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Regulation of NK Cell Function by Human Granulocyte Arginase

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The arginine-hydrolyzing enzyme arginase is constitutively expressed by human polymorphonuclear granulocytes (PMN). Upon PMN cell death arginase is liberated and depletes arginine in the microenvironment. This amino acid depletion suppresses T cell proliferation and cytokine secretion and emerges as a key mechanism of immunosuppression during chronic inflammation and tumor growth. Here we show that PMN arginase also severely impairs key functions of primary human NK cells as well as IL-2-activated NK cells. In the absence of arginine, NK cell proliferation and IL-12/IL-18-induced secretion of IFN-γ are severely diminished. In contrast, NK cell viability, granule exocytosis, and cytotoxicity are independent of extracellular arginine. The mechanism of NK cell suppression by arginine depletion is posttranscriptional since mRNA transcript frequency is unaffected upon NK cell activation in the absence of arginine. Finally, we demonstrate that human purulent exudate ex vivo inhibits NK cell functions exclusively due to liberated arginase. Arginase inhibitors are therefore promising pharmacological agents to treat unwanted suppression of the innate (NK cell) as well as the adaptive (T cell) immune system. The Journal of Immunology, 2009, 182: 5259–5267.

Inflammation is often associated with immunosuppression locally within the micromilieu of the inflammatory focus as well as systemic impairment of immune functions (1). Although inflammation-induced immunosuppression has likely evolved as a homeostatic mechanism to prevent excessive tissue destruction (2), this essential feature of the mammalian immune system might be detrimental in situations of infection- and tumor-associated inflammation. On the one hand, immunosuppression during infection impedes clearance of the relevant microorganism and predisposes to novel infections (2, 3). On the other hand, malignant tumors are able to evade an effective tumor-cytotoxic immune response by inducing immunosuppressive pathways within the tumor micromilieu as a consequence of host immune system-induced selection pressure on the population of individual tumor cell clones (4). The presence and phenotype of the peri- and intratumoral inflammatory infiltrate is significantly associated with the prognosis in animal models and cancer patients (5–7). One key regulatory parameter that influences immune reactivity is the availability of the amino acid arginine (8). Arginine can be metabolized by inducible NO synthase to the cytotoxic, tumoricidal, and anti-microbial effector molecule NO. It can also be hydrolyzed to ornithine and urea by the enzyme arginase. The dominant cellular localization of arginase is different in human vs murine peripheral monocytes and granulocytes. In humans, two arginase isoenzymes (designated arginase I and II) exist that differ in cellular expression, regulation, and subcellular location (9, 10). Arginase I is constitutively expressed in granules of polymorphonuclear neutrophils (11) and is liberated during cell death in vitro as well as during granulocyte-dominated, purulent inflammation in vivo (12). The hepatic isofrom arginase I is inducibly expressed in all murine myeloid cells (13–16) and is crucially involved in various animal models of immunopathology (17–20). Arginase depletes the inflammatory micromilieu of arginine and this induces profound suppression of human T lymphocyte effector functions (12, 21–25). In humans, arginase has been demonstrated in inflammatory cells and in the inflammatory micromilieu of patients with asthma (26), renal cell carcinoma (27), lung cancer (28), and prostate carcinoma (22).

The composition of a cellular inflammatory infiltrate clearly differs depending on the circumstances (e.g., infection, tumor, autoimmunity). The early inflammatory infiltrate is normally dominated by inflaming granulocytes and NK cells (29). Granulocytes have phagocytic and microbicidal effector functions and constitute the first line of defense against invading microorganisms. NK cells secrete cytokines and chemokines, kill infected or malignant cells without prior sensitization in an Ag-independent manner via different effector pathways (perforin/granzyme, death receptors, Ab-dependent cellular cytotoxicity via Fc receptor expression), and shape the evolving innate and adaptive immune response by reciprocal interaction with APCs and T lymphocytes (30, 31). Human NK cell activation is controlled by a variety of stimulatory (e.g., NKG2D, CD16, CD244 Nkp46, Nkp44, Nkp30) or inhibitory (e.g., KIR family, CD94-NKG2A) receptors (32). Also, the cytokine microenvironment (e.g., IL-2, IL-12, IL-15, IL-18) influences the activation state of NK cells (30).

Our present study expands this concept by demonstrating that the availability of the amino acid arginine in the micromilieu significantly influences the activation state of human NK cells. We show a complex regulation of NK cell functions by arginine...
availability. Arginine depletion by polymorphonuclear granulocyte (PMN) arginase induces a complete loss of NK cell proliferation and a posttranscriptional suppression of IFN-γ secretion. In contrast, NK cells remain viable and show no defect in granule exocytosis and cytotoxicity in an arginine-free environment. Our data clarify important aspects of immune suppression by arginase-mediated arginine depletion in the context of inflammation associated with tumors, autoimmunity, and infection.

**Materials and Methods**

**Human subjects**

Human studies were approved by the Ethics Committee of the University of Heidelberg, and informed consent was obtained from all subjects.

**Reagents**

Unless stated otherwise, chemicals were purchased from Sigma-Aldrich. N^\text{ω}-hydroxy-nor-L-arginine (nor-NOHA) was from Bachem. RPMI 1640 medium without arginine and RPMI 1640 medium without tryptophan were purchased from PromoCell and supplemented with MnCl\textsubscript{2} at a physiological concentration (4 \(\mu\text{M}\)) to allow for full arginase activity.

**Isolation of human PBMC, T cells, and NK cells**

Human PBMC were purified exactly as described (11) from heparin-anticoagulated peripheral blood (for isolation of T cells) or from buffy coat preparations (for isolation of NK cells) of healthy human donors. T cells were purified from PBMC by negative selection (MACS technology; Miltenyi Biotec) and were >95\% positive for CD3. Human NK cells were separated from PBMC by negative selection (Dynal Biotech) and were >95\% positive for CD56 and <3\% positive for CD3. IL-2-activated NK cells were generated from primary NK cells as previously described (33). The cells were cultured with irradiated JY cells and 200 U/ml IL-2 for 2–4 wk until used for experiments.

**Isolation of human PMN and generation of PMN sonicates**

PMN were isolated from peripheral blood as previously described (11). The purified PMN were resuspended in PBS (4 \times 10^5 cells/ml) and sonicated for 3 min (amplitude 80) in a Vibra-Cell sonicator (Sonics & Mac- Biosciences) after 48 h and incubated for another 16 h. Cells were harvested and 51Cr release was measured in a gamma-counter (Berthold Technologies). The percentage of specific lysis was calculated as follows: (experimental release − spontaneous release)/(maximum release − spontaneous release) \times 100.

**Arginase enzymatic assay**

Arginase activity was measured in cell lysates as previously described (11). One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 \(\mu\text{mol}\) of urea per minute.

**In vitro proliferation assay and cytokine determinations**

All cell culture experiments were performed with arginine-free medium (Arg\textsuperscript{−} medium) that was supplemented with 1 mM arginine as indicated (Arg\textsuperscript{+} medium). The medium was further supplemented with 10\% dialyzed, arginine-free FCS (PAA Laboratories), penicillin/streptomycin, and 2\% glutamine. Purified NK cells or T cells (1 \times 10^5 well in 200 \(\mu\text{l}\) for each cell type) were cultured in triplicate in round-bottom 96-well plates at 37°C and 5\% CO\textsubscript{2}. For stimulation, T cells were activated with paramagnetic microspheres that are coupled with anti-CD3 and anti-CD28 (T cell ex- pander; Dynal Biotech). NK cells were stimulated with 200 U/ml IL-2 (provided by Department of Medicine 5, University Hospital Heidelberg) or with the combination of IL-12 (5 ng/ml) and IL-18 (20 ng/ml) (both from PromoCell). For cytokine determinations, supernatants of stimulation cultures were harvested after 48 h, diluted 1/3 with PBS/10\% FCS, and cytokine concentrations were measured by specific capture ELISA according to the manufacturer’s instructions (BD Biosciences). For proliferation analysis, the cells were pulsed with 1 \(\mu\text{Ci}/\text{well}\) [\(^3\text{H}\)]thymidine (Amersham Biosciences) after 48 h and incubated for another 16 h. Cells were harvested on glass fiber filters using an automatic cell harvester and the incorporation of [\(^3\text{H}\)]thymidine was measured in a microplate scintillation counter (Packard Instruments).

Real-time RT-PCR quantification

RT-PCR was performed exactly as previously described (12). Briefly, 2 \times 10^5 IL-2-activated NK cells were collected in 300 \(\mu\text{l}\) of lysis buffer from the MagNA Pure mRNA isolation kit I (Roche Diagnostics) and mRNA was isolated with the MagNA Pure LC device using the mRNA-L standard protocol. After reverse transcription a primer set specific for the sequence of human IFN-γ was used to perform PCR with the LightCycler FastStart DNA SYBR Green I Kit (Roche) according to the manufacturer’s protocol. The calculated copy numbers were normalized according to the average expression of two housekeeping genes, cyclophilin B and β-actin. Values were thus given as input adjusted copy number per microliter of cDNA.

**Apoptosis measurement**

To analyze apoptosis and cell death, 10^5 human T cells or NK cells were resuspended in saline containing 0.05\% annexin V (Roche) and 100 ng/ml propidium iodide (PI). After incubation at 4°C for 30 min the cells were analyzed by flow cytometry with a FACScan (BD Biosciences).

**NK cell degranulation and cytotoxicity**

NK cell degranulation was measured by flow cytometry as described elsewhere with slight modifications (34). Briefly, 2 \times 10^5 human NK cells and 2 \times 10^5 cells of the erythroleukemia cell line K562 (American Type Culture Collection) were mixed by gentle pipetting, spun down for 1 min at 1000 rpm, and incubated for 2 h at 37°C in 200 \(\mu\text{l}\) of the respective medium as indicated. NK cells were then stained with a FITC-labeled anti-CD56-Ab, and degranulation was detected with a PE-Cy5-labeled anti-CD107a-Ab (all Abs from BD Biosciences). After incubation for 45 min on ice, the cells were analyzed by flow cytometry (FACScan; BD Biosciences). NK cell cytotoxicity was analyzed by chromium release assay as previously described (35). Briefly, K562 cells were grown in mid-log phase in assay medium (IMDM with 10\% FCS and penicillin/streptomycin) and 5 \times 10^5 cells (volume, 100 \(\mu\text{l}\) were labeled with 0.1 \(\mu\text{Ci}/\text{ml}\) ^51Cr for 1 h at 37°C. After washing twice in Arg\textsuperscript{−} medium they were incubated with IL-2-activated NK cells in different E:T ratios in triplicates for 4 h. Spontaneous release was determined by incubating target cells in medium alone, and maximum release was determined by adding 100 \(\mu\text{l}\) of 2\% Triton X-100 to the target cell suspension. After incubation, supernatants were harvested and ^51Cr release was measured in a gamma-counter (Berthold Technologies). The percentage of specific lysis was calculated as follows: (experimental release − spontaneous release)/(maximum release − spontaneous release) \times 100.

**Immunoblot analysis**

Cells were lysed for 30 min at 4°C in lysis buffer (11), cell debris was spun down at 18,000 \(\times \text{g}\) for 10 min at 4°C, and SDS-PAGE was done as previously described (15). The proteins were transferred to a Hybond-P polyvinylidene difluoride membrane (Amersham Biosciences). After blocking with 5\% nonfat dry milk in TBST buffer for 2 h, the membranes were incubated with a polyclonal rabbit antibody to human GCN2 or anti-human phospho-GCN2 Ab (Cell Signaling Technology). Ab reactivity was moni- tored with HRP-conjugated anti-rabbit IgG (Santa Cruz Biotechnology), followed by visualization with the ECL Plus detection system (Amersham Biosciences).

**Statistical analysis**

Statistical analysis was done with InStat software (GraphPad Software). Comparison of two different parameters was done using paired Student’s t test, and comparison of four different conditions was done using paired ANOVA with Bonferroni correction.

**Results**

**Suppression of NK cell proliferation by human PMN arginase**

The early inflammatory response is dominated by PMN, followed by the recruitment of innate immune cells like dendritic cells, macrophages, and NK cells. Upon PMN cell death the azurophil granule enzyme arginase I is liberated and this leads to arginine depletion in the PMN microenvironment (12). We first wanted to study the proliferative potential of primary human NK cells in the setting of arginine depletion. Cellular activation was analyzed in cell culture medium without arginine

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3 Abbreviations used in this paper: PMN, polymorphonuclear granulocytes; Arg\textsuperscript{−} medium, arginine-free medium; Arg\textsuperscript{+} medium, medium containing 1mM arginine; 1-NMMA, N^\omega- monomethyl-L-arginine; nor-NOHA, N^\omega- hydroxy-nor-L-arginine; NOS, nitric oxide synthase; PI, propidium iodide; PMN-S, PMN sonicate; PUS-SN, cell-free supernatant of human pus; TAM, tumor-associated macrophage.
(Arg(−) medium) or in the presence of 1 mM arginine (Arg(+) medium). PMN cell death was mimicked in vitro within our published cell culture system (12). To standardize cell death, PMN were sonicated to liberate intracellular constituents followed by measurement of arginase activity in the sonicate. Mean arginase activity of PMN sonicates (PMN-S) used separately from seven different blood donors was 4106 ± 1037 mU/ml. PMN sonicates were then diluted to a defined arginase activity of 300 mU/ml in RPMI 1640 cell culture medium containing 1 mM arginine. This arginase activity is physiologically meaningful since it is equivalent to the amount of enzyme liberated upon cell death of ~5 × 10⁶ PMN/µl. The cell culture medium was preincubated for 16 h to allow for arginase-mediated arginine depletion. This medium (Arg(+) medium plus PMN-S) was then used for experiments of proliferation and cytokine secretion. When indicated, the specific arginase inhibitor nor-NOHA was added to the preincubation step to prevent arginine degradation via arginase (Arg(+) medium plus PMN-S plus nor-NOHA). Primary NK cells and IL-2-activated NK cells were stimulated with IL-2 in the different media, and proliferation was measured after 48 h by [³H]thymidine incorporation (Fig. 1A). T cells were stimulated with anti-CD3/anti-CD28-coated microbeads in parallel as controls (Fig. 1A) (12). NK cell proliferation was strongly suppressed when arginine was absent (Arg(−) medium) during cellular activation as compared with normal cell culture medium (Arg(+)) medium. Similarly, PMN arginase led to significant reduction of proliferation in comparison to Arg(+) medium in primary NK cells (reduction of 91.7 ± 8.3%, p < 0.001) as well as in IL-2-activated NK cells (71.0 ± 38.5%, p < 0.001 in 10 independent experiments). This suppression of proliferation was due to loss of arginine availability via liberated PMN arginase, since the arginase inhibitor nor-NOHA completely restored proliferation (comparison with Arg(+)) medium, p < 0.001 for primary NK cells and IL-2-activated NK cells). The impaired NK cell proliferation in the context of arginine depletion was already seen after 24 h (data not shown). Finally, we compared the influence of different arginine concentrations on T cell and NK cell proliferation (Fig. 1B) and saw a pronounced difference between both cell types in their sensitivity toward arginine depletion. While human T lymphocytes show half-maximal proliferation at an arginine concentration of 20 µM, human NK cells preserve proliferation at lower arginine concentrations and show half-maximal proliferation at an arginine concentration of 2 µM (see Fig. 1B).

**NK cell viability and early activation is unaffected by PMN arginase**

Suppression of NK cell proliferation might have been caused by a reduced viability of the cells upon arginine depletion. We therefore analyzed the frequency of apoptosis and cell death after activation of human NK cells (and T cells as controls) in the presence or absence of arginine. Cells were stimulated and cultured for 48 h as in the proliferation assays and were then stained with annexin V-FITC and PI to determine the frequency of apoptotic (annexin V-FITC⁺/PI⁻) and dead (annexin V-FITC⁻/PI⁺) cells by flow cytometry. As shown in Fig. 2A, primary NK cell viability was not affected by arginine depletion (frequency of annexin V-FITC⁺ and PI⁺ (viable) cells in Arg(−) medium, 71.4 ± 20.9%; in Arg(+) medium, 68.2 ± 18.3%; in Arg(+) medium plus PMN-S, 70.9 ± 21.2%; and in Arg(+) medium plus PMN-S plus nor-NOHA, 70.6 ± 18.6%; means of three independent experiments). Statistically, there was no difference in viability between all conditions analyzed together (p = 0.53) and in individual comparisons (Arg(−)/Arg(+), p = 0.41; Arg(+)/Arg(+) plus PMN-S, p = 0.38; Arg(+) plus PMN-S/Arg(+) plus PMN-S plus nor-NOHA, p = 0.89). Similarly, IL-2-activated NK cell and T cell viability (12) remained unchanged in the absence of arginine (data not shown).

We then analyzed the expression of CD69, an early activation marker and triggering receptor of NK cells (36). Resting NK cells do not express CD69 on the cell surface. Upon stimulation with IL-2 for 24 h, the expression of CD69 was up-regulated on primary human NK cells but there was no significant difference between arginine-free or arginine-containing medium (mean fluorescence intensity in Arg(−) medium, 23.4; mean fluorescence intensity in Arg(+) medium, 20.9; p = 0.31; seven independent experiments; see Fig. 2B). In summary, NK cell viability and early activation are unimpaired in the absence of arginine and do not account for the severe impairment of proliferation.
Depletion of arginine leads to reduced IFN-γ secretion by a posttranscriptional mechanism

One of the major NK cell functions is the secretion of the proinflammatory cytokine IFN-γ. During the early phase of inflammation NK cells are the main source of IFN-γ. This cytokine has stimulatory effects on the innate and adaptive immune system and is involved in effective antitumor immune responses (30). The combination of IL-12 and IL-18 secreted by activated macrophages and other innate immune cells stimulates NK cells to produce high amounts of IFN-γ (31). We therefore wanted to determine the ability of NK cells to secrete IFN-γ in the absence of arginine. Primary NK cells and IL-2-activated NK cells were stimulated with IL-12 (5 ng/ml) and IL-18 (20 ng/ml) in the indicated media. Supernatant was harvested after 24 h, and IFN-γ was measured by ELISA. The data shown are the means of two (primary NK cells) and six (IL-2-activated NK cells) independent experiments in triplicates. The IFN-γ secretion of NK cells stimulated in Arg(+) medium in each experiment was set to 100% to allow comparison between different donors.

FIGURE 3. Human PMN arginase reduces NK cell IFN-γ secretion by a posttranscriptional mechanism. A, Purified human NK cells and IL-2-activated NK cells were stimulated with IL-12 (5 ng/ml) and IL-18 (20 ng/ml) in the indicated media. Supernatant was harvested after 24 h, and IFN-γ was measured by ELISA. The data shown are the means of two (primary NK cells) and six (IL-2-activated NK cells) independent experiments in triplicates. The IFN-γ secretion of NK cells stimulated in Arg(+) medium in each experiment was set to 100% to allow comparison between different donors. *, p < 0.05; **, p < 0.01. B, Human IL-2-activated NK cells (2 × 10⁶) were stimulated as in A. At the indicated time points (3 and 6 h) mRNA was prepared and reverse-transcribed. The frequency of IFN-γ transcripts was quantified by LightCycler technology. The mean of three independent experiments is shown. To allow comparison between the different experiments, the amount of transcripts in Arg(+) medium after 3 h was set to 100%. No induction of IFN-γ transcripts was detected in unstimulated NK cells (data not shown).
induced pronounced transcription of the cytokine (Fig. 3B). In contrast to the significantly reduced IFN-γ protein in the supernatant (Fig. 3A), arginine depletion did not lead to a quantitative reduction of IFN-γ mRNA transcript numbers ($p = 0.59$ for 3 h and $p = 0.53$ for 6 h), suggesting a posttranscriptional mechanism of regulation.

**Arginine depletion does not impair NK cell degranulation and cytotoxicity**

We next wanted to analyze the influence of arginine on the cytotoxic function of human NK cells. NK cells store cytotoxic effector proteins (e.g., perforin and granzymes) in secretory lysosomes, which are surrounded by a lipid bilayer that contains CD107a (lysosomal-associated membrane glycoprotein 1, LAMP-1). Upon contact with microbes or tumor cells, granule lysosomal membranes fuse with the cell membrane, leading to the release of the granule content into the local microenvironment and the death of the eliciting cell. In this process CD107a is now exposed on the cell surface and can be detected by flow cytometry as a read-out for granule exocytosis (34). IL-2-activated NK cells were therefore coincubated with K562 tumor cells at an E:T-ratio of 1:1. After 2 h of incubation in medium with or without arginine, the expression of CD107a on the NK cell surface was analyzed by flow cytometry. The absence of arginine did not diminish the degranulation capacity of human IL-2-activated NK cells in three independent experiments (frequency of CD107a expressing cells: unstimulated NK cells, $1.3 \pm 0.1\%$; stimulated NK cells in Arg(−) medium, $11.2 \pm 5.4\%$; stimulated NK cells in Arg(+) medium, $11.9 \pm 5.1\%$; $p = 0.25$ for the comparison of stimulated NK cells in Arg(−) vs Arg(+) medium; see one individual experiment in Fig. 4A). Although degranulation is just one step of NK cell cytotoxic effector function, the expression of CD107a on the cell membrane correlates with NK cell killing activity (34). NK cells can efficiently kill target cells without prior stimulation and without the need for Ab recognition. After having demonstrated unimpaired granule exocytosis, we next analyzed the effect of arginine depletion on NK cell cytokotoxicity. As target cell we used the tumor cell line K562 at different E:T ratios and analyzed NK cytokotoxicity by chromium release of K562 cells. Incubation of IL-2-activated NK cells with K562 cells in Arg(+) medium and Arg(−) medium showed no difference in cytotoxic effector function of NK cells ($p = 0.27$ for the comparison of specific lysis in Arg(−) vs Arg(+) medium, summary of eight independent experiments) (Fig. 4B). When NK cells were preincubated for 20 h in medium with or without arginine and K562 cells were then added to measure cytokotoxicity, CD107a expression was also not significantly altered upon arginine depletion, and killing activity was unaffected as well (data not shown). To summarize, NK cell degranulation and cytotoxic activity are independent of arginine concentration in the microenvironment.

**NO does not participate in PMN-mediated inhibition of human NK cells**

Arginine can be metabolized by NO synthases (NOS) of different myeloid cells to NO and citrulline. Specifically, murine and human granulocyte-like tumor-induced myeloid-derived suppressor cells express the enzymes arginase and/or inducible NOS, and both enzymes have been shown to participate in the suppression of T lymphocyte functions (8). To determine whether NO is involved in NK cell suppression in the context of PMN cell death and arginase liberation, we measured nitrite, a stable reaction product of NO, in the supernatant of our NK cell cultures cocultivated with PMN-S. In six different experiments we could never detect nitrite in the context of PMN-S-induced inhibition of NK cell proliferation (stimulation with IL-2) or IFN-γ synthesis (stimulation with IL-12 and IL-18) (data not shown). We also directly compared pharmacological arginase inhibition (by nor-NOHA) and NOS inhibition (by $N^\omega$-monomethyl-L-arginine, L-NMMA, 500 μM) in the NK cell activation experiments in the context of granulocyte sonicate (PMN-S) (Fig. 5). Arginase inhibition with nor-NOHA completely reconstituted NK cell proliferation (Fig. 5A; see also Fig. 1A) and IFN-γ synthesis ($p < 0.05$ for the comparison of Arg(+) plus PMN-S plus nor-NOHA vs Arg(+) plus PMN-S in the three individual experiments for both proliferation and cytokine synthesis shown in Fig. 5; see also Fig. 3A). In contrast, inhibition of NOS by L-NMMA had no significant influence on the profound suppression of both T cell functions (comparison of Arg(+) plus PMN-S plus L-NMMA vs Arg(+) plus PMN-S in three individual experiments; $p = 0.8$ for proliferation and $p = 0.6$ for IFN-γ synthesis; Fig. 5). In summary, these data argue against an involvement of NOS and NO in the PMN arginase-induced NK cell suppression.

**The absence of arginine does not lead to phosphorylation of the eukaryotic amino acid starvation detector system GCN2 in human NK cells**

Lower eukaryotes have developed specific mechanisms to cope with biochemical stress such as amino acid starvation. This leads to an increase of unbound tRNA, which induces phosphorylation and activation of the kinase GCN2. Activated GCN2 induces phosphorylation of eIF2α (eukaryotic translation initiation factor 2α),
which inhibits protein translation via blocking of eIF2B (8). Mammalian cells express a GCN2 homolog, and phosphorylation of GCN2 mediates the down-regulation of murine CD8 T cell proliferation upon tryptophan depletion (37) and is detectable in human T lymphocytes upon TCR-mediated activation (M. Bhairo and M. Munder, manuscript in preparation). We therefore analyzed the possible involvement of GCN2 in the context of arginine deficiency-mediated suppression of human NK cells. Using human IL-2-activated NK cells stimulated with IL-2 (200 U/ml) and tryptophan-depleted media (final concentration, 1 mM), we never saw phosphorylation of GCN2 upon activation of human NK cells (Fig. 6) in four independent experiments. These results are compatible with a mechanism of human NK cell suppression upon arginine depletion that is independent of GCN2 phosphorylation.

**FIGURE 5.** NO synthase is not involved in PMN arginase-mediated NK cell suppression. A. Freshly isolated human NK cells were stimulated with IL-2 (200 U/ml) in the presence of arginine (Arg(+), concentration 1 mM). Alternatively, Arg(+)-medium was preincubated for 16 h with an aliquot of human PMN-S, corresponding to a final arginase activity of 300 mU/ml, in the presence or absence of the specific arginase inhibitor nor-NOHA (1 mM) or the specific NOS inhibitor L-NMMA (500 μM). NK cells were then stimulated in these different media and proliferation was assessed by [3H]thymidine incorporation after 48 h for additional 16 h. B. NK cells were stimulated with IL-12 (5 ng/ml) and IL-18 (20 ng/ml) in the indicated media (as in A). Supernatant was harvested after 24 h, and IFN-γ was measured by ELISA. The data shown are representative of three different experiments performed in triplicate.

**FIGURE 6.** Phosphorylation of GCN2 does not correlate with suppression of NK cell proliferation upon arginine depletion. IL-2-activated NK cells were stimulated with IL-2 for 24 h in the indicated media (or frozen at the beginning of the experiment) and were then lysed in SDS-PAGE lysis buffer. After SDS-PAGE and Western blotting, expression of phosphorylated GCN2 (p-GCN2) and GCN2 (molecular mass, 220 kDa) was detected by specific Abs. One representative experiment of four independently performed experiments is demonstrated.
Amino acid specificity of NK cell suppression: no influence of tryptophan as opposed to arginine deficiency

Tryptophan deficiency due to induced IDO was shown to exert important immunosuppressive functions in the murine immune system (37). We therefore wanted to analyze if human NK cell suppression due to arginine depletion can be recapitulated also by tryptophan deficiency. Primary human NK cells were activated with IL-2 in arginine- or tryptophan-deficient media and proliferation was measured after 48 h by \[^{3}H\]thymidine incorporation. The data shown are from one representative independently performed experiment (of three total experiments).

NK cell suppression due to purulent inflammation

Having demonstrated differential regulation of human NK cell function by extracellular PMN arginase I, we wanted to analyze NK cell behavior within an in vivo-generated prototypical PMN-dominated inflammatory milieu. PMN-mediated inflammation in vivo leads to the generation of pus as a correlate of massive PMN cell death and liberation of intracellular molecules. We therefore prepared cell-free supernatant of pus (PUS-SN) derived from surgical abscess drainage. Within this PUS-SN, arginase I protein as well as high arginase activity are detectable (12). This arginase activity within the human PUS-SN (ex vivo) was 42,677 mU/ml, >100 times higher than the activity (300 mU/ml) that reproducibly leads to complete arginine depletion and suppression of T cell (12) and NK cell (Fig. 1) proliferation in vitro. To test the functional potential of PMN-derived arginase I from human pus, we preincubated Arg(+) medium with PUS-SN (with or without nor-NOHA), diluted to a final arginase activity of 300 mU/ml. Human NK cells were then activated in the respective media by IL-2, and proliferation was assessed after 48 h. IL-2-activated NK cells were also stimulated with IL-12 and IL-18 to measure IFN-\(\gamma\) secretion by ELISA. When the cells were activated in medium preincubated with PUS-SN, NK cell proliferation (Fig. 8A) and IFN-\(\gamma\) secretion (Fig. 8B) were diminished significantly (proliferation, \(p < 0.001\) for Arg(-)/Arg(+) or Arg(+) plus PMN-S; IFN-\(\gamma\) synthesis, \(p < 0.05\) for Arg(-)/Arg(+) or Arg(+)/Arg(+)) plus PMN-S; summary of three independent experiments). This suppression was due to liberated PMN arginase within the PUS-SN since proliferation and cytokine secretion could be completely rescued by inhibition of arginase within PUS-SN by nor-NOHA from the start of the preincubation (\(p < 0.001\) for Arg(+) plus PMN-S/Arg(+)) plus PMN-S plus nor-NOHA for both proliferation and IFN-\(\gamma\) synthesis; summary of three independent experiments).

These results demonstrate that NK cell suppression via arginase-mediated arginine depletion is a mechanism operating physiologically within the human purulent inflammatory milieu.

Discussion

Human PMN arginase constitutes a homeostatic immunoregulatory mechanism that limits excessive immune activation. In a PMN-dominated inflammatory milieu like human pus, arginase I is liberated. This leads to local arginine depletion and consecutive inhibition of proliferation and cytokine secretion by invading T cells (12) and NK cells (this paper). Upon PMN cell death, mimicked in the present study by sonication, a variety of potentially toxic products and enzymes are liberated into the extracellular milieu (1). Nevertheless, arginase accounts for the impairment of NK cell functions, since they are completely restored with the specific arginase inhibitor nor-NOHA. Our analysis also clearly shows that arginase deficiency does not globally suppress all aspects of NK cell activation. While certain functions (transcription of cytokine mRNA, CD69 expression, granule exocytosis, and cellular cytotoxicity) are completely preserved in the absence of arginase, other NK cell effector functions (cytokine secretion, proliferation) are severely compromised in an arginine-depleted micromilieu. We used a physiologically relevant arginase activity for our in vitro experiments (equivalent to \(~5 \times 10^6\) PMN/ml). Arginase-regulated NK cell activity is further complicated since PMN cell death in vivo likely generates an arginase concentration gradient around the epicenter of inflammation. In human purulent inflammation, arginase activity in vivo is >100 times higher than the activity needed in vitro to shut down T cell effector functions completely (12). Arginine concentration within an inflamed tissue is also likely to be regulated dynamically by blood supply, venous and lymphatic drainage, as well as cellular metabolism. Finally, different cells of the immune system shut down identical effector functions at differing arginine concentrations (Fig. 1B). NK and T cell reactivity in vivo is consecutively regulated depending on the location of the cells within the micromilieu and the differing arginine concentrations that are present during cellular activation.

How does arginine depletion induce suppression of NK cell functions? The suppression of proliferation of murine CD8\(^+\) T cells upon tryptophan depletion (due to IDO) is mediated by phosphorylation and activation of the eukaryotic stress response kinase

**FIGURE 8.** Arginase within human pus ex vivo suppresses NK cell proliferation and secretion of IFN-\(\gamma\). Cell-free supernatant was prepared from human pus (PUS-SN) derived from a parastomal skin abscess of a patient with Crohn disease. Arginase activity was measured (42,677 mU/ml), pus was diluted to a final arginase activity of 300 mU/ml in Arg(+) medium, and the medium was incubated for 16 h. When indicated, nor-NOHA was added from the start of the incubation to inhibit arginase activity within PUS-SN. A, Human IL-2-activated NK cells were stimulated with 200 U/ml IL-2 in the respective media, and proliferation was assessed after 48 h by \[^{3}H\]thymidine incorporation. The data shown are from one representative independently performed experiment (of two total experiments). B, Human IL-2-activated NK cells were stimulated with IL-12 and IL-18 for 48 h, and IFN-\(\gamma\) was measured in the supernatants by ELISA. One representative experiment is shown (of two total experiments).
GCN2 (37). While human T lymphocytes also respond to arginine starvation with GCN2 phosphorylation (M. Bhalroa and M. Munder, manuscript in preparation), this amino acid sensing pathway is not detectable in human NK cells upon IL-2 stimulation in the absence of arginine or tryptophan (Figs. 6 and 7). In contrast, human NK cell activation is specifically impaired only by arginine deficiency and not by tryptophan depletion (Fig. 7), and this fundamental functional difference does not correlate with differences in GCN2 phosphorylation in human NK cells. We therefore found no evidence that GCN2 phosphorylation is involved in the response of human NK cells to cellular stress due to amino acid deprivation. It remains to be analyzed if this phosphorylated GCN2 phenotypic difference (as compared with murine and human T lymphocytes) reflects cell-type, nutrient-specific, or species-specific differences in the response to exogenous stress.

Our findings of arginase-associated NK cell inhibition have significant implications for the role of NK cells in cancer. Tumor growth is supported by myeloid-derived suppressor cells within the tumor micromilieu. Murine GR-1+CD11b+ as well as human tumor-induced myeloid-derived suppressor cells express the enzymes arginase and/or inducible NO synthase, metabolize arginine and/or synthesize the reactive oxygen product peroxynitrite, and suppress T lymphocyte proliferation and effector functions (38). In a murine mammary adenocarcinoma tumor model, myeloid-derived suppressor cells expand and suppress NK cell cytotoxicity in a cell contact-dependent manner, involving the inhibition of perforin synthesis and an interference with Stat5 signaling (39). NK cell interaction with different arginase-expressing myeloid cell types has already been described during an evolving murine antitumor immune response. The phenotype of murine tumor-associate macrophages (TAM) dictates the influx and tumor-cytotoxic potential of NK cells. While classically activated TAM secrete IL-12, which leads to NK cell influx and activation with consecutive tumor lysis, alternatively activated, arginase-expressing TAM are associated with unimpaired tumor growth (40). Upon NK depletion, murine arginase-expressing TAM cells predominate, impair CD8+ CTL activity, and thereby promote tumor growth (41). Murine splenic NK cell cytotoxicity in a myeloma tumor model is also impaired by i.v. arginase injection, correlating with a shorter survival of the mice (42). Based on the data in our present study, we hypothesize that arginase within the tumor microenvironment, either myeloid cell-associated (28), passively liberated during PMN cell death (12), or by active secretion (43), suppresses the human antitumor NK cell response by inhibition of NK cell IFN-γ secretion and proliferation.

Our demonstration of unimpaired NK cell-mediated cytotoxicity in the absence of arginine (Fig. 4B) is in contrast to earlier studies that showed partial inhibition of NK cell-mediated killing upon arginine depletion (42, 44). Similarly, cytotoxicity of naive or PHA-activated splenocytes was inhibited in arginase-preincubated medium (45). The discrepant results might be due to differences in the source (spleen vs blood) and purity (unpurified vs highly purified), species (murine vs human), and the level of cytotoxicity (much higher in our present study) of the analyzed human NK cells. The unimpaired NK cell cytotoxicity in the context of complete arginine deficiency ensures efficient local microbial defense early in an inflammatory focus while uncontrolled NK cell proliferation and cytokine secretion in inflamed peripheral tissues are suppressed. PMN arginase also does not affect NK cell viability (Fig. 2A), and suppressed NK functions can be reconstituted upon activation of the cells in arginine-supplemented conditions (data not shown). This was similarly demonstrated for NK cell suppression by IDO-generated tryptophan metabolites (46). The arginase-induced NK cell hyporesponsiveness is therefore likely a functional defect amenable to therapeutic intervention in vivo. While specific arginase inhibitors are not yet available for pharmacological use in humans, several studies have demonstrated a positive effect of arginine supplementation as immunonutrition to support immune function (47). In small patient cohorts, arginine supplementation led to increased in vitro NK cell functions in healthy volunteers (48), after pneumococcal vaccination (49), and in breast cancer patients (50).

In summary, we have shown a selective inhibition of certain NK cell functions within a proinflammatory environment. Human PMN have the potential to suppress NK cell proliferation and cytokine secretion via liberation of arginase I and consecutive arginine depletion. In contrast, NK cell viability, granule release, and cytotoxicity are independent of the availability of arginine in the microenvironment. Our findings highlight the complexity of immune regulation in the context of inflammatory reactions and add further impetus to the development of arginase inhibitors for treatment of patients with inflammation-associated immunosuppression in general and specifically in the context of cancer.

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Disclosures

The authors have no financial conflicts of interest.

References


