Original article

Expression of activated FcγRII discriminates between multiple granulocyte-priming phenotypes in peripheral blood of allergic asthmatic subjects

Deon Kanters, BSc,[a]* Willem ten Hove, MSc,[a,b]* Bart Luijk, MD, PhD,[a]* Corneli van Aalst, BSc,[a] René C. Schweizer, MD, PhD,[a] Jan-Willem J. Lammers, MD, PhD,[a] Hubert G. M. Leufkens, PharmD, PhD,[b] Jan A. M. Raaijmakers, PhD,[b] Madelon Bracke, PhD,[a,b] and Leo Koenderman, PhD[a] *Utrecht, The Netherlands*

Background: Allergic asthma is associated with chronic airway and systemic immune responses. Systemic responses include priming of peripheral blood eosinophils, which is enhanced after allergen challenge. In a subpopulation of asthmatic subjects, neutrophils are associated with bronchial inflammation.
Objective: We sought to monitor systemic granulocyte priming in allergic asthmatic subjects as a consequence of chronic and acute inflammatory signals initiated by allergen challenge.
Methods: Blood was taken at baseline and 6 to 24 hours after allergen challenge in asthmatic subjects with and without late asthmatic responses. Systemic granulocyte priming was studied by using expression of cellular markers, such as α-chain of Mac-1 (αm)/CD11b, L-selectin/CD62L, and an activation epitope present on FcγRII/CD32 recognized by monoclonal phage antibody A17.
Results: Eosinophils of asthmatic subjects have a primed phenotype identified by cell-surface markers. Neutrophils of these patients were subtly primed, which was only identified after activation with N-formyl-methionyl-leucyl-phenylalanine. After allergen challenge, an acute increase in eosinophil priming characterized by enhanced expression of activated FcγRII was found in patients experiencing a late asthmatic response and not in patients with a single early asthmatic response. In contrast, expression of αm/CD11b and L-selectin on granulocytes was not different between control and asthmatic subjects and was not affected by allergen challenge. Interestingly, expression of both adhesion molecules was positively correlated, and αm expression on eosinophils and neutrophils correlated positively with bronchial hyperresponsiveness.
Conclusion: Different phases, phenotypes, or both of allergic asthma are associated with distinct priming profiles of inflammatory cells in peripheral blood.
Clinical implications: Insight in differences of systemic innate responses will lead to better definition of asthma subtypes and to better designs of new therapeutic options. (J Allergy Clin Immunol ■■■■;■■■:■■■-■■■.)

Key words: *Priming, allergen challenge, neutrophils, eosinophils, allergic asthma, gene expression, peripheral blood*

Allergic asthma is accompanied by a chronic inflammation in the airways.[1] This inflammation is characterized by the presence of T_H2-type cytokines, including IL-3, IL-4, IL-5, IL-13, and GM-CSF. These cytokines stimulate growth, differentiation, and functionality of inflammatory cells that have been implicated in asthma, such as B/T cells, mast cells, basophils, and eosinophils.[2,3] In addition to these typical T_H2 cytokines, increasing evidence indicates the involvement of other cytokines, such as TNF-α, in the chronic inflammation of asthma.[3,4]

Next to eosinophils, neutrophils are also implicated in the pathogenesis of asthma.[5-10] The presence of neutrophils is associated with an increase in the concentration of the proteolytic enzyme matrix metalloproteinase 9 in bronchoalveolar lavage fluid, tissue, and sputum,[5,11-13] suggesting a role of neutrophils in the remodeling process in asthma.

In several studies eosinophils of asthmatic patients have been reported to exhibit a preactivated or primed phenotype in peripheral blood, which can be demonstrated by an enhanced expression of priming-associated epitopes.[14,15] Priming of these inflammatory cells is particularly associated with an enhanced functionality of adhesion-associated responses that facilitates the recruitment of eosinophils from the blood to the airways. This was shown by increased chemotactic responses and transendothelial movement of these cells, which were in part mediated by upregulation of adhesion molecules.[16-20]

From [a]the Department of Pulmonary Diseases, University Medical Center Utrecht (UMCU), and [b]the Division of Pharmacoepidemiology and Pharmacotherapy, Utrecht Institute for Pharmaceutical Sciences, Faculty of Science, Utrecht University.
*These authors contributed equally to this work.
Supported by a research grant of GlaxoSmithKline and the Netherlands Asthma Foundation (NAF) project numbers 3.2.99.16, 3.2.98.40, and 3.2.01.49.
Disclosure of potential conflict of interest: L. Koenderman and J.-W. J. Lammers have received grant support from GlaxoSmithKline. J. A. M. Raaijmakers is employed by GlaxoSmithKline. The rest of the authors have declared that they have no conflict of interest.
Received for publication June 22, 2006; revised June 1, 2007; accepted for publication June 7, 2007.
Reprint requests: Leo Koenderman, PhD, Department of Pulmonary Diseases, University Medical Center, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands. E-mail: L.Koenderman@umcutrecht.nl.
0091-6749/$32.00

doi:10.1016/j.jaci.2007.06.021

Abbreviations used
αm: α-chain of Mac-1
AHR: Airway hyperresponsiveness
EAR: Early asthmatic response
FITC: Fluorescein isothiocyanate
fMLF: N-formyl-methionyl-leucyl-phenylalanine
LAR: Late asthmatic response
MoPhab: Monoclonal phage antibody

In contrast, priming of neutrophils in peripheral blood of allergic asthmatic subjects has not been described as clearly as for eosinophils. In comparison with healthy donors, neutrophils of asthmatic subjects are not characterized by differences in expression of (pre-)activation markers, such as the α-chain of Mac-1 (αm)/CD11b and L-selectin/CD62L.[15,21] However, we have previously shown that preactivation of peripheral blood neutrophils can be measured by means of gene expression analysis in asthma[22] and chronic obstructive pulmonary disease.[23] The determination of priming of neutrophils probably needs these very sensitive measures because (primed) neutrophils are thought to quickly leave the peripheral blood in response to inflammation induced after, for example, exposure to allergens.[24-26]

Little is known regarding priming phenotypes of inflammatory cells in the context of different phases, phenotypes, or both of allergic asthma. Our first study on the kinetics of priming of inflammatory cells showed that allergen challenge lead to a short burst of priming of eosinophils, but not of neutrophils, in the peripheral blood after 6 hours.[15] The data corroborated other data showing differential activation of inflammatory cells in response to allergen challenge in allergic asthmatic subjects.[27,28] Allergen exposure is characterized by 2 phenomena in the airways of allergic asthmatic patients, the early asthmatic response (EAR) and the late asthmatic response (LAR), which are associated with airflow limitation and an increase of airway hyperresponsiveness (AHR).[28] The LAR, which is maximal after 6 to 8 hours,[29] exhibits characteristics that resemble the chronic inflammatory phase of allergic asthma in the bronchial tissue, such as infiltration of inflammatory cells starting as early as 3 hours after allergen challenge.[27,30] Systemic inflammatory responses during the LAR have been described in several studies and were associated with an increase of blood eosinophil numbers that likely had a primed phenotype.[30-32]

In this study the mechanisms involved in the systemic innate immune responses of allergic asthmatic subjects were investigated before and after allergen challenge. The allergen-induced priming of peripheral blood eosinophils and neutrophils was measured in atopic subjects with mild asthma with and without allergen-induced LAR.

METHODS

Cloning of CD32 into pMT2SM_VSV

CD32 (FcγRIIA) was cloned into a pMT2 vector containing a vesicular stomatitis virus (VSV)-epitope tag. U937 cDNA was used as a template, and a PCR reaction was performed with the following primers: CD32_Fw, TCCCCCGGGATGGCTATGGAGACCCAA; CD32_Rev, TAAAGCGGCCGAGTTATTACTGTTGACATG. The PCR product was digested with *Sma*I and *Not*I restriction enzymes for cloning into the pMT2SM_VSV vector. Therefore the existing pMT2_FcαRI_VSV[33] was used to replace the FcαRI/CD89 insert by the FcγRIIA/CD32 PCR product. This new construct was verified by means of sequencing.

Generation of stable transfectants

A Ba/F3 cell line expressing FcγRIIA/CD32 was generated similarly as described for Ba/F3_FcαRI/CD89 cell lines, as described by Bracke et al.[33] In short, Ba/F3 cells were cultured at a cell density of 10^5 to 10^6 cells/mL in RPMI 1640 supplemented with 8% Hyclone serum (Gibco, Carlsbad, Calif) and recombinant mouse IL-3. For the generation of polyclonal transfectants, pMT2_VSV containing FcγRIIA was electroporated into Ba/F3 cells (0.28 V; capacitance, 960 μ-Farad [μFD]) together with pSG5-CMV-Hygro containing the hygromycin resistance gene. Cells were cultured in the presence of IL-3 and selected in 500 μg/mL hygromycin (Boehringer Mannheim, Mannheim, Germany). After 2 weeks of selection, cells were tested for CD32 expression, and positive cells were sorted with a FACSvantage flow cytometer (Becton-Dickinson Immunocytometry Systems, Mountain View, Calif). Briefly, CD32-transfected Ba/F3 cells were incubated with the CD32 mAb IV.3 for 30 minutes at 4°C and subsequently with a fluorescein isothiocyanate (FITC)–conjugated secondary antibody. Fluorescence of the cells was quantified with the flow cytometer, and IV.3-positive cells were sorted and cultured. Polyclonal cell lines were generated expressing CD32. Stable cell lines were grown continually on murine IL3 (mIL3) and hygromycin. Expression of FcγRII/CD32 was checked regularly with the flow cytometer.

Patients and healthy control subjects

We included 45 nonsmoking, steroid-naive patients with a diagnosis of asthma according to the Global Initiative for Asthma guidelines (Table I).[34] All patients had stable asthma without a respiratory tract infection in the last 4 weeks before entering the study. They had positive allergen skin prick test responses,[35] with at least 1 positive reaction to common inhaled allergens, and they all had documented AHR (PC_{20} methacholine, <8 mg/mL). When patients used low doses of inhaled or nasal corticosteroids, this medication was stopped at least 4 weeks before study entry. No other antiasthma drugs were allowed during the study, except short-acting β_2-agonists. Patients receiving an inhalation challenge had a baseline FEV_1 of greater than 70% of the predicted value after withholding β_2-agonists for 8 hours at both the control visit and on the day of the allergen challenge. Healthy subjects without asthma symptoms or presence of atopy (Table I) were selected from the laboratory and clinical staff. The medical ethics committee of the University Medical Center Utrecht (Utrecht, The Netherlands) approved the study, and all patients provided written informed consent.

Inhalation challenges

Methacholine challenge. AHR was measured by using an inhalation provocation test with methacholine. Methacholine was inhaled in doubling concentrations at 5-minute intervals in a range starting from 0.038 mg/mL to a maximum of 8 mg/mL methacholine, according to a standardized challenge protocol, to determine PC_{20} methacholine.[36] FEV_1 was measured at 30 and 90 seconds after 2-minute tidal breathing through a calibrated nebulizer (model 646; Devilbiss, Inc, Somerset, Pa; 0.13 mL/min) while the nose was clipped. The challenge with inhaled methacholine was performed until the FEV_1 decreased by at least 20% from baseline FEV_1 to determine

TABLE I. Characteristics (mean [± SEM]) of control subjects and subjects with mild asthma

	Control subjects	Asthmatic subjects
No.	26	45
Age (y)	30.1 (3.0)	24.5 (1.1)
Sex (M/F)	14/12	33/12
Atopy	−	+
Baseline FEV_1 (% predicted)	−	93.9 (1.8)
PC_{20} methacholine (mg/mL)	−	1.4 (0.3)

M, Male; *F*, female.

PC_{20}. Methacholine (Sigma-Aldrich, St Louis, Mo) was dissolved in saline (0.9%) solution.

Allergen challenge. All patients were admitted to the hospital in the morning. They underwent an allergen challenge after a short rest period according to a standardized challenge procedure.[36] Each subject's specific skin sensitivity, together with the measured baseline PC_{20} methacholine, were used to apply the Cockcroft formula to calculate the lowest dose of allergen to start with during the allergen challenge in each subject.[35] FEV_1 was measured 10 minutes after 2-minute tidal breathing in the same calibrated nebulizer as used for measurement of the PC_{20} methacholine while the nose was clipped. All FEV_1 measurements were performed with a digital spirometer (Sensorloop; SensorMedics Corp, Yorba Linda, Calif).

For safety reasons, we started 2 concentrations below the calculated PC_{20} allergen value. Thereafter, the patients inhaled doubling concentrations of allergen with 10-minute intervals until the FEV_1 decreased more than or equal to 15% from the baseline FEV_1. When a decrease in FEV_1 of more than or equal to 15% was reached, this was recorded as the EAR. FEV_1 measurements were continued every 10 minutes for the first hour, then every 30 minutes until 2 hours, and subsequently every hour until 7 hours after the allergen challenge. Then all patients inhaled salbutamol, and when they were stable, they were dismissed from the hospital with instructions to record symptoms and their peak flow at regular intervals. The LAR was defined as a decrease in FEV_1 of more than or equal to 15% from baseline.[36] Subjects with both an EAR and a LAR were defined as dual responders, and the subjects with only an EAR were defined as single responders.

Modulation of granulocyte phenotypes by allergen provocation

We investigated leukocyte priming in peripheral blood before and 6 and 24 hours after allergen challenge.[15] Patient characteristics were assessed within 1 week before the allergen challenge. Physical examination and measurement of vital parameters were performed together with measurement of baseline FEV_1, methacholine challenge, and skin prick testing. Furthermore, a sham allergen challenge with saline inhalation was performed. Blood samples were taken to measure leukocyte priming at baseline (15 minutes before start) and at 6 hours after sham challenge. In addition, FEV_1 was measured at the same time points. AHR assessed by means of a methacholine challenge test was measured at the end of this clinical assessment day (7 hours after sham challenge). On a second admission day, within a week after the assessment day, an allergen challenge was performed with repeated FEV_1 measurements. Blood samples were taken at the same time points as during sham challenge. At the third day of admission, 24 hours after allergen challenge, patients returned to the hospital for assessment of symptoms, and a methacholine challenge was performed. FEV_1 and peripheral blood leukocyte priming measurements were performed 24 hours after allergen challenge.

Procedure for staining granulocytes with monoclonal antibodies directed against FcγRII (CD32: monoclonal phage antibody A17), αm (CD11b: 44A), and L-selectin (CD62L: Dreg56)

Blood was collected in tubes containing sodium heparin as anticoagulant and put on ice immediately after venipuncture. From each tube, 2 samples of 50 μL were taken per FACS analysis. Blood samples were stained with either of the different antibodies. The first sample remained on ice until further processing. The second sample was stimulated with 10^{-6} mol/L N-formyl-methionyl-leucyl-phenylalanine (fMLF; Sigma) for 10 minutes at 37°C to determine the expression of epitopes after maximal *in vitro* stimulation. Thereafter, the cells were kept on ice during the remainder of the analysis.

For staining of monoclonal phage antibody (MoPhab) A17, we used a directly FITC-labeled MoPhab A17, as described previously.[15,37] In short, MoPhab A17 was diluted 1:10 with PBS containing 4% milk powder. One hundred microliters of this mix was added to whole-blood samples of 50 μL each and incubated for 60 minutes on ice. For staining of αm and L-selectin, 1 μL of CD11b antibody (clone 44A, 10 μg/mL) and 1 μL of CD62L antibody (clone dreg56, 10 μg/mL) were added to 50 μL of blood samples and incubated for 30 minutes on ice. These mAbs were isolated from the supernatant of hybridomas obtained from the American Type Culture Collection (Rockville, Md).

After incubation with the different antibodies, erythrocytes were lysed in isotonic ice-cold NH_4Cl solution, followed by centrifugation at 4°C. In the case of anti-αm/CD11b and anti-L-selectin/CD62L antibodies, cells were counterstained with 1 μL of goat antimouse (GAM)-FITC (Becton-Dickinson). For all conditions, the cells were washed at 4°C and resuspended in ice-cold PBS containing 1% human serum albumin (Sanquin, Amsterdam, The Netherlands) for analysis. Cells were analyzed in a FACSvantage flow cytometer (Becton-Dickinson). Neutrophils and eosinophils were identified according to their specific side-scatter and forward-scatter signals.[20,23] Data are reported as median fluorescence intensity in arbitrary units.

Statistics

The results are expressed as means ± SEM. Differences between study groups were compared by using the Mann-Whitney *U* test or the Wilcoxon signed-rank test. We used repeated-measures ANOVA for statistical evaluation of the effect of allergen exposure (baseline or 6 and 24 hours after allergen challenge). Correlation studies were performed with Spearman correlation tests. All statistical tests were performed with the Statistical software package SPSS version 13.0 (Chicago, Ill). *P* values of less than .05 were considered statistically significant (*$P < .05$, **$P < .005$, ***$P < .001$).

RESULTS

Phage antibody A17 recognizes FcγRIIA (CD32) in an activated state

We have developed a MoPhab A17 that recognizes innate immune cells activated *in vitro* and *in vivo*.[15,37] Here we show that this antibody recognizes FcγRII in an active conformation. As can be seen from Fig 1, murine Ba/F3 cells expressing active human FcγRIIA are specifically recognized by A17, whereas Ba/F3 cells expressing human FcαRI[33] are not. This phage antibody does not recognize FcγRII on unprimed granulocytes while this receptor is nicely expressed (Fig 2). Activation of granulocytes

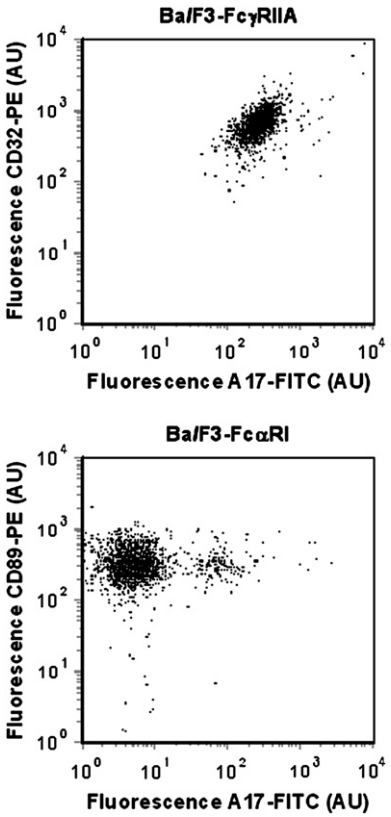

FIG 1. MoPhab A17 specifically recognizes FcγRIIA (CD32) on the murine pre–B-cell line Ba/F3. Ba/F3 cells were stably transfected with FcγRIIA or FcαRI (CD89) as a control. Thereafter, the cells were double stained with MoPhab A17 (FITC labeled) and with either IV.3/CD32 (phycoerythrin *[PE]* labeled) or A59/CD89 (PE labeled). Fluorescence is depicted in arbitrary units *(AU)*.

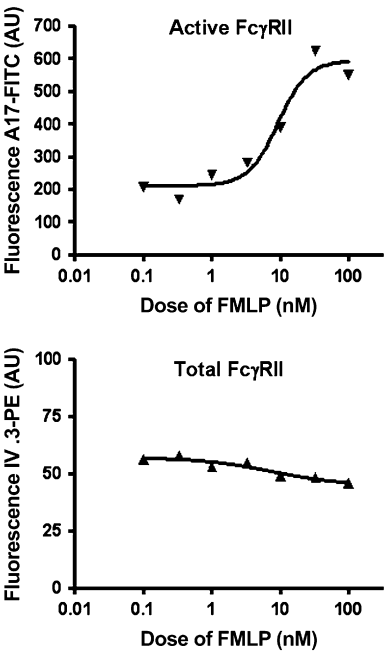

FIG 2. MoPhab A17 only recognizes FcγRIIA (CD32) when neutrophils are activated with fMLF. Median channel fluorescence is depicted in arbitrary units *(AU)*. Whole blood was activated with different amounts of fMLF before erythrocytes were lysed, and the leukocytes were stained with MoPhab A17 or mAb IV.3 (CD32). Neutrophils were identified according to their specific side-scatter and forward-scatter signals. Fluorescence is depicted in arbitrary units (AU). Data shown are means of 3 different experiments.

with different concentrations of fMLF is associated with rapid expression of the activation epitope on FcγRII recognized by A17, whereas the expression of the receptor *per se* identified by mAb IV.3[38] stays constant. Similarly, as in primary granulocytes, transfected Ba/F3 cells need cytokine priming to bind to IgG-coated particles (results not shown).

Priming of eosinophils and neutrophils is associated with differential expression of activation epitopes

Eosinophils from allergic asthmatic subjects exhibited a higher expression of activated FcγRII compared with cells from healthy donors (Fig 3, *A* and *B*).[15] In marked contrast to eosinophils, neutrophil expression of active FcγRII did not differ between healthy control and asthmatic subjects (Fig 3, *A* and *C*). Further characterization of granulocyte priming was performed by staining for the integrin subunit αm/CD11b and L-selectin/CD62L. Both markers on eosinophils and neutrophils did not differ significantly between healthy control and asthmatic subjects (Fig 3, *D* and *E*, respectively), making these markers less useful for the determination of granulocyte priming in the systemic compartment in allergic asthma. Interestingly, a clear positive correlation was found between the expression of αm/CD11b and L-selectin/CD62L on both eosinophils and neutrophils from asthmatic subjects (Fig 4).

Eosinophils of asthmatic subjects showed an enhanced responsiveness to the innate immune stimulus fMLF in the context of expression of active FcγRII compared with those of control subjects (Fig 3, *B*). Interestingly, neutrophils also showed an enhanced responsiveness to fMLF (Fig 3, *C*). Comparable with earlier studies,[39,40] activation of eosinophils and neutrophils by fMLF *in vitro* increased the expression of αm/CD11b and decreased the expression of L-selectin/CD62L on granulocytes of both asthmatic subjects (Fig 3, *F*) and control subjects (data not shown). In agreement with the unstimulated samples, the fMLF-stimulated granulocytes also did not show differences between control and asthmatic subjects on CD11b or L-selectin expression (data not shown).

Different priming phenotypes of granulocytes found in single and dual responders after allergen challenge

We next investigated the effect of bronchial allergen challenge on the systemic inflammatory responsiveness of granulocytes. This was investigated in allergic patients who had both an EAR and a LAR and in patients who only had an EAR (Table II). No differences were found on expression of active FcγRII on neutrophils and eosinophils

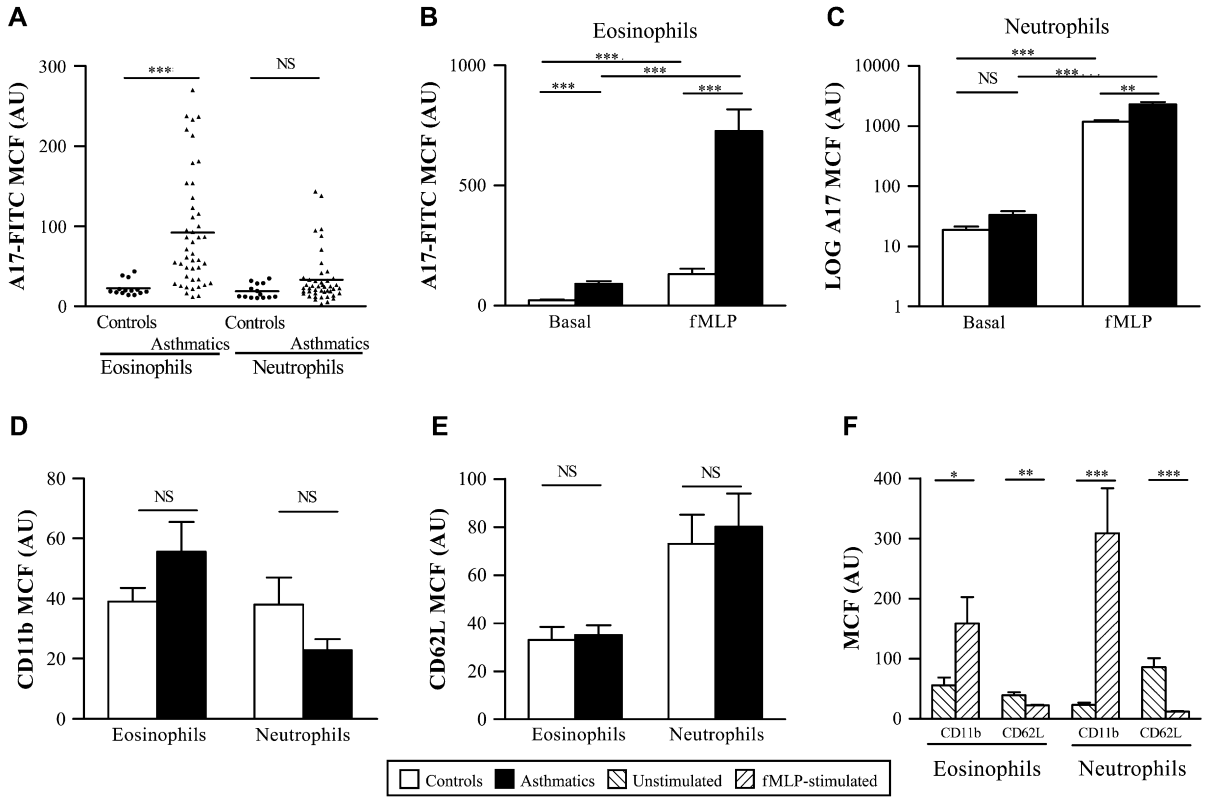

FIG 3. *In vivo* priming of eosinophils (**A**, **B**, and **D-F**) and neutrophils (**A** and **C-F**) in asthmatic subjects compared with cells from control subjects. Priming was measured by means of determination of expression of MoPhab A17 (Fig 3, *A-C*), αm (CD11b; Fig 3, *D* and *F*), or L-selectin (CD62L; Fig 3, *E* and *F*). Statistics was performed with Mann-Whitney *U* tests. Median channel fluorescence is depicted in arbitrary units *(AU)*.

between dual and single responders (Fig 5). Six hours after allergen challenge, eosinophils showed an increased expression of active FcγRII in dual responders, whereas no effect was seen in single responders. Interestingly, allergen challenges had no effect on expression of active FcγRII on neutrophils in both single and dual responders (Fig 5).

As expression of active FcγRII on neutrophils was not affected at basal levels, and 6 hours after allergen challenge, we investigated the responsiveness of the cells to fMLF. As shown in Fig 6, the responsiveness for fMLF of neutrophils and eosinophils was differentially modulated by allergen challenge in dual and single responders. In dual responders eosinophils did not show an enhanced responsiveness to fMLF in the context of expression of active FcγRII after allergen challenge (Fig 6, *A*). In contrast, on allergen challenge neutrophils of these dual responders showed an enhanced responsiveness to *in vitro* stimulation with fMLF (Fig 6, *B*). In single responders, on the other hand, eosinophils showed a decreased responsiveness to fMLF stimulation after allergen challenge (Fig 6, *C*), whereas neutrophils were not affected in these patients (Fig 6, *D*). In contrast to expression of active FcγRII, no differences in basal or fMLF-stimulated expression of CD11b or L-selectin were found 6 hours after allergen challenge in dual or single responders (data not shown). All priming responses induced by allergen exposure in fMLF-stimulated granulocytes were normalized to baseline 24 hours after allergen challenge (data not shown).

Different inflammatory responses are reflected by differences in granulocyte priming phenotypes

The correlation between priming markers and long-term characteristics of the asthma phenotype, such as enhanced AHR, was studied. Previous studies have shown that αm/CD11b expression on eosinophils in peripheral blood correlated inversely with low levels of AHR to histamine.[21] We could confirm the correlation between CD11b and eosinophil AHR in our patients (Fig 7, data shown are from dual responders; Table II). In addition to the correlation in eosinophils, we also identified a strong correlation between AHR and CD11b expression levels on neutrophils (Fig 7, *B*, data shown are from dual responders; Table II). In the same patient group no correlation could be found between AHR and L-selectin levels on eosinophils ($r = -0.50$, $P = .22$) and neutrophils ($r = -0.67$, $P = .06$), but this might be caused by the low power of the study. In addition, no correlation was found between AHR and MoPhab A17 expression on eosinophils ($r = -0.32$, $P = .41$) and neutrophils ($r = -0.30$, $P = .44$).

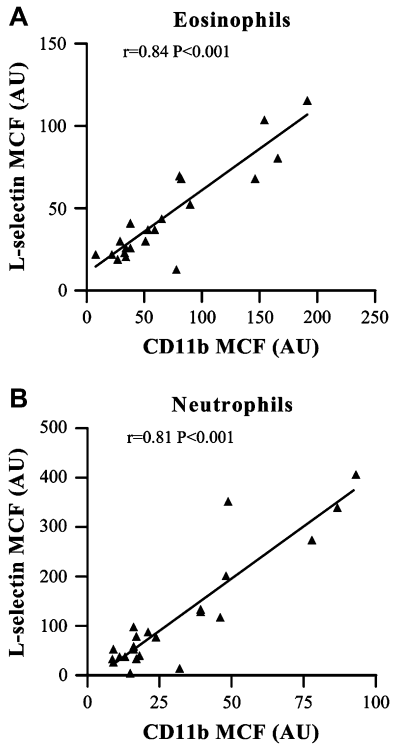

FIG 4. Integrin subunit αm/CD11b expression correlates positively with L-selectin/CD62L expression on eosinophils (**A**; n = 25) and neutrophils (**B**; n = 23) in peripheral blood of asthmatic subjects. Data regarding αm/CD11b expression levels are plotted against the L-selectin/CD62L expression values within the same patient. Median cell fluorescence *(MCF)* is presented in arbitrary units *(AU)*.

TABLE II. Characteristics (mean [± SEM]) of single and dual responders

	Single responders	Dual responders
No.	7	10
Age (y)	23.6 (3.4)	22.7 (0.9)
Sex (M/F)	7/0	4/6
Baseline FEV_1 (% predicted)	94.1 (3.8)	87.9 (2.8)
PC_{20} methacholine (mg/mL)*	0.79 (0.08-5.22)	0.66 (0.08-4.90)
Allergen challenge with HDM/cat/grass	2/3/2	8/2/0
EAR (% decrease in FEV_1 from baseline)	19.8 (2.3)	24.9 (2.5)
LAR (% decrease in FEV_1 from baseline)	1.8 (1.1)	30.0 (4.1)
Average onset LAR	–	5 h

M, Male; *F*, female; *HDM*, house dust mite.
*Geometric mean of baseline PC_{20} methacholine (range).

DISCUSSION

Priming of inflammatory cells in peripheral blood is a general process in chronic inflammatory diseases, such as allergic asthma.[15,16,40,41] Important inflammatory processes, such as chemotaxis, adhesion, and transendothelial migration, can all be preactivated *in vivo* in peripheral blood cells. This process can be mimicked by adding T_H2 cytokines to nonprimed granulocytes from healthy individuals *in vitro*.[16,41,42] Therefore priming of peripheral blood granulocytes reflects part of the systemic inflammatory response in allergic asthma.[16,43] We used this priming response to study the systemic innate immune response in different phenotypes and phases of allergic asthma, with a focus on modulation of both eosinophils and neutrophils.

The expression of the integrin subunit αm/CD11b, L-selectin/CD62L, or both is modulated by activation *in vitro* and is therefore used as a marker of leukocyte activation.[39,40,44] Previous studies have shown that no differences in expression of αm or L-selectin were found on granulocytes of subjects with mild (to moderate) asthma (with or without treatment) compared with cells of control subjects.[21,40] We developed the MoPhab A17 as a tool to detect cytokine-primed leukocytes *in vitro* and *in vivo*.[15,37] A17 recognizes FcγRII (CD32) only in the context of activated cells (Figs 1 and 2). Ba/F3 cells stably transfected with FcγRIIA express an activated FcγRII when cytokines are present in the culture medium and are clearly recognized by the A17 antibody (Fig 1). The binding is specific because Ba/F3 cells stably transfected with FcαRI (CD89)[33] are not recognized by A17 under the same conditions. The A17 antibody only recognizes active FcγRII on neutrophils because activation of cells is essential for A17 to bind (Fig 2). The expression of FcγRII *per se* visualized with the antibody IV.3 is constant, irrespective of the presence of activators. The data are in concordance with our earlier findings showing that the functionality of FcγRII on primary eosinophils,[45] as well as in transfected Ba/F3 cells,[46] is under the control of inside-out signals induced by both chemoattractants and cytokines. FcγRII-mediated binding of these cells to IgG-coated targets is critically dependent on the presence of these stimuli. Although the epitope recognized by A17 remains to be identified, binding of A17 to active FcγRII is inhibited by preincubation of the cells with an excess of mAb IV.3, which recognizes the D2γ domain of the extracellular portion of the receptor.[38]

We studied the expression of αm and L-selectin on granulocytes to compare and validate the kinetics of the expression of active FcγRII on these cells. Expression of active FcγRII seems to be more sensitive to priming stimuli compared with αm/CD11b, because the A17 antibody could recognize eosinophil priming in asthmatic subjects even without allergen challenge (Fig 3),[15] whereas αm/CD11b and L-selectin/CD62L expression levels were similar on eosinophils or neutrophils of both asthmatic and control subjects (Fig 3).

Much to our surprise, strong positive correlations were found between L-selectin/CD62L and αm/CD11b expression levels on both eosinophils and neutrophils from asthmatic subjects (Fig 4). These data suggest that expression of αm/CD11b and L-selectin/CD62L is controlled differently compared with the modulated expression of these markers on cells activated *in vitro* (Fig 1, *F*)[39,40] or in the lung tissue *in vivo*.[47] Our data are consistent with

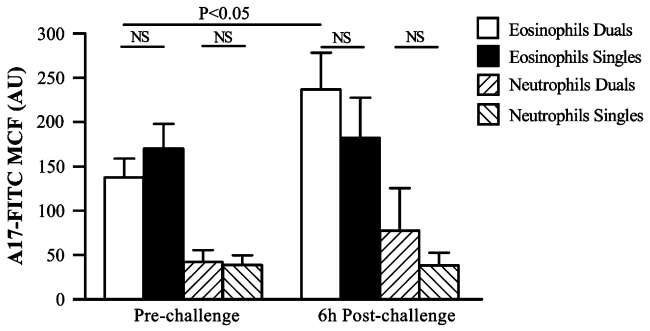

FIG 5. Differential MoPhab A17 expression before and after allergen challenge on eosinophils and neutrophils between dual (n = 10) and single (n = 7) responders. The differences in priming were analyzed with the Mann-Whitney *U* test. Differences between prechallenge and postchallenge MoPhab A17 expressions were analyzed with Wilcoxon signed-rank tests. Data are depicted as median channel fluorescence *(MCF)* and given in arbitrary units *(AU)*.

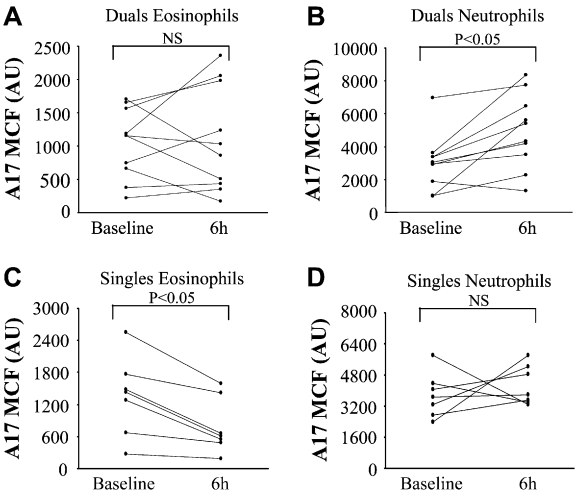

FIG 6. Differential regulation of responsiveness for fMLF in neutrophils and eosinophils isolated from peripheral blood of dual and single responders. Priming was measured on the basis of expression of MoPhab A17 on eosinophils and neutrophils of dual responders (**A** and **B**, respectively) and single responders (**C** and **D**, respectively) before and 6 hours after allergen challenge. Data were analyzed with Wilcoxon signed-rank tests. *NS*, Not significant; *MCF*, median channel fluorescence; *AU*, arbitrary units.

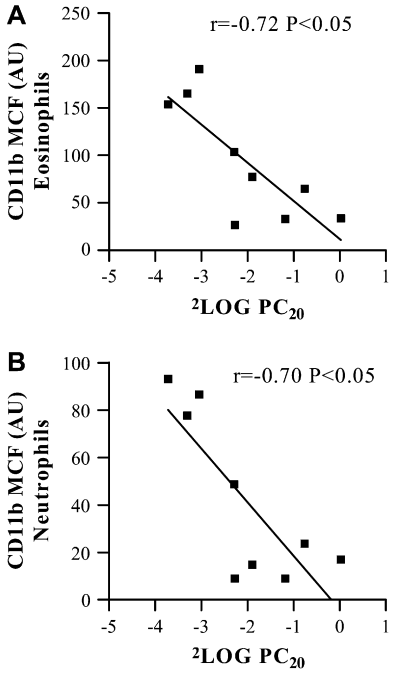

FIG 7. Low baseline (before allergen challenge) $\log_2$ PC_{20} methacholine values (<1.5 mg/mL) correlate with αm/CD11b expression on eosinophils **(A)** and neutrophils **(B)** isolated from peripheral blood of dual responders (n = 9). Data regarding αm/CD11b expression levels are plotted against the baseline PC_{20} methacholine in the same patient. Median channel fluorescence *(MCF)* is given in arbitrary units *(AU)*.

a model that αm^{bright}/L-$selectin^{bright}$ cells comprise a phenotype that facilitates adhesion and homing in allergic asthmatic subjects. During subsequent extravasation, L-selectin is shed from the surface, whereas αm is even more increased.[47]

In marked contrast to eosinophils, no differences were found in basal expression of active FcγRII on peripheral blood neutrophils of asthmatic and healthy control subjects.[15] However, these cells exhibit an enhanced responsiveness toward the chemoattractant fMLF (Fig 3, *C*), demonstrating that neutrophils in these patients are subtly primed *in vivo*.

We next addressed the question of whether differences in asthma phenotypes were associated with differences in systemic innate immune responses. Priming was measured before and after allergen challenges in 2 types of subjects with mild allergic asthma: single and dual responders. These 2 groups of patients did not distinguish themselves at baseline in terms of disease severity, baseline FEV_1, or AHR. The only apparent difference was the presence of an allergen-induced LAR in dual responders. In marked contrast to single responders, the LAR in dual responders is characterized by an increased expression of active FcγRII on peripheral blood

eosinophils 6 hours after allergen challenge. In the same patients the neutrophil compartment was not changed in this context. A similar activation phenotype was found for FcαRI (CD89) expressed on eosinophils isolated from asthmatic patients.[48] The consequence of the enhanced expression of activated FcRs on human eosinophils for the pathology seen in asthma remains to be established, but it is clear that FcRs are important effector molecules in the activation of cytotoxic mechanisms used by these cells. By means of inside-out activation of these receptors, the cells acquire a primed state that is required for optimal interaction with immunoglobulin-coated targets.

It was surprising that the neutrophil compartment did not seem to be targeted during the allergen-induced systemic activation of the innate immune system because neutrophils are recruited to the lung after allergen provocation[24,26] and these cells are involved in the persistent inflammation in asthmatic subjects.[5,6,8,9] Therefore we decided to study the priming of the neutrophil compartment in more detail. We showed that neutrophils were more sensitive for fMLF stimulation after allergen challenge in dual responders, whereas neutrophils in the blood of single responders were not influenced (Fig 6).

The present study cannot explain the mechanisms involved in the differences in fMLF responsiveness of granulocytes from single and dual responders. It is, however, tempting to speculate that asthmatic patients only experience a late-phase asthmatic reaction when both eosinophil and neutrophil compartments are functionally upregulated. This situation is similar in both male and female asthmatic patients (results not shown).

In marked contrast to expression of active FcγRII, which is rapidly upregulated during acute responses (eg, allergen challenge), αm/CD11b expression on eosinophils and neutrophils significantly correlated with bronchial hyperresponsiveness of asthmatic subjects (Fig 7), as was also seen by In't Veen et al.[21] These findings are in line with the hypothesis that the expression of αm/CD11b and L-selectin/CD62L on eosinophils and neutrophils better reflects a more continuous inflammatory tone caused by chronic tissue inflammation.

In conclusion, multiple priming phenotypes of peripheral blood granulocytes can be identified by means of analysis of differential expression of activation markers on granulocytes. Different types of allergic asthma (eg, single EAR or dual EAR/LAR after allergen challenge) are characterized by different priming phenotypes of eosinophils and neutrophils. The data underline the presence of differences in persistent chronic inflammation and acute allergen-induced inflammation. The differences in innate responses will have consequences in the diagnosis of multiple disease phenotypes and the design of new therapeutic options for these different classes of patients.

We thank Pieter Zanen, MD, PhD, for statistical analysis of the data and Hanneke van Deutekom, BSc, and Anne van Lindert, MSc, from the Department of Pulmonary Diseases (University Medical Center, Utrecht, The Netherlands) for excellent technical support.

REFERENCES

1. Busse WW, Lemanske RF Jr. Asthma. N Engl J Med 2001;344:350-62.
2. Ritz SA, Stampfli MR, Davies DE, Holgate ST, Jordana M. On the generation of allergic airway diseases: from GM-CSF to Kyoto. Trends Immunol 2002;23:396-402.
3. Holgate ST. Cytokine and anti-cytokine therapy for the treatment of asthma and allergic disease. Cytokine 2004;28:152-7.
4. Thomas PS. Tumour necrosis factor-alpha: the role of this multifunctional cytokine in asthma. Immunol Cell Biol 2001;79:132-40.
5. Wenzel SE, Balzar S, Cundall M, Chu HW. Subepithelial basement membrane immunoreactivity for matrix metalloproteinase 9: association with asthma severity, neutrophilic inflammation, and wound repair. J Allergy Clin Immunol 2003;111:1345-52.
6. Jatakanon A, Uasuf C, Maziak W, Lim S, Chung KF, Barnes PJ. Neutrophilic inflammation in severe persistent asthma. Am J Respir Crit Care Med 1999;160:1532-9.
7. Douwes J, Gibson P, Pekkanen J, Pearce N. Non-eosinophilic asthma: importance and possible mechanisms. Thorax 2002;57:643-8.
8. European Network for Understanding Mechanisms of Severe Asthma. The ENFUMOSA cross-sectional European multicentre study of the clinical phenotype of chronic severe asthma. Eur Respir J 2003;22:470-7.
9. Gibson PG, Simpson JL, Saltos N. Heterogeneity of airway inflammation in persistent asthma: evidence of neutrophilic inflammation and increased sputum interleukin-8. Chest 2001;119:1329-36.
10. Wenzel SE, Szefler SJ, Leung DY, Sloan SI, Rex MD, Martin RJ. Bronchoscopic evaluation of severe asthma. Persistent inflammation associated with high dose glucocorticoids. Am J Respir Crit Care Med 1997;156:737-43.
11. Kelly EA, Busse WW, Jarjour NN. Increased matrix metalloproteinase-9 in the airway after allergen challenge. Am J Respir Crit Care Med 2000;162:1157-61.
12. Cataldo DD, Bettiol J, Noel A, Bartsch P, Foidart JM, Louis R. Matrix metalloproteinase-9, but not tissue inhibitor of matrix metalloproteinase-1, increases in the sputum from allergic asthmatic patients after allergen challenge. Chest 2002;122:1553-9.
13. Cundall M, Sun Y, Miranda C, Trudeau JB, Barnes S, Wenzel SE. Neutrophil-derived matrix metalloproteinase-9 is increased in severe asthma and poorly inhibited by glucocorticoids. J Allergy Clin Immunol 2003;112:1064-71.
14. Koenderman L, van der Bruggen T, Schweizer RC, Warringa RA, Coffer P, Caldenhoven E, et al. Eosinophil priming by cytokines: from cellular signal to in vivo modulation. Eur Respir J Suppl 1996;22:119s-25s.
15. Luijk B, Lindemans CA, Kanters D, van der Heijde R, Bertics P, Lammers JW, et al. Gradual increase in priming of human eosinophils during extravasation from peripheral blood to the airways in response to allergen challenge. J Allergy Clin Immunol 2005;115:997-1003.
16. Warringa RA, Mengelers HJ, Raaijmakers JA, Bruijnzeel PL, Koenderman L. Upregulation of formyl-peptide and interleukin-8-induced eosinophil chemotaxis in patients with allergic asthma. J Allergy Clin Immunol 1993;91:1198-205.
17. Moser R, Fehr J, Olgiati L, Bruijnzeel PL. Migration of primed human eosinophils across cytokine-activated endothelial cell monolayers. Blood 1992;79:2937-45.
18. Ebisawa M, Liu MC, Yamada T, Kato M, Lichtenstein LM, Bochner BS, et al. Eosinophil transendothelial migration induced by cytokines. II. Potentiation of eosinophil transendothelial migration by eosinophil-active cytokines. J Immunol 1994;152:4590-6.
19. Lantero S, Alessandri G, Spallarossa D, Scarso L, Rossi GA. Stimulation of eosinophil IgE low-affinity receptor leads to increased adhesion molecule expression and cell migration. Eur Respir J 2000;16:940-6.
20. Mengelers HJ, Maikoe T, Brinkman L, Hooibrink B, Lammers JW, Koenderman L. Immunophenotyping of eosinophils recovered from blood and BAL of allergic asthmatics. Am J Respir Crit Care Med 1994;149:345-51.
21. In't Veen JC, Grootendorst DC, Bel EH, Smits HH, van der Keur M, Sterk PJ, et al. CD11b and L-selectin expression on eosinophils and neutrophils in blood and induced sputum of patients with asthma compared with normal subjects. Clin Exp Allergy 1998;28:606-15.
22. Pals CM, Verploegen SA, Raaijmakers JA, Lammers JW, Koenderman L, Coffer PJ. Identification of cytokine-regulated genes in human leukocytes in vivo. J Allergy Clin Immunol 2000;105:760-8.

23. Oudijk EJ, Nijhuis EH, Zwank MD, van de Graaf EA, Mager HJ, Coffer PJ, et al. Systemic inflammation in COPD visualised by gene profiling in peripheral blood neutrophils. Thorax 2005;60:538-44.
24. Nocker RE, Out TA, Weller FR, Mul EP, Jansen HM, van der Zee JS. Influx of neutrophils into the airway lumen at 4 h after segmental allergen challenge in asthma. Int Arch Allergy Immunol 1999;119:45-53.
25. Koh YY, Dupuis R, Pollice M, Albertine KH, Fish JE, Peters SP. Neutrophils recruited to the lungs of humans by segmental antigen challenge display a reduced chemotactic response to leukotriene B4. Am J Respir Cell Mol Biol 1993;8:493-9.
26. Taube C, Dakhama A, Rha YH, Takeda K, Joetham A, Park JW, et al. Transient neutrophil infiltration after allergen challenge is dependent on specific antibodies and Fc gamma III receptors. J Immunol 2003;170:4301-9.
27. Aalbers R, Kauffman HF, Vrugt B, Koeter GH, de Monchy JG. Allergen-induced recruitment of inflammatory cells in lavage 3 and 24h after challenge in allergic asthmatic lungs. Chest 1993;103:1178-84.
28. Weersink EJ, Postma DS, Aalbers R, de Monchy JG. Early and late asthmatic reaction after allergen challenge. Respir Med 1994;88:103-14.
29. Cockcroft DW, Murdock KY. Changes in bronchial responsiveness to histamine at intervals after allergen challenge. Thorax 1987;42:302-8.
30. Bentley AM, Kay AB, Durham SR. Human late asthmatic reactions. Clin Exp Allergy 1997;27(suppl 1):71-86.
31. Grutters JC, Brinkman L, Aslander MM, van den Bosch JM, Koenderman L, Lammers JW. Asthma therapy modulates priming-associated blood eosinophil responsiveness in allergic asthmatics. Eur Respir J 1999;14:915-22.
32. Bancalari L, Dente FL, Cianchetti S, Prontera C, Taccola M, Bacci E, et al. Blood markers of early and late airway responses to allergen in asthmatic subjects. Relationship with functional findings. Allergy 1997; 52:32-40.
33. Bracke M, Lammers JW, Coffer PJ, Koenderman L. Cytokine-induced inside-out activation of FcalphaR (CD89) is mediated by a single serine residue (S263) in the intracellular domain of the receptor. Blood 2001;97: 3478-83.
34. National Heart, Lung, and Blood Institute, Global strategy for asthma management and prevention NHLBI/WHO Workshop Report. Bethesda (MD): National Institutes of Health; 1995. (updated April 2002). Publication no. 95-3659.
35. Cockcroft DW, Murdock KY, Kirby J, Hargreave F. Prediction of airway responsiveness to allergen from skin sensitivity to allergen and airway responsiveness to histamine. Am Rev Respir Dis 1987;135:264-7.
36. Sterk PJ, Fabbri LM, Quanjer PH, Cockcroft DW, O'Byrne PM, Anderson SD, et al. Airway responsiveness. Standardized challenge testing with pharmacological, physical and sensitizing stimuli in adults. Report Working Party Standardization of Lung Function Tests, European Community for Steel and Coal. Official Statement of the European Respiratory Society. Eur Respir J Suppl 1993;16:53-83.
37. Koenderman L, Kanters D, Maesen B, Raaijmakers J, Lammers JW, de Kruif J, et al. Monitoring of neutrophil priming in whole blood by antibodies isolated from a synthetic phage antibody library. J Leukoc Biol 2000;68:58-64.
38. Ierino FL, Hulett MD, McKenzie IF, Hogarth PM. Mapping epitopes of human Fc gamma RII (CDw32) with monoclonal antibodies and recombinant receptors. J Immunol 1993;150:1794-803.
39. Neeley SP, Hamann KJ, White SR, Baranowski SL, Burch RA, Leff AR. Selective regulation of expression of surface adhesion molecules Mac-1, L-selectin, and VLA-4 on human eosinophils and neutrophils. Am J Respir Cell Mol Biol 1993;8:633-9.
40. Chung KF, Mann BS. Blood neutrophil activation markers in severe asthma: lack of inhibition by prednisolone therapy. Respir Res 2006;7:59.
41. Warringa RA, Mengelers HJ, Kuijper PH, Raaijmakers JA, Bruijnzeel PL, Koenderman L. In vivo priming of platelet-activating factor-induced eosinophil chemotaxis in allergic asthmatic individuals. Blood 1992;79: 1836-41.
42. Schweizer RC, Welmers BA, Raaijmakers JA, Zanen P, Lammers JW, Koenderman L. RANTES- and interleukin-8-induced responses in normal eosinophils: effect of priming with interleukin-5. Blood 1994;83: 3697-704.
43. Bochner BS. Systemic activation of basophils and eosinophils: markers and consequences. J Allergy Clin Immunol 2000;106(suppl): S292-302.
44. Thorne KJ, Richardson BA, Mazza G, Butterworth AE. A new method for measuring eosinophil activating factors, based on the increased expression of CR3 alpha chain (CD11b) on the surface of activated eosinophils. J Immunol Methods 1990;133:47-54.
45. Koenderman L, Hermans SW, Capel PJ, van de Winkel JG. Granulocyte-macrophage colony-stimulating factor induces sequential activation and deactivation of binding via a low-affinity IgG Fc receptor, hFc gamma RII, on human eosinophils. Blood 1993;81:2413-9.
46. Bracke M, Dubois GR, Bolt K, Bruijnzeel PL, Vaerman JP, Lammers JW, et al. Differential effects of the T helper cell type 2-derived cytokines IL-4 and IL-5 on ligand binding to IgG and IgA receptors expressed by human eosinophils. J Immunol 1997;159:1459-65.
47. Mengelers HJ, Maikoe T, Hooibrink B, Kuypers TW, Kreukniet J, Lammers JW, et al. Down modulation of L-Selectin expression on eosinophils recovered from bronchoalveolar lavage fluid after allergen provocation. Clin Exp Allergy 1993;23:196-204.
48. Bracke M, van de Graaf E, Lammers JW, Coffer PJ, Koenderman L. In vivo priming of FcalphaR functioning on eosinophils of allergic asthmatics. J Leukoc Biol 2000;68:655-61.