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Human Activated T Lymphocytes Modulate IDO Expression in Tumors through Th1/Th2 Balance

Jessica Godin-Ethier,† Sandy Pelletier,† Laïla-Aïcha Hanafi, Philippe O. Gannon, Marie-Andrée Forget, Jean-Pierre Routy, Mohamed-Rachid Boulassel, Urszula Krzemien, Simon Tanguay, Jean-Baptiste Lattouf, Nathalie Arbour, and Réjean Lapointe

Previous cancer vaccination approaches have shown some efficiency in generating measurable immune responses, but they have rarely led to tumor regression. It is therefore possible that tumors emerge with the capacity to down-regulate immune counterparts, through the local production of immunosuppressive molecules, such as IDO. Although it is known that IDO exerts suppressive effects on T cell functions, the mechanisms of IDO regulation in tumor cells remain to be characterized. Here, we demonstrate that activated T cells can induce functional IDO expression in breast and kidney tumor cell lines, and that this is partly attributable to IFN-γ. Moreover, we found that IL-13, a Th2 cytokine, has a negative modulatory effect on IDO expression. Furthermore, we report IDO expression in the majority of breast and kidney carcinoma samples, with infiltration of activated Th1-polarized T cells in human tumors. These findings demonstrate complex control of immune activity within tumors. Future immune therapeutic interventions should thus include strategies to counteract these negative mechanisms. The Journal of Immunology, 2009, 183: 7752–7760.

Immune cell infiltration is frequently observed in numerous types of tumors during both development and progression of cancer, including tumor Ag-specific T lymphocytes. These tumor-infiltrating immune cells (TIICs) have been linked to prognostic value in several cancer types, such as ovarian, colorectal, breast, hepatocellular, renal cell, and prostate carcinomas. However, the functions of Ag-specific T lymphocytes appear to be negatively controlled, indicating that mechanisms of immune tolerance to human tumors are dominant in patients with progressive disease. Although successful immune vaccinations leading to increasing numbers of peripheral tumor-specific T lymphocytes have been demonstrated, beneficial clinical responses are generally observed in a small proportion of patients. This suggests that tumors can negatively affect T lymphocyte antitumor activity. Therefore, tolerizing immune mechanisms not only impact the natural course of the disease but also interfere with the eradication of tumors after vaccination.

Tumor Ag-targeted immunotherapy is certainly effective from an immunological point of view. The actual challenges are to increase the proportion of clinical responders, improve the duration of the response, and extend immunotherapy to common and aggressive cancers, such as breast carcinoma and renal cell carcinoma (RCC). In the last few years, this has led to an intense search, for major mechanisms mediating immune tolerance controlled by tumors. Potential mechanisms are diverse and include the expression of IDO in the tumor environment.

IDO is a tryptophan-catabolizing enzyme with immunosuppressive effects on T cell functions. It has been described as a key molecule in protecting the fetus against the mother's T cell aggression. In murine models, IDO has been demonstrated to be an important mediator of tumor escape when expressed by tumor cells and APCs found in tumor-draining lymph nodes. IDO mediates its immunosuppressive functions through the depletion of tryptophan from the microenvironment and the accumulation of toxic tryptophan metabolites. This culminates in the inhibition of T cell proliferation and functions, and in the induction of apoptosis. IDO has been detected in different cancer cell lines and in a variety of human primary tumors. Moreover, in ovarian, endometrial and colorectal carcinomas, IDO expression by tumor cells has been associated with poor prognosis. Some studies have linked IDO-expressing endometrial tumors with reduced lymphocyte infiltration and in human melanoma, the presence of IDO-expressing cells in tumor-draining lymph node correlates with decreased long-term survival.

However, factors regulating IDO expression in human tumor cells and the possible influence of T cells on IDO expression in tumors are currently unknown. In some normal cell types, IDO expression, which is known to be induced by both type I (α) and type II (γ) IFNs, can also be modulated by the Th1/Th2 cytokine profile. Anti-inflammatory cytokines, such as TGF-β, IL-4, and IL-13 have been shown to control IDO expression by antagonizing the IFN-γ effect in human fibroblasts and monocytes, whereas the opposite has been reported in cosinophils and microglia. Th1 and Th2 cytokines were reported in human

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Abbreviations used in this paper: TIIC, tumor-infiltrating immune cell; RCC, renal cell carcinoma; FRSQ, Fonds de la Recherche en Santé du Québec; Treg, regulatory T cell; DC, dendritic cell; 1MT, 1-methyltryptophan; CD82L, L-selectin.

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breast tumors, but the impacts of this cytokine mixture on IDO expression in tumor cells are currently unknown. A better understanding of these interactions is crucial, considering that tumor-derived IDO can negatively control T cell-mediated specific responses and thus impair immune-mediated tumor destruction.

In this study, we first characterized TIICs from human breast and kidney carcinomas and found that tumor-associated T lymphocytes bore a Th1-activated phenotype. We then demonstrated that activated T lymphocytes induced IDO in human breast and kidney tumor cell lines. This induction was partly mediated by IFN-γ secretion from T lymphocytes, but was negatively modulated by IL-13, a Th2 cytokine. These findings indicate that the Th1/Th2 balance provided by activated T lymphocytes may influence immunomodulatory mechanisms in tumors and thus could have a tremendous impact on immunotherapies aimed at fighting cancers. Our study constitutes the first analysis of IDO expression regulation by activated T cells, which needs to be better understood.

Materials and Methods

Patients and samples

This study was approved by the institutional Ethics Committee, and informed consent was obtained from each donor or patient before collection of the samples.

Freshly resected breast cancer specimens from 61 patients, who had undergone radical or partial mastectomy, were obtained from the Fonds de la Recherche en Santé du Québec (FRSQ)-Centre Hospitalier de l’Université de Montréal breast cancer tissue bank of the FRSQ Cancer Network. RCC tissues were obtained after radical or partial nephrectomy from 29 patients of the McGill University Health Center (Montreal, Canada). Collected tissues were mechanically homogenized with a Medimachine (Dako Cytomation), and single-cell suspensions were centrifuged on a lymphocyte separation medium cushion (Wisent). TIICs were recovered from the interface and stained for flow cytometry. PBMCs from healthy donors recruited by the Division of Hematology and Immunodeficiency at the Royal Victoria Hospital (Montreal, Canada) and from cancer patients were isolated as described previously (34).

Flow cytometry

Conjugated mAbs used: CD3-FITC, CD3-Alexa Fluor 700, CD4-Pacific Blue, CD8-allophycocyanin-Cy7, CD8-FITC, HLA-DR-PE, L-selectin-FITC from BD Biosciences; CD3-allophycocyanin, CD4-FITC, CD8-PE, CD45RA-PE, and CD45RO-allophycocyanin from Caltag; and CCR7-PE-conjugated mAbs used: CD3-FITC, CD3-Alexa Fluor 700, CD4-Pacific Blue, CD8-allophycocyanin-Cy7, CD8-FITC, HLA-DR-PE, L-selectin-FITC from BD Biosciences; CD3-allophycocyanin, CD4-FITC, CD8-PE, CD45RA-PE, and CD45RO-allophycocyanin from Caltag; and CCR7-PE from R&D Systems. Dead cells were excluded by staining with 7-aminoactinomycin D (BD Biosciences). For Th1/Th2 polarization and lytic granule content analysis, TIICs and PBMCs were thawed and incubated for 5 h with 20 ng/ml PMA (Sigma-Aldrich), 1 μg/ml ionomycin (Sigma-Aldrich), and 5 μg/ml brefeldin A (Sigma-Aldrich). Intracellular staining was performed as described previously (34). Samples analysis was performed on a BD Biosciences FACSCalibur or LSRII instruments.

Cells and reagents

All cell lines were obtained from American Type Culture Collection. MDA-MB-231 and MCF-7 cells were cultured in RPMI 1640 complete medium containing 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin/100 μg/ml streptomycin, and 10 μg/ml gentamicin (all from Wisent). KTCL and 786-0 cell lines were cultured in DMEM complete medium (Wisent), whereas Eagle’s modified MEM complete medium (Wisent) was used to cultivate the BT-20 cell line.

T lymphocytes were purified from PBMCs by negative selection with EasySep human CD4 or CD8 cell enrichment kits (Stem Cell Technologies). Isolated T cell subset purity was between 90 and 98%.

Monocyte-derived dendritic cells (DC) were generated from PBMCs of healthy donors (35), followed by 24 h of stimulation with 500 ng/ml soluble trimeric CD40L (Immunex) and/or 10 μg/ml IFN-γ (Pierce Endogen) and/or 5 μg/ml LPS (Sigma-Aldrich). IL-13 was from PeproTech, and GM-CSF was from Pierce Endogen. Neutralizing anti-human IFN-γ and IL-13 Abs were obtained from R&D Systems. The IDO inhibitor 1β-methyltryptophan (1βMT) was purchased from Sigma-Aldrich. CFSE was obtained from Molecular Probes.

Coculture assays

TIICs isolated from RCC patients or T cells isolated from healthy donor PBMCs were incubated for 24 h on 5 μg/ml plate-bound anti-CD3 (clone OKT3; eBioscience) or isotopic control, at a concentration of 2 × 104 cells/ml in Iscove’s modified Dulbecco’s complete medium (Invitrogen) supplemented with 7.5% human serum (heat-inactivated, prepared from at least three normal donors). Unstimulated or activated T cells or TIICs were then cultured with MDA-MB-231 cells at a 2:1 ratio in V-bottom 96-well plates (Costar; Corning Life Sciences) for 24 h. Neutralizing Abs were added to T cells 20 min before coculture. Supernatants were harvested after 24 h of incubation, and human IFN-γ was evaluated by ELISA with 0.4 μg/ml anti-IFN-γ mAb and 0.2 μg/ml biotinylated anti-IFN-γ Ab (Pierce Endogen; Ref. 34). Cells were collected for RNA or protein extraction.

RT-PCR

RNA from cocultures was prepared with RNEasy mini- or microkits (Qiagen) from breast and kidney specimens (34), and cDNA was synthesized from 500 ng of mRNA (34). Sequences of the PCR primers spanning distinct exons were: β-actin (5′, AAGGCAACACGGCAGAGATGAC; 3′, TAATGTCCACCGACTTTCCCG, amplicon 300 bp); 18S ribosomal subunit (5′, ATCAACCTTGGTGGTAGTGCG; 3′, TCTTTGAGTAGGGTAGCGC, amplicon 111 bp); and IDO (5′, CGCCTTGTTGAAATAGCTTCTTG; 3′, CTTCCCCGAAACCTTCTACAC, amplicon 167 bp). Classical RT-PCR amplification was undertaken with HotStartTag DNA polymerase (Qiagen). Cycling conditions were 15 min at 95°C, 24 (β-actin) or 32 (IDO) cycles of 45 s at 94°C, 45 s at 55°C, and 1 min at 72°C, then 10 min at 72°C, in a T3 Thermocycler system (Biometra).

Quantitative real-time RT-PCR, amplifications were performed with 0.5 μM concentrations of each IDO or 18S primer, 10 μM of 2× SYBR Green mix (Qiagen), 4 μl of cDNA (1/20 dilution), and water (36). The cycling conditions were 10 min at 95°C, then 35 cycles of 30 s at 94°C, 45 s at 60°C, 30 s at 72°C for 18S or 40 cycles of 30 s at 94°C, 60 s at 55°C, 30 s at 72°C for IDO, and a melting curve from 72 to 95°C. IDO expression was established relative to the housekeeping gene 18S, according to Pfaffl’s method (37).

Western blotting

Protein extracts from the above-mentioned pelleted cell lines were prepared, quantified, resolved by 10% SDS-PAGE, and transferred to polyvinylidene difluoride membranes (36). Membranes were incubated with specific Abs for β-actin (36) and IDO (sheep polyclonal anti-human IDO Ab 1/100 from Hyclut Biotechnology, and 1 h with a secondary peroxidase-conjugated rabbit anti-sheep 1/50,000 from Chemicon). Proteins were detected as described previously (36).

Functional assessment of IDO impact

To evaluate the enzymatic activity of IDO, the effects of conditioned media from tumor/IDO− or tumor/IDO+ on T cell functions were evaluated. Briefly, supernatants from activated T cells were prepared from CD8+ T cells, stimulated for 24 h with anti-CD3 or an isotype-matched control. To induce IDO expression in tumor cells, the KTCL cell line was incubated for 24 h in activated T cell supernatant. To prepare IDO-conditioned medium, the supernatant was replaced by fresh medium in which 250 μM l-1MT was added or not to confirm IDO specific activity. To assess effects on T lymphocytes, CFSE-labeled CD4+ responder T cells were activated with 5 μg/ml anti-CD3 and 300 μM IL-2 (PeproTech) and cultured in tumor/IDO− or/IDO+ -conditioned medium for 5 days. Cells were harvested, and CFSE dilution was assessed by flow cytometry.

Statistical analyses

Group comparisons for flow cytometry data were analyzed by Tukey’s parametric multiple comparison test. Normal distribution and homogeneity of variance for these values were satisfied. To compare experimental groups to controls in the IDO-conditioned medium experiment, the non-parametric Mann-Whitney U test was used. Statistically significant differences were determined as p values of <0.05. Statistics were performed with SPSS 13.0 software for Windows (LEAD Technologies).

Results

Infiltration of activated immune cells in tumors

To determine whether infiltrating immune cells in human breast carcinomas and RCCs display an activated or resting/anergic state, we undertook an extensive phenotyping assessment of their activation status. Expressions of several surface markers on TIICs and
PBMCs from cancer patients were compared by flow cytometry. A representative flow cytometric profile is provided (supplemental Fig. 1). The infiltrates comprised a diversity of immune cells, and the most abundant cells present in TIICs were T cells (56.9% in breast vs 53.4% in RCC; Fig. 1A, top). Among the CD3+ T cell subset, we observed significantly increased expression of the activation marker HLA-DR, in both breast carcinoma (p = 0.001) and RCC (p < 0.001) TIICs in comparison with PBMC (Fig. 1A, bottom). Tumor-infiltrating T cells had a memory phenotype when considering the significantly higher CD4+CD45RO+/CD4+CD45RA− ratio in TIICs than in PBMCs (45.3 ± 39.0% and 6.6 ± 11.5%; p = 0.006 for breast carcinoma vs 39.9 ± 52.4% and 1.1 ± 0.9%, p = 0.001 for RCC; Fig. 1B, top). These data were also supported by a decreased proportion of CD4+CCCR7+/CD62L− in TIICs from breast cancer vs PBMC from the same donors (p = 0.021; Fig. 1C, top). A similar profile was noted among CD8+ T cells (Fig. 1, B and C, bottom). Taken together, these data strongly suggest that tumor-infiltrating T lymphocytes have an activated phenotype: HLA-DR+CD45RO−CCR7+/CD62L−.

Th1-polarized T cells infiltrate RCC

To dissect the nature of tumor-infiltrating T cells, we selected one prototypic Th1 cytokine (IFN-γ), one prototypic Th2 cytokine (IL-4), and one lytic enzyme (perforin) to evaluate the effector functions of kidney TIICs. Intracellular flow cytometric analysis revealed a significantly higher percentage of IFN-γ-secreting CD4+ T cells in TIICs (53.0%) than in PBMCs (19.3%) from RCC patients (Fig. 2A, left). However, the percentage of IL-4+CD4+ T cells was similarly low in each group (Fig. 2A, right). Moreover, IFN-γ-secreting CD8+ T cells were in significantly higher proportion in TIICs and PBMCs from cancer patients compared with healthy donor PBMC (Fig. 2B, left). We found a significantly higher number of perforin-producing CD8+ T cells in PBMCs from RCC patients than from healthy donors (Fig. 2B, right). Altogether, these data show that T cells infiltrating kidney tumors display an IFN-γ-Th1 profile. However, it is still unresolved how tumor cells respond to such cytokines from activated T cells present in the tumor environment.

Immune cells prepared from RCC induce IDO expression in human renal carcinoma cells

To evaluate the effects of these TIICs on tumor cells upon interaction, in vitro experiments have been performed coculturing RCC TIICs with human renal carcinoma cells to evaluate the expression of immunosuppressive genes. Given the increased proportion of IFN-γ-expressing T cells that we detected in TIICs, and that IDO is an IFN-γ-inducible gene, we sought to determine whether TIICs isolated from renal cell carcinomas could lead to IDO expression in tumor cells. These experiments revealed the induction of IDO in tumor cells after coculture with TIICs from three donors pretreated with isotype control (Fig. 3, top; RCC53, RCC70, and RCC75). This induction was specific to the tumor environment because PBMCs did not induce similar levels of IDO expression. As expected, TIICs as well as PBMCs from RCC53 and RCC70 cancer patients

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*The online version of this article contains supplemental material.*

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**FIGURE 1.** Infiltration of activated T lymphocytes in breast and kidney tumors. Single-cell suspensions obtained from tumor tissues and peripheral blood of breast and kidney cancer patients were incubated with Abs specific to various lymphocyte markers to evaluate the proportion of CD3+ and CD3+ HLA-DR+ (A), CD4+CCR7+CD62L+ and CD8+CCR7+CD62L+ cells (C), or CD4+CD45RO/RA and CD8+ CD45RO/RA ratios (B). Analyses were gated on the lymphocytic population and dead cells were excluded by 7-aminoactinomycin D staining. Dotted lines link samples from the same patient and the mean is represented by a bar.

**FIGURE 2.** Th1-polarized T cells infiltrate kidney cancers. TIICs and PBMCs from kidney cancer patients and PBMCs from healthy donors were stained for flow cytometry, and percentages of IFN-γ- or IL-4-producing cells among CD3+CD4+ T cells (A) and IFN-γ or perforin CD3+CD8+ T cells (B) are represented by dots. Bar, Mean value for each group. TIICs and PBMCs from the same patients are linked by dotted lines.
activated with anti-CD3 induced IDO expression in the tumor cell line (Fig. 3, bottom), whereas TIICs from donor RCC75 failed to induce similar levels of IDO expression, after activation with anti-CD3.

In summary, this experiment illustrates that TIICs prepared from different RCC patients had the capacity to up-regulate IDO.

**Activated T lymphocytes induce IDO expression in human tumor cells**

Although parameters measured from freshly resected tumors provided crucial information, TIICs extraction rarely leads to sufficient amount of cells to study the effects of TIICs interaction with tumor cells. Because we wanted to further investigate tumor cell responses to human activated T cells, we pursued our study with in vitro experiments using human activated T lymphocytes similar to lymphocytes found in human tumors to evaluate the expression of immunosuppressive genes. In our experimental system, we detected IDO mRNA in MDA-MB-231 cocultured with activated CD4$^+$ and CD8$^+$ T cells, and also at a lower level with unstimulated CD8$^+$ T cells (Fig. 4A). Surprisingly, IFN-γ levels in supernatants from activated CD8$^+$ T cell and MDA-MB-231 cocultures were low in comparison to levels obtained from CD4$^+$-activated T cells (Fig. 4A), suggesting that IFN-γ is not the only molecule produced by activated T cells responsible for IDO induction. These data support other findings in this paper shown in Fig. 7B.

To evaluate whether our model was representative of what can be encountered in breast and kidney cancers, we next investigated IDO induction in different breast and kidney cancer cell lines. We found IDO in all cell lines after coculture with activated CD8$^+$ T cells. IDO mRNA was also detected in both kidney cancer cell line (Fig. 3), whereas TIICs from donor RCC75 failed to induce similar levels of IDO expression, after activation with anti-CD3.

To assess IDO functional activity, we then evaluated the consequences of IDO-expressing tumor cells on T cell functions. Activated T cells induce IDO expression in human breast and kidney cancer cell lines. A–C, CD4$^+$ and/or CD8$^+$ T cells isolated from healthy donor PBMCs were stimulated or not with plate-bound anti-CD3 for 24 h and cocultured with kidney or breast cancer cell lines for 24 h. A and B, Immunosuppressive genes (IDO, arginase 2, PGE synthase) and β-actin mRNA expression were evaluated by RT-PCR, and IFN-γ levels in coculture supernatants were determined by ELISA. MDA-MB-231 cells stimulated with plate-bound anti-CD3 or IFN-γ were used as negative and positive controls for IDO expression. C, IDO protein expression was evaluated by Western blotting. DC and HCT-116 were, respectively, positive and negative controls for IDO expression. C/L/L, CD40L/LPS/IFN-γ.

**IDO expressed by human cancer cells inhibits T cell functions**

To assess IDO functional activity, we then evaluated the consequences of IDO-expressing tumor cells on T cell functions. Activated CD4$^+$ T cells were cultured in conditioned medium from IDO-expressing KTCL cells. Proliferation and IFN-γ production were then examined. When cultured in tumor/IDO$^-$-conditioned medium, activated T cell proliferation was decreased by 33.3% compared with T cells cultured in control medium (p = 0.04), as indicated by the CFSE dilution (Fig. 5B). Importantly, T cell proliferation was rescued to the control level by adding the IDO inhibitor 1MT (p < 0.05), therefore illustrating a direct role of IDO in the reduced T cell proliferation (Fig. 5A). Similar results were obtained for IFN-γ secretion (data not shown). Thus, enzymatically active IDO protein detected in tumor cell lines in response to activated T cells exerted an inhibitory effect on T cell functions.
IDO expression in breast and kidney cancers

Considering the Th1-polarized nature of tumor-infiltrating activated T cells (Fig. 2) and IDO up-regulation in cancer cell lines in response to activated T lymphocytes, we next wanted to confirm whether IDO was detected in a substantial proportion of human breast and kidney cancer clinical samples. We evaluated IDO expression in cancer samples and normal tissues, by quantitative real-time RT-PCR. IDO mRNA was detected in several normal tissues (Fig. 6A), including breast and kidney. IDO expression in breast sample. Almost one-half (27 of 57) of the breast cancer samples expressed twice the IDO level found in the normal reference breast sample (Fig. 6B). Furthermore, 24 RCC clinical samples were analyzed. The IDO mRNA level was 2-fold higher in RCC samples than in an untreated kidney cancer cell line (Fig. 6C). No correlation was found between IDO mRNA expression and the patients’ clinicopathological parameters for breast carcinoma samples. Among RCC samples, IDO expression correlated with the clear cell histological type, because clear cell constituted 89% (16 of 18) of IDO-positive samples (Spearman correlation coefficient = 0.766, p < 0.001). All of the six IDO-negative clinical samples were not clear cell RCC, for instance, they were papillary, chromophobe, or carcinoid. These data indicate that IDO is present in a high proportion of cancer samples. As such, we must better understand the mechanisms by which activated T lymphocytes modulate IDO expression in tumor cells, and the potential involvement of IFN-γ, which was previously shown to be an important IDO inducer.

Activated T cells provide both positive and negative signals for IDO expression in cancer cells

To understand the relative importance of IFN-γ in the induction of IDO in tumors from the interaction with activated T cells, we assessed IDO expression while blocking IFN-γ with a neutralizing Ab. When stimulated with 100 U/ml exogenous recombinant IFN-γ (corresponding to 4 ng/ml), IDO expression by MDA-MB-231 was completely abolished by anti-IFN-γ (Fig. 7A), thus determining the threshold of the blocking Ab. In contrast, IDO expression was only partly inhibited by the same amount of anti-IFN-γ in MDA-MB-231 cocultured with activated CD4+ T cells secreting a measured level of 500 pg/ml IFN-γ (Fig. 7B). Similar results were obtained with CD8+ T cells (data not shown). We thus suspected that other factors may be implicated in the regulation of IDO expression in tumor cells.

To explore whether IDO induction was dependent on cell-cell contact or solely on soluble factors, we incubated the MDA-MB-231 cell line in activated T cell supernatant, or with activated T cells separated by a tissue culture insert. These results revealed that IDO induction in tumor cells in response to activated T cells was cell contact independent (supplemental Fig. 3).

To identify soluble factors modulating IDO expression, we performed a cytokine array on supernatant from activated CD4+ T cells vs control CD4+ T cells. We observed an up-regulation of IL-13 and GM-CSF secretion by activated T cells (supplemental Fig. 4). We evaluated their impact on IDO modulation by treating the MDA-MB-231 cell line with IFN-γ alone or in combination with these cytokines. The results revealed that neither IL-13 nor GM-CSF alone was able to induce IDO in MDA-MB-231 (Fig. 7C). However, when stimulated with exogenous IFN-γ, decreases of 58 and 84% in IDO relative expression were observed with the addition of IL-13 (at 10 and 50 U/ml, respectively). A 57% decrease of IFN-γ-induced IDO expression was also seen with GM-CSF (100 U/ml; Fig. 7C). Although IL-4 shares a common receptor with IL-13, no difference in IFN-γ-dependent IDO mRNA levels was observed in response to IL-4 (data not shown).

To further establish the role of IL-13 in modulating IDO expression in cancer cells from activated T cells, we used an IL-13-blocking Ab. Our results demonstrate that the IL-13-blocking Ab increased IDO mRNA expression (Fig. 7D). We thus confirmed the inhibitory action of IL-13 on IDO expression by the MDA-MB-231 breast cancer cell line. Taked together, these results indicate that cytokines (GM-CSF and IL-13) produced by activated T cell can attenuate IFN-γ-mediated IDO expression induced in human tumor cells. Thus, IDO expression appears to be regulated by a complex balance of Th1/Th2 cytokines secreted by activated T cells.

Discussion

The inability of tumor-infiltrating lymphocytes to control tumor progression highlights the importance of understanding factors...
emerging from tumors, which negatively control the antitumoral response. IDO, an important immunosuppressive molecule, has been identified as one of the mechanisms leading to immune response down-regulation. Indeed, the negative feedback provided by IDO expression in DC after contact with activated T cells (39) is a necessary immunoregulatory mechanism of T cell deactivation. It is now clear that this control mechanism can also emerge in tumor cells, leading to the inhibition of the T cell response and proliferation. Such an active process of tolerance induction in the tumor microenvironment has an obvious negative impact on immune functions, and potentially on the effectiveness of clinical manipulation of the antitumor response by immunotherapeutic approaches. Inhibiting IDO or the factors controlling its expression may increase the efficacy of immune-based cancer therapy. However, such factors are not well characterized in human cancer. This study is the first report on the mechanisms of IDO regulation in tumor cells in response to activated T cells in human cancer.

We first examined the phenotype of immune cells infiltrating breast cancer and RCC, and observed memory T cells expressing the surface marker CD45RO. These T cells also had an activated phenotype, which was illustrated by their high expression of HLA-DR. These results are in line with previous reports on the activation marker HLA-DR from the surface of breast carcinoma (40) and RCC (41) TIIcs. Moreover, the memory profile among T cells from TIIcs is consistent with the intense CD45RO expression seen by Cózar et al. (42) in RCC. Considering the phenotypically activated T cells present in the tumor microenvironment, inhibitory mechanisms must be responsible for their impaired functions.

From the ineffectiveness of the antitumor response and the activated state of TIIcs from RCC samples that we analyzed by flow cytometry, we hypothesized that activated T cells could induce immunosuppressive genes expression by tumor cells. Of the different suppressive genes we tested, we found that only the expression of IDO was induced in tumor cell lines in response to activated T cells in our experimental model. The protein expression of IDO was confirmed in a subset of cell lines, and we also corroborated IDO-negative biological effects on T lymphocyte functions. In their study, Brandacher et al. (27) noted that high IDO expression in colorectal cancer correlated with a decrease in CD3\(^{+}\)-infiltrating T cells. To explain this observation, they proposed that IDO expression in human tumors could result from IFN-\(\gamma\)
IDO expression by Tumors is Regulated by T Cells

Figure 7. IDO expression in tumors is modulated by Th1/Th2 cytokine balance.

A. The MDA-MB-231 cell line was cultured with various amounts of exogenous recombinant human IFN-γ (1000, 100, 10, and 1 U/ml) to evaluate the blocking threshold of anti-IFN-γ neutralizing Ab. B, MDA-MB-231 was cocultured with unactivated or anti-CD3-activated CD4+ T cells in the presence of anti-IFN-γ Ab or isotype control. A and B, IDO mRNA expression was determined by RT-PCR.

C. The MDA-MB-231 cell line was treated or not with 50 U/ml IFN-γ alone or in combination with different amounts of recombinant human IL-13 or recombinant human GM-CSF. D, CD4+ T cells were treated with anti-CD3 or isotype control and cocultured with MDA-MB-231 in the presence of a neutralizing Ab to IL-13 (50 μg/ml). C and D, Relative IDO expression was assessed by real-time quantitative RT-PCR with 18S as reference. Monocyte derived DC untreated or treated with 10^5 U/ml IFN-γ were used respectively as negative or positive controls.

Produced by host immune cells after the recognition of early-stage tumor cells, which could lead to tryptophan starvation and inhibition of T cell proliferation and functions in the tumor environment. We thus consider that our study supports their hypothesis, the role of IFN-γ in this process needing to be determined.

We expected IFN-γ to play a major role in the expression of IDO by cancer cell lines in response to activated T cells. Using a neutralizing Ab, we demonstrated the importance of IFN-γ for IDO induction in our cocultures. However, our data suggest the presence of one or multiple factors, other than IFN-γ, produced by activated T cells and which could contribute to IDO induction in tumor cells. These factors have not been identified and are currently under investigation, but we speculate that type I IFNs could be involved in this process.

We did identify two factors, GM-CSF and IL-13, which negatively control IFN-γ-induced IDO expression. Strikingly, even if the Th2 cytokine IL-13 was found to down-regulate IDO, we did not observe any regulatory functions for IL-4 on IDO expression. The different roles of IL-4 and IL-13 may reflect the involvement of different pathways in the signaling events leading to IDO expression (43). The effects of IL-13 on IDO expression correlated with the antagonizing effect of Th2 cytokines on IFN-γ that has been noted in other cell types, such as fibroblasts and monocytes. Moreover, in a recent study, IL-13 has been detected in breast tumor clinical samples (44, 45). Aspord et al. (44) also demonstrated that CD4+ T cells infiltrating breast tumors produce IFN-γ as well as IL-4 and IL-13. Obviously, IDO expression in tumors is modulated by several factors from activated T lymphocytes, which need to be identified.

Although Th cells have been shown to play a major role in antitumor immunity, both in mice and humans, the nature of this Th response is of major importance. In murine cancer models, it has been established that predominant Th1 responses are associated with tumor resistance, whereas Th2 or Th1/Th2 mixed responses correlate with tumor progression (46). However, such correlation has not been strongly shown in humans. Th1 infiltration frequently correlates with better prognostic value (47). Also, Th1 responses are linked to a stronger antitumor immunity but without being associated with tumor regression. Failure of Th1 responses to wane tumors might be explained by different immunosuppressive mechanisms, such as IDO, which could block effector functions of T cells. Our results suggest that Th2 cytokines down-regulate IDO expression in cancer cells. This does not support evidence suggesting that Th1 responses are more capable of generating an effective antitumor immune response. Because IDO is thought to preferentially induce Th1 cell apoptosis (32), its expression in tumor cells could subvert the immune response toward a Th2 profile. We thus think that a Th1 antitumor response with the concomitant inhibition of IDO expression or activity to overcome immunosuppression, would still be the most appropriate strategy to eradicate tumors, rather than using Th2-polarizing antitumor immunizations.

We analyzed IDO mRNA in breast carcinoma and RCC clinical samples and found a high expression in a large proportion of tumor specimens. However, because IDO expression was measured from bulk tumor specimens, we cannot exclude the possibility that part of the IDO in our breast and kidney cancer clinical samples originated from infiltrating immune cells. Although the analysis of RCC samples revealed a positive correlation between IDO expression and clear cell histological type, we could not correlate IDO mRNA expression in breast cancer specimens with clinical parameters. In other studies, IDO expression was correlated with a poor clinical outcome in different human cancers, such as melanoma (18), endometrial (26), colorectal (27), and ovarian (25) cancers.
Our inability to link IDO to clinicopathological parameters could be attributed to the bias introduced in our tissue bank toward high grade and large tumor size.

Our data show that activated T cells can induce IDO expression in tumor cells and negatively impact the immune response. In melanoma, it has been demonstrated that IDO can affect DC-based vaccines given that IDO-positive cells were detected by immunohistochemistry in tumor biopsies 24 h after the administration of a DC-based vaccine (48). Furthermore, IDO expression led to increased expression of FoxP3, and thus possibly T regulatory T (Treg) cells (48). The complementarities between these two immunosuppressive mechanisms have been demonstrated recently by Munn and colleagues (49) as they showed that IDO-expressing plasmacytoid DC can activate resting Treg in mice. Inversely, Treg can induce IDO expression in DC after CTLA-4 ligation (50). IDO is certainly not the sole immunosuppressive factor facilitating tumor escape from the immune system, but these evidences indicate that immunosuppressive mechanisms could be closely related.

Considering that immunotherapy leading to accumulation of activated specific T cells at tumor sites could result in IDO-induced tolerance, its clinical relevance is obvious, and this should be considered in the development of vaccination strategies.

In conclusion, although the immunosuppressive role of IDO is already established, it is still unclear to what extent host IDO-expressing APCs or tumor cells contribute to the induction of tolerance in cancer. IDO+ DCs may exert their immunoregulatory effects, not only at the tumor site but also in lymph nodes, where APCs prime specific T cells (51). This could explain the powerful effects of IDO in down-regulating the immune response against tumors. The present study demonstrates for the first time that human activated T cells can positively and negatively modulate IDO expression in tumor cell lines through Th1 and Th2 cytokine secretion. Furthermore, these IDO-positive tumor cells can impair T cell responses. This mechanism of IDO induction provides new evidence as to the complexity and vast potency of this immunosuppressive molecule in human cancer. Our study also gives clear evidence that the Th1/Th2 cytokine balance provided to the tumor is an important aspect to consider in future immunotherapies.

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Disclosures

The authors have no financial conflict of interest.

References

IDO EXPRESSION BY TUMORS IS REGULATED BY T CELLS


**Supplemental Figure 1 Phenotyping of immune cell populations in breast and renal cell carcinoma samples.** TIIIC and PBMC obtained from breast and kidney cancer patients were incubated with conjugated specific antibodies. The expression of various lymphocyte markers was evaluated by FACS to determine the proportion of (A) CD4\(^+\) and CD8\(^+\), (B) CD3\(^+\)HLA-DR\(^+\), (C) CD45RA\(^+\) and CD45RO\(^+\), (D) CCR7 and CD62L, cells. Dead cells were excluded by 7-AAD staining. A, B and D are gated on CD3\(^+\) cells, and C is gated on CD4\(^+\) cells. Typical flow cytometric profiles are illustrated.

**Supplemental Figure 2 IDO is expressed in breast and kidney cancer lines in response to IFN-γ.** Kidney (786-O or KTCL) and breast (BT-20, MCF-7 or MDA-MB-231) cancer cell lines were either untreated, treated with OKT3-conditioned supernatant without T cells or with 1 x 10\(^3\) U/ml IFN-γ for 24 h. The cells were harvested, and IDO, Arg2, COX-2, PGES and β-actin mRNA was detected by RT-PCR. IFN-γ levels from supernatants were determined by ELISA. Legend: p.b.: plate-bound.

**Supplemental Figure 3 IDO induction in tumor lines is cell-contact independent.** CD4\(^+\) or CD8\(^+\) T cells from PBMC were activated with anti-CD3 or isotype-matched control for 24 h. MDA-MB-231 were left untreated, co-cultured with activated T cells or their supernatant alone, or cultured with activated T cells in 2-compartment wells. Cells were harvested, and IDO mRNA expression was quantified relative to 18S by real time RT-PCR. Legend: p.b.: plate-bound.

**Supplemental Figure 4 Cytokine secretion profile of activated T cells.** Isolated CD4\(^+\) T cells were stimulated for 24 h with anti-CD3 or isotype-matched control. Supernatants were collected and cytokines produced were determined using a RayBio Human Cytokine Antibody Array (RayBiotech, Inc.).