Activated CD69+ T Cells Foster Immune Privilege by Regulating IDO Expression in Tumor-Associated Macrophages

Qiyi Zhao, Dong-Ming Kuang, Yan Wu, Xiao Xiao, Xue-Feng Li, Tuan-Jie Li and Limin Zheng

J Immunol 2012; 188:1117-1124; Prepublished online 19 December 2011;
doi: 10.4049/jimmunol.1100164
http://www.jimmunol.org/content/188/3/1117

Supplementary Material
http://www.jimmunol.org/content/suppl/2011/12/19/jimmunol.1100164.DC1.html

References
This article cites 45 articles, 12 of which you can access for free at:
http://www.jimmunol.org/content/188/3/1117.full#ref-list-1

Subscriptions
Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscriptions

Permissions
Submit copyright permission requests at:
http://www.aai.org/ji/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/cgi/alerts/etoc
Activated CD69⁺ T Cells Foster Immune Privilege by Regulating IDO Expression in Tumor-Associated Macrophages

Qiyi Zhao,*†,1 Dong-Ming Kuang,*† Yan Wu,* Xiao Xiao,* Xue-Feng Li,* Tuan-Jie Li,† and Limin Zheng*‡

Substantial evidence indicates that immune activation at stroma can be rerouted in a tumor-promoting direction. CD69 is an immunoregulatory molecule expressed by early-activated leukocytes at sites of chronic inflammation, and CD69⁺ T cells have been found to promote human tumor progression. In this study, we showed that, upon encountering autologous CD69⁺ T cells, tumor macrophages (MΦs) acquired the ability to produce much greater amounts of IDO protein in cancer nests. These cells isolated from the hepatocellular carcinoma tissues expressed significantly more CD69 molecules than did those on paired circulating and nontumor-infiltrating T cells; these tumor-derived CD69⁺ T cells could induce considerable IDO in monocytes. Interestingly, the tumor-associated monocytes/MΦs isolated from hepatocellular carcinoma tissues or generated by in vitro culture effectively activated circulating T cells to express CD69. IL-12 derived from tumor MΦs was required for early T cell activation and subsequent IDO expression. Moreover, we found that conditioned medium from IDO⁺ MΦs effectively suppressed T cell responses in vitro, an effect that could be reversed by adding extrinsic IDO substrate tryptophan or by pretreating MΦs with an IDO inhibitor 1-methyl-DL-tryptophan. These data revealed a fine-tuned collaborative action between different types of immune cells to counteract T cell responses in tumor microenvironment. Such an active induction of immune tolerance should be considered for the rational design of effective immune-based anticancer therapies.

Tumor progression is now recognized as the product of evolving cross-talk between different cell types within tumors. Although normal stroma is nonpermissive for tumor progression, cancer cells can modulate adjacent stroma to generate a supportive microenvironment (1–3). Clinical and experimental studies showed that dysfunctional APCs repurpose the T cell responses away from antitumor immunity (4, 5). In contrast, inflammatory mediators released by T cells, including IL-4, IL-10, and IL-17, can alter the polarization of APCs to promote tumor progression (6–8). These observations are in accordance with the growing evidence that adaptive immune cells have an unexpected role in regulating innate immunity (9, 10), but the exact underlying mechanisms in tumors are not yet clear.

Macrophages (MΦs) markedly outnumber other APCs and represent an abundant population of stroma cells in tumors (11, 12). These cells are derived almost entirely from circulating monocytes; in response to environmental signals, they acquire special phenotypic characteristics that are associated with diverse functions (11–13). Despite generalized immunosuppressive status of MΦs in most solid tumors, we recently found that soluble factors derived from cancer cells can trigger transient activation of newly recruited monocytes in the peritumoral stroma (14). These activated monocytes express B7-H1 molecules to suppress tumorspecific T cell function (15, 16), suggesting that activation of monocytes in tumors may not necessarily represent a host-defense mechanism against the malignancy but rather a novel strategy for tumor immune editing. Such a mechanism may also be used by other immune cells. For example, CD69 is generally recognized as the early activation marker on leukocytes (17). However, recent studies showed that accumulation of CD69⁺ T cells in lung is associated with a metastatic phenotype in human pulmonary carcinomas and that activated CD69⁺ T cells can function as a regulatory subset in mouse tumors (18, 19). Thus, the immune tolerance may also be actively induced by triggering immune activation in tumor microenvironments.

IDO is a rate-limiting enzyme for tryptophan catabolism (20, 21). In humans and mice, IDO inhibits Ag-specific T cell proliferation in vitro and suppresses T cell responses to fetal alloantigens during murine pregnancy (22, 23). Expression of IDO is often induced or maintained by many inflammatory cytokines, of which IFN-γ is the most potent (20). In addition to being expressed in APCs, most human cancers also express high levels of IDO protein, which correlates with poor prognosis in some cases (22). In contrast, low or rare IDO expression is observed in most mouse and human tumor cell lines, possibly due to the lack of a complete cancer microenvironment in cell lines in vitro (22, 24, 25). At present, little is known about the regulating mechanisms of

*Key Laboratory of Gene Engineering of the Ministry of Education, State Key Laboratory of Biocontrol, School of Life Sciences, Sun Yat-sen University, Guangzhou 510 275, People’s Republic of China; †Department of Infectious Diseases, Third Affiliated Hospital, Sun Yat-sen University, Guangzhou 510 275, People’s Republic of China; and ‡State Key Laboratory of Oncology in Southern China, Sun Yat-sen University Cancer Center, Guangzhou 210 275, People’s Republic of China.

1Q.Z. and D.-M.K. contributed equally to this work.

Received for publication January 19, 2011. Accepted for publication November 21, 2011.

This work was supported by project grants from the National Basic Research Program of China (“973” Program) (2010CB529904 and 2011CB811305), the National Natural Science Foundation of China (30730086, 81171982, and 91029737), and the Fundamental Research Funds for the Central Universities (11lgzd12).

Address correspondence and reprint requests to Prof. Limin Zheng and Prof. Dong-Ming Kuang, School of Life Sciences, Sun Yat-sen University, 135 XinGang Xi Lu, Guangzhou, Guangdong 510 275, People’s Republic of China. E-mail addresses: zhenglim@mail.sysu.edu.cn (L.Z.) and kdmimg@mail.sysu.edu.cn (D.-M.K.)

The online version of this article contains supplemental material.

Abbreviations used in this article: HCC, hepatocellular carcinoma; HLA-DR, HLA D-related; MΦ, macrophage; 1-MT, 1-methyl-DL-tryptophan; TAM, tumor-associated macrophage; TSN, tumor culture supernatant.

Copyright © 2012 by The American Association of Immunologists, Inc. 0022-1767/12S16/00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1100164
IDO in tumor-associated macrophages (TAMs) at stroma of human tumors in situ.

The present study showed that IDO protein was selectively highly expressed in MΦs in several types of human tumor tissues. The expression pattern of IDO coincided with the infiltration of CD69+ T cells in tumor environments. Accordingly, the CD69+ T cells isolated from hepatocellular carcinoma (HCC) tissues induced significant levels of IDO in autologous monocytes in vitro. Moreover, we provide evidence that IL-12 derived from tumor MΦs was required for early T cell activation and subsequent IDO expression. Therefore, activated CD69+ T cell-mediated MΦ IDO expression may represent a novel mechanism by which the adaptive activation is linked to immune tolerance in the tumor milieu.

Materials and Methods

Patients and specimens

Tumor tissues and peripheral blood samples were obtained from the Cancer Center of Sun Yat-sen University. One hundred and ten patients with hepatocellular (n = 80), pulmonary (n = 10), gastric (n = 10), or cervical (n = 10) carcinomas underwent curative resection between 2000 and 2004, and samples from these patients were used for immunohistochemistry. Blood samples, as well as paired fresh nontumor and tumor tissues, from 10 HCC patients were used for the isolation of peripheral and tissue-infiltrating leukocytes. All samples were anonymously coded in accordance with local ethical guidelines (as stipulated by the Declaration of Helsinki), and written informed consent was obtained. The protocol was approved by the Review Board of Sun Yat-sen University Cancer Center.

Abs and reagents

Mouse anti-human IDO was purchased from Chemicon (Temecula, CA). Blocking mAbs against human TNF-α, IFN-γ, IL-6, IL-10, and IL-12p70 (clone: 24910) and control Ab were obtained from R&D Systems (Abingdon, U.K.). Mouse anti-human CD68, Envision System, and Doublestain System for immunohistochemistry were from Dako (Glostrup, Denmark). Abs against CD3, CD15, CD20, and CD56 were purchased from Neomarkers (Fremont, CA), and CFSE and cell isolation and tissue culture reagents were from Invitrogen (Grand Island, NY). All other reagents were obtained from Sigma-Aldrich (St. Louis, MO), unless indicated otherwise.

Cell lines and preparation of culture supernatants

Human cervical cancer (HeLa) and hematoma (SK-HeP1 and HepG2) cell lines were obtained from the American Type Culture Collection; human normal liver cells (LO2) and lung cancer (95D) cell line were from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences. All cells were tested for mycoplasma contamination using a single-step PCR method, and they were maintained in DMEM supplemented with 10% FBS (HyClone Laboratories, Logan, UT) (14). Tumor cell supernatants (TSNs) and supernatant from LO2 were prepared as previously described (15).

Isolation of mononuclear cells from peripheral blood and tissues

PBMCs were isolated by Ficoll density-gradient centrifugation (14). Fresh tumor- and nontumor-infiltrating lymphocytes were obtained as previously described (15). In short, tissue specimens were cut into small pieces and digested in RPMI 1640 supplemented with 0.05% collagenase IV, 0.002% DNase I (Roche), and 20% FBS at 37°C for 20 min. Dissociated cells were filtered through a 150-μm mesh and separated by Ficoll centrifugation to obtain mononuclear cells.

Isolation and culture of monocytes

Monocytes were purified from PBMCs or tumor-infiltrating leukocytes using anti-CD14 magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer’s instructions. The CD14+ cells from healthy blood were cultured in DMEM containing 10% human AB serum in the presence or absence of 15% TSN or culture supernatant from LO2 for 6 d to obtain TAMs (15) or control MΦs. MΦs incubated with IFN-γ (100 IU/mL) for 2 d served as IDO+ control.

In vitro coculture model

Circulating or tumor-infiltrating T cells were purified using the Pan T Cell Isolation Kit II (Miltenyi Biotec), followed by incubation with PE-conjugated anti-CD69 (Beckman Coulter, Brea, CA) and anti-PE MicroBeads (Miltenyi Biotec), according to the manufacturers’ instructions (26). Culturing or tumor-infiltrating CD14+ cells, in vitro cultured MΦs or TSN-treated MΦs (TAMs), were cultured with autologous purified T cells, CD69+ T cells, or CD69+ T cells in RPMI 1640 supplemented with 5 IU/ml IL-2 (eBioscience, San Diego, CA) at a ratio of 1:4 for the indicated times. The medium was changed every other day. In some experiments, cells were pretreated with a specific blocking mAb (5 μg/ml) against TNF-α, IFN-γ, IL-6, IL-10, or IL-12p70 or with a control Ab. To detect IDO activity, 100 μM supernatants from coculture were harvested, and kynuren e was detected using the absorbance method, as previously described (27).

In vitro T cell culture model

To generate conditioned media, MΦs were left untreated or cocultured with autologous T cells in the presence or absence of an IDO inhibitor, 1-methyl-tryptophan (1-MT, 100 μM) for 3 d. Thereafter, the supernatants were harvested, centrifuged, and stored in aliquots at −80°C. To determine the IDO activities in TAMs, CFSE-labeled purified T cells (2 × 10^5 cells/well in 96-well round-bottom plates) were activated by anti-CD3/CD28 (2 μg/ml for each; eBioscience) in the presence of conditioned media for 2 d. Thereafter, the cells were maintained in the same conditioned media supplemented with 20 IU/ml IL-2 every other day. In some experiments, tryptophan (25 μM) was added to the culture.

Flow cytometry

MΦs were stained with fluorochrome-conjugated mAbs for CD66, B7-H1, or HLA D-related (HLA-DR) or control Ab (BD Pharmingen, San Diego, CA). T cells from in vitro culture, PBLs, and tumor- and nontumor-infiltrating lymphocytes were left untreated or stimulated at 37°C for 5 h with Leukocyte Activation Cocktail (BD Pharmingen). Thereafter, cells were stained with CD3, CD4, CD8, CD69, and TGF-β, fixed and permeabilized with IntraPre Reagent, and stained with IL-2, TNF-α, Foxp3, or IFN-γ (Beckman Coulter). Data were acquired on a Gallios flow cytometer (Beckman Coulter) and analyzed with FlowJo software.

ELISA

Concentrations of TNF-α, IFN-γ, and IL-12p70 (cat no. 88-7126) in the conditioned media were detected using ELISA kits, according to the manufacturer’s instructions (eBioscience) (14).

Immunohistochemistry and immunofluorescence

Paraffin-embedded and frozen samples were cut into 5-μm sections and processed for immunohistochemistry and immunofluorescence, as previously described (15). Following incubation with the Ab against human IDO, CD68, CD3, CD15, CD20, or CD56, the sections were stained with dianiba benzidine using the EnVision System. For immunofluorescence analysis, samples were stained with mouse anti-human IDO, rabbit anti-human CD3, mouse anti-human CD69 (R&D Systems), mouse anti-human CD68 (Dako), or rabbit anti-human CD68 (Santa Cruz Biotechnology), followed by Alexa Fluor 488- or 555-conjugated goat anti-mouse IgG and Alexa Fluor 488- or 555-conjugated goat anti-rabbit IgG (Invitrogen). Positive cells were quantified by ImagePro Plus software (Media Cybernetics) and expressed as the percentage of positive cells (mean ± SD) in 10 high-powered fields, as detected by confocal microscopy.

Evaluation of immunohistochemical variables

Analysis was performed by two independent observers. In low-power fields (×100), the tissue sections were screened, and the five most representative areas were manually selected using a LeicaDMIRB inverted microscope. For evaluating the density of CD3, CD15, CD20, CD56, or CD68 cells, the respective areas were measured at high power (×600, ~0.10 mm^2/field). The number of the above cells was counted manually and expressed as cells/field.

Immunoblotting

The proteins were extracted, as previously described (14), and separated by 10% SDS-PAGE, immunoblotted with an Ab against GAPDH (Abcam, Cambridge, MA) or IDO, and visualized with an ECL kit.

Statistical analysis

Results are expressed as mean ± SEM, unless otherwise indicated. Statistical significance was determined by the Student t test. A value of p < 0.05 was considered statistically significant.
Results

Distinct expression patterns of IDO in TAMs

To evaluate the potential role of MΦ-derived IDO in tumor immunopathology, we first analyzed its level in adjacent sections of HCC tissues stained for CD68 (marker for MΦs) and IDO. The results showed that IDO protein was expressed mainly in tumor MΦs and was only weakly expressed on stromal and hepatoma cells (n = 80; Fig. 1A). In contrast, little or no IDO signal was detected in nontumor tissues, although CD68+ cells did accumulate in these areas (n = 20; Fig. 1A). Using confocal microscopy, we confirmed that most IDO+ cells in cancer nests were also positive for CD68 (72 ± 13%; n = 10; Fig. 1B). Of note, most CD68+ cells in cancer nests of HCC tissues exhibited an IL-10+ HLA-DRlow suppressive phenotype (14). Similar results were obtained in pulmonary, gastric, and cervical carcinomas tissues, including the accumulation of IDO+ MΦs in the cancer nests (Fig. 1C, data not shown).

We recently observed that tumor environments can induce the formation of suppressive MΦs in the cancer nest (14, 15). To investigate whether such a mechanism is also responsible for the selective accumulation of IDO+ MΦs in cancer nests, we incubated normal blood monocytes with the culture supernatants from hepatoma or normal liver cell lines. On day 6, exposure of monocytes to 15% supernatants obtained from hepatoma cells, including HepG2 and SK-Hep-1 cells, resulted in MΦs with an increased expression of B7-H1 and reduced expression of HLA-DR and CD86 (Fig. 1E, data not shown). Unexpectedly, such treatments failed to induce IDO expression in these suppressive MΦs (Fig. 1D), suggesting that additional factors within the tumor milieu are required for inducing IDO expression in tumor MΦs.

Tumor-infiltrating CD69+ activated T cells induced IDO+ monocytes/MΦs

Tumor milieus comprise both cellular and noncellular components (1, 2). Immunohistochemical detection of 43 HCC tumor samples revealed that MΦs and CD3+ T cells are prominent components of immune cell infiltrates in HCC tissues (Supplemental Fig. 1A, 1B). Interestingly, a significant portion of the CD68+ monocytes/MΦs in the cancer nests that express IDO (Fig. 1A, 1B), but not those in nontumor tissues, are in close contact with CD3+ T cells (Fig. 2A), suggesting that T lymphocytes might exert a functional impact on monocytes/MΦs in the tumor milieu. Therefore, our next objective was to determine the effect of tumor-infiltrating T cells on IDO expression in monocytes/MΦs.

We first examined the activation status and cytokine production profile of T cells isolated from HCC tissues. In all of the samples (n = 7) analyzed, ~50% of the tumor-infiltrating T cells were CD69+ cells that were considered to be early-activated T cells (Fig. 2B, 2C). CD69 was weakly expressed on paired circulating and nontumor-infiltrating T cells (Fig. 2B, 2C). The activation of tumor T cells in situ was further confirmed by dual staining of CD69 and CD3 in HCC tissues, showing that most T cells in the cancer nest, but not in nontumor tissues, stained positively for CD69 (Fig. 2D). Compared with tumor-infiltrating CD69− T cells, CD69+ T cells isolated from the same tumor tissues had more potential to produce IL-2, TNF-α, and IFN-γ (Fig. 2E). Notably, IFN-γ is the most potent cytokine for IDO induction in both APCs and epithelial cells (20) (Fig. 1D). In addition, further characterization of CD69+ T cells isolated from HCC tissues showed that only a fraction of tumor-infiltrating CD69+ T cells could express regulatory T cell marker Foxp3 and membrane-bound TGF-β at levels comparable to those exhibited by CD69− T cells (Supplemental Fig. 1C, 1D). These data suggested that CD69 is expressed by activated T cells but not selectively by regulatory T cells in human HCC tissues.

To evaluate the effect of CD69+ T cells on monocyte/MΦ IDO induction, we purified both tumor-infiltrating CD69− and CD69+ T cells from the same HCC patient and cultured those cells with purified autologous circulating monocytes for 48 h. The result showed that coculturing with tumor-infiltrating CD69+ T cells, but not with CD69− T cells, significantly induced IDO expression in monocytes/MΦs (Fig. 3A). Consistent with the hypothesis that IFN-γ induces IDO expression in tumors, such upregulation of IDO protein was effectively attenuated by adding an IFN-γ-specific blocking mAb (Fig. 3A). These results indicated that CD69+ T cells in tumor environments contribute to IFN-γ-mediated IDO expression in monocytes/MΦs.

**FIGURE 1.** Tumor-suppressive MΦs differentially expressed IDO in vivo and in vitro. A, Adjacent sections of paraffin-embedded HCC samples and paired liver tissues (n = 80) were stained for Ab against IDO or CD68. B, Frozen HCC sections were double stained for IDO (red) and CD68 (green) and examined by confocal microscopy. One of 10 representative samples is shown. C, Adjacent sections of paraffin-embedded pulmonary and gastric cancer samples (n = 10 for each) were stained for IDO or CD68. Purified monocytes were left untreated or were cultured for 6 d with 15% culture supernatant from hepatoma (HepG2 and SK-Hep-1) or normal liver (L02) cells, and the expression of IDO and surface markers was determined by Western blot (D) and flow cytometry (E). The IFN-γ-treated MΦs served as a positive control in D. Results in D are representative of at least six separate experiments. Results in E show the mean ± SD of six separate experiments.
Cross-talk between tumor MΦs and T cells triggered IDO expression in MΦs

Most tumor T cells, but not circulating or nontumor T cells, expressed a significant level of CD69, which suggested that tumor environment can educate infiltrating T cells to adopt an activated phenotype. APCs are critical for initiating and maintaining tumor-specific T cell responses, and monocytes/MΦs markedly outnumber other APCs in tumors (14, 15). Thus, we investigated whether tumor monocytes/MΦs could activate circulating T cells in vitro. Purified blood CD69^+ T cells were cocultured with autologous circulating or tumor-infiltrating CD14^+ cells for 2 d. The result showed that exposure of T cells to tumor CD14^+ cells resulted in ∼10-fold upregulation of CD69 on both CD4^+ and CD8^+ T cells (47 ± 14% and 49 ± 16%, respectively; n = 3; Fig. 3B). In contrast, CD14^+ cells isolated from nontumor tissue or blood only had marginal effects (Fig. 3B).

The results described above indicated that tumor environments induce the formation of suppressive MΦs, which, in turn, leads to early T cell activation and subsequent MΦ IDO expression in cancer nest. To test this, we incubated monocytes with the culture supernatants from hepatoma cells to generate suppressive TAMs and then cultured those cells with purified autologous T cells. Consistent with the above results, exposure of T cells to TAM for 48 h elicited marked expression of CD69 at levels comparable to those exhibited by circulating T cells after their coculture with autologous CD14^+ cells from HCC tissues (45 ± 15% and 40 ± 12% in CD4^+ and CD8^+ T cells; n = 10; Fig. 4A). Measuring cytokines produced by cocultured cells over time revealed a delayed accumulation of IFN-γ in the culture supernatants, reaching a maximum within 5 d (Fig. 4B). Parallel with the kinetics of IFN-γ production, we detected considerable levels of IDO protein in the cocultured cells and its catalyzed product kynurenine in culture supernatant after 3 d (Fig. 4C, Supplemental Fig. 2A). In accordance with this, control MΦs, which did not trigger significant T cell activation and IFN-γ production, failed to induce IDO expression or kynurenine production in the coculture system (Fig. 4, Supplemental Fig. 2A). Similar results were obtained from coculture of autologous T cells and MΦs that had been exposed to supernatant from SK-Hep-1, HeLa, or 95D cells but not from normal liver (LO2) cells (Fig. 4D, data not shown). Comparison of the kinetics of T cell activation, IFN-γ production, and IDO expression in coculture revealed that early T cell activation preceded IFN-γ production, and IFN-γ production preceded IDO expression (Fig. 4A–C). Supporting this hypothesis, conditioned medium from the coculture of TAMs and T cells induced marked IDO expression in TAMs without coculture (Fig. 4E). Moreover, confocal microscopic analysis further confirmed the expression of IDO protein in TAMs but not in CD3 T cells or control MΦs (Supplemental Fig. 2B).

FIGURE 3. Tumor-infiltrating CD69^+ T cells induced MΦ IDO expression. A, Purified CD69^+ and CD69^- T cells from HCC tissues were cultured for 2 d with autologous blood CD14^+ cells in the presence or absence of Ab against IFN-γ at a ratio of 4:1. The production of IFN-γ in T cells and the expression of IDO in monocytes were detected by ELISA and Western blot, respectively. ELISA results, expressed as mean ± SEM, are from three individuals, and the Western blot is representative of three separate experiments. B, Purified circulating, nontumor, or tumor-infiltrating CD14^+ cells were cultured for 3 d with autologous circulating sorted CD69^- T cells at a ratio of 4:1. One of three representative results is shown.
IL-12 was required for early T cell activation and MΦ IDO expression

To study the mechanisms involved in the induction of early T cell activation by TAMs, we investigated the initial effects of TAMs on freshly isolated circulating T cells; the results showed that direct coculture of TAMs and T cells led to a rapid accumulation of TNF-α and IL-12p70 in the culture supernatants by 24 h (Fig. 5A). However, such accumulated cytokine production, as well as IDO expression in TAMs, was totally lost when seeding T cells in the upper chamber of Transwell plates, which suggested a cell contact-dependent mechanism involving the T cell–MΦ interactions in tumor environments (Figs. 4E, 5B). No measurable level of TNF-α or IL-12p70 was detected in the culture supernatants derived from control MΦs plus T cells or from TAMs alone (Fig. 5A). To ascertain whether these cytokines contribute to early T cell activation, we used specific neutralizing Abs that effectively abolish their effects in our coculture systems. Blockade of IL-12 effectively inhibited both upregulation of CD69 protein and production of IFN-γ in T cells and subsequent IDO expression in TAMs, whereas the anti–TNF-α Ab had only marginal effects (Fig. 5C, 5D). Interestingly, a combination of Abs against TNF-α and IFN-γ was superior to the anti–IFN-γ Ab alone in inhibiting T cell-mediated IDO expression in TAMs (Fig. 5E). These data implied that IL-12 is involved in IFN-γ induction, and TNF-α potentiates IFN-γ–mediated IDO expression.
IDO in TAMs induced T cell dysfunction

Studies in mouse models revealed that expression of IDO in dendritic cells helped dormant tumor cells to evade cytotoxic T cell responses (28). To test the effect of TAM-derived IDO on tumor T cell immunity, we labeled purified T cells with CFSE and then cultured them in conditioned medium from control TAMs or IDO+ TAMs (T cell-exposed TAMs) in the presence of polyclonal stimulations. Exposure to conditioned medium from IDO+ TAMs for 5 d induced dysfunctional T cells with impaired capacities for proliferation, as well as production of IFN-γ, TNF-α, and IL-2 (Fig. 6A, 6B). Supporting the general view that IDO exerts its function during T cell activation (20, 21, 29), such impairments of cytokine production were present in the proliferating T cells (CFSEdim) but not in the resting ones (Fig. 6B, 6C). Addition of extrinsic IDO substrate tryptophan and pretreatment of TAMs with an IDO inhibitor 1-MT during the preparation of conditioned medium markedly restored T cell proliferation and cytokine production (Fig. 6A–C). Collectively, these findings suggested that TAM-derived IDO might suppress adaptive immunity by preferential inhibition of inflammatory T cell proliferation.

Discussion

IDO has been recognized as an important mediator for tumor immune escape, but the precise regulating mechanisms in human cancer are not clear (30–32). The present study showed that IDO is selectively expressed at high levels in MΦs in situ in several types of human solid tumors but not in tumor-educated suppressive MΦs generated in vitro. Data from both clinical sample analysis and in vitro study demonstrated that CD69+ T cells effectively promote IDO expression in monocytes/MΦs by releasing IFN-γ. Moreover, we provide evidence that, upon encountering autologous T cells, tumor monocytes/MΦs produced IL-12 to activate T cells, which, in turn, led to IDO expression in MΦs and, in that way, created favorable conditions for tumor growth.

Studies in other systems showed that CD69 could exert multiple regulatory functions (17, 33). CD69-deficient mice develop an exacerbated form of arthritis with reduced TGF-β level, whereas engagement of CD69 stimulates TNF-α production and ERK activation on T cells (34, 35). The present study showed that CD69+-activated T cells are required for induction of MΦ IDO expression in tumor environments. This conclusion is based on the following evidence. First, most CD68+ cells in tumor tissues, but not in nontumor tissues, exhibited an IDO+ phenotype, which coincided with the significantly higher levels of CD69+ T cells found in tumor tissues. Second, the CD69+ T cells derived from HCC tissues induced marked IDO expression in monocytes/MΦs, whereas tumor-infiltrating CD69– T cells had no effect. Third, CD69+ T cells were the most potent IFN-γ–producing T cells in HCC tissues, and blockade of IFN-γ effectively abolished the induced expression in monocytes/MΦs. In accordance with these findings, TAMs generated in culture supernatants from solid tumors plus autologous T cells showed significant IDO expression; by contrast, those cultured solely in tumor supernatants only exhibited a suppressive phenotype, without IDO activities. These observations suggested that immune activation can be rerouted into immune tolerance by a fine-tuned collaborative action between different types of immune cells in tumor environments. This notion is supported by our recent studies showing that proinflammatory IL-17–producing cells in HCC tissues fostered immune privilege by inducing B7-H1 on APCs (16, 36).

Although TAMs originate from prototypical inflammatory cells, they are strongly impaired with regard to various functions related to inflammation (37, 38). We recently showed that tumors dynamically regulate the activation of recruiting monocytes at distinct sites, which leads to the formation of suppressive MΦs in cancer nests (14, 15). In the current study, we observed that, in contact with autologous T cells, the suppressive MΦs regained their ability to produce IL-12 in tumors and, thereby, activated T cells to produce IFN-γ, which, in turn, led to IDO expression in MΦs and ultimately impaired antitumor T cell immunity. However, abolishing the direct coculture of TAMs and T cells attenuated IL-12 production and subsequent IDO expression. Thus, these data suggested that the IL-12 produced by TAMs in cancer nests may not represent the host reaction to the malignancy but rather promote tumor evasion via a feedback-inhibitory mechanism involving the IFN-γ–IDO–T cell dysfunction axis.
notion is supported by our recent findings that the high density of monocytes/MFs in the cancer nests correlated with advanced disease stages and could serve as an independent predictor of poor survival in HCC patients (39). Consistent with our observations, recent studies showed that intratumoral delivery of exogenous IL-12 could elicit an IFN-γ-dependent IDO counterregulation in the mouse model (40).

IDO is a tryptophan-degradation enzyme with a profound regulatory effect on T cell responses. Although IDO protein is often found in dendritic cells and various human carcinomas, the regulatory mechanisms of IDO in TAMs remain to be defined (20, 22, 41). The present study showed that IFN-γ and TNF-α released by activated CD69+ T cells stimulated IDO expression in monocytes/MFs. Furthermore, in vitro study using mAbs against TNF-α and IFN-γ indicated that IFN-γ was essential for IDO induction and that proinflammatory TNF-α acted synergistically with IFN-γ to enhance IDO expression in TAMs. This hypothesis is compatible with our previous studies showing that the proinflammatory cytokine TNF-α can significantly enhance IL-10–dependent B7-H1 expression on monocytes (15).

The suppressive mechanisms of TAMs are less well understood in humans (11, 37, 38). In addition to producing IDO, tumor MFs express several members of the B7 family of costimulating molecules. Both B7-H1 and B7-H4 are selectively expressed by various regulatory T cells can trigger production of IL-10 by MFs, which, in turn, stimulates such cells to express B7-H4 in an autocrine manner and renders them immunosuppressive via the B7-H4 molecules (43). Therefore, manipulating the expression and signaling through these molecules may open new avenues for developing novel immune-based therapies to enhance antitumor immunity in human cancer.

Tumors can mimic some of the signaling pathways of the immune system to propagate conditions that favor immune tolerance and so escape tumor immunity (30, 44). Our results provide important new insights into the collaborative action of tumor stroma cells that is exercised to counteract effective T cell responses. Tumor monocytes/MFs educate the newly recruited T lymphocytes to take on a CD69+ proinflammatory phenotype, which, in turn, leads to expression of IDO in monocytes/MFs; in this way, they create conditions of unresponsiveness in tumors. Such an active induction of tolerance in the tumor environment has an obvious negative impact on the effectiveness of clinical manipulation of the antitumor response by immunotherapeutic approaches. Thus, a better understanding of the inflammatory context might provide a novel strategy for the rational design of anticancer therapies (45).

Acknowledgments
We thank R.Q. Weaver for linguistic revision of the manuscript.

Disclosures
The authors have no financial conflicts of interest.

References


