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Reduced Cytotoxic Function of Effector CD8+ T Cells Is Responsible for Indoleamine 2,3-Dioxygenase-Dependent Immune Suppression1

Hanzhong Liu,* Li Liu, † Kaifeng Liu,* Peyman Bizargity,* Wayne W. Hancock, ‡ and Gary A. Visner2*

Indoleamine 2,3-dioxygenase (IDO), a potent immunosuppressive enzyme, contributes to tumoral escape, immune tolerance, and protection against allograft injury. In this paper, we report that inhibition of CD8+ T cell-mediated cytotoxic function is an important mechanism behind IDO’s immune-modulating property. The experimental rat lung allograft proved attractive for evaluating effector CD8+ T cells. Enhanced IDO activity achieved by using a lung-tissue-targeted nonviral human IDO gene transfer approach reduced, but did not eliminate, infiltrating CD8+ T cells. Although CD8+ T cells existed in the IDO-high lung allografts, CD8+ T cells remained viable and could proliferate for an extended period. However, cells lost their ability to attack allogeneic donor lung cells in vivo and allogeneic target cells in vitro. The impaired cytotoxic function seen in the IDO-treated CD8+ T cells was accompanied by defects in production of granule cytotoxic proteins, including perforin and granzyme A and B. Furthermore, we discovered that IDO leads to an impaired bioenergetic condition in active CD8+ T cells via selective inhibition of complex I in the mitochondrial electron transfer chain. These intriguing findings provide a base for establishing a novel mode of IDO’s immune-suppressing action. Additionally, donor lung IDO delivery, a direct and/or leukocyte passenger effect, impaired CD8+ T effector cell function. The Journal of Immunology, 2009, 183: 1022–1031.

1 Abbreviations used in this paper: IDO, indoleamine 2,3-dioxygenase; 1-mT, 1-methyltryptophan; ACR, acute cellular rejection; BLT, brain-lung transfer; C57BL/6, C57BL/6J mouse strain; CD8, CD8+ T cells. Enhanced IDO activity achieved by using a lung-tissue-targeted nonviral human IDO gene transfer approach reduced, but did not eliminate, infiltrating CD8+ T cells. Although CD8+ T cells existed in the IDO-high lung allografts, CD8+ T cells remained viable and could proliferate for an extended period. However, cells lost their ability to attack allogeneic donor lung cells in vivo and allogeneic target cells in vitro. The impaired cytotoxic function seen in the IDO-treated CD8+ T cells was accompanied by defects in production of granule cytotoxic proteins, including perforin and granzyme A and B. Furthermore, we discovered that IDO leads to an impaired bioenergetic condition in active CD8+ T cells via selective inhibition of complex I in the mitochondrial electron transfer chain. These intriguing findings provide a base for establishing a novel mode of IDO’s immune-suppressing action. Additionally, donor lung IDO delivery, a direct and/or leukocyte passenger effect, impaired CD8+ T effector cell function.

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2 Address correspondence and reprint requests to Dr. Gary A. Visner, Division of Pulmonary Medicine, Children’s Hospital Boston, Harvard Medical School, 300 Longwood, Hunnewell Room 268, Boston, MA 02115. E-mail address: gary.visner@childrens.harvard.edu

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Materials and Methods

Materials

All chemicals and reagents were purchased from Sigma-Aldrich unless otherwise specified.

Plasmid construction and establishment of hIDO-overexpressing endothelial cell line

The hIDO-containing plasmid with an ET-1 promoter was constructed and is referred to as pET1-hIDO. The empty vector (pET-null) served as the control vector. Constructs were confirmed by restriction and sequence analysis (5, 13). A stable hIDO-overexpressing endothelial cell line was established by integrating hIDO gene into the genomic DNA of HUVEC (American Type Culture Collection) using the Sleeping Beauty-based nonviral gene transfer system as described previously (13).

Animal model, in vivo gene delivery, lung function and histopathology

Specific pathogen-free male Lewis and Sprague-Dawley rats (~300 g) were purchased from Harlan Sprague Dawley and housed and cared for by Animal Care Services at the Children’s Hospital Boston. The orthotopic left lung transplants were performed using Lewis donors and Sprague-Dawley recipient (allografts) or the same strain (isografts), as described previously (4, 13). Some rats receiving allografts were given IDO inhibitor 1-mT in drinking water (5 mg/ml, pH 7) immediately after surgery. Left (transplanted) lungs were harvested at 6 days posttransplantation in all animals (4, 5).

For in vivo gene delivery, 450 µg of plasmid DNA was complexed with in vivo jetPEI (Qbiogene) using a charge ratio of 1:10. The DNA/polyethyleneimine (PEI) complex was delivered to donor animals by tail vein injection 24 h before transplantation (13). No gene therapy was given to recipient animals at any time. All animals were allowed to recover and killed 6 days posttransplantation.

In vivo assessment of transplanted lung function was performed by measuring peak airway pressures (PawP) and partial pressure of oxygen (PaO2), as previously described (5, 14). In brief, animals were mechanically ventilated with 2% isoflurane at 80 breaths/min, 10 ml/kg, and a FiO2 <0.6. Temperatures were maintained above 37°C. Left (transplanted) lungs were excised and minced into small pieces under sterile conditions. The pieces were digested for 60 min at 37°C in RPMI 1640 (2 mM L-glutamine, 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin) and either with irradiated allogeneic donor splenocytes or without stimulator cells (stimulator/responder cell ratio was 1:1) at 37°C, 5% CO2, in a humidified atmosphere. The purity of T cells was estimated to be 90–95% by FACS analysis.

To measure the quantitative extent of proliferation in the purified CD8+ T cells from untreated and IDO-high lung allograft, the cells were labeled by incubation for 10 min at room temperature with CFSE (2 µM) and then cultured in complete RPMI 1640 (2 mM L-glutamine, 1% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin) and either with irradiated allogeneic donor splenocytes or without stimulator cells (stimulator/responder cell ratio was 1:1) at 37°C, 5% CO2, in a humidified atmosphere. T cell proliferation was assessed by FACS analysis of CFSE dilution after 72 h (16).

For allogeneic cytotoxicity assay, CD8+ T cells collected from untreated and IDO-high lung allografts were prepared as effector cells and target cells were prepared with 2% irradiating allogeneic Lewis or isogenic Sprague-Dawley rat splenocytes (negative control) with Con A (2.5 µg/ml) for 48 h. The effector cells were cocultured with the target cells (106 per well) in a white opaque 96-well round-bottom plate for 4 h at 37°C in a humidified atmosphere of 5% CO2. Lysis of target cells was assessed using a CellTox bioluminescence cytotoxicity assay kit (Cell Technology).

Real-time quantitative PCR

Total cellular RNA was extracted using RNeasy mini kit (Qiagen), reverse-transcribed into cDNA, and amplified using TaqMan reverse transcription reagents (Applied Biosystems). Quantitative analysis of gene expression was performed using the ABI Prism 7700 sequence detection system with 18S rRNA used as an endogenous reference, as previously described (4, 13). Specific primers used for detection of message for IDO, perforin, and granzyme A and B were purchased from Applied Biosystems.

Biochemical analyses and Western blotting

Kynurenine concentration in lung tissue supernatant or cell culture medium was measured at 480 nm by a spectrophotometer with purified t-kynurenine (0–100 µM) used as a standard, as previously described (4).

Protein levels of hIDO, perforin, and granzyme A and B in cell lysate or tissue supernatant were evaluated by an ECL Western blotting analysis kit (Amersham Biosciences). Mouse monoclonal anti-hIDO Ab (1/1000 dilution; Millipore Corporation), anti-perforin Ab, and anti-granzyme A and B anti-Ab (1/500; Santa Cruz Biotechnology) were used to detect hIDO, perforin, and granzyme A and B protein, respectively (4, 5).

To mimic the in vivo condition of IDO-high environment containing active CD8+ T cells, coculture study was performed using the BD Falcon Cell Culture Insert Kit (BD Biosciences) (4). Normal or IDO-overexpressing HUVECs were seeded onto 35-mm diameter wells at 80% confluence, 10 µg/ml PEI, and 1.0. A laparotomy-sternotomy was performed and the right main bronchus occluded using a microvascular clip. PawP from the left (transplanted) lung was measured with a pressure transducer. Meanwhile, PaO2 was measured from blood removed from the abdominal aorta using an i-STAT portable analyzer (Heska Corporation).

For lung morphological assessment, the paraffin-embedded lung block was cut into 4-µm sections and stained with H&E. The severity of acute cellular rejection (ACR) was graded as A0–A4 based on the revised work of the Children’s Hospital Boston. The orthotopic left lung transplants were performed using Lewis donors and Sprague-Dawley recipient (allografts) or the same strain (isografts), as described previously (4, 13). Some rats receiving allografts were given IDO inhibitor 1-mT in drinking water (5 mg/ml, pH 7) immediately after surgery. Left (transplanted) lungs were harvested at 6 days posttransplantation (13).

To mimic the in vivo condition of IDO-high environment containing active CD8+ T cells, cell coculture study was performed using the BD Falcon Cell Culture Insert Kit (BD Biosciences) (4). Normal or IDO-overexpressing HUVECs were seeded onto 35-mm diameter wells at 80% confluence, 10 µg/ml PEI, and 1.0. A laparotomy-sternotomy was performed and the right main bronchus occluded using a microvascular clip. PawP from the left (transplanted) lung was measured with a pressure transducer. Meanwhile, PaO2 was measured from blood removed from the abdominal aorta using an i-STAT portable analyzer (Heska Corporation).

Mitochondrial pathophysiology

Commercial kits were used to determine intracellular ATP level (Molecular Probes), to isolate mitochondrial protein (BioChain Institute) and to assess citrate synthase activity in isolated CD8+ T cells according to the manufacturer’s instruction.

To mimic the in vivo condition of IDO-high environment containing active CD8+ T cells, cell coculture study was performed using the BD Falcon Cell Culture Insert Kit (BD Biosciences) (4). Normal or hIDO-overexpressing HUVECs were seeded onto 35-mm diameter wells at 80% confluence, 10 µg/ml PEI, and 1.0. A laparotomy-sternotomy was performed and the right main bronchus occluded using a microvascular clip. PawP from the left (transplanted) lung was measured with a pressure transducer. Meanwhile, PaO2 was measured from blood removed from the abdominal aorta using an i-STAT portable analyzer (Heska Corporation).

For lung morphological assessment, the paraffin-embedded lung block was cut into 4-µm sections and stained with H&E. The severity of acute cellular rejection (ACR) was graded as A0–A4 based on the revised working formulation for the classification of pulmonary allograft rejection according to the presence and extent of perivascular and interstitial mononuclear cell infiltrates, as described previously (5, 14, 15). The experimental protocols were approved by the Animal Care Committee of the Children’s Hospital Boston.

T cell isolation, proliferation and alloccytotoxicity assay

Left (transplanted) lung was excised and minced into small pieces under sterile conditions. The pieces were digested for 60 min at 37°C in RPMI 1640 containing 0.7 mg/ml collagenase IV and 0.3 mg/ml Dnase I (Roche). A single-cell suspension was prepared by passing the digested lungs through a 70-µm BD Falcon cell strainer (BD Biosciences), as described previously (13). Purified CD8+ T cells were isolated from the single-cell suspension by immunomagnetic-activated sorting using a rat anti-CD8+ T cell isolation kit (Milteny Biotec). The purity of T cells was estimated to be 90–95% by FACS analysis.

To measure the quantitative extent of proliferation in the purified CD8+ T cells from untreated and IDO-high lung allograft, the cells were labeled by incubation for 10 min at room temperature with CFSE (2 µM) and then cultured in complete RPMI 1640 (2 mM L-glutamine, 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin) and either with irradiated allogeneic donor splenocytes or without stimulator cells (stimulator/responder cell ratio was 1:1) at 37°C, 5% CO2, in a humidified atmosphere. T cell proliferation was assessed by FACS analysis of CFSE dilution after 72 h (16).

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succinate, 0.3 mM EDTA, 1 mM KCN, 100 μM oxidized cytochrome c, and purified mitochondrial protein (30 μg/ml). Specific complex II/III activities were determined by calculating the slope of the reaction in the linear range in the presence or absence of 10 μM antimycin A; 4) complex III (ubiquinol:cytochrome c reductase) activity was measured by following the reduction of oxidized cytochrome c at 550 nm in a reaction mixture containing 25 mM potassium phosphate (pH 7.4), 1 mM EDTA, 1 mM KCN, 0.5% BSA, 100 μM oxidized cytochrome c, and purified mitochondrial protein (25 μg/ml). The reaction was initiated by adding 50 μM decylubiquinol, which was made by dissolving decylubiquinone in acidified ethanol and using sodium dithionite as a reducing agent (19). Specific complex III activities were determined by calculating the slope of the reaction in the linear range in the presence or absence of 10 μM antimycin A (complex III inhibitor); 5) complex IV activity was determined using a rat complex IV assay kit according to the manufacturer’s instruction (MitoSciences).

Statistics Data are expressed as mean ± SEM, and statistical analyses performed with the Prism statistical program (GraphPad Software). One-way ANOVA with the Newman-Keul’s test was used to evaluate differences between groups. A p value of less than 0.05 was considered significant.

Results

Enhanced local IDO activity reduces, but does not eliminate, the number of effector CD8⁺ T cells in lung allografts

To create an in vivo environment with enhanced IDO activity, we delivered a plasmid with an endothelial cell specific promoter, ET-1, encoding pET-hIDO to lung grafts by using a pharmacological-grade reagent, polymer PEI, as a gene carrier delivered by i.v. injection. PEI has been shown to transfect lung tissue in vivo with high efficiency and specificity. Moreover, PEI at the concentrations used causes little, if any, lung inflammation (4, 5, 13). The pET-hIDO/PEI complex was given to donor lung 24 h before surgery and a high level of hIDO mRNA and protein was detected in hIDO-treated lung allografts 6 days after transplantation, whereas no hIDO mRNA or protein was found in normal lung, lung isografts, and untreated or vehicle-treated lung allografts (Fig. 1 A).

The hIDO transgene expression is sufficient to create an IDO-high tissue environment, as endogenous kynurenine content was significantly elevated in the pET-hIDO/PEI-treated allografts in comparison to untreated or vehicle-treated allografts. Notably, the hIDO-induced high level of kynurenine was blocked using the pharmacologic inhibitor of IDO, 1-mT (Fig. 1 A), confirming that increased kynurenine level was due to enhanced IDO activity provided by hIDO transgene.

Considering that effector CD8⁺ T cells are major effector cells responsible for acute lung allograft injury in rodents (12), we determined whether alloreactive CD8⁺ T cells can survive in the IDO-high tissue environment. We confirmed the previous study showing a high density of CD8⁺ T cells in rodent lung allografts (12), as a large number of CD3⁺- (Fig. 1 B) and CD8⁺-positive T cells (Fig. 1 C) were found in untreated allografts; this finding was not affected by treatment with vehicle or pET-hIDO/PEI in combination with 1-mT. In contrast, the active hIDO transgene

![Fig. 1.](http://www.jimmunol.org/.../IDO%20inhibits%20CD8%20T%20cell%20cytotoxicity.png)
significantly reduced the absolute number of CD3⁺ and CD8⁺ T cells detected in lung allografts, which is consistent with IDO’s proapoptosis biological features. However, this effect is incomplete, as the number of CD3⁺ (Fig. 1B) and CD8⁺ T cells (Fig. 1C) in the IDO-high allograft was still significantly higher than that from normal lungs or isografts. Intriguingly, the remaining CD8⁺ T cells in the IDO-high allografts are still viable, as determined by trypan blue dye exclusion technique (data not shown). To further confirm that CD8⁺ T cells can survive in the IDO-high environments, we evaluated proliferation of alloreactive CD8⁺ T cells infiltrating untreated and pET-hIDO/PEI-treated lung allografts. Freshly isolated CD8⁺ cells from the two different allografts (hIDO treated and untreated) were labeled with CFSE to track cell division in vitro for 72 h. As shown in Fig. 2A, we observed that CD8⁺ T cells obtained from IDO-high lung allografts remained viable with a low level of proliferation for at least 3 days in experimental conditions without any additional stimulation, thus excluding the possibility that the cells have initiated an apoptotic death process (20). Extensive proliferation was observed in CD8⁺ T cells from untreated allografts, but far less in cells from hIDO-high allografts. Isolated CD8⁺ T cells from untreated allografts were highly responsive to allogeneic stimulation (Fig. 2A); however, allogeneic stimulated CD8⁺ T cell proliferation from IDO-treated allografts was markedly reduced. Moreover, we observed that the diminished proliferation of the hIDO-treated CD8⁺ cells is associated with impaired biochemical conditions, as a restricted ATP production (Fig. 2B) and a lower level of citrate synthase activity (Fig. 2C) was seen in cells obtained from hIDO-treated allografts as compared with those from untreated allografts. These results support the hypothesis that alloreactive CD8⁺ cells can survive in IDO-high in vivo conditions; however, the proliferative property of CD8⁺ T cells function was drastically reduced.

**IDI-high tissue microenvironments abrogate cytotoxicity of alloreactive CD8⁺ T cells**

We next asked whether alloreactive CD8⁺ cells lost their cytotoxic function in the IDO-high environment. To clarify this issue, we first assessed rat lung allograft rejection/injury based on lung function parameters and histologic analysis. Similar to our previous experiments in which hIDO transgene was delivered intratracheally (5), i.v. delivery of hIDO also resulted in a remarkable reduction in ACR and improved lung function. Untreated lung allografts exhibited widespread tissue necrosis and destruction of bronchovascular architecture at 6 days posttransplantation. Notably, the morphological appearance of the IDO-high allografts displayed little or no obvious pathologic lesions and closely resembled those of normal left lungs or lung isografts (Fig. 3A). As shown in Fig. 3B, the ACR grade in IDO-high allografts was significantly lower than that from untreated or vehicle-treated allografts. Essentially consistent with the histological observations, quantitative analysis of pulmonary function revealed that hIDO transgene expression significantly reduced PawP (Fig. 3C) and increased PaO₂ in blood taken from left pulmonary vein (Fig. 3D) in lung allografts. These clinical observations suggest that, although the active hIDO transgene could not entirely eliminate infiltrating CD8⁺ T cells from lung allografts, the CD8⁺ T cells within the allograft do not seem to have a potent cytotoxic effector function and consequently failed to attack the allogeneic donor lung cells.

To further confirm that the IDO-high environment inhibited cytotoxic activity of effector CD8⁺ T cells, we performed a direct cytotoxicity assay of CTL activity. CD8⁺ T cells were isolated and collected from normal and IDO-high lung allografts and assessed for the ability to lyse Con A-treated Lewis (allogeneic) or Sprague-Dawley (isogeneic control) splenocytes. Our results showed that CD8⁺ T cells obtained from untreated or vehicle-treated allografts potently lyse allogeneic target cells, whereas the cells from
hIDO-high allografts were significantly less effective. In contrast, all the CD8+ cells had no effect on lysis of isogeneic target (negative control) cells (Fig. 3E). These results confirm that enhanced IDO activity induced by the hIDO transgene is able to prevent acute lung allograft injury not only by inhibiting allogeneic stimulated proliferation but also through its inhibition of CD8+ T cell cytotoxic function. The inhibition on CD8+ T cell-mediated cytotoxicity was attributed to the increased IDO activity, as the improved histological appearance and functional property seen in the hIDO-high allografts was reversed by IDO inhibitor 1-mT (Fig. 3, B–E).

The IDO-dependent reduction on alloreactive CD8+ T cell-mediated cytotoxicity resulted from impaired granule exocytosis pathway

The above findings raise an alternate possibility that IDO-high environments hamper cytotoxic function of infiltrating CD8+ T cells through blocking of TCR signaling. To address this issue, freshly isolated CD8+ T cells from lung allografts were analyzed for transcript expression of perforin (Fig. 4A) and granzyme A (Fig. 4B) and B (Fig. 4C), which are key indicators for TCR activation of effector CD8+ T cells (20). We first confirmed that CD8+ cells obtained from normal lungs or lung isografts are not in an activated state, as these cells showed little perforin mRNA expression and no detectable level of mRNA for granzyme A and B. In contrast, a dramatic increase in these signals was seen in CD8+ T cells taken from lung allografts and notably, mRNA abundance for these genes was not affected by the active hIDO transgene expression (Fig. 4, A–C). These results suggest that CD8+ T cells infiltrating to hIDO-high lung allografts, like the cells infiltrating to the untreated allografts, were indeed triggered by engagement of T cell receptors. Moreover, the initial and proximal CD8+ TCR-signaling pathway affecting activities of transcription factors were not blocked by IDO.

Although CD8+ T cells obtained from IDO-high allografts express abundant mRNA for perforin and granzyme A and B, these cells have lost their cytotoxic effector functions against donor target cells (Fig. 3). We therefore further analyzed these cells for expression of the major components in cytotoxic granules at the protein level. Western blot analysis revealed that resting CD8+ T cells isolated from normal or lung isografts constitutively express perforin protein, which was further up-regulated in CD8+ T cells obtained from untreated or vehicle-treated lung allografts. Unlike mRNA level, however, the active hIDO transgene significantly reduced perforin protein expression (Fig. 4A). Similarly, CD8+ T cells purified from untreated or vehicle-treated lung allografts exhibited stronger granzyme A (Fig. 4B) and B (Fig. 4C) protein expression compared with CD8+ T cells from IDO-high lung allografts, whereas these proteins were not detectable in cells from

![Figure 3](http://www.jimmunol.org/content/pubapi/100/9/1026/F3.large.jpg)
normal lung or lung isografts. Quantitative assessment for the content of total granule enzymes mirrored the granzyme A and B protein expression, in which CD8$^+$ T cells obtained from untreated allografts contained a significantly higher level of granule enzymes compared with cells from IDO-high lung allografts. The reduction in granule enzyme content was reversed by 1-mT (Fig. 4D). These

FIGURE 4. Effector CD8$^+$ T cells obtained from IDO-high tissue environment have defects in production of cytotoxic proteins. Level of mRNA and expression of protein of perforin (A), granzyme (Gr) A (B), and granzyme B (C) in CD8 T cells from the various lung tissues is indicated. The Western blot was reprobed with an antiactin Ab and quantified by densitometry using a Bio-Rad Gel Doc system. In D, Quantification of the content of total granule enzyme in freshly purified CD8$^+$ T was determined by cleavage of BLT with 5,5'-dithiobis(2-nitrobenzoic acid) as coloring agent. Graph or data represent assessment in a minimum of four independent experiments. Results are mean ± SEM. *, $p < 0.05$; **, $p < 0.01$ vs all other allograft groups.
IFN-γ, there was ~60% reduction of TNF-α production and nearly complete inhibition of IL-2 production in CD8⁺ T cells from IDO-high lung allografts (Fig. 5, B and C).

Enhanced IDO leads to an impaired bioenergetic condition in active CD8⁺ T cells

The findings that ATP production and citrate synthase activity are inhibited in CD8⁺ T cells obtained from IDO-high lung allografts (Fig. 2) yields potential clues about the mechanisms behind IDO-dependent inhibition on effector CD8⁺ T cell proliferation, cytokine production, and protein synthesis of cytotoxic granule enzymes. We began to address this issue by establishing and cultivating a stable hIDO-overexpressing cell line. As shown in Fig. 6A, HUVECs transfected with hIDO gene expressed active IDO enzymatic activity, as judged by the accumulation of L-kynurenine in the culture medium in the presence of an excessive amount of L-tryptophan (400 μM). Then we cocultured hIDO-overexpressing HUVECs with alloreactive CD8⁺ T cells purified from lung allografts and monitored the possible changes of mitochondrial oxidative phosphorylation (OXPHOS) in these cells. This study was prompted by the notion that OXPHOS is the core mitochondrial pathway responsible for transferring electrons through a series of acceptor cytochromes to generate ΔΨm, setting-up a driving force for ATP synthesis. We observed that exposure of alloreactive CD8⁺ T cells to hIDO-overexpressing HUVECs elicited a significant decrease in ΔΨm, whereas concomitant treatment with 1-mT abolished these changes (Fig. 6B). In a separate study, CD8⁺ T cells were cultured in conditioned medium with exogenously added L-kynurenine (0–400 μM). The results showed that L-kynurenine dose-dependently reduced ΔΨm. Notably, reduction on ΔΨm occurred at L-kynurenine concentration ~200 μM (Fig. 6C), which corresponds to concentrations achieved in culture medium with hIDO-overexpressed HUVECs (Fig. 6A). Thus, IDO via production of kynurenine in hIDO-expressing cells could interfere with mitochondrial bioenergetics of neighboring CD8⁺ T cells.

Because mitochondrial ΔΨm is generated by a functional electron transport chain containing four multiprotein complexes, next we conducted more detailed studies on the ability of IDO-overexpressing cells to affect activity of individual complexes in active CD8⁺ T cells. Mitochondria was isolated from CD8⁺ T cells that have been cultured with normal or hIDO-overexpressing HUVECs for 48 h and analyzed for activity of complex I, II, III, linked II-III, and IV. Our results showed that IDO selectively reduced activity of complex I, whereas the activity of complex II, III, linked II-III, and IV remained intact (Fig. 6D). These in vitro observations are in line with our in vivo study showing that hIDO transgene limited ATP production in the infiltrating CD8⁺ T cells. In contrast, these results also demonstrated that the majority of the electron transport chain components are resistant to IDO-associated mitochondrial toxicity. We thus conclude that the survival of the infiltrating CD8⁺ T cells in the hIDO-high allografts as observed (Fig. 2A) is, at least in part, of the results of still-intact complexes II-IV.

Discussion

The role of IDO as an immunosuppressive enzyme contributing to tumoral escape, immune tolerance, and providing protection against allograft injury has been established. However, molecular mechanisms responsible for the IDO-dependent suppression on immune responses are still being investigated. Munn et al. proposed that the inhibitory effect displayed by IDO on T cell reaction can be due to depletion of tryptophan, an essential amino acid for T cell growth (1). Frumento et al. further showed that IDO-mediated tryptophan-derived metabolites, such as kynurenine, are proapoptotic to T cells (21). In the present

results indicate that IDO is able to block translation of perforin and granzyme mRNAs, even though TCR signaling in the IDO-treated CTLs appears normal.

IDO-high tissue microenvironment differentially inhibits cytokine production by effector CD8⁺ T cells

Cytokine production is a characteristic of CD8⁺ T cell response to activation. In agreement with this and the above findings of induced mRNA of the granule exocytosis pathway, we found that abundant IFN-γ, which is another major activation marker for effector CD8⁺ T cells, was produced in CD8⁺ T cells from lung allografts, regardless of treatment with hIDO. Again, this is in contrast to the observation that CD8⁺ T cells taken from normal lung or lung isografts contained little IFN-γ (Fig. 5A). Similarly, the cytokines TNF-α and IL-2 were dramatically increased in CD8⁺ T cells isolated from untreated lung allografts as compared with untransplanted control lungs and isografts. However, unlike

FIGURE 5. Effect of IDO on cytokine profile in CD8⁺ T cells. The CD8⁺ T cells were purified from lung tissue and the indicated cytokine measured in cell lysates with commercial ELISA kits. Results are mean ± SEM (n = 4 in each group). *, p < 0.05; **, p < 0.01 vs other allograft groups.
study, we demonstrate in a rat model of orthotopic vascularized lung transplantation that a substantial amount of alloreactive effector CD8⁺/H11001 T cells exist in IDO-high lung allografts. Moreover, these allografts containing CD8⁺/H11001 T cells, like lung isografts, show little if any clinical and histological evidence for ACR. We also observed that CD8⁺/H11001 T cells with no signs of apoptosis were found within other type of tissue expressing high level of IDO, i.e., placenta from mice with successful allogeneic pregnancies (our unpublished data). These intriguing findings indicate that T cell apoptosis/death induced by tryptophan starvation and/or production of tryptophan-derived T cell toxic metabolites may not be the only mechanism responsible for IDO-dependent immune suppression.

We provide several lines of evidence showing that an IDO-high environment inhibits all three areas of effector CD8⁺/H11001 T cell function with impaired allogeneic stimulated proliferation, cytokine production, and degranulation/cytotoxicity. First, MLR assay showed that CD8⁺/H11001 T cells obtained from lung allografts strongly proliferate in response to allogeneic stimulator cells, whereas proliferation of CD8⁺/H11001 T cells from IDO-high allografts was weak by the same challenge. Second, CD8⁺/H11001 T cells from IDO-high allografts lost their ability to lyse allogeneic target cells, even though these CD8⁺/H11001 T cells remain viable and can proliferate in response to TCR or allogeneic challenge. Third, the reduced cytotoxicity of CD8⁺/H11001 T cells taken from the IDO-high allografts was mirrored by the acceptance of IDO-high allografts. Fourth, the major component of cytotoxic lymphocyte granules, including perforin and granzyme A and B, were significantly reduced in CD8⁺/H11001 cells from IDO-high allografts compared with that from untreated allografts. Last, the IDO-high environment resulted in differential cytokine production in effector CD8⁺/H11001 T cells. The reduced effector CD8⁺/H11001 T cell functions were due to enhanced IDO activity, as pharmacologic inhibition of IDO by 1-mT reversed these effects. The above data, taken together with a recent study showing that CD8⁺/H11001 T cells play a predominant role in mediating lung allograft rejection (12), suggests that IDO-dependent inhibition on cytotoxic effector function of infiltrating CD8⁺/H11001 T cells represent an additional mechanism underlying IDO-induced immune suppression. It should be noted, however, that the present study does not exclude the possibility that active hIDO transgene expression may also impact CD4⁺/H11001 T cell responses or passenger leukocyte activity which in turn contribute to the observed CD8⁺/H11001 T dysfunction.

FIGURE 6. IDO inhibits mitochondrial function of CD8⁺/H11001 T cells by decreasing complex I electron transfer activity. A, Normal or IDO-overexpressing HUVECs were cultured in complete MEM containing excess L-tryptophan (400 μM) for up to 48 h. Kynurenine (kyn) released to the culture medium was determined at time points indicated. B, Purified CD8⁺/H11001 T cells obtained from lung allografts were cocultured with normal or IDO-overexpressing HUVECs in the absence or presence of 1-mT (1 mM) in mixed medium (50/50 MEM/RPMI 1640). The ∆Ψm of the CD8⁺/H11001 T cells was evaluated 48 h after coculturing. C, CD8⁺/H11001 T cells were cultured in the complete RPMI 1640 with exogenously added L-kynurenine (0–400 μM). After incubation for 48 h at 37°C, ∆Ψm of CD8⁺/H11001 T cells was determined with a commercial kit using a unique fluorescent probe, JC-1, to signal the loss of ∆Ψm. D, In separate experiments, mitochondrial protein was prepared from the CD8⁺/H11001 T cells and the activity of mitochondrial membrane-bound protein complexes that participate in the electron transfer was measured. Results are shown as mean ± SEM of four independent experiments. In A, **, p < 0.01 vs the other two groups at the time point indicated. In B and D, *, p < 0.05 vs all other groups. In C, **, p < 0.01 ∆Ψm at 200 μM vs ∆Ψm at 0–100 μM; †, p < 0.05 ∆Ψm at 400 μM vs other groups.
IDO may create a local microenvironment that can lead to impairment of Ag-dependent activation of CD8\(^+\) T cells, thereby contributing to immune tolerance to donor Ags. Evidence collected from the present study, however, disfavors this notion or at least suggests it is only part of the story. We explored the effect of IDO on production of alloantigen-specific cytokine release and found that IFN-\(\gamma\) expression was induced and similar in freshly isolated CD8\(^+\) T cells from both IDO-high and untreated lung allografts. In keeping with these observations, transcripts encoding major cytotoxic mediators, including perforin and granzyme A and B, were up-regulated in IDO-treated CD8\(^+\) T cells. This contrasts sharply with the findings that the IDO-treated CD8\(^+\) T cells expressed a very low level of the three cytotoxic proteins. In an experiment similar to our own, resting NK cells were found to contain little perforin and granzyme B, but the mRNA for these two genes were abundant (22). Therefore, the IDO-dependent inhibition on cytotoxic/degranulation effector function of active CD8\(^+\) T cells is achieved by posttranscriptional regulation on perforin and the granzyme genes.

The common pathway that may affect T cell viability, proliferation, and effector function is the mitochondrial OXPHOS by a functional electron transport chain (23). The present study provides the first evidence that IDO can limit the energy supply in active CD8\(^+\) T cells by reducing the mitochondrial membrane \(\Delta \Psi \text{m}\). Moreover, we provide evidence showing that the IDO-associated mitochondrial dysfunction is the result of impaired complex I function. Exactly why IDO selectively inhibits complex I activity is not evident from the present study. However, it was found that NADH can be transferred to NAD\(^+\) via complex I and ATP synthesis is dependent on a continuous supply of NAD\(^+\). In contrast, tryptophan is the sole source of substrate for de novo NAD\(^+\) synthesis and enhanced IDO can boost intracellular NAD\(^+\) concentration (24). Thus, there is a possible negative feedback loop to regulate complex I activity by IDO via production of kynurenine and NAD\(^+\) inside the activated CD8\(^+\) T cells.

Mitochondrial function, specifically an intact electron transport complex I function, has been shown to be critical for CD8\(^+\) T cell function (25). Rotenone, a potent inhibitor of electron transport complex I, was shown to block stimulated proliferation and degranulation and result in differential cytokine production in effector CD8\(^+\) T cells (25). Similarly we found that an IDO-high environment resulted in a loss in CD8\(^+\) T cell function along with an inhibition of mitochondrial function and more specifically electron transport complex I. Interestingly, TNF-\(\alpha\) production in effector CD8\(^+\) T cells was found to be much more sensitive to rotenone inhibition of complex I than IFN-\(\gamma\)-was. The IDO-high environment also resulted in differential expression of TNF-\(\alpha\) and IFN-\(\gamma\)-in effector CD8\(^+\) T cells. In addition, we found that IL-2 production was the most sensitive to this effect, with nearly complete inhibition of IL-2 production. Given the fact that the only signal shown to up-regulate perforin and granzyme A and B is IL-2 (26), the IDO-induced defects in production of cytotoxic proteins in effector CD8\(^+\) T may also be attributed to the loss of IL-2 signal. In addition, several reports showed that activation of general control non-derepressible 2 (GCN2), a stress-responsive kinase, led to profound inhibition of mRNA translation and impaired T cell function (27, 28). It is thus plausible that the impaired bioenergetic condition created by IDO may activate GCN2, and consequently inhibit cytoxicity of effector CD8\(^+\) cells. Obviously, more studies are required to clarify the issues.

It should be noted that the present study does not exclude the possibility that the depletion of tryptophan and/or the production of tryptophan metabolites are responsible for the inhibition of the immune response by IDO. Rather, we expanded the above theories by showing that IDO can limit effector function of activated CD8\(^+\) T cells before IDO-induced cell apoptosis/death. These intriguing findings prompt us to propose a novel model of IDO action suppressing immune response. IDO creates a tissue environment that can initially reduce the functional property and proliferation of T cells by limiting their energy demand via an incomplete electron transport chain, which in turn sensitizes activated cells to tryptophan starvation- or tryptophan metabolites-induced apoptosis/death. This conclusion is drawn from the experimental results based on a lung transplant model. However, it might have far-reaching implications for other important phenomenon, i.e., IDO-induced tumoral immune escape.

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**Disclosures**

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**References**


