

Granulocyte-macrophage colony-stimulating factor stimulates macrophages to respond to IgE via the low affinity IgE receptor (CD23)

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We have found increased concentrations of granulocyte-macrophage colony-stimulating factor (GM-CSF) in the bronchoalveolar lavage fluid of 11 patients with nocturnal asthma (15.3 ± 4.6 pg/ml) compared with normal subjects (2.3 ± 6.1 pg/ml) ($p = 0.03$). In contrast to patients with asthma, low affinity IgE receptors (FcεRII or CD23) are not expressed on monocytes obtained from healthy, nonatopic donors. FcεRII expression was induced by the cytokines GM-CSF and interleukin (IL)-4 either alone or in combination. As assessed by flow cytometry, the combination of IL-4 and GM-CSF was found to be synergistic, inducing up to $54.8\% \pm 4.6\%$ FcεRII-positive monocytes compared with a maximum of $27.4\% \pm 5.0\%$ and $30.0\% \pm 4.0\%$ with IL-4 and GM-CSF alone, respectively ($p < 0.05$ compared with either cytokine alone). Human monocytes from the peripheral blood of seven normal subjects were cultured for 24 hours with and without IL-4 or GM-CSF. With IL-4, addition of IgE/anti-IgE complexes failed to induce IL-1 secretion and inhibited IL-1 secretion induced by lipopolysaccharides. The addition of GM-CSF or IgE immune complexes alone resulted in no additional IL-1 secretion in supernatants of the untreated monocytes, whereas the IgE complexes did stimulate IL-1 secretion by monocytes cultured in GM-CSF, as measured by ELISA (from 0.7 ± 0.2 ng/ml to 2.3 ± 0.5 ng/ml; $p < 0.01$). This could be confirmed to represent an FcεRII-dependent process insofar as blocking of the FcεRII receptors abrogated the capacity of the IgE complexes to induce IL-1β (1.0 ± 0.4 ng/ml IL-1; $p = NS$ in comparison with untreated monocytes). We conclude that GM-CSF is increased in the bronchoalveolar lavage fluid of patients with asthma and may be important in macrophage activation via induction of the low affinity IgE receptor, thereby making cells susceptible to IgE-dependent activation. (J ALLERGY CLIN IMMUNOL 1994;93:650-7.)

Key words: Asthma, granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-4, low affinity IgE receptors (FcεRII or CD23), bronchoalveolar lavage, monocytes, interleukin-1

Activation of alveolar macrophages (AMs) may have an important role in the pathophysiology of asthma.¹⁻³ Secretion of AMs of preformed and newly synthesized mediators, including cytokines such as interleukin (IL)-1β and tumor necrosis factor-α, contribute to the inflammation charac-

Abbreviations used

AMs:	Alveolar macrophages
BAL:	Bronchoalveolar lavage
BSA:	Bovine serum albumin
GM-CSF:	Granulocyte-macrophage colony-stimulating factor
HBSS:	Hank's balanced salt solution
IL:	Interleukin

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teristic of allergic inflammation.³⁻⁶ Our previous observations have established the presence of AM-derived IL-1β in the airways of patients with asthma³ and the ability of mononuclear phagocytic cells to be stimulated in an allergen-specific fashion to secrete both IL-1 and TNF-α.⁷ Stimu-

lation of monocytes by allergen may be accomplished in an IgE-specific fashion through the expression on these cells of low affinity IgE receptors (FcεRII or CD23). These receptors are not expressed on monocytes or AMs obtained from healthy, nonatopic subjects but are greatly upregulated on the monocytes and AMs of patients with asthma and may be induced by several cytokines, including IL-4 and GM-CSF.^{8, 9} We therefore speculated that induction of these receptors on mononuclear phagocytic cells may contribute to the pathophysiology of asthma by rendering these cells susceptible to subsequent activation by allergen. The present studies were therefore performed to further investigate the regulation of FcεRII on monocytes and the capacity of cytokines to render monocytes capable of secreting IL-1 in response to allergen.

METHODS

Subjects

Monocyte studies were performed on a group of normal volunteers who lacked a medical history consistent with atopic disease including asthma, allergic rhinitis, or eczema. Skin prick test results to timothy, short ragweed, oak, cat, and *Der p1* were negative, and at the time of study results of spirometry were normal. Cytokine assays were performed on bronchoalveolar lavage (BAL) fluid obtained from 11 patients with asthma and seven normal control subjects. The patients with asthma met the criteria of the American Thoracic Society American College of Chest Physicians for asthma and had reversible airway obstruction.¹⁰ All subjects reported a history of seasonal allergic rhinitis and demonstrated positive skin test results (wheal > 3 mm) to at least 3 of 13 common Colorado allergens. Subjects with asthma were selected on the basis of documented nocturnal exacerbations of asthma. On the night that BAL was performed, nocturnal worsening of peak expiratory flows between 8:00 PM and 4:00 AM ranged from 24% to 41%. Subjects' asthma was controlled with inhaled albuterol (11 of 11) and theophylline (10 of 11) at the time of study and bedtime forced expiratory volume in 1 second ranged from 58% to 101% of predicted values. Subjects were excluded if they had other medical illnesses or recent upper respiratory infections or if they had taken steroids (oral or inhaled) or cromolyn within 6 weeks of the study. All subjects gave informed consent, which was approved by the Institutional Review Board at the National Jewish Center for Immunology and Respiratory Medicine.

BAL

Normal subjects and patients with asthma were admitted to the sleep laboratory at the National Jewish Center. BAL was performed at 4:00 AM. The right

middle lobe was used for each BAL. Five 60 ml aliquots were infused with gentle hand suction used to produce the effluent from each aliquot, as previously described.¹¹ BAL fluid was centrifuged, and the supernatant was collected. Fluid was dialyzed, lyophilized, and resuspended 10 times concentrated for the GM-CSF ELISA.

ELISA

Evaluation of GM-CSF within the BAL fluid and IL-1β production from stimulated monocytes was assessed by ELISA with commercially available kits. The IL-1β ELISA (Cistron Biotechnology, Piscataway, N.J.) used two monoclonal antibodies recognizing nonoverlapping epitopes. The sensitivity of this ELISA is 10 pg/ml and the assay does not detect IL-1α, IL-2, tumor necrosis factor-α, interferon-γ, IL-6, or fibroblast growth factors.¹² The GM-CSF ELISA (Genzyme, Boston, Mass.) is also a sandwich technique, however, between a monoclonal mouse antibody bound to the wells and a polyclonal rabbit anti-human GM-CSF as the second antibody. The sensitivity of this ELISA is also 10 pg/ml, and BAL fluid does not interfere with the capacity to measure GM-CSF (unpublished observations). For both ELISAs, flat-bottomed microtiter plates were coated with anti-cytokine antibodies. Authentic cytokine standards and samples (neat for BAL fluid and 1:10 and 1:100 dilution from monocyte supernatants) were applied in duplicate and allowed to bind overnight at 4° C. After washing, enzyme-linked anti-cytokine antibodies were applied. Finally, substrate was introduced and color change was determined on a Dynatech MR580 ELISA reader (Dynatech Corp., Burlington, Mass.). Data are expressed as the concentration of cytokine produced per milliliter.

Mononuclear phagocytes

Complete medium consisted of RPMI 1640 (Gibco, Grand Island, N.Y.) supplemented with penicillin (200 U/ml), streptomycin (200 μg/ml), L-glutamine (4 mmol/L), N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid buffer (25 mmol/L; pH 7.20) and 10% Nu serum (Collaborative Research, Bedford, Mass.). Heparinized venous blood was obtained from normal subjects and patients with asthma. Monocytes were isolated as described¹³ via the sequential application of Ficoll-hypaque (Pharmacia, Uppsala, Sweden) centrifugation and plastic adherence of peripheral blood. This preparation was greater than 90% monocytes as determined by Giemsa staining.

B lymphocytes

Parallel experiments were performed on B lymphocytes. B lymphocytes were enhanced in the nonadherent mononuclear cell population. These cells sequentially underwent negative selection of T lymphocytes through rosetting with neuraminidase-treated sheep erythrocytes as described by Galili and Schlesinger¹⁴

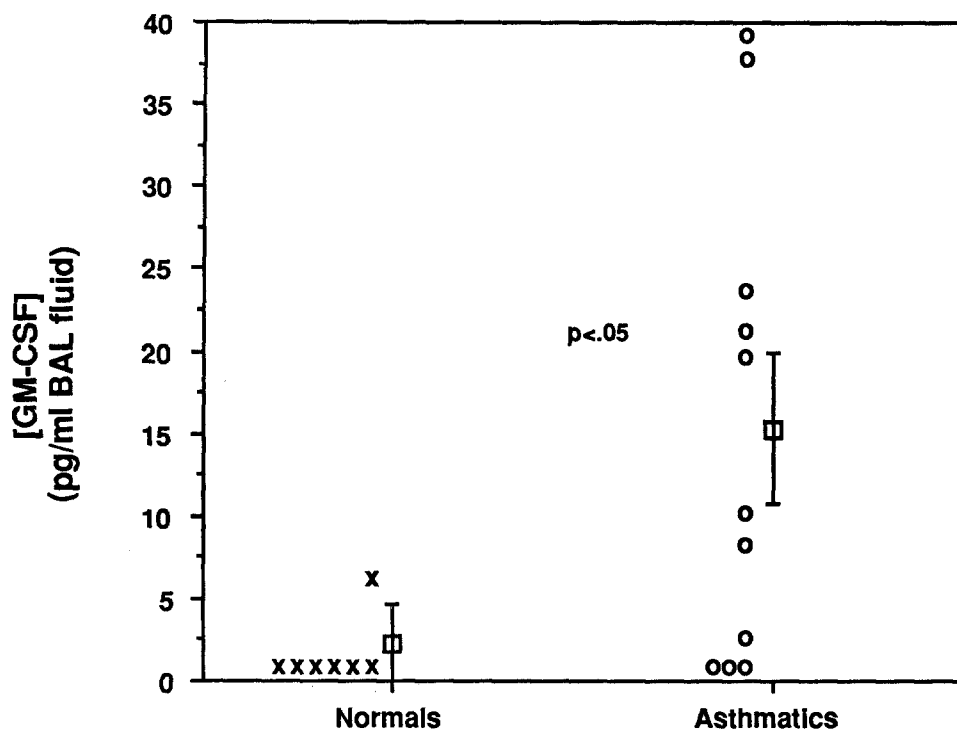


FIG. 1. GM-CSF in BAL fluid. Normal subjects ($n = 7$) and patients with asthma ($n = 11$) with documented nocturnal exacerbations of asthma were admitted to the sleep laboratory. BALs were performed at 4:00 AM. Five 60 ml aliquots of saline solution were infused and aspirated by means of gentel suction. After centrifugation, supernatants were collected, lyophilized, and resuspended 10-fold concentrated. GM-CSF concentrations were determined by ELISA.

and elimination of natural killer cells by exposure to L-leucine methyl ester (5 mmol/L) (Sigma Chemical Co., St. Louis, Mo.).¹⁵ These cells were less than 1% macrophages or T lymphocytes as determined by CD14 and CD3 staining, respectively.

FcεRII (CD23) expression

Monocytes or B lymphocytes were allowed to interact with a range of concentrations of IL-4 (10 to 1000 U; Genzyme), or GM-CSF (10 to 1000 U; Genzyme) alone or in combination. Cocultures were performed in complete medium in a 37° C, 5% CO₂ atmosphere. Surface expression of FcεRII (CD23) was determined via flow cytometric analysis after 48-hour coculture. Mononuclear cells were stained with a commercially available biotin-conjugated monoclonal mouse anti-human FcεRII antibody (1:100 dilution; The Binding Site, San Diego, Calif.). Antibodies were diluted in Hanks' balanced salt solution (HBSS) supplemented with bovine serum albumin (BSA) (0.1%; Sigma) and sodium azide (0.01%; Sigma) (HBSS/BSA/azide). Because B lymphocytes may constitutively express FcεRII (CD23),^{8, 16, 17} monocytes were additionally stained for the mononuclear phagocyte marker CD14 (LeuM3; Becton-Dickinson, Mountain View, Calif.). For the B

lymphocyte experiments, cells were dually stained with a fluorescein-conjugated anti-human CD19 antibody (Becton-Dickinson). An isotype-matched fluorescein-conjugated control mouse antibody was used to determine nonspecific binding (Becton-Dickinson). Binding was allowed to occur for 30 minutes at 4° C with continuous agitation. After washing two times in HBSS/BSA/azide, monocytes were allowed to interact with streptavidin-phycoerythrin (avidin-phycoerythrin; Becton-Dickinson). The binding of the avidin conjugate in the absence of primary antibody was performed to determine background fluorescence. After 30 minutes at 4° C, cells were again washed two times in HBSS/BSA/azide and finally resuspended in fixative (2.5% paraformaldehyde; Sigma). Dual immunofluorescence was performed on monocytes on a Coulter Epics Profile fluorescence-activated cell sorter (Coulter Corp., Hialeah, Fla.), and data were collected only for cells that stained with the LeuM3 or CD19. The flow cytometer's windows were set to demonstrate ~1% background fluorescence. Data were analyzed as the percent of cells expressing FcεRII (CD23), after subtracting out binding with the control antibodies. Experiments were performed in duplicate, and data represent the mean ± SD of seven subjects.

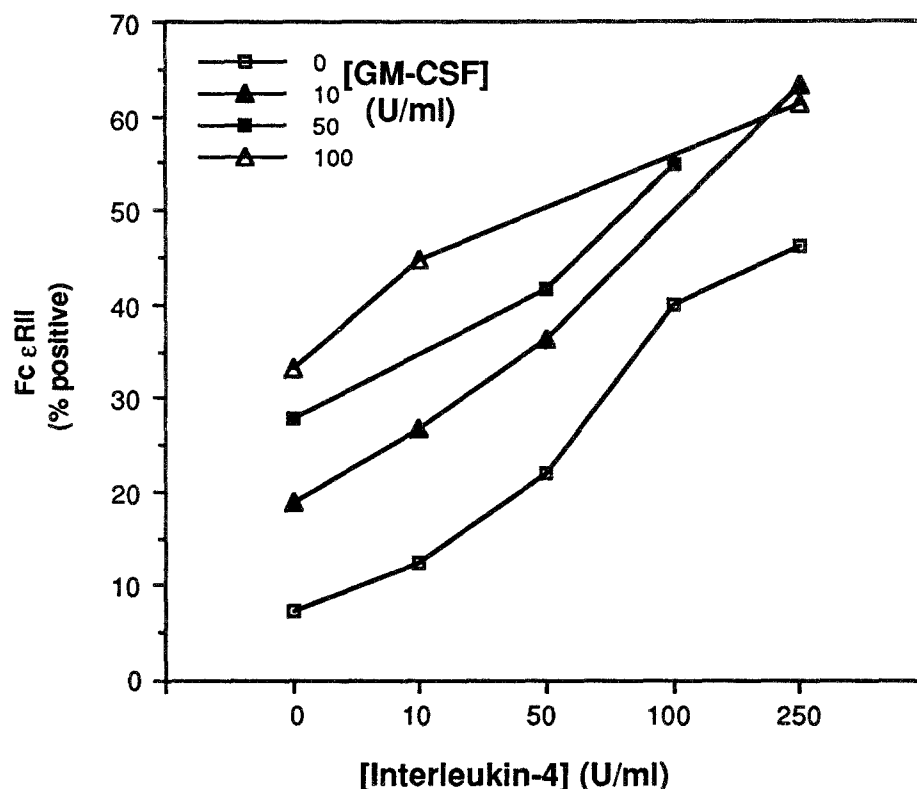


FIG. 2. GM-CSF and IL-4 modulation of FcεRII expression on monocytes. Monocytes (>90% pure) were isolated from peripheral venous blood from nonatopic subjects via Ficoll-hypaque centrifugation and plastic adherence. Cells were allowed to interact with a range of concentrations of IL-4 and GM-CSF as shown either alone or in combination for 48 hours. Surface expression of FcεRII was determined via flow cytometry with dual immunofluorescence to gate on the monocytic (CD14+) population.

IL-1 induction

The IgE-FcεRII-dependent activation of cytokine-stimulated monocytes was investigated by ELISA. Monocytes (10^6) were incubated with or without the additional presence of IL-4 (1000 U/ml) or GM-CSF (500 U/ml). After 24 hours, the monocytes were stimulated with the combination of IgE (0.1 μ mol/L) and affinity-purified goat anti-human IgE antibody (1:100; Kirkegaard & Perry Laboratories Inc., Gaithersburg, Md.) in the presence of cytokine. Lipopolysaccharide (10 μ g/ml; Sigma) was also used as a stimulus as a positive control. IgE was obtained from the U266 human IgE myeloma line via affinity purification as described previously.⁷ Contaminating endotoxin in the IgE and in anti-IgE preparations was removed with an endotoxin-removing gel (Pierce Chemical Co., Rockford, Ill.). IgE was quantified and equilibrated with RPMI 1640 before use. To assure that monocyte activation was FcεRII-dependent, experiments were performed in the additional presence of the anti-FcεRII antibody (at 1:100 dilution) and with the use of F(ab')₂ anti-human IgE antibodies (DiaMed, Inc., Windham,

Maine). Both the IgE and the α IgE contained less than 1 ng endotoxin as determined by the limulus lysate assay. Polymyxin B (10 μ g/ml; Sigma) was used to stoichiometrically bind contaminating endotoxin. After an additional 24 hours at 37° C, supernatants were collected and evaluated by ELISA for IL-1 β . Experiments were performed in quadruplicate, and seven subjects were studied for each cytokine.

Statistics

Results are presented as means $\pm$ SD for the replicate data points. Statistical analyses were performed with Student's *t* test on a Macintosh IICx computer (Apple Computer, Inc., Cupertino, Calif.) with the "Statview" software program (Brain Power, Inc., Calabasas, Calif.).

RESULTS

GM-CSF in BAL fluid

BAL fluid was obtained at 4:00 AM from normal subjects ($n = 7$) and patients who had known

TABLE I. Effect of GM-CSF on FcεRII expression by B lymphocytes

Resting	GM-CSF (1000 U/ml)	IL-4 (1000 U/ml)
6.2% ± 2.2%	7.1% ± 2.5%	26.9% ± 0.4%*

FcεRII expression was determined on CD19-positive B lymphocytes by flow cytometry of T cell, monocyte, and natural killer cell depleted mononuclear cells by dual fluorescence.

* $p < 0.05$.

nocturnal exacerbations of asthma ($n = 11$). Cell-free supernatants were dialyzed and concentrated 10-fold, and GM-CSF concentrations were determined by ELISA. Data are shown in Fig. 1 and represent means ± SD. Only one of the seven normal subjects had demonstrable GM-CSF, and for the nonasthmatic cohort there was 2.3 ± 6.1 pg GM-CSF per milliliter of concentrated BAL fluid. Eight of 11 patients with asthma had measurable GM-CSF in BAL fluid, and for this cohort there was 15.3 ± 4.6 pg GM-CSF ($p < 0.05$ compared with normal subjects).

Synergy between GM-CSF and IL-4 modulation of monocyte FcεRII (CD23 receptors)

Peripheral blood monocytes obtained from healthy, nonallergic subjects do not express FcεRII. We and others have previously demonstrated that both IL-4 and GM-CSF are able to induce low levels of FcεRII (CD23) expression on monocytes. The present studies confirm a dose-dependent induction of FcεRII expression via these cytokines, which at 1000 U/ml of cytokines achieved $27.4\% \pm 5.0\%$ expression with IL-4 and $30.0\% \pm 4.0\%$ expression with GM-CSF ($n = 7$). Because these figures are significantly lower than the level of expression observed on AMs derived from the asthmatic lung ($69.2\% \pm 6.3\%$), we investigated the capacity of IL-4 and GM-CSF to function synergistically to induce FcεRII expression. Monocytes were incubated with 10 to 1000 U/ml of each cytokine, and FcεRII expression was assayed via flow cytometry. A representative experiment is displayed in Fig. 2. The combination of cytokines induced a level of FcεRII expression ($54.8\% \pm 4.6\%$) significantly greater than that observed with either IL-4 or GM-CSF alone ($p < 0.05$ compared with either cytokine alone). As previously demonstrated,¹⁷ resting B lymphocytes expressed a low level of FcεRII ($6.2\% \pm$

2.2%), and increased expression of receptors was induced by IL-4. The present study demonstrated that in contrast to IL-4, GM-CSF does not induce FcεRII expression in purified B-lymphocyte populations (Table I).

Cytokine priming of monocytes to respond via FcεRII

Monocytes were preincubated with either IL-4 or GM-CSF for 24 hours and then exposed to the combination of IgE and anti-IgE antibodies. After an additional 24 hours, supernatants were collected and assayed for IL-1β. As we have previously shown, the IgE immune complexes fail to induce IL-1 production in the absence of FcεRII. The induction of these receptors by IL-4 did not lead to IL-1 production after exposure to the IgE complexes (the mean data from seven subjects are displayed in Fig. 3). As previously demonstrated, this may reflect the antiinflammatory effects of IL-4 on human mononuclear phagocytes insofar as IL-4 inhibited the induction of IL-1 induced by lipopolysaccharide. In contrast to IL-4, induction of FcεRII with GM-CSF did "prime" mononuclear phagocytic cells ($n = 7$) to respond to the IgE immune complexes (Fig. 3). Basal IL-1 secretion in untreated monocytes was 0.7 ± 0.2 ng/ml. Neither IgE immune complexes nor GM-CSF induced significant IL-1 secretion (Table II). However, after pretreatment with GM-CSF for 24 hours, subsequent exposure of the monocytes to the IgE complexes resulted in secretion of 2.3 ± 0.5 ng/ml IL-1 ($p < 0.01$ in comparison with untreated monocytes). This could be confirmed to represent an FcεRII-dependent process insofar as blocking of the FcεRII receptors with anti-FcεRII antibodies abrogated the capacity of the IgE complexes to induce IL-1β (1.0 ± 0.9 ng/ml IL-1; $p = \text{NS}$ in comparison with untreated monocytes) (Table II). In addition, F(ab')₂ fragments of the anti-human IgE were equally effective inducers of IL-1 production (Table II).

DISCUSSION

The allergen-specific activation of inflammatory cells, including AMs, may contribute to the pathophysiology of asthma.¹⁻³ Such activation of AMs in patients with asthma can be demonstrated by the presence of IL-1β transcripts.³ AMs may be activated in an antigen-dependent fashion by either pinocytosis or immunoglobulin-mediated phagocytosis. Pinocytosis is an inefficient process requiring relatively high concentrations of antigen

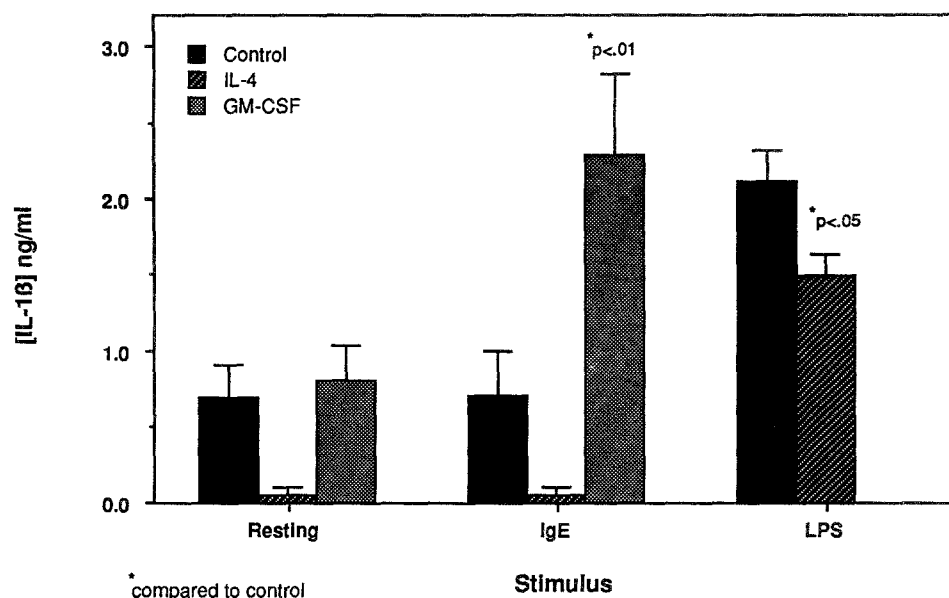


FIG. 3. IL-1 production by IL-4- and GM-CSF-treated monocytes. Monocytes were incubated with or without cytokine. After 24 hours, cells were either left in a resting state or stimulated with IgE immune complexes or lipopolysaccharides. After an additional 24 hours, supernatants were collected, and IL-1 β concentration was determined by ELISA.

TABLE II. Stimulation of IL-1 by IgE immune complexes is Fc ϵ RII-dependent

Resting	IgE complexes	GM-CSF* + IgE immune complexes		
		+ Anti-IgE	+ F(ab') ₂ Anti-IgE	+ Anti-CD23
0.68 $\pm$ 0.22	1.07 $\pm$ 0.32†	2.29 $\pm$ 0.53‡	2.75 $\pm$ 0.52‡	1.04 $\pm$ 0.90‡

Monocytes stimulated in the additional presence of GM-CSF (500 U/ml).

†*p* = NS compared with resting cell.

‡*p* < 0.01 compared with resting cell.

and mass action effects for an immune response to be initiated. Antigen may be more efficiently internalized by immunoglobulins on the surface of the antigen-presenting cells.¹⁸⁻²¹ The efficiency of immunoglobulin-mediated internalization of antigen is demonstrated by the capacity of a B lymphocyte to effectively mediate T-cell proliferation at a concentration of antigen 100 to 1000 times less than that required by an antigen-presenting cell lacking surface immunoglobulin. Such efficient processing of antigen may be critically relevant in states such as allergic asthma in which vanishingly low concentrations of antigen are present. Immunoglobulin may also be important in antigen processing by AMs as suggested by the observation that AMs fail to stimulate T-cell proliferation in response to *Listeria* species unless

first coated by immunoglobulin.²² Mononuclear phagocytic cells may process antigen through their expression of specific immunoglobulin receptors, including those for IgG, IgE, and possibly for IgA. Fc γ receptors²³ are critical to macrophage function but may be less important to antigen presentation. Only Fc γ R type I can efficiently bind monomeric IgG.²³ However, the high concentrations and broad specificity of serum and tissue IgG would render this process nonselective. Thus several studies have suggested that IgG bound to Fc γ receptors does not mediate the presentation of antigen to T lymphocytes.^{24, 25}

Fc ϵ RII receptors²⁶⁻²⁹ are expressed on Langerhans cells, follicular dendritic cells, B lymphocytes, and mononuclear phagocytes, supporting a role in antigen presentation. Fc ϵ RII-dependent

uptake of the antigen into pinocytic vesicles and phagolysosomes has been demonstrated.³⁰ In atopic dermatitis IgE bound to FcεRII on epidermal Langerhans cells functions to "capture" antigens, before antigen processing and presentation to T lymphocytes.³¹⁻³³ Similarly, on B lymphocytes, allergen-specific IgE bound to FcεRII allows antigen nonspecific B cells (B cells with surface immunoglobulin directed toward an unrelated antigen) to specifically bind and present antigen to T lymphocytes.²⁵

Support for a role for FcεRII in asthma is derived from the observations that expression of this receptor is substantially upregulated on asthmatic AMs.^{9, 34-36} Expression of FcεRII is induced by several cytokines including IL-4 and GM-CSF.^{8, 9} IL-4 is synthesized at low concentrations, and identification of IL-4 protein in human tissue has proved problematic. However, IL-4-specific messenger RNA transcripts can be identified in the asthmatic lung.³⁷ Previous studies have demonstrated the presence of GM-CSF transcripts and protein in the asthmatic lung after an allergen challenge.³⁸ The present study extends this observation to naturally occurring asthma (Fig. 1). Although both IL-4 and GM-CSF induced FcεRII expression on monocytes, neither of these cytokines induced receptor concentrations similar to those we had observed on AMs obtained from patients with asthma.⁹ This suggested that additional factors must be involved in the maturation of AMs in asthma. We therefore investigated the possibility that IL-4 and GM-CSF may act in synergy in regard to FcεRII expression. These studies demonstrated that the combination of these two cytokines is capable of inducing a level of expression of these receptors comparable to that observed on asthmatic AMs (Fig. 2).

We reasoned that after the induction of FcεRII expression on mononuclear phagocytic cells in asthma, exposure of these cells to allergen and allergen-specific IgE leads to cell activation. We therefore investigated the capacity of IL-4 and GM-CSF to "prime" mononuclear phagocytic cells from nonatopic, non-FcεRII-expressing subjects to secrete IL-1 in an FcεRII-dependent fashion. The combination of human myeloma IgE and goat anti-human IgE (as a surrogate antigen) was used to stimulate the monocytes, a combination that we have established to represent an FcεRII-dependent stimulus.⁷ When these monocytes were preincubated with IL-4, no IL-1 secretion was observed when the cells were subse-

quently exposed to the IgE complexes (Fig. 3). Furthermore, the IL-4-treated cells secreted less IL-1 when subsequently stimulated with lipopolysaccharides. These observations are consistent with previous reports that IL-4 functions as an antiinflammatory factor on mononuclear phagocytes.³⁹⁻⁴⁰ When GM-CSF was used to prime the monocytes, subsequent exposure to the IgE complexes resulted in significant stimulation of IL-1 production (Fig. 3). This could be further confirmed as representing an FcεRII-dependent process through the capacity of an anti-IgE receptor blocking antibody to abrogate IL-1 secretion and through the effectiveness of F(ab')₂ fragments of the anti-IgE antibody to induce IL-1 secretion (Table II).

In summary, these studies confirm the presence of GM-CSF in naturally occurring asthma. As previously demonstrated, both GM-CSF and IL-4 induce FcεRII expression on monocytes, but in contrast to IL-4, this activity of GM-CSF did not extend to the B lymphocyte. GM-CSF and IL-4 synergize to induce a level of expression of FcεRII on monocytes similar to that which is observed on AMs derived from patients with asthma. Pretreatment of monocytes with GM-CSF, but not IL-4, "primes" monocytes such that subsequent exposure to IgE-containing immune complexes leads to cellular activation and secretion of IL-1. These observations support a model in which a stimulus that induces FcεRII expression on mononuclear phagocytes (including allergen or possibly virally derived signals) renders them susceptible to activation and secretion of inflammatory mediators after a subsequent exposure to allergen.

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