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Antimicrobial and immunoregulatory properties of human tryptophan 2,3-dioxygenase

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In mammals, the regulation of local tryptophan concentrations by the IFN-γ-i inducible enzyme IDO is a prominent antimicrobial and immunoregulatory effector mechanism. Here, we show for the first time that another tryptophan-degrading enzyme, the liver-specific tryptophan 2,3-dioxygenase (TDO), is also capable of mediating antimicrobial and immunoregulatory effects. Using a tetracycline inducible eukaryotic system, we were able to express recombinant TDO protein, which exhibits functional properties of native TDO. We found that HeLa cells expressing recombinant TDO were capable of inhibiting the growth of bacteria (*Staphylococcus aureus*), parasites (*Toxoplasma gondii*) and viruses (herpes simplex virus). These TDO-mediated antimicrobial effects could be blocked by the addition of tryptophan. In addition, we observed that, similar to IDO-positive cells, TDO-positive cells were capable of inhibiting anti CD3-driven T-cell proliferation and IFN-γ production. Furthermore, TDO-positive cells also restricted alloantigen-induced T-cell activation. Here, we describe for the first time that TDO mediates antimicrobial and immunoregulatory effects and suggest that TDO-dependent inhibition of T-cell growth might be involved in the immunotolerance observed *in vivo* during allogeneic liver transplantation.

Key words: Kynurenine · T cells · Tolerance · Tryptophan · Tryptophan 2,3-dioxygenase

Introduction

L-tryptophan (L-trp) is an essential amino acid that is not only required for the synthesis of proteins, but also for the biosynthesis of neurotransmitters such as serotonin and melatonin. Nevertheless, most of the dietary L-trp is catabolised *via* the kynurenine pathway to kynurenines and these are eliminated in the urine (hence their names). A small amount of the dietary tryptophan is used to produce the physiological relevant NAD[1, 2].

In mammals, the first and rate-limiting step of the kynurenine pathway, namely the oxidation of tryptophan to *N*-formyl kynurenine, is catalysed by the hepatic tryptophan 2,3-dioxygenase (TDO, EC 1.13.11.11) and the extra-hepatic IDO (EC 1.13.11.52). Recently, a third tryptophan-degrading enzyme, IDO2, was described, however, the *in vivo* function of this enzyme remains speculative [3, 4].

The function of IDO has been most intensively analysed and shown to be involved in several essential processes. Being an immunoregulated enzyme with antimicrobial and immunoregulatory function, IDO regulates T-cell responses and induces maternal tolerance towards the allogeneic foetus [5]. Interestingly, IDO also seems to play a role in cancer progression as the magnitude of its expression, for example, correlates with the overall survival of serous-type ovarian cancer patients, specifying IDO as a marker for a poor prognosis [6].

IDO mediates its activity locally, in inflamed tissue or lymph nodes. In contrast, TDO activity is mainly expressed in the liver and is not regulated by the immune system but does have

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systemic effects by controlling the tryptophan levels in the blood. This role in tryptophan homeostasis is supported by the fact that the tryptophan concentration itself influences TDO activity by stabilising the enzyme complex [7].

Although TDO, IDO and IDO2 catalyse the same 2,3-dioxygenase function, TDO shares little sequence identity with either IDO or IDO2, both of which show significant conserved sequences at the amino acid level, especially in the region important for the catalytic activity [8, 9].

Although the crystal structure of IDO2 has not yet been published, crystallographic analysis revealed a high structural similarity of IDO and TDO with respect to the heme-binding motif, despite the fact that IDO was found to be a monomeric enzyme and TDO a tetramer [9]. Recently, resonance Raman and optical absorption spectroscopy were used to compare structural and functional properties of IDO and TDO. The data obtained by Batabyal *et al*. suggest that the dioxygenase reaction mediated by both enzymes is carried out *via* distinct mechanisms [10].

Additionally, it has been shown that IDO has a broader substrate spectrum, including L- and D-tryptophan, serotonin, tryptamine and L-5-hydroxykynurenine [11]. In contrast, TDO was initially discovered to be an L-trp-specific enzyme and only later shown to cleave related derivatives such as 6-fluoro-tryptophan [12, 13].

Despite all these differences, the result of the L-trp catabolism, executed by all 2,3-dioxygenases, is the generation of *N*-formyl kynurenine, which is then converted to further important metabolites such as quinolinic acid, which causes neuronal death in the brain due to the activation of a subpopulation of NMDA receptors, whereas another tryptophan metabolite, kynurenic acid, has an antagonistic effect on the same receptors [14]. Moreover, the metabolites kynurenine and 3-hydroxykynurenine have been shown to be involved in the regulation of T-cell growth [15].

In summary, TDO and IDO share the capacity to mediate an oxidative cleavage of tryptophan and to initiate the kynurenine pathway. However, both enzymes differ in nearly all other aspects ranging from their amino acid sequence, tissue distribution, substrate specificity and regulation of enzyme activity. The important role of IDO in the regulation of local immune responses is generally accepted. Here, we analyse whether or not the liver-specific TDO, in addition to its role in systemic tryptophan homoeostasis, might also mediate antimicrobial and immunoregulatory effects within liver tissue.

Results

Inducible TDO gene expression in HeLa cells

HeLa T-Rex cells were transfected with pcDNA4-TDO vector containing human liver TDO cDNA. We obtained several different cell lines, which degraded tryptophan and produced kynurenine after treatment with tetracycline. Thereafter, we obtained multiple cell clones, which were all able to produce kynurenine after tetracycline stimulation (Fig. 1A).

To show IDO or TDO expression in stimulated cells immunofluorescence (Fig. 1B) and Western blot analyses (Fig. 1C) were performed. These data indicate that unstimulated HeLa-TDO cells did not express IDO or TDO protein. After stimulation with tetracycline, TDO protein was detected using an anti-myc or a TDO-specific monoclonal antibody. No IDO protein was detected in tetracycline-stimulated HeLa-TDO cells. As a control HeLa-TDO cells were stimulated with IFN-γ and showed a strong IDO expression while the TDO protein was absent.

In addition, HPLC analysis indicated that TDO-positive HeLa cells indeed cleaved tryptophan. Furthermore, quantitative analysis suggested that there is nearly an equimolar conversion of tryptophan to kynurenine since the sum of both metabolites is not significantly different in all experimental groups (Fig. 2A). As additional control, the substrate specificity of TDO was characterised biochemically. As shown in Fig. 2B, TDO-positive cells, as expected, are able to metabolise L-trp while these cells fail to cleave D-tryptophan. In contrast, IFN-γ-activated IDO-positive HeLa cells are capable of metabolising both forms of tryptophan. In addition, 1-methyl-L-trp (1-MT), a specific inhibitor of IDO, reduces kynurenine production by IFN-γ-stimulated, IDO-expressing HeLa cells, whereas the same amount of 1-MT did not significantly reduce the kynurenine content in the supernatants of tetracycline stimulated, TDO-expressing cells (Fig. 2C).

Antimicrobial effects mediated by TDO

Since the publication of data by Pfefferkorn [16], it has been recognised that tryptophan degradation mediated by the IFN-γ inducible IDO is involved in the defense against several pathogens, including *Toxoplasma gondii*, *Staphylococcus aureus* and herpes simplex virus [16–18]. In order to show whether or not TDO-mediated tryptophan degradation is also sufficient to mediate antimicrobial effects, we used HeLa-TDO cells as host cells for analysing antiparasitic, antibacterial and antiviral effects. Interestingly, as shown in Fig. 3, HeLa-TDO cells, after stimulation with tetracycline, are capable of restricting the growth of *T. gondii*, *S. aureus* and herpes simplex virus. In all three settings TDO-mediated tryptophan degradation is responsible for this antimicrobial effect, since supplemental tryptophan blocks the antimicrobial effects. Done as a control, untransfected HeLa cells stimulated with the same amount of tetracycline did not influence the growth of the microorganisms used (data not shown).

Immunoregulatory effects mediated by TDO

The regulation of the local tryptophan concentration by IDO was found to be involved in the mediation of immunoregulatory effects [19]. In analogy, we therefore were interested in analysing potential immunoregulatory effects of TDO. Thus, tetracycline stimulated or unstimulated HeLa-TDO cells were co-cultured with PBL and a mitogenic antibody against CD3

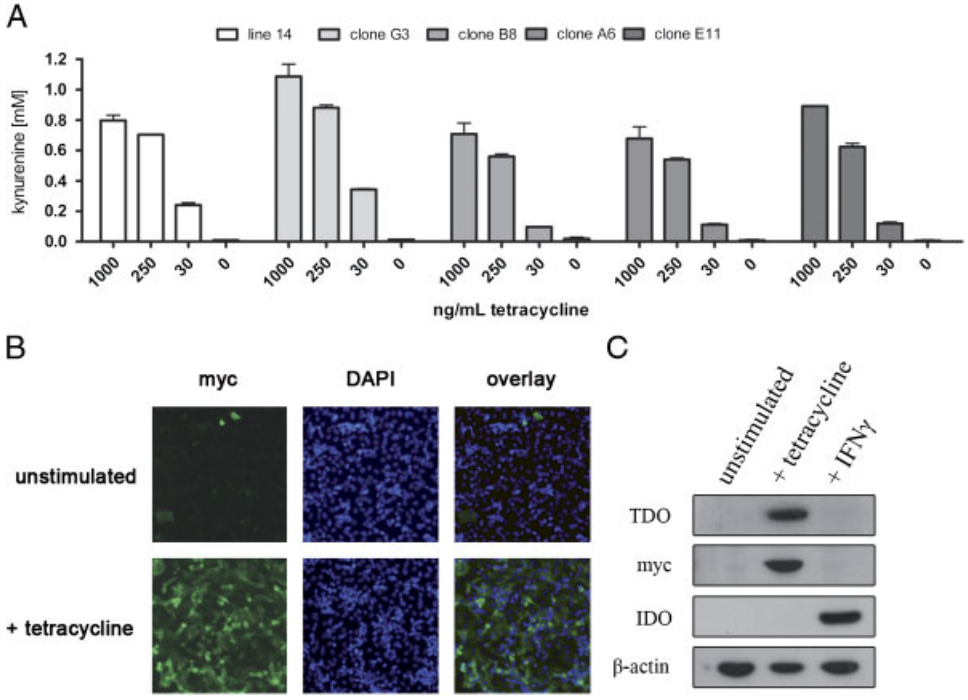

Figure 1. Tetracycline-stimulated HeLa-TDO cells express active TDO protein. (A) HeLa-TDO cell clones metabolise L-trp to kynurenine upon stimulation with different concentrations of tetracycline. Data are given as mean kynurenine concentration found in supernatants from triplicate cultures ± SEM. (B) Immunofluorescence analysis of transfected HeLa-TDO cells with an anti-myc antibody. Cells prestimulated with 100 ng/mL tetracycline for 48 h display a strong increase of TDO-myc immunoreactivity. (C) Western blot analysis of HeLa-TDO cells using anti-myc-, anti-β-actin-, anti TDO- or anti IDO-antibodies. Cells remained unstimulated or were stimulated with 1000 U/mL IFN-γ or 30 ng/mL tetracycline for 24 h. TDO protein was detectable with polyclonal anti-TDO antibody as well as with the anti-myc antibody. As expected, IDO protein was only detectable in IFN-γ-stimulated cells.

either in the presence or in the absence of tryptophan. As shown in Fig. 4A, pre-treatment of HeLa-TDO cells with tetracycline resulted in a decreased proliferation of co-cultured T cells which was abrogated by supplemental tryptophan. However, as seen in Fig. 4a, the addition of tryptophan into the untreated control group also results in an enhanced T-cell proliferation. These data suggest that, as described earlier by others, HeLa cells, present as third party cells during T-cell activation, inhibit T-cell proliferation. This immunosuppressive effect was ascribed to IDO induction by IFN-γ produced by T cells within the cell culture [20]. Therefore, the antiproliferative effect, seen in the tetracycline-treated group, is possibly a combined effect of IDO- and TDO-activity. In order to prove a possible synergistic effect of IDO and TDO, we stimulated HeLa-TDO cells with low concentrations of tetracycline or IFN-γ which were on their own unable to induce an antibacterial effect. However, the combination of both resulted in the induction of a strong antibacterial effect, which could be blocked by the addition of tryptophan as shown in Fig. 4B. In order to avoid IDO-TDO synergistic effects during T-cell activation, we excluded HeLa cells from our experimental setting by changing the culture conditions. Hence, we used supernatants harvested from IDO- or TDO-positive HeLa-TDO cells as culture medium for T cells. As shown in Fig. 5, supernatants harvested from IFN-γ or tetracycline-stimulated HeLa-TDO cells are both capable of restricting T-cell growth. This antiproliferative effect was abrogated in the presence of supplemental tryptophan. Furthermore, the presence of 1-MT during the stimulation of HeLa cells completely blocks IDO-mediated inhibition of T-cell proliferation, whereas it did not antagonise the TDO-mediated antiproliferative effect. Furthermore, T cells activated by a stimulatory antibody against CD3, did not only show a reduced proliferative response, but also failed to produce IFN-γ (Fig. 6) when they are stimulated in supernatants harvested from IDO- or TDO-positive HeLa cells.

In order to analyse TDO-mediated inhibition of T-cell growth in a more physiological setting, we also examined the influence of TDO activation on allogeneic T-cell response. As shown in Fig. 7, we found that supernatants from TDO-positive HeLa-TDO cells are capable of inhibiting alloantigen-driven T-cell proliferation, which was also blocked by the addition of exogenous tryptophan. We therefore conclude that TDO is capable of mediating antimicrobial and immunoregulatory effects.

Discussion

The precise regulation of local tissue concentrations of tryptophan was found to be important during infection with *T. gondii in vivo* [21]. In addition, a reduced tryptophan availability is responsible for IFN-γ-mediated antiparasitic effects against *T. gondii in vitro*. In 1989, IDO was found to be the key regulator of tryptophan concentrations responsible for antiparasitic effects

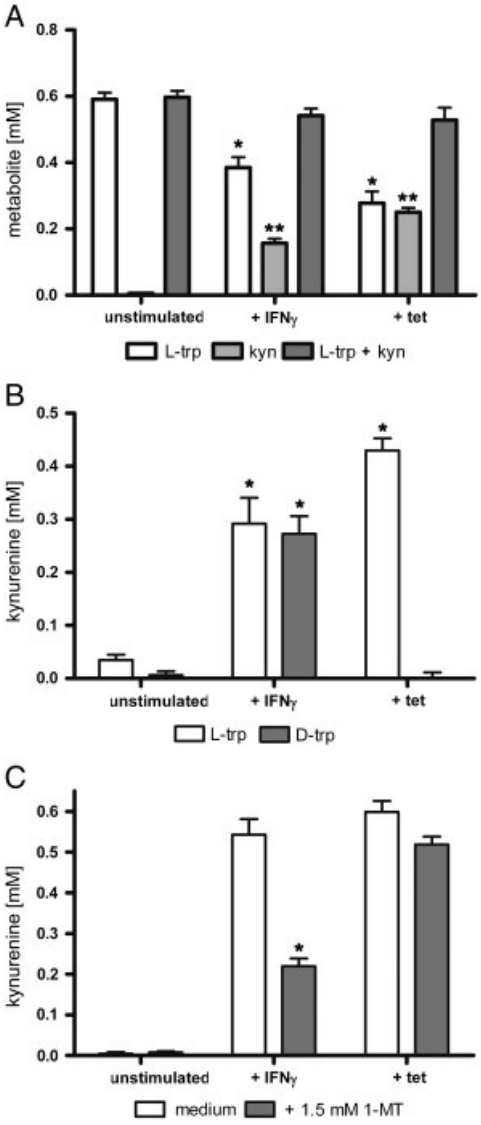

Figure 2. TDO and IDO display distinctive characteristic traits. (A) HPLC analysis shows that upon stimulation with tetracycline (30 ng/mL for 3 days) the TDO, as well as upon stimulation with IFN-γ (1000 U/mL for 3 days), the IDO enzyme metabolise L-trp (100 μg/mL L-trp) to kynurenine in an equimolar range. Data are given as mean ± SEM tryptophan or kynurenine concentration of five independent experiments. A significant reduction of the tryptophan concentration ($p<0.01$) and a significant increase of the kynurenine concentration ($p<0.01$) in comparison to unstimulated cells is marked with one (L-trp) or two kynurenine asterisks. (B) IFN-γ stimulation (500 U/mL for 36 h) of HeLa-TDO cells led to the expression of IDO enzyme that was capable of cleaving both tryptophan enantiomers (L-trp and D-trp), whereas a tetracycline (3 ng/mL) stimulation caused expression of TDO, which only metabolised L-trp. The kynurenine concentration in the cell supernatants is given as mean ± SEM of three experiments. A significant ($p<0.01$) increase of the kynurenine amount in the supernatant of stimulated cells, compared with unstimulated cells, is marked with an asterisk. (C) 1-MT, the IDO-specific inhibitor, inhibited exclusively IDO activity, whereas TDO activity was not significantly altered. HeLa-TDO cells were stimulated with 1000 U/mL IFN-γ or 50 ng/mL tetracycline and IDO and TDO activity was determined by measuring the kynurenine concentration in cell supernatants. Data are given as mean ± SEM kynurenine concentration in cell supernatants from five independent experiments. A significant ($p<0.01$) decrease of kynurenine production in 1-MT-treated cells is marked with an asterisk.

mediated by human fibroblasts [16]. In subsequent analyses, different human cells were found to use IDO as an antiparasitic effector molecule. In these experiments, cells were usually stimulated with IFN-γ to produce IDO and therefore it seemed likely that, in addition to IDO, also other IFN-γ-induced mechanisms might be involved in the observed antiparasitic effect. Subsequently, however, it has been shown that recombinant IDO expressed in the absence of IFN-γ mediates the same antiparasitic effect. In addition, further data obtained with parasites expressing the *trpB* gene from *Escherichia coli*, and therefore independent of exogenous tryptophan, are able to replicate in IDO-positive cells [22]. In summary, there is no doubt that IDO-mediated antiparasitic effects result from a depletion of tryptophan.

In the case of antibacterial and antiviral effects, mediated by IDO, this is not quite so clear, but the abrogation of the antimicrobial effects by excess tryptophan or by the IDO inhibitor 1-methyl-tryptophan argues strongly in its favour [23]. In addition, we found recently that the minimal tryptophan concentration necessary for T-cell and bacterial growth was different since bacteria need up to 40-fold higher tryptophan concentrations [24].

Here, we show that cells expressing recombinant TDO, an enzyme which is different from IDO in nearly all aspects other than the basic enzyme function, are capable of mediating antiparasitic, antibacterial and antiviral effects. We therefore conclude that in this setting only the reduction of the local tryptophan concentration is responsible for the observed antimicrobial effects and no additional effects mediated by IFN-γ or by the IDO protein itself are involved. This raises the question, whether or not this might also occur *in vivo*. The role of IDO in antimicrobial defense is generally accepted since IDO is expressed in inflamed tissues under control of proinflammatory cytokines such as IFN-γ, TNF-α and IL-1. TDO, in contrast, is constitutively expressed in the liver, and the main mediator enhancing TDO expression is cortisone, which has an anti-inflammatory effect. Furthermore, TDO activity appears to be reduced during infectious diseases, in which a strong IDO induction is found [25]. Therefore, we suggest that the mediation of antimicrobial effects is unlikely to be a primary function of liver TDO. However, new data concerning expression of *tdo* have recently been published in which TDO protein was found in rat skin and in the human central nervous system [26, 27]. In these tissues, the functional role of TDO and the regulation of TDO activity are unknown, and this finding leaves the possible involvement of TDO in antimicrobial defence open.

The key observation that tryptophan degradation is involved in tolerance against allogeneic foetuses lead to a flood of data characterising the role of IDO in the regulation of T-cell responses in autoimmune diseases, tumour immunology and transplant medicine. Today, there is no doubt that IDO is one of the key players in the regulation of T-cell proliferation. The main mechanism by which IDO activity mediates these inhibitory effects on T-cell activation is unclear, but tryptophan starvation,

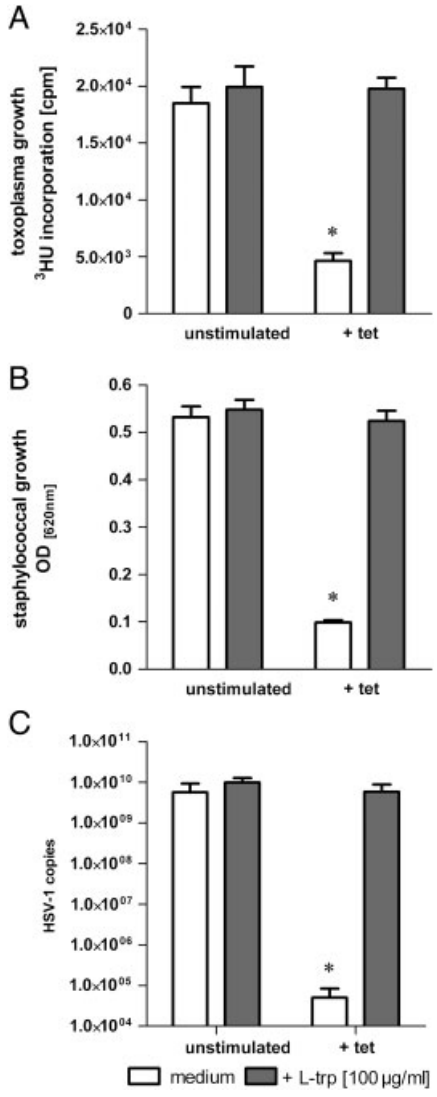

Figure 3. Expression of TDO inhibits microbial growth. Transfected HeLa-TDO cells were stimulated with 50 ng/mL tetracycline or remained unstimulated. Microorganisms were added in medium with or without supplemental L-trp (L-trp, 100 µg/mL). A significant ($p<0.01$) reduction of the pathogen growth is marked by an asterisk. (A) *T. gondii* growth was determined by the incorporation of ^{3}H-uracil. HeLa-TDO cells expressing TDO inhibited the growth of the intracellular parasite. The addition of L-trp abrogated the antiparasitic effect. Data are given as mean cpm+SEM of five independent experiments. (B) *S. aureus* growth was measured by optical density at 620 nm of the culture supernatant. The TDO-mediated antibacterial effect was blocked by the addition of L-trp. Data are given as mean OD+SEM of five independent experiments. (C) HSV-1 replication, identified by quantitative real-time PCR, was similarly suppressed by the expression of TDO in transfected HeLa-TDO cells. The viral replication was restored after addition of L-trp. Data are given as mean HSV-1 copy number + SEM of three independent experiments.

the induction of an integrated stress response by empty tRNA arriving at the ribosome, the mediation of toxic effects by tryptophan degradation products and the induction of inhibitory DC, for example by kynurenine, are contenders [15, 28–31]. In this manuscript, we show for the first time that cells expressing TDO are capable of inhibiting mitogen- as well as alloantigen-driven T-cell responses. Furthermore, we show that TDO-positive cells not only inhibit T-cell proliferation but also block IFN-γ production. In addition, we found that supplemental tryptophan abrogates these TDO-mediated immunoinhibitory effects. Therefore, it is at least possible that TDO is involved in immunoregulation. Indeed, Suzuki and Tatsumi were first in suggesting a putative immunoregulatory role for TDO. Both groups described an unexpected TDO expression in early murine concepti and in the mouse placenta and suggested that this tryptophan-degrading enzyme, similar to IDO, might be involved in the tolerance against allogeneic foetuses [32, 33]. However, to date no data on the impact of TDO-mediated tryptophan degradation in these locations have been published and TDO knockout mice are not yet available to address this question directly. Furthermore, IDO was found to be involved in the induction of maternal tolerance, however, the outcome of allogeneic pregnancy in IDO knockout mice is not altered, suggesting that IDO is not the only factor involved in this setting [34]. Since the IDO inhibitor 1-methyl-tryptophan, used to show the involvement of IDO in the induction of tolerance in pregnancy, is specific for IDO, this would argue against the possible involvement of TDO in the experiments conducted by Munn *et al.* [19]. Thus, the definitive answer to the question of the involvement of TDO in maternal tolerance and pregnancy is not yet out.

TDO in general is described to be a constitutively expressed liver-specific enzyme and the liver displays inherent tolerogenic properties. These tolerogenic properties of the liver are suggested to be involved in the development of oral tolerance and in the immune privilege of hepatic allografts [35]. For example, in different animal models, it has been observed that liver transplants are successful even across major histocompatibility complex barriers. Moreover, liver transplants were described to induce donor-specific tolerance [36]. In humans, the tolerogenic effects mediated by liver transplants are controversially discussed [37, 38]. The underlying mechanisms responsible for these tolerogenic effects are not completely understood, and many factors may be involved.

The reduction of local tryptophan concentration has been described to be involved in the induction of local immunosuppression and additionally to induce systemic tolerance. For example, data obtained with corneal cells, constitutively expressing recombinant IDO, show that tryptophan degradation *in vivo* mediates tolerance against allogeneic cornea transplants [39]. This is mediated by the induction of local inhibitory effects on T-cell growth, but also in part by the induction of systemic tolerance. We suggest that TDO-mediated tryptophan degradation might act in a similar way during liver transplantation. TDO, expressed by hepatocytes might influence the cellular immune reaction within the liver by two different, not mutually exclusive mechanisms. First of all, it could be assumed that TDO, or TDO in combination with IDO, reduces the availability of tryptophan, which then inhibits T-cell responses in liver parenchyma. This would be of importance for the regulation of the effector phase of T cells, as well as the stimulation of effector-memory cells in the

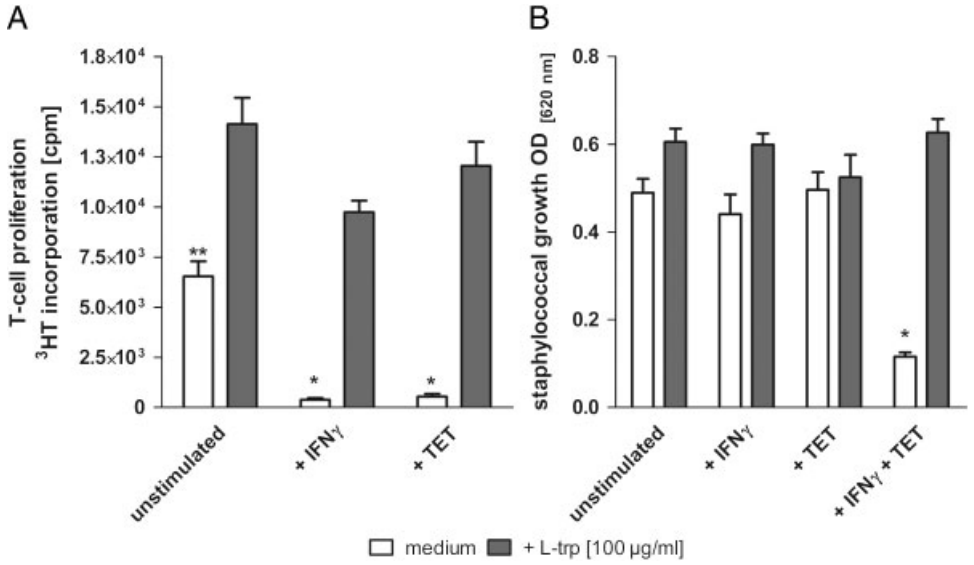

Figure 4. Co-expression of IDO and TDO has synergistic effects on T-cell proliferation and bacterial growth. (A) Expression of TDO inhibits T-cell proliferation. T cells were added to prestimulated transfected HeLa-TDO cells and OKT3-driven T-cell proliferation was determined by the incorporation of ^{3}H-thymidine. IDO activity (stimulation with 500 U/mL IFN-γ) as well as TDO activity (stimulation with 50 ng/mL tetracycline) inhibited T-cell proliferation significantly ($p<0.01$, marked with an asterisk) and the inhibition could be abrogated by the addition of 100 µg/mL L-trp. Data are given as mean cpm ± SEM of four independent experiments. Note that the T-cell growth in the medium control in unstimulated cells is significantly lower than in medium with additional L-trp (marked by two asterisks). (B) The co-expression of IDO (stimulation with 31 U/mL IFN-γ and TDO (stimulation with 3.75 ng/mL tetracycline) synergistically inhibits bacterial growth in supernatants of co-stimulated cells ($p<0.01$ marked with an asterisk) in contrast to single-stimulated or unstimulated cells. Data are given as mean OD ± SEM of four independent experiments.

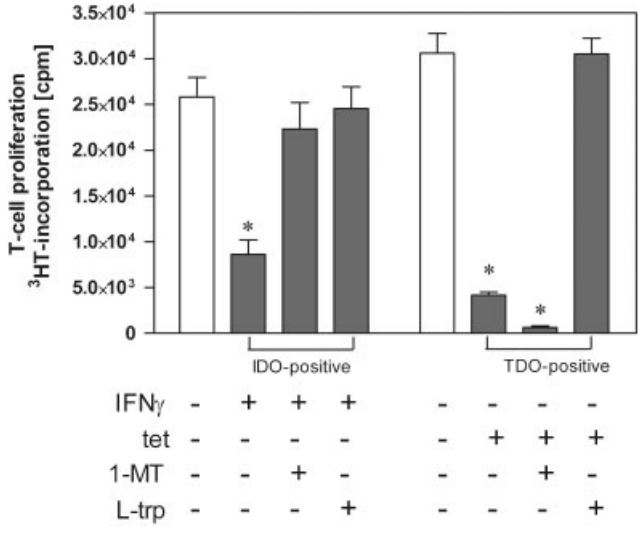

Figure 5. HeLa-TDO cells were stimulated with IFN-γ or tetracycline in the absence or presence of 1-MT for 3 days and culture supernatants were used as culture medium for OKT3-stimulated PBL. Supernatants of stimulated HeLa-TDO cells inhibit T-cell proliferation. OKT3-activated T cells proliferate in supernatants of unstimulated cells (white bars), whereas supernatants from IFN-γ or tetracycline stimulated HeLa-TDO cells expressing IDO- or TDO-activity did not support T-cell growth. The presence of 1-MT only antagonised the immunoregulatory effect of IFN-γ-activated cells, whereas supplemental tryptophan blocked the immunosuppressive effect of IDO- or TDO-expressing cells. Data are given as mean cpm ± SEM of three independent experiments. A significant inhibition of T-cell proliferation ($p<0.01$) in comparison to the unstimulated group is marked with an asterisk.

liver. On the other hand, it is known that tryptophan degradation products, for example kynurenine, can render immunostimulatory DC into tolerogenic DC and it is well described that the liver harbours a number of immunocompetent dendritic cells [31, 40]. Due to the uptake of kynurenine, produced and secreted by TDO-positive hepatocytes, these DC might become suppressive DC and after migration might inhibit T-cell responses in the regional lymph nodes or favour the development of regulatory T cells.

A further aspect arguing for a possible involvement of TDO in the protection of allogeneic liver transplants might be derived from the use of glucocorticoids in the classical immunosuppressive therapy used after liver transplantation [35]. Since corticoids are the main inducers of TDO activity, the protective effect of corticoids in this setting might at least in part, be mediated by the induction of an immunosuppressive TDO activity.

In summary, our data show that cells expressing TDO are capable of inhibiting the growth of several pathogens and are also able to downregulate cellular immune responses. These *in vitro* data are difficult to transfer into the *in vivo* situation. Presently, no TDO knockout animals are available to study the tolerogenic properties of TDO in the liver directly. However, since TDO is a conserved gene, and thus most likely essential for survival, TDO deficiency might be lethal. Unfortunately no TDO-specific inhibitors are available to date to use experimentally *in vivo*. However, despite the unclear *in vivo* function, our *in vitro* data clearly indicate that TDO might be one of the factors explaining the tolerogenic properties of liver tissue.

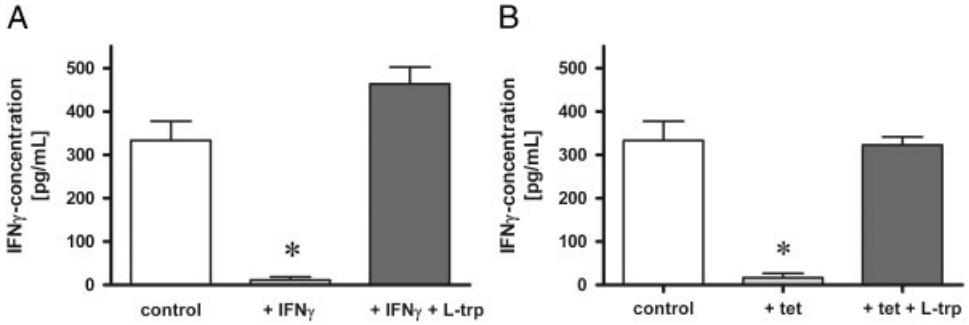

Figure 6. Conditioned medium harvested from differential stimulated HeLa-TDO cells, was used as culture medium for PBL. T cells activated in supernatants from unstimulated HeLa-TDO cells produce IFN-γ, whereas supernatants from IDO-positive (A: IFN-γ stimulated) or TDO-positive (B: tetracycline stimulated) cells inhibit IFN-γ production by T cells. This inhibitory effect could be blocked by the supplementation of tryptophan. PBL were stimulated as shown in Fig. 5. After 3 days, IFN-γ content in T-cell cultures was determined by ELISA. Data are given as mean IFN-γ production ± SEM of three independent experiments. A significant inhibition of IFN-γ production is marked with an asterisk.

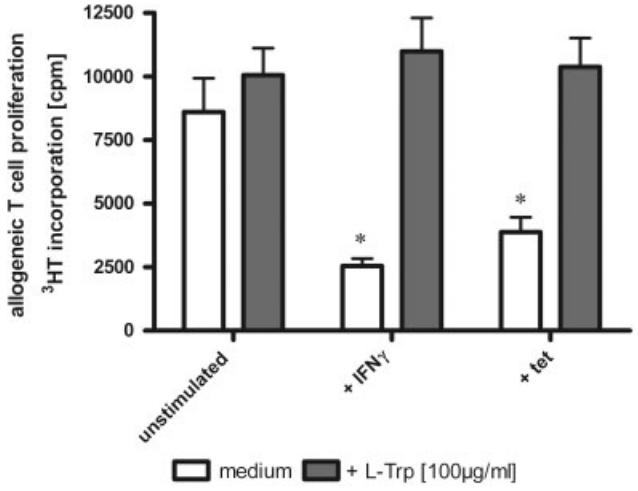

Figure 7. Conditioned medium harvested from IFN-γ or tetracycline-stimulated HeLa-TDO cells inhibits allogeneic T-cell proliferation. T-cell proliferation was induced by irradiated allogeneic PBL and determined by the incorporation of ^{3}H-thymidine after 5 days of culture. Data are shown as mean cpm ± SEM of three independent experiments. T-cell growth was observed in supernatants of unstimulated HeLa-TDO cells, whereas in supernatants of IDO-positive (stimulated with 1000 U/mL IFN-γ) or TDO-positive (stimulated with 75 ng/mL tetracycline) HeLa-TDO cells T-cell proliferation was abolished. This effect could be abrogated by the addition of L-trp. A significant inhibition of T-cell proliferation ($p<0.01$) in comparison to the unstimulated group is marked with an asterisk.

Materials and methods

Media, cells and reagents

Iscove's modified Dulbecco's medium (Gibco, Grand Island, USA), with and without tryptophan, supplemented with 5–10% heat-inactivated FBS was used as the cell culture media. T-REx-HeLa™ cells were purchased from Invitrogen (Karlsruhe, Germany).

Cells were cultured in supplemented Iscove's medium in culture flasks (Costar, Cambridge, USA) and split weekly in 1:10 ratios by using trypsin/EDTA (Gibco). Mycoplasma contamination was regularly excluded, using both culture methods and PCR.

PBL were prepared from heparinised blood of healthy donors after density gradient centrifugation.

Recombinant human IFN-γ was purchased from Tebu-Bio (Offenbach, Germany). L-trp, D-tryptophan, kynurenine and 1-methyl-L-trp were purchased from Sigma-Aldrich (Deisenhofen, Germany).

Production of recombinant HeLa-TDO cells

TDO cDNA was synthesised from human liver mRNA. The coding region, corresponding to 406 amino acids, was amplified *via* PCR, using the forward primer 5′-ATAATAGGTACCATGAGT GGGTGCCCA-3′ and the reverse primer 5′-ATAATACTCGAGATCT-GATTCATCACTG-3′, encoding for a 5′ KpnI or 3′ XhoI restriction site, respectively. The amplified DNA fragment was inserted into the inducible eukaryotic expression plasmid pcDNA4/TO/*myc*-HisA (Invitrogen). An aliquot of 1 µg of the plasmid was transfected into the stably expressing tetracycline repressor cell line T-REx-HeLa™ using the manufacture transfection kit Lipofectamine 2000 Reagent (Invitrogen) to establish HeLa-TDO cells. The cloning of positive transfected cells (after immunofluorescence, Western blot and kynurenine assay analysis) was carried out by limiting dilution. The expression of TDO was induced by adding tetracycline 0–1000 ng/mL to the cells for 24–72 h at 37°C. In first experiments, we found that 30–50 ng/mL of tetracycline were sufficient to induce maximal antibacterial effects, whereas tetracycline in doses below 5 ng/mL induce no or only moderate inhibition of bacterial growth.

Immunofluorescence analysis

In total 2×10^5 transfected HeLa cells were stimulated with 100 ng/mL tetracycline for 48 h in 24-well plates or left unstimulated as a control. Subsequently, an anti-myc antibody (Invitrogen), 1:500 diluted in PBS/0.2% horse serum, was used to detect the expression of TDO. Thereafter, the cells were incubated with DAPI, fixed and analysed by UV-microscopy (Nikon Eclipse TE 200).

Western blot analysis

In total 1×10^6 HeLa-TDO cells were stimulated with 30 ng/mL tetracycline or 1000 U/mL IFN-γ for 24 h. Thereafter, cells were harvested, and lysed by three freeze/thaw cycles in the presence of a protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). Separation of the proteins was performed by electrophoresis using 10% NuPAGE Novex *bis*-Tris mini gels and the appropriate electrophoresis system from Invitrogen. Seeblue Plus2 marker was used as molecular mass standard. After the proteins were semi-dry blotted on nitrocellulose membranes (CarboGlas, Schleicher & Schüll, Dassel, Germany), the membranes were blocked in 3% w/v skim milk powder in TBS. An overnight incubation at 4°C with the primary mouse anti c-myc antibody (1:500, Serotec, Düsseldorf, Germany), anti-β-actin antibody (1:10 000, Sigma, St. Louis, USA), anti-TDO-A antibody (1:750, kindly gifted by C. L. Miller, Johns Hopkins University, Baltimore, USA) or anti human IDO antibody (1:3000, Chemicon, Hofheim, Germany), diluted in 3% w/v skim milk powder in TBS was performed [25]. The membrane was thereafter incubated for 2 h at room temperature with goat anti-mouse HRP-conjugated or goat anti-rabbit HRP-conjugated IgG (1:10 000–70 000, Jackson Immuno Research Laboratories, Dianova, Hamburg, Germany), diluted in 3% w/v skim milk powder and 0.05% v/v Tween 20 in TBS. After additional washes, bands were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Freiburg, Germany).

Kynurenine assays

Photometric detection of kynurenine: supernatants harvested from IFN-γ-stimulated or tetracycline-stimulated cells were analysed for their kynurenine content, using Ehrlich reagent as described previously [41]. 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 were used.

Detection of tryptophan and kynurenine *via* HPLC: supernatants, harvested from HeLa-TDO cells that were incubated for 3 days in the presence or absence of tetracycline or IFN-γ and with or without L-trp, were precipitated with 1/10 v/v 25% sulfosalicylic acid. After centrifugation, 500 μL ofthe supernatant was adjusted with 85 μL 1 M NaOH to a pH of 7.2. The tryptophan and kynurenine amount was quantified, using the Gold-Universal-Grad System (Beckman Coulter, Krefeld, Germany), containing of a 126 solvent module and the 166 detector, together with a 5 μm reversed-phase column (Grom-Sil FMOC-2, 100 mm × 4 mm). The separation was performed in sodium acetate buffer (50 mM/pH 4.2) with an increasing gradient of acetonitrile, using a flow rate of 1.0 mL/min. The absorbance of the column effluent was monitored at 254 nm. The tryptophan peak was identified by means of comparison with the retention time of standards which were previously determined.

Determination of bacterial growth

For the analysis of bacterial growth in conditioned medium, *S. aureus*, obtained from routine diagnostic specimens was used. The IDO sensitivity of this strain has been analysed previously [42]. Bacteria were grown on brain heart infusion agar (Difco, Hamburg, Germany), containing 5% sheep blood and incubated at 37°C in 5% CO_2-enriched atmosphere. For use in experiments, a 24 h old single bacterial colony was picked and resuspended in RPMI 1640 (Gibco) without tryptophan. Bacteria were serially diluted in the same medium and 10 μL were added to 200 μL of conditioned medium. After incubation for 16–24 h, bacterial growth was monitored using a microplate photometer (SLT Lab instruments, Crailsheim, Germany) by measuring the optical density at 620 nm.

Determination of toxoplasma growth

T. gondii tachyzoites of the BK strain (obtained from Drs. Seitz and Saathoff, Institut für Medizinische Parasitologie, Bonn, Germany) were maintained in L929 murine fibroblasts (American Type Culture Collection, Rockville, USA) in IMDM containing 5% v/v FBS. Tachyzoites were usually harvested after 3–5 days of incubation, resuspended in tryptophan-free medium RPMI 1640 (Gibco) and then used for infection experiments.

After cytokine stimulation for 72 h, HeLa-TDO cells were infected with 2×10^4 toxoplasma tachyzoites *per* well. Toxoplasma growth was measured by the ^{3}H-uracil incorporation method [43].

Determination of viral replication

Supernatants of fibroblasts infected with HSV-I (kindly provided by K.E. Schneeweis, University Bonn, Germany) were stored in aliquots of 200 μL at −80°C. Before infection of HeLa-TDO cells, the virus containing supernatant was thawed and diluted accordingly in tryptophan-free RPMI 1640 medium.

After 72 h incubation at 37°C, duplicate cultures were infected with 1.45×10^5 HSV-I copies (equal to a $TCID_{50}$ of about 1×10^2/well) and viral replication was monitored as described previously [44]. A plasmid containing the amplified region was used as a standard. The amplification was carried out using an iCycler and analysed with iCycler iQ Version 3.0a (Bio-Rad, München, Germany).

Preparation of conditioned medium

In total 1.25×10^6 HeLa-TDO cells were cultured in 5 mL culture medium in culture flasks. Cells were stimulated for 3 days with tetracycline (30–75 ng/mL) or IFN-γ (1000 U/mL) in the presence or absence of additional 1-MT (1.2 mM). Thereafter, supernatants were utilised as culture medium for bacteria or

peripheral blood lymphocytes in the presence or absence of additional tryptophan (100 μg/mL). For allogeneic T-cell supernatants X-Vivo15 lymphocyte medium (Gibco) was used with additional 1 μL/mL β-mercaptoethanol and 200 mM L-glutamine.

T-cell proliferation assay

In total 3×10^4 HeLa-TDO cells, irradiated with 5200 Gy, were incubated in the presence or absence of IFN-γ or tetracycline for 72 h. Thereafter, PBL (1.5×10^5/well) were added with or without supplemental tryptophan or 1-MT (final concentration 100 or 300 μg/mL, respectively). T-cell proliferation was determined after 3 days by adding ^{3}H-thymidine for 14–18 h. PBL were stimulated with a monoclonal anti-CD3 antibody for 3 days. In other experiments, proliferation of 1.5×10^5 PBL cells/well was activated by addition of allogeneic PBL (1.5×10^5/well, irradiated with 2600 Gy). In additional experiments, PBL were activated by the use of an anti-CD3 monoclonal antibody and medium conditioned by HeLa-TDO cells served as culture medium.

IFN-γ assay

Conditioned medium served as culture medium for PBL (1.5×10^5/well) stimulated with monoclonal anti-CD3 antibody. After 3 days, the supernatants were harvested and the IFN-γ concentration measured using the human IFN-γ ELISA development Kit, DuoSet from R&D Systems (Minneapolis, USA). The amount of recombinant IFN-γ used to stimulate HeLa-TDO cells was subtracted from the IFN-γ content determined in respective experimental groups.

Statistical analysis

All experiments were performed in duplicates (virus growth) or triplicates (all other experiments) and data are given as mean ± SEM of a minimum of three independent experiments. For statistical analysis, the unpaired *t*-test was used and *p*-values <0.01 were accepted as significant. The analysis was performed with GraphPad Prism software.

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Abbreviations: **L-trp:** L-tryptophan · **1-MT:** 1-methyl-L-trp · **TDO:** tryptophan 2,3-dioxygenase

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