Indoleamine 2,3-dioxygenase specific, cytotoxic T cells as immune regulators

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Running Title: IDO-specific T cells

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Scientific Category: Immunobiology
Abstract

Indoleamine 2,3-dioxygenase (IDO) is an immunoregulatory enzyme that is implicated in suppressing T-cell immunity in normal and pathological settings. Here, we describe that spontaneous cytotoxic T-cell reactivity against IDO exists not only in cancer patients but also in healthy individuals. We show that the presence of such IDO-specific CD8+ T cells boosted T-cell immunity against viral or tumor-associated antigens by eliminating IDO+ suppressive cells. This had profound effects on the balance between IL-17-producing CD4+ T cells and regulatory T cells. Furthermore, this caused an increase in the production of the pro-inflammatory cytokines IL-6 and TNF-α while decreasing the IL-10 production. Finally, the addition of IDO-inducing agents (i.e. the TLR9 ligand CpG, soluble CTLA4 or IFN-γ) induced IDO-specific T cells among PBMC from cancer patients as well as healthy donors. In the clinical setting, IDO may serve as an important and widely applicable target for immunotherapeutic strategies where IDO play a significant regulatory role. The present describe for the first time effector T cells with a general regulatory function that may play a vital role for the mounting or maintaining of an effective adaptive immune response. We suggest terming such effector T cells “supporter T cells”.

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**Introduction**

Induction of tolerance, which is a central mechanism counteracting tumor-specific immunity and preventing effective anti-cancer immune therapy, requires a specific environment in which tolerogenic dendritic cells (DC) play an essential role deviating the immune response away from effective immunity. It was recently shown that IDO provides a potential mechanism for the development of DC mediated T-cell tolerance. IDO+ DC inhibit T-cell proliferation due to tryptophan depletion and accumulation of toxic tryptophan metabolites. IDO+ DC have been shown to induce T-cell anergy and/or generation of regulatory T cells (Tregs). In cancer patients, IDO elevation occurs in a subset of plasmacytoid DC in tumor-draining lymph nodes. In addition, most human tumors overexpress IDO. Activation of IDO in either tumor cells or nodal regulatory DC each appears to be sufficient to facilitate tumoral immune escape. IDO may help tilting the tumor microenvironment from hostile to supportive for tumor cells, and also elaborate a peripheral mechanism of immune escape that could facilitate tumor progression.

Tregs have been defined as a specialized subpopulation of T cells that act to suppress activation of the immune system and thereby maintain immune system homeostasis and tolerance to self-antigens. Subsequently, they are additionally termed suppressor T cells. Tregs exist to down regulate immune responses in various inflammatory circumstances and ultimately assure peripheral T-cell tolerance. The best characterized subset of these immune suppressive cells are CD4+CD25highCD127Foxp3+ T cells. Over the past years, additional regulatory T-cell subsets including CD8+ suppressor T cells have been described in humans and mice. Recently, we identified very potent antigen-specific CD8+ suppressor T cells in PBMC from cancer patients. These natural occurring HLA-A2-restricted CD8+ T cells were specific for the anti-inflammatory molecule Heme Oxygenase-1 (HO-1). The data linked the cellular stress response to the regulation
of adaptive immunity and added a new dimension to the role of antigen-specific CD8+ T cells in the regulation of cellular immune responses.

We have recently described that IDO are spontaneously recognized by cytotoxic T cells (CTL) in cancer patients. Thus, IDO-specific T cells were present in peripheral blood as well as in the tumor microenvironment. These IDO-reactive T cells were able to recognize and kill tumor cells, including directly isolated AML blasts, as well as IDO-expressing DC, i.e. one of the major immune suppressive cell populations. We could not detect spontaneous responses against IDO in the control group of healthy individuals. Thus, albeit IDO has an immune suppressive effect, the up regulation of IDO expression seems to induce a specific cytotoxic T-cell response. However, we found it quite astonishing that the T cells in the patients did not exhibit tolerance towards IDO, since IDO is inducible under normal physiological conditions. We speculated that this could suggest a more general role of IDO-specific T cells in the regulation of the immune system. IDO may play a critical role for the strength and duration of a given immune response due to its inflammation-induced counter-regulatory function. Hence, IDO-specific CD8+ T cells may play an important role in the early phase of an immune response by eliminating IDO+ cells thereby delaying local immune suppression. With this hypothesis, we continued our analysis of possible IDO-specific T-cell responses in healthy donors as well as in cancer patients and examined the role of IDO-specific T cells in the adaptive immune system.
Materials and methods

Donors

Peripheral Blood Mononuclear Cells (PBMC) were collected from healthy individuals and cancer patients (renal cell carcinoma, melanoma, and breast cancer). Blood samples from cancer patients were drawn a minimum of four weeks after termination of any kind of anti-cancer therapy. The majority of renal cell carcinoma patients had previously been treated with IL2 and IFN-α, most melanoma patients had received high dose IL2 and IFN-α, while all breast cancer patients were pre-treated with several kinds of chemotherapy, (e.g. epirubicin, docetaxel, cabecitabine), trastuzumab, and/or endocrine therapy. Informed consent was obtained from the patients prior to any of these measures in accordance with the Declaration of Helsinki. All protocols were approved by the Herlev University Hospital ethics committee.

ELISPOT assay

The ELISPOT assay was used to quantify peptide-specific IFN-γ releasing effector cells as described previously 17;18. The spots were counted using the ImmunoSpot Series 2.0 Analyzer (CTL Analyzers).

MHC-tetramer staining

PBMC were stained with PE coupled MHC-tetramers, followed by antibody staining with CD8-allophycocyanin (APC) and CD3-FITC (BD Biosciences). 7-Amino-Actinomycin D (7AAD) was used for exclusion of nonviable cells in all samples (BD Biosciences). MHC-tetramers were prepared as described 19;20. The MHC-tetramer complexes used were: HLA-A2/IDO5 (IDO199-207; ALLEIASCL), HLA-A2/CMV pp65 495-503 (NLVPMVATV), HLA-A2/CMV IE1 316-324
(VLEETSVM), HLA-A2/ Flu matrix p58-66 (GILGFVFTL), HLA-A2/ MART-1 26-35
(EAAIIGILTV), HLA-A2/HIV-1 pol476-484 (ILKEPVHGV), and HLA-A3/HIV-1 nef73-82
(QVPLRPMTYK). The samples were analyzed and occasionally sorted on FACS Aria or FACS
Canto II, using DIVA software (BD Biosciences).

Establishment of antigen-specific T-cell cultures and clones

PBMC were stimulated with irradiated (25 Gy), IDO5 (IDO199-207; ALLEIASCL)-loaded
autologous DC with β2m, IL-12 (PeproTech), and U/ml IL-7 (PeproTech) in X-vivo with 5%
human AB serum. The cultures were restimulated every seven-ten days with IL-2. After four to five
weeks, growing cultures were tested for specificity for IDO5 and specific cultures were cloned by
limiting dilution in the presence of IDO5-loaded PBMC and IL-2. Growing clones were expanded
with IL-2 and IDO5-loaded PBMC or Dynabeads CD3/CD28 T-cell expander (Dynal).

Cytotoxicity assay

Conventional 4 hour 51Cr-release assays for CTL-mediated cytotoxicity was carried out as described
elsewhere 21. Target cells were peptide-loaded T2-cells, the colon cancer cell lines HCT116 and
SW480 (ATCC), the melanoma cell line FM55M 22, in vitro generated autologous immature Dc
(iDC) and matured DC (mDC), and allogeneic ex vivo isolated CD14+ monocytes (isolated using
MACS CD14+ microbeads). In some assays, CD14+ monocytes were treated with 100 U/ml IFN-γ
for two days prior to analysis. Lysis were blocked using the HLA class I specific mAb W6/32 (2
µg/100 µl). 23

Down-regulation of IDO in cancer cells
Human SW480 cancer cells were transfected with indicated short hairpin RNA (ShRNA) plasmids obtained from SuperArray using FuGene6 (Roche) according to manufacturers instructions. Blots were developed with the ECL system obtained from Amersham and a CCD camera (LAS-1000, Fujifilm). Antibodies used: anti-Cdk7 (MO-1) (Santa Cruz) and anti-IDO (Millipore Corporation).

Co-culturing with autologous IDO-specific T cells

PBMC were stimulated in vitro with 50 µg/ml viral peptide (CMV pp65495-503 (NLVPMVATV), CMV IE1316-324 (VLEETSVML) or Flu matrix p58-66 (GILGFVFTL)). 40 U/ml IL-2 was added on day 2 and 6. The PBMC were either cultured alone or added autologous IDO5-specific T cells (in a PBMC to IDO5-specific T cell ratio of 2000:1) on day 6. On day 9, the cultures were stimulated with 120 U/ml IL-2. After 12 days in culture, the number of viral-specific T cells in the cultures, either cultured alone or added IDO5-specific T cells was compared by MHC-tetramer staining. The number of Tregs, IL-17A producing T cells and the CD4/CD8 cell ratio in the cultures were also compared. As a control, PBMC were co-cultured with autologous CD8+ T cells of irrelevant specificity.

Co-stimulation with IDO peptide

PBMC were stimulated in vitro with 25 µg/ml viral or tumor-associated antigens (CMV pp65495-503 (NLVPMVATV), CMV IE1316-324 (VLEETSVML), or MART-126-35 (EAAGIGILTV)), either in co-culture with 25 µg/ml IDO5 peptide or an irrelevant peptide (HIV-1 pol476-484 (ILKEPVHGV)). 40 U/ml IL-2 was added every third day. Every seven days, the cultures were stimulated with a mixture of CMV- or MART-1 peptide plus IDO5 peptide, or a mixture of CMV or MART-1 peptide plus HIV-1 pol476-484 peptide, respectively. Cells were stimulated with 10-, 100-, and 1000-fold diluted peptides for the second, third and fourth peptide stimulation, respectively. After three to four
stimulations, the number of CMV- or MART-1-specific T cells in the cultures, either co-cultured with IDO5 peptide or HIV-1 pol476-484 peptide, was compared by MHC-tetramer staining. The number of Tregs, IL-17A producing T cells and the CD4/CD8 cell ratio in the cultures were also compared.

*Intracellular staining for CD4<sup>+</sup>CD25<sup>high</sup>CD127<sup>+</sup>Foxp3<sup>+</sup> Tregs*

Cells were stained with the following antibodies: CD3-APC-Cy7, CD4-PerCP, CD25-APC, and CD127-FITC (BD Biocience and eBioscience). After fixation and permeabilization, cells were stained with PE anti-human Foxp3 (eBioscience). Isotype controls were used to enable correct compensation and confirm antibody specificity. Cells were analysed using FACS Canto II flow cytometer (BD Bioscience).

*Intracellular staining for IL-17 producing T cells*

PBMC were stimulated with Leukocyte Activation Cocktail containing PMA, ionomycin and Brefaldin A (BD Bioscience) for five hours. Cells were stained with FITC anti-human IL-17A (eBioscience) after fixation and permeabilization according to manufacturer’s instructions, after surface staining with CD3-APC-Cy7 and CD4-PerCP (BD Bioscience). Isotype controls were used to enable correct compensation and confirm antibody specificity. Stained cells were analysed using FACS Canto II flow cytometer (BD Bioscience).

*Cytokine ELISA*

Cell culture supernatants were collected and stored at -80°C. Amounts of IL-6, IL-10, IL-17A, IFN-γ, and TNF-α were measured by standard sandwich ELISA using commercially available antibodies and standards according to the manufacturer’s protocols (eBioscience).
ELISA for quantitative determination of Tryptophan

Cell culture supernatants were collected and stored at -80°C. After precipitation and derivatization, Tryptophan concentrations were quantitatively determined by competitive ELISA according to manufactures instructions (Labor Diagnostika Nord). Quantification of unknown samples was achieved by comparing their absorbance with a reference curve prepared with known standards.

Induction of IDO-specific T cells by IFN-γ, CTLA4-Ig or CpG ODN

PBMC were stimulated with either 100 U/ml IFN-γ, 1 ug/ml CpG ODN (Type B CpG oligodeoxynucleotide specific for human TLR9; InvivoGen) or 1 ug/ml cytotoxic T lymphocyte associated antigen 4 (CTLA4)-IgG2a fusion protein (Research Diagnostics) once a week. 40 U/ml IL-2 was added every third day. After four weeks, the cultures were tested for the presence of IDO-specific T cells by MHC-tetramer staining.

RNA preparation and reverse transcription–coupled PCR

Resulting cDNA was tested using primers for GAPDH (5′-AGGGGGGAGCCAAAAGGG-3′, 5′-GAGGAGTGGGTGTCGCTGTTG-3′, positions 440 and 980, respectively; product size 558 bp). Primers suited for amplification were as follows: IDO (5′-TGTCGTAAGGTCTTGCCAGG-3′; 5′-CGAAATGAGAACAAACGTCC-3′, positions 408 and 557, respectively; product size, 170 bp).

Statistical analysis

The percentages of antigen-specific T cells between cultures were compared using one-tailed two sampled paired t-tests with a significance level at 0.05. The fold increase of antigen-specific T cells between PBMC cultures was defined as percentage of antigen specific T cells in cultures co-cultured with IDO peptide divided by percentage in cultures co-cultured with HIV-1 peptide.
Results

**IDO5-specific T cells are detectable in healthy donors**

First, we examined PBMC from healthy donors for the presence of T cells specific for the HLA-A2 restricted IDO-derived epitope IDO5 (IDO\textsubscript{199-207}; ALLEIASCL). We found that IDO-reactivity could readily be detected by IFN-\(\gamma\) ELISPOT and HLA-A2/IDO5 tetramer staining (Fig. 1A and B). All in all we examined 28 healthy donors for spontaneous T-cell reactivity against the HLA-A2 restricted IDO epitope and identified specific T cells in three individuals (Fig. 1A). As a control of the HLA-A2/IDO5 tetramer, an IDO5-specific T-cell clone were stained (Fig. 1C).

**IDO5-specific T cells are specifically able to kill IDO-expressing cells**

IDO-specific T-cell clones were established from bulk cultures by limiting dilution cloning from cancer patients\textsuperscript{16} as well as healthy donors. The lytic capacity of representative clones from a healthy donor and a breast cancer patient are depicted in Figure 1D-I. The IDO-specific T cells effectively killed IDO5-pulsed TAP-deficient T2-cells, whereas T2-cells pulsed with an irrelevant peptide (HIV-1 pol\textsubscript{476-484}) were not lysed (Fig. 1D). Importantly, IDO-specific T cells also killed the HLA-A2\(^+\)/IDO\(^+\) colon cancer cell line SW480 (Fig. 1E). In contrast, IDO-specific T cells did not lyse the HLA-A2\(^+\)/IDO\(^-\) colon cancer cell line HCT116 (Fig. 1E). HLA-restriction was confirmed by blocking HLA class I using the HLA class I specific mAb W6/32, which completely abolished lysis of the SW480 cells (Fig. 1F). Using IDO ShRNA we down-regulated IDO protein expression in SW480 and thereby rescued these tumor cells from being killed by IDO-specific T cells, whereas cells transfected with irrelevant control ShRNA were killed (Fig. 1F). Cold target inhibition assays using unlabeled T2-cells pulsed with IDO5 peptide confirmed HLA-A2/peptide specificity of the killing: The addition of cold (unlabeled) IDO5-pulsed T2-cells completely abrogated the killing of FM55M melanoma cells, whereas the addition of cold T2-cells pulsed with the irrelevant HIV-1
pol476-484 peptide did not have an effect on the killing of FM55M (Fig. 1G). IDO expression is not restricted to tumor cells, but can also be induced in immune cells. In this regard, IDO-specific T cells specifically killed autologous IDO+ in vitro generated mature DC, while IDO− immature DC and IDO− CD14+ monocytes were not killed (Fig. 1H and I). IDO is known to be induced by both type I and II interferons, which are found at sites of immune activation. Thus, IFN-γ is a well described inducer of IDO in many cell types including fibroblasts, endothelial cells, tumour cells, monocyte-derived macrophages and DC. We treated CD14+ monocytes with IFN-γ, which indeed induced IDO expression (data not shown). These IDO-expressing CD14+ monocytes were susceptible to killing by autologous IDO-specific T cells (Fig. 1I).

IDO-specific T cells boost viral immunity

We utilized IDO-specific T-cell clones to examine a potential role of such cells in enhancing immune responses. Hence, we added IDO-specific T cells from a healthy donor to autologous PBMC cultures (in ratio 1:2000) that were stimulated with an HLA-A2 restricted epitope from cytomegalovirus (CMV). The addition of IDO-specific T cells resulted in a vast increase in the number of tetramer positive, CMV-specific CD8+ T cells in the cultures; based on 4 independent experiments the number of CMV-specific CD8+ T cells increased significantly (p < 0.05); on average from 18% to 36% (Fig. 2A and B). Furthermore, we observed a notable reduction of CD4+CD25highCD127Foxp3+ Tregs in cultures with added IDO-specific T cells (Fig. 2C). Importantly, these changes did not correspond to similar differences in the percentage of CD8+ and CD4+ T cells between the cultures (Fig. 2D).

Likewise, we added IDO-specific T cells from a breast cancer patient to autologous PBMC cultures (in ratio 1:2000) stimulated with an HLA-A2 restricted epitope from influenza (Flu). Again, the addition of IDO-specific T cells resulted in a vast increase in the number of tetramer positive,
antigen-specific CD8⁺ T cells in the cultures. This was observed in three independent experiments. In one experiment the number of Flu-specific CD8⁺ T cells almost doubled from 7% to nearly 14% (Fig. 3A). The addition of IDO-specific T cells at the same time decreased the amount of CD4⁺CD25hiCD127Foxp3+ Tregs in the cultures to the half (Fig. B), while increasing the number of IL-17A producing CD4⁺ T cells from 0.2% to 0.6% (Fig. 3C). Importantly, these changes did not correspond to similar differences in the percentage of CD8⁺ and CD4⁺ T cells between the cultures (Fig. 3D). We additionally compared the activity of IDO between the cultures by measuring the amount of Tryptophan in the cell culture supernatants. Interestingly, the highest concentration of Tryptophan, i.e. least IDO activity, was found in cultures with added IDO-specific T cells (Fig. 3E). Addition of IDO-specific T cells additionally reduced the concentration of IL-10 in the cell culture supernatants, while increasing concentrations of IL-17A, IL-6 and TNFα as measured by standard cytokine ELISA (Fig 3F). Notably, addition of autologous CD8⁺ T-cell clones of unknown specificities to PBMC from the same patient did not increase the number of tetramer positive, Flu-specific CD8⁺ T cells (data not shown). Likewise, we added a T-cell clone specific for the tumor-associated antigen ML-IAP²⁶ to autologous CMV peptide stimulated PBMC (in ratio 1:2000). The addition of ML-IAP-specific T cells did not change the number of CMV-tetramer positive CD8⁺ T cells (Fig. 3G), or the concentrations of IL-10, IL-17A, IL-6 and TNFα in the cell culture supernatants (data not shown). The amount of IFN-γ in the cell culture supernatants increased both by addition of IDO-specific T cells and by addition of ML-IAP-specific T cells (data not shown).

**Co-stimulation with IDO peptide boosts T-cell reactivity against viral and tumor-associated antigens**

To further analyze the supporting effect of IDO-specific T cells on T-cell responses, PBMC from 15 HLA-A2⁺ healthy donors and cancer patients were stimulated with an HLA-A2 restricted CMV or
MART-1 epitope either in co-culture with IDO5 peptide or an irrelevant HLA-A2 restricted epitope from HIV-1 in the presence of IL-2. After three to four in vitro stimulations IDO-specific T cells could be detected in cultures stimulated with IDO5 peptide (0.05%-0.1% IDO-specific CD8+ T cells). In comparison, IDO-reactivity could not be detected in any of the HIV-1 peptide stimulated cultures.

The increase in T-cell reactivity towards CMV- or MART-1 was calculated for each donor/patient as fold increase of MHC-tetramer-specific CD8+ T cells in co-culture with IDO5 peptide (Fig 4A). Notably, we observed from 1.5 to eight fold increases of tetramer-specific CD8+ T cells in 2/3 of the cultures with IDO-reactivity (Fig 4A, B and C). Importantly, co-stimulation with IDO5 peptide did not result in a decrease in CMV- or MART-1-reactivity in any of the examined individuals (Fig. 4A). Thus, co-activation of IDO-specific T cells not only boosted T-cell immunity towards viral antigens but also towards the well-known tumor-associated antigen MART-1.

Stimulation of IDO-specific T cells reduces Tregs numbers while boosting IL17 production

We examined the amount of Tregs and IL-17 producing CD4+ T cells in PBMC from melanoma patients stimulated with MART-1 peptide either in co-culture with IDO5 peptide or an irrelevant HIV-1 peptide in the presence of IL-2 as described above. We observed that activation of IDO-specific T cells with IDO5 peptide, not only boosted T-cell immunity towards MART-1 (Fig. 4A), but at the same time decreased the amount of CD4+CD25^highCD127^-Foxp3^+ Tregs (Fig. 5A and B), and increased the number of IL-17A producing CD4+ T cells (Fig. 5C and D). This was observed in all four examined patients. In cultures where co-stimulation with IDO5 peptide did not boost T-cell immunity towards MART-1 (Fig. 4A), we could not detect any differences in the number of Tregs (data not shown). Importantly, changes in the amount of MART-1-specific T cells, Tregs and IL-17A producing CD4+ T cells did not correspond to similar changes in the percentage of CD8+ and
CD4⁺ T cells between the cultures (Fig. 5E). Hence, activation of IDO-specific T cells with IDO5 peptide seemed to change the overall composition of regulatory cells. Finally, we compared the secretion of cytokines between cell culture supernatants by cytokine ELISA. PBMC cultures composing IDO-specific T cells, i.e. PBMC co-cultured with IDO5 peptide, showed higher concentrations of IL-17A, IL-6 and TNFα in the cell culture supernatants, whereas a lower amount of IL-10 was detected (Fig. 5F).

**IDO-inducing agents stimulate IDO-specific T cells with supporter functions**

IDO expression may be either constitutive or secondarily induced by mediators produced as a result of a local immune response. IFN-γ is a potent inducer of IDO, which could point to a scenario where IFN-γ indirectly induce or boost IDO-specific T cells. Therefore, PBMC from seven different healthy donors were treated with IFN-γ in the presence of IL-2, and subsequently examined for IDO-specific T cells. Indeed, we were able to detect HLA-A2/IDO5 tetramer positive CD8⁺ T cells (0.05%-0.1%) in four of the donors after treatment with IFN-γ (Fig. 6A). Triggering of functional IDO requires ligation of B7-1/B7-2 molecules on DC by CTLA4/CD28 expressed on T cells. TLR9 ligation activates DC to up regulate surface expression of B7 ligands and thereby increases expression of IDO. To determine whether this TLR9 ligand-induced up regulation of IDO expression in DC also results in activation of IDO-specific T cells, PBMC from three cancer patients were treated with the TLR9 ligand CpG ODN in the presence of IL-2 and, subsequently, examined for IDO-specific T cells. TLR9 signaling with CpG ODN induced a measurable number of IDO-specific T cells (0.1%) in two of three patients (Fig. 6B). Likewise, CTLA4 mediated up regulation of IDO has been demonstrated. In this regard, soluble CTLA4 (CTLA4-Ig) has been shown to induce IDO expression in DC which are competent to express functional IDO and mediate...
IDO-dependent T-cell suppression. To assess if CTLA4-Ig-induced IDO up-regulation indeed stimulates IDO-specific T cells, we co-cultured PBMC with CTLA4-Ig and IL-2, and, subsequently, examined the cultures for the presence of IDO-specific T cells. In two of three patients HLA-A2/IDO5 tetramer positive CD8+ T cells (0.1%-0.2%) were detectable after exposure to CTLA4-Ig (Fig. 6C).

Finally, we isolated IFN-γ-induced, MHC-tetramer positive T cells by Fluorescence-Activated Cell Sorting (FACS) and added these to CMV peptide stimulated PBMC from the same donor. The addition of the tetramer-sorted IDO-specific T cells boosted T-cell immunity towards CMV compared to cultures with the addition of a similar amount of FACS-sorted, autologous CD8+ T cells (Fig. 6D). Next, we examined the effect of co-culturing PBMC from another healthy donor with standard in vitro matured, autologous IDO+ DC. This indeed induced IDO-specific T cells in the PBMC culture (data not shown). Finally, we sorted HLA-A2/IDO5 tetramer positive T cells from a healthy donor after two in vitro IDO5 peptide stimulations. The tetramer positive IDO-specific T cells were co-cultured with autologous CMV peptide stimulated PBMC, which resulted in an increase in the number of CMV-specific T cells compared to cultures with the addition of a similar number of FACS-sorted, autologous CD8+ T cells (Fig. 6E). Thus, addition of HLA-A2/IDO5 tetramer positive T cells boosted T-cell immunity towards CMV antigens similar to the IDO-specific T-cell clones described above.
Discussion

In the present study, we show that circulating IDO-specific, cytotoxic CD8+ T cells are present in healthy donors although not as frequent as in patients with cancer. Even more important, we show that co-activation of IDO-specific cytotoxic T cells boosted T-cell immunity towards viral and tumor-associated antigens. We show that IDO-specific T cells were capable of killing IDO-expressing regulatory cells thereby directly targeting the IDO-dependent counter-regulatory pathway. This “supportive” effect on T-cell immunity by IDO-specific T cells might be mediated in several direct and indirect manners. First of all, IDO is a major inhibitor of the effector phase of the immune response. IDO expression can suppress effector T cells directly by degradation of the essential amino acid tryptophan or via enhancement of local Treg-mediated immunosuppression. With respect to the former, some of the biological effects of IDO are mediated through local depletion of tryptophan, whereas others are mediated via immunomodulatory tryptophan metabolites. Effector T cells starved of tryptophan are unable to proliferate and go into G1 cell cycle arrest. These cells are in addition more sensitive to apoptosis. We show that the level of tryptophan was elevated after the addition of IDO-specific T cells. This indicates that the addition of IDO-specific T cells directly decreased IDO activity, presumably due to the lysis of IDO-expressing cells. When IDO+ DC are injected in vivo, they create suppression and anergy in antigen-specific T cells in the LN draining the injection site. Constitutive IDO expression in DC provides T cells with regulatory properties that block T-cell responses to antigenic stimulation. The B7 receptors on IDO+ DC bind to CTLA4 on Tregs causing them to proliferate and induce antigen-specific anergy. Hence, IDO does not only suppress effector T cells directly but also influence Tregs bystander suppressor activity. Recently, it has been described that exposure of Tregs to IL-6 and other pro-inflammatory cytokines induces reprogramming of mature Tregs to acquire a phenotype resembling pro-inflammatory Th17 cells. It has been shown that IDO play a vital
role in this conversion of Foxp3+ Tregs to Th17-like effector cells 6,37. IDO stimulates Treg bystander suppressor activity and simultaneously blocks the IL-6 production that is required to convert Tregs into Th17-like T cells 6,37. Tumor-infiltrating Th17 cells express other cytokines in addition to IL-17, which might be functionally relevant 41,42. A large fraction of Th17 cells produce high levels of effector cytokines such as IL-2, GM-CSF, IFN-γ as well as TNF 40. In the study of Sharma et al. it was described that the phenotype of reprogrammed Tregs after IDO-blocking was similar to that of activated Th17 cells or to “polyfunctional” T helper cells 37. Hence, such Th17-like cells co-expressed IL-17, IL-22, IL-2 as well as TNF-α 37. These findings suggested that inducible or pre-existing IDO activity at local sites of inflammation may dominantly suppresses pro-inflammatory processes and block effector T-cell responses to antigens encountered. Conversely, when IDO is absent even strong pro-inflammatory stimuli do not elicit local Treg suppression, and Tregs are reprogrammed to acquire a pro-inflammatory Th17-like phenotype. In accordance hereof, we show that the frequency of Tregs decreased, the frequency of IL-17 producing cells increased while the overall number of CD4+ T cells were constant or decreased slightly when IDO-specific T cells were present, all suggesting an overall decrease in IDO activity. Furthermore, IDO-specific T cells increased the production of both IL-6 as well as the other pro-inflammatory cytokine TNF-α. Additionally, since IDO activation in DC have shown to result in IL-10 production and generation of Tregs 43 we examined the effect on IL-10 production. We observed a decrease in IL-10 when IDO-specific T cells were present. Finally, it should be noted that the metabolites of tryptophan are directly toxic to CD8+ T-cells and CD4+ Th1 cells 44, but not Th2 cells. Hence, increased IDO activity seems to tilt helper T-cell polarization toward a Th2 phenotype 45. It is therefore in addition possible that IDO-specific T cells by the killing of IDO-expressing cells were skewing the Th-response in a Th1-direction.
Taken together, these different mechanism-of-actions could explain how IDO-specific T-cells were able to boost CD8⁺ T-cell immunity in general; the direct killing of IDO-expressing cells diminished the direct IDO-mediated suppression of effector T cells and decreased the local Treg suppression by reprogramming Tregs to acquire a pro-inflammatory Th17-like phenotype under influence of an increased IL-6 production. It should further be noted, that IDO⁺ cells may be suppressive by other means than IDO. Hence, IDO-specific T cells may not only reduce IDO-mediated suppression but in addition further immune suppression mediated by IDO⁺ regulatory cells.

All in all our results suggest that IDO-specific CD8⁺ T cells are a natural part of the T-cell repertoire in man. IDO-specific T cells might interact with IDO expressing cells hereby eliminating or delaying the local immune suppression and thereby supporting the ongoing immune response. We were able to directly link the up regulation of IDO with IDO-specific T cells by showing that the addition of the IDO-inducing agents IFN-γ, CTLA4-Ig or CpG ODN generated measurable numbers of IDO-specific T cells among PBMC. Thus, it seems apparent that IDO-specific T cells play a supportive role, which might be crucial for the mounting or keeping of an effective immune response during infection. Consequently, we suggest giving such effector T cells with an immune enhancing function the general term “supporter T cells”.

Interestingly, CD14⁺ monocytes are major CMV target cells in vivo. Monocytes are responsible for dissemination of the virus throughout the body during acute and late phase of infection. CMV has been shown to induce IDO expression in monocytes, which has been suggested to confer an advantage to CMV-infected monocytes to escape T-cell responses. CMV is the most immunodominant antigen to be encountered by the human immune system. The CD8⁺ T-cell response to CMV typically constitutes a sizeable percentage of the CD8⁺ T-cell repertoire in CMV-
seropositive individuals. In light of this, it seems possible that IDO-specific T cells might have evolved to function as supporter T cells for the constitutive anti-CMV CD8+ T-cell response. Notably, we detected the IDO-specific T-cell responses in three healthy donors, which in addition all had strong CMV-specific CD8+ T-cells responses. Furthermore, we describe that CMV-specific CD8+ T-cell responses are strongly boosted in the presence of IDO-specific T cells.

In the clinical setting, the targeting of IDO could have synergistic effects in anti-viral immune therapy, e.g. in HIV vaccines. In this regard, it has been shown that HIV inhibits CD4+ T-cell proliferation by inducing IDO in plasmacytoid DC and macrophages. In cancer immune therapy, the boosting of IDO-specific immunity could have both direct and in-direct effects. Hence, we describe that IDO-specific T cells are able to recognize and kill IDO+ cancer cells. In fact, it may be possible that the sizable reactivity to this antigen in normal individuals contributes to immune surveillance against cancer. However, the induction of IDO-specific immune responses by therapeutic measures could function highly synergistic with additional anti-cancer immune therapy not only by eliminating cancer cells but in addition suppressive DC. By definition almost any successful anti-cancer immune therapy strategy aims of inducing immunological activation and inflammation. Within the limits of acceptable toxicity, as much immune activation as possible is the goal; hence, counter-regulation is not desired. Naturally, one should be cautious of the possible introduction of autoimmunity when targeting a tolerogenic molecule like IDO. However, the circulation of a measurable number of IDO-specific T cells does not seem to cause autoimmunity. Furthermore, since IDO-specific T cells can be introduced by IDO-inducing agents this appears to be under tight control. In this regard, an interesting aspect of IDO is that systemic inactivation at the organism level, either pharmacologically or genetically, does not appear to cause autoimmunity. IDO may not be involved in tolerance to self but rather in tolerance to nonself antigens where immune non-responsiveness may be important e.g. fetal antigens. Induction of IDO+ tolerogenic
DC occurs during infection of DC with viruses and intracellular pathogens, such as *L. monocytogenes*. Such IDO+ DC seem to be involved in protection of the host from granuloma breakdown and pathogen dissemination in advanced human listeriosis. This might have major implications for IDO-based immune therapy as boosting immunity to neoantigens but not normal self antigens by triggering IDO-specific T cells is very attractive. This can naturally only be examined in a clinical setting. Hence, we believe that our data justify and warrant clinical testing to evaluate the efficiency and safety of IDO-based vaccinations. Consequently, a phase I vaccination study is ongoing (from June 2010) at Center for Cancer Immune Therapy, Herlev University Hospital in which patients with non small cell lung cancer (NSCLC) are vaccinated with the IDO5 peptide with Montanide adjuvant (NCT01219348).

In conclusion, IDO may serve as an important and widely applicable target for immunotherapeutic strategies where IDO constitute a significant counter-regulatory mechanism induced by IFN-γ or other IDO-inducing signals. Until this day Tregs have been defined as suppressor T cells. The data described here add a new dimension to Tregs by illustrating effector T cells acting as Tregs; herein defined as “supporter T cells”. Our data suggest that antigen-specific T cells play a vital role in immune regulation. We find it realistic that additional proteins are targets for antigen-specific Tregs depending on the function and expression of these antigens.
Acknowledgements

We would like to thank Merete Jonassen and Tina Seremet for excellent technical assistance.

Supported by grants from the The Novo Nordisk Foundation, The Danish Cancer Society, Danish Medical Research Council, The Lundbeck Foundation, The John and Birthe Meyer Foundation, and Herlev University Hospital.

Authorship and Conflict of Interest Statements

The authors Mads Hald Andersen and Per thor Straten have previously filed a patent application based on the use of IDO for vaccination. The rights of the patent application have been transferred to Herlev University Hospital through the Capital Region of Denmark.

Rikke Bæk Sørensen: performed and designed research, analyzed and interpreted data, co-wrote manuscript

Sine Reker Hadrup performed research, contributed vital new reagents and analytical tools,

Inge Marie Svane: contributed vital new reagents and analytical tools,

Mads Christian Hjortsø: performed research

Per thor Straten: interpreted data and gave conceptual advice

Mads Hald Andersen: developed the concept and designed the experiments, collected data, analyzed and interpreted data, and wrote the manuscript.
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Figure legends

Figure 1: Spontaneous cytotoxic T-cell reactivity against IDO

Spontaneous T-cell reactivity against IDO5 (IDO199-207; ALLEIASCL) in PBMC, from HLA-A2+ healthy donors (HD), visualized by IFN-γ ELISPOT assay (A) and flow cytometry (B) after one in vitro peptide stimulation. For IFNγ ELISPOT assay, PBMC were plated at 4 x 10^5 PBMC in duplicates in specialized ELISPOT wells either alone or added IDO5 peptide. The average number of IDO5-specific spots (after subtraction of spots in wells without added peptide) was calculated per 4 x 10^5 PBMC for each donor (black circles) (A). For flow cytometry, IDO5-specific T cells were identified using the MHC-tetramer complex HLA-A2/IDO5 and CD8 mAb. For comparison, cells were stained with the MHC-tetramer complex HLA-A2/HIV-1 pol476-484 and CD8 mAb (B). As control, an IDO5-specific T-cell clone was stained with the HLA-A2/ HIV-1 pol476-484-PE and HLA-A2/IDO5-PE complexes (C). Lytic capacity of representative IDO5-specific T-cell clones from a healthy donor (HD) or a breast cancer patient (BC) assayed by ^51Cr-release assay. Target cells were TAP-deficient T2-cells pulsed with IDO5 or an irrelevant peptide (HIV-1 pol476-484) (D), the HLA-A2+/IDO+ colon cancer cell line SW480 and the HLA-A2+/IDO- colon cancer cell line HCT116 (E), SW480 blocked with the HLA class I specific mAb W6/32 (F), SW480 transfected with IDO ShRNA for down-regulation of IDO protein expression and SW480 transfected with control ShRNA as a positive control (F), the HLA-A2+/IDO+ melanoma cell line FM55M (G), FM55M added cold T2-cells pulsed with IDO5 peptide or irrelevant peptide (HIV-1 pol476-484) in a inhibitor to target ratio of 20:1 (G), autologous in vitro immatured and matured DC (H), and ex vivo isolated autologous IDO+CD14+ monocytes as well as IFN-γ treated IDO+CD14+ monocytes (I). All ^51Cr-release assays were performed in effector to target ratio of 5:1, except the experiments regarding ShRNA, which were performed in effector to target ratio of 15:1. Data are mean ± s.d. (n = 3).
Figure 2: IDO-specific T cells boosted specific immunity towards CMV in PBMC from a healthy donor

PBMC from an HLA-A2+ healthy donor cultured with CMV IE1_{316-324} (VLEETSVML) peptide either alone (top) or added an autologous, IDO5 (IDO_{199-207}; ALLEIASCL)-specific T-cell clone (in a PBMC to clone ratio of 2000:1) (bottom). The percentage of CMV IE1_{316-324}-specific CD8+ T cells in each culture was identified by flow cytometry using the MHC-tetramer complex HLA-A2/CMV IE1_{316-324} and CD8 mAb. For comparison, cells were stained with the MHC-tetramer complex HLA-A2/HIV-1 pol_{476-484} and CD8 mAb. Data from two representative experiments are shown (A). Percentage of CMV IE1_{316-324}-specific T cells found in PBMC cultured alone (white bars) or added an IDO5-specific T-cell clone (black bars). Data are mean ± s.d. (n = 4) *(p < 0.05)* (B). The percentage of CD4+CD25^{high}CD127 Foxp3+ Tregs in each culture was identified by flow cytometry using intracellular staining for Foxp3. For comparison, cells were stained with isotype controls. The data shown are from one donor, representative of 4 experiments (C). Distribution of CD4+ and CD8+ T cells in the cultures. Data are mean ± s.d. (n = 4) (D).

Figure 3: IDO-specific T cells boosted specific immunity towards Flu in PBMC from a cancer patient

PBMC from an HLA-A2+ breast cancer patient cultured with Flu matrix p_{58-66} (GILGFVFTL) peptide either alone (top) or added an autologous, IDO5 (IDO_{199-207}; ALLEIASCL)-specific T-cell clone (in a PBMC to clone ratio of 2000:1) (bottom). The percentage of Flu matrix p_{58-66}-specific CD8+ T cells in each culture was identified by flow cytometry using the MHC-tetramer complex HLA-A2/Flu matrix p_{58-66} and CD8 mAb. For comparison, cells were stained with the MHC-tetramer complex HLA-A2/HIV-1 pol_{476-484} and CD8 mAb (A). The percentage of
CD4⁺CD25<sup>hi</sup>CD127<sup>−</sup>Foxp3<sup>+</sup> Tregs (B) and IL-17A producing CD4⁺ T cells (C) in each culture were identified by flow cytometry using intracellular staining for Foxp3 and IL-17A, respectively. For comparison, cells were stained with isotype controls. Distribution of CD4⁺ and CD8⁺ T cells in the cultures (D). Tryptophan concentrations in cell culture supernatants pre and post addition of the IDO5-specific T-cell clone measured by competitive ELISA (E). Secreted cytokines (IL-10, IL-17A, IL-6 and TNFα) in cell culture supernatants quantified by ELISA (F). All data shown are from one patient. Data are mean ± s.d. (n = 3). White bars: Flu matrix p<sub>58-66</sub> stimulated PBMC cultured alone. Black bars: Flu matrix p<sub>58-66</sub> stimulated PBMC added an IDO5-specific T-cell clone (A-F). PBMC from an HLA-A2⁺ melanoma cancer patient cultured with CMV pp65<sub>495-503</sub> (NLVPMVATV) peptide either alone (top) or added an irrelevant autologous, ML-IAP<sub>280-289</sub> (QLCPICRAPV)-specific T-cell clone (in a PBMC to clone ratio of 2000:1) (bottom). The percentage of CMV pp65<sub>495-503</sub>-specific CD8⁺ T cells in each culture was identified by flow cytometry using the MHC-tetramer complex HLA-A2/CMV pp65<sub>495-503</sub> and CD8 mAb. For comparison, cells were stained with the MHC-tetramer complex HLA-A2/HIV-1 pol<sub>476-484</sub> and CD8 mAb. The data shown are from one patient, representative of 3 experiments (G).

**Figure 4: Co-stimulation with IDO peptide increased frequencies of CMV- and MART-1-specific T cells**

PBMC from HLA-A2⁺ healthy donors and HLA-A2⁺ cancer patients (melanoma and renal cell carcinoma patients) stimulated in vitro with CMV peptide (CMV pp65<sub>495-503</sub> (NLVPMVATV) or CMV IE1<sub>316-324</sub> (VLEETSVML)) or MART-1<sub>26-35</sub> (EAAGIGILTV) peptide either in co-culture with IDO5 (IDO<sub>199-207</sub>; ALLEIASCL) peptide or an irrelevant peptide (HIV-1 pol<sub>476-484</sub>). The percentage of CMV- or MART-1<sub>26-35</sub>-specific CD8⁺ T cells in each PBMC culture was identified by flow cytometry using the MHC-tetramer complexes HLA-A2/CMV pp65<sub>495-503</sub> (NLVPMVATV), HLA-
A2/CMV IE1316-324 (VLEETSVML) or HLA-A2/ MART-126-35 (EAAGIGILTV) and CD8 mAb.

The differences in tetramer-specific CD8⁺ T-cell percentages between the cultures is given, for each donor/patient, as fold increase of tetramer-specific CD8⁺ T cells in co-culture with IDO5 peptide. Data are mean differences, n=15 (A). Example of MHC-tetramer staining of PBMC from a healthy donor stimulated in vitro with CMV IE1316-324 peptide either in co-culture with an irrelevant peptide (HIV-1 pol476-484) (top) or IDO5 peptide (bottom). The data shown are from one donor, representative of 6 different donors/patients (B). Example of MHC-tetramer staining of PBMC from a melanoma patient stimulated in vitro with MART-126-35 peptide either in co-culture with an irrelevant peptide (HIV-1 pol476-484) (top) or IDO5 peptide (bottom). The data shown are from one patient, representative of 4 different patients (C). In all experiments, cells were stained with the MHC-tetramer complex HLA-A2/HIV-1 pol476–484 and CD8 mAb for comparison.

Figure 5: Co-stimulation of IDO-specific T cells reduced Treg numbers while boosting IL-17, IL-6 and TNF-α production

PBMC from HLA-A2⁺ melanoma cancer patients stimulated in vitro with MART-126-35 (EAAGIGILTV) peptide either in co-culture with IDO5 peptide or an irrelevant peptide (HIV-1 pol476-484). The percentage of CD4⁺CD25⁺CD127⁻Foxp3⁺ Tregs (A, B) and IL-17A producing CD4⁺ T cells (C, D) in each culture was identified by flow cytometry using intracellular staining for Foxp3 and IL-17A, respectively. For comparison, cells were stained with isotype controls.

Examples of Treg staining (A) and IL-17A staining (C) of PBMC stimulated in vitro with MART-126-35 peptide either in co-culture with an irrelevant peptide (HIV-1 pol476-484) (top) or IDO5 peptide (bottom). Examples shown are from one patient, representative of 4 different patients (A, C).

Distribution of CD4⁺ and CD8⁺ T cells in the cultures (E). Secreted cytokines (IL-10, IL-17A, IL-6 and TNFα) in cell culture supernatants quantified by ELISA (F). Data are mean ± s.d. (n = 4
patients). White bars: MART-1<sub>126-35</sub> stimulated PBMC in co-culture with an irrelevant peptide (HIV-1 pol<sub>476-484</sub>). Black bars: MART-1<sub>126-35</sub> stimulated PBMC in co-culture with IDO5 peptide.

**Figure 6: IDO-inducing agents expanded IDO-specific T cells with supporter functions**

Example of reactivity against IDO5 (IDO<sub>199-207</sub>; ALLEIASCL) in PBMC, from an HLA-A2<sup>+</sup> healthy donor, stimulated *in vitro* with IL-2 and IFN-γ. The percentage of IDO5-specific CD8<sup>+</sup> T cells was identified by flow cytometry, *ex vivo (top)* and after stimulation (*bottom*), using the MHC-tetramer complex HLA-A2/IDO5 and CD8 mAb. The data shown are from one donor, representative of 4 different donors (*A*). Examples of reactivity against IDO5 in PBMC, from an HLA-A2<sup>+</sup> renal cell carcinoma patient, stimulated *in vitro* with IL-2 and CTLA4-Ig (*B*), or CpG ODN (*C*). The percentage of IDO5-specific CD8<sup>+</sup> T cells was identified by flow cytometry, *ex vivo (top)* and after stimulation (*bottom*), using the MHC-tetramer complex HLA-A2/IDO5 and CD8 mAb. The data shown are from one patient, representative of 2 different patients (*B, C*). PBMC, from an HLA-A2<sup>+</sup> healthy donor, stimulated *in vitro* with CMV pp65<sub>495-503</sub> (NLVPMVATV) peptide and co-cultured with either autologous, isolated CD8<sup>+</sup> T cells (*top*) or autologous, isolated IFN-γ-induced IDO5-specific T cells (*bottom*). The percentage of CMV pp65<sub>495-503</sub>-specific CD8<sup>+</sup> T cells in each culture was identified by flow cytometry using the MHC-tetramer complex HLA-A2/CMV pp65<sub>495-503</sub> and CD8 mAb (*D*). PBMC, from an HLA-A2<sup>+</sup> healthy donor, stimulated with CMV IE1<sub>316-324</sub> (VLEETSVML) peptide and co-cultured with either autologous, isolated CD8<sup>+</sup> T cells (*top*) or autologous, IDO5-specific T cells isolated after two *in vitro* peptide stimulations (*bottom*). The percentage of CMV IE1<sub>316-324</sub>-specific CD8<sup>+</sup> T cells in each culture was identified by flow cytometry using the MHC-tetramer complex HLA-A2/CMV IE1<sub>316-324</sub> and CD8 mAb (*E*). In all experiments, cells were stained with the MHC-tetramer complex HLA-A2/HIV-1 pol<sub>476-484</sub> and CD8 mAb for comparison.
Figure 1

A. No peptide vs. ID05 peptide

B. HIV-1 specific spots per 4 x 10^5 cells

C. ID05 specific spots per 4 x 10^5 cells

D. IDO-specific T cells

E. Lysis of HD and BC

F. Lysis of HD and BC

G. Lysis of HD and BC

H. Lysis of HD and BC

I. Lysis of HD and BC

Legend:
- HD (n=28)
- T2 cells + irrelevant peptide
- T2 cells + ID05 peptide
- HCT116
- SW480
- SW480 + IDO SHRNA
- SW460 + control SHRNA
- FM65
- FM65 + 20% T2 + irrelevant peptide
- FM65 + 20% T2 + ID05 peptide
- mDC
- ID05
- IFN-g treated CD4 cells
- CD4 cells
Figure 2

A

Experiment 1

CMV stimulated PBMC

CMV stimulated PBMC + IDO-specific T cells

B

Experiment 2

CMV stimulated PBMC

CMV stimulated PBMC + IDO-specific T cells

C

CMV stimulated PBMC

CMV stimulated PBMC + IDO-specific T cells

D

CMV stimulated PBMC

CMV stimulated PBMC + IDO-specific T cells

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Figure 4

A

B
CMV stimulated PBMC

CMV stimulated PBMC + IDO peptide

C
MART-1 stimulated PBMC

MART-1 stimulated PBMC + IDO peptide

Donors/patients (n=15)

Log scale of Fold increase with IDO peptide

0.1 1 10

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Figure 5
PBMC ex vivo

IFNγ stimulated PBMC

IFNγ stimulated PBMC

CTLA4-Ig stimulated PBMC

CpG stimulated PBMC

CMV stimulated PBMC + CD8+ T cells

CMV stimulated PBMC + CD8+ T cells

CMV stimulated PBMC + IDO-specific T cells

CMV stimulated PBMC + IDO-specific T cells
Indoleamine 2,3-dioxygenase specific, cytotoxic T cells as immune regulators

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