

The missing link between indoleamine 2,3-dioxygenase mediated antibacterial and immunoregulatory effects

Anika Müller, Kathrin Heseler, Silvia K. Schmidt, Katrin Spekker, Colin R. MacKenzie, Walter Däubener*

Institute of Medical Microbiology and Hospital Hygiene, Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany

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Abstract

The interferon (IFN)- γ -inducible tryptophan degrading enzyme indoleamine 2,3-dioxygenase (IDO) has not only been recognized as a potent antimicrobial effector molecule for the last 25 years but was recently found also to have potent immunoregulatory properties. In this study, we provide evidence that both tryptophan starvation and production of toxic tryptophan metabolites are involved in the immunoregulation mediated by IDO, whereas tryptophan starvation seems to be the only antibacterial effector mechanism. A long-studied controversy in the IDO research field is the seemingly contradictory effect of IDO in the defence against infectious diseases. On the one hand, IFN- γ -induced IDO activity mediates an antimicrobial effect, while at the same time IDO inhibits T-cell proliferation and IFN- γ production. Here, we suggest that both effects, dependent on the threshold for tryptophan, cooperate in a reasonable coherence. We found that the minimum concentration of tryptophan required for bacterial growth is 10–40-fold higher than the minimum concentration necessary for T-cell activation. Therefore, we suggest that during the first phase of infection the IDO-mediated tryptophan depletion has a predominantly antimicrobial effect whereas in the next stage, and with ongoing tryptophan degradation, the minimum threshold concentration of tryptophan for T-cell activation is undercut, resulting in an inhibition of T-cell growth and subsequent IDO activation.

Keywords: indoleamine 2,3-dioxygenase • T cell • bacteria • interferon- γ • kynurenine • tryptophan

Introduction

It is generally accepted that the antigen-driven cellular immune response directed against a particular pathogen, begins in the lymph node by the interaction of naïve T cells with dendritic cells. In the past dendritic cells were regarded as immunostimulatory cells only. However, several different subsets of dendritic cells have been described recently, some of which are capable of inhibiting T-cell responses [1, 2].

According to data by the group of Mellor and Munn [3], the induction of the tryptophan degrading enzyme indoleamine 2,3-dioxygenase has been recognized as an immunoregulatory mechanism. Indoleamine 2,3-dioxygenase (IDO)⁺ dendritic cells were characterized as either immunodownregulatory or tolerogenic dendritic cells [4]. Different pathways leading to IDO induction in

dendritic cells have been described and several cytokines (types I and II interferons [IFNs], tumour necrosis factor [TNF]- α , granulocyte macrophage-colony stimulating factor [GM-CSF], prostaglandin E [PGE]₂) [5–8] and surface molecules (CTLA4, CD80/86, CD40, CD200) [9–12] have been implicated in IDO induction. In general, most of them are dependent on the presence or production of IFN- γ [13], while only a few pathways appear to be independent of IFN- γ [14]. Type-1 IFNs seem to be important in specialized CD19⁺ DC subsets and IDO is additionally involved in the induction of type-1 IFNs in these cells [15]. Most data have been obtained from macrophages/dendritic cells and therefore the focus has been on the primary or secondary activation of T cells especially within the lymph node.

Dendritic cells are the most prominent antigen-presenting cells (APC) in the primary activation of resting, naïve T cells. In secondary T-cell activation and especially also the triggering of the effector phase of T-cell activation in the inflamed tissue, several other cell types can interact with T cells as APC. In human beings, IDO expression can be induced in several non-professional APC (NP-APC) and it can be assumed that IDO activity within these cells is involved in the control of the local T-cell effector phase. Much attention was paid to the role of IDO expression in tumour cells

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*Correspondence to: Prof. Dr. Walter DÄUBENER,
Institute of Medical Microbiology and Hospital Hygiene,
Heinrich-Heine-Universität Düsseldorf, Universitätsstraße 1 Geb. 22.21,
40225 Düsseldorf, Germany.
Tel.: +49 (0) 211-81-12464
Fax: +49 (0) 211-81-15906
E-mail: daeubene@uni-duesseldorf.de

and it was found that a surprisingly high proportion of cells within human tumours express IDO activity [16]. In line with the fact that IDO expressing tumour cells are capable of inhibiting T-cell proliferation, *in vivo* studies in mice showed that IDO⁺ tumour cells become resistant to immune eradication [16]. Histological data, obtained with human ovarian carcinoma cells, showed that IDO expression correlates with a worse clinical outcome [17]. Therefore, it would appear that IDO has a double role in tumour immunology: IDO-expressing DC, especially in tumour draining lymph nodes, are active in the afferent arm of the immune system and inhibit T-cell activation, whereas IDO⁺ tumour cells themselves down-regulate the efferent arm of the immune system by inhibiting T-cell effector function [18]. The molecular mechanism causing IDO-mediated inhibition of T-cell responses is not absolutely clear, but it has been suggested that T cells might use the GCN2 pathway as a sensor for tryptophan depletion and that the induction of an integrated stress response is responsible for inhibition of T-cell proliferation [19]. Alternatively, it was assumed that toxic metabolites produced along the kynurenine pathway, especially kynurenine and 3OH-kynurenine, might inhibit T-cell responses either directly [20, 21] or *via* dendritic cells [22]. Other theories involve the preferred induction of regulatory T cells or the development of regulatory dendritic cells [23].

It is well known that minor subsets of dendritic cells are capable of expressing IDO; however, the constitutive and inducible expression of this enzyme in several other cell types has also been described. For example, IDO expression has been found in mucosal surfaces of the lung and the lower gastrointestinal tract. Both organ systems are involved in autoimmune and/or chronic inflammatory of allergic diseases and IDO might play a role in the regulation of these pathological processes [24, 25]. Starting with the initial finding of Pfefferkorn *et al.* [26], a set of different human pathogens have been found to be inhibited by IDO. The exact mechanism of this IDO-mediated antimicrobial effect remains unclear, but it is widely accepted that depletion of tryptophan is responsible for the antiparasitic effect [27]. Until now, there have been no data available showing the role of tryptophan metabolites or of the GCN2 pathway in the IDO-mediated control of pathogens. The antimicrobial effect of IDO in human cells is expressed mainly in NP phagocytes such as endothelial cells [28], epithelial cells [29], fibroblasts [26] and astrocytes [30], the same cells which might influence the effector phase of T cells as mentioned above. Thus, it would appear that IDO might have two contradictory roles in the immune defence against infectious agents; on the one hand, inhibiting the growth of pathogens directly, and on the other hand, inhibiting the effector phase of the T-cell response to these agents. In this manuscript, we show that the minimum tryptophan concentration, required for T-cell growth, is up to 40-fold lower than the minimum concentration, required for the growth of bacteria. Therefore, during an ongoing immune response at the site of a local infection, tryptophan degradation results first in an inhibition of bacteria and then later, at a lower concentration, an inhibition of the T-cell effector phase is reached, resulting in a reduction of IFN- γ production. This may result in a protective

attenuation of the ongoing IDO activation, thus ensuring adequate tryptophan levels for tissue cells.

Materials and methods

Media and cells

Iscove's modified Dulbecco's medium and Roswell Park Memorial Institute (RPMI) 1640 (Gibco, Grand Island, NY, USA), with and without tryptophan, supplemented with 2-mM L-glutamine and 5–10% heat inactivated foetal calf serum were used as culture media for all cell lines. 86HG39 cells (human astrocytoma cells) [31] were obtained from Dr. Bilzer (Neuropathologie, Universität Düsseldorf, Germany), A549 cells (lung carcinoma, type II alveolar cells), HeLa cells (human cervix carcinoma cells) and human foreskin fibroblasts (HFF) were obtained from American Type Culture Collection (Rockville, MD, USA).

All cell lines were cultured in supplemented Iscove's medium in culture flasks (Costar, Cambridge, MA, USA) and split weekly in 1 : 3 to 1 : 10 ratios by using trypsin/EDTA (Gibco). In all cell lines, mycoplasma contamination was regularly excluded, using both culture methods and PCR.

Peripheral blood lymphocytes (PBL) were prepared from heparinized blood of healthy donors after density gradient centrifugation. Alloreactive T-cell lines directed against 86HG39 cells were obtained by coculturing mitomycin-treated 86HG39 cells and PBL. Before using in the experiments T cells were restimulated using mitomycin-treated 86HG39 cells and IL-2 (5 ng/ml) (R&D, Rüsselsheim, Germany).

Preparation of conditioned medium

A total of 5×10^6 86HG39 or A549 cells were cultured in 20-ml culture medium in culture flasks. Alternatively, 1.25×10^6 HeLa cells or HFF were cultured in 5-ml culture medium. Cells were stimulated for 3 days with IFN- γ (500 U/ml) in the presence or absence of additional tryptophan (25–100 μ g/ml) (Sigma-Aldrich, Deisenhofen, Germany) or 1-methyl-L-tryptophan (1-MT, 1.5 mM). 1-methyl-L-tryptophan was used since its efficiency in IDO inhibition was about 10-fold higher than efficiency of the D-isomer.

Thereafter, supernatants were harvested and kynurenine content was determined. Supernatants were utilized as culture medium for bacteria or PBL. In some experiments, supernatants were supplemented with different amounts of tryptophan.

Kynurenine assay

Supernatants harvested from IFN- γ -stimulated cells were analysed for their kynurenine content, using Ehrlich reagent as described before [32]. As a standard we diluted kynurenine (Sigma-Aldrich) in culture medium. For the calculation of the kynurenine content in the supernatant, linear regression and GraphPad Prism software was used.

Determination of bacterial growth

For the analysis of bacterial growth in conditioned medium, several strains of staphylococci or enterococci, obtained from routine diagnostic specimens

were used. The IDO sensitivity of these strains has been previously analysed [33, 34]. Bacteria were grown on brain heart infusion agar (Difco, Hamburg, Germany), containing 5% sheep blood and incubated at 37°C in 5% CO₂-enriched atmosphere. For use in experiments, a 24-hr-old single bacterial colony was picked and resuspended in RPMI 1640 without tryptophan. Bacteria were serially diluted in the same medium and 10 µl were added to 200 µl of conditioned medium.

Bacterial growth in Iscove's medium with or without supplemental tryptophan or kynurenine was used as a control. After incubation for 16–24 hrs, bacterial growth was monitored using a microplate photometer (SLT Labinstruments, Crailsheim, Germany) by measuring the optical density at 620 nm. In some experiments, the bacterial population present in the cultures was enumerated by counting colony forming units (CFU) after plating 10 µl aliquots of serially diluted culture supernatants on blood agar.

Proliferation assays

A total of $0.3\text{--}3 \times 10^4$ mitomycin-treated 86HG39 cells were incubated in the presence or absence of IFN- γ for 24–72 hrs. Thereafter, alloreactive T cells (1×10^5 /well in 10 µl tryptophan-free RPMI 1640 medium) were added with or without supplemental tryptophan (final concentration 100 µg/ml). T-cell proliferation was determined after 3 days by adding ³H-thymidine for 14–18 hrs.

In other experiments mitomycin-treated 86HG39 cells, irradiated HFF or A549 cells were activated with IFN- γ as described above and then 1.5×10^5 PBL were added in 10 µl tryptophan-free RPMI 1640 medium and stimulated with either a monoclonal anti-CD3 antibody, staphylococcal enterotoxin B (SEB, 10 ng/ml, Sigma-Aldrich) or phytohemagglutinin A (PHA 2 µg/ml, Sigma-Aldrich) for 3 days. As a control, some cultures were supplemented with L-tryptophan or 1-methyl-L-tryptophan at the time of IFN- γ stimulation. In additional experiments, PBL were activated by the use of an anti-CD3 monoclonal antibody and conditioned medium served as culture medium. As a control, PBL stimulated in Iscove's Medium in the presence or absence of additional tryptophan or kynurenine were used.

The ³H-thymidine incorporation method was also used to determine the growth of 86HG39 or A549 cells in the presence or absence of different amounts of tryptophan or kynurenine. In these experiments, 1×10^4 tumour cells were cultured for 3 days in 96-well flat-bottomed culture plates prior to the addition of ³H-thymidine.

High-performance liquid chromatography (HPLC) analysis

Degradation of tryptophan was also measured by HPLC (Gold-Universal-Grad System, BeckmanCoulter, Krefeld, Germany). Before measurement, the culture medium was deproteinized by the treatment with 25% sulphosalicylic acid (1:10 vol/vol). After precipitation the supernatant was adjusted to a pH of 7.2 by adding NaOH and mixed with FMOC (9-fluorenyloxycarbonylchloride)-reagent (Alltech Grom, Rottenburg, Germany). After a 1.5-fold dilution with sodium acetate (50 mM / pH 4.2), 50 µL of the sample were injected into a 5-µm reversed phase column (Grom-Sil FMOC-2, 100 mm × 4 mm) and analyses were carried out at a flow rate of 1.0 ml/min. The separation was performed in sodium acetate buffer (50 mM / pH 4.2) with an increasing gradient of acetonitrile. The absorbance of the column effluent was monitored at 263 nm. The tryptophan peak was identified by means of comparison with the retention time of standards which were previously determined.

Statistical analysis

All experiments were done in triplicate and data are given as mean \pm S.E.M. of 2 to 18 independent experiments, as indicated in the legends of the figures. For statistical analysis, dependent on the type of the experiment the unpaired or paired t-test was used and *P*-values <0.01 were accepted as significant. The threshold tryptophan concentrations for bacteria and T cells were determined using linear regression and GraphPad Prism software.

Results

Inhibition of the efferent arm of the immune system by IDO⁺ bystander cells

It is well described that IDO⁺ NP-APC, such as mesenchymal stroma cells, fibroblasts and epithelial cells, as well as cells from different tumour lines, are capable of mediating inhibitory effects on T cells in an IDO-dependent fashion, when they are present as bystander cells during T-cell activation [35–37]. We assume that this immunoregulatory effect, mediated by NP-APC, does not have an incidental bystander function but rather is ubiquitously present as a part of the IFN- γ stimulation, resulting from inflammation. We have previously shown that immortalized human brain microvascular cells (HBMEC) are able to inhibit T-cell responses in an IDO-dependent fashion [38]. In addition, Figs 1–3 demonstrate that different tumour cells for example astrocytoma cells (86HG39), lung carcinoma cells (A549) or cervix carcinoma cells (HeLa) as well as native tissue cells (HFF) are, subsequent to IDO induction by IFN- γ , capable of inhibiting mitogen (PHA), superantigen (SEB), anti-CD3 and alloantigen-driven T-cell activation, when present as bystander cells during T-cell activation. These inhibitory effects could be blocked by the addition of tryptophan as well as by the addition of the IDO specific inhibitor 1-methyl-tryptophan. Further experiments indicated that the inhibitory effect mediated by NP-APC, present as bystander cells, was dependent on the number of cells present during T-cell activation. For example 3×10^4 86HG39 cells stimulated with IFN- γ for 72 hrs are able to inhibit the T-cell response by 70%, whereas 3×10^3 cells have no effect. In addition, we found that the IFN- γ pre-stimulation time was also critical in the mediation of this anti-proliferative effect: 3×10^4 86HG39 cells pre-treated with IFN- γ for only 24 hrs were unable to inhibit T-cell proliferation (Fig. 2). Using double culture systems, separated by a semi-permeable membrane, we found that this IDO-mediated anti-proliferative effect was independent of a cell–cell contact. Even culture supernatants, conditioned by IFN- γ activated cells, used as culture medium for subsequent T-cell activation, mediated this anti-proliferative effect (Fig. 3). As a control, supernatants from IFN- γ -activated Jurkat cells or from Epstein-Barr virus (EBV)-transformed B-cells, which do not express IDO activity, were used and did not influence T-cell proliferation (data not shown).

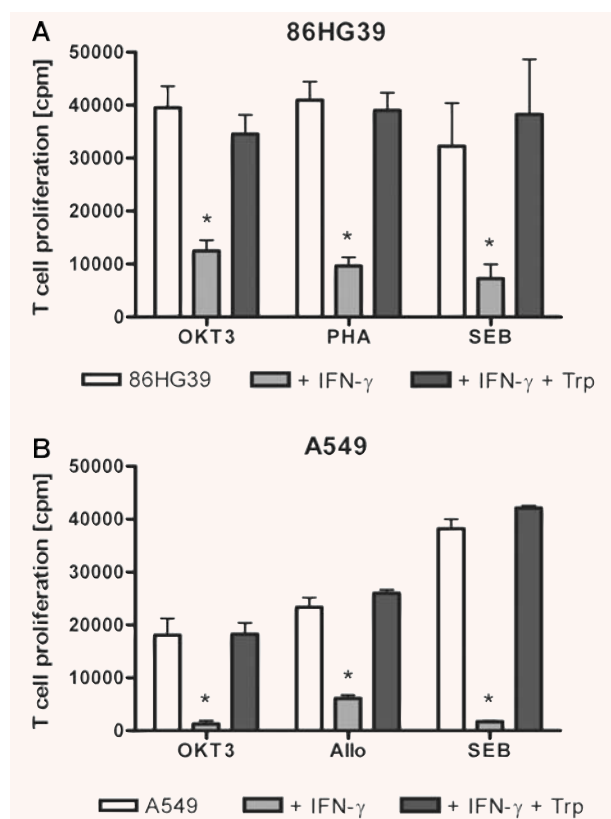


Fig. 1 IDO⁺ tumour cells are capable of inhibiting mitogen-driven T-cell proliferation: Mitomycin-treated 86HG39 cells (A) or irradiated A549 cells (B) were incubated with or without IFN- γ (500 U/ml). After 3 days, 1.5×10^5 PBL were added and stimulated with anti-CD3 antibodies, phytohemagglutinin A or staphylococcal enterotoxin B for additional 3 days in the absence or presence of tryptophan (100 μ g/ml). T-cell proliferation was determined by ³H-thymidine incorporation after 3 days. Data are given as mean \pm S.E.M. of two independent experiments, each done in triplicate. Asterisks indicate significant inhibition of T-cell responses ($P < 0.005$).

In conclusion, these data confirm and expand the previously published data of several different groups, including ours, that indicate that IDO⁺ bystander cells can mediate anti-proliferative effects. In addition, we have previously shown that the cells, used in the experiments, shown in Figs 1–3 are capable of mediating an ido-dependent antimicrobial effect. In subsequent studies we analysed the respective roles of tryptophan depletion and accumulation of toxic kynurenine pathway metabolites in the mediation of immunoregulatory and antimicrobial effects.

Mechanism of IDO-mediated antibacterial and immunoregulatory effects

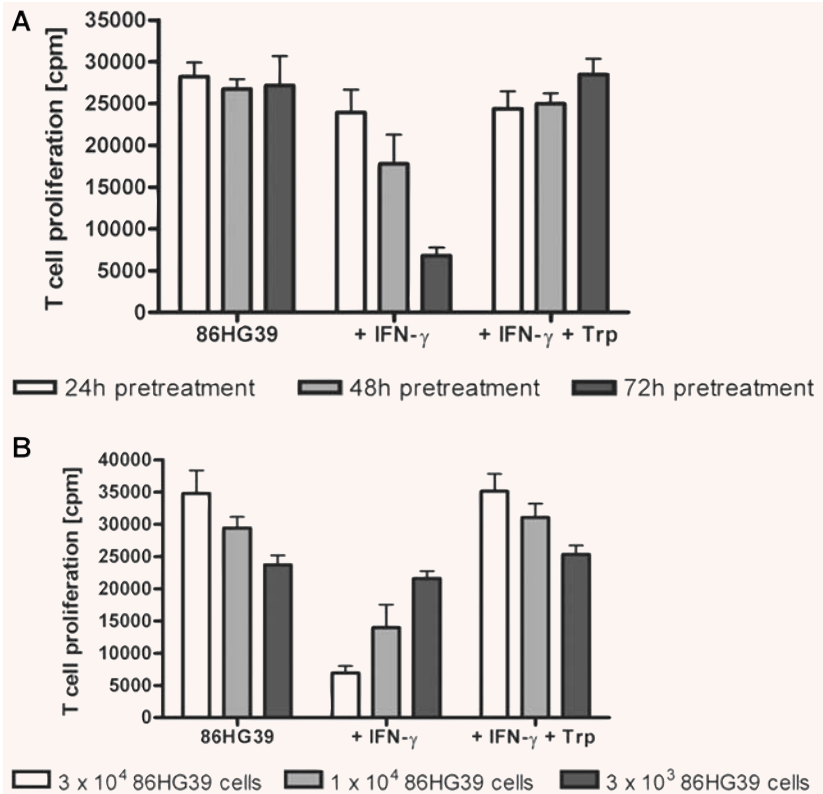
Several data from different groups using diverse read-out systems indicate that toxic kynurenines, as well as tryptophan starvation

are capable of mediating immunoregulatory effects, while the antiparasitic effect, mediated by IDO, seems to depend on tryptophan starvation alone. Having found that supernatants of IDO⁺ cells inhibit T-cell growth we have attempted to analyse the mechanism of IDO-mediated antimicrobial and anti-proliferative effects in parallel. To achieve this, we stimulated 86HG39 cells with IFN- γ (500 U/ml) in the presence or absence of additional tryptophan (up to 10-fold of the normal tryptophan concentration). After 3 days of incubation, conditioned medium was harvested and used as culture medium for bacteria and T cells. In addition, the concentration of the tryptophan metabolite kynurenine was measured in the culture supernatants. Data with supernatants from stimulated 86HG39 cells were shown in Fig. 4 and similar results were also obtained with supernatants from other cells (HBMEC, A549). All data are given as mean \pm S.E.M. of three independent experiments all done in triplicate estimations. Figure 4A indicates that independently of the amount of tryptophan initially present in the culture medium all tryptophan was converted into kynurenine within 3 days of stimulation. This is in line with the known high efficiency of IDO in tryptophan cleavage. Furthermore, the near equimolar production of kynurenine excludes the possibility that other tryptophan metabolites were produced in significant amounts by 86HG39 cells. Using HPLC analysis we found that the tryptophan content in media harvested from IFN- γ stimulated 86HG39 cells is below the detection limit of this method (<15 nM).

Having shown that the supernatant of IFN- γ stimulated cells was depleted of tryptophan, this conditioned medium was used to analyse bacterial growth. As indicated in Fig. 4B, we found that, independent of the amount of tryptophan initially added to the cultures, a strong antibacterial effect could be detected. To show that the lack of bacterial growth in the cell culture supernatants is due to IDO-induction and subsequent tryptophan degradation, we added large amounts of tryptophan (100 μ g/ml) to the supernatants at the time of infection. As also shown in Fig. 4B, this results in a complete restoration of bacterial growth, indicating that the kynurenine concentration present in the cultures did not influence bacterial growth. This was also supported by the finding that kynurenine, added in different amounts into bacterial cultures, did not influence bacterial growth (Fig. 5A).

In a third experimental setting, the same supernatants that were used to determine kynurenine content and to measure bacterial growth, were also used to analyse T-cell activation induced by cross-linking anti-CD-3 antibodies. As shown in Fig. 4C, T-cell proliferation was inhibited in medium, conditioned by IFN- γ -activated, IDO⁺ cells, and there is a trend to an enhanced inhibitory effect in media, initially supplemented with high amounts of tryptophan or containing large amounts of kynurenine. In order to analyse the effector mechanism involved, we supplemented the media at the time-point of T-cell stimulation with large amounts of tryptophan. As indicated by data in Fig. 4C, this second tryptophan supplementation restores T-cell proliferation in supernatants containing small amounts of kynurenine only, while the tryptophan supplementation did not influence the inhibitory effect, mediated by conditioned medium containing high amounts of kynurenine. These data suggest that both tryptophan starvation and the increase of the kynurenine

Fig. 2 Time and dose dependency of IDO-mediated inhibition of T-cell growth by 86HG39 cells. Mitomycin-treated 86HG39 cells (3×10^4 /well) were incubated for 24, 48 or 72 hrs in the presence or absence of IFN- γ (500 U/ml). After 3 days, 1×10^5 alloreactive T cells were added for additional 3 days in absence or presence of tryptophan (100 μ g/ml). T-cell proliferation was determined by 3 H-thymidine incorporation after 3 days. Data are given as mean \pm S.E.M. of two independent experiments, each done in triplicate (A). The identical read-out system was used in part B, but different amounts of 86HG39 cells/well were used and stimulated for 3 days.



concentration mediate inhibitory effects on T-cell growth. This is supported by data obtained by analysing T-cell proliferation in the presence or absence of different amounts of kynurenine with or without supplemental tryptophan, as shown in Fig. 5B. In summary, these experiments combine the tryptophan starvation and the toxic metabolites hypotheses and therefore confirm that both hypotheses are not mutually exclusive. Furthermore, this is the first report, to our knowledge, which documents an analysis of the mechanism of IDO-mediated antibacterial and immunoregulatory effects in parallel since most groups focus either on the immunoregulatory or the antimicrobial function of this enzyme.

A difference in the tryptophan requirement combines IDO-mediated antimicrobial and immunoregulatory effects

We have recognized that IDO⁺ NP-APCs are capable of inhibiting both T-cell proliferation and bacterial growth. These two IDO-mediated effects seem to be contradictory in terms of a unifying immune defence hypothesis. To analyse the relationship between IDO-mediated antimicrobial and immunoregulatory effects in more detail, we aimed to define the threshold concentration of tryptophan, necessary to mediate both effects. Therefore, we once again used supernatants harvested from IDO⁺ cells to determine bacterial growth and T-cell growth and supplemented these conditioned media with

different amounts of tryptophan. Figure 6B shows the bacterial growth, measured by determining optical density. These data indicate that bacterial growth is blocked in the supernatant of IFN- γ -stimulated cells and that this antibacterial effect could be restored by the addition of tryptophan. A 50% inhibition of bacterial growth was found at a tryptophan concentration of about 0.38 μ g/ml (1.9 μ M). The same supernatants were also used to culture T cells and the data in Fig. 6A indicate that T-cell proliferation in medium, conditioned by IFN- γ activated A549 cells, is reduced by about 70%. A half-maximal inhibition of T-cell growth was achieved at a tryptophan concentration of about 0.02 μ g/ml (0.1 μ M) (Fig. 6). Hence, we found a 19-fold difference in the tryptophan need for T cells compared to bacteria. In order to analyse this difference in more detail, we determined CFU as a second and a more sensitive readout system, to quantify bacterial growth in four additional experiments. We noticed that bacterial growth in conditioned medium, harvested from IFN- γ activated cells, was dramatically reduced from 1×10^9 CFU/ml in the supernatant of untreated cells to 1 CFU/ml in the supernatant of IFN- γ activated cells (Fig. 6C). A half-maximal reduction of bacterial growth, using the CFU method, was ascertained at a tryptophan concentration of 0.82 μ g/ml (4.1 μ M), which is 41-fold higher than the threshold concentration for T cells. All experiments shown in Fig. 6 were done with one strain of *Staphylococcus aureus*. To ensure that the comparatively high threshold concentration is consistent with other strains we also analysed four additional *Staphylococcus aureus* isolates and

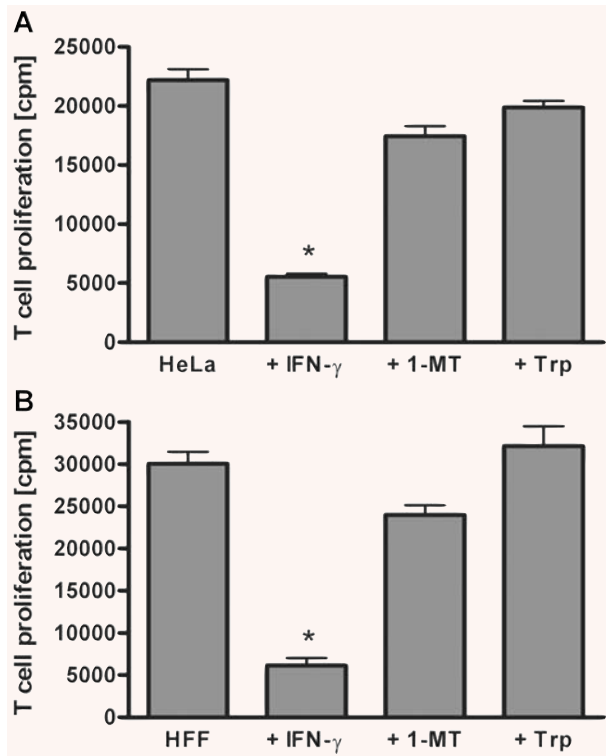


Fig. 3 Inhibition of T-cell growth in media, conditioned by IDO⁺ cells. 1.25×10^6 HeLa cells (A) or human foreskin fibroblasts (B) were incubated in the presence of IFN- γ (500 U/ml) or IFN- γ and 1-methyl-L-tryptophan (1-MT) for 3 days. Thereafter, supernatants were collected and used as culture medium for PBL (1×10^5 /well) and stimulated with anti-CD3 antibodies either in presence or absence of tryptophan (100 μ g/ml). T-cell proliferation was determined by ³H-thymidine incorporation after 3 days and data are given as mean \pm S.E.M. of two independent experiments, each done in triplicate. Asterisks indicate significant inhibition of T-cell responses ($P < 0.001$).

obtained comparable results. Here, we detected again a 50% inhibition of bacterial growth at a tryptophan concentration of about 0.3 μ g/ml. Furthermore, four strains of enterococci were tested and showed a 50% inhibition of bacterial growth at a tryptophan concentration of 0.2 μ g/ml (1.0 μ M) (Fig. 7). Altogether, these data indicate that the tryptophan need of bacteria is higher than the tryptophan need of T cells. This led us to the conclusion that a comparably lesser tryptophan degradation mediates an antibacterial effect whereas a 10–40-fold stronger tryptophan depletion is necessary to mediate an immunoregulatory effect.

Discussion

The widely accepted understanding that IDO is an antimicrobial effector mechanism is derived from *in vitro* data published by different

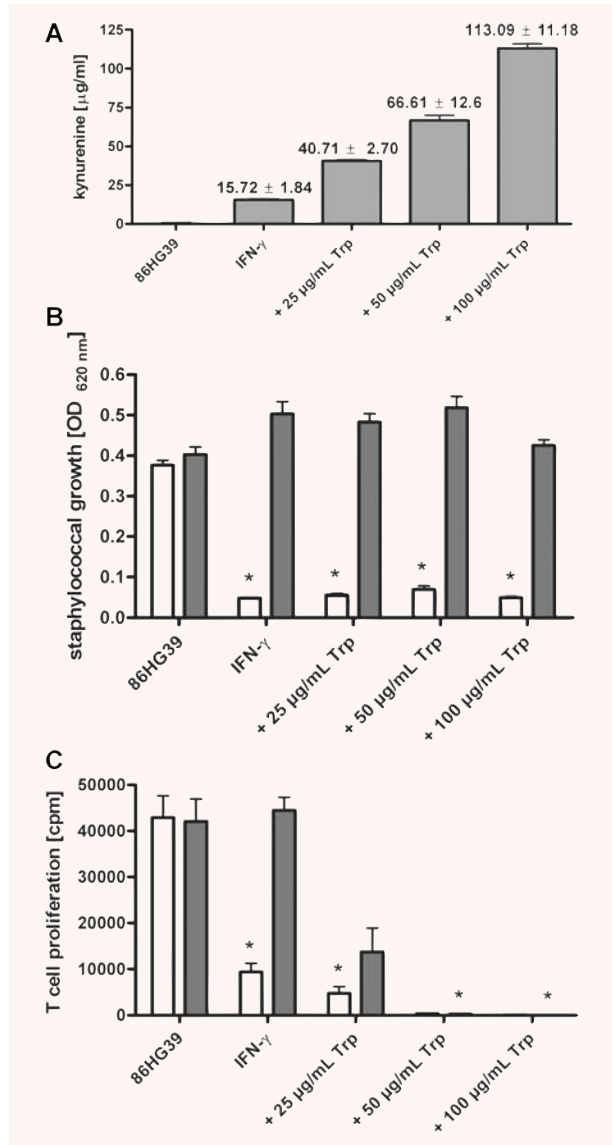


Fig. 4 Differences in the tryptophan antagonism of antibacterial and anti-proliferative effects mediated by IDO⁺ 86HG39 cells. A total of 5×10^6 86HG39 cells were incubated with or without IFN- γ (500 U/ml) in the presence of different concentrations of additional tryptophan (0, 25, 50 or 100 μ g/ml). After 3 days of incubation, supernatants were harvested and the kynurenine content was measured as described in materials and methods (A). In addition, *Staphylococcus aureus* (10–50 CFU/well) was added to the supernatants in the absence (white bars) or presence (grey bars) of tryptophan (100 μ g/ml). Bacterial growth was determined photometrically after 24 hrs (B). Furthermore, cell supernatants were used as culture medium for PBL (1×10^5 /well, suspended in 10 μ l of tryptophan-free RPMI 1640 medium), stimulated with anti-CD3 antibodies either in presence (grey bars) or absence (white bars) of tryptophan (100 μ g/ml) (C). Data were given as mean \pm S.E.M. of three independent experiments, each done in triplicate. Asterisks indicate significant inhibition of bacterial growth or of T-cell responses ($P < 0.001$).

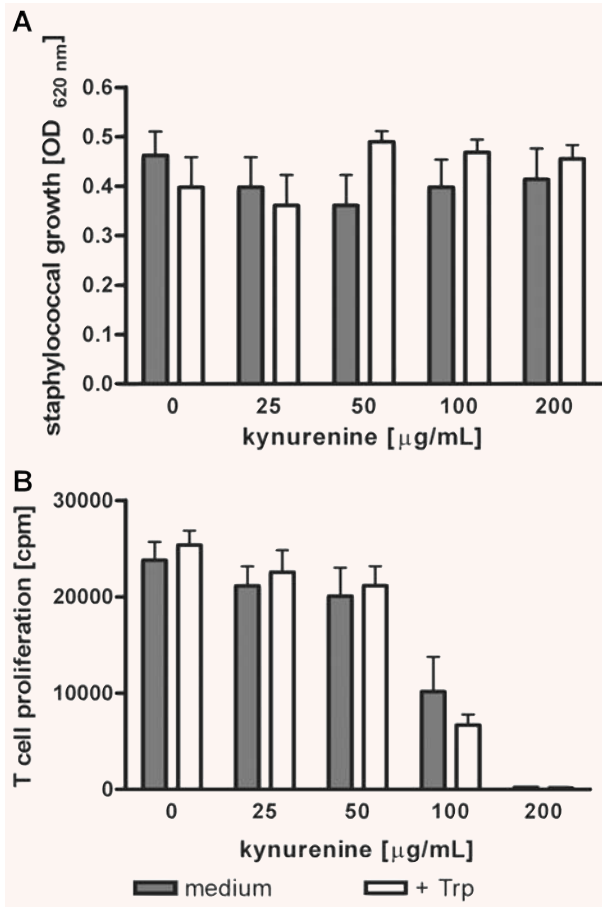


Fig. 5 Different effects of kynurenine on the proliferation of T cells and bacteria. Staphylococcal growth in presence of different concentrations of kynurenine was analysed photometrically (A). Anti-CD3-driven T-cell activation was analysed in absence or presence of different concentrations of kynurenine (B). In both experiments, a part of the cultures was supplemented with tryptophan (100 $\mu\text{g/ml}$). Data are given as mean \pm S.E.M. of three independent experiments, each done in triplicate.

groups showing IDO-dependent growth inhibition of parasites [26, 39], bacteria [40, 41] and viruses [29, 42]. However, to date only indirect *in vivo* evidence for the involvement of IDO in the antimicrobial defence has been described. For example, increased concentrations of kynurenines were found in the cerebrospinal fluid of patients with HIV-encephalitis [43]. In mice infected with *Plasmodium berghei*, IDO protein was detected immunohistochemically within endothelial cells [44]. Furthermore, in toxoplasma-infected mice a dramatic drop in the tryptophan concentration in lung tissue was accompanied by an increase in kynurenine concentration [45]. In contrast, IDO-mediated effects in mice *in vivo* have been described in the fields of transplant immunology, tumour immunology and in autoimmune and allergic diseases. For example, the inhibition of IDO by 1-MT *in vivo* resulted in a worsening of the symptoms in an experimental autoimmune

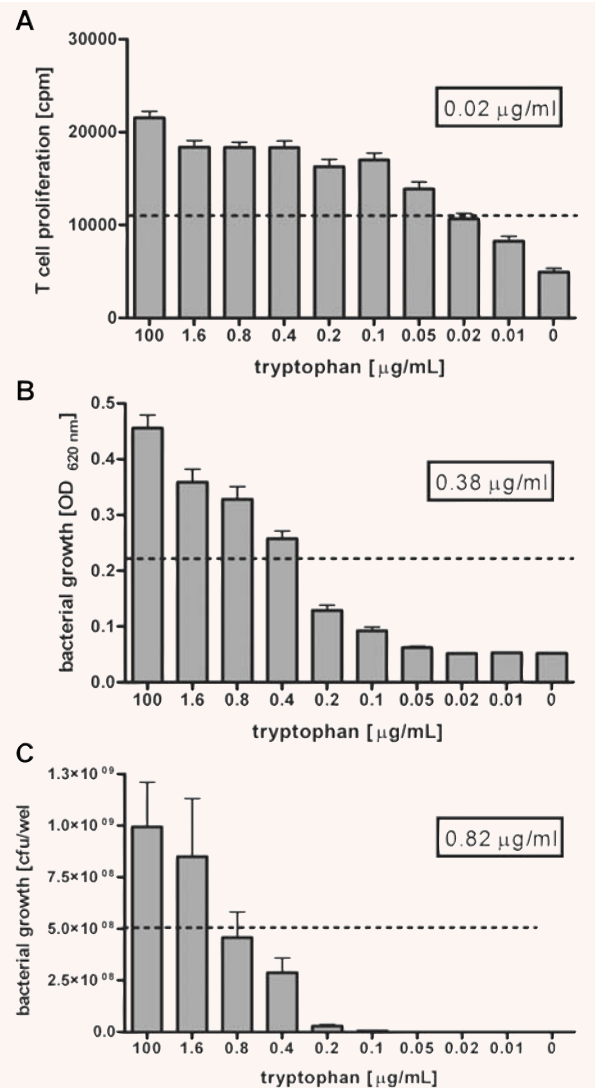


Fig. 6 Differences in the tryptophan requirement between T cells and bacteria. Supernatants harvested from A459 cells, incubated with or without IFN-g (1000 U/ml), were used as culture medium. Anti-CD3-driven T-cell proliferation in conditioned medium in the presence or absence of different concentrations of supplemental tryptophan (A). Staphylococcal growth in conditioned medium in the presence or absence of different concentrations of supplemental tryptophan was determined photometrically (B) or using the cultivation method (C). Data are given as mean \pm S.E.M. of 18 independent experiments, each done in triplicate. A dotted line indicates half maximal responses. The paired t-test indicates a significant lower tryptophan requirement for T cells in comparison to bacteria for all tryptophan concentrations below 0.8 mg/ml ($P = 0.0014$).

encephalomyelitis model [46] and the induction of IDO in dendritic cells has been shown to block the development of diabetes in non-obese-diabetic (NOD) mice [47]. In both examples, IDO induction in professional APC is necessary. In transplant immunology and

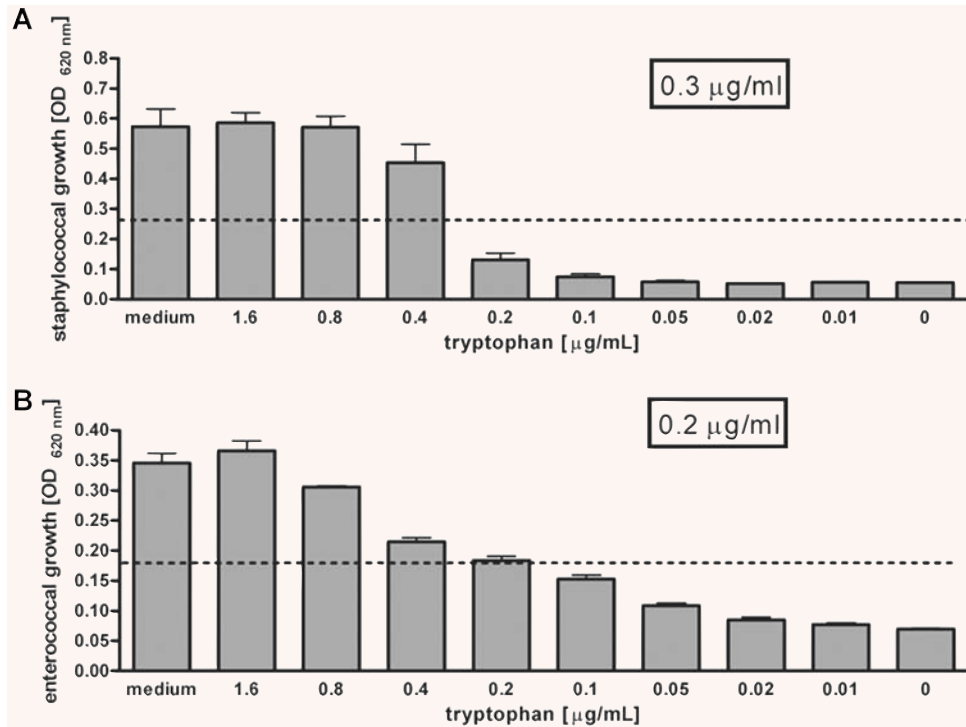


Fig. 7 Determination of the tryptophan requirement of different bacteria. Four different strains of *Staphylococcus aureus* (A) or four different strains of enterococci (B) were cultured in conditioned medium from IFN- γ (500 U/ml) stimulated A549 cells in absence or presence of different concentrations of tryptophan. Data are shown as mean \pm S.E.M. of four independent experiments, each done in triplicate. A dotted line indicates half maximal proliferation.

tumour immunology IDO-mediated effects by professional and by NP-APC have been specified. For example, Beutelsbacher *et al.* described that corneal cells expressing IDO inhibit the rejection of allogeneic cornea transplants [48]. Even in tumour immunology, IDO⁺ professional APC, as well as tumour cells, have been involved in the induction of T-cell tolerance. Here, we show, as in previous reports, that IDO⁺ tumour cells (86HG39 and A549), as well as native cells such as fibroblasts (HFF), are capable of inhibiting T-cell proliferation. In addition, we reveal that the inhibition of T-cell growth by 86HG39 and A549 cells is predominantly mediated by tryptophan depletion. Nevertheless, under special conditions tumour cells are capable of producing kynurenine in quantities sufficient to mediate an inhibition of T-cell growth, as displayed in Figs 4 and 5. This effect, mediated by the tryptophan degradation product kynurenine, could not be abrogated by supplemental tryptophan. The toxic effects of kynurenine on T-cell proliferation have been described independently by the groups of Terness and Frumento [20, 21]. Both groups defined, as we did, that about 100 μ g/ml of kynurenine were necessary to mediate a half maximal inhibition of anti-CD3 antibody-driven T-cell proliferation.

In addition, we revealed that IDO-mediated antibacterial effects are solely due to tryptophan depletion, while the production and accumulation of tryptophan metabolites do not influence the growth of bacteria. Furthermore, we found that T cells are more sensitive to the toxic effects of kynurenine than tumour cells [27]. This opens up the possibility that tumour cells benefit from tryptophan degradation, which might protect them from an undesirable anti-tumour T-cell response.

In this manuscript, we aimed to compare IDO-mediated antimicrobial and immunoregulatory effects. Bacteria usually prefer to use amino acids from their environment rather than synthesize them *de novo*, since amino acid synthesis has a high cost in terms of energy expenditure. Especially the biosynthesis of tryptophan requires approximately seven times the energy which is necessary to produce other amino acids, *e.g.* glycine or alanine [49]. Many human cells are capable of controlling the growth of pathogens after IFN- γ -induced expression of IDO activity, while murine NP-APC cells fail to express IDO activity.

Besides the different role of IDO in antimicrobial defence in human and murine cells, there are also differences in IDO induction and regulation between professional and NP-APC. For example, murine and human dendritic cell subsets have been described, which produce an enzymatically inactive IDO protein. Thus, a second signal is seemingly necessary to initiate IDO activity. It was suggested that in human, as well as in murine macrophages, signals mediated *via* the costimulatory molecules B7-1 and B7-2 might be involved in IDO activation and/or induction. These costimulatory molecules are usually absent in human fibroblasts and astrocytes [50]. However, in these cells stimulation with IFN- γ alone is sufficient to induce IDO expression and enzyme activity. Analysing NP-APC of human origin, we and others have found that these cells, after stimulation with IFN- γ , always express IDO protein and demonstrate IDO activity.

We decided to define the tryptophan threshold concentration necessary to mediate an inhibition of bacterial and T-cell growth, since the bacteria used in this study (staphylococci and enterococci)

are capable of growing in culture medium even in the absence of feeder cells. This is in striking contrast to other pathogens, described to be under the control of IDO, since *Toxoplasma gondii* and viruses depend on host cells for growth. In our culture system, these host cells should themselves respond to the IFN- γ present in the conditioned medium, display IDO activity. Hence, the feeder cells (present in the culture or separated by a semi-permeable membrane) would degrade tryptophan during the 72 hrs necessary for parasite or virus growth. This would thus result in a dramatic overestimation of the threshold tryptophan concentration.

In a recent review, Mellor *et al.* stated that the fact that IDO slows pathogen replication does not necessarily mean it would be a useful host defence mechanism, since the IDO-mediated inhibition of T-cell responses might benefit the pathogen more than the host [1, 23]. Using an *in vivo* model of HIV-1 encephalitis in NOD/severe combined immunodeficiency disorder (SCID) mice, reconstituted with human PBL, Potula *et al.* showed that the inhibition of IDO by 1-MT results in an enhanced T-cell response and a more efficient elimination of virus-infected macrophages [51]. Analysing the role of IDO in infection and immunoregulation a recent review suggested that antimicrobial effects, mediated by IDO, might be beneficial in acute infection and deleterious in chronic infection [52].

Data covering the quantitative aspects concerning tryptophan concentrations during T-cell activation are rare. In independent experiments Munn *et al.* and Lee *et al.* found a threshold concentration of tryptophan for a 50% inhibition of T-cell growth of about 0.5–1 μ M (corresponding to about 0.1–0.2 μ g/ml), which is about 5–10 times higher than the threshold concentration found in our experimental settings [53, 54]: In 1999, Munn *et al.* used medium conditioned by M-CSF derived MDM, cultured for 48 hrs in the presence of OKT3 and T cells. Using this cell supernatant, they observed that supplementation with about 0.5–1 μ M of tryptophan was necessary to reach half maximal T-cell proliferation in comparison to T-cell proliferation in tryptophan-rich medium. They used 4×10^4 M-CSF differentiated macrophages/well and 2×10^5 corresponding homologous T cells in the presence of soluble OKT3 antibodies and the T-cell response was determined using ^3H -thymidine after 72 hrs. In this setting a comparably high number of macrophages were present during T-cell activation and it was shown by the same authors that macrophages, in contrast to monocytes, which were present in our culture system, are far more potent mediators of an

IDO-dependent tryptophan degradation. Therefore, we assume that the macrophages, present in the second round of T-cell proliferation, were capable of cleaving the added tryptophan with high efficiency and thus, the minimum threshold concentration of tryptophan was overestimated. The same effect might have occurred in the experimental setting, described by Lee *et al.* [54], consisting of murine T lymphocytes, stimulated by alloantigens in tryptophan-free chemically defined medium. These authors, too, found a threshold tryptophan concentration of about 1 μ M. However, also in this paper, an extremely high number of cells (4×10^6 responder and 4×10^6 stimulator splenocytes per millilitre) were cultured together, which may have likewise resulted in an overestimation of the minimum tryptophan threshold concentration.

Nevertheless, the 1- μ M tryptophan concentration threshold, necessary to mediate a 50% inhibition of T-cell growth, determined by the groups of Mellor and Lee [53, 54], is still 4 times lower than the threshold concentration for bacteria, measured by us using the CFU-method (Fig. 6C).

In summary, our data indicate that IDO expression in human NP-APC plays several roles in inflammatory diseases. In human NP-APC, tryptophan degradation, caused by IDO, is capable of mediating both, antibacterial and immunoregulatory effects. While immunoregulatory effects are mediated *via* tryptophan starvation and by the production of the toxic metabolite kynurenine, antibacterial effects are mediated by tryptophan depletion only. In the case of tumour cells, which frequently express IDO activity, tryptophan starvation and the production of kynurenines might represent an escape mechanism that inhibits the induction of a T-cell-mediated anti-tumour response. Therefore, we postulate a new model, linking both IDO-mediated effects, occurring during bacterial infections of soft tissue: Firstly, the growth of microorganisms is inhibited and secondly, at a later time-point, the T-cell effector phase is inhibited, acting as a protective feedback mechanism against a possible overshooting T-cell response.

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