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Giardia duodenalis arginine deiminase modulates the phenotype and cytokine secretion of human dendritic cells by depletion of arginine and formation of ammonia[1]

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Running title: *Giardia ADI enzyme modulates human DC response*

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Abstract

Depleting arginine is a recognized strategy of pathogens to evade immune effector mechanisms. Depletion depends on microbial enzymes such as arginases which are considered as virulence factors. The effect is mostly interpreted as being a consequence of successful competition with host enzymes for the substrate. However, both arginases and arginine deiminases (ADI) have been associated with pathogen virulence. Both deplete arginine but their reaction products differ. An ADI has been implicated in the virulence of *Giardia duodenalis,* an intestinal parasite that infects humans and animals causing significant morbidity. Dendritic cells (DC) play a critical role in host defense, also in a murine *G. duodenalis* infection model. Functional properties of these innate immune cells depend on the milieu in which they are activated. Here, the dependency of the response of these cells on arginine was studied using Giardia ADI and LPS-stimulated human monocyte-derived DC. Arginine depletion by ADI significantly increased TNF-α and decreased IL-10 and IL-12p40 secretion. It also reduced up-regulation of surface CD83 and CD86 molecules that are involved in cell-cell interactions. Arginine depletion also reduced phosphorylation of S6K in DC suggesting the involvement of the mTOR signaling pathway. The changes were due to arginine depletion and reaction product formation, in particular ammonium ions. Comparison of NH_4^+ and urea revealed distinct immunomodulatory activities for these products of deiminases and arginases, respectively. The data suggest that a better understanding of the role of arginine-depleting pathogen enzymes for immune evasion will have to take enzyme class and reaction products into consideration.

Introduction

Many pathogens are thought to compete with the host for arginine as part of their virulence mechanisms. This is best known for pathogens expressing arginases that compete for arginine with host nitric oxide synthases and thereby are considered to prevent anti-microbial nitric oxide (NO) formation (1, 2). However, also other arginine-metabolizing enzymes have been implicated in microbial virulence, in particular arginine deiminases (ADI). The latter enzymes are thought to be relevant in streptococcal infections (3, 4) and infections with the non-invasive, gastrointestinal protozoan parasite *Giardia duodenalis* (5, 6), a medically significant cause of diarrheal syndromes and malabsorption (7, 8).

Arginine is not only necessary for generation of NO but it plays also other important roles during immune response. Lack of arginine was shown to inhibit T-cell function (9), and arginine levels affect signaling via the mammalian target of rapamycin (mTOR) pathway as reported for other cells (10, 11). The mTOR pathway, in turn, was shown to contribute to the regulation of co-stimulatory surface marker levels on dendritic cells (DC; (1, 12, 13)). These cells play a crucial role during induction of an adaptive immune response by secreting cytokines and by cell-contact dependent interactions with T- and other immune cells. Although DC are important for adaptive immunity against microbial infections, the effect of pathogen-mediated arginine-depletion on their function is not known.

Arginine-dependent virulence mechanisms of pathogens rely on enzymes such as arginases or deiminases that deplete arginine but also generate reaction products at the same time. Of note, these products differ between these classes of enzymes that generate ornithine and urea or citrulline and NH_4^+, respectively. Commonly, changes of immune cell responses due to different arginine levels have been studied by comparing responses in the presence or absence of arginine. However, this does not reflect the situation when arginine is depleted by an enzymatic reaction as it can be the case during infections. Yet, the combined effect of arginine

depletion by an enzymatic reaction and the ensuing product formation on immune cells has largely been ignored.

Referring to *G. duodenalis* as a relevant model, we studied here the immunomodulatory effects of arginine-depletion by exposing human monocyte-derived DC to recombinant *G. duodenalis* ADI during DC activation with LPS. The effect of this treatment on IL-10, IL-12p40, and TNF-α secretion as well as cell surface expression of CD83 and CD86 was monitored. We show that both, arginine-depletion and NH_4^+ formation by the active parasite enzyme are immunomodulatory on monocyte-derived dendritic cells (moDC) causing an increase in TNF-α, as opposed to a decrease in IL-10 and IL12p40 production and a reduction of surface located CD86 and CD83. In particular the latter effect correlated with an inhibition of the mTOR pathway since phosphorylation of the mTOR S6K target protein was decreased. We furthermore show that NH_4^+ but not urea exacerbated the inhibition of IL-10 production and surface marker up-regulation compared with arginine depletion alone, suggesting a difference between the immunomodulatory activities of the products of arginases and deiminases.

Material and methods

Cell culture

DC were cultured in RPMI 1640 (Sigma, Germany) supplemented with 10% FCS (Biochrom, Germany) or arginine-free RPMI (PanBiotech, Germany) supplemented with 10% dialyzed FCS (Biochrom). Both media contained 10 mM Hepes (Biochrom), penicillin (100 U/mL) - streptomycin (100 µg/mL) (PAA, Germany) and 50 µM 2-mercaptoethanol (Roth, Germany).

G. duodenalis trophozoites, strain WB-C6 (ATCC 50803), were propagated in TYI-S-33 medium as previously described (14). Genomic DNA was isolated with the DNeasy Blood & Tissue kit (Qiagen, Germany).

Generation of recombinant proteins

The ADI-coding sequence (*Gd*-adi) was amplified from genomic DNA of *G. duodenalis* strain WB-C6 by PCR using *Pfu* polymerase (Fermentas, Germany) and specific primers (5'-aatgactgacttctccaaggataaaga-'3 and 5'- tccctcacttgatatcgacgcagatgtca-'3). The resulting 1.8 kb fragment was cloned into the expression vector pASG-IBA35 (StarGate cloning, IBA GmbH, Germany) according to the manufacturer's manual to produce a recombinant protein with an N-terminal His_6-tag. After transformation into *E. coli* DH5αZ1 cells (15), the His_6-tagged ADI (pASG-IBA35_ADI) was purified from cultures grown in LB medium supplemented with 100 µg/mL ampicillin (Roth) and 50 µg/mL spectinomycin (Sigma) overnight at 37°C without induction by anhydrotetracycline. Cells were harvested by centrifugation (8200 x *g*, 10 min, 4°C) and then washed in ice-cold PBS. The pellet was resuspended in 25 mM Hepes

(Fluka, Germany), 150 mM NaCl (Merck, Germany), 5 mM imidazole (Merck) plus EDTA-free protease inhibitor cocktail (Roche, Germany), pH 7.5 (Buffer A) and disrupted using a high pressure homogenizer (EmulsiFlex, Avestin, Germany). After centrifugation (15000 x *g*, 30 min, 4°C), the supernatant was passed through a 0.45 µm pore size filter (Sartorius, Germany) and loaded onto a HisTrap^{TM}FF column (GE Healthcare, Germany) pre-equilibrated with Buffer A on an ÄktaFPLC system (GE Healthcare). Finally, protein was eluted in 25 mM Hepes, 150 mM NaCl, and 200 mM imidazole, pH 7.5 (Buffer B). Finally, imidazole was removed by desalting using a PD-10 column (GE Healthcare) and residual LPS by an EndoTrap® kit (Hyglos, Germany). Recombinant protein in 1x PBS was finally concentrated using a Vivaspin concentrator (5 kDa PES membrane, Sartorius). To obtain a catalytically inactive arginine deiminase mutant (ADI_{C424A}), cysteine424 was changed to alanine using the QuikChange® mutagenesis kit (Stratagene). Site-directed mutagenesis PCR was performed using the primers 5'-gtacggctctctgcacgccgcatctcaggttgtt-'3 and 5'-aacaacctgagatgcggcgtgcagagagccgtac-'3 and, the vector pASG-IBA35_ADI as template. Recombinant ADI_{C424A} was expressed and purified as described for the wild-type enzyme. Purity of both recombinant proteins was controlled by SDS-PAGE and verified using Western blot. Protein concentration was determined using a BCA protein assay kit (Thermo Scientific Fisher, Germany). Enzymatic activity of recombinant ADI was measured by colorimetric determination of citrulline formation as described (16).

Production of polyclonal antibody

An alpaca was immunized four times (day 0, 21, 35, 49; Preclinics, Potsdam, Germany) with 300 µg of purified recombinant ADI resuspended in 100 µL PBS plus adjuvant (complete Freund's adjuvant for the 1st injection and incomplete Freund's adjuvant for subsequent booster injections). Pre-immune serum was collected prior to the first immunization. After the last booster injection, the antiserum was obtained.

For Western blot analysis the alpaca antiserum was diluted 1:2500 and the secondary horseradish-peroxidase-conjugated goat anti-llama IgG antibody (Bethyl Laboratories, USA) 1:20000, both in 5% non-fat dried milk (Roth), 1xPBS, 0.05% Tween20 (Roth).

Generation of human moDC

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of healthy volunteers (German Red Cross, Berlin, Germany) by density gradient centrifugation (Ficoll-Paque^{TM} Plus, GE Healthcare). Monocytes were separated by magnetic cell sorting using CD14 MicroBeads (Miltenyi Biotec, Germany). Typically, cells were thereby enriched to ≥90% CD14^{+} as determined by flow cytometry. To generate moDC, 3.0x10^{6} monocytes were seeded into 6-well tissue culture plates. DC growth medium additionally contained 1000U/mL human rGM-CSF (Bayer, USA) and 10 ng/mL human rIL-4 (R&D Systems, Germany). After 6 days, immature DC were harvested and the cell population was characterized by analyzing an aliquot by flow cytometry. Staining of surface markers revealed low levels of CD14 and CD86 and high levels of HLA-DR, whereas CD25 and CD83 were not expressed.

DC stimulation and analysis of cytokine production and cell surface markers

$5x10^5$ immature DC/mL were seeded into a 12-well tissue culture plate and cultured at 37°C either in DC growth medium or in arginine-free DC growth medium supplemented or not with 2 mM of arginine (Merck), or citrulline (Sigma), and/or ammonium chloride (Sigma), respectively. Cells were exposed to different concentrations of recombinant ADI or ADI_{C424A} and 1 µg LPS/mL (*Escherichia coli* 026:B6 from Sigma) was added at the same time to trigger DC maturation. Cell-culture supernatants were collected after 24 h and were frozen at -80°C until determination of TNF-α, IL-10, IL-12p70 (all from eBioscience, Germany), IL-23 (U-CyTech, Netherlands), and IL-12p40 (Biolegend, Germany) by ELISA following manufacturers' protocols. DC were stained with anti-human FITC-CD14 (Immunotools), PerCP-CD86 (Abcam), PE-HLA-DR (Immunotools), AlexaFluor488-CD83 (Biolegend), or Dyomics647-CD25 (Immunotools) conjugated mAb or the relevant isotype controls (Immunotools). Data were acquired on a LSR II flow cytometer (Becton Dickinson, Germany) and analyzed using FlowJo software (Tree Star, Inc.). Dead cells were excluded by gating according to FSC/SSC characteristics.

Western blot analysis

One million immature DC/mL were seeded into a 12-well tissue culture plate in arginine-free DC medium containing rGM-CSF and rIL-4, further supplemented or not with 2 mM of arginine, citrulline and/or ammonium chloride. After 30 min of resting, cells were treated with 2 µM rapamycin (Sigma), recombinant ADI or ADI_{C424A} and further cultured at 37°C for 90 min. The immature cells were then exposed to 1 µg/mL LPS for 30 min. Cells were harvested, centrifuged at 300 x *g* at 4°C for 10 min, and supernatants were then collected for citrulline detection. Cell pellets were washed twice in ice-cold PBS before resuspension in

lysis buffer [10 mM Tris-HCl (Roth) pH 7.2, 150 mM NaCl (Merck), 1% Triton X-100 (Merck), 1% sodium deoxycholate (Sigma), EDTA-free protease inhibitor cocktail (Roche)]. To remove cell debris, samples were centrifuged for 5 min at 14000 x *g* at 4°C. Protein concentration of the supernatant was determined by using BCA protein assay (Thermo Scientific Fisher, Germany) according to the manufacturers' protocol. A total of 50 µg protein was separated by SDS-PAGE and transferred to nitrocellulose membranes (Biorad, Germany). Membranes were blocked for 1 h in 5% non-fat dried milk (Roth), 1xTBS, 0.1% Tween20 (Roth) and incubated over night at 4°C with primary antibodies (Cell signaling, Germany) against p70-S6K (#9202), phospho-p70-S6K (#9205), E4-BP1 (#9452), phospho-E4-BP1 (#9455) and β-actin (#4967) diluted 1:1000 in 5% BSA (Roth), 1xTBS, 0.1% Tween20. Bound antibodies were detected with horseradish peroxidase-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, USA) diluted 1:2000 in 5% BSA, 1xTBS, 0.1% Tween20 and visualized on X-ray films (GE Healthcare) using ECL Plus Western Blotting Detection System (GE Healthcare). Quantification of Western blot signals was performed with ImageJ 1.42q software (NIH, USA).

Descriptive statistics

Data are given as mean ± SD. Statistical significance was assessed by paired t-test (two-tailed). All analyses were performed using GraphPad Prism 5 software (GraphPad Software, Inc., USA) at the level of $p < 0.05$.

Results

Defining experimental conditions for arginine-depletion by recombinant, enzymatically active G. duodenalis ADI during DC activation in vitro

The average daily intake of arginine by humans has been estimated to be 5 g of arginine, corresponding to 28 mmol, (17). It has been reported that an infected person sheds up to 10^9 *G. duodenalis* organisms per day (18). We first aimed to estimate the effect of ADI activity on the amount of arginine that may be available to *G. duodenalis* during an infection. Thus, we determined the ADI activity per million trophozoites to be equivalent to approx. 0.02 U by preparing lysates from different isolates and determining their ADI activity (data not shown). Therefore, at least 20 units of enzyme may be produced per day of infection. One unit corresponds to 1 µmol arginine metabolized per minute, hence >28 mmol could potentially be turned over in one day. Thus, the infection with *Gd* has the potential to substantially deplete arginine in the gastrointestinal tract.

We cloned the adi gene present in strain WB-C6 and produced the respective recombinant enzyme as well as an enzymatically inactive mutant ADI_{C424A} (19), in which Cys at residue 424 was replaced by Ala, as hexahistidine-tagged proteins in *E. coli*. Analysis by SDS-PAGE and Western blot of the two affinity-purified proteins demonstrated that the recombinant proteins were highly pure and behaved similar to the endogenous ADI parasite protein of 66 kDa (Fig. 1). That was also described by others, but unlike we could not confirm the previously described higher molecular weight of 85 kDa for native *Gd* ADI (20). The purified wild type protein had an ADI activity of 6.8 U/mg.

To achieve arginine-depletion by recombinant ADI in a standard 24 h DC stimulation assay enabling to study the consequences of arginine-depletion on DC, we calculated that 4 µg of active recombinant ADI will convert all free arginine in 43 min (culture volumes of 1 ml

RPMI containing 1.15 mM arginine), 10-fold less enzyme will achieve this in approx. 7 h and 100-fold less enzyme would need nearly 72 h assuming a constant activity over time. Thus, we choose these amounts of the active enzyme to study dose dependence in the following DC activation experiments and used the inactive mutant to control for possible effects unrelated to enzyme activity.

Enzymatic arginine depletion by ADI modifies pro- and anti-inflammatory cytokine secretion of LPS-activated moDC

In the intestine, DC project cellular extensions between epithelial cells (IEC) into the lumen of the gut to sample antigens (21, 22). Through these projections they are thought to recognize microbe-associated molecular patterns by pattern recognition receptors and become stimulated to mature and migrate to peripheral lymph nodes where they initiate antigen-specific immunity (23, 24). Maturing DC up-regulate cell surface markers (e.g. MHC, CD83, CD86) and release cytokines (e.g. IL-12, IL-10, TNF-α) to enable communication with and activation of T- and other immune cells (25). To model such conditions and investigate potential immunomodulatory effects of arginine limitation on DC function, we used human moDC that can be readily prepared in the necessary quantities, stimulated them with LPS, and exposed the cells to ADI. Citrulline concentrations in the culture supernatants were determined at the end of the 24 h assay period and confirmed the calculated effects of the ADI dilutions (see above and Fig. 2A) on arginine turn-over. As expected, addition of ADI_{C424A} did not result in citrulline formation at all tested concentrations (Fig. 2A and data not shown). Cytokine concentrations in these supernatants were then determined by ELISA (Fig. 2B). LPS-activation of cells stimulated roughly a 100-fold increase in IL-12p40, TNF-α and IL-10 in arginine-replete medium compared to non-activated controls (data not shown). In contrast, LPS-stimulated moDC exposed to 4 µg/mL of ADI produced significantly less IL-10 and IL-

12p40. These values were 45% ($P < 0.001$, n = 7) and 17% ($P < 0.01$, n = 6) lower compared to control cells exposed to mutant ADI_{C424A}, respectively. In contrast, TNF-α secretion by ADI-treated and LPS-activated moDC was increased and values were on average 74% ($P < 0.01$, n = 6) higher then values of LPS-stimulated, mutant enzyme exposed control cells. Both, the decreased IL-10 and IL-12p40 and the increased TNF-α production, depended on ADI activity, indicating that the kinetics of arginine-depletion did matter. These data show that arginine depletion by ADI modulates cytokine secretion of LPS-activated moDC and, importantly, that the effects differ depending on the cytokine analyzed.

Enzymatic arginine depletion by ADI reduces upregulation of surface CD83 and CD86 levels of LPS-activated moDC

To investigate the influence of arginine depletion by ADI on the phenotype of maturing DC, moDC were stimulated with LPS and treated with ADI or its mutant ADI_{C424A} as described above and analyzed for selected surface marker proteins levels by flow cytometry. Activation of immature moDC with LPS induced up-regulation of CD83 and CD86 (data not shown) as expected. In contrast, LPS-stimulated moDC treated with arginine-depleting levels of ADI expressed significantly less CD83 and CD86 than control cells. Surface CD83 was on average 22% ($P < 0.05$, n = 3) and that of CD86 15% ($P < 0.01$, n = 4) lower than on LPS-treated, mutant enzyme-exposed control cells. The effects were again ADI dose dependent (Fig. 3B). The reduced up-regulation of surface CD83 and CD86 unlikely reflects a general effect on surface protein levels since HLA-DR abundance on LPS-activated moDC was not affected by ADI (data not shown).

ADI immunomodulatory effects on LPS-activated moDC result from both arginine depletion and product formation

We asked next whether the modulatory effects of ADI on moDC functional phenotype resulted from arginine depletion and/or the formation of ADI reaction products citrulline and/or NH_4^+. To investigate possible cause effect relationships, moDC were generated as before, harvested and then seeded into arginine-free culture medium that was only subsequently supplemented with arginine, citrulline, and/or ammonium chloride, respectively. Cells were then treated as before with 4 µg of ADI or ADI_{C424A} and were activated with LPS. After 24 h of incubation, supernatants were collected and citrulline content and cytokine concentration were determined. Furthermore, moDC were harvested and cell surface markers were analyzed.

IL-10 and TNF-α secretion of cells grown in arginine-replete media were modulated as described above, i.e. ADI-treated LPS-activated moDC produced less IL-10 and more TNF-α than control cells (Fig. 4). MoDC stimulated in the absence of arginine showed a small decrease in IL-10 production but levels remained significantly higher than those of cells treated with ADI in the presence of arginine where citrulline and NH_4^+ could be formed (Fig. 4). The addition of the ADI products, in particular of NH_4^+ in the form of NH_4Cl, reduced IL-10 levels to those observed in the presence of arginine and ADI. Similarly, CD83 and CD86 surface levels were also reduced, although this did not reach statistical significance for the latter marker (data not shown). In contrast, increase in TNF-α production was mainly driven by arginine depletion (Fig. 4) and not affected by NH_4^+. The increase due to arginine depletion was reduced by citrulline, indicating that citrulline could substitute for arginine in this respect (Fig. 4). Supplementing DC with NH_4^+ during stimulation in the presence of arginine but with no ADI present also reduced IL-10 but had no effect on TNF-α production (Fig. 5).

Thus, the immunomodulation of moDC by ADI resulted from a combination of distinct effects of arginine depletion and/or citrulline and NH_4^+ product formation.

NH_4^+ and urea, the reaction products of ADI and arginases, differ in their effect on cytokine secretion and surface marker profile of LPS-stimulated moDC

As mentioned before, many pathogens are thought to evade NO-mediated immune clearance through arginine depletion by arginases, which will result in the formation of ornithine and urea (2). Immunomodulatory effects may thus be different from conditions where deiminases are relevant. We were therefore interested in comparing the immunomodulatory effects of NH_4^+ and urea. LPS-stimulated cells were incubated in medium devoid of arginine but supplemented with ammonium chloride or urea for 24 h and then supernatants were collected for the detection of IL-10 and TNF-α by ELISA (Fig. 6A) and CD83 and CD86 proteins by flow cytometry (Fig. 6B). Notably, cells stimulated in medium supplemented with urea produced significantly more IL-10 and displayed significantly higher levels of CD83 and CD86 surface proteins than cells stimulated in the presence of NH_4^+. In contrast and corroborating our result that TNF-α production was not affected by NH_4^+ (Fig. 4), no differences were noted between cells treated with NH_4^+ or urea with respect to TNF-α release.

Arginine turn-over by ADI results in decreased phosphorylation of the mTOR-signaling pathway target S6K in LPS-activated moDC

Previous studies suggested an inhibitory effect of branched-chain amino acids on the mTOR/S6K-signaling pathway, resulting in impaired maturation of moDC and particularly affecting CD83 expression (26). mTOR is a serine/threonine kinase that is present in two distinct protein complexes, mTORC1 and mTORC2. Activation of mTORC1 leads to

phosphorylation of the protein S6 kinase (S6K) and the eukaryotic initiation factor 4E (eIF-4E) binding protein 1 (4E-BP1) that both are involved in the regulation of protein translation (27). Arginine levels have been shown to affect mTOR signaling in T-cells (10, 11). Since ADI by arginine depletion had immunomodulatory effects on moDC, we hypothesized that this may also involve the mTOR pathway in moDC. MoDC were seeded in arginine-free medium, then supplemented or not with arginine. The cells were treated with recombinant ADI, catalytically inactive ADI_{C424A} or, as a positive control, with the mTOR inhibitor rapamycin and were activated with LPS. Cells were incubated for 24 h before determining CD83 surface levels (Fig. 7A). In the presence of arginine CD83 levels were lower on cells treated with ADI than on cells exposed to the mutant enzyme, as shown before. MoDC stimulated in the absence of arginine or in the presence of arginine and rapamycin also showed reduced levels of CD83 protein (Fig. 7A).

To investigate whether the reduced CD83 surface levels caused by ADI activity correlated with mTOR signaling, treated cells were harvested 30 min after LPS stimulation to assess mTOR-dependent phosphorylation events. Cells were lysed and equal amounts of total protein were separated by SDS-PAGE. The abundance of phosphorylated protein of the mTOR target S6K was then determined by Western blot analysis and results were quantified. Suppression of S6K phosphorylation in moDC correlated with reduced CD83 surface protein levels and this depended on arginine levels (Fig. 7B). Control cells treated with rapamycin showed the expected suppression of S6K phosphorylation. Phosphorylation of 4E-BP1, however, did not depend on arginine availability (data not shown).

These data suggest that arginine levels, similar to what has been shown for branched-chained amino acids (26), affect mTOR activity in moDC but also indicate a difference between amino acids.

Discussion

We show here that arginine depletion by *G. duodenalis* ADI modulates surface markers and cytokine response of in vitro activated human moDC. The immunomodulation depended on both, depletion of arginine and the ADI reaction products, in particular NH_4^+. By investigating the consequences of arginine depletion we found that the mTOR pathway is implicated in the molecular signaling and leads to the modulation of DC responses. These findings add further evidence to the hypothesis suggested by others (28) that ADI is a molecularly defined virulence and pathogenicity factor of *G. duodenalis*. Arginine-depleting enzymes of pathogens have been implicated in immune evasion mechanisms because of competing for the same substrate as host NO synthases. Little attention has been paid to date to effects beyond this inhibition of microbial effector function. Our data implicate that arginine-metabolizing enzymes of pathogens are more widely involved in immunomodulation and suggest distinct roles for the reaction products of different enzyme classes in this process.

Currently, the relevance of our in vitro findings for the understanding of the pathogenesis of giardiasis must remain speculative and further studies are required. Nonetheless, we would like to discuss their potential implications: Firstly and as mentioned before, DC in the intestine project extensions between epithelial cells into the lumen of the gut to sample antigens (21, 22). Although only two studies with murine cells have been published to date that investigated the interaction of Giardia parasites with DC (29, 30), they provided evidence for the importance of DC in controlling Giardia infection (30). The ability to modulate DC function could thus provide a selective advantage to the parasite and our calculations suggest that arginine depletion may indeed occur in situ.

Secondly, arginine-dependent response modulation would be consistent with observations on the pathophysiology of human giardiasis. Atrophy of villi has been detected microscopically in intestinal biopsies of chronically infected patients, and symptomatic disease has been

correlated with a dysfunction of the epithelial barrier (31) but the process leading to this is not understood. These sequelae can, however, also be observed when intestinal biopsies are treated with TNF-α (32). TNF-α may thus have pathogenic properties in this context. Studies in mice showed that peak level Giardia parasite loads were around 10-fold higher in animals devoid of TNF-α (33) hence it was proposed that it has a protective function in giardiasis. However, in the same study transepithelial resistance was reduced to the same extent despite a much lower parasite burden in TNF-α responsive mice. An explanation consistent with the results from human biopsies exposed to TNF-α (31) could be that reduced epithelial integrity during giardiasis is due to parasite factors and TNF-α, the latter exerting a dual (protective and pathogenic) role.

Thirdly, children suffering from symptomatic giardiasis were shown to have increased mucosal levels of pro-inflammatory cytokines including TNF-α, which decreased after anti-parasitic treatment and resolution of symptoms while local levels of IL-10 increased after treatment (34). This is in agreement with our in vitro findings and may indicate that parasite ADI mediated arginine depletion impaired IL-10 and enhanced TNF-α secretion by mucosal DC in situ in these children. The relative abundance of IL-10 and TNF-α is recognized as a critical parameter in intestinal diseases in mice (35) and in humans (36, 37).

Fourthly, although *G. duodenalis* ADI is found intracellularly as part of the arginine dehydrolase (ADH) pathway and this pathway is thought to exploit arginine as an energy source to produce ATP in these amitochondrial organisms (38), the protein is also released by the parasite. Microarray analysis of the transcriptional response to host cell contact had revealed an up-regulation of *ADI* mRNA (28), and ADI as well as ornithine carbamoyl transferase (OCT, the next enzyme in the ADH pathway) were released by the parasite in contact with intestinal cells in vitro and in vivo ((39) and S.B. unpublished data). The facts that ADI and OCT were detected in these assays and are also immunodominant antigens during infection (40, 41) indicate that significant amounts of the enzymes are extracellular

and therefore free citrulline may even be further metabolized to ornithine. It is tempting to speculate that this may exacerbate TNF-α production because the negative feedback of citrulline on this parameter would be reduced. Thus, release of ADH compounds could have evolved in part due to selective pressure by the host's immune response.

To our knowledge this is the first report showing an immunomodulatory effect of arginine depletion and NH_4^+ formation on the response of DC other than on NO formation. Precedence for a clinically relevant role of amino acids in modulating immune responses exists (42, 43). For DC it has been shown that branched-chain amino acids affect maturation, and it has been proposed that this modulation is a consequence of inhibition of the mTOR pathway (26). Our results are consistent with this hypothesis but extend the concept to arginine. Of note, NH_4^+ has recently been reported to modulate mTOR activity in yeast cells (44). This suggests that reaction products formed by arginine-depleting enzymes could further affect mTOR signaling. However, additional signaling pathways are likely to be involved in mediating the distinct effects of arginine depletion alone and NH_4^+ formation. The latter has recently also been invoked in the T cell inhibition mediated by *Salmonella* L-asparaginase II (45). Further studies are required to understand this comprehensively.

In summary, we describe immunomodulatory effects of arginine depletion on human DC. Using ADI from *G. duodenalis* we show that this immunomodulation depends on arginine depletion and the products formed by the enzyme. This reveals novel facets of DC response modulation by arginine-metabolizing enzymes and may have implications for the general understanding of arginine-dependent virulence mechanisms of pathogens.

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Figure Legends

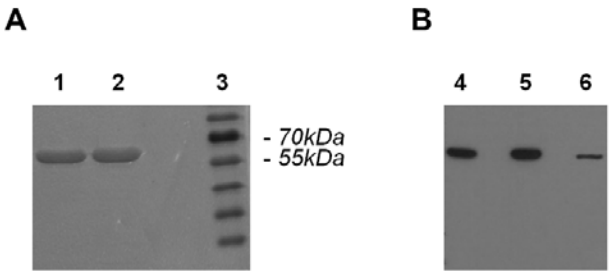


Figure 1. SDS-PAGE and Western blot analysis of purified recombinant *G. duodenalis* arginine deiminase

A, Coomassie-stained SDS-PAGE loaded with affinity-purified recombinant catalytically inactive ADI_{C424A} (lane 1), recombinant enzymatically active ADI (lane 2) and molecular mass standard (lane 3). B, Antigenic identification of 0.5 µg affinity-purified recombinant ADI (lane 4), 0.5 µg catalytically inactive ADI_{C424A} (lane 5) and native ADI in 2.9 µg of *G. duodenalis* (strain WB-C6) lysate (lane 6) by Western blot analysis using a polyclonal alpaca antiserum raised against ADI. Pre-immune serum used as control did not react with any *G. duodenalis* protein (data not shonw).

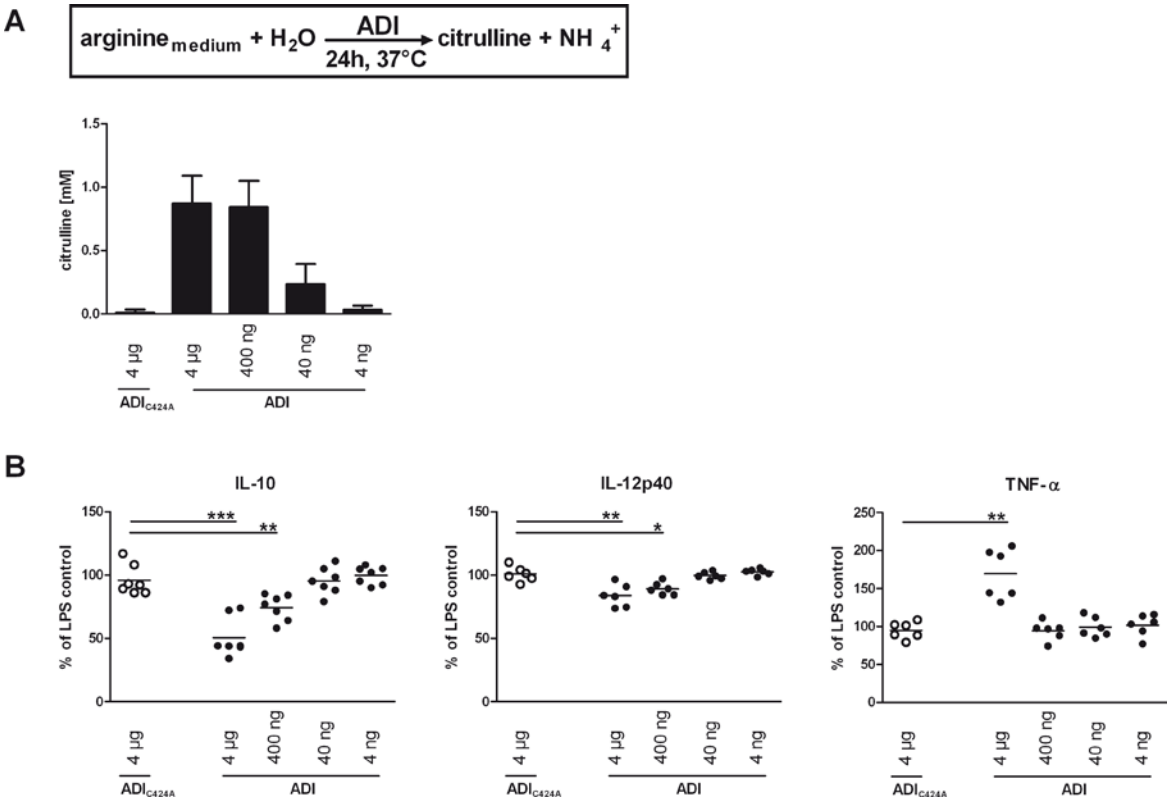


Figure 2. Enzymatic arginine depletion by ADI modulates cytokine secretion of LPS-activated human moDC

Immature moDC (5 x 10^5 per sample) were exposed to the indicated amounts of recombinant ADI or, as control, corresponding levels of ADI_{C424A} (only highest amount shown) and 1 µg/mL LPS was added. Citrulline content was determined as a measure of the cumulated ADI activity and arginine depletion over the 24 h assay time (A). DC cytokine secretion into supernatants was assessed by ELISA and is expressed as percentage of the amount secreted by LPS stimulated cells not exposed to any ADI protein (B). Bars in (A) represent the mean ± SD from experiments with DC prepared from seven different donors. Symbols in (B) represent values from individual donors with means indicated by horizontal lines. Differences between the amount of cytokines secreted by cells exposed to mutant (control) or active ADI were analyzed by paired t test (two-tailed); $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

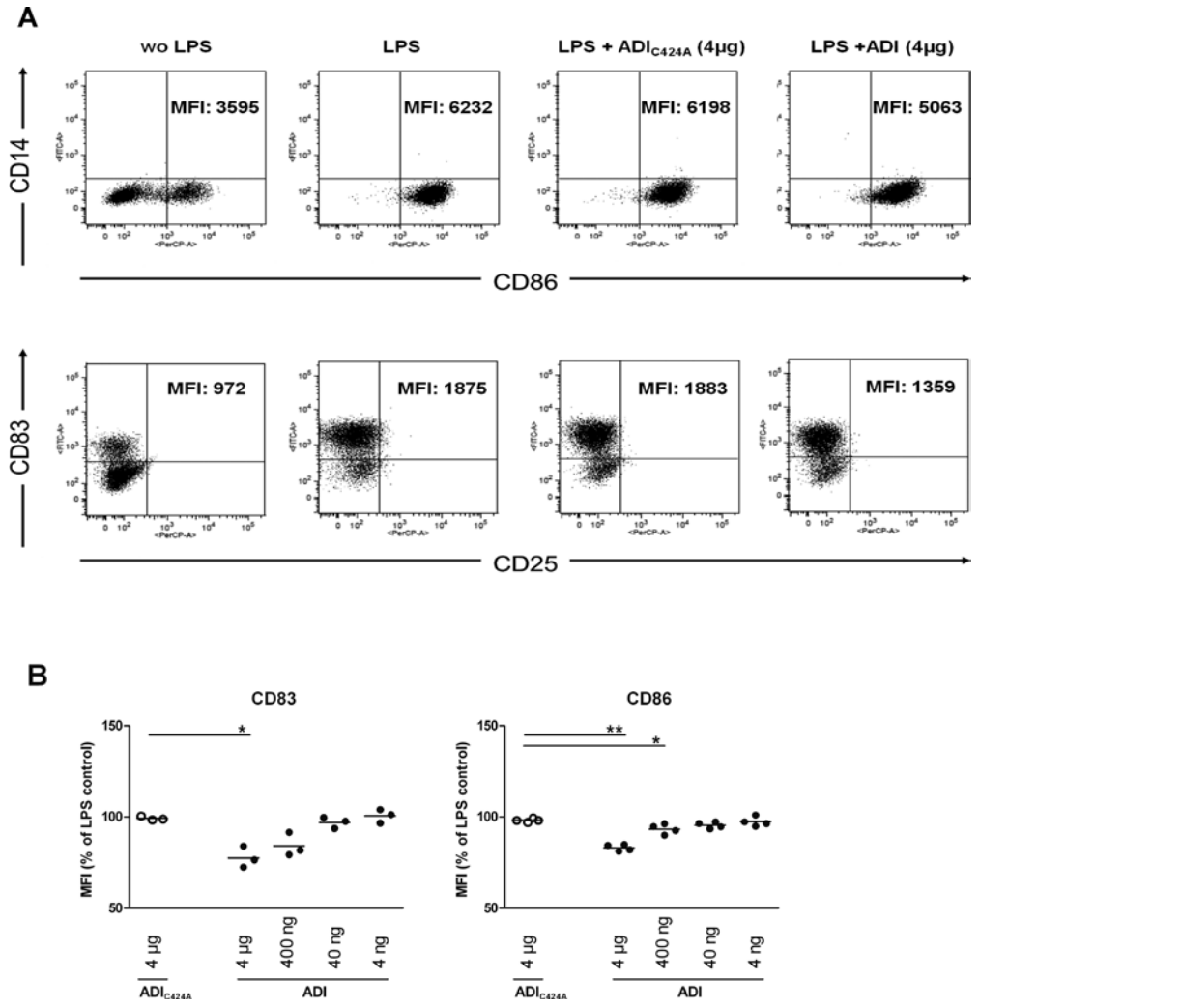


Figure 3. Enzymatic arginine depletion by ADI reduces CD83 and CD86 surface markers induction by LPS activation on moDC

Cells were treated as described in Fig. 1 and all cells, except the non-stimulated control cells, were treated with 1 µg/mL LPS. After incubation for 24 h at 37°C, moDC were harvested, stained with cell surface marker-specific antibodies and analyzed by flow cytometry. (A) Representative dot plots for CD14/CD83 (top panel) and CD25/CD86 (bottom panel) expression with the respective mean fluorescence intensity (MFI) values for CD86 and CD83 are shown. (B) Relative MFI in % of control LPS-stimulated cells for CD83 (n=3) and CD86 (n=4) with moDC from different donors are shown. Horizontal lines correspond to mean values and differences between the respective MFI on cells exposed to mutant (control) or active ADI were analyzed by paired t test (two-tailed); *$p < 0.05$, **$p < 0.01$.

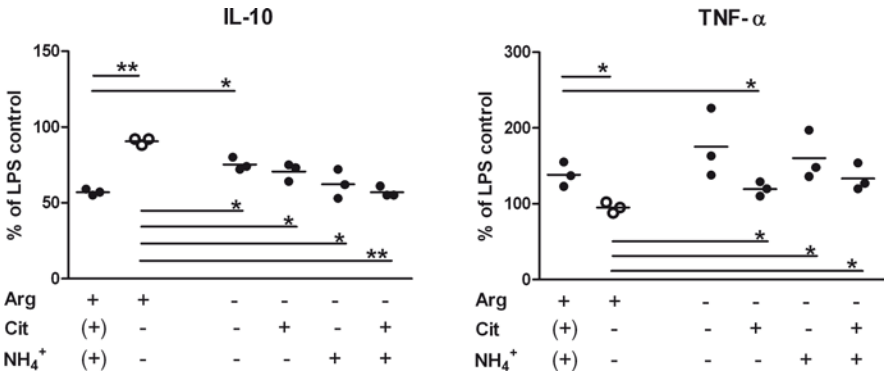


Figure 4. Depletion of arginine and formation of ADI reaction products citrulline and ammonium ions modulate moDC response to LPS activation

Immature moDC after harvest were seeded into arginine-free growth medium. The medium was then supplemented as indicated with arginine, citrulline and/or ammonium chloride (2mM each; brackets indicate addition of ADI to arginine-replete medium to reflect formed, not supplemented products). Cells were activated with LPS and treated with ADI (●) or mutant ADI_{C424A} (○) for control. Values correspond to % of the respective parameter determined for cells stimulated with LPS only in arginine-replete medium. After 24 h, supernatants were collected and cytokine concentrations determined by ELISA. Horizontal lines correspond to mean values and differences between the respective parameter determined with cells exposed to mutant (control) or active ADI were analyzed by paired t test (two-tailed); $*p < 0.05$, $**p < 0.01$.

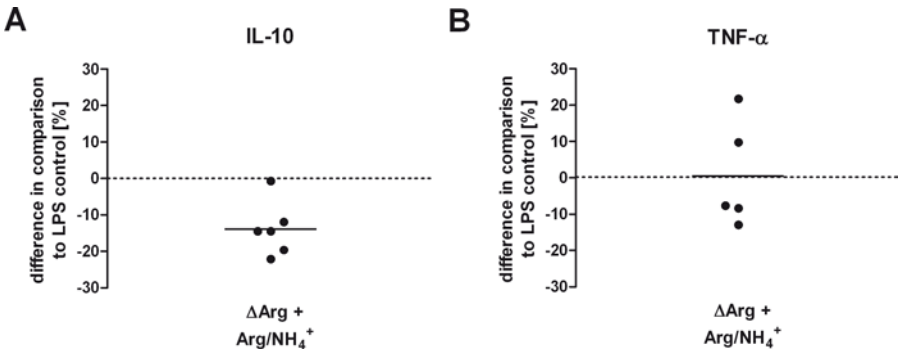


Figure 5. Addition of NH_4^+ in the presence of arginine reduces IL-10 secretion of moDC after LPS activation

Immature moDC were prepared and stimulated as described (Fig. 4) and medium was supplemented with arginine or arginine and ammonium chlorid. After 24 h, supernatants were taken and cytokine concentrations determined by ELISA. Each dot represents an independent experiment with moDC prepared from an individual donor and values are expressed as % difference to cytokine amounts produced by arginine supplemented, LPS-stimulated cells. Significance was tested against the null hypothesis that there is no differences between cells stimulated in the presence of arginine or arginine plus ammonium ion using paired t test (two-tailed); P values were $p < 0.01$ for IL-10 levels and non-significant for TNF-α..

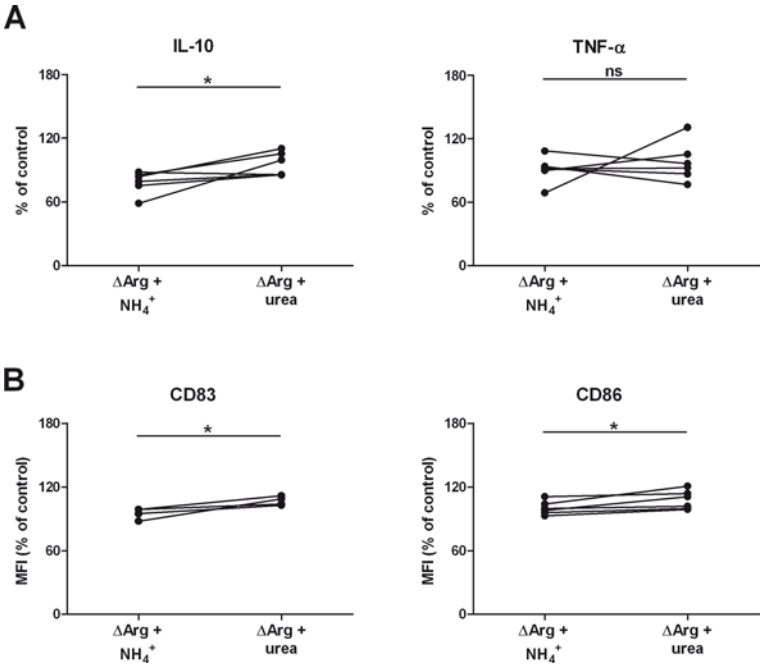
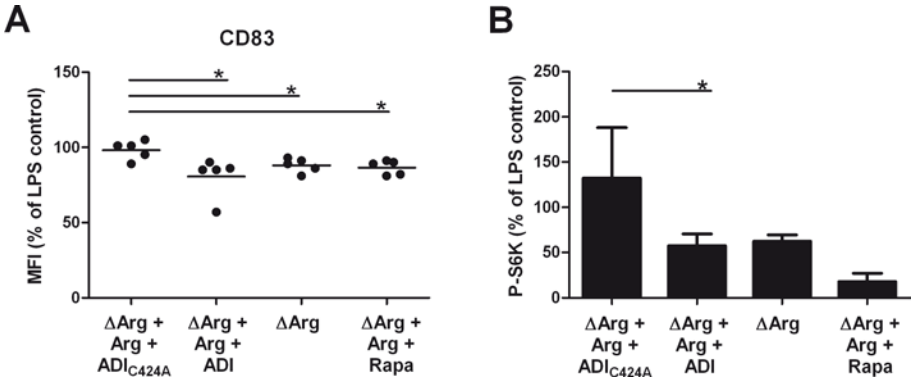


Figure 6. Immunomodulation of LPS-activated moDC under arginine starvation is different between NH_4^+ and urea.

MoDC were prepared as described in Fig. 4 and stimulated in arginine-free medium supplemented with either 2 mM of ammonium chloride or urea. After 24 h, supernatants were collected and cytokine concentrations determined by ELISA (A). The moDC were harvested and surface marker proteins were analyzed by flow cytometry (B). Dots represent values from experiments with cells from six (A) or four (B) individual donors and are expressed as % of control values obtained with LPS-activated cells with no supplement (paired t test (two-tailed); *$p < 0.05$).



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677 **Figure 7. ADI-mediated arginine depletion decreases mTOR-signaling in LPS-**
678 **stimulated moDC**

679 10^6 immature moDC were seeded in arginine-free growth medium into each well of a 12-well
680 tissue culture plate. Arginine-free medium (ΔArg) was supplemented or not with 2 mM
681 arginine and cells were treated or not with 4 µg of ADI, 4 µg of ADI_{C424A}, or 2 µM
682 rapamycin. For activation 1 µg/mL LPS was added after 2 h to each sample. (A) MoDC CD83
683 surface marker levels 24 h after stimulation compared to stimulated control cells are shown.
684 Bars represent the mean ± SD of independent experiments with five different donors (paired
685 t test (two-tailed), $*p < 0.05$ compared with the respective control). (B) In parallel, 30 min
686 after LPS stimulation, cells were harvested, washed and lysed. 50 µg of cell extracts were
687 separated by SDS-PAGE and p70-S6K, phospho-p70-S6K and β-actin were detected by
688 immunoblotting and quantified by image analysis. Phosphorylated p70-S6K levels were
689 normalized relative to β-actin and compared to stimulated control cells. Levels of total p70-
690 S6K levels were not significantly different between the different experimental groups. Bars
691 represent the mean ± SD from five individual experiments with cells from different donors
692 (paired t test (two-tailed), $*p < 0.05$, $**p < 0.01$ compared with the respective control).

Footnotes

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