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Blockade of IDO Inhibits Nasal Tolerance Induction

Arnold P. J. van der Marei,* Janneke N. Samsom,* Mascha Greuter,* Lisette A. van Berkel,† Tom O’Toole,*, Georg Kraal,* and Reina E. Mebius‡*

The amino acid tryptophan is essential for the proliferation and survival of cells. Modulation of tryptophan metabolism has been described as an important regulatory mechanism for the control of immune responses. The enzyme IDO degrades the indole moiety of tryptophan, not only depleting tryptophan but also producing immunomodulatory metabolites called kynurenines, which have apoptosis-inducing capabilities. In this study, we show that IDO is more highly expressed in nonplasmacytoid dendritic cells of the nose draining lymph nodes (LNs), which form a unique environment to induce tolerance to inhaled Ags, when compared with other peripheral LNs. Upon blockade of IDO during intranasal OVA administration, Ag-specific immune tolerance was abrogated. Analysis of Ag-specific T cells in the LNs revealed that inhibition of IDO resulted in enhanced survival at 48 h after antigenic stimulation, although this result was not mediated through alterations in apoptosis or cell proliferation. Furthermore, no differences were found in CD4+ T cells expressing FoxP3. Our data suggest that the level of IDO expression in dendritic cells, present in nose draining LNs, allows for the generation of a sufficient number of regulatory T cells to control and balance effector T cells in such a way that immune tolerance is induced, whereas upon IDO blockade, effector T cells will outnumber regulatory T cells, leading to immunity. The Journal of Immunology, 2007, 179: 894–900.

Regulation of immune responses by naturally occurring CD4+CD25+ regulatory T (Treg) cells is of major importance for avoidance of allergy, maintenance of tolerance against harmless foreign Ags, and suppression of autoimmune diseases (1–10). In addition, T cells with regulatory capacity can be induced when harmless Ag is presented via mucosal tissues (11–17). Furthermore, targeting subimmunogenic amounts of peptide to dendritic cells (DCs) results in the induction of Treg cells (18, 19). The exact mechanism for the induction of these adaptive Treg cells in the periphery and regulation of immune responses is still largely unknown, although an inverse relationship between cell division and the induction of Treg cells has been suggested (18). In a variety of studies, it has been shown that Treg cells can suppress the proliferation and/or differentiation of effector T cells. Whether this effect is based on interaction of Treg and effector T cells, immunomodulatory cytokine production like IL-10 or TGF-β, and/or modulation of APCs by Treg cells is not yet clear (16).

DCs are pivotal players in steering differentiating T cells toward a tolerogenic or an immunogenic response. DCs may directly exert immunomodulatory functions by expressing regulatory ligands such as PD-L1 and PD-L2, inducing inhibitory signals in the opposing T cells (20–22). This tolerogenic function may be acquired by DCs through prior T cell interaction by ligation of CD80/CD86 to CTLA-4 on Treg cells (23–26). Ligation of CD80/CD86 on DCs resulted in up-regulation of IDO, an intracellular enzyme that degrades the indole moiety of tryptophan, serotonin, and melatonin (27–31). Tryptophan is an essential amino acid, crucial for the proliferation of cells, and its depletion results in inhibition of T cell activation. Not only the depletion of tryptophan may account for this inhibitory effect, but also the metabolites called kynurenines, which are formed upon degradation of tryptophan, have immunomodulatory properties (32–35). It has been suggested that kynurenines can induce apoptosis via modulation of mechanisms involving oxygen free radicals (25).

The importance of IDO in immunomodulation can be inferred from several studies in which it was shown that expression of IDO is essential for prevention of autoimmune diseases and fetal rejection during pregnancy (36–39). In the case of tumor development, it has been shown that tumors may induce expression of IDO in surrounding DCs (31, 40, 41). This response might in turn suppress antitumor responses by host T cells, and in consequence favor tumor cell survival.

In light of these data on the role of IDO in immune regulation and tolerance, we set out to study its role in mucosal tolerance induction. Earlier studies have shown that when harmless Ag is delivered via the nasal mucosa, tolerance induction occurs in the draining lymph nodes (LNs), i.e., the cervical LNs (CLNs), and that this ability is unique to these CLNs, suggesting a specific microenvironment (17, 42, 43). Similarly, such a microenvironment that favors the induction of tolerance has been proposed for the intestinal immune system (44). In this study, we show that IDO is differentially expressed in DCs from CLNs vs DCs from nonmucosal peripheral LNs (PLNs), and by inhibiting its function in vivo, we demonstrate a crucial role for IDO in mucosal tolerance induction.

Materials and Methods

Mice

Female BALB/c mice age 8–12 wk were purchased from Charles River Breeding Laboratories and kept under standard animal housing conditions. The DO11.10 transgenic mice were bred at our own facilities and used at 8–12 wk of age. The Animal Experiments Committee of the VU University
ences). IDO-specific primers and primers for housekeeping genes were used in a RevertAid First Strand cDNA Synthesis kit (Fermentas Life Sciences). Total RNA was isolated by precipitation with isopropanol, and cDNA was synthesized from total RNA and lysed in TRIzol (Invitrogen Life Technologies). RNA was isolated by extracting a reaction mixture with SYBR Green (Life Technologies) and 18 S RNA. A standard curve was generated for each primer set with cDNA from a pool of enriched cell suspension.

Cell isolation and staining

CLNs (internal jugular and superficial) and/or PLNs (popliteal) were isolated, and single-cell suspensions were made by cutting LN s with scissors. These LN pieces were further digested at 37°C using Blyenzyme 2 (Roche) and 100 U/ml DNase I (Roche) for 20 min while stirring continuously. Cell clumps were removed by pipetting the cells over a nylon mesh. The LN cells were washed and resuspended in PBS with 2% newborn calf serum. To stain for DCs, Alexa Fluor 488-conjugated anti-MHC class II (MHCII, clone M5/114) and PE-conjugated anti-CD11c (clone N418; eBioscience) were used. For analysis of the DC subpopulations, FITC-conjugated anti-CD8 (clone 53-6.7; Coulter), goat anti-mouse DO11.10 TCR (KJ1-26; Caltag Laboratories), and biotinylated anti-CD4 (clone GK1.5; Pharmingen), and biotinylated anti-CD19 (clone MB19-1; eBioscience) were used. For analysis of the CD19, anti-mouse DO11.10 TCR (KJ1-26; Caltag Laboratories) were used to stain for OVA-specific T cells. Allophycocyanin-conjugated annexin V (Molecular Probes) and 7-aminoactinomycin D (7AAD; Molecular Probes) were used to stain for early and late apoptotic cells. Staining for FoxP3 was performed according to the manufacturer’s protocol (eBioscience).

Cells were analyzed by flow cytometry (FACSCalibur; BD Biosciences). A total of 2–5 × 10^6 cells were analyzed per high-sensitivity experiment.

Real-time PCR

MHCII^*^CD11c^*^ DCs were sorted using a MoFlo sorter (DakoCytomation) and lysed in TRIzol (Invitrogen Life Technologies). RNA was isolated by precipitation with isopropanol, and cDNA was synthesized from total RNA using a RevertAid First Strand cDNA Synthesis kit (Fermentas Life Sciences). IDO-specific primers and primers for housekeeping genes were used to generate a reaction mixture. Total volume of reaction mixture was 20 μl, containing cDNA, 300 nM each of primer, and SYBR Green Master Mix (Applied Biosystems). To correct for primer efficiency, a standard curve was generated for each primer set with cDNA from a pool of nonactivated CLN, PLN, and mesenteric LNs and expression of transcripts was related to β-actin and 18 S RNA.

Experimental animal model for tolerance induction and inhibition of IDO

Four groups of seven mice were used in these experiments. For tolerance induction, 400 μg of OVA (Albumin Chicken Egg 5X Crystalline; Calbiochem) in 10 μl of saline was administered intranasally. Control mice received 10 μl of saline intranasally. To inhibit IDO, mice were treated with 1-methyl-l-tryptophan (1-MT) slow-release pellets (43.2 mg/pellet, 2-day release; Innovative Research of America) 7 h before tolerance induction. To place pellets, mice were anesthetized with 40 μl of a 7:4:3 mixture of saline to ketamine (Aescoket) to xylazine (Sedazine; Prodelab Pharma), a small incision between the scapulas was made, and a 1-MT pellet or placebo pellet was s.c. implanted. Seven hours later, OVA or saline was administered intranasally.

Sensitization and measurement of delayed-type hypersensitivity (DTH) responses

Mice were sensitized for a Th1 response as described earlier (45). Four days after intranasal OVA administration or 1 day after transfer of CD4^+^ T cells, mice received 100 μg of OVA in 25 μl of saline, mixed with 25 μl of IFA (Difco) s.c. administered in the tail base. Five days later, ear thickness was measured with an engineer’s micrometer (Mitutoyo), and a challenge of 10 μg of OVA in 10 μl of saline was given in the auricle of each ear. After 24 h, the mean increase in ear thickness of both ears was expressed as the DTH response.

Transfer experiments

To assess whether functional Treg cells had differentiated in tolerized and sensitized mice, spleens from these mice were collected and pooled 8 days after ear challenge. Spleens were minced through a 100-μm gauze to obtain single-cell suspensions. To deplete erythrocytes from this cell suspension, cells were incubated for 2 min on ice in lysis buffer (150 mM NH₄, 1 mM NaHCO₃ (pH 7.4)). Cells were washed and resuspended in PBS with 2% newborn calf serum, and CD4^+^ T cells were enriched using the CD4-negative selection kit (Dynal Biotech). The enriched cell suspension contained 50–60% CD4^+^ T cells, as determined by flow cytometry (FACSCalibur; BD Biosciences). A total of 2–5 × 10^6 CD4^+^ T cells were i.v. injected via the lateral tail vein in naïve BALB/c receptor mice. The next day, these mice were sensitized and challenged with OVA as described. These mice did not receive OVA intranasally.
PLNs and of DCs in CLNs 24 and 72 h after stimulation with OVA is the expression of IDO in DCs under nonstimulated conditions in CLNs and BALB/c mice were i.v. injected with and were subsequently stimulated by intranasal administration of 400 µg of OVA in IFA in the auricle of each ear and after 1 day, mice were sensitized with OVA or saline intranasally and were sensitized 4 days later with 10³ g of OVA in the auricle of each ear. Four days after sensitization, mice were challenged with 10³ g of OVA in IFA in the tail base. Four days after sensitization, mice were challenged with 10³ g of OVA in IFA in the tail base and challenged in the ears 5 days later. The DTH response was determined by measuring the increase in ear thickness. Data are from one representative experiment of two performed. Mice that did not receive CD4⁺ cells were used as controls for tolerance (OVA) and DTH (NaCl) response. Data are mean increase of both ears of the animals. Bar indicates the mean of each group. When 1-MT-treated mice (CD4⁺ + 1MT) were transferred, no tolerance to OVA could be measured, whereas transfer of CD4⁺ T cells from placebo mice (CD4⁺ + PLAC) resulted in immune tolerance to OVA. *p < 0.05 shows significant difference between tolerized and nontolerized or IDO-inhibited groups are indicated. Data are from one representative experiment of two performed.

Statistics

Groups of mice in the DTH experiments with ear swelling responses were compared using an one-way ANOVA followed by Bonferroni’s multiple comparison test. A value of p < 0.05 was considered significant.

Differences in percentage of 7AAD⁺ cells at 48 and 72 h after Ag administration in the 1-MT vs the placebo group were compared by two-tailed Student’s t test and considered significant when p < 0.05.

Results

DC populations in CLN and PLN are identical

We have shown that CLNs are unique in their capacity to induce tolerance to intranasally administered Ags when compared with PLNs (17), and that within these CLNs, Ag-specific Treg cells are generated (16). In several studies, plasmacytoid DCs (pDCs) have been identified as tolerogenic DCs, producing IDO and inhibiting T cell responses (26, 31, 46, 47). To see whether pDCs are differentiated in both sets of LNs. By staining for MHCII, CD11c, and GR-1, we could distinguish pDCs from conventional interstitial DCs and compare the relative contribution of each subset to the DC population in CLNs and PLNs (Fig. 1A). Plasmacytoid DCs with MHCII⁺B220⁺ expression (Fig. 1A, population 1) are equally represented in CLNs and PLNs. Interstitial DCs (Fig. 1A, populations 2 and 3) that enter the LNs by afferent lymphatics are also equally represented. Thus, with respect to the distribution of DC subsets in CLNs vs PLNs, no differences could be found.

IDO is differentially expressed in DCs from CLNs compared with DCs from PLNs

As several studies have shown that IDO is important for the regulation of T cell activation and tolerance induction, we addressed whether IDO is expressed in DCs from CLNs and compared its expression with that in DCs from PLNs. To this end, we sorted CD11c⁺ MHCII⁺ DCs (gated as indicated in Fig. 1) were sorted, RNA was isolated and cDNA was synthesized. Mice were i.v. injected with 1-MT (1-MT or placebo pellets). These cells were i.v. injected into groups of seven naive mice to assess for the presence of functional Treg cells. One day after injection of the CD4⁺ T cells, mice were sensitized with OVA in IFA in the tail base and challenged in the ears 5 days later. The DTH response was determined by measuring the increase in ear thickness. Data are from one representative experiment of two performed. Mice that did not receive CD4⁺ cells were used as controls for tolerance (OVA) and DTH (NaCl) response. Data are mean increase of both ears of the animals. Bar indicates the mean of each group. When 1-MT-treated mice (CD4⁺ + 1MT) were transferred, no tolerance to OVA could be measured, whereas transfer of CD4⁺ T cells from placebo mice (CD4⁺ + PLAC) resulted in immune tolerance to OVA. *p < 0.05 shows significant difference between tolerized and nontolerized or IDO-inhibited groups are indicated. Data are from one representative experiment of two performed.

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expression to DCs from PLNs. Because studies in CD62L-deficient mice have shown that pDCs can only enter the LNs via high endothelial venules (48) and are thus not directly involved in the transport of OVA from the nasal mucosa to the draining LN after intranasal administration of OVA, we focused on differences in expression of IDO within the MHCII<sup>high</sup>CD11<sup>chigh</sup> interstitial DCs (Fig. 1B). These DCs take up the Ag at the site of entry (in this case the nasal mucosa), migrate to the draining LNs, mature, and become MHCII<sup>high</sup>CD11<sup>chigh</sup> DCs with high Ag-presenting capabilities. Cell sorting of MHCII<sup>+</sup>/I<sup>H<sub>1</sub></sup>CD11c<sup>+</sup>/I<sup>H<sub>1</sub></sup> cells from CLNs and PLNs gave a DC population of 90% purity. Upon analysis, IDO expression was increased in DCs derived from CLNs when compared with PLNs. Furthermore, upon intranasal administration of Ag, IDO expression in CLN DCs could be further enhanced (Fig. 2). Within activated PLN DCs, induction of IDO could not be detected (data not shown). However, the route of Ag administration (i.m.) for activation of PLN DCs differs from the intranasal Ag administration that results in mucosal tolerance, impeding a proper comparison of activated DCs from PLNs vs CLNs. The results suggest that this differential expression of IDO may underlie the specific capacity of mucosa draining CLNs to induce tolerance.

Inhibition of IDO prevents tolerance induction

To investigate whether the difference in IDO expression between DCs from CLNs and PLNs is related to the crucial distinction in the capacity of the two types of LNs to induce tolerance, IDO was inhibited in vivo during nasal tolerance induction. By implanting slow-release pellets, which released the competitive IDO inhibitor 1-MT at a steady state for 48 h, IDO could be inhibited during the intranasal administration of OVA, the stage at which Treg cells are induced. In this experiment, the induced Treg cells are able to suppress a Th1 response against OVA (16). As control for the 1-MT pellets, placebo pellets were implanted. Intranasal administration of OVA followed by systemic sensitization led to the induction of tolerance as shown by a significant reduction of the DTH response after ear challenge in the placebo group (Fig. 3). However, when IDO was inhibited by 1-MT during the intranasal administration of OVA, these animals showed a prominent DTH that was as high as that of animals that had received NaCl intranasally (either with placebo or 1-MT pellets). These data clearly show that the activity of IDO is crucial for the induction of tolerance to intranasally administered Ags.

Inhibition of IDO during tolerance induction prevents the development of functional Treg cells

Because IDO inhibition led to prevention of tolerance induction, we addressed whether this result was due to the lack of induction of specific Treg cells in IDO inhibited animals. An effective way of determining the presence of Treg cells in tolerized animals is by isolating CD4<sup>+</sup> cells from tolerized mice and determining whether they can transfer tolerance to naïve acceptor mice (16, 49). Hereto
naive recipients were injected with CD4+ cells purified from animals, which had received OVA intranasally in the presence or absence of 1-MT pellets, and they were subsequently sensitized and challenged. As can be seen in Fig. 4, CD4+ cells from mice tolerized in the absence of 1-MT could effectively transfer tolerance to naive animals as DTH responses were suppressed. However, CD4+ cells from mice that had undergone the tolerization protocol in the presence of 1-MT failed to do so, indicating the absence or impaired function of Treg cells in the donor mice.

**Inhibition of IDO does not lead to a reduced expression of FoxP3 by mucosally activated CD4+ cells**

Naturally arising Treg cells and some subsets of adaptive Treg cells express FoxP3, a transcription factor that is necessary for their development (49). Recently it has been shown that IDO regulates the differentiation of a subset of FoxP3+ adaptive Treg cells in vitro (50). To see whether IDO inhibition prevents mucosally activated CD4+ cells from differentiating toward the regulatory phenotype by inhibiting FoxP3 expression, we analyzed CD4+ cells from mice shown in Fig. 2. Spleen cells, which contained Treg cells that transferred immune tolerance in the previous experiment were analyzed at 7 and 14 days after sensitization in the ear (Fig. 5). No differences between the expression of FoxP3 in CD4+CD25+ and CD4+CD25− cells from mice with or without 1-MT pellets could be found. FoxP3 expression was highest on CD4+CD25+ cells, which is consistent with the observation that FoxP3 is required for the generation of these naturally occurring Treg cells. Only a small amount of the CD4+CD25− cell population, which we have previously shown to contain the induced OVA-specific Treg cells (51), expressed FoxP3, and these FoxP3 expressing cells had disappeared by 14 days after sensitization.

**In vivo inhibition of IDO does not alter proliferation and cell death of Ag-specific T cells**

By depleting tryptophan, IDO inhibits cell division and may induce cell death, this way executing its regulatory role (25, 52, 53). Moreover, immunosuppressive metabolites of tryptophan, kynurenines, can also influence induction of tolerance and/or cell death (32–35). As both mechanisms, i.e., blockade of cell division and cell death, should be impaired when IDO is inhibited, we decided to study cell division as well as cell death simultaneously in vivo in the CLN where mucosally induced Tregs differentiate. In this experiment, mice were enriched for OVA-specific T cells by adoptive transfer of CFSE labeled DO11.10 cells as previously described (16). Thereafter 1-MT or placebo pellets were placed in the acceptor mice and 7 h later OVA was administered. Cell division and apoptosis vs cell death of the Ag-specific DO11.10 cells was determined in the nose draining CLN at 48 and 72 h after intranasal OVA treatment. In this analysis, the percentage of OVA-specific T cells that had just gone into apoptosis (annexin V+/7AAD−) could be distinguished from the cells that were undergoing cell death (annexin V+/7AAD−) and those that were dead (annexin V−/7AAD−). Both at 48 and 72 h, we could not observe a significant difference in apoptotic cell numbers in the 1-MT vs the placebo group; however, at 48 h significantly more 7AAD+ cells could be observed in the placebo group (29.3%) when compared with the 1-MT-treated group (22.4%) (Fig. 6A). In addition, no difference in the number of cell divisions between both groups could be observed at 48 and 72 h (Fig. 6B). These findings suggest that IDO activity is required for the generation of Treg cells during intranasal tolerance induction, and that the mechanism by which they are induced involves regulation of cell death, but that this is not mediated through the induction of apoptosis or by measurable inhibition of cell division.

**Discussion**

This study shows for the first time that IDO is of critical importance in nasal tolerance induction. Although pDCs are a major producer of IDO and have shown their importance for tolerance induction (26, 31, 46, 47), we showed that no specific differences in pDC numbers could be found when CLNs and PLNs were compared. This finding, together with their immature phenotype and the fact that pDCs enter the LNs through high endothelial venules (48) suggests that pDCs are not the determining factor when it comes to tolerance induction in our experimental model. We show that IDO is differentially expressed in interstitial MHCIiblow/CD11cint DCs in CLNs when compared with PLNs and that IDO activity is crucially involved in the induction of immune tolerance to intranasally administered Ags. The differential expression of IDO in DCs isolated from CLNs compared with DCs isolated from PLNs correlates with previous findings that show the uniqueness of the CLNs in their capacity to induce nasal tolerance (17, 42, 43). During this process, DCs play an important role in
determining whether tolerance or immune activation will be induced. We hypothesize that the microenvironment of the CLNs is responsible for up-regulating IDO expression in DCs, steering an immune response directed against a harmless Ag toward immune tolerance.

In our studies, inhibition of IDO only at the time that the induction of tolerance takes place clarified its role in the induction of Treg cells upon intranasal administration of Ag. This regulatory function of IDO agrees with several other studies (29, 38, 54–56) showing the importance of IDO in preventing autoimmune diseases and tissue rejection. Also during fungi infection, regulation of IDO expression was shown to contribute to the induction of Treg cells. In these studies, inhaled fungi were able to control inflammation by stimulating IFN-γ induced IDO production, resulting in the induction of Treg cells (57, 58). It has also been reported that tumors are able to create a microenvironment that results in the induction of Treg cells (57, 58). It has also been hypothesized that the presence of IDO is crucial for mediating nasal tolerance through the induction of Treg cells. The microenvironment created by stromal cells within the draining LNs may provide unique signals that determine the outcome of an immune response by influencing APCs.

In conclusion, we can say that the presence of IDO is crucial for mediating nasal tolerance through the induction of Treg cells. The microenvironment created by stromal cells within the draining LNs may provide unique signals that determine the outcome of an immune response by influencing APCs.

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Disclosures

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References


