (sens OVA), but not after stimulation with ragweed (sens RW). AM from nonsensitized mice displayed only a small increase after OVA stimulation (ns OVA). Each group contains data from four different wells. Mean  $\pm$  SEM are reported. #,  $p < 0.01$  compared to ns med, ns RW, sens med, sens RW; \*,  $p < 0.001$  to all other groups.

levels of total IgE (mean  $\pm$  SEM) ( $212.4 \pm 48.2$  ng/ml), OVA-specific IgE ( $661.2 \pm 196.7$  EU/ml), and OVA-specific IgG1 ( $634.9 \pm 199.2$  EU/ml) compared to the nonsensitized mice ( $106.3 \pm 17.2$  ng/ml,  $<10$  EU/ml, and  $<10$  EU/ml, respectively). When stimulated with OVA in vitro, alveolar macrophages from sensitized mice released significantly higher amounts of KC, MIP-2, and TNF- $\alpha$  compared to alveolar macrophages from nonsensitized mice (Fig. 8). This effect was allergen-specific, since stimulation with ragweed did not elicit release of KC, MIP-2, and TNF- $\alpha$  by alveolar macrophages from OVA-sensitized mice (Fig. 8). Macrophages from sensitized Fc $\gamma$ R $^{-/-}$  mice, following in vitro OVA stimulation, showed no increase in production of KC, MIP-2, and TNF- $\alpha$  ( $344.6 \pm 78.6$ ,  $201.6 \pm 21.3$ , and  $213.8 \pm 22.4$  pg/ml, respectively) when compared to macrophages from nonsensitized Fc $\gamma$ R $^{-/-}$  mice ( $419.3 \pm 96.9$ ,  $211.6 \pm 22.9$ , and  $232.4 \pm 18.8$  pg/ml, respectively), but were significantly ( $p < 0.001$ ) lower when compared to macrophages from sensitized C57BL/6 wild-type mice ( $1203.1 \pm 119.9$ ,  $911.7 \pm 42.9$ , and  $702.4 \pm 22.4$  pg/ml, respectively).

## Discussion

Following allergen challenge of sensitized hosts, neutrophils are the first inflammatory cells detected in the airways. This has been described in several species, including humans (10), horses (20), rats (21), and mice (13). The mechanism underlying this initial inflammatory response as well as the biologic relevance of this transient phenomenon in the development of AHR is not well-defined. It has been suggested that this transient neutrophilia in the airways is nonspecific, simply a response to the introduction of allergen (22). In contrast, different observations in humans (12) and in animal models (13) suggest that prior sensitization to the allergen is critical to the development of neutrophil accumulation after allergen challenge. In the present study, we show that in sensitized and challenged mice, levels of MIP-2, KC, and TNF- $\alpha$  are rapidly increased in BAL fluid as early as 2 h and peak at 8 h after the allergen challenge. Neutrophil influx into the airway begins at about 2–4 h, peaking at 8 h after allergen challenge. At 24 h, neutrophil numbers decreased by 50% in BAL fluid, neutrophil chemokine levels have returned to baseline values; 48 h after the challenge, neutrophils are no longer detected in the airways.

The neutrophilic inflammatory phase after single intranasal allergen challenge is dependent on previous sensitization of the mice with the concordant allergen. Nonsensitized mice challenged with allergen show only low numbers of neutrophils in the BAL fluid and significantly lower levels of the neutrophil chemokines (MIP-2, KC) and TNF- $\alpha$  compared to the sensitized and challenged mice.

Because prior sensitization of the mice was critical in evoking the neutrophil response after allergen challenge, we investigated whether this effect was allergen-specific. Sensitized mice only displayed this early neutrophil inflammatory response when they were challenged with the corresponding allergen, in contrast to sensitized mice challenged with a different allergen. Such specificity of the response suggests a possible and essential role for allergen-specific Abs. This was confirmed in Ab-deficient mice. Indeed, mice with a disruption of the H chain gene (lacking B cells and serum Igs (15)) did not develop an increase in neutrophil chemokines and did not develop lung neutrophilia. In contrast, it has been shown that B cell-deficient mice were capable of developing the later, eosinophilic response (23).

Mice sensitized to allergen have been shown to display high levels of different allergen-specific Ab isotypes, predominantly allergen-specific IgE and IgG1 (24), BALB/c mice are able to produce higher levels of these Abs than C57BL/6 mice (25). Following sensitization and single intranasal challenge, levels of allergen-specific IgE and IgG1 were significantly increased in the serum of sensitized mice compared to nonsensitized mice. This was true for all three strains used in this study, B6/129 and C57BL/6 mice and to a greater extent in BALB/c mice. In general, Ab molecules bound to allergen can induce neutrophil inflammation either by activating the complement system and generating peptide fragments C3a and C5a (which are highly chemotactic for neutrophils (26, 27), or by binding to FcRs on different cell surfaces triggering cellular activation (28) and production of proinflammatory cytokines (29). Macrophages and neutrophils can directly interact with Ag-Ab immune complexes through their Fc $\gamma$ R (30) and express three classes of Fc $\gamma$ Rs, Fc $\gamma$ RIIB, Fc $\gamma$ RIII, and Fc $\gamma$ RI after activation (31). The  $\gamma$  subunit is common to the high affinity (Fc $\gamma$ RI) and low affinity activation receptor (Fc $\gamma$ RIII) as well as the high affinity IgE receptor (Fc $\epsilon$ RI) (28). In this study, mice lacking the  $\gamma$  subunit did not develop a neutrophil response after allergen challenge, suggesting that Abs are acting as a stimulus via the Fc $\gamma$ Rs. These results are similar to models of immune complex-mediated diseases where the presence of Fc $\gamma$ Rs, especially Fc $\gamma$ RI and Fc $\gamma$ RIII are required to invoke a predominant neutrophilic inflammatory response (28).

IgG1 has been shown to bind to Fc $\gamma$ RIII (32, 33), similar to IgG2a and IgG2b, whereas only IgG2a binds to Fc $\gamma$ RI (34, 35). In the present study Fc $\gamma$ RIII $^{-/-}$  mice did not develop the early neutrophil inflammation and increase in neutrophil chemokine levels, suggesting that this response is mediated by Fc $\gamma$ RIII. These results are similar to findings in models of immune-complex disease. Chouchakova et al. (36) showed that Fc $\gamma$ RIII $^{-/-}$  mice demonstrated lower neutrophil counts and MIP-2 and TNF- $\alpha$  levels in BAL fluid following induction of immune complex alveolitis. Interactions through Fc $\gamma$ RIII appear to synergize with those triggered via complement activation (37).

It has been reported that in a mouse model of allergic airway inflammation, allergen-specific IgE is present in airway secretions of sensitized mice and results in immune complexes with allergen after allergen challenge (38). These immune complexes were shown to be more potent than allergen alone in inducing airway inflammation, including an increase in BAL fluid neutrophil numbers; this effect was dependent on Fc $\epsilon$ RI. Mast cells have been

reported to produce ENA-78, which can function as a potent neutrophil chemoattractant during allergic airway inflammation (39). In patients with asthma, the high affinity IgE receptor (FcεRI) may be expressed on human blood neutrophils (40), and activation through this receptor can trigger IL-8 secretion, suggesting that these cells may have an autocrine effect at the site of inflammation through the release of neutrophil chemotactic mediators. However, in contrast to humans, rodent FcεRI is only expressed on basophils and mast cells (41). In the present study, mice lacking FcεRI developed the same degree of neutrophil inflammation and release of neutrophil chemokines after allergen challenge as their wild-type littermates, indicating, at least in this model, that the presence of FcεRI is not essential to this early neutrophilia, a finding which is supported by studies in immune complex-mediated alveolitis, where mast cells were not necessary for the development of an Arthus reaction in the lung (37). Furthermore, mast cells and IgE are not necessary for the development of later events such as AHR and lung tissue inflammation (42, 43). Together, the data implicate allergen-specific IgG in the triggering of the lung neutrophilia. This is in keeping with models of active anaphylaxis, where IgG-mediated, but not IgE-mediated, pathways were shown to be involved in the inflammatory responses (44). A role for allergen-specific IgE in mediating this neutrophil inflammation can not be completely ruled out as the low affinity receptors for IgG (FcγRII and III) are also low affinity receptors for IgE (45).

Our findings that allergen and allergen-specific IgG1 together in a complex can induce lung neutrophilia after intranasal administration to naive mice further supports a role for allergen-specific IgG in mediating the early neutrophil accumulation. We used a concentration of allergen-specific IgG1 which was previously shown to passively induce AHR in vivo when given i.v. (46). This same concentration of Ab, preincubated with allergen, and administered as a s.i.n. was able to induce a neutrophil influx into the airways, confirming that allergen in combination with allergen-specific IgG can trigger neutrophil inflammation. This is similar to induction of immune complexes in the lungs in vivo using BSA or OVA and anti-BSA or anti-OVA IgG, which leads to neutrophil inflammation and AHR, an effect associated with activation of the complement system (36, 47–49). Interestingly, in this model AHR to i.v. administered MCh peaked at 1 h and was no longer detectable 24 h after the administration of the Ab, and was not associated with an influx of eosinophils and lymphocytes. In the model of airway inflammation and AHR used in the present study, AHR to inhaled MCh was detected 24 h after the allergen challenge in combination with an increase in tissue eosinophil numbers (13). These differences in the time course of AHR development might be due to different mechanisms responsible for the development of AHR, either an allergen-unspecific one, caused by acute lung injury following complement activation, or a pathway which is allergen-specific and T cell-mediated.

In the present study, OVA challenge of sensitized mice resulted in a rapid increase in neutrophil chemokine levels in the BAL fluid, well before detection of neutrophils in the airways. This suggests that resident airway cells were directly activated by allergen challenge and initiated the release of neutrophil chemokines resulting in the influx of neutrophils into the airways. Alveolar macrophages are the predominant cell type (96% of total cells) in BAL fluid in sensitized but nonchallenged mice and murine macrophages express FcγRI and FcγRIII (50). In allergen-sensitized hosts, cell-bound Ab may couple to allergen immediately following challenge, resulting in cell activation. Furthermore, macrophages from sensitized mice demonstrate high levels of MIP-2, KC, and TNF-α intracellularly, shortly following the allergen challenge, confirming that these cells at least in the initial phase of the response, are

a source of neutrophil chemokines in this model. Alveolar macrophages isolated from OVA-sensitized mice and stimulated with OVA in vitro were triggered to release significantly higher levels of MIP-2, KC, and TNF-α compared to macrophages isolated from nonsensitized or ragweed-sensitized mice. However, macrophages from nonsensitized mice, when challenged with OVA (but not ragweed) in vitro, also showed a small, but significant, increase in MIP-2, KC, and TNF-α levels, perhaps due to direct activation of these cells by OVA, which, in contrast to ragweed, is strongly mannoseylated and may activate the cells directly through Toll-like receptors. Following the initial migration of neutrophils into the airways, allergen-IgG complexes may bind directly the FcγR on the neutrophil surface, further stimulating production of chemokines in the local environment (51). This could explain the parallel increases in neutrophil numbers in the BAL fluid and levels of MIP-2, KC, and TNF-α, 8 h after the allergen challenge.

Neutrophils are regarded as the first line of defense against microorganisms, and critical effector cells in both innate and humoral immunity (52) and are capable of releasing a number of different substances, including proteases, reactive oxygen species, and mediators, which may contribute to the development of AHR (53–55). Indeed, an increasing number of clinical investigations in patients with allergic and nonallergic asthma have also described the accumulation of neutrophils in the airways (2–11). In the present study, we demonstrate that neutrophil inflammation after challenge may be driven by allergen recognition through allergen-specific IgG and FcγRs on cells capable of rapidly secreting neutrophil chemokines. This may be the first step in a cascade of inflammatory events which follow sensitization and challenge. This initial neutrophilic inflammation is followed by eosinophil and lymphocyte accumulation and altered airway function (13). The contribution of this early and transient neutrophil phase to the development of these subsequent events is under investigation.

Taken together, a paradigm emerges which may play an important role in furthering our understanding of the heterogeneity of asthma as well as pathological findings and responses to therapy. Two distinct phases result from allergen exposure of sensitized hosts. The first phase, which is dominated by a transient accumulation of neutrophils in the bronchoalveolar space, is dependent on cytophilic, allergen-specific Abs, triggered as a result of prior exposure, and FcγRIII interactions on cells capable of rapidly synthesizing and secreting neutrophil chemoattractants. This phase is presumably independent of local T cell effects and overrides some of the controversial issues of a Th1 vs Th2 unbalance. Moreover, neutrophils are resistant to the effects of corticosteroids (56). This initial phase is followed by a second, but equally distinct phase, where eosinophils and lymphocytes predominate. In contrast to the initial phase, this later phase is sustained, is dependent on T cells (CD4<sup>+</sup>), specific cytokines (e.g., IL-5 (57), IL-13 (58–59), GM-CSF (60)), and is more sensitive to corticosteroids (61). The relative roles for each phase in the development of airway pathology and altered airway function and symptoms remains to be determined. Nonetheless, one can envision differing scenarios emerging depending on which phase predominates, and which will also dictate the response to therapy.

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