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Synovial Autoreactive T Cells in Rheumatoid Arthritis Resist IDO-Mediated Inhibition¹

Lingqiao Zhu,* Fang Ji,* Yuan Wang,[†] Yi Zhang,[‡] Qiang Liu,*[†] Jingwu Z. Zhang,*[§] Kouji Matsushima,^{||} Qi Cao,* and Yanyun Zhang^{2*}

A hallmark of T cell-mediated autoimmunity is the persistence of autoreactive T cells. However, it remains to elucidate the manner in which synovial T cells are sustained in patients with rheumatoid arthritis (RA). We found that dendritic cells (DC) and tissues from the synovial joints of RA patients expressed higher levels of IDO than DC from healthy donors. Interestingly, T cells derived from the joint synovial fluid (SF) of RA patients proliferated in response to either autologous or allogeneic IDO-positive DC, an outcome that was not affected by the addition of IDO inhibitor 1-methyl-D-tryptophan (1-MT). In contrast, addition of 1-MT to the culture stimulated with allogeneic or autologous IDO-positive DC significantly enhanced the proliferation of T cells derived from peripheral blood of healthy donors or from peripheral blood of RA patients. Furthermore, we found that functionally active tryptophanyl-tRNA-synthetase (TTS) was significantly elevated in T cells derived from the SF of RA patients, leading to enhanced storage of tryptophan in T cells and to subsequent resistance to IDO-mediated deprivation of tryptophan. The RA SF enhancement of TTS expression in T cells was blocked by mAb to IFN- γ and TNF- α . These results suggest that the resistance of T cells to IDO-mediated deprivation of tryptophan represents a mechanism by which autoreactive T cells are sustained in vivo in RA patients. Specifically, blocking of the up-regulation of TTS expression in T cells presents an avenue for development of a novel therapeutic approach to treatment of RA. *The Journal of Immunology*, 2006, 177: 8226–8233.

Rheumatoid arthritis (RA)³ is a chronic inflammatory disorder of the autoimmune response that mainly targets joints (1). RA develops when autoreactive T cells are activated and recruited to joints, where they mediate synovial inflammation and eventually cause tissue destruction and cartilage and bone invasion (1–5). Previous studies have demonstrated that synovial fluid (SF) from RA patients contains large numbers of autoreactive T cells expressing high levels of CD45RO (4, 6), indicating that T cells infiltrating the synovial joints of RA patients are activated and have adopted an effector/memory phenotype (7). Adoptive transfer of synovial infiltrating T cells derived from RA patients causes inflammatory arthritis in SCID mice. Partial elim-

ination or inhibition of T cells by immunosuppressive drugs or anti-T cell Abs ameliorates the disease (8). Thus, it is clear that autoreactive T cells play important roles in initiating and sustaining synovial inflammation in RA patients. However, the manner in which these autoreactive T cells are sustained during the progression and evolution of RA remains poorly investigated.

Tryptophan is an essential amino acid that is important to cell survival and proliferation (9, 10). Tryptophan can be catabolized by IDO, the initial and rate-limiting enzyme of tryptophan degradation pathways, to generate kynurenine, a molecule with the ability to induce T cell apoptosis. Furthermore, deprivation of tryptophan by IDO halts the proliferation of T cells at mid-G₁ phase, which, in concert with proapoptotic activity of kynurenine, leads to diminishing T cell-mediated immune responses and subsequent development of immune tolerance (11–14). IDO is expressed in various tissues in cells such as dendritic cells (DC) and activated macrophages. Although DC play a critical role in initiating a primary T cell response (15), IDO-positive DC are seen to be important in the generation and maintenance of peripheral tolerance via depletion of autoreactive T cells and induction of regulatory T cell responses (16). Recent studies have demonstrated that increased expression of IDO by tumor cells may account for their escape from immune attack (17–19). Impaired IDO-mediated tryptophan catabolism has also been observed in experimental autoimmune disease models, including NOD mice (20), in which autoreactive T cells persist during disease progression. In contrast, tryptophanyl-tRNA-synthetase (TTS) is a constitutively expressed cytoplasmic enzyme with the ability to mediate the association of tryptophan with its specific tRNA (21). The complex of tryptophan with its tRNA forms a reservoir of tryptophan for protein synthesis, which then nullifies IDO-mediated deprivation of tryptophan (22, 23). However, whether dysregulated tryptophan catabolism contributes to the persistence of autoreactive T cells that then can mediate persistent tissue injury in RA patients remains unknown.

Using samples from RA patients and healthy donors, we investigated a possible mechanism by which autoreactive T cells persist

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³ Abbreviations used in this paper: RA, rheumatoid arthritis; 1-MT, 1-methyl-D-tryptophan; C_T, cycle threshold; DC, dendritic cell; PB, peripheral blood; PI, propidium iodide; SF, synovial fluid; ST, synovial tissue; TTS, tryptophanyl-tRNA-synthetase.

in RA patients. We found that RA patient-derived SF contained inflammatory factors (e.g., IFN- γ and TNF- α). These cytokines were able to enhance the expression of TTS in T cells, leading to the resistance of RA patient-derived SF T cells to IDO-mediated deprivation of tryptophan. This means that blocking of TTS may prove valuable for further development of novel approaches to the restoration of IDO-mediated immune tolerance and the diminishment of autoreactive T cell-mediated chronic immune responses.

Materials and Methods

Patient samples

A group of 35 patients with definitive RA (based on the criteria of the American Rheumatism Association) was investigated in this study, which included 24 females and 11 males whose ages ranged from 24 to 73 years (mean age, 43 years), with a disease duration of 9 ± 6 years. Erythrocyte sedimentation rate, C-reactive protein, and rheumatoid factors were 56.4 ± 20.6 mm/h, 38.0 ± 23.7 mg/L, and 99.8 ± 88.2 IU/ml, respectively. An age-matched group of 36 healthy individuals was included as a control. Peripheral blood (PB) specimens were collected from RA patients or healthy donors, and SF from RA patients during routine knee joint aspirations. Samples of synovial tissue (ST) were obtained from RA patients during wrist synovectomy or arthroplasty. Control ST was obtained from patients with traumatic lesions during knee arthroscopy. No immunosuppressive or immunomodulatory drugs had been taken by patients for at least 3 mo when samples were collected. Informed consent was obtained from all study subjects before sample collection. The study protocol was approved by the institutional review board of the Institute of Health Sciences (Shanghai, China).

Cell preparation

Rheumatoid SF supernatants were collected following centrifugation at 1800 rpm for 20 min and depletion of cells and particulate debris. Supernatants of SF were then aliquoted and immediately stored at -80°C . Mononuclear cells from PB (PBMC) and SF (SF mononuclear cells) were isolated by Ficoll density gradient centrifugation (Lymphoprep; Axis-shield PoC). Cells were incubated with allophycocyanin-conjugated mouse anti-human CD3 (HIT3a) mAb and a mixture of mAbs against lineage markers, including FITC-labeled CD14 (M5E2), CD16 (3G8), CD19 (H1B19), and CD20 (2H7) mAbs and PE-labeled CD56 (B159) mAb. CD3 $^{+}$ T cells and lineage-negative (Lin $^{-}$) cells (CD3 $^{-}$, CD14 $^{-}$, CD16 $^{-}$, CD19 $^{-}$, CD20 $^{-}$, and CD56 $^{-}$) were then sorted using a FACS (FACSARIA; BD Biosciences). The Lin $^{-}$ cells were seen to be a DC-enriched population (24). In some experiments, to avoid CD3-specific Ab-mediated T cell activation, we sorted CD4 $^{+}$ and CD8 $^{+}$ cells following the staining with allophycocyanin-conjugated mouse anti-human CD4 (RPA-T4) and anti-CD8 (RPA-T8) mAbs using FACS. The purity of sorted cells was consistently $>98\%$, as revealed by immunofluorescence analysis.

In some experiments, IDO-positive DC (termed IDO $^{+}$ DC) were generated from healthy donor-derived PBMC, as previously described, with some modification (12, 15). In brief, monocytes separated from PBMC were cultured for 7 days with 25 ng/ml GM-CSF (R&D Systems) and 50 ng/ml IL-4 (R&D Systems) in serum-free X-vivo15 medium (BioWhittaker). Optimal conditions were maintained by splitting these cultures at day 5 and every 2–3 days replacing 50% of the medium with new medium containing fresh cytokines. The nonadherent cells from the culture were stimulated with TNF- α (50 ng/ml), IL-1 β (2 ng/ml), IL-6 (100 ng/ml; PeproTech), and PGE $_2$ (1 $\mu\text{g}/\text{ml}$; Sigma-Aldrich) for an additional 2–4 days. The nonadherent (IDO $^{+}$) fraction was harvested by gentle aspiration. Analysis by flow cytometry showed that IDO $^{+}$ cells constituted up to 65% of these cells. This was determined by staining, after fixation and permeabilization, with purified mouse anti-IDO (10.1) mAb (Chemicon International) and subsequently with FITC-labeled goat anti-mouse IgG (Chemicon International). All Abs used for separation and staining were purchased from BD Pharmingen, unless otherwise indicated.

Quantitative PCR

Total RNA was extracted from the sorted CD3 $^{+}$ T cells and DC using the guanidinium thiocyanate-phenol-chloroform method modified for TRIzol (Invitrogen Life Technologies). IDO transcripts were detected through RT-PCR, whereas cDNA of TTS was quantified through real-time PCR technique, all with GAPDH as control. Primers for IDO were as follows: forward, 5'-ACTGGAGGCACTGATTGA-3' and reverse, 5'-ATTAGTTTGTGGCTCTGTTA-3'. Real-time PCR were performed using a SYBR green PCR mix

and conducted with the ABI Prism 7900HT (Applied Biosystems). Thermocycler conditions included an initial holding at 50°C for 2 min, then 95°C for 10 min; this was followed by a two-step PCR program: 95°C for 15 s and 60°C for 60 s for 40 cycles. Data were collected and quantitatively analyzed on an ABI PRISM 7900 sequence detection system (Applied Biosystems). The primer sequences were designed using the Primer Express Software version 2.0 provided with the ABI Prism 7900HT (TTS, forward, 5'-GAAAGGCA TTTTCGGCTTCA-3' and reverse, 5'-CAGCCTGGATGGCAGGAA-3'; GAPDH, forward, 5'-GTGAAGTCCGAGTCAACG-3' and reverse, 5'-TG AGGTCAATGAAGGGGTC-3'). The GAPDH gene was used as an endogenous control to normalize for differences in the amount of total RNA in each sample. All values were expressed as fold increase or decrease relative to the expression of GAPDH. The mean value of the replicates for each sample was calculated and expressed as cycle threshold (C_T ; cycle number at which each PCR reaches a predetermined fluorescence threshold, set within the linear range of all reactions). The amount of gene expression was then calculated as the difference (ΔC_T) between the C_T value of the sample for the target gene and the mean C_T value of that sample for the endogenous control (GAPDH). Relative expression was calculated as the difference ($\Delta\Delta C_T$) between the ΔC_T values of the test sample and of the control sample. Relative expression of genes of interest was calculated and expressed as $2^{-\Delta\Delta C_T}$.

Western immunoblot analysis

Cells were harvested, pelleted by centrifugation, and resuspended in lysis buffer. Equal amounts of protein (20 μg) were loaded onto a 5% acrylamide stacking gel and separated by SDS-PAGE using a 10% separating gel. Following transfer of separated proteins, nitrocellulose membranes were blocked and probed overnight at 4°C with mouse anti-IDO mAb (Chemicon International). The membrane was then probed for 1 h at room temperature with goat anti-mouse peroxidase-conjugated IgG (Kirkegaard & Perry Laboratories), and the immunoreactivity was detected by chemiluminescence. To quantify IDO proteins, each band density was normalized to actin protein.

Histology and immunohistochemical staining

Cryosections of ST from RA patients or patients with traumatic lesions were routinely stained with H&E. For IDO staining, cryosections were fixed in 10% formalin for 10 min and then treated with mouse anti-IDO mAb (Chemicon International), followed by staining of peroxidase-conjugated rabbit anti-mouse IgG (1:200; Kirkegaard & Perry Laboratories), and development with 3,3'-diaminobenzidine. Sections were finally counterstained with hematoxylin and examined microscopically.

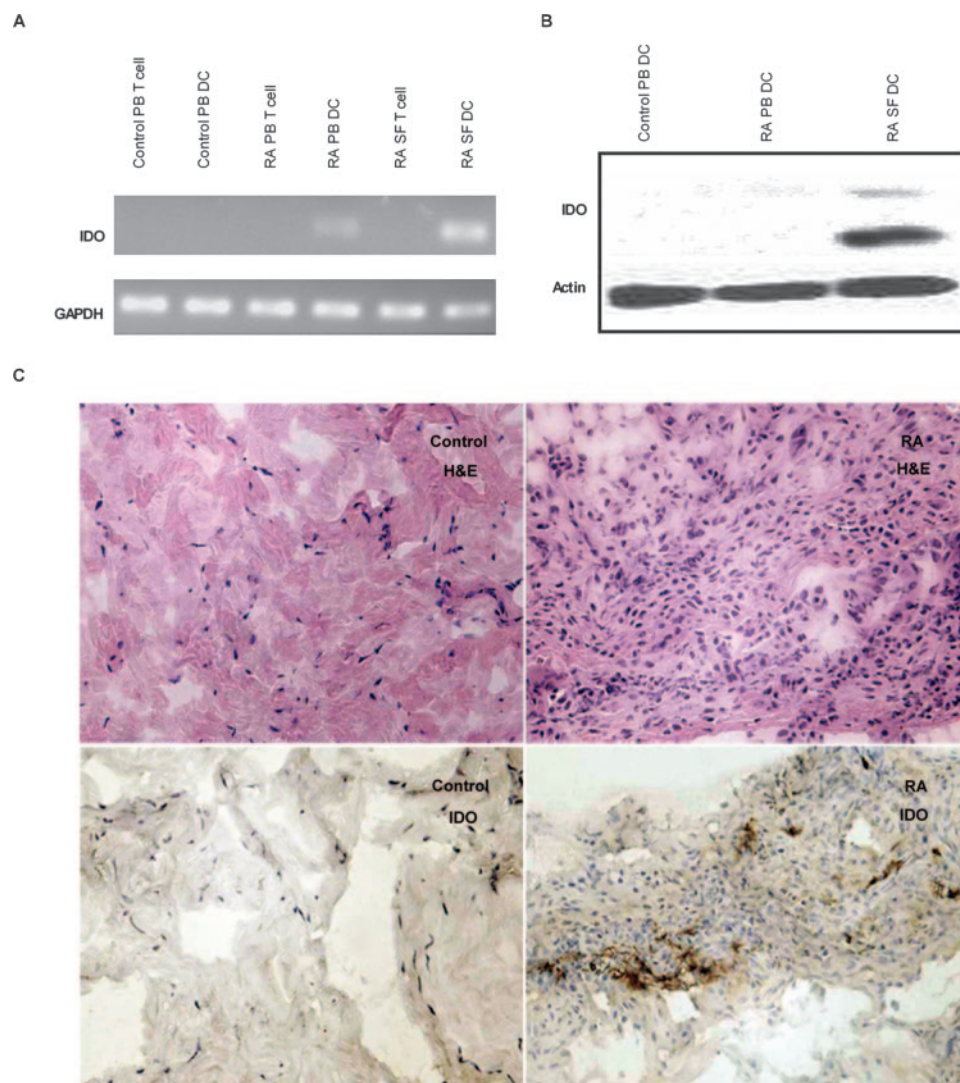
HPLC assay

DC were cultured in the RPMI 1640 medium supplemented with 10% FCS, 100 IU/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 2 mM L-glutamine (complete medium). Twenty-four hours after culture, supernatant was collected and its kynurenine concentration was measured with HPLC. HPLC was performed, as previously described (25), with minor modification. In brief, 200 μl of supernatant was diluted with 200 μl of potassium phosphate buffer (0.05 M (pH 6.0)), and protein was precipitated with 50 μl of 2 M trichloroacetic acid. One hundred fifty microliters of supernatants was then injected into an RP $_{18}$ column and eluted with a degassed potassium phosphate solution (0.015 M; pH 6.4) containing 27 ml/L acetonitrile at a flow rate of 0.8 ml/min. Kynurenine was detected using a UV detector at a wavelength of 360 nm. The values were referred to a standard curve with defined kynurenine concentrations (0–60 μM). To analyze cellular tryptophan use, purified T cells were cultured in complete medium with 50 μM added tryptophan at 37°C in 5% CO $_2$. Twenty-four hours after culture, 100 μl of supernatant was collected for determination of tryptophan concentration. Preparation of protein-free supernatant was the same as for kynurenine measurement. Tryptophan concentration was measured using a fluorescence detector at the excitation wavelength of 285 nm and emission wavelength of 365 nm.

Mixed leukocyte reaction

MLR was performed by addition of DC (1×10^4 cells/well) to allogeneic or autologous T cells (1×10^5 cells/well) in RPMI 1640 medium supplemented with 10% FCS without addition of tryptophan in 96-well U-bottom microtest tissue culture plates (BD Biosciences). Cultures were set up in the presence or absence of IDO inhibitor, 1-methyl-D-tryptophan (1-MT, 150 μM ; Sigma-Aldrich). For T cells stimulated by autologous DC, 1 $\mu\text{g}/\text{ml}$ purified anti-human CD3 (OKT3) mAb (eBioscience) was added to the medium. Triplicate wells were cultured for each group in MLR. Cells were stimulated *ex vivo* for 5 days, and 1 $\mu\text{Ci}/\text{well}$ [^3H]thymidine was added 18 h before the end of culture. The cells were then harvested onto

FIGURE 1. IDO expression in DC of joint synovium from RA patients. *A*, IDO gene expression was analyzed through RT-PCR. RNA was prepared from FACS-sorted DC and CD3⁺ T cells derived from SF or PB of RA patients, with cells from PB of healthy donors as controls. Expression of IDO mRNA was normalized to GAPDH. *B*, PB or SF DC from RA patients and PB DC from healthy donors were subjected to Western blot analysis using mouse anti-human IDO mAb. As a normalized control, expression of actin was analyzed in parallel. *C*, IDO expression of ST was detected in situ by immunohistochemistry. Cryosections of ST from patients with RA were prepared and immunostained, as described in *Materials and Methods*. ST from patients with traumatic lesions was used as controls. Tissues were stained with H&E or immunohistochemically stained for IDO. IDO staining appeared brown, and sections were counterstained with hematoxylin. Original magnification, $\times 100$. These results are representative of five independent experiments.



glass fiber filters for measurement of [³H]thymidine incorporation based upon scintillation counting. In parallel with each MLR, T cells alone were cultured as controls for [³H]thymidine incorporation. In some experiments, cellular apoptosis was determined using an annexin V-FITC apoptosis detection kit I, according to the manufacturer's instructions (BD Biosciences). In brief, after 5-day MLR, cells were harvested, stained with FITC-labeled annexin V and propidium iodide (PI) for 15 min at room temperature, and analyzed by FACS within 1 h. Necrotic cells were excluded by gating on PI-negative (PI⁻) cells, and cell externalization of phosphatidylserine was monitored by its binding to annexin V.

TTS induction

PBMC from healthy donors were cultured in complete medium with IFN- γ (PeproTech) (23), or with SF supernatant at the indicated dilutions, as previously described (26), in 24-well plates at a concentration of 1×10^6 cells/ml/well. Forty-eight hours after culture, cells were harvested and T cells were sorted using FACS for RNA abstraction, tryptophan concentration detection, and MLR assay. TTS expression in induced T cells was measured by real-time PCR. In some experiments, additional neutralizing mouse anti-human mAb were added to the SF-treated cultures to counter IL-18 (125-2H), IL-10 (23738), IFN- γ (25718), and/or TNF- α (6401) (5 μ g/ml each; R&D Systems), with mouse IgG as control.

Statistics

Significant differences were evaluated with Student's *t* tests, except that multiple treatment groups were compared within individual experiments by ANOVA or Kruskal-Wallis test. Data are expressed as the mean $\pm$ SD, and probability values of <0.05 were considered significant.

Results

DC from joint SF of RA patients express functional IDO

To determine the role of IDO in autoreactive T cell persistence in RA patients, we first measured the expression of IDO in DC and CD3⁺ T cells that were derived from RA patients, and compared it with that of cells from healthy control donors. Both RT-PCR and Western blot analysis showed that IDO was detected only in DC derived from joint SF (SF DC) and, to a lesser extent, PB (PB DC) of RA patients. In contrast, IDO was not detected in the control PB DC derived from healthy donors, T cells that were derived from the PB (PB T cells) and SF (SF T cells) of RA patients, or the control PB T cells of healthy donors (Fig. 1, *A* and *B*). Immunohistochemical staining demonstrated that macrophage-like cells in the joint ST from RA patients expressed IDO (Fig. 1*C*). Culture of RA patient-derived SF DC in complete medium containing tryptophan induced the production of significantly higher levels of kynurenine (27.7 ± 10.1 μ M) than did DC from the PB of RA patients (1.9 ± 0.6 μ M) and those from healthy donors (2.2 ± 0.6 μ M; Fig. 2). These data suggest that IDO expressed by DC of joint SF from RA patients is functionally active.

We next tested the ability of RA patient-derived IDO-positive SF DC and IDO-negative PB DC to stimulate the proliferation of allogeneic PB T cells of healthy donors, with healthy donor-derived IDO-negative PB DC as controls. Both IDO-negative PB

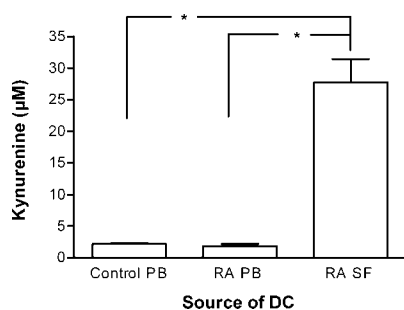


FIGURE 2. IDO activity in DC from SF of RA patients to functionally metabolize tryptophan. SF DC derived from patients with RA were sorted by FACS, as described in *Materials and Methods*, and cultured in complete medium with additional tryptophan for 24 h, with PB DC from healthy donors or from patients with RA as controls. Kynurenine in the culture supernatant was measured by HPLC. Results are expressed as the mean $\pm$ SD of 10 samples in each group. *, $p < 0.05$ significance compared between the groups.

DC of RA patients and PB DC of healthy donors induced the proliferation of allogeneic PB T cells from healthy donors. Addition of IDO inhibitor 1-MT did not affect the proliferation of allogeneic T cells in these ex vivo cultures (Fig. 3A). In contrast, whereas RA patient-derived SF DC that expressed high levels of IDO also stimulated allogeneic T cell proliferation, addition of 1-MT greatly enhanced the proliferation of these allogeneic T cells (Fig. 3A). These results suggest that SF DC of RA patients express functional IDO that is able to catabolize tryptophan, leading to the inhibition of T cell proliferation.

RA patient-derived SF T cells resist IDO-mediated suppression

We next determined the ability of SF T cells derived from RA patients to proliferate in response to IDO-positive DC. RA patient-derived SF T cells were cultured in the presence of allogeneic IDO-positive SF DC, with stimulation of PB T cells from RA patients and PB T cells from healthy donors, respectively, with the same allogeneic SF DC as controls. We found that all cultured T cells proliferated in response to allogeneic IDO-positive SF DC (i.e., $29,136 \pm 3,600.2$ cpm for SF T cells of RA patients; $33,990.2 \pm 5,411.7$ cpm for PB T cells of RA patients; and $45,385.2 \pm 6,725.4$ cpm for PB T cells of healthy donors; Fig. 3B). Interestingly, addition of 1-MT to the cultures significantly enhanced the proliferation of PB T cells of healthy donors (75.3%) and PB T cells of RA patients (71.6%), when compared with SF T cells of RA patients (18.7%; Fig. 3B). These results suggest that the inhibitory effect of SF DC-derived IDO on T cell proliferation is markedly ablated in SF T cells from RA patients. This conclusion was further confirmed by the culture of RA patient-derived SF T cells stimulated with autologous SF IDO-positive DC together with anti-CD3 mAb, with or without addition of 1-MT. As shown in Fig. 3C, whereas both PB T cells of healthy donors and SF T cells of RA patients proliferated to a similar extent in response to IDO-positive autologous DC plus anti-CD3 mAb, addition of 1-MT significantly enhanced the proliferation of PB T cells from healthy donors 4-fold more than that of SF T cells from RA patients. Taken together, these data suggest that, although the expression of functional IDO is increased in synovial DC and tissues of RA patients, RA patient-derived SF T cells have developed the ability to resist IDO-mediated tryptophan deprivation.

Enhanced expression of TTS in synovial T cells from RA patients

Previous studies have demonstrated that TTS induces the association of tryptophan with its specific tRNA. This tryptophan-tRNA

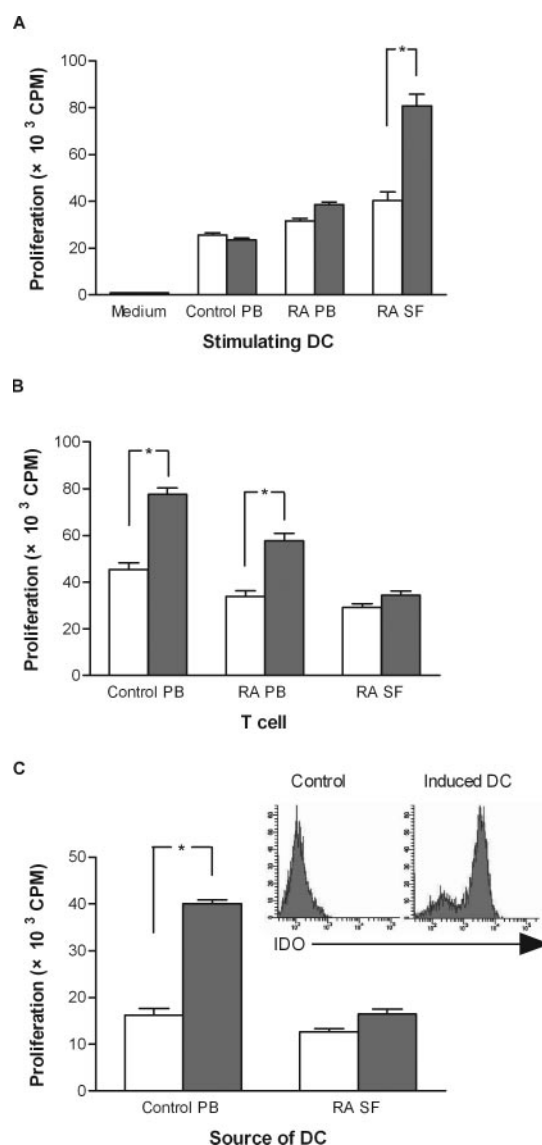


FIGURE 3. Synovial T cells from RA patients are resistant to IDO-mediated suppression. The indicated DC (1×10^4 cells/well) and T cells (1×10^5 cells/well) were incubated in 200 μ l of complete medium per well in 96-well U-bottom microtest tissue culture plates in the presence or absence of 150 μ M 1-MT, an inhibitor of IDO. T cell proliferation was measured by incorporation of [³H]thymidine added during the last 18 h of the 5-day incubation. A, SF DC and PB DC derived from RA patients were used as stimulators and were cultured with allogeneic PB T cells from healthy donors, with PB DC from healthy donors as controls. B, SF T cells derived from RA patients as responders were stimulated with allogeneic SF DC, with PB T cells from RA patients or from healthy donors as controls. C, RA-SF T cells were stimulated with autologous SF DC in the presence of 1 μ g/ml anti-CD3 mAb; PB T cells from healthy donors were stimulated in parallel with autologous IDO⁺ DC, which were ex vivo induced from PBMC-derived monocytes, as described in *Materials and Methods*. Inset, Representative histogram of IDO expression of the ex vivo induced DC. Results are expressed as the mean $\pm$ SD of triplicate cultures of 10 samples in each group. $\square$, Represent cultures without 1-MT; $\blacksquare$, with 1-MT. *, $p < 0.05$ significance compared between the groups.

complex enhances the storage of tryptophan and subsequent protein synthesis (22, 23). To determine whether TTS-mediated formation of tryptophan-tRNA complex accounts for the resistance of SF T cells from RA patients to IDO-mediated deprivation of tryptophan, RNA was prepared from SF T cells and PB T cells of RA

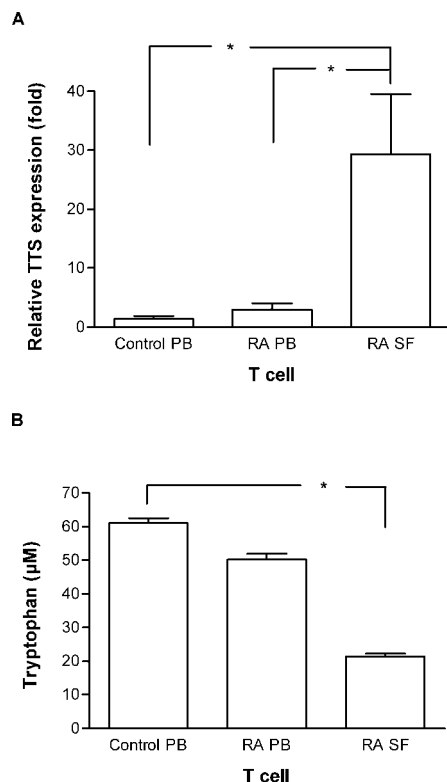


FIGURE 4. Analysis of TTS expression and tryptophan consumption in T cells from SF of RA patients. **A**, Real-time PCR analysis of TTS expression in T cells isolated from SF and paired PB of RA patients ($n = 12$), with a panel of PB T cells of healthy donors as control. TTS expression was normalized to endogenous GAPDH in the same samples and expressed as $2^{-\Delta\Delta CT}$ as instructed in *Materials and Methods*. **B**, Tryptophan using capacity of T cells from SF, paired PB of RA patients, or PB of healthy donors was measured through detection of tryptophan in their culture supernatant. T cells were cultured in complete medium with 50 μM added tryptophan for 24 h, and levels of tryptophan concentration were analyzed using HPLC. Results are expressed as the mean $\pm$ SD of triplicate cultures of eight samples in each group. In all cases, asterisks indicate statistically significant differences between the groups ($p < 0.05$).

patients, with PB T cells of healthy donors used as controls. Real-time PCR showed that SF T cells of RA patients expressed significantly more TTS transcripts than PB T cells from healthy donors or from RA patients (Fig. 4A). Furthermore, when T cells were cultured in medium with additional tryptophan for 24 h, SF T cells from RA patients consumed more tryptophan than did PB T cells from RA patients or healthy donors (21.4 ± 2.4 vs 50.1 ± 5.2 and 60.9 ± 4.3 μM ; Fig. 4B). These results suggest that increased expression of TTS in SF T cells from RA patients is tightly correlated with their enhanced consumption of tryptophan.

Previous studies have demonstrated that IFN- γ is able to enhance the expression of TTS in T cells (23). To determine whether enhanced expression of TTS augments the consumption of tryptophan by T cells, PBMC from healthy donors were treated with IFN- γ for 48 h, with untreated PBMC as controls. Real-time PCR showed significantly increased expression of TTS in sorted T cells from IFN- γ -treated PBMC as compared with untreated T cells ($p < 0.05$; Fig. 5A). We referred to these IFN- γ -treated T cells expressing increased level of TTS as TTS^{high} T cells, and those T cells left IFN- γ untreated as TTS^{low} T cells. When cultured in medium with additional tryptophan, there was significantly less tryptophan in the supernatant from TTS^{high} T cell cultures than in that of TTS^{low} T cells (18.4 ± 2.8 vs 61.0 ± 1.4 μM ; Fig. 5B). Addition of TTS

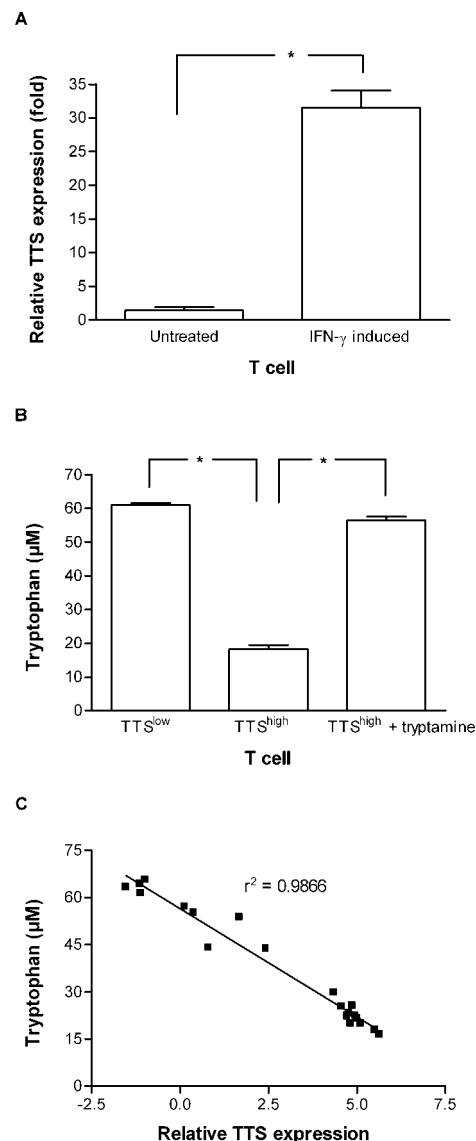


FIGURE 5. Tryptophan consumption of T cells was associated with their levels of TTS expression. **A**, TTS expression in IFN- γ -induced T cells. PBMC from healthy donors were incubated in complete medium with or without IFN- γ for 48 h; T cells were then isolated by FACS. TTS mRNA expression of the isolated T cells was calculated through real-time PCR by comparing with GAPDH and was expressed as $2^{-\Delta\Delta CT}$. **B**, Tryptophan concentration of the culture supernatant of IFN- γ -induced T cells was determined by HPLC. IFN- γ -treated T cells expressing increased levels of TTS were termed TTS^{high} T cells, whereas those left IFN- γ untreated were termed TTS^{low} T cells. Cells were cultured for 24 h in complete medium with 50 μM added tryptophan. In parallel, tryptamine, a TTS inhibitor, was added to the TTS^{high} T cell cultures to suppress the function of TTS. **C**, Correlation curve between tryptophan consumption and level of TTS expression. Data are taken from experiments on the in vitro induced TTS^{high} and TTS^{low} T cells, and TTS expression is expressed as $-\Delta\Delta CT$; $r^2 = 0.9688$. Results are presented as the mean $\pm$ SD of 21 samples. In all cases, asterisks indicate statistically significant differences between the groups ($p < 0.05$).

inhibitor tryptamine inhibited the consumption of more tryptophan by TTS^{high} T cells (Fig. 5B). Statistical analysis showed the close correlation between tryptophan consumption and the expression of TTS in these cultured T cells ($r^2 = 0.9688$; Fig. 5C). Interestingly, when stimulated with allogeneic IDO-positive DC, IFN- γ -treated TTS^{high} T cells proliferated more vigorously than did TTS^{low} T cells (Fig. 6A). Furthermore, addition of IDO inhibitor 1-MT to the

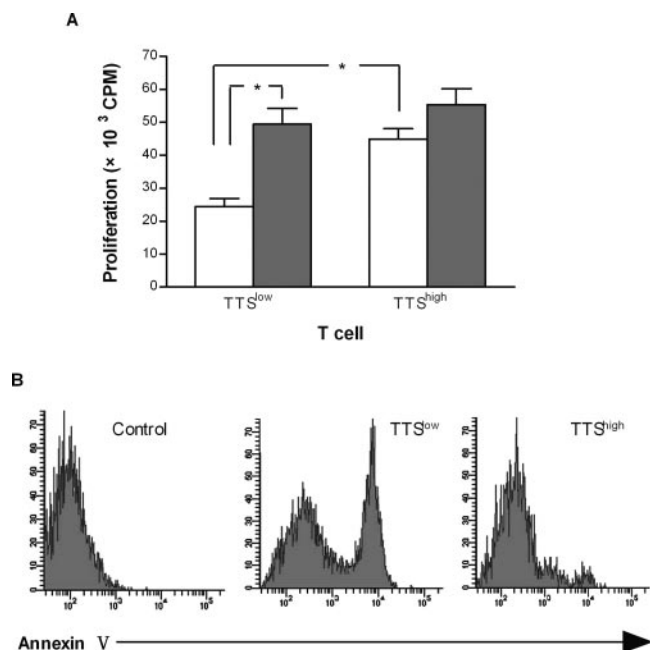


FIGURE 6. Enhanced expression of TTS in T cells allows resistance to IDO-mediated suppression. *A*, IDO⁺ DC and TTS^{high} T cells were induced in vitro, as described in *Materials and Methods*. IDO⁺ DC were used to stimulate allogeneic TTS^{high} T cells or TTS^{low} T cells in the presence or absence of 1-MT. Cells were cultured for 5 days, and [³H]thymidine was added to each well 18 h before the end of the incubation. Shown is [³H]thymidine incorporation into T cells. Results are expressed as the mean $\pm$ SD of six samples in each group. □, Represent cultures without 1-MT; ■, with 1-MT. *B*, After 5-day MLR, cells were washed and stained with annexin V-FITC and PI using annexin V-FITC apoptosis detection kit. The frequency of PI⁻ annexin V⁺ apoptotic cells was analyzed by FACS. Representative results of three independent experiments are shown. *, $p < 0.05$ significance compared between the groups.

cultures markedly enhanced the proliferation of TTS^{low}, but not TTS^{high} T cells (Fig. 6A). Supernatants from the culture of TTS^{high} T cells stimulated with IDO-positive DC contained a markedly reduced amount of tryptophan catabolite kynurenine (5.7 μ M) as compared with that of TTS^{low} T cells (14.9 μ M). Flow cytometric analysis further revealed that more T cells underwent apoptosis in the TTS^{low} group stimulated by IDO-positive DC than in the TTS^{high} T cell group (44.1 vs 10.0%; Fig. 6B). The findings indicated that enhanced expression of TTS in T cells was responsible for their resistance to IDO-mediated tryptophan deprivation and apoptotic cell death.

RA patient-derived SF enhances TTS expression in T cells

Finally, we tested whether factors in SF of RA patients play an important role in up-regulating the expression of TTS in SF T cells. As shown in Fig. 7A, stimulation of PBMC from RA patients or healthy donors with RA patient-derived SF, but not with sera from the same patient, increased the expression of TTS in T cells as many as 9-fold as compared with unstimulated T cells. This SF-mediated up-regulation of TTS expression was SF dose dependent (Fig. 7B). Addition of neutralizing mAb to IFN- γ or to TNF- α , but not to IL-18 and IL-10, blocked the elevation of TTS in healthy donor-derived T cells stimulated by RA patient-derived SF (Fig. 7C). Thus, proinflammatory cytokines in the SF of RA patients, including IFN- γ and TNF- α , play pivotal roles in up-regulating TTS in T cells.

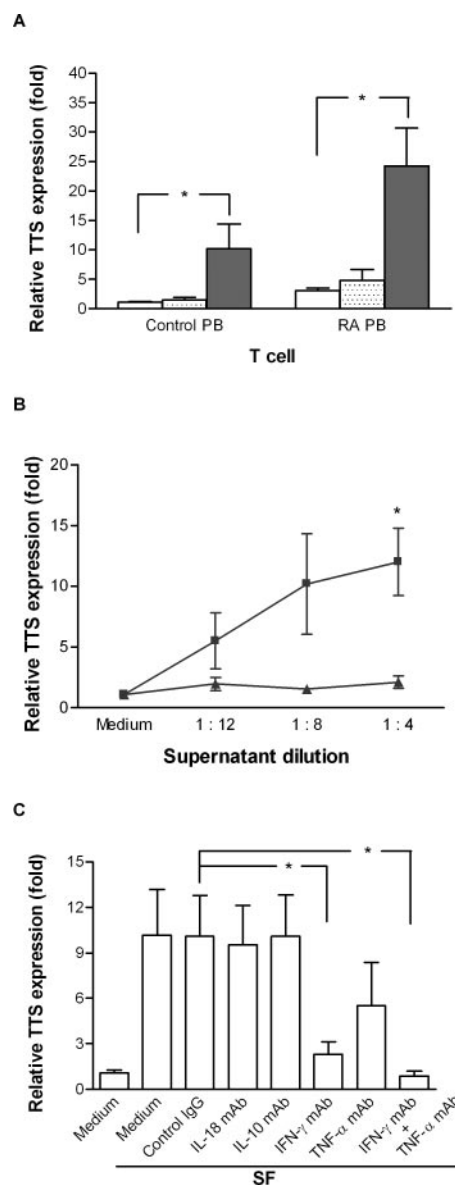


FIGURE 7. Elevated expression of TTS in T cells resulted from SF of RA patients. PBMC derived from healthy donors were cultured with SF supernatant from RA patients for 48 h. Cells were then collected, and T cells were isolated by FACS to analyze TTS expression through real-time PCR. *A*, SF supernatant or sera from RA patients was added to cultures of PBMC from healthy donors and from RA patients at a dilution of 1/5. □, Represent cultures with medium alone; ▤, with sera from RA patients; ■, with paired SF supernatant from RA patients. *B*, The cultures were performed in the presence of various dilutions of SF or paired sera from RA patients. Triangles represent sera from RA patients; squares, the paired SF supernatant from RA patients. *C*, Neutralizing mouse anti-human mAbs against IL-18, IL-10, IFN- γ , and/or TNF- α were added to the culture with RA SF supernatant to block specific cytokine(s) in SF. Results are expressed as the mean $\pm$ SD of eight samples analyzed. In all cases, asterisks indicate statistically significant differences between the groups ($p < 0.05$).

Discussion

Catabolism of tryptophan by IDO plays an important role in regulating T cell-mediated immune responses and inflammation (27, 28). We demonstrated that whereas DC freshly isolated from PB of both healthy donors and RA patients did not express detectable IDO protein, SF DC from RA patients expressed high levels of functionally active IDO. Although IDO produced by SF DC inhibited the proliferation of PB T cells derived either from healthy

donors or RA patients, synovial T cells from RA patients resisted the suppressive effect of IDO. We found that these synovial T cells from RA patients expressed high levels of TTS and were able to preserve tryptophan, which appears to be responsible for their resistance to IDO-mediated tryptophan deprivation. Furthermore, blocking proinflammatory cytokines, including TNF- α and IFN- γ , abrogated the effect of RA patient-derived SF on up-regulating TTS expression in T cells. Compared with TTS^{low} T cells, T cells with increased expression of TTS gained the ability to resist IDO-mediated tryptophan deprivation. Thus, it is likely that proinflammatory cytokines in SF of RA patients play important roles in up-regulating TTS expression in T cells, leading to the resistance of T cells to tryptophan deprivation by IDO and the subsequent persistence of inflammatory synovial T cells in RA patients.

Tryptophan is an essential amino acid that is required by all cells to synthesize proteins. The depletion of tryptophan and the accumulation of proapoptotic tryptophan-derived catabolite may arrest proliferating cells in mid-G₁ phase and block effector activity of T cells (29). Data from previous studies indicate that IDO⁺ DC is involved in the development of tolerance (30–35). IDO⁺ DC suppressed Ag-specific T cell responses in vitro (36). Induction of IDO in DC by CTLA-4-Ig resulted in blockade of clonal T cell expansion (37). Furthermore, in vivo administration of 1-MT to mice, the specific inhibitor of IDO, accelerated the diseases of experimental autoimmune encephalomyelitis (38) and T cell-mediated colitis (39), in which autoreactive T cells persist during disease progression. Autoreactive T cells capable of recognizing tissue-specific Ags are not necessarily deleted in the thymus as they can be cloned from lymph nodes of mice and from the circulation of humans (40). However, the manner in which these self-reactive T cells are exquisitely regulated remains controversial. Data from recent studies indicate that regulatory DC expressing high levels of IDO play important roles in modulating autoimmune responses (27). In our studies, we found that although SF DC from patients with RA expressed functional IDO, synovial T cells isolated from these patients resisted IDO-mediated suppression. These observations suggest that the resistance of synovial T cells to IDO-mediated tryptophan deprivation is an important mechanism responsible for the persistence of inflammatory T cells in the joints of RA patients.

The resistance of synovial T cells to IDO-mediated deprivation of tryptophan is associated with the increased expression of TTS. Data from other investigators suggest that TTS protects T cells from IDO-mediated cell injury by inducing the formation of tryptophan-tRNA complex. This outcome benefits from the lower Michaelis constant (K_m) of TTS for tryptophan than that of IDO (18, 41–43). This may result not only in significantly increased preservation of intracellular tryptophan-tRNA complex for protein synthesis, but also in the reduction of cytotoxic tryptophan catabolites mediated by IDO. Indeed, our findings indicate that stimulation of TTS^{high} T cells with IDO-positive DC leads to more markedly decreased tryptophan catabolites kynurenines (5.7 μ M) than does stimulation of TTS^{low} T cells (14.9 μ M). Such stimulation was accompanied by substantially reduced apoptosis in TTS^{high} T cells as compared with that of TTS^{low} T cells. This supports the observations from previous studies that the tryptophan catabolites are cytotoxic to T cells (44). It is likely that by use of tryptophan, TTS has dual protective effects on T cells toward IDO-mediated cell suppression, which increases survival of inflammatory T cells in inflamed rheumatoid synovium.

The exact causal relationship between IDO and TTS is not entirely known. Previous studies have demonstrated that whereas IDO is mainly expressed in macrophages, DC, and placental trophoblasts (22), TTS is constitutively expressed in various types of

cells, but can be markedly up-regulated in cells such as T cells, fibroblasts, and monocytes (21, 45–48). Interestingly, both IDO and TTS can be induced by IFN- γ (21, 22). It is conceivable that cells expressing an increased level of TTS are protected in an environment in which increased IDO leads to cell suppression. Indeed, there is recent evidence indicating that TTS protects both DC and CD8⁺ T cells against IDO-mediated tryptophan deprivation and cytotoxic catabolites of tryptophan (23). Similar protective effects on host cells expressing high levels of TTS against IDO-mediated cell suppression have also been reported in host cells infected by pathogens (49). In our study, we showed that whereas synovial DC expressed high levels of active IDO, synovial, but not peripheral T cells from RA patients had substantially higher TTS expression. It appears that the protective mechanism of TTS toward the suppressive effect of IDO plays an important role in survival of pathogenic T cells in inflamed rheumatoid synovium.

It is intriguing to investigate how TTS is regulated in RA-SF T cells. Data from prior studies have demonstrated that IFN- γ can enhance the expression of TTS in T cells (46, 50). We found that RA patient-derived SF contained high levels of inflammatory cytokines IFN- γ and TNF- α that are responsible for up-regulating the expression of TTS in T cells. Although SF supernatant derived from RA patients increased expression of TTS in T cells from healthy donors, addition of neutralizing mAbs to IFN- γ and/or TNF- α , but not mAb to IL-18 or IL-10, markedly suppressed the expression of TTS in SF-treated T cells. Furthermore, because TTS was up-regulated in normal T cells in response to RA patient-derived SF, we suggest that the synovial environment of RA patients may be another major target for potential therapeutic intervention toward synovial inflammatory T cells in RA.

It is also of interest to understand that IFN- γ has dual functions in immune response. In rheumatoid synovium, although IFN- γ increases IDO in DC, it can also induce T cells to express high levels of TTS. Previous studies have demonstrated that IFN- γ is able to increase the expression of TTS in various types of cells as early as 6 h after exposure to IFN- γ (21, 51). Notably, treatment with IFN- γ for 18 h led to a 4-fold increase in the expression of TTS in human fibroblasts as opposed to that of control cells (45). These findings collectively suggest that TTS induction by IFN- γ can be mediated independently from de novo protein synthesis (21). Our results described in this study confirm that human T cells, upon treatment with IFN- γ , substantially up-regulate the expression of TTS within 24–48 h, which is in agreement with previous reports (21, 45). Thus, although IFN- γ can also increase IDO in DC, the superior ability of TTS to IDO in competing for tryptophan in IFN- γ -activated T cells (18, 41–43) results in an augmented intracellular reservoir of tryptophan for protein synthesis and in decreased cytotoxic catabolites mediated by IDO. Further investigation of molecular mechanisms by which IFN- γ increases the expression of TTS in inflammatory cells will be important for understanding the pathological role of synovial inflammatory T cells in RA.

In summary, our data provide evidence for a link between impaired T cell response toward tryptophan depletion and environmental autoreactivity in RA patients. We demonstrated that increased TTS expression in T cells allows resistance to IDO-mediated tryptophan deprivation, which may contribute to the persistence of autoreactive T cells in local tissues of patients with autoimmune disease. Given the importance of tryptophan metabolism in regulating T cell-mediated immune response, our findings provide new insight into the pathophysiology of synovial inflammation in RA patients and development of novel therapeutic approaches for RA.

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Disclosures

The authors have no financial conflict of interest.

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