Arginase I is constitutively expressed in human granulocytes and participates in fungicidal activity

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Abstract

The balance of arginine metabolism via nitric oxide synthase (NOS) or arginase is an important determinant of the inflammatory response of murine macrophages and dendritic cells. Here we analysed the expression of the isoform arginase I in human myeloid cells. Using healthy donors and arginase I-deficient patients, we found that in human leukocytes arginase I is constitutively expressed only in granulocytes and is not modulated by a variety of pro- and antiinflammatory stimuli in vitro. We demonstrate that arginase I is localised in azurophil granules of neutrophils and constitutes a novel antimicrobial effector pathway, likely through arginine depletion in the phagolysosome. Our findings demonstrate important differences between murine and human leukocytes with respect to regulation and function of arginine metabolism via arginase.
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Introduction

In the mouse, the metabolism of arginine is essential for many immunological functions. In leukocytes, arginine can be metabolized by inducible nitric oxide synthase (iNOS) to the cytotoxic, tumoricidal and antimicrobial effector molecule nitric oxide (NO). Myeloid cells also hydrolyse arginine to urea and ornithine with the enzyme arginase, and this leads to the synthesis of polyamines and proline. Two arginase isoenzymes exist (arginase I and II), which differ in subcellular localization, regulation and possibly function. Arginase I is a cytosolic enzyme, which is expressed mainly in the liver as part of the urea cycle, whereas arginase II is a mitochondrial protein found in a variety of tissues. In the murine immune system, arginase and iNOS undergo reciprocal induction in several cell types by a variety of inflammatory and anti-inflammatory agonists. In murine macrophages and dendritic cells the polarization of arginine metabolism is driven by cytokines. T helper (T\(_H\)) 1 cytokines induce iNOS, whereas T\(_H\)2 cytokines upregulate arginase I.

Several important immunological functions of arginase have been clarified recently, including a direct and indirect role in inducing T cell hyporesponsiveness and the induction of T\(_H\)2-mediated immunopathology in murine schistosomiasis. The T\(_H\)2-induced arginase I of murine macrophages supports the growth of various microorganisms, most likely by competing with iNOS for arginine and by providing polyamines, and arginase is also found in murine inflammatory cell infiltrates in experimental autoimmune encephalomyelitis, glomerulonephritis, Herpes simplex virus infection of the eye and asthma.

In contrast to the wealth of knowledge regarding arginine metabolism in the murine immune system, very little is known about a possible role of arginase in human immunology. Arginase was detected in human mononuclear cells after injury and found in inflammatory cells of bronchoalveolar lavage fluid of asthmatic patients or in psoriatic lesions. We have therefore analysed the role of arginase in the human immune system. Surprisingly, the expression and function of arginase is strikingly different in human leukocytes when
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compared with murine leukocytes. Although arginase remains a crucial player in host defense, we demonstrate that within the human immune system arginase I is selectively expressed in granulocytes (polymorphonuclear leukocytes; PMN), quantitatively the largest subpopulation of myeloid cells. We show that in human PMN the enzyme is not cytosolic but localizes to the azurophil granules. Finally, we provide evidence that arginase I is a novel antimicrobial effector mechanism of human PMN, likely operating through arginine depletion within the phagolysosome.
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Materials and Methods

Blood donors and mice.
Experiments were performed in compliance with the relevant laws and institutional guidelines, human studies were approved by the ethics committee of the University of Heidelberg. Informed consent was provided according to the Declaration of Helsinki. Mice of strain C57BL/6 were obtained from the specific pathogen-free animal facilities of the Max-Planck-Institute for Immunobiology and were used between 6-8 wk of age.

Reagents and cells.
If not otherwise stated, chemicals were purchased from Sigma (St Louis, MO). N-ω-Hydroxy-nor-L-arginine (nor-NOHA) was from Bachem (Bubendorf, Switzerland). Recombinant murine cytokines: IL-10 and G-CSF were from Pepro-Tech (London, U.K.), IL-4 was from R&D Systems (Minneapolis, MN). All recombinant human cytokines were from PromoCell (Heidelberg, Germany). The L-arginine auxotroph strain of \( S. \) cerevisiae is an isogenic derivative of the laboratory strain FL100 (ATCC 28583) with a deficiency in ornithine transcarbamoylase (OTC, arg3), generously provided by Jacky de Montigny and Ives Lombard (both Université Louis Pasteur, Strasbourg, France). In some experiments wild type \( C. \) albicans strain ATCC 90028 were used.

Generation of murine bone marrow-derived macrophages (BMDM) and PMN.
Murine BMDM were generated as previously described\(^3,4\). Murine PMN were generated from bone marrow\(^21\) with slight modifications. Briefly, bone marrow cells (1×10\(^6\)/ml) were cultured in RPMI 1640 medium (20% horse serum, 5 ng/ml G-CSF, 5 nM hydrocortisone, 2 mM L-glutamine, 60 µM 2-mercaptoethanol, 1 mM sodium pyruvate, 1× non-essential amino acids, 100 U/ml penicillin and 100 µg/ml streptomycin). The cells were fed on d 4 with fresh
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medium (without G-CSF and hydroxycortisone) and harvested on d 7. After 1 h plastic adherence, the non-adherent fraction was harvested. This procedure led to a PMN purity of 90–95%, assessed by microscopy and flow cytometry analysis (GR1hi cells with characteristic forward/side scatter profile).

Generation of human macrophages and isolation of human peripheral blood mononuclear cells (PBMC) and PMN.

CD14+ monocytes were purified from peripheral blood of healthy human donors with the MACS®-system (Miltenyi-Biotec, Bergisch-Gladbach, Germany) and cultured for 10 days with M-CSF (5 ng/ml) in hydrophobic teflon bags3,4 to yield macrophages. To isolate human PBMC and PMN, EDTA-anticoagulated peripheral blood of healthy human donors was layered on top of Ficoll. After 20 min centrifugation (700 g), the PBMC (= interphase) were harvested. The pellet, containing erythrocytes and PMN, was resuspended in Hanks’ Balanced Salt Solution (HBSS, without Ca2+ and Mg2+) and mixed at a ratio of 1:1 with 3% dextran. After sedimentation of erythrocytes (20 min) the PMN-rich supernatant was harvested and the remaining erythrocytes were subjected to hypotonic lysis (155 mM NH4Cl, 10 mM KHCO3, 0.1 mM EDTA, pH 7.4) for 15 min on ice. After washing, cell purity and viability were checked by flow cytometry and microscopy. The method reproducibly yields PMN with a purity and viability > 95%. When indicated, PMN were further purified by flow cytometry on a Vantage Flow Cytometer (BD Biosciences, Heidelberg, Germany) according to forward and side scatter, yielding purities > 99.5%. PBMC were separated in CD14+ (monocyte fraction, purity: 95%) and CD14− cells (lymphocyte fraction, purity 90%) with the MACS®-system.
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**Measurement of PMN fungicidal activity**

PMN were preincubated with inhibitors for 30 min at 37 °C. Yeast and PMN (5×10^6 each) were cultured in 1 ml HBSS (+ 10% human serum) for 60 min. Viability of yeast was evaluated with the XTT assay exactly as described and with the FUN-1 Yeast Viability Kit® (Molecular Probes, Göttingen, Germany). In the latter case, PMN were lysed in ice-cold water and in case of *S. cerevisiae* the cell pellet was resuspended for 10 min at 34°C in 50 ul of DNAse I (1 U/µl, Invitrogen, Carlsbad, CA) and Streptolysin O (2 mg/ml) for permeabilization. 50 µl of FUN-1 cell stain (0.125 mM) + Calcofluor White M2R (0.05 mM) in HBSS/4% glucose were added. After 30 min at 30°C in the dark, at least 200 yeast cells per sample were analyzed by fluorescence microscopy. The percentage of fungal cell damage (“% Kill”) was defined by the following equation: 100 – (100 × [OD_{405} of fungi with PMN] / [OD_{405} of fungi]). The percentage of reduction of fungal cell damage (“% Kill inhibition”) upon treatment with inhibitors was defined by the following equation: 100 – (100 × [% Kill of fungi with PMN with inhibitor] / [% Kill of fungi with PMN]).

**Metabolism of ^14^C-L-arginine and thin-layer chromatography (TLC).**

After washing, 2×10^5 cells were incubated for 2 h at 37°C with arginine-free Dulbecco’s Modified Eagle’s Medium containing 2% FCS and 0.1 μCi of L-(U-^14^C) arginine (Amersham Biosciences, Freiburg, Germany). Cells were subsequently lysed by two freeze-thaw cycles. 20 µl of the lysate were spotted onto TLC plates (Merck, Darmstadt, Germany), dried for 1 h at 42°C and developed in the solvent system chloroform/methanol/ammonium hydroxide/water 0.5/4.5/2.0/1.0 (vol/vol). Spots were developed with Ninhydrin by heating at 120°C for 5 min and analyzed by scintillation counting.

**Arginase enzymatic assay.**
Arginase activity was measured in cell lysates as previously described with slight modifications. Briefly, cells were lysed with 0.5% Triton X-100, 25 mM Tris-HCl, pH 7.5, containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and 2 g/ml each of aprotinin, leupeptin and pepstatin (lysis buffer). To 100 µl of this lysate 20 µl of 10 mM MnCl₂ was added, and the enzyme was activated by heating for 10 min at 56°C. In case of determination of native arginase activity, these steps (addition of exogenous manganese and heating) were omitted. Arginine hydrolysis and measurement of urea concentration were performed exactly as previously described. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 µmol of urea per min.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western Blot analysis.

Cells were lysed for 30 min on ice in lysis buffer (see above). Cell debris was spun down at 18,000 g for 5 min at 4°C. SDS-PAGE was done as previously described. The proteins were transferred to a Hybond-P polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences). After blocking with 5% nonfat dry milk in TBST-buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) for 2 h, the membranes were incubated with the following primary antibodies (1:5000 in TBST, 5% bovine serum albumin): polyclonal rabbit anti-rat arginase I antiserum which is cross-reactive to mouse and human arginase I and anti-ERK1/2 (extracellular signal regulated kinase, Cell Signaling Technology, Beverly, MA). Antibody reactivity was monitored with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Santa Cruz Biotechnology Inc, Santa Cruz, CA), followed by visualization with the ECL detection system (Amersham Biosciences).

Subcellular fractionation of neutrophils.

Subcellular fractionation of neutrophils was performed as previously described. Briefly, resting neutrophils isolated from freshly heparinized human peripheral blood were
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resuspended in 50 mM Tris-HCl, pH 7.5 containing 2 mM PMSF and then disrupted by repeated freeze-thaw. Homogenates were centrifuged at 1200 rpm in a Sorvall T 6000D centrifuge for 10 min, and the supernatant, representing the postnuclear extract, was saved. After centrifugation of the postnuclear extract at 45000 rpm in a TLA rotor for 90 min at 4°C using an Optima TL Ultracentrifuge (Beckman Instruments, Palo Alto, CA), supernatant (soluble fraction) and pellet (membrane fraction, resuspended in 50 mM Tris-HCl, pH 7.5, containing 2 mM PMSF) were saved. To prepare the distinct subcellular fractions, freshly prepared neutrophils (about 3-5×10⁸) were gently disrupted, and the postnuclear fractions were fractionated in 15%-40% continuous sucrose gradients as described previously²⁵. Subcellular fractions were analyzed for marker proteins for each organelle as described²⁵.

Immunoelectron microscopy.

Resting human neutrophils and exudate neutrophils from skin window chambers after phagocytosis of latex beads²⁶ were fixed for 24 h in 4% paraformaldehyde in PHEM buffer (60 mM PIPES, 25 mM HEPES, 2 mM MgCl₂, 10 mM EGTA, pH 6.9) and then processed for ultrathin cryosectioning as previously described²⁷. Ultra-thin frozen sections were incubated at room temperature with the indicated antibodies and 10 and/or 15 nm protein A gold²⁷, embedded in a mixture of methylcellulose and uranyl acetate and examined with a Philips CM 10 electron microscope (Eindhoven, The Netherlands). For controls, the primary antibody was replaced by an irrelevant rabbit antibody. Antibodies used in double immunolabeling were rabbit anti-human lactoferrin (Lf) (Cappel Laboratories, Durham, NC) and rabbit anti-human myeloperoxidase (MPO) (DAKO, Glostrup, Denmark).

Results

Arginase in human myeloid cells: specific expression in PMN.
We analysed the expression of arginase in various human myeloid cell types treated or not with several agonists. We stimulated the cell populations with the recombinant human cytokines IL-1\(\alpha\), IL-1\(\beta\), IFN-\(\gamma\), TNF-\(\alpha\), IL-4, IL-10, IL-13 as well as LPS, PGE\(_2\), dexamethasone and the cAMP-enhancing agents dibutyryl-cAMP and forskolin, alone and in various combinations. Human monocyte-derived macrophages and dendritic cells showed no arginase activity in the resting state. In contrast to the murine system, we were also unable to induce arginase activity or protein in human macrophages (Figure 1), dendritic cells and monocytes (data not shown) with T\(_h\)2 cytokines over a wide range of individual concentrations (0.01-100 ng/ml for each cytokine) or any of the above mentioned agonists.

We also stimulated various immortalized human myeloid cell lines (U937, HL-60, THP-1, with or without induction of differentiation by phorbol myristate acetate or retinoic acid) in the same way with the same negative results (data not shown). We then turned to leukocytes, which we derived directly from the blood of healthy donors. In the PMN of all the blood donors \((n = 31)\) we consistently found high arginase activity (mean: 1644 ± 423 mU/mg protein, range: 667-2189 mU/mg protein) (Figure 2A). In contrast, in 19/25 PBMC fractions (involving lymphocytes and monocytes) no arginase activity was detectable, while in the remaining 6 PBMC samples we found low arginase activity (mean: 111 ± 46 mU/mg protein, range: 35-156 mU/mg protein, Figure 2A). In order to rule out any contamination of the PBMC fraction with PMN or vice versa, we corroborated our findings by FACS\textsuperscript{®} sorting a pure PMN population according to forward/side scatter pattern from the conventional PMN fraction (purity of FACS\textsuperscript{®} sorted PMN > 99.5\%). Alternatively, we eliminated contaminating PMN from the PBMC fraction by FACS\textsuperscript{®} sorting. In three independent experiments we only demonstrated arginase activity and protein in the PMN fraction, while PBMC, purified monocytes (CD14\(^+\), purity 95\%) or lymphocytes (CD14\(^-\), purity 90\%) completely lacked enzymatic activity and protein (Figure 2B). We also analysed PMN of two patients with complete arginase I-deficiency (OMIM 207800, patient 1: 18 year old male, patient 2: 5 year old female). The PMN of these patients had neither arginase activity nor expression of
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arginase I protein (Figure 2C). Therefore we concluded that resting human PMN express solely the hepatic isoform (arginase I) and have no compensatory upregulation of arginase II even in the face of complete arginase I deficiency.

Since arginase I is a Th2-inducible protein in murine macrophages and dendritic cells we wondered if human PMN arginase would be further augmented by Th2 cytokine stimulation. We treated human PMN with the Th2 cytokine IL-4 or a combination of IL-4 and IL-10, treatments which yield maximal arginase activity in murine myeloid cells (see also Figure 1). In six separate experiments (four with conventionally purified PMN and two with FACS® sorted PMN) we found no significant change in human PMN arginase activity, protein expression (Figure 3A) or arginase I mRNA levels (data not shown) upon Th2 cytokine stimulation. Finally, we analysed the expression of arginase in murine PMN. Unlike their human counterparts, unstimulated murine PMN show no arginase protein or activity (Figure 3B). The Th2 cytokine IL-4 either alone or in cooperation with IL-10, induces the hepatic isoform arginase I and considerable arginase activity (mean of three experiments: 1153 mU/mg protein, range: 1162-1290 mU/mg protein) in murine PMN (Figure 3B). These cells therefore follow the same pattern of arginase I regulation as murine macrophages and dendritic cells. We conclude that murine and human PMN differ fundamentally in terms of basal expression and cytokine-mediated regulation of arginase I.

PMN do not metabolize L-arginine despite high arginase activity.

Upon Th2 stimulation, murine macrophages upregulate arginase I and consume arginine present in the local microenvironment. Since human PMN express high arginase activity constitutively, we wondered if these cells were constantly hydrolysing arginine after uptake from the extracellular milieu. We therefore incubated resting human PMN with radioactively labeled 14C-L-arginine and analysed the conversion of this substrate into ornithine and spermine by TLC. As a control we used resting and IL-4 stimulated murine BMDM. Whereas resting BMDM showed no arginase activity and no significant hydrolysis of arginine, the Th2
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stimulated, arginase-expressing murine BMDM metabolized arginine and produced ornithine and spermine. Surprisingly, human PMN were very inefficient in degrading arginine (Table 1). This led us to question whether, in contrast to the cytosolic location of arginase within murine macrophages\(^1\,\text{,}\,\text{28}\), the enzyme might be sequestered in a different compartment in human PMN with impaired arginine accessibility.

**Arginase I is present in azurophil granules of human PMN.**

In order to determine the localization of the enzyme within human PMN, we performed subcellular fractionation experiments. Firstly, we separated resting human PMN in a soluble and a membrane fraction and found that arginase I is not a membrane-bound protein in resting human PMN (Figure 4A). Subsequent subcellular fractionation assays (Figure 4B), that resolved cytosol, plasma membrane, tertiary granules, specific granules and azurophil granules\(^24\,\text{,}\,\text{25}\) showed that arginase I protein is mainly present in fraction 8 (Figure 4C), the myeloperoxidase-positive azurophil granules of resting human PMN. Arginase activity assays with aliquots of the various PMN fractions confirmed these results (Figure 4C). Since arginase is enriched in this subcellular compartment, we observed an even higher enzyme activity (mean of 3 experiments: 3650 ± 97 mU/mg protein in fraction 8) compared to whole cell PMN lysates (see Figure 2A).

To confirm the biochemical data, resting human neutrophils were immunolabeled for arginase I and analyzed by immunogold electron microscopy. Arginase I localized to a granular compartment, where it was present in the matrix (Figure 5A). To define further the subtype of granule in which arginase I is present, cryosections of resting human neutrophils were double labeled with anti-arginase I and anti-MPO (for azurophil granules) or anti-lactoferrin (Lf, for specific granules). With this technique, almost all arginase I-positive granules were also MPO-positive (Figure 5B), while only very few arginase I-positive granules were positive for lactoferrin (Figure 5B). We found that in human eosinophils arginase I is also a granular enzyme, which is present in cristalloid-containing and in cristalloid-free granules (Figure 5C).
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We conclude from these morphological data and the biochemical subcellular fractionation results (see Figure 4) that arginase I is present almost exclusively in azurophil granules of human PMN as well as in human eosinophils.

These findings explain the lack of constitutive arginine degradation by human PMN, since the content of azurophil granules of resting PMN is kept separate from the extracellular milieu to protect the cell. Indeed, a constant uptake and degradation of L-arginine by human PMN would deplete the plasma of L-arginine, so that this important amino acid would be unavailable for other cell types. We therefore hypothesized that the function of PMN arginase probably differs from that of arginase-expressing murine macrophages.

**Human PMN arginase: a novel fungicidal effector mechanism.**

The function of neutrophil azurophil granules, which contain a large number of proteolytic and microbicidal enzymes, is mainly to fuse with the phagosome during phagocytosis. Following fusion, the contents of the granules enter the phagosome and kill invading microorganisms. We therefore wondered whether arginase I might fulfill an antimicrobial function. If this were the case then the enzyme should localize to the phagosome during phagocytosis. We addressed this by performing immunogold electron microscopy on human PMN after phagocytosis of latex beads. Arginase I localized to the phagolysosome of human PMN after uptake of the beads (Figure 6A). It has recently been shown that the phagosome of human PMN is depleted of arginine during phagocytosis. By demonstrating that arginase I is present in the azurophil granule fraction (Figure 4 and 5) and in the phagosome during phagocytosis (Figure 6A) we provide a mechanistic explanation for phagosomal arginine depletion. In order to investigate the relevance of this in defence to infection, we first used an arginine-auxotroph strain of *S. cerevisiae* during phagocytosis and fungicidal killing by human PMN. In vitro, this mutant depends on the presence of arginine in the medium in order to grow and survive (data not shown). We hypothesized that it should provide a sensitive test of the effectiveness of arginine depletion in the phagosomal environment. We coincubated
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human PMN with *S. cerevisiae* and assessed the fungicidal activity of the phagocytes in the presence or absence of the potent arginase inhibitor N-ω-Hydroxy-nor-L-arginine (nor-NOHA) using two different methods to assess the viability of *S. cerevisiae* after phagocytosis. Both assays demonstrated a small but significant reduction of neutrophil fungicidal activity when arginase activity is blocked during phagocytosis (mean reduction of kill by arginase inhibition: 11.3%, standard error (SE) 0.07 by XTT assay and 13.9%, SE 0.29 by fluorescence microscopy, Figure 6B and 6C). PMN possess multiple oxidative and non-oxidative effector pathways for microbial killing which cooperate and also yield a certain degree of redundancy. To test the role of arginase further, we inhibited the oxidative fungicidal activity of human neutrophils in the presence or absence of arginase inhibition. MPO inhibition by NaN₃ alone reduced the killing activity of human neutrophils (mean reduction of kill 9.2%, SE 0.08 and 19.4%, SE 0.09 as assessed by XTT assay and fluorescence microscopy, respectively, Fig. 6B and 6C). But when arginase was also inhibited, the fungicidal activity is further impaired (mean reduction of kill by combined inhibition: 21.3%, SE 0.08 by XTT assay and 29.8%, SE 0.27 by fluorescence microscopy, Fig. 6B and 6C), demonstrating the additive effect of inhibiting both neutrophil effector pathways. We then analysed the significance and contribution of arginase to the fungicidal activity of PMN for a wild-type, non-arginine auxotroph strain of *C. albicans*. In contrast to the arginine-auxotroph strain of *S. cerevisiae* we saw no impairment of PMN fungicidal activity when PMN arginase activity was blocked during phagocytosis (Figure 7A). As a control, we again blocked the formation of reactive oxygen species by inhibiting myeloperoxidase with NaN₃ or alternatively NADPH oxidase with diphenyleneiodonium (DPI) and significantly inhibited PMN fungicidal activity (Figure 7A). Like other members of the arginase family, human PMN arginase activity critically depends on the presence of manganese, most probably as part of the active center of the enzyme. Since human PMN lysates show only about 5-10% of arginase activity without addition of exogenous manganese during the enzymatic assay (“native arginase activity”, data not shown), we concluded that
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Manganese might be a limiting factor for the activity of the enzyme also in the cellular context. RPMI medium 1640 does not contain manganese by itself. In our phagocytosis experiments the concentration of manganese was therefore only about 10-20% of the physiologic situation corresponding to the fraction of human serum used for yeast opsonization. We therefore added various non-toxic concentrations of manganese during phagocytosis of *C. albicans* and saw a pronounced induction of PMN arginase activity in 7 independent experiments (Figure 7B). More importantly, this induction of enzymatic activity always correlated with an increase in PMN fungicidal killing activity (Figure 7C) which could be reversed upon blocking with the arginase inhibitor nor-NOHA (mean reduction of kill 28.7%, Figure 7D). As demonstrated with the arginine-auxotroph strain of *S. cerevisiae* (Figure 6B and C), the reduction of fungicidal kill upon blocking of myeloperoxidase with azide (mean reduction of kill 29.2%) can be further augmented upon additional arginase inhibition (mean reduction of kill 47.4%), while no additive effect is observable when NADPH oxidase is blocked with DPI (without arginase inhibition: 45.5% kill reduction, with arginase inhibition: 47.3%, Figure 7D). All the substances that increased killing activity of PMN (i.e. MnCl₂, nor-NOHA, NaN₃ and DPI) didn’t impair microbial growth by themselves when tested at the respective concentrations.

In summary, we have demonstrated that phagosomal arginase I constitutes a novel antimicrobial effector mechanism in the phagolysosome of human PMN probably by depleting arginine from the local environment.
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Discussion

The cytokine-driven regulation of arginine metabolism via iNOS and arginase in the murine immune system is well established\(^2-7,33\). In this study we have analysed the expression, subcellular localization and function of arginase in the human immune system. We have uncovered important differences between human and murine leukocyte arginase. In the murine system the enzyme is not expressed in unstimulated leukocytes but is inducible in all major myeloid cells by TH2 stimulation\(^2-4\) (Figure 3B). In human leukocytes this is not the case. We found that neither arginase activity nor protein is present in human monocytes, macrophages or dendritic cells, either resting or activated \textit{in vitro} by a variety of pro- and antiinflammatory stimuli. Human resting PMN, on the other hand, show high constitutive arginase activity (mean: 1644 mU/mg protein), which is of the same order of magnitude as TH2 stimulated murine macrophages (Figure 1) or dendritic cells\(^3,4\) and human hepatocytes (Figure 1). This arginase expression is not further modulated by TH2 cytokines (Figure 3A).

Only a few reports have addressed the expression of arginase in cells of the human immune system. Early reports described a protein with arginase activity that was isolated from PMN of a patient with chronic myeloid leukemia\(^34\) and secreted arginase was found in the supernatant of maturing malignant cells from two patients with acute myeloid leukemia\(^35\). Arginase was induced in human mononuclear cells after injury\(^19\) or by stimulation with an immunomodulatory peptide\(^36\). The enzyme was also detected in a subpopulation of inflammatory cells found in the bronchoalveolar lavage fluid of asthmatic patients\(^18\) or isolated from psoriatic lesions\(^20\). Here we have analysed highly purified human leukocyte subsets and demonstrated that leukocyte arginase activity is mainly, if not exclusively, localized in PMN.

While apopotosis of human PMN degrades the various toxic granule constituents in a highly regulated way, this might not be the case under conditions of chronic inflammation or necrosis. We hypothesize that a dysregulated liberation of arginase into the local
Arginase in granulocytes depletes arginine and participates in local immnosuppression via T cell hyporesponsiveness\textsuperscript{8} or in fibrosis via enhanced proline synthesis\textsuperscript{11}.

Arginase I deficiency is a rare autosomal recessive genetic defect (incidence 1:350,000) which leads to hyperargininemia, hyperammonemia, neurological impairment and progressive dementia due to the compromised hepatic urea cycle\textsuperscript{1,37}. The severity of symptoms depends mainly on the degree of enzymatic deficiency (reduction or total absence of arginase I) and the timely diagnosis with institution of dietary treatment. Two patients with arginase I deficiency were assessed and their PMN showed no arginase activity or protein. This both confirms that arginase activity in human PMN is only due to the isoenzyme arginase I and demonstrates that a compensatory increase of arginase II, as described for kidney tissue from hyperargininemic patients\textsuperscript{37,38} and in the arginase I-deficient mouse\textsuperscript{39} does not occur in human PMN (Figure 2C).

What is the function of human PMN arginase? Myeloid cells selectively deplete the phagosome of essential nutrients for microbial pathogens\textsuperscript{40}. Upon phagocytosis, microorganisms respond by inducing and repressing transcription of a variety of genes in order to adapt to the new environment. The transcriptional profile of the ingested microbe therefore reflects the microenvironment of the phagosome. It was recently demonstrated that \textit{S. cerevisiae} and \textit{C. albicans} upregulate genes of their endogenous arginine biosynthetic pathways upon phagocytosis by human neutrophils\textsuperscript{30}. This transcriptional response is most likely due to an arginine-deprived surrounding within the phagosome. Interestingly, upon phagocytosis by human monocytes no upregulation of yeast arginine biosynthetic genes was noted. The authors concluded from this discrepancy that, in contrast to human PMN, the phagosomal environment of monocytes is not depleted of arginine. Our findings of a selective expression of arginase in human PMN phagosomes (and not in monocytes) offer a likely explanation for the arginine deprivation encountered by yeast upon PMN phagocytosis.

Numerous reports have clarified that various oxidative and non-oxidative antimicrobial or antitumor effector pathways exist in PMN\textsuperscript{29,32,41}. These pathways cooperate and might...
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provide redundancy in PMN effector function. Depending on the specific microorganism, individual effector mechanisms are more important than others\textsuperscript{32,41}. In this study we have worked with a model organism that is exquisitely sensitive to arginine deprivation due to its inability to synthesize the amino acid itself. We used this strain of \textit{S. cerevisiae} as a functional bioprobe to proof the existence of arginase-mediated arginine deprivation within the human PMN phagosome even under suboptimal conditions of limited manganese availability and consecutive low arginase activity. More importantly, we show that arginase activity and fungicidal potential of human PMN increase during phagocytosis of wildtype \textit{C. albicans} with the availability of manganese, an essential constituent of active arginase. When manganese is not limiting, arginase and myeloperoxidase contribute comparably to the killing of \textit{C. albicans} (Figure 7D). Human arginase I, like all mammalian arginases, has a basic (pH 8.5-9) pH optimum\textsuperscript{1}. The vacuolar pH of human neutrophils initially becomes basic upon phagocytosis before it gets acidic after 15-30 min of ingestion\textsuperscript{41,42}. The rise in pH from about 6.0 to 7.8-8.0 soon after phagocytosis depends on the activity of NADPH oxidase, since it is due to consumption of protons by the protonation of O$_2^-$ and O$_2$$^2$. This alkalinization reaches an optimal level for the activity of granule proteases\textsuperscript{41}. The rise in pH might also be necessary for the enzymatic activity of arginase in the phagosome during the early phase of phagocytosis. We have seen pronounced inhibition of fungicidal activity by arginase inhibition especially during the first 30-90 min of phagocytosis whereas later on the effect of arginase inhibition disappeared (data not shown). Similarly, when NADPH oxidase is blocked by DPI, arginase does not further add to the killing of \textit{C. albicans} (Figure 7A and D) and \textit{S. cerevisiae} (data not shown), likely because the necessary phagosome alkalinization is missing.

The antimicrobial effector function of intraphagosomal arginase needs to be evaluated with additional pathogenic microbes. Arginine deprivation might enhance the sensitivity of the parasite towards killing by other effector pathways. We further hypothesize that arginine
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might be necessary for the synthesis of anti-phagocyte defense mechanisms in certain pathogens.

A review of published cases of patients with arginase I deficiency as well as the clinical history of the two patients analysed in this study did not reveal an increased incidence of severe infectious problems. Furthermore, we were able to do one phagocytosis experiment with PMN of a patient with arginase I deficiency and saw no impairment in fungicidal activity of arginase I-deficient PMN (data not shown). Obviously, human PMN are able to compensate for lack of the enzyme due to the redundancy of multiple antimicrobial pathways. The immunological competence of patients with arginase I deficiency is reminiscent of patients with MPO deficiency of PMN. Although this genetic defect is associated with impairment of in vitro PMN fungicidal activity, it is usually clinically silent.

In summary, we have clarified the cellular distribution, subcellular localisation, regulatory aspects and function of arginase in human leukocytes. We have demonstrated for the first time that only human PMN constitutively express arginase I, that the enzyme is localized in the azurophil granules and that it works as a novel fungicidal effector mechanism of human PMN.
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References


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Figure legends

Figure 1.

Arginase I is not inducible by Th2 cytokines in human macrophages. Murine bone marrow-derived macrophages or human monocyte-derived macrophages were stimulated with the indicated cytokines (10 ng/ml each) for 24 h and cell lysates were analyzed by immunoblotting for arginase I and ERK1/2 (to control for equal protein loading). The results of parallel determinations of arginase activities (in mU/mg protein) in aliquots of the same cell lysates are noted above the respective lanes of the arginase blots.

Figure 2.

Among human leukocytes, arginase I is specifically expressed in PMN. (A) Arginase activity is found in the PMN fraction of human peripheral blood leukocytes. Leukocytes of healthy human blood donors were separated into PBMC and PMN. Mean arginase activity of 31 PMN preparations was \(1644 \pm 423\) mU/mg protein and of 25 PBMC preparations \(26 \pm 52\) mU/mg protein (\(P < 0.0001\) for comparison between PMN and PBMC; 19/25 PBMC preparations showed no arginase activity). (B) Arginase I is specifically expressed in FACS\(^\circledR\)-purified human PMN. PBMC and PMN were FACS\(^\circledR\)-purified according to forward/side scatter characteristics. The highly pure populations (purity > 99.5%) were analyzed by immunoblotting. Results shown are representative of three independent experiments. (C) Absence of arginase I protein and arginase activity in PMN of a patient with arginase I deficiency. Purified PMN of a 18 year old male patient with arginase I-deficiency (ARGI\(^{-/-}\)) and a control healthy blood donor (ARGI\(^{+/+}\)) were lysed and analyzed by immunoblotting. Two experiments yielded identical results. (B and C): The results of parallel determinations of arginase activities (in mU/mg protein) in aliquots of the same cell lysates are noted above the respective lanes of the arginase blots. To control for equal protein loading, Western Blots for ERK1/2 were done in parallel.
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Figure 3.

Human and murine PMN differ in the regulation of arginase I expression. (A) Constitutively expressed arginase I in human PMN is not further induced by TH2 cytokines. Human PMN were stimulated with the indicated cytokines for 14 h. Arginase I protein expression was analyzed by immunoblotting. (B) Induction of arginase I protein in murine PMN by TH2 stimulation. Bone marrow-derived murine PMN were stimulated with the indicated cytokines. After 14 h, the cells were harvested and analyzed by immunoblotting. (A) and (B): The results of parallel determinations of arginase activities (in mU/mg protein) in aliquots of the same cell lysates are noted above the respective lanes of the arginase blots. To control for equal protein loading, Western Blots for ERK1/2 were done in parallel. Results shown are representative of four independent experiments.

Table 1.

Human PMN do not metabolize arginine despite constitutive arginase activity. Human PMN were incubated in 14C-L-arginine-containing medium. After cell lysis, the radioactively labeled metabolic products were analysed by thin layer chromatography and quantified by scintillation counting. The values for each compound are expressed as percentage of the total radioactivity measured in triplicate cultures ± standard deviation. As controls, murine bone marrow-derived macrophages (BMDM) were analysed in parallel. The murine BMDM were left unstimulated or preincubated with IL-4 to induce arginase activity. L-arginine metabolism was then assessed as described above for human PMN. Arginase activity of the various cell populations is also shown in the table.

Figure 4.

Subcellular fractionation of resting human PMN demonstrates that arginase I is present in azurophil granules. (A) Equal amounts of postnuclear extract (E), soluble (S) and
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membrane (M) proteins from resting human PMN were run on SDS-PAGE and analyzed by immunoblotting for arginase I. Human liver protein extract (L) was used as a positive control.

(B) Postnuclear fractions from resting neutrophils were subjected to subcellular fractionation and analyzed for specific markers for the distinct subcellular organelles, which are plotted normalized to the fraction with maximal activity. The following markers were assayed: cytosol, lactate dehydrogenase (open diamonds, CYT); plasma membrane, HLA (closed triangles, PM); tertiary granules, gelatinase (open triangles, TG); specific granules, lactoferrin (closed circles, SG); azurophil granules, myeloperoxidase (open squares, AG). (C) Proteins from subcellular fractions 1-8 of resting human neutrophils were assayed for arginase I protein by immunoblotting and in parallel for enzymatic activity. All data shown are representative of four separate experiments.

Figure 5.

Localization of arginase I in human resting PMN analysed by immunogold electron microscopy. (A) Cryosections of PMN were immunogold labeled with polyclonal rabbit anti-arginase I Ab (10-nm gold). Arginase I localized to the matrix of granules (arrows). (B) Arginase I colocalizes with myeloperoxidase. To characterize the arginase I-positive granules, PMN were double-labeled with anti-arginase I (10-nm gold) and anti-myeloperoxidase (MPO) or anti-lactoferrin (Lf, both 15-nm gold), respectively. After double-labeling for MPO, almost all arginase I-positive granules were also MPO-positive (arrows), whereas in most granules there was no colocalisation of arginase I (arrowheads) and lactoferrin (thin arrow). (C) Localization of Arginase I in human eosinophils. In the same cryosections as above, some eosinophils were present. Arginase I is again found in the matrix of granules with (c) or without crystalloid (arrows). Bars: 200 nm.

Figure 6.
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Arginase I participates in the fungicidal activity of human PMN. (A) Arginase I localizes to the phagosome in human PMN. Exudate neutrophils from skin window chambers after phagocytosis of latex beads were analysed by immunogold electron microscopy. Cryosections of neutrophils were immunogold labeled with polyclonal rabbit anti-arginase I. Arginase I (arrows) localizes to the phagolysosomes (ph). Bar 200 nm. Inset: area of another cell showing a small phagolysosome. (B) and (C) Inhibition of arginase inhibits the fungicidal activity of human PMN. Human PMN were cocultured with an arginine-auxotroph strain of S.cerevisiae. The viability of S.cerevisiae after phagocytosis was monitored with the XTT assay in 12 independent experiments (B) and in 6 experiments in parallel with the FUN-1 Cell viability kit® (C). PMN arginase was inhibited by preincubation with N-ω-Hydroxy-nor-L-arginine (nor-NOHA, 200 µM). PMN myeloperoxidase was inhibited by preincubation with sodium azide (NaN₃, 1 µM). To allow for comparison between individual experiments, the percentage of kill inhibition (compared to the viability of S.cerevisiae after phagocytosis without the use of inhibitors) is shown. The mean reduction of PMN fungicidal activity of 12 (B) and 6 (C) separate experiments is shown. The inhibition of fungicidal activity was statistically significant with the indicated P values, analyzed with the two-tailed Student’s t test.

Figure 7.

The fungicidal activity of human PMN against C.albicans is partially due to manganese-dependent activity of arginase I. (A) When manganese is limiting (RPMI medium 1640 with 10% serum) arginase does not contribute to the fungicidal activity of human PMN against C. albicans. Human PMN were cocultured for 1h with a wild-type strain of C. albicans. The viability of C. albicans after phagocytosis was monitored with the XTT assay in 7 independent experiments. PMN arginase was inhibited by preincubation with N-ω-Hydroxy-nor-L-arginine (nor-NOHA, 200 µM) and PMN myeloperoxidase with sodium azide.
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(NaN₃, 1 µM) in 7 independent experiments, while in 3 separate experiments NADPH oxidase was blocked with diphenylene iodonium (DPI, 20 µM). To allow for comparison between individual experiments, the percentage of kill inhibition (compared to the viability of C. albicans after phagocytosis without the use of inhibitors) is shown. The inhibition of fungicidal activity was statistically significant with the indicated P values, analyzed with the two-tailed Student’s t test. n.s.: not significant. (B) Arginase activity in human PMN depends on the manganese concentration present during phagocytosis of C. albicans. Experimental set-up was as in (A). During phagocytosis increasing concentrations of exogenously added MnCl₂ were present. Arginase activity was determined in lysates of PMN-C. albicans coincubations after 1h. The generation of reactive oxygen intermediates was inhibited by the presence of 1µM NaN₃ during phagocytosis. No exogenous manganese was added during the enzymatic assay (“native arginase activity”). One representative example of four independent experiments is shown. r=correlation coefficient. (C) Fungicidal activity of human PMN against C. albicans correlates with PMN arginase activity. The representative experiment is the same as in (B). The percentage of C. albicans kill (% kill after 1h of coincubation compared to the viability of C. albicans alone) is shown in correlation to the different PMN arginase concentrations induced by manganese as demonstrated in (B). (D) Upon manganese supplementation (RPMM medium 1640 with 10% serum + 50 µM MnCl₂) arginase does participate in the fungicidal activity of human PMN against C. albicans. Experimental set-up and statistical analysis is as in (A).
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Figure 1
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Figure 2
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Table 1. Arginine metabolism of human PMN

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<th>Human PMN</th>
<th>Murine BMDM</th>
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<tr>
<td>Ø</td>
<td>1005 ± 280</td>
<td>39 ± 1.5</td>
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<tr>
<td>Arginase activity (mU/mg protein)</td>
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<td>Spermine</td>
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Table 1
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Figure 4
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Figure 7
Arginase I is constitutively expressed in human granulocytes and participates in fungicidal activity

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