

Human eosinophil granulocytes do not express the enzyme arginase

Claudia Luckner-Minden,^{*,†} Ina Fischer,[‡] Claus-Dieter Langhans,[§] Martin Schiller,^{*} Pascale Kropf,^{||} Ingrid Müller,^{||} Jens M. Hohlfeld,[‡] Anthony D. Ho,^{*} and Markus Munder^{*,†,1,2}

^{*}Department of Hematology, Oncology and Rheumatology, University Hospital Heidelberg, and [†]Institute of Immunology and

[§]Children's Hospital, University of Heidelberg, Heidelberg, Germany; [‡]Fraunhofer-Institute for Toxicology and Experimental Medicine, Hannover, Germany; and ^{||}Department of Immunology, Faculty of Medicine, Imperial College London, United Kingdom

RECEIVED NOVEMBER 17, 2009; REVISED FEBRUARY 11, 2010; ACCEPTED FEBRUARY 13, 2010. DOI: 10.1189/jlb.1109741

ABSTRACT

Human polymorphonuclear PMN constitutively express the enzyme arginase I, which hydrolyzes arginine to ornithine and urea. This arginine consumption has been recognized as a key pathway of myeloid cell-mediated suppression of the adaptive immune system during inflammation, infection, and tumor growth. Eos granulocytes are crucial immunoregulatory and effector cells of allergic inflammation and infections with parasites and helminths and in a variety of tumors. Here, we analyzed if human Eos also express arginase with its potential immunosuppressive consequences. We show that human peripheral blood Eos do not express arginase I or II protein or arginase enzymatic activity. Correspondingly, no metabolism of arginine to ornithine can be detected in Eos-S. Neither Eos apoptosis nor cytokine-mediated cellular activation induces arginase in human Eos in vitro. Finally, we show that arginase activity and protein are also undetectable in Eos of allergic patients from peripheral blood or from BALF activated in vivo during allergic pulmonary inflammation. This work demonstrates a fundamental difference between neutrophil and Eos granulocytes. As Eos are not equipped with the immunosuppressive enzyme arginase, they cannot participate, via arginine limitation, in the suppression of the evolving adaptive immune response in allergy, infections, or tumor immunity. *J. Leukoc. Biol.* 87: 000-000; 2010.

Introduction

The evolving adaptive immune response is initiated and regulated by cells and effector pathways of the innate immune system. Myeloid cells, like macrophages, dendritic cells, and granulocytes, are essential for the preservation of host integrity

against microbial pathogens via cytotoxicity, antigen presentation, and secretion of a variety of soluble mediators, such as cytokines and chemokines. Balancing their immunostimulatory activities, myeloid cells are also known to suppress the adaptive immune response. This has likely evolved as a homeostatic regulatory capacity to prevent detrimental tissue damage and overwhelming inflammatory responses [1]. One of the prime mechanisms of myeloid cell-associated immunosuppression is based on the modulation of extracellular arginine concentration via the enzyme arginase, which hydrolyzes arginine to the products ornithine and urea. Two mammalian arginase isoenzymes (designated arginase I and II) exist, which catalyze the same biochemical reaction but differ in cellular expression, regulation, and subcellular localization [2]. The hepatic isoform arginase I can inducibly be expressed in all types of murine myeloid cells, mainly by Th2 cytokines [3]. In the human immune system, arginase I is constitutively expressed only in polymorphonuclear PMN [4]. Upon liberation during PMN cell death, the enzyme can lead to extracellular arginine depletion [5]. This was characterized as a key immunosuppressive mechanism during inflammation and within the tumor microenvironment [6], as the function of T lymphocytes [7] and NK cells [8] is compromised severely when the extracellular arginine concentration is low. Arginase I expression has also become a defining biochemical hallmark of MDSC. In mice with tumors, as well as in cancer patients, these monocyte- or granulocyte-like cells expand systemically and can be found locally within the inflammatory stromal microenvironment of the tumor [9]. MDSC can inhibit the anti-tumor immune response by arginase-mediated arginine depletion in murine tumor models and in human cancer patients [9]. Arginase is also involved in several important downstream metabolic pathways, which start from ornithine, the product of the arginase enzymatic reaction. Ornithine can be metabolized via ornithine

Abbreviations: BALF=bronchoalveolar lavage fluid, Eos=eosinophil(s), Eos-S=sonicate of human eosinophil(s), FSC=forward-scatter, MDSC=myeloid-derived suppressor cell(s), nor-NOHA=N- ω -hydroxy-nor-L-arginine, PI=propidium iodide, PMN=neutrophil granulocyte(s), PMN-S=sonicate of human neutrophil granulocyte(s), SSC=side-scatter

1. Current address: Department of Hematology, Oncology and Pulmonology, Johannes Gutenberg-University, Mainz, Germany.

2. Correspondence: Department of Hematology, Oncology and Pulmonology, Johannes Gutenberg-University, Langenbeckstrasse 1, 55131 Mainz, Germany. E-mail: munder@uni-mainz.de

decarboxylase to polyamines (putrescine, spermidine, spermine), small polycationic molecules that participate in a variety of fundamental cellular functions [3]. Alternatively, ornithine can be metabolized via ornithine aminotransferase to proline, which is an essential precursor for the synthesis of collagen [3]. In summary, myeloid cell-associated arginase is a key enzyme involved in immune regulation, cellular proliferation, tissue regeneration, and fibrosis. A better understanding of its expression and regulation in the human immune system is therefore of significant importance.

A prominent member of the granulocyte family is the Eos granulocyte. Despite their relatively low numbers in peripheral blood, Eos are a quantitatively important cell type in various human tissues (bone marrow, lymphoid tissue, uterus, gastrointestinal tract) [10]. Eos are multifunctional effector and immunoregulatory cells, mainly involved in allergic diseases and the immune response against parasites, helminths, and tumors [11]. They are equipped with several proteins that can be released by degranulation and mediate cytotoxicity (major basic protein), antiviral, and pore-forming activity in target membranes (Eos cationic protein; Eos-derived neurotoxin) and oxidation of halides or pseudohalides to hypohalous acids with consecutive antibacterial activity (Eos peroxidase). They also contain a variety of granule-stored enzymes such as acid phosphatase, phospholipase D, catalase, nonspecific esterases, collagenase, histaminase, and matrix metalloproteases. Eos can secrete proinflammatory lipid mediators (e.g., platelet-activating factor; leukotriene C₄) and a multitude of mainly preformed and therefore, rapidly mobilized cytokines (e.g., IL-2, IL-4, IL-5, IL-10, IL-13, IL-16, IL-18, TGF- β) and chemokines (e.g., CCL5, CCL11) [10, 11]. Eos communicate with other immune cells via this plethora of different soluble mediators and participate in the regulation of the evolving phenotype of the adaptive immune system, regulate the expression of adhesion receptors, and modulate cellular trafficking [10]. Eos therefore fulfill key immunoregulatory functions within the inflammatory response.

In this study, we analyzed whether Eos also express arginase with its potential immunosuppressive consequences. Peripheral blood Eos of healthy human donors were purified, and arginase expression was analyzed biochemically (enzymatic activity and measurement of amino acid concentrations) by intracellular flow cytometry and by Western blot. Finally, Eos were purified directly *ex vivo* from the BALF of allergic patients after segmental allergen provocation. We show that in contrast to human PMN, human peripheral blood Eos do not express arginase activity or arginase protein. Arginase was also not expressed in Eos undergoing apoptosis, after stimulation *in vitro* with a variety of inflammatory cytokines and *in vivo*, during allergic asthma. In summary, we demonstrate an important biological difference between neutrophil and Eos granulocytes with regard to the key immunosuppressive enzyme arginase.

MATERIALS AND METHODS

Human subjects

All work involving human subjects was carried out in accordance with the Declaration of Helsinki. Human studies with healthy normal blood donors

(21–55 years) were approved by the Ethics Committee of the University of Heidelberg (Germany) and were conducted with the understanding and informed consent from all subjects. For experiments with blood granulocytes from allergic donors, nonsmoking subjects (22–52 years) with a clinically manifest allergy against grass pollen were recruited. For experiments with cells from BAL, subjects (23–54 years) with mild, intermittent asthma and allergy to grass pollen were identified. Subjects had normal laboratory test findings and elevated total IgE levels (>100 IU/mL) and were hyper-responsive to methacholine (provocation concentration of 20% <8 mg/mL). They had a positive airway response to inhaled allergen. All subjects were volunteers and gave their written consent after being fully informed about the purpose and nature of the studies, which were approved by the Ethics Committee of Hannover Medical School (Germany).

Reagents and media

If not otherwise stated, chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). nor-NOHA was from Bachem (Weil am Rhein, Germany) and used at a final concentration of 1 mM. RPMI-1640 medium without arginine was purchased from PromoCell (Heidelberg, Germany) and supplemented with MnCl₂ at a physiological concentration (10 μ M) to allow for full arginase activity. All cytokines (IFN- γ , IL-4, IL-5, IL-10, IL-13, GM-CSF) were purchased from PromoCell. A polyclonal rabbit anti-human arginase I antiserum was generated by immunization of rabbits with human recombinant arginase I [5]. Cell culture studies were done in arginine-free RPMI-1640 medium (PromoCell), supplemented with dialyzed FCS (10% v/v, PAA Laboratories, Pasching, Austria), glutamine, sodium pyruvate, penicillin, and streptomycin (all from Invitrogen, Karlsruhe, Germany).

Isolation of human PMN and Eos from peripheral blood of healthy donors

Total granulocytes were isolated from peripheral heparin-anticoagulated blood as described previously by density gradient centrifugation using Ficoll-Paque™ (GE Healthcare, Freiburg, Germany) and dextran sedimentation, followed by erythrocyte lysis with ammonium chloride buffer (155 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA) [4]. Human Eos were isolated from this fraction by negative selection using the Eos isolation kit (Miltenyi Biotec, Bergisch-Gladbach, Germany), according to the instructions of the manufacturer. Briefly, the granulocyte pellet was resuspended in buffer (PBS, 0.5% BSA, 2 mM EDTA, pH 7.2) and incubated with a mixture of biotin-conjugated antibodies against CD2, CD14, CD16, CD19, CD56, CD123, and CD235a, followed by incubation with anti-biotin microbeads. The antibody-bound cells (non-Eos) were separated from the Eos fraction by magnetic separation using a LS column (Miltenyi Biotec). The isolated, untouched Eos were resuspended in cell culture medium and analyzed for viability by PI staining (>95% negative, i.e., viable) and purity (>98%) by flow cytometry (CD15⁺, CD16⁻ cells) and cytospin preparations. The negative fraction (comprised of >98% neutrophils) was used as PMN control for the respective experiments.

Generation of cell lysates and sonicates and measurement of arginase enzymatic assay

To prepare cell lysates, cells were lysed in 1% Brij-96 for 20 min, the lysate was centrifuged for 30 min at 20,000 g and 4°C, and the supernatant was stored for further use. For the generation of cell sonicates, purified PMN or Eos were resuspended in PBS (50×10⁶ cells/ml), sonicated for 3 min (amplitude 80) in a Vibracell sonicator (Sonics & Materials, Newtown, CT, USA), and centrifuged for 30 min at 20,000 g and 4°C. The supernatant was filtered (0.2 μ m), protein concentration and arginase activity were determined, and aliquots were frozen at –80°C until further use. Arginase activity was measured in cell lysates or sonicates as described previously [5]. Briefly, cells were lysed with 100 μ L 0.1% Triton X-100. After 30 min on a shaker, 100 μ L 25 mM Tris-HCl was added. To 100 μ L of this lysate, 10 μ L 10 mM MnCl₂ was added, and the enzyme was activated by heating for 10 min at 56°C. Arginine hydrolysis was conducted by incubating the lysate

with 100 μ L 0.5 M arginine (pH 9.7) at 37°C for 15–120 min. The reaction was stopped with 800 μ L H₂SO₄ (96%)/H₃PO₄ (85%)/H₂O (1:3:7, v/v/v). The urea concentration was measured at 540 nm after addition of 40 μ L α -isonitrosopropiophenone (dissolved in 100% ethanol), followed by heating at 95°C for 30 min. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μ mol urea/min. The lower limit of detectable arginase activity in this assay is 40 mU/mg cellular protein.

Immunoblot analysis

Cells were lysed for 30 min at 4°C in lysis buffer [5], cell debris was spun down at 20,000 g for 15 min at 4°C, and SDS-PAGE was done as described previously [12]. The proteins were transferred to a Hybond-P polyvinylidene difluoride membrane (GE Healthcare). After blocking with 5% non-fat dry milk in TBST buffer for 1.5 h, the membranes were incubated with a polyclonal rabbit anti-human arginase I antiserum (diluted 1:5000 in TBST, 5% BSA), a rabbit anti-arginase II antibody (clone H-64, Santa Cruz Biotechnology, Santa Cruz, CA, USA), and an anti-ERK1/2 antibody (clone K-23, Santa Cruz Biotechnology). Antibody reactivity was monitored with HRP-conjugated anti-rabbit IgG (Santa Cruz Biotechnology), followed by visualization with the ECL Plus detection system (GE Healthcare).

Flow cytometry, intracellular arginase I staining, apoptosis measurement

Antibodies used for flow cytometry were as follows: anti-arginase I (clone 6G3, HyCult Biotech, Uden, The Netherlands), the respective isotype control (clone MOPC21, BD Biosciences, Heidelberg, Germany), anti-CD15 (Beckman Coulter, Krefeld, Germany), and anti-CD16 and the respective isotype controls (all from BD Biosciences). Anti-arginase I antibody and the respective isotype control antibody were coupled with Alexa Fluor® 488 using the Zenon® labeling kit, according to the instructions of the manufacturer (Invitrogen). Human peripheral blood leukocytes (1×10^6), derived from normal blood donors after hypotonic erythrocyte lysis, were stained with fluorescently labeled mAb against CD15 or CD16. After 20 min at 4°C, the cells were washed, fixed with 2% paraformaldehyde for 15 min, and permeabilized in PBS/0.5% saponin. FcR blocking reagent (20 μ L, Miltenyi Biotec) was added to the cells for 20 min. Then, 0.2 μ g Alexa Fluor® 488-conjugated anti-arginase I or Alexa Fluor® 488-conjugated murine IgG1 was added to the cells, and 20 min later, the cells were washed [13]. To analyze apoptosis and cell death, 10^5 human Eos were resuspended in saline containing 0.05% annexin V (Roche Diagnostics, Mannheim, Germany) and 100 ng/mL PI. After incubation at 4°C for 30 min, the cells were analyzed by flow cytometry with a FACScan (BD Biosciences). Flow cytometric analyses for secreted cytokines as controls for Eos stimulation experiments were performed using an EPICS XLTM flow cytometer (Coulter, Hialeah, USA) using the FlowCytomix kit (Bender MedSystems, Vienna, Austria).

Amino acid measurements

Suspensions were sonicated, and sulfosalicylic acid was added. The samples were allowed to stand for 10 min at 4°C and then centrifuged. The supernatant (300 μ L) was diluted with 100 μ L physiologic sample dilution buffer. Amino acid analysis was performed in the physiologic mode of an automatic amino acid analyzer (Biotronic LC 3000, Eppendorf/Biotronik, Hamburg, Germany). Ion exchange chromatography was used to separate amino acids by increasing pH, ion strength, and temperature of the elution buffers. After post-column derivatization with ninhydrine, amino acids were detected at 440 nm and 570 nm. For data collection and processing, ChromStar (SCPA GmbH, Weyhe-Leeste, Germany) was used.

Isolation of granulocytes from blood and BALF of allergic subjects

Blood granulocytes were isolated from heparin-anticoagulated venous blood using Percoll density gradient centrifugation following selection of

CD16⁺ neutrophils and CD16⁺ Eos by MACS technology [14]. Separated Eos and neutrophils were resuspended in RPMI cell culture medium, cell number was determined, and cell pellets were frozen (–80°C) until they were used for protein analysis. The bronchoscopy procedure with segmental allergen provocation for the isolation of Eos from BAL has been described before [15]. Briefly, lung segments were lavaged 24 h after local instillation of allergen (mixed grass allergens, SQ299; house dust mite allergens, SQ503; ALK-Abelló, Wedel, Germany) with 100 mL sterile saline, and the BALF was collected on ice. Total BAL cells were counted and differentiated by standard techniques. When Eos percentages were <80%, granulocytes were separated from monocytes and lymphocytes by Percoll gradient. Eos were not separated further from the minor ($2.5 \pm 0.8\%$) contaminating neutrophil subpopulation. The granulocyte pellet was resuspended in culture medium, the cell number was determined, and cell pellets were frozen (–80°C) until they were used for protein analysis. The purity of blood neutrophils was $98 \pm 0.7\%$, and purity of blood and BAL Eos was $96.6 \pm 3.2\%$ and $84.7 \pm 3.4\%$, respectively.

RESULTS

Human peripheral blood Eos do not express arginase activity

We have shown previously that human PMN constitutively express high arginase activity as a result of the isoform arginase I [4]. Eos granulocytes are key myeloid effector cells in the inflammatory defense against helminths and parasites, during allergic inflammation, and in a variety of tumors. We therefore wanted to study the possible expression of the enzyme arginase in human Eos. Eos and PMN were purified from the peripheral blood of healthy blood donors. Arginase activity was determined in PMN and Eos lysates of 10 individual donors (Fig. 1). High arginase activity was detected in all individual human PMN lysates (mean activity: 1122 ± 217 mU/mg protein; range: 839–1471 mU/mg protein), confirming our data published previously [4]. In contrast, no arginase activity was detectable in 10 individual Eos lysates (Fig. 1).

No constitutive expression of arginase I and II protein in human Eos

Two different isoforms of arginase have been characterized in mammalian cells [2, 3]. The high arginase activity of human

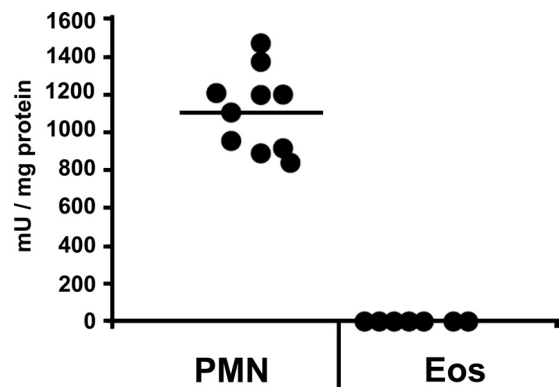


Figure 1. Human peripheral blood Eos do not express arginase activity. Human PMN and Eos were purified from peripheral blood of 10 healthy blood donors. Arginase activity was determined in cell lysates and is depicted as mU/mg cellular protein.

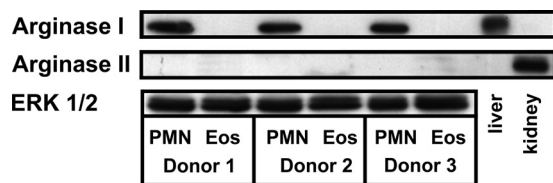


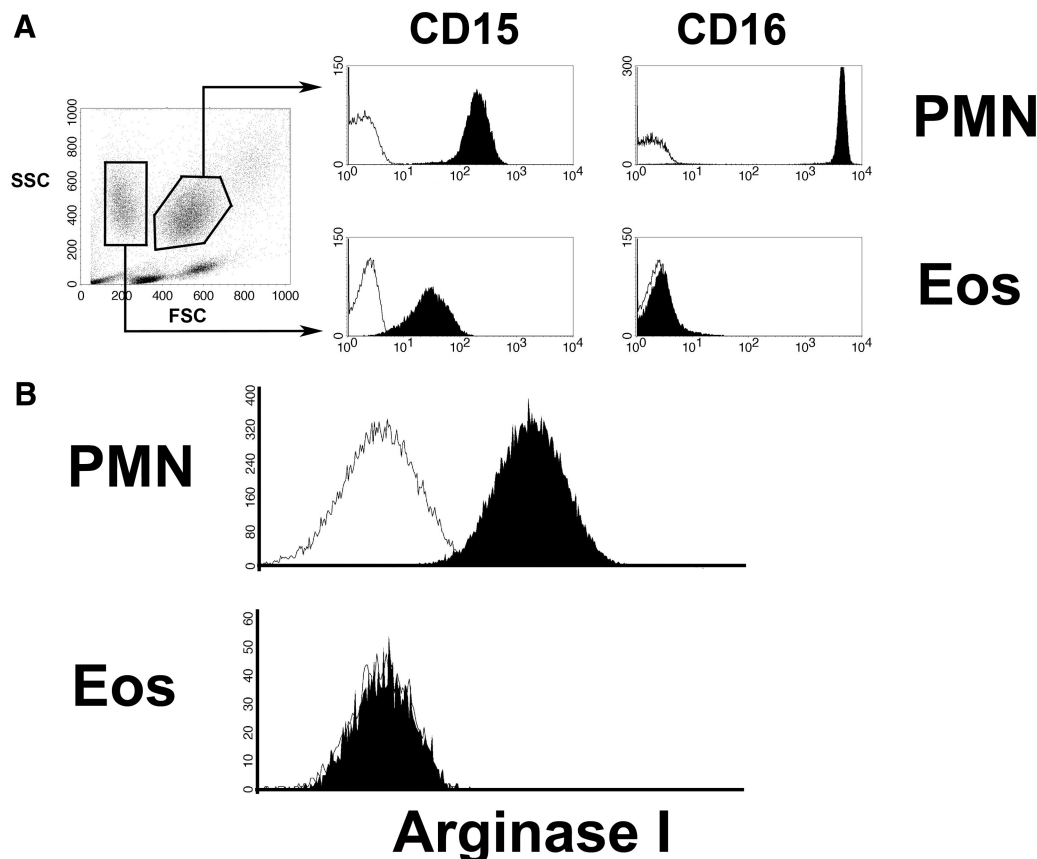
Figure 2. No expression of both arginase isoenzymes in human peripheral blood Eos. Human PMN and Eos were purified from peripheral blood of 10 healthy blood donors. Expression of arginase I and II was analyzed in cellular lysates of PMN and Eos by immunoblotting. To control for equal protein loading, Western blots for ERK1/2 were done in parallel. Expression of arginase I in human liver homogenate and of arginase II in human kidney homogenate is also demonstrated as a positive control. The results of three representative blood donors are demonstrated.

PMN is exclusively a result of the expression of the hepatic isoform arginase I [4], and the extrahepatic isoform arginase II can be found at lower expression levels in a variety of mammalian tissues [2]. As arginase enzymatic activity might be undetectable in Eos lysates as a result of endogenous inhibitory mechanisms, we analyzed the expression of both arginase isoforms at the protein level in human Eos by Western blot (Fig. 2). Arginase I was detected in human liver (positive control) and human PMN, as published previously [4]. No arginase I protein was detectable in highly purified human Eos of 10 individual donors (in each case, corresponding to no mea-

surable arginase enzymatic activity). Although human PMN and Eos fundamentally differ with respect to the expression of arginase I, both cell types do not express the extrahepatic isoform arginase II (Fig. 2).

We then analyzed the expression of arginase I by flow cytometry after intracellular staining. This technique is ideal for the analysis of antigen expression on a per-cell basis and might allow the detection of potential arginase I-negative subpopulations within the whole peripheral blood PMN fraction. This approach can also reveal potential minor arginase I-expressing Eos subpopulations that might escape detection in the unfractionated, whole Eos analysis by Western blot. Furthermore, no cellular separation (with its potential for cellular activation and possible secretion of intracellular constituents such as arginase) is necessary, and a different anti-arginase I antibody (recognizing the native protein) is used for the intracellular flow cytometric detection of arginase I in human myeloid cells [13]. Human peripheral blood leukocytes, derived from normal blood donors after hypotonic erythrocyte lysis, were analyzed for surface expression of CD15 and CD16 as well as for intracellular expression of arginase I. As demonstrated and marked by boxes in Figure 3A, after fixation of the leukocytes with 2% paraformaldehyde, two separate granulocytic CD15⁺ SSC^{high} populations can be identified in the FSC/SSC plot, corresponding to CD15⁺CD16^{low/-} Eos and CD15⁺⁺CD16⁺⁺ PMN. Arginase I protein is only detectable in PMN, whereas Eos are homogeneously negative (Fig. 3B). No

Figure 3. No detection of intracellular arginase I in human Eos by flow cytometry. Human peripheral blood leukocytes were stained with fluorescently labeled anti-CD15 and anti-CD16 antibodies. After fixation and permeabilization, the intracellularly expressed arginase I was detected by staining with an anti-arginase I antibody and subsequent analysis by flow cytometry. Filled graphs represent the staining with the respective antibody; staining with the isotype control antibody is represented by the line graphs. (A) Two distinct SSC^{high} populations appear in the FSC/SSC plot, and analysis of surface marker expression of the respective gated populations (as marked by boxes) identified CD15⁺CD16^{low/-} Eos granulocytes and CD15⁺⁺CD16⁺⁺ PMN. (B) Intracellular arginase I expression is only detected in the PMN subpopulation of peripheral blood leukocytes. One representative experiment (total: six individual blood donors) is demonstrated.



separate, arginase I-negative subpopulation was detectable within the PMN fraction in all analyzed leukocyte donors, and no arginase I-positive minor subpopulation could be demonstrated among Eos in all analyzed samples (total: six individual experiments with different blood donors).

Human Eos do not express arginase protein and activity upon induction of apoptosis or inflammatory stimulation

After having demonstrated that there was no constitutive expression of arginase protein and enzymatic activity in resting human Eos, we next addressed whether there was induction of the enzyme in Eos undergoing apoptosis as a result of growth factor withdrawal in vitro. Apoptosis is a complex process, and recognition of apoptotic cells is a trigger for various anti-inflammatory pathways such as the release of lipid mediators (e.g., sphingosine-1-phosphate) or cytokines (e.g., TGF- β), which can both induce arginase in myeloid cells [16, 17]. We

therefore incubated human primary Eos in normal cell culture medium and monitored cell viability and apoptosis at different time-points by flow cytometry after staining with annexin V and PI (Fig. 4A). Simultaneously, we prepared cell lysates for the analysis of arginase protein expression by Western blot and determination of arginase activity. Summarizing three independent experiments, the frequency of viable (annexin V⁻, PI⁻) Eos declined from $91.5 \pm 5.6\%$ (t=0 h; data not shown) to $27.0 \pm 4.5\%$ (t=24 h), $9.5 \pm 7.4\%$ (t=48 h), $3.7 \pm 1.0\%$ (t=72 h; data not shown), and $0.7 \pm 0.2\%$ (t=96 h) upon growth factor withdrawal (Fig. 4A). The frequency of apoptotic (annexin V⁺, PI⁻) Eos reached a maximum after 24 h ($59.4 \pm 6.3\%$) and then declined to $29.1 \pm 12.1\%$ at 48 h, $11.1 \pm 0.6\%$ at 72 h, and $5.1 \pm 3.0\%$ at 96 h, and the percentage of dead cells (annexin V⁺, PI⁺) increased steadily until it reached a maximum of $94.2 \pm 3.3\%$ at 96 h. At all time-points tested, we never detected arginase activity or protein in the heterogenous Eos population undergoing apoptosis in vitro (data not shown).

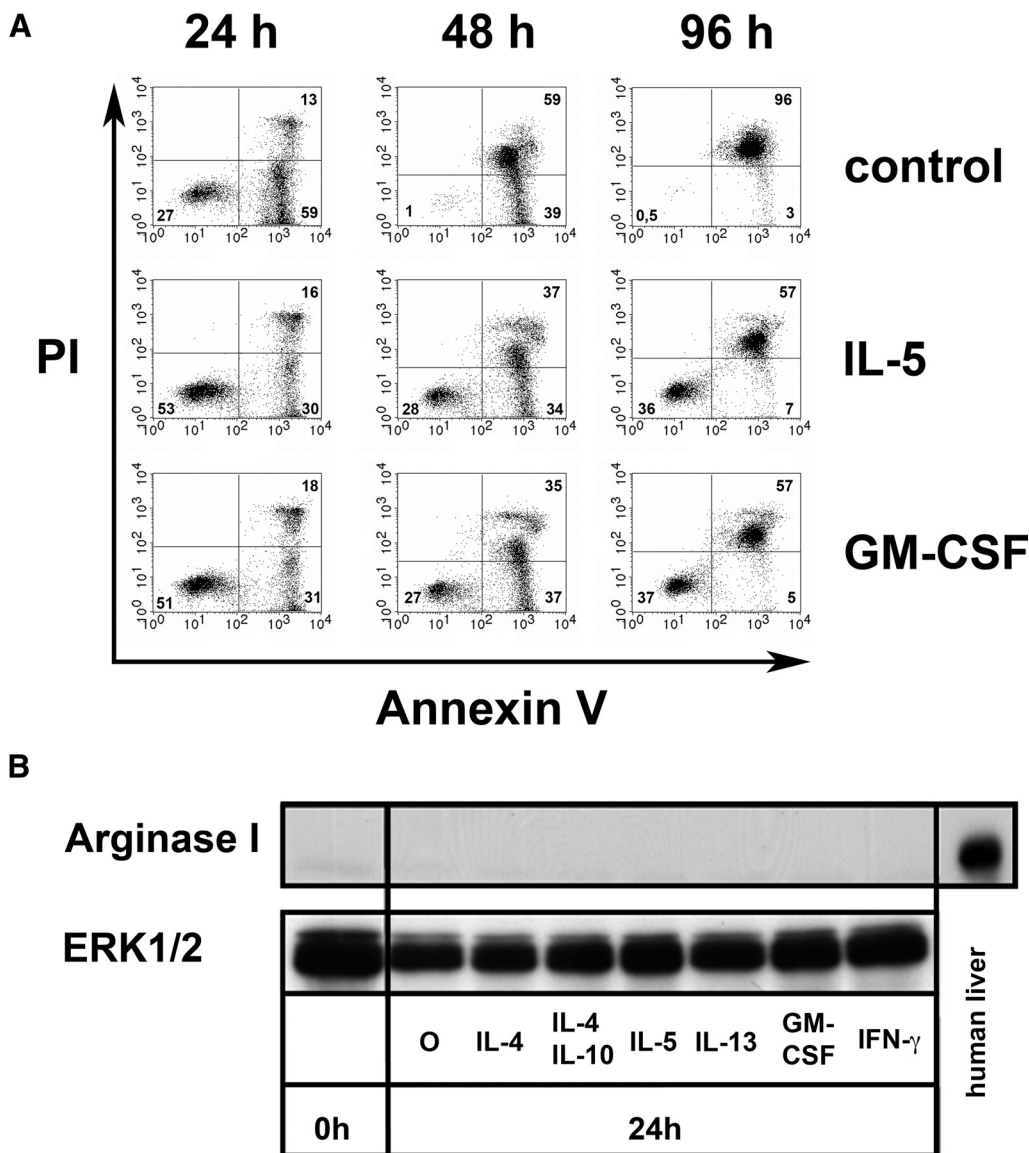


Figure 4. No induction of arginase in human Eos during apoptosis and by cytokine-mediated activation.

(A) Human Eos were incubated in vitro in normal cell culture medium without exogenous growth factors (upper row: control) or in medium supplemented with IL-5 (20 ng/ml) or GM-CSF (20 ng/ml). At the indicated time-points, cells were harvested, stained with annexin V and PI, and analyzed by flow cytometry. The frequency of viable (annexin V⁻, PI⁻), apoptotic (annexin V⁺, PI⁻), and dead cells (annexin V⁺, PI⁺) is shown in the respective quadrants. One representative experiment (total: three) is demonstrated. No detectable arginase activity or arginase I protein could be found in Eos at all time-points tested. (B) Eos were purified from peripheral blood of healthy donors. The cells were incubated in the presence of the indicated cytokines (concentration: 20 ng/ml each). After 24 h, cells were harvested and lysed, and protein concentration as well as arginase activity was measured (result: no arginase activity in all Eos samples). Cell lysates were separated by SDS-PAGE (20 μ g protein/lane), and Western blots were done with anti-arginase I antibody as well as anti-ERK1/2 antibody as a loading control. A lysate of human liver was included as a positive control for arginase I expression. One representative experiment (total: seven) is demonstrated. Identical results (no arginase activity or protein) were found at 48 h, 72 h, and 96 h (total: three experiments; data not shown).

In the murine system, arginase I is also not expressed in resting myeloid cells but can be induced in all different myeloid cell types, typically by Th2 inflammatory cytokines such as IL-4, IL-10, or IL-13 [3]. We therefore stimulated highly purified human Eos with different inflammatory cytokines (Fig. 4B) known to prolong Eos viability and activate human Eos for a variety of cellular functions (cytokine synthesis, chemotaxis, degranulation) or are prototypical arginase-inducing cytokines in the murine system. To control for viability and activation of the cells, we analyzed annexin V and PI staining (Fig. 4A) and measured cytokine secretion in the supernatants and surface membrane expression of activation markers (data not shown). As expected, Eos viability was increased *in vitro* by various growth factors, most prominently, by IL-5 and GM-CSF (Fig. 4A). In seven (for 24 h) or three (for 48 h, 72 h, and 96 h; data not shown) separate experiments with different blood donors, we never saw arginase activity or protein induction in the Eos by all stimuli tested (Fig. 4B). We conclude that in human Eos, arginase is neither constitutively expressed nor inducibly up-regulated by a variety of prototypical Eos stimulatory agonists.

No alteration of arginine concentration upon incubation of cell culture medium with Eos-S

Based on the absence of arginase activity and protein in human Eos, we hypothesized that sonicates of these cells would not induce alterations of extracellular arginine and ornithine concentrations. To address this, we prepared Eos-S and as positive controls, PMN-S, which induce arginine depletion and de novo ornithine synthesis in cell culture medium as a result of the presence of arginase I [5]. We then determined arginase activity in the cell-free sonicates (mean arginase activity in four PMN-S: 1259 ± 78 mU/mg protein; no arginase activity measurable in four Eos-S). Cell culture medium [500 μ M arginine=Arg(+)] was incubated for 18 h at 37°C, alone or with a PMN-S aliquot of a defined final arginase activity (300 mU/ml) or an aliquot of Eos-S of equivalent protein concentration (250 μ g/ml; no arginase activity measurable). In parallel, arginase activity was blocked with nor-NOHA (1 mM) during the incubation of the cell culture media with the different sonicates. We first determined arginine and ornithine concentrations by HPLC in the different cell culture media after incubation with PMN-S or Eos-S $\pm$ nor-NOHA after the 18-h incubation step (Fig. 5). Incubation of normal cell culture medium without FCS (no ornithine present) with PMN-S led to a complete depletion of arginine (mean concentration: 0.7 μ M) and consecutive arginase-mediated synthesis of ornithine (mean concentration: 422 μ M), and this could be prevented by addition of the specific arginase inhibitor nor-NOHA during the incubation period. Also, a similar de novo synthesis of urea, the second metabolic product of arginase-mediated hydrolysis of arginine, could be detected only upon incubation with PMN-S (mean concentration: 45 μ M; data not shown), and this could again be prevented by addition of nor-NOHA (data not shown). In contrast, no decrease of arginine concentration and no de novo synthesis of ornithine were detectable upon incubation of cell culture medium with Eos-S, which correlates with the absence of arginase in human Eos.

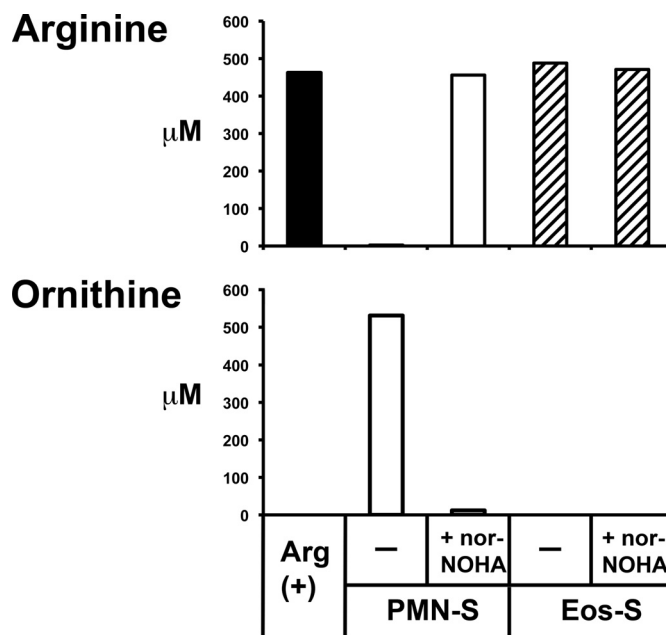


Figure 5. Human Eos-S do not alter extracellular arginine and ornithine concentrations. After purification from peripheral blood, human PMN and Eos were sonicated to induce release of intracellular constituents. Normal cell culture medium [500 μ M arginine; Arg(+)] was incubated for 24 h with an aliquot (corresponding to a final arginase activity of 300 mU/ml) of the filtered PMN-S or an aliquot of Eos-S of equivalent protein concentration (with no arginase activity measurable). This incubation was carried out in the absence or presence of the specific arginase inhibitor nor-NOHA. Concentrations of arginine and ornithine were determined at the end of the incubation period in the respective media. One representative experiment (total: four) is shown.

Human Eos from the inflammatory BAL of asthmatic patients do not express arginase

Cellular activation states might differ fundamentally between *in vitro*-stimulated cells and the *in vivo* situation within an inflammatory infiltrate. It is therefore conceivable that human Eos might express the enzyme arginase *in vivo* upon appropriate stimulation with consecutive enzyme liberation and arginine depletion. We therefore analyzed human primary Eos isolated from the peripheral blood and the BALF of allergic donors after induced allergic responses via segmental allergen provocation. PMN and Eos were purified from the peripheral blood, and Eos were also purified from the BALF 24 h after local instillation of mixed grass allergens. Again, no arginase activity (Fig. 6A) or arginase I protein (Fig. 6B) was detectable in purified Eos from the peripheral blood (10 individual samples) or BALF (six individual samples). In contrast, peripheral blood PMN of the allergic donors demonstrated arginase activity (mean: 1255 ± 260 mU/mg, eight individual samples), which was comparable with PMN arginase activity of healthy blood donors. In six allergic donors, alveolar macrophages were also purified from the BALF prior to segmental allergen provocation. These cells did not express arginase activity or arginase I protein (data not shown). Arginase has been found

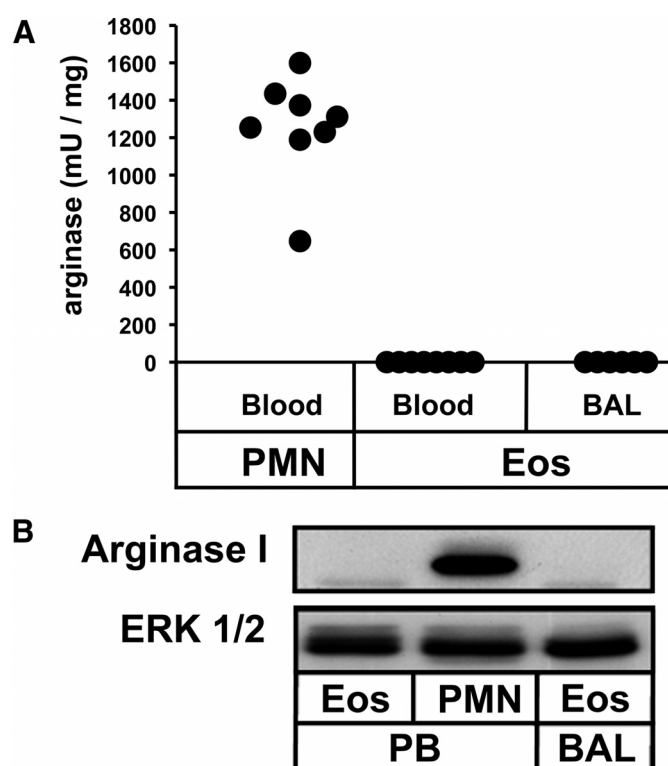


Figure 6. No arginase expression in peripheral blood and BAL Eos of allergic donors. PMN and Eos were purified from the peripheral blood, and Eos were also purified from the BALF 24 h after local instillation of mixed grass or house dust mite allergens (segmental allergen provocation). (A) Arginase activity was determined in cellular lysates of the respective cell isolates ($n=8$ donors for blood PMN; $n=10$ donors for blood Eos; $n=6$ donors for BAL Eos). (B) A representative Western blot for arginase I expression in peripheral blood (PB; PMN and Eos) as well as BAL (Eos) is demonstrated. Expression of ERK1/2 is shown as a loading control.

to be highly expressed in the local bronchopulmonary inflammatory infiltrate of murine and human asthma [18]. We conclude from our data that human Eos do not contribute to the release of arginase and the consecutive arginine depletion, ornithine and polyamine generation, and reduced NO synthesis with ensuing bronchial hyper-responsiveness that is characteristic of allergic asthma [19].

DISCUSSION

Human Eos are involved in the inflammatory response of allergic diseases and parasitic and helminth infections; they participate in antibacterial and antiviral immunity as well as in anti-tumor immune mechanisms. In contrast to human PMN, which express high arginase activity homogeneously as a result of constitutive arginase I expression [4], this study demonstrates that human Eos do not express arginase enzymatic activity or arginase protein. We tried to induce arginase in human Eos by induction of apoptosis or by stimulation with various inflammatory cytokines, which are known to regulate important Eos functions [10, 20–22]. Neither arginase protein

nor enzymatic activity could be induced in human Eos under all tested conditions in vitro. This lack of induction is identical to the situation in human PMN, in which the constitutive expression level of arginase I also cannot be modulated by a variety of stimuli [4]. In an earlier study, immunoelectron microscopy on inflammatory Eos detected low levels of arginase I associated with granules [4]; however, the current experiments demonstrate that this does not have a measurable impact on the local environment when compared with arginase I derived from neutrophils.

Our findings of an absence of arginase in human Eos clearly have implications for the understanding of disease pathogenesis in allergic asthma [19]. This disease is characterized by complex interactions between local cells of the bronchial tree and invading inflammatory immune cells, leading to airway hyper-responsiveness, smooth muscle cell hyperplasia and hypertrophy, goblet cell hyperplasia, and subepithelial fibrosis, summarized as airway remodeling [19]. Among the inflammatory infiltrate in human asthma, Eos are one of the predominant cell types. Asthmatic inflammation is characterized by a dysregulation of arginine homeostasis as a result of increased expression of arginase within resident bronchial cells as well as infiltrating inflammatory cells. Arginase I and II have been found as key up-regulated gene products in the inflamed lung of murine asthma models as well as in human patients [18]. Also, an increase of arginase activity in serum and concomitantly reduced plasma arginine levels has been described in patients with exacerbations of severe asthma [23]. Arginase-mediated arginine depletion and consecutive NO deficiency contribute to airway hyper-responsiveness in animal models and in patients with asthma [24]. Arginase is also involved in airway remodeling via generation of proline and subsequently, collagen [24]. Although increased Eos and arginase activity within the asthmatic lung coincide, no published evidence for arginase expression in asthmatic Eos exists so far. We cannot exclude in our current study the possibility that all Eos within the inflammatory BAL infiltrate might have already secreted induced arginase in vivo as a result of activating stimuli. Nevertheless, in light of the complete absence of arginase protein and enzymatic activity in all BAL Eos of all tested individuals, we consider this option rather unlikely. Our data do not support a role of Eos-associated arginase as a mechanism of Eos-induced fibrosis or asthmatic airway remodeling. Neutrophilic and Eos asthma describe two partially overlapping human asthma disease phenotypes that are defined by the composition of the local pulmonary inflammatory infiltrate. As we did not find evidence for a role of Eos or alveolar macrophages (data not shown) as source of arginase in human asthma, this highlights the potential role of arginase-expressing neutrophils in asthmatic inflammation. Increased PMN numbers are found in induced sputum of asthma patients and are associated with more severe airflow obstruction [25]. Also, there is a clear relationship between airway neutrophilia and airflow obstruction in asthma [26]. We hypothesize that chronic airway remodeling in human asthma is linked to arginase-expressing PMN and not Eos.

In summary, the absence of arginase in human Eos contrasts with its high-level expression in human PMN. Human Eos

therefore lack the endogenous immunosuppressive effector system of arginase-mediated arginine depletion. This finding has implications for Eos-dominated inflammatory reactions in allergic asthma, parasite, and helminth infections and for their participation in the anti-tumor immune response.

AUTHORSHIP

Claudia Luckner-Minden: designed and performed experiments and assisted in writing the manuscript. Ina Fischer: designed and performed experiments (data of allergic donors) and assisted in writing the manuscript. Claus-Dieter Langhans: performed experiments (amino acid measurements) and assisted in writing the manuscript. Martin Schiller: performed experiments (cytokine controls). Pascale Kropf: assisted in designing experiments and writing the manuscript. Ingrid Müller: assisted in designing experiments and writing the manuscript. Jens M. Hohlfeld: designed the BAL experiments and performed the clinical studies in atopics and asthmatics. Anthony D. Ho: assisted in writing the manuscript. Markus Munder: conceived the total project, designed all experiments, and wrote the manuscript.

ACKNOWLEDGMENTS

This work was supported by the Deutsche Forschungsgemeinschaft (MU1547/3-1 to M. M. and SFB587/B8 to J. M. H.) and by the Dietmar Hopp-Stiftung. The authors thank Manuel Modolell (Max-Planck Institute for Immunobiology, Freiburg, Germany) and L. B. Nicholson (University of Bristol, UK) for critical review of the manuscript.

REFERENCES

- Nathan, C. (2002) Points of control in inflammation. *Nature* **420**, 846–852.
- Jenkinson, C. P., Grody, W. W., Cederbaum, S. D. (1996) Comparative properties of arginases. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **114**, 107–132.
- Munder, M. (2009) Arginase: an emerging key player in the mammalian immune system. *Br. J. Pharmacol.* **158**, 638–651.
- Munder, M., Mollinedo, F., Calafat, J., Canchado, J., Gil-Lamagnere, C., Fuentes, J. M., Luckner, C., Doschko, G., Soler, G., Eichmann, K., Muller, F. M., Ho, A. D., Goerner, M., Modolell, M. (2005) Arginase I is constitutively expressed in human granulocytes and participates in fungicidal activity. *Blood* **105**, 2549–2556.
- Munder, M., Schneider, H., Luckner, C., Giese, T., Langhans, C. D., Fuentes, J. M., Kropf, P., Mueller, I., Kolb, A., Modolell, M., Ho, A. D. (2006) Suppression of T-cell functions by human granulocyte arginase. *Blood* **108**, 1627–1634.
- Bronte, V., Zanovello, P. (2005) Regulation of immune responses by L-arginine metabolism. *Nat. Rev. Immunol.* **5**, 641–654.
- Rodriguez, P. C., Zea, A. H., Culotta, K. S., Zabaleta, J., Ochoa, J. B., Ochoa, A. C. (2002) Regulation of T cell receptor CD3 ζ chain expression by L-arginine. *J. Biol. Chem.* **277**, 21123–21129.
- Oberlies, J., Watzl, C., Giese, T., Luckner, C., Kropf, P., Muller, I., Ho, A. D., Munder, M. (2009) Regulation of NK cell function by human granulocyte arginase. *J. Immunol.* **182**, 5259–5267.
- Gabrilovich, D. I., Nagaraj, S. (2009) Myeloid-derived suppressor cells as regulators of the immune system. *Nat. Rev. Immunol.* **9**, 162–174.
- Rothenberg, M. E., Hogan, S. P. (2006) The eosinophil. *Annu. Rev. Immunol.* **24**, 147–174.
- Akuthota, P., Wang, H. B., Spencer, L. A., Weller, P. F. (2008) Immunoregulatory roles of eosinophils: a new look at a familiar cell. *Clin. Exp. Allergy* **38**, 1254–1263.
- Munder, M., Eichmann, K., Moran, J. M., Centeno, F., Soler, G., Modolell, M. (1999) Th1/Th2-regulated expression of arginase isoforms in murine macrophages and dendritic cells. *J. Immunol.* **163**, 3771–3777.
- Kropf, P., Baud, D., Marshall, S. E., Munder, M., Mosley, A., Fuentes, J. M., Bangham, C. R., Taylor, G. P., Herath, S., Choi, B. S., Soler, G., Teoh, T., Modolell, M., Muller, I. (2007) Arginase activity mediates reversible T cell hyporesponsiveness in human pregnancy. *Eur. J. Immunol.* **37**, 935–945.
- Erpenbeck, V. J., Fischer, I., Wiese, K., Schaumann, F., Schmiedl, A., Nasenstein, C., Krug, N., Hohlfeld, J. M. (2009) Therapeutic surfactants modulate the viability of eosinophils and induce inflammatory mediator release. *Int. Arch. Allergy Immunol.* **149**, 333–342.
- Erpenbeck, V. J., Hagenberg, A., Dulkys, Y., Elsner, J., Balder, R., Krentel, H., Discher, M., Braun, A., Krug, N., Hohlfeld, J. M. (2004) Natural porcine surfactant augments airway inflammation after allergen challenge in patients with asthma. *Am. J. Respir. Crit. Care Med.* **169**, 578–586.
- Johann, A. M., Barra, V., Kuhn, A. M., Weigert, A., von Knethen, A., Brune, B. (2007) Apoptotic cells induce arginase II in macrophages, thereby attenuating NO production. *FASEB J.* **21**, 2704–2712.
- Freire-de-Lima, C. G., Xiao, Y. Q., Gardai, S. J., Bratton, D. L., Schiemann, W. P., Henson, P. M. (2006) Apoptotic cells, through transforming growth factor- β , coordinately induce anti-inflammatory and suppress pro-inflammatory eicosanoid and NO synthesis in murine macrophages. *J. Biol. Chem.* **281**, 38376–38384.
- Zimmermann, N., King, N. E., Laporte, J., Yang, M., Mishra, A., Pope, S. M., Muntel, E. E., Witte, D. P., Pegg, A. A., Foster, P. S., Hamid, Q., Rothenberg, M. E. (2003) Dissection of experimental asthma with DNA microarray analysis identifies arginase in asthma pathogenesis. *J. Clin. Invest.* **111**, 1863–1874.
- Maarsingh, H., Zaagsma, J., Meurs, H. (2009) Arginase: a key enzyme in the pathophysiology of allergic asthma opening novel therapeutic perspectives. *Br. J. Pharmacol.* **158**, 652–664.
- Hartnell, A., Kay, A. B., Wardlaw, A. J. (1992) IFN- γ induces expression of Fc γ RIII (CD16) on human eosinophils. *J. Immunol.* **148**, 1471–1478.
- Hogan, S. P., Rosenberg, H. F., Moqbel, R., Phipps, S., Foster, P. S., Lacy, P., Kay, A. B., Rothenberg, M. E. (2008) Eosinophils: biological properties and role in health and disease. *Clin. Exp. Allergy* **38**, 709–750.
- Tamura, N., Ishii, N., Nakazawa, M., Nagoya, M., Yoshinari, M., Amano, T., Nakazima, H., Minami, M. (1996) Requirement of CD80 and CD86 molecules for antigen presentation by eosinophils. *Scand. J. Immunol.* **44**, 229–238.
- Morris, C. R., Poljakovic, M., Lavrisa, L., Machado, L., Kuypers, F. A., Morris Jr., S. M. (2004) Decreased arginine bioavailability and increased serum arginase activity in asthma. *Am. J. Respir. Crit. Care Med.* **170**, 148–153.
- Maarsingh, H., Zaagsma, J., Meurs, H. (2008) Arginine homeostasis in allergic asthma. *Eur. J. Pharmacol.* **585**, 375–384.
- Woodruff, P. G., Khashayar, R., Lazarus, S. C., Janson, S., Avila, P., Boushey, H. A., Segal, M., Fahy, J. V. (2001) Relationship between airway inflammation, hyperresponsiveness, and obstruction in asthma. *J. Allergy Clin. Immunol.* **108**, 753–758.
- Shaw, D. E., Berry, M. A., Hargadon, B., McKenna, S., Shelley, M. J., Green, R. H., Brightling, C. E., Wardlaw, A. J., Pavord, I. D. (2007) Association between neutrophilic airway inflammation and airflow limitation in adults with asthma. *Chest* **132**, 1871–1875.

KEY WORDS:

arginine metabolism • immunosuppression • leukocyte