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The role of indoleamine 2, 3-dioxygenase in lepromatous leprosy immunosuppression

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Summary

To elucidate further the possible role of the tryptophan, rate-limiting enzyme indoleamine 2, 3-dioxygenase (IDO) in leprosy, the distribution of IDO-positive cells and IDO activity in the skin biopsies and sera of these patients representing the entire spectrum of the disease were studied. An increased number of macrophages/dendritic cells (DC–lineage IDO$^+$ cells were found in lepromatous (LL) compared to tuberculoid (BT) and reversal reaction (RR) patients. IDO-positive cells showing CD68 and CD86 surface markers predominated in LL lesions, while higher levels of IDO activity were observed in the sera of LL *versus* BT patients. Tests revealed an increased IDO message in *Mycobacterium leprae*-stimulated peripheral blood mononuclear cells (PBMC) by real-time polymerase chain reaction (PCR) and increased IDO expression in *M. leprae*-stimulated CD14$^+$ cells of both healthy controls (HC) and LL patients, as evaluated via flow cytometry. Increased *M. leprae*-induced IDO–protein synthesis was also confirmed by Western blot. Based on our *in vitro* studies, it was confirmed that *M. leprae* up-regulated IDO expression and activity in HC and LL monocytes. Interferon (IFN)-γ synergized with *M. leprae* in promoting IDO expression and activity in monocytes. IDO expression induced by both IFN-γ and *M. leprae* was abrogated by 1-methyltryptophan (1-MT). Our data suggest that *M. leprae* chronic infection activates the suppressive molecule IDO which, in turn, contributes to the specific immunosuppression observed in LL leprosy.

Keywords: IDO, IFN-γ, lepromatous leprosy, monocytes, *Mycobacterium leprae*

Introduction

While investigated extensively both *in vivo* and *in vitro*, the fact that lepromatous (LL) patients do not present a T cell response to *Mycobacterium leprae* remains undecipherable [1,2]. In these patients, disseminated skin lesions with high bacterial loads and negative lepromin skin tests are associated with the absence of both T cell proliferation and interferon (IFN)-γ production *in vitro* by *M. leprae*-stimulated blood cells [3,4].

It is known that T cell responsiveness to *M. leprae* is progressively reduced, resulting in an immunological and clinical spectrum of the disease ranging from high T helper type 1 (Th1) responder patients at the tuberculoid pole towards the complete absence of the cellular immune response at the lepromatous end of the spectrum [3,5]. As a whole, the intrinsically unstable borderline forms present a gradually decreasing immune response. These patients may show spontaneous immune reactivation at any time during the course of *M. leprae* infection. The emergence of a *M. leprae*-specific, T cell response and higher Th1 cytokine production has been documented during these episodes [6,7].

The emergence of cell immunity attracts newly activated macrophages towards previously borderline LL lesions that substitute the inactive macrophages. In a previous study, it was demonstrated that CD68-positive macrophages isolated from LL lesions suffered morphological and functional changes in culture, suggesting an environmental-dependent state [8], which the naturally occurring reversal reaction (RR) phenomenon seems to confirm [9,10].

Recent data indicate that the mononuclear phagocytic system *in vivo* differentiates and migrates in response to a combination of factors leading to the development of a

variety of cell phenotypes associated with the inflammatory phase [10,11].

Evidence of a provoked reversion of the specific anergy to *M. leprae* observed in LL cells has been reported, having motivated attempts at therapeutic interventions in the past [12,13]. *M. leprae* components, genetic background and long-lasting antigen stimulation are among the factors capable of explaining the anergy observed in LL patients [14,15]. Secretions from the adherent cells of LL patients have been shown to inhibit lymphocyte proliferation in healthy volunteers [16]. Recent data have upheld the idea that macrophage deactivation is controlled by inflammatory suppressor genes [10]. Indeed, in leprosy, both interleukin (IL)-10 and cyclo-oxygenase-2 (COX-2) have clearly been identified as suppressor genes [17,18].

Several recent studies have described indoleamine 2, 3-dioxygenase (IDO)-dependent T cell suppression by antigen-presenting cells (APCs) in many infectious and inflammatory conditions, indicating that biochemical changes due to tryptophan catabolism have a profound effect on T cell proliferation and effector functions in tissue microenvironments [19,20]. IDO plays an active role in the human immune response by catalyzing the first and also rate-limiting step in the kynurenine pathway of tryptophan degradation, the end product being N-formyl-kynurenine in conjunction with the production of 3-hydroxyanthranilic and quinolinic acids. These products are known to function as intercellular messengers with neuroactive, pro-apoptotic and immunoregulatory properties. However, the pro-apoptotic effects seem to be more effective in the Th1 than the Th2 lymphocytes [21,22].

The suppressor activity of the downstream tryptophan metabolites due to rate-limiting IDO enzymatic activity is assumed to be exerted by many pathways, such as the generation of regulatory T cell (T_{reg}) $CD4^+CD25^+$forkhead box P3 ($FoxP3^+$), the dependence on cytotoxic T lymphocyte-associated protein 4 (CTLA4)-expressing cells and IL-10 production [23,24]. Besides inhibiting intracellular pathogens [25,26], the local tryptophan-deficient environment and even the direct effect of accumulating tryptophan metabolites have also been demonstrated to induce T cell tolerance during immunosurveillance in pregnancy [27] and tumours [19,28].

The IFN-γ-dependent IDO induction by dendritic cells (DCs) and macrophages so often seen during infection represents an innate pathogen elimination mechanism [26] through anti-microbial molecular synthesis and tryptophan privation. Conversely, IDO induction could also represent an effective adaptation to an intracellular microorganism such as *M. leprae* which, although presenting massive gene decay in its genome, can still maintain the ability to synthesize a complex amino acid-like tryptophan [29]. It has been reported that prolonged secretion of inflammatory cytokines such as the presence of tumour necrosis factor (TNF) in foci of chronic inflammation seems to play an anti-inflammatory role by supporting IDO expression [30,31]. In this context, it has been hypothesized that, in chronic infections, effective immunity could be impaired by the generation of IDO-mediated suppression [32].

In the present study, it was seen that IDO expression was significantly higher in LL lesions than in either borderline tuberculoid (BT) or RR lesions. Increased IDO activity along with lower levels of L-tryptophan and higher levels of L-kynurenine in LL than in BT patient sera were also found. It was observed similarly that *M. leprae* increased proinflammatory cytokine levels in monocytes as well as in IDO expression and activity.

These combined data strongly support the view of IDO as an additional mechanism in determining the gradual development of T cell–*M. leprae*-specific anergy across the clinical spectrum of leprosy and, as such, creating a myriad of possibilities for future interventions.

Materials and methods

Patients and clinical specimens

The acquisition of all specimens was approved by the Human Ethics Committee of the Oswaldo Cruz Foundation in Brazil. Leprosy patients (20 lepromatous, 19 BT and 13 RR) were classified according to the Ridley and Jopling classification scale [33].

Buffy coats were obtained from normal donors (healthy controls: HCs) at the Hemotherapy Service of the Clementino Fraga Filho University Hospital, associated with the Federal University of Rio de Janeiro, RJ, Brazil, in accordance with the guidelines set down in the Declaration of Helsinki.

Immunohistochemical studies

Leprosy patient (seven BT, 14 LL) skin biopsies were obtained prior to treatment at diagnosis. Four RR biopsies were collected at the onset of reversal reaction. For routine histopathological analyses, all skin tissues were stained with haematoxylin and eosin (H&E) in addition to Wade stains.

To detect IDO and the phenotype of IDO^+ cells, immunoperoxidase labelling of cryostat sections was performed. Sections were then incubated with anti-IDO (1:50), anti-S100 (1:200), anti-CD86 (1:50), anti-CD11c (1:25), anti-CD68 (1:100) and anti-human leucocyte antigen D-related (HLA-DR) (1:100) in 0·1% bovine serum albumin (BSA) and in $Ca^{2+}Mg^{2+}$-free phosphate-buffered saline (PBS) for 1 h at room temperature. Biotinylated horse or goat secondary antibodies were incubated for 1 h at room temperature. Primary antibodies were visualized with the Vector Elite avidin–biotin complex kit (ABC, Vector Laboratories, Burlingame, CA, USA), which uses an avidin–peroxidase conjugate for signal amplification. The ABC reagent was incubated for 40 min, washed, and then incubated with substrate (3-amino-9-ethylcarbazole) for 10 min. Slides were counter-

stained with Mayer's haematoxylin and mounted with paramount medium (Dako, Thousand Oaks, CA, USA). Images were obtained via a Nikon Eclipse microscope with Image ProPlus software.

Double immunofluorescences were also performed to confirm the macrophage subtype of the IDO$^+$ cells. Cryostat sections (LL, $n = 3$) were fixed in acetone and incubated with 10% normal goat serum. Sections were incubated overnight with primary antibody anti-IDO (1:100), CD68 (1:100), CD86 (1:50) or CD209 (1:50), followed by goat anti-rabbit labelled with fluorochrome Alexa Fluor 488® (1:1000, Molecular Probes, Eugene, OR, USA) or by goat anti-mouse labelled with fluorochrome Alexa Fluor 568® (1:1000, Molecular Probes). After washing, slides were mounted with Vectashield (Vector Laboratories). Images were obtained via confocal laser microscopy (LSM 510 META scanning; Zeiss, Göttingen, Germany).

Cell culture and stimulation

Peripheral blood mononuclear cells (PBMCs) were isolated under endotoxin-free conditions from heparinized venous blood by Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ, USA) density centrifugation. PBMC, suspended in RPMI-1640 medium containing 2 mM L-glutamine, 10% fetal calf serum (FCS) and antibiotics, were cultured overnight in tissue culture plates at 37°C/5% CO_2. Monocyte purification was performed by cell sorting using the CD14-positive selection magnetic cell sorting kit (Miltenyi Biotec, Auburn, CA, USA). Monocytes were recovered, resuspended in complete culture medium and cultured in 24-well plates (Costar, Cambridge, MA, USA) at 1×10^6 cells/well. Monocyte cultures were > 90% pure, as judged by flow cytometry analysis. Irradiated, armadillo-derived *M. leprae* sonicate was added to the cultures at a multiplicity of infection (MOI) of two bacteria : cell or 10:1 for 24 h. When necessary, monocytes were treated with 10 ng/ml IFN-γ (R&D Systems, Minneapolis, MN, USA) overnight. For evaluation of cytokine secretion, supernatants from *M. leprae*-stimulated monocytes were harvested after 2 days of culture and stored at –20°C until future use.

RNA preparation and real-time polymerase chain reaction (PCR)

RNA was extracted from frozen skin fragments or *M. leprae*-stimulated PBMC cultures. Total RNA was prepared using the Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA purity was verified and cDNA synthesis carried out using the Superscript III first-strand RT–PCR kit (Invitrogen). *Taq*man PCRs were performed via the universal PCR Master Mix (×2) and specific primers and probes (Applied Biosystems, Bedford, MA, USA). PCR was performed in the ABI Prism 7000-sequence detection system (Applied Biosystems) at 50°C for 5 min, 95°C for 10 min, 50 cycles of 95°C for 15 s, and 60°C for 1 min. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an endogenous control. *IDO* (Hs00158032), *TNF* (Hs00174128) and *IFN*-γ mRNA (Hs00174143) were quantified using the $2^{-\Delta\Delta Ct}$ method for PBMC samples and $2^{-\Delta Ct}$ (in which Ct is a threshold cycle) for biopsies [34].

Estimation of IDO activity in patient sera and supernatants from monocyte cell cultures by high performance liquid chromatography analysis (HPLC)

To detect IDO activity, serum samples from leprosy patients and supernatants from *M. leprae*-stimulated CD14$^+$ cell cultures were collected and frozen in –20°C until HPLC analysis. Tryptophan (Trp) and kynurenine (Kyn) concentrations were measured by HPLC, as described previously [35].

Immunoblotting

IDO expression was quantified by Western blot analysis. Protein extracts were obtained from skin biopsies (four BT and four LL) and cellular cultures. The skin fragments (four BT and four LL) and monocytes were both washed twice with ice-cold PBS and incubated for 30 min with 50 µl of lysis buffer [50 mM Tris–HCl, pH 7·5, 5 mM ethylenediamine tetraacetic acid (EDTA), 10 mM ethyleneglycol tetracetic acid (EGTA), 50 mM sodium fluoride (NaF), 20 mM β-glycerophosphate, 250 mM NaCl, 0·1% Triton X-100, 1 mg/ml BSA, and 1:1000 of protease inhibitor cocktail II (Calbiochem, San Diego, CA, USA)] adapted from Scheinman *et al.* [36]. Afterwards, the extracts were sonified for 90 s followed by centrifugation (15 000 ***g***, 20 min). Proteins (30 µg) were resolved on 12% sodium dodecyl sulphate-polyacrylamide gels and blotted onto nitrocellulose membranes (Bio-Rad) with a semidry transfer cell (Bio-Rad). After blocking with 5% BSA and 0·15% Tween (TBS-T), blots were incubated for 1 h at room temperature with 1 µg/ml antibody against IDO (H-110) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Nitrocellulose membranes were then washed three times for 5 min each with TBS-T. Anti-rabbit horseradish peroxidase-conjugated immunoglobulin (Ig)G (1:2000) was used as a secondary antibody. An enhanced chemiluminescence detection system (ECL; Amersham Biosciences, Piscataway, NJ, USA) was used in all blots to detect the related secondary antibody. Blots were then stripped and reprobed for α-tubullin and used as controls of the protein load.

Fluorescence activated cell sorter (FACS) analysis of monocyte phenotypes and intracellular staining of IDO-expressing cells

Monocytes were collected with a cell scraper after 24 h of culture. Cells were harvested and stained with anti-CD14,

anti-CD80, anti-CD86 and anti-HLA-DR antibodies. For IDO intracellular staining after fixation and permeabilization (Fixation/Permeabilization Buffer; eBioscience, San Diego, CA, USA), cells were stained with rabbit anti-IDO polyclonal antibody (Santa Cruz Biotechnology) followed by phycoerythrin (PE)-conjugated goat anti-rabbit secondary antibody (Santa Cruz Biotechnology). Normal rabbit IgG was used as the corresponding isotype antibody control. Flow cytometry analyses used a Cyan flow cytometer (Dako). Gates were set for collection and analysis of 20 000 live events. Data were analysed via Summit Software (Dako).

Cytokine detection by immunoassay or enzyme-linked immunosorbent assay (ELISA)

Cytokine production in *M. leprae*-stimulated monocyte cultures [TNF, IL-10, transforming growth factor (TGF)-β, IL-12p40, IL-6 and IL-1β] was measured by ELISA according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

Statistical analysis

Results are reported as pooled data from the entire series of experiments. All experimental data are mean ± standard error (s.e.). For comparison of patient and control data, the Friedman analysis of variance (ANOVA), Kendall's coefficient of concordance test and Wilcoxon's signed-rank test were used whenever appropriate. The adopted statistical significance level was $P \leq 0{\cdot}05$.

Results

High IDO protein expression in LL skin lesions

Routine histopathological analyses of skin biopsies were performed to select representative specimens of the polar forms of leprosy and RR lesions. BT lesions were characterized by an organized dermal granuloma composed of phagocytic cells with evident epithelioid differentiation surrounded by lymphocytes of giant cells (Fig. 1a). LL lesions were characterized by massive macrophage infiltration containing a variable number of Virchow cells full of bacilli accompanied by few lymphocytes, separated from the flat epidermis by a clear zone (Fig. 1b). RR lesions showed activated macrophages with an epithelioid appearance, organized or not as granuloma (Fig. 1e).

Immunohistochemistry was performed to identify IDO protein expression in leprosy skin lesions (BT, $n = 7$; LL, $n = 14$; and RR, $n = 4$). The number of IDO-positive cells was higher in the LL (50–75%) than BT lesions (< 25%), in which only scattered positive cells in the periphery and in the subepidermal area outside the granuloma could be detected (Fig. 1c). In LL lesions, besides the high positivity in the LL cellular infiltrate in which foamy cells predominated, isolated IDO^+ cells with a dendritic-like morphology were detected on the dermis (Fig. 1d) and in some endothelial cells. In RR lesions and, as is often the case, in BT lesions as well, very few IDO^+ cells (< 25%) were observed in the dermal granuloma (Fig. 1e).

To characterize the IDO^+ cell phenotype, leprosy skin tissues were also immunostained for S100, CD86, CD11c and HLA-DR, which are surface markers present in the macrophage/DC lineage. Almost all cells constituting the LL dermal infiltrate were positive for HLA-DR, CD11c, CD86 (Fig. 1f,h,j, respectively) and CD68 (data not shown) (> 75% for all markers). These patterns differed from the ones in the BT lesions, in which $CD11c^+$ and $CD86^+$ cells (25–50%) were detected in the centre of the granuloma corresponding to the epithelioid macrophages and surrounded by a lymphocyte mantle similar to that found in RR lesions (Fig. 1g,i, respectively). In the BT and RR lesions, most cells were negative for IDO staining (Fig. 1c,e, respectively). However, in both types of lesions, some dendritic-like-shaped, S100-positive cells, which may be IDO-positive, were discerned on the dermis itself (Fig. 1c) (data not shown).

Double-immune labelling and confocal laser microscopy were used to examine the nature of the cells expressing IDO in LL skin lesions. About 70% of the IDO-expressing cells in the inflammatory infiltrate co-localized with CD68 (50–75%) (Fig. 2a), while approximately 50% co-expressed CD86, indicating that IDO-positive cells have their origin in macrophages. Within the CD86 population (50–75%), both positive and negative IDO cells were present, suggesting the simultaneous occurrence of two different $CD86^+$ cells (Fig. 2b). Single cells with a dendritic-like cell morphology revealed the presence of CD86 in the cellular membrane and IDO in the cytoplasm (insert in Fig. 2b).

To confirm the increased IDO protein expression in LL skin biopsies observed by immunohistochemistry, the IDO protein content in LL and BT biopsies was evaluated via immunoblotting. IDO expression was higher in LL in comparison to BT biopsies (Fig. 2c). This result confirms that IDO protein is highly expressed in LL patient cells.

IDO mRNA transcripts were assessed in tissue samples by real-time PCR. The mRNA expression of IDO was observed in all groups tested, although the IDO message in the LL lesions increased only moderately in comparison to what was seen to occur in the BT and RR lesions (Fig. 3a). IFN-γ and TNF mRNA expression in leprosy skin lesions were analysed similarly. The LL and BT samples showed no differences in IFN-γ and TNF mRNA expression, except for being significantly higher in the RR lesions (Fig. 3b,c). IDO protein expression was reduced in BT and RR lesions compared to LL, and a similar trend was also observed with respect to IDO mRNA, although it failed to reach statistical significance.

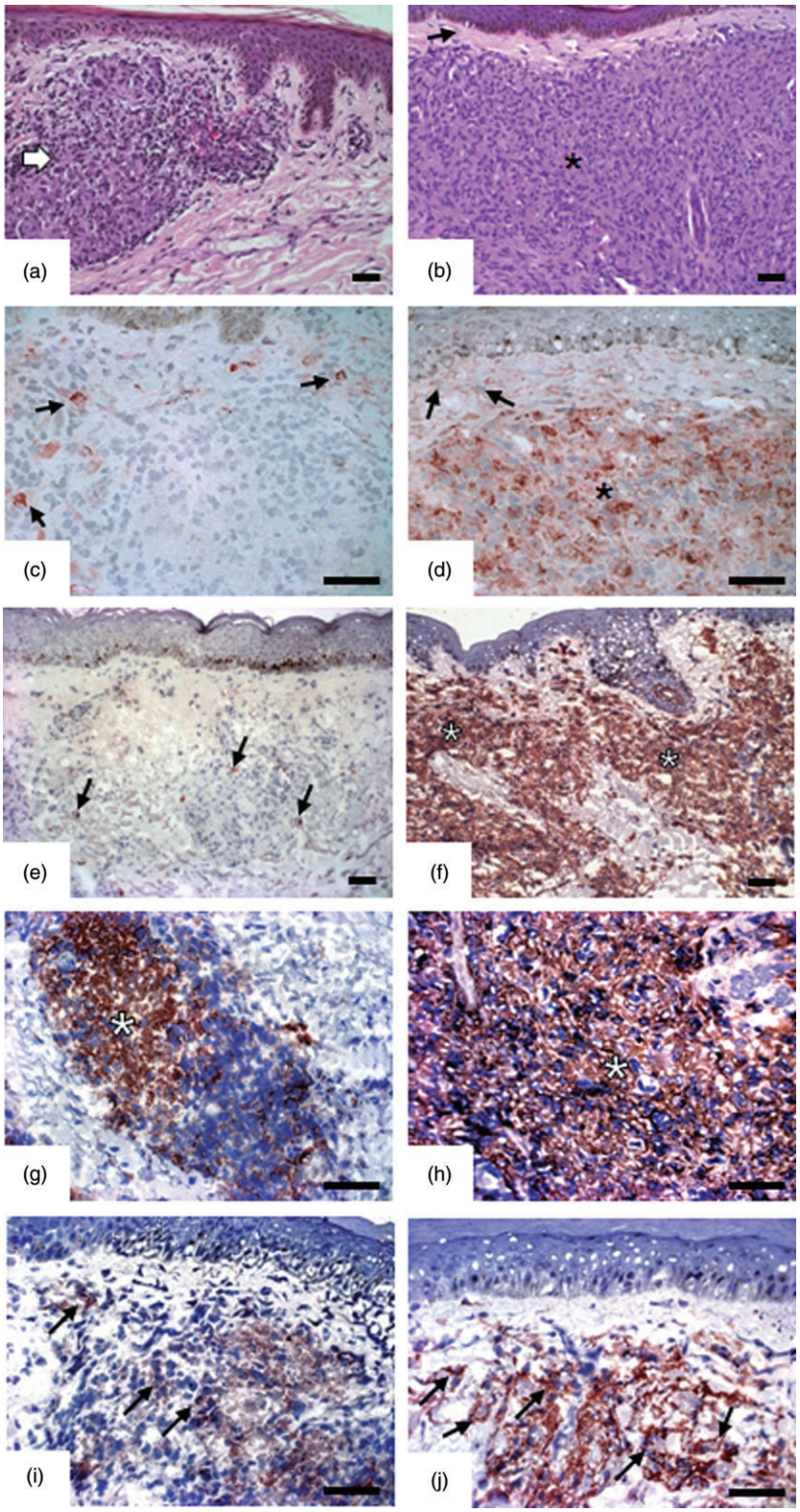

Fig. 1. Indoleamine 2, 3-dioxygenase (IDO) protein expression in leprosy lesions (LL) *in vivo* and cell phenotypes. Borderline tuberculoid (BT) lesions are characterized by the presence of dermal epithelioid granuloma (a, white arrow) and LL lesions by the presence of massive, foamy macrophage infiltration and scattered lymphocytes (b, black asterisk) separated from the flat epidermis by a clear zone (b, black arrow). In BT, a few scattered IDO^+ cells are detected within the subepidermal area but are almost entirely absent in the dermal granuloma (c, black arrows). In LL, high numbers of IDO^+ cells are detected in the dermal inflammatory infiltrates in both lesions (d, black asterisk). IDO-positive cells and dendritic cell (DC)-like shaped cells can be seen within the subepidermic zone (d, black arrows). Similarly to the BT lesions, very few IDO^+ cells are detected in the reversal reaction (RR) lesions (e, black arrows). In BT, CD11c expression is detected mainly inside the granuloma (g, white asterisk) while, in the LL lesions, CD11c and human leucocyte antigen D-related (HLA-DR) expression are observed inside the dermal inflammatory infiltrate (h,f, respectively, white asterisks). Some scattered $CD86^+$ cells were observed inside the dermal granuloma in a BT lesion (I, black arrows). In LL, CD86 expression is detected in the dermal inflammatory infiltrate in the majority of cells (j, black arrows). The findings shown are representative of BT, LL ($n = 14$), and RR ($n = 4$) samples. (a–b) Haematoxylin and eosin staining was used for routine diagnosis. (c–j) The immunoperoxidase method was counterstained with haematoxylin. Scale bars = 50 μm. All images were obtained via Nikon E400 Eclipse and processed by Image ProPlus software.

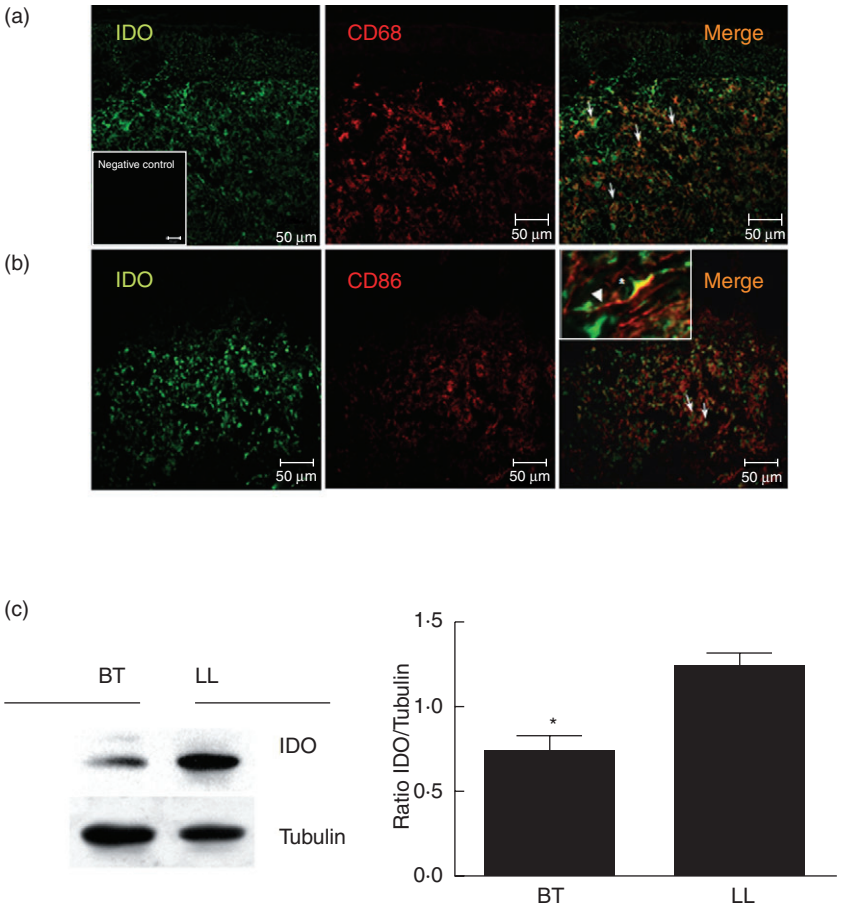

Fig. 2. Indoleamine 2, 3-dioxygenase (IDO) protein expression in lepromatous skin lesions (LL) by immunofluorescence and immunoblotting assays. (a) Double-immunofluorescent images revealed that most IDO$^+$ cells co-localize to inflammatory macrophages (CD68$^+$ cells) (white arrows). (b) Some single CD86$^+$ cells also express IDO, exhibiting a dendritic-like cell morphology (white arrows and insert). Isolated cells present dendritic-like cell morphology in which IDO is located in the cytoplasm (white asterisk) and CD86 is inside the cellular membrane (white arrowhead) (see insert). IDO (green, Alexa Fluor 488®) is double-stained with CD68 or CD86 antibodies (red, Alexa Fluor 568®). Double-positive cells are yellow. Photographs of the single stainings for IDO, CD68 and CD86 are also shown. Data shown are representative of three LL cryostat samples. Images were visualized and obtained by a laser confocal microscope (Zeiss). Scale bars = 50 μm. (c) The expression of IDO in skin biopsies was evaluated by immunoblotting. Extracts of four LL and four borderline tuberculoid (BT) skin lesions were evaluated, and a representative blot of one LL and one BT extract was demonstrated. A densitometry analysis of the blots was performed, showing an IDO/tubulin ratio. Results are expressed as mean ± standard error of the mean ($n = 4$); *$P < 0{\cdot}05$.

High IDO activity is detected in the sera of lepromatous leprosy patients

The tryptophan : kynurenine ratio and kynurenine concentration level(s) observed in the serum are indices that reflect the systemic state of IDO activity, an important aspect that could broaden our understanding of the sometimes stark differences in the spectral clinical forms of leprosy. The IDO-activity index was measured in the sera of leprosy patients. IDO activity increased dramatically in LL patient sera relative to the findings of HCs and BT patients. The major differences observed in the tryptophan : kynurenine ratio (0·09 ± 0·008 in LL *versus* 0·051 ± 0·008 in BT, $P = 0{\cdot}002$) and kynurenine concentration (1·4 ± 0·07 in LL *versus* 0·82 ± 0·16 in BT, $P < 0{\cdot}0001$) indicate that IDO activity could be an important marker and, in this capacity, might also be useful in analysing differences in cellular immune activity and cellular microbicidal oxygen species at the two opposite poles of leprosy disease (Fig. 4a,b).

The activity level of IDO in the sera of BT patients was higher than in RR, although not significant. The RR levels were the lowest in all groups, including among HCs (Fig. 4a).

The patterns of the proinflammatory cytokines in leprosy patient sera were also examined. In this connection, the serum levels of two known IDO inducers – IFN-γ and TNF – were analysed. In the same set of experiments reported in Fig. 4a,b, it was evident that IFN-γ serum levels were higher in the BT *versus* the HC and RR sera but, importantly, not in the LL sera (24·63 ± 1·36 in HC *versus* 66·86 ± 19·10 in BT, $P = 0{\cdot}0012$ and 66·86 ± 19·10 in BT *versus* 25·68 ± 2·80 in RR, $P = 0{\cdot}0093$), while TNF was higher in the LL patients (173·1 ± 63·9 in LL *versus* 49·90 ± 4·63 in BT, $P = 0{\cdot}02$) (Fig. 4d).

M. leprae increases IDO expression and activity in naive and lepromatous monocytes

In order to verify whether *M. leprae* increases IDO expression *in vitro*, PBMC from HCs were exposed to *M. leprae* at a low MOI of 2:1 (bacilli : monocyte) for 1, 3, 6, 12 and 24 h. As observed in Fig. 5a, *M. leprae* alone successfully increased IDO mRNA expression in HC-derived PBMC after 12 h of culture, maintaining a higher IDO expression for 24 h after exposure to *M. leprae*. Because increased IDO gene expression in PBMC (Fig. 5a) and monocytes was observed (not shown) in the present study and because macrophages and DCs are the major IDO-producing cells, it was deemed appropriate to evaluate the percentage of IDO-positive cells

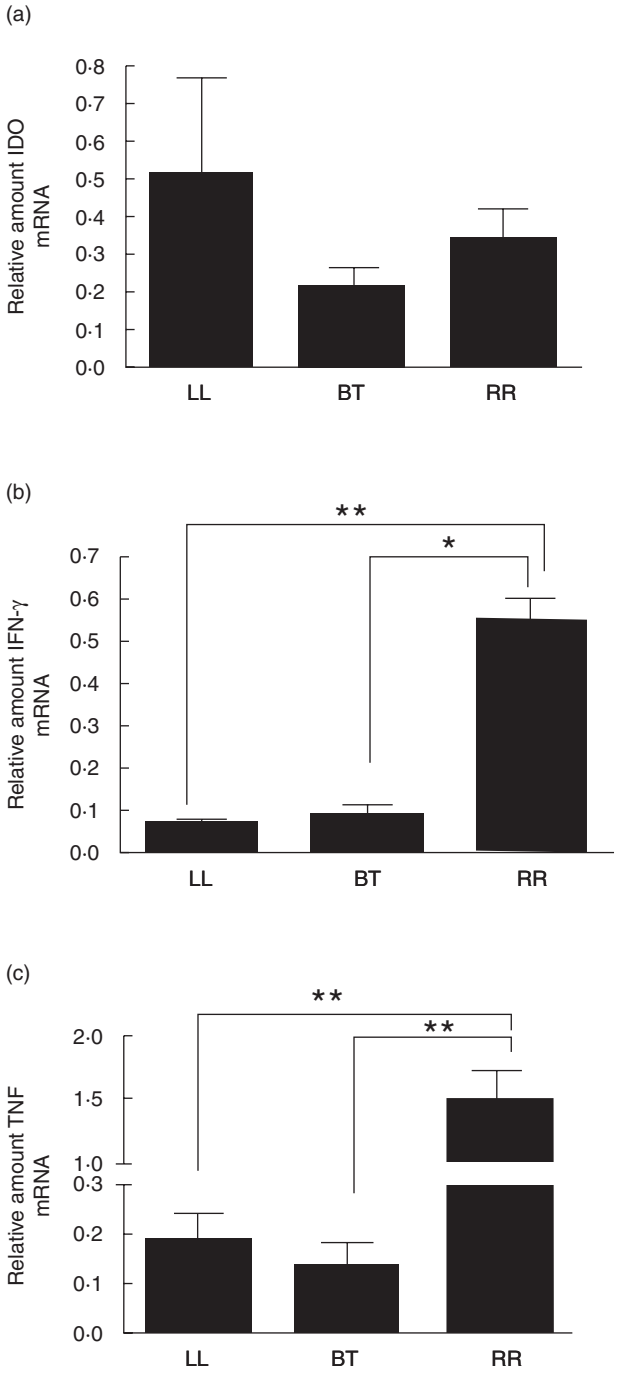

Fig. 3. Differential cytokines and indoleamine 2, 3-dioxygenase (IDO) expression in skin biopsies of leprosy patients. Biopsies were obtained from lepromatous lesions (LL) ($n = 11$), borderline tuberculoid (BT) ($n = 10$) and reversal reaction (RR) leprosy ($n = 5$) patients. Real-time polymerase chain reaction (PCR) was performed to measure the mRNA levels of indoleamine 2, 3-dioxygenase (IDO) (a), interferon (IFN)-γ (b), and tumour necrosis factor (TNF) (c). Results are the means ± standard error from four independent experiments. A Mann–Whitney *t*-test was used for statistical analysis, in which (*) means *P* value < 0·05 and (**) means *P* value < 0·01.

and IDO activity in naive and LL monocytes. The capacity of IFN-γ to modulate IDO expression and activity in LL cells was also evaluated.

The percentage of IDO-positive cells was similar in naive and LL non-stimulated monocytes (2·22 ± 0·33% in HC *versus* 1·52 ± 0·69% in LL) (Fig. 5b,c). By adding IFN-γ to the culture, the percentage of IDO^+ cells in both HC and LL monocytes increased (4·11 ± 0·52% in HC *versus* 7·39 ± 3·07% in LL). Similarly, *M. leprae* increased IDO expression in both HC and LL monocytes.

As described in Materials and methods, the induction of IDO expression by *M. leprae* was more effective in 10:1 MOI. IDO activity was also evaluated in culture supernatants by HPLC. Curiously, IFN-γ increased IDO activity in HC but not in LL supernatants in comparison to the non-stimulated cultures (0·14 ± 0·04 in IFN-γ *versus* 0·063 ± 0·03 in non-stimulated cells in HC and 0·051 ± 0·037 in IFN-γ *versus* 0·043 ± 0·029 in non-stimulated cells in LL supernatants) (Fig. 5b,c). *M. leprae* increased IDO activity in the supernatants of both HC and LL monocytes *versus* the supernatants of non-stimulated cells. In naive monocytes, *M. leprae* 2:1 increased IDO activity to levels similar to those induced by IFN-γ (Fig. 5b). In LL monocyte supernatants, there was an increase, although not significantly, of IDO activity in both 2:1 and 10:1 MOI when compared to non-stimulated cultures (Fig. 5c). Immunoblotting analysis demonstrated that, in LL monocytes, both *M. leprae* and IFN-γ increased IDO protein synthesis (Fig. 5d). However, 10:1 *M. leprae* MOI was, according to our flow cytometry data, more effective in inducing IDO than 2:1 MOI.

As IFN-γ is a known IDO inducer and our results suggested clearly that *M. leprae* could also increase IDO expression and activity, it was decided to evaluate the effect of the simultaneous addition of *M. leprae* and IFN-γ in evaluating IDO expression.

In naive monocytes, a synergistic effect on IDO expression was seen when cells were stimulated with *M. leprae* at a high MOI 50:1 in the presence of IFN-γ (not shown), whereas no significant changes were observed when using lower MOI 2:1 or 10:1 (Fig. 5e). In LL monocytes, the synergistic effect on IDO expression could be verified when cells were stimulated with a low *M. leprae* MOI 2:1 in the presence of IFN-γ (Fig. 5f). IDO expression induced by both *M. leprae* and IFN-γ was abrogated by the IDO inhibitor, 1-methyltryptophan (1-MT) (not shown).

In order to verify the proinflammatory cytokine levels known to interfere with IDO induction [37], the TNF and IL-1β levels in monocyte supernatants were analysed. In naive monocytes, *M. leprae* was able to increase TNF levels, as demonstrated previously by the present authors [38,39]. In the supernatants of LL monocytes, *M. leprae* at 2:1 MOI increased TNF production when compared to non-stimulated cultures (7610 ± 380 pg/ml in *M. leprae* 2:1 treated *versus* 3850 ± 2100 pg/ml in non-stimulated cultures). Interestingly, higher concentrations of *M. leprae*

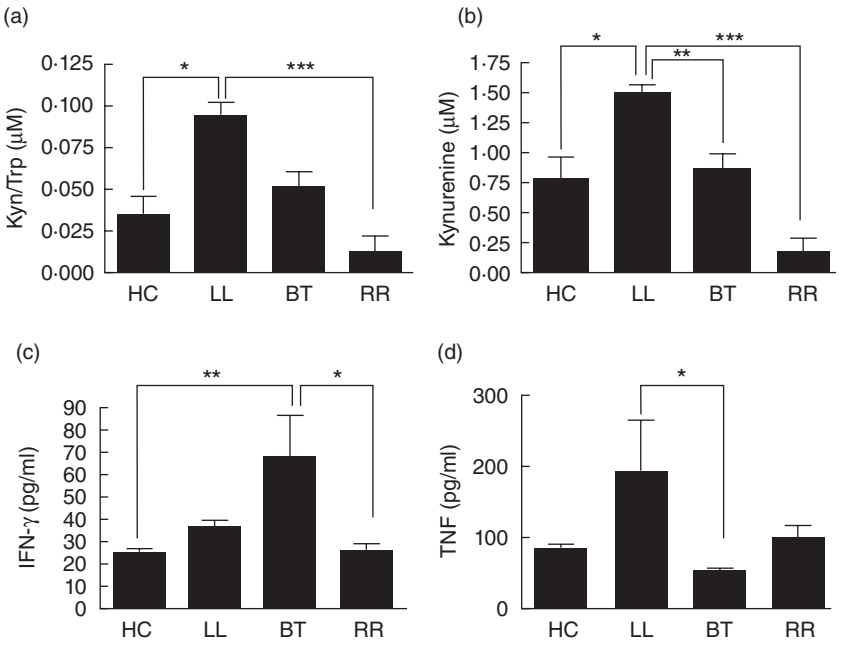

Fig. 4. Higher indoleamine 2, 3-dioxygenase (IDO) activity in lepromatous (LL) patient sera. The rate of IDO activity was evaluated by determining the amounts of kynurenine and tryptophan (kyn/trp), as determined by high performance liquid chromatography analysis (HPLC) analysis in the sera of: [healthy controls (HC)] $n = 10$, (LL) $n = 10$ [borderline tuberculoid (BT)], $n = 9$ and reversal reaction (RR) $n = 10$; (a). (b) Sera kynurenine levels are shown. (c–d) Serum levels of interferon (IFN)-γ and tumour necrosis factor (TNF) were evaluated by enzyme-linked immunosorbent assay (ELISA). Results are the means ± standard error of at least four independent experiments. A Kruskal–Wallis test/Dunn's multiple comparison test was used for statistical analysis, in which (*) means P value < 0·05 (**) means P value < 0·01 and (***) means P value < 0·001.

did not lead to increased TNF levels in comparison to non-stimulated cultures (Fig. 6a). TNF levels seemed to correlate with higher IDO activity in the same culture. No significant differences were observed in IL-10 production in the *M. leprae*-stimulated cells as opposed to the non-stimulated cells (Fig. 6c). Conversely, IL-1β levels increased at the higher *M. leprae* concentrations of 10:1 (Fig. 6b) and 50:1 MOI (data not shown).

Discussion

The role of APCs in the outcome of the immune response has been reviewed recently [40]. Leprosy is a unique human disease presenting a typical clinical spectrum in correlation with the degree of the specific immune response to the infectious agent, *M. leprae*. Indeed, an antigen-specific anergy has been reported in LL [1,2]. Although genetic factors have been implicated decisively in the susceptibility of leprosy, the immunological spectrum is most probably dependent upon other interacting factors during the course of infection [41].

The present study has demonstrated for the first time that the tryptophan rate-limiting enzyme IDO was associated with the LL form of the disease and, even more importantly, that *M. leprae* was able to induce IDO expression in LL and naive monocytes *in vitro*. Moreover, high IDO expression was evident in the resident macrophages of LL lesions. At the same time, it was possible to confirm an increase in the IDO protein via immunoblotting. Nonetheless, higher IDO activity was detected by HPLC in the sera of LL patients than that found in BT and RR patients. In the LL lesions, almost all macrophages in the infiltrate were IDO$^+$, in contrast to the presence of few positive cells in the BT and RR lesions.

Our study also observed that most IDO$^+$ cells co-localized with CD68 and CD86. IDO was detected similarly in dendritic-like-shaped cells in both BT and LL lesions. In the latter, two subpopulations of macrophages (CD209$^+$ CD1b$^-$ and CD209$^-$ CD16$^+$) have been described [42]. It was shown that the inflammatory infiltrates in LL skin lesions presented large amounts of CD11c$^+$, CD86$^+$, HLA-DR$^+$ and CD68$^+$ cells. Altogether, these data suggest that the infiltrate contains a mixture of macrophages and potential DCs with either a stimulatory or inhibitory function. The functional activity of IDO seems to be dependent upon CD86 occupation [43]. It has been reported that IDO DC-positive cells are also positive for CD11c, HLA-DR and CD86. However, with regard to the human macrophage, this remains a subject of debate. The availability of more well-defined surface markers capable of discriminating between the origins and differentiation stages of these populations would be welcome.

It was also shown that the expression of IDO mRNA occurred in both BT and LL lesions, being higher in the latter. It was demonstrated that normal control tissue is virtually negative for IDO [44]. Our data are in correlation with the protein levels analysed by immunohistochemistry and immunoblotting analyses. Although the IDO transcript was seen to increase only moderately in LL patients, the higher tissue protein and IDO activity levels found in the sera suggest that the regulatory steps followed in IDO function, transcription, translation and enzymatic activity distinguish the two polar forms of leprosy because they are regulated independently [45]. Moreover, the low activity found in RR sera reinforces the idea that RR is the result of immune activation in previously non-responding patients [9,46].

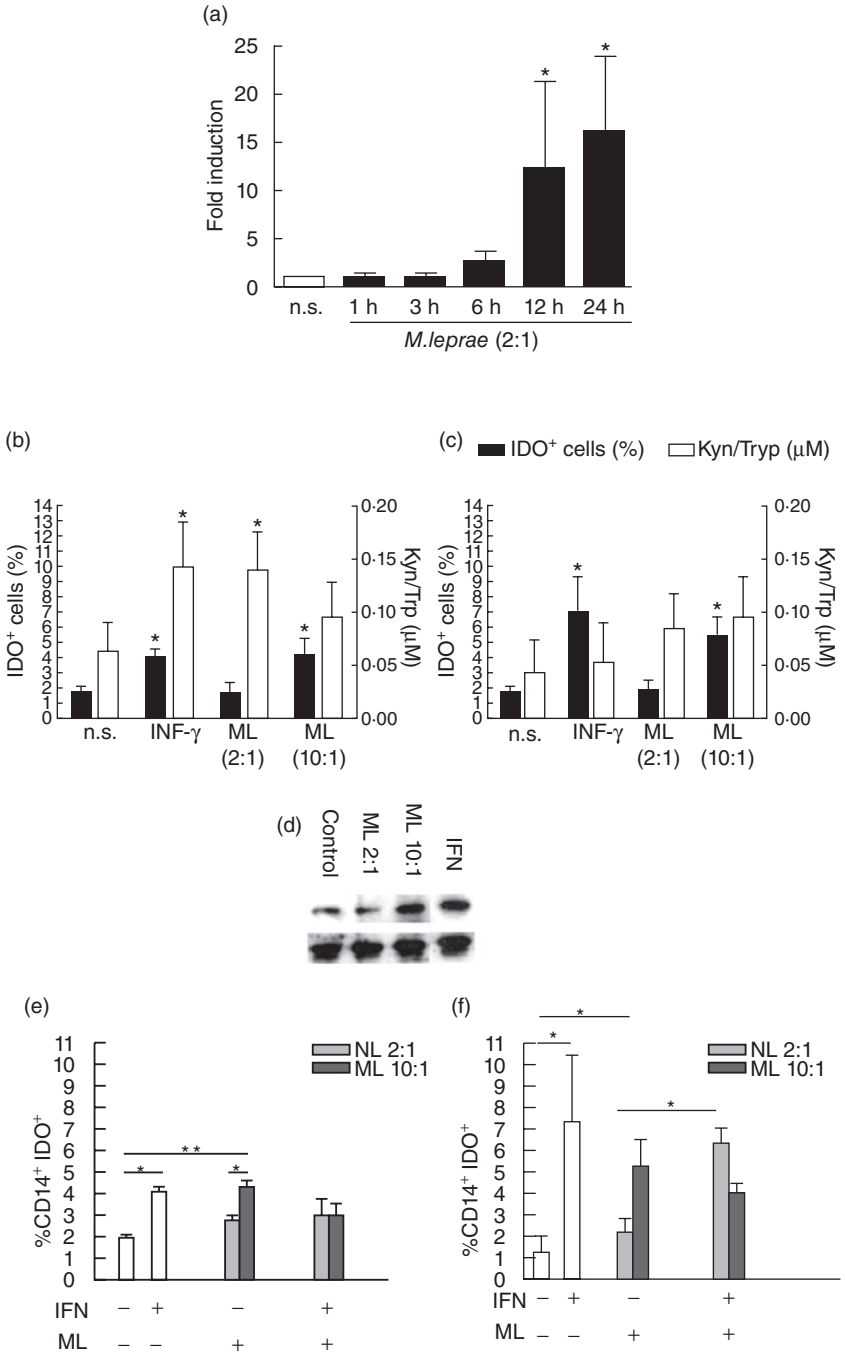

Fig. 5. *Mycobacterium leprae* increases indoleamine 2, 3-dioxygenase (IDO) expression in blood cells. Peripheral blood mononuclear cells (PBMCs) from healthy controls (HCs) were stimulated or not with *M. leprae* at a multiplicity of infection (MOI) of 2:1 (bacilli : cells) for 1, 3, 6, 12 and 24 h, respectively. (a) Cells were processed, as described in Materials and methods, and analysed by real-time polymerase chain reaction (PCR). Results are expressed as a fold induction in a mean ± standard error of five independent experiments. A Wilcoxon test was used for statistical analysis in which (*) means P value < 0·05 and (**) means P value < 0·01. (b–c) Monocytes of lepromatous (LL) patients ($n = 5$) and HCs ($n = 4$) were stimulated with *M. leprae* at a low MOI of 2:1 (bacilli : monocyte) and an MOI of 10:1 for 24 h. Alternatively, monocytes were stimulated with interferon (IFN)-γ (10 ng/ml). The relationship between IDO expression, observed by flow cytometry analysis (black bars), and IDO activity, as evaluated by the kynurenine/tryptophan ratio via high performance liquid chromatography analysis (HPLC) (white bars), in HCs (b) and LL patients (c). (*) means P value < 0·05 in relation to non-stimulated cultures. (d) Cell lysates from *M. leprae* or IFN-γ-stimulated LL monocytes were analysed by immunoblotting with an antibody against IDO or tubulin. A representative of three independent blottings is shown. (e–f) The percentage of IDO$^+$ cells in both *M. leprae* and IFN-γ-stimulated cells was ascertained. (e) HC monocytes and (f) LL monocytes. A Mann–Whitney t-test was used for statistical analysis, in which (*) means P value < 0·05 (**) means P value < 0·01, and (***) means P value < 0·001; n.s.: non-stimulated cells.

Although no significant differences were seen in TNF and IFN-γ gene expression when comparing LL and BT biopsies, analysis of cytokine production in leprosy patient sera demonstrated that BT patients had increased IFN-γ levels, whereas the TNF levels were higher among the LL. Despite the low IFN-γ levels in their sera, however, the involvement of IFN-γ in IDO induction in these patients cannot be excluded, as it is known that the presence of sub-optimal IFN-γ levels at the infection site can induce persistent bacterial growth due to insufficient tryptophan depletion [45]. Unfortunately, sera IDO activity has only rarely been investigated in infectious diseases. In this connection, Becerra *et al.* [47] found increased IDO activity in the sera of dengue virus (DENV)-infected patients during the most febrile days of the disease, indicating that IDO activity was at least partially dependent upon IFN-γ.

It is known that, although they have opposite effects, IDO and inducible nitric oxide synthase (iNOS) are both IFN-γ-inducible genes. Nitric oxide (NO) has an inhibitory effect

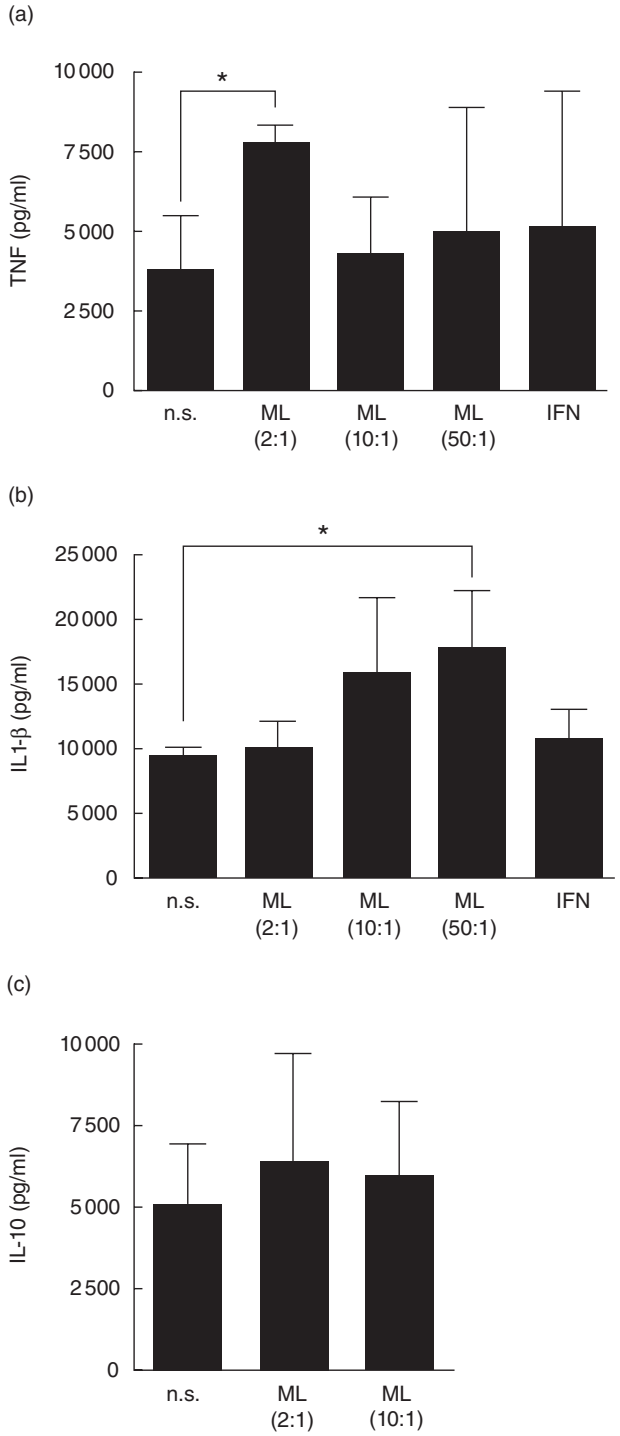

Fig. 6. Cytokine production in lepromatous (LL) patient monocytes stimulated with *Mycobacterium leprae* at a different multiplicity of infection. LL patient monocytes were stimulated with *M. leprae* at 10:1. Cell supernatants were collected 24 h post-stimulation and assayed by enzyme-linked immunosorbent assay (ELISA) for the presence of cytokines. Results are shown for tumour necrosis factor (TNF) (a), interleukin (IL)-1β (b) and IL-10 (c). Results are the means ± standard error of three independent experiments performed in duplicate. A Mann–Whitney *t*-test was used for statistical analysis in which (*) means P value < 0·05; n.s.: non-stimulated cells.

on IDO activity but not on its production [45]. There is a possibility that this same mechanism occurs in leprosy infection. Our previous data indicated that NO predominates in BT lesions and that macrophages isolated from LL lesions acquire the capacity to produce NO [8]. While responsible for inducing tissue damage and inflammation, iNOS and other reactive oxygen species (ROS) from the respiratory burst are hypothesized to be the most efficient bactericidal mechanism against *M. leprae*. One physiological response to superoxide generation is the induction of IDO [48,49], which detoxifies superoxide by using it to break up the pyrole ring in tryptophan [50,51]. IDO could also inhibit intracellular oxidative stress consuming superoxide anions during the oxidative cleavage of tryptophan into N-formylkynurenine. iNOS is also inhibited by IDO activity through activation of haem oxygenase 1 (HO-1) [52], responsible for dismutating haem in iron, biliverdin and carbon monoxide (CO). CO is an endogenous inhibitor of iNOS involved, together with bilirubin, in T_{reg} tolerance induction [53]. As such, IDO would also act as a systemic attenuator of the inflammatory phase, inhibiting excessive T cell proliferation and inducing apoptosis [53].

It could be hypothesized that *M. leprae* has advantages over this anti-oxidant axis of IDO activity. It could also be hypothesized that, in BT patients, the response for the most part takes place via iNOS and ROS induction by IFN-γ, which maintains a low bacterial load. In contrast, high bacterial loads in LL patients could contribute to increased proinflammatory cytokines such as TNF capable of inducing relative amounts of ROS, which are then degraded by IDO activity.

Because previous studies have described monocytes as a major source of active IDO in normal peripheral blood [51], IDO protein expression was evaluated in *M. leprae* and IFN-γ-stimulated monocytes by flow cytometry and immunoblotting. *M. leprae* increased both IDO expression and activity in HC and LL monocytes. In contrast, in BT monocytes neither *M. leprae* nor IFN-γ was able to increase IDO expression and activity (not shown). Furthermore, IFN-γ triggered an increase in IDO^+ cells in HC monocytes and LL patient cells. However, IDO activity was only increased in HC monocytes but not LL monocytes, despite increased expression. IDO enzymatic activity is regulated on both the post-transcriptional and post-translational levels [54,55], while several mechanisms are capable of affecting its functions [56]. Again [56], a discrepancy between IDO expression and activity in human monocytes was also found. As observed in human immunodeficiency virus (HIV) models [57], it can be concluded that IFN-γ did not increase IDO activity in LL cells due to the fact that, in leprosy, IDO induction may also occur in an IFN-γ-independent pathway.

Overall, our results suggest that, in LL, *M. leprae* is capable of inducing IDO gene expression and enzymatic activity which, in turn, may perhaps lead to the unresponsiveness of

T cells to *M. leprae*, precisely the opposite of what is observed in BT and RR cells. It could also be speculated that, in the early stages of *M. leprae* infection, IFN-γ-induced IDO could have an anti-microbial effect through tryptophan deprivation. However, the tryptophan metabolites might also function as either suppressors of specific T cell clones or inducers of T regulatory cells and IL-10 secretion, leading to the subsequent loss of responsiveness.

In addition to taking control of pathogen proliferation provoked by tryptophan depletion, IDO works as a counter-regulatory mechanism in avoiding the harmful effects of the inflammatory response [45,58]. In so doing, IDO could down-regulate the adaptive immune response as a negative feedback loop for Th1 cells.

Moreover, in both HC and LL patient monocytes, *M. leprae* increased TNF levels, suggesting the involvement of a TNF-dependent pathway. It is curious that our *in vitro* data demonstrated that even though IL-1β levels increased in intermediate MOI 10:1, low MOI 2:1 was able to augment TNF levels in LL cells.

The capacity of IFN-γ to modulate IDO expression induced by *M. leprae* was investigated because it was able to induce IDO expression in both HC and LL patient monocytes. In HC cells, a synergistic effect between *M. leprae* and IFN-γ was observed only in the presence of increased MOI 50:1 (not shown), indicating that different mycobacterial components may have an influence on IDO expression. Conversely, in LL cells, an increase in IDO expression induced by both *M. leprae* and IFN-γ occurred in the presence of low MOI 2:1.

In summary, the present study demonstrated that *M. leprae* was able to induce IDO expression and activity in human cells, implying that the immunosuppression presented by LL patients is a multi-faceted and multi-step phenomenon in which the clinical presentation of each patient across the entire spectrum of leprosy depends upon preserving a tight balance among the activating and deactivating factors. Considering that the IDO inhibitor has shown promising results in clinical trials [59], therapeutic interventions in LL may thus soon be forthcoming.

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Disclosure

The authors have no duality of interest to declare.

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