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*J Immunol* 2003; 171:3947-3954; doi: 10.4049/jimmunol.171.8.3947

http://www.jimmunol.org/content/171/8/3947
Mediation of Enhanced Transcription of the IL-10 Gene in T Cells, Upon Contact with Human Glioma Cells, by Fas Signaling Through a Protein Kinase A-Independent Pathway

Bei-Chang Yang,1* Heng-Kai Lin,* Wei-Shio Hor,* Jun-Yen Hwang,* Yu-Ping Lin,* Ming-Yie Liu,† and Ying-Jan Wang†

Elevated expression of IL-10 has been frequently observed in tumor tissues and tumor-infiltrating cells. We show herein that transcription of the IL-10 gene in primary peripheral T cells and T cell lines is up-regulated upon contact with glioma cells without an induction of apoptosis in those T cells. Glioma-associated IL-10 induction was suppressed by interrupting the engagement of Fas and its ligand (Fas-L) with the antagonistic Ab, ZB4, by reducing Fas-L expression of glioma cells using the Fas-L-specific ribozyme, or by preventing cell-to-cell contact in a Transwell culture setting. Cross-linking of Fas with the agonistic Ab, CH-11, triggered apoptosis and enhanced the expression of IL-10 in Jurkat cells at the transcriptional and translational levels. Inhibiting caspase activities by caspase inhibitors, Z-VAD (Z-Val-Ala-Asp(Ome)-fluoromethylketone) and Z-IETD (Z-Ile-Glu(Ome)-Thr(Ome)-Asp(Ome)-fluoromethylketone), abolished this IL-10 induction in Jurkat cells. Intracellular staining detected IL-10 proteins in Fas-cross-linked Jurkat cells and in PHA-activated T cells. However, few IL-10 proteins were detectable in Jurkat cells cocultured with glioma cells, indicating a requirement of other factors for IL-10 production. Direct activation of protein kinase A (PKA) by forskolin elevated the transcription of IL-10 in Jurkat cells. However, KT5720, a selective PKA inhibitor, reduced neither anti-Fas-triggered nor glioma-associated IL-10 expression. Phosphorylation of cAMP response element binding protein and activating transcription factor-1 in Jurkat cells was not affected by coculturing with glioma cells or by anti-Fas treatment, further suggesting a PKA-independent pathway. In summary, our results demonstrate nonlethal cross-talk between tumor and immune cells leading to IL-10 dysregulation in T cells, which might contribute to Fas-L+ tumor-associated immunosuppression. The Journal of Immunology, 2003, 171: 3947–3954.

F as receptor (Fas, CD95, APO-1)-mediated apoptosis plays an important role in controlling cell numbers during embryonic development and immune homeostasis. Upon trimerization by its ligand (Fas-L),3 the Fas signal is propagated through a caspase cascade by activation of caspase-8, which results in apoptosis of Fas+ cells (1). However, Fas activation does not always bring about apoptosis. Resistance to Fas cross-linking has frequently been observed in malignant cells of diverse cellular origins, including breast, brain, colon, liver, and pancreas (2–6). Immune cells may also undergo Fas activation without cell death in physiological conditions. For instance, freshly isolated memory T cells, despite the presence of membrane Fas, are resistant to agonistic Fas Abs (7). Fas-L costimulates the proliferation of CD8+ T cells and resting T lymphocytes by augmenting IL-2 production (8, 9). Fas-mediated transcriptional activation of the IL-10 gene in monocytes was followed by the release of large amounts of cytokines and occurred in the absence of apoptosis (10). Moreover, apoptosis in lymphoid cells upon Fas activation led to rapid production of IL-10, which is probably responsible for the induction of immune deviation during Ag presentation (11). These results suggest that the Fas signal may be linked to the production of cytokines in immune cells that consequently shapes the immune response.

IL-10 is an anti-inflammatory cytokine and immunosuppressive factor, and it has been implicated in autoimmunity, tumorigenesis, and transplantation tolerance (12–14). An elevated level of IL-10 is commonly found in the serum of tumor patients (14, 15). An excessive amount of IL-10 might contribute to tumor-associated T cell anergy, reduced MHC-I expression, and antagonism of T cell expansion (16–18). Either tumors themselves or a variety of host cells in the tumor environment produce IL-10. Infection of normal human B cells with EBV leads to immortalization of these B cells and accompanying IL-10 production (19, 20). The expression of IL-10 in oral and oropharyngeal squamous cell carcinomas is positively correlated with the expression of Fas-L (21). Recently, uncharacterized glioma-generated soluble factors have been reported to suppress T cell responses to and enhance IL-10 expression by PBMC (22). Gliomas are common tumors in the CNS and express high levels of Fas-L (23, 24). These findings raise the questions of whether cross-talk between tumor and immune cells through the Fas system is connected to IL-10, and whether this is the way in which tumor cells evade immune surveillance.

1 This work was supported by grants from the National Science Council, Taiwan, Republic of China (NSC90-2320-B006-MB099) and from the Minister of Education, Republic of China (91-FA09-1-4, to B.-C.Y.).
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3 Abbreviations used in this paper: Fas-L, Fas ligand; AMC, 7-amino-4-methyl coumarin; ATF-1, activating transcription factor-1; CREB, cAMP response element binding protein; EGF/P-N1, enhanced green fluorescent protein-N1; PI, propidium iodide; PKA, protein kinase A; Z-IETD, (Z-Ile-Glu(Ome)-Thr(Ome)-Asp(Ome)-fluoromethylketone; Z-VAD, Z-Val-Ala-Asp(Ome)-fluoromethylketone.
In this paper we investigated the mechanism of glioma-associated IL-10 induction in T cells. We provide detailed evidence showing a role for the Fas/Fas-L system in cross-talk between glioma and T cells using an in vitro coculture system. Our data demonstrate that tumor Fas-L effectively induces transcriptional activity of the IL-10 gene in T cells without causing cell death. Furthermore, an association of the PKA pathway with the Fas signal in T cells is demonstrated.

Materials and Methods

Cell culture and treatments

Human glioma cell lines, U-373MG and U-118MG, were obtained from American Type Culture Collection (Manassas, VA). U373(R) and U118(R) expressing low levels of Fas-L were established from U-373MG and U-118MG, respectively, by transfection of a plasmid that codes for a Fas-L-specific ribozyme (Fas-L-ribozyme) as described previously (25, 26). Both U373(V) and U118(V) carry the enhanced green fluorescent protein-N1 (EGFP-N1) vector plasmid and served as the nonribozyme controls. Allogeneic human peripheral circulating T cells were isolated from venous blood obtained from healthy volunteers by Ficoll-Paque gradient centrifugation according to the manufacturer’s instructions (Amersham Pharmacia Biotech, Uppsala, Sweden). Harvested mononuclear cells were washed once with PBS and resuspended in RPMI 1640 medium. T cells in the suspension were isolated by running them through a nylon wool column. Usually, the T cell preparation obtained had a purity of about 80%, which was calculated by CD3 staining combined with flow cytometric analysis (using a CD3-specific Ab purchased from Calbiochem Laboratories, Burlingame, CA). The human T cell leukemia lines, Jurkat and Molt-4 (obtained from American Type Culture Collection), were cultured in RPMI 1640 medium (Life Technologies-BRL Life Technologies, Grand Island, NY) supplemented with 10% FBS and incubated at 37°C in a humidified atmosphere (5% CO2). Glioma cells were maintained in 10% FBS/DMEM (Life Technologies-BRL Life Technologies-BRL Life Technologies-BRL). The morphology of Jurkat cells harvested from coculture experiments was routinely checked under a microscope to ensure no contamination by glioma-derived cells. In coculture experiments using U118(V)/R or U373(V)/R, contamination of tumor cells in harvested Jurkat samples for RNA extraction could be ruled out, because they are EGFP positive and appear green under a fluorescence microscope. To demonstrate the requirement for cell-to-cell contact for IL-10 induction, glioma cells and T cells were cultured together in a chamber of Transwell plates or separated by insertion of a 0.4-μm pore size semipermeable membrane (six-well plate; Costar, Bodenheim, Germany). The engagement of Fas and Fas-L was interfered with by the mouse mAb, ZB4 (Upstate Biotechnology, Lake Placid, NY), at a working concentration of 125 ng/ml. Fas-mediated apoptosis and IL-10 induction in Jurkat cells were achieved using the agonistic Ab of Fas (CH-11; Upstate Biotechnology). The amount of CH-11-induced apoptosis in Jurkat cells was achieved by the general caspase inhibitor, Z-VAD (Z-Val-Ala-Asp(Ome)-fluoromethylketone; Calbiochem-Novabiochem), or by the caspase-8 specific inhibitor, II Z-IETD (Z-Ile-Glu(Ome)-Thr(Ome)-Asp(Ome)-fluoromethylketone; Calbiochem-Novabiochem) (29).

RT-PCR

Total RNA was prepared using the RNeasy kit according to the manufacturer’s instructions (Qiagen, Hilden, Germany). cDNA was generated using oligo(dT) as a primer and StrataScrp-H reverse transcriptase in the presence of RNasin (Stratagene, La Jolla, CA). RT-PCR was performed on cDNA transcribed from glioma RNA using primers specific for the IL-10 and β-actin genes as described previously (27). The cDNA template was subjected to PCR amplification in a DNA thermal cycler (Hybaid Omnigene, Middelflex, U.K.). β-Actin served as a quantitative control. PCR products were fractionated by agarose electrophoresis, stained with ethidium bromide, and visualized under UV light.

Intracellular staining of IL-10 protein

Intracellular IL-10 protein in T cells was detected as described previously (28). Cells were washed once in cold PBS, fixed in 4% paraformaldehyde, resuspended in PBS/0.1% PMSF, and then stored at −20°C for 24 h. Aliquots of stimulated and fixed cells were thawed and washed in PBS/BSA. Cells were permeabilized in PBS with 0.1% saponin, 0.1% BSA, 1 mM Ca2+, and 1 mM Mg2+ with 5% skim milk/106 cells for 1 h to block nonspecific binding. The R-PE-conjugated anti-IL-10 Ab (BD Pharmingen, San Diego, CA) was then added to samples at an optimal concentration according to the manufacturer’s instructions. Stained BJAB cells expressing a high level of IL-10 protein served as a staining control. Cells with arbitrary FL-2 values of >570 in flow cytometric analysis were considered IL-10 positive.

Immunoblotting

Cells (~5 × 106) were extracted with lysis buffer containing 0.5% Triton X-100, 50 mM Tris-Cl (pH 7.4), 25 mM KCl, 5 mM MgCl2, 0.15 M NaCl, 1 mM EGTA, 1 μg/ml aprotinin, and 1 mM PMSF. Proteins were separated with 10–15% SDS-PAGE and electrophoretically transferred onto a polyvinylidifluoride membrane (MSI, Westboro, MA). Subsequently, nonspecific binding sites were blocked with 5% skimmed milk and 0.1% Tween 20. Blots were probed with rabbit Abs specific for a FasL response element binding proteins (FLIPs), anti-CREBs (Upstate Biotechnology) or phosphorylated CREBs (anti-phospho-CREBs, which also cross-react with phosphorylated activating transcription factor-1 (ATF-1); Upstate Biotechnology), followed by peroxidase-linked Ig-specific secondary Abs (Dako, Carpentaria, CA). To detect caspase-8 protein, blots were probed with mouse mAb recognizing full-length and cleaved caspase-8 (567, p43/42, and p18; 1C11; Cell Signaling Technology, Beverly, MA). Immunoblots were made visible by fluorography with an ECL detection kit (Amersham Pharmacia Biotech). The amount of α-tubulin-specific Ab (NeoMarker, Fremont, CA) and served as a protein-loading control.

Measurement of activities of caspases

Jurkat cells were either treated with 10 ng/ml CH-11 or cocultured with glioma cells for the indicated time periods. Cells were then extracted with a buffer containing 25 mM HEPES, 5 mM MgCl2, 5 mM EDTA, 5 mM DTT, 2 mM PMSF, 10 μg/ml pepstatin A, 10 μg/ml leupeptin, and 2 mM PMSF. Caspase-3 (Ac-DEVD-7-amino-4-methyl coumarin (Ac-DEVD-AMC), caspase-8 (Ac-IETD-AMC), and caspase-9 (Ac-LeHD-AMC) levels were quantitatively measured using the fluorometric CaspACE assay system (Biovision, Mountain View, CA). The labeled fluorochrome AMC is released from the substrate upon cleavage by caspase. Free AMC, its amount reflecting the amount of caspase activity, produces a yellow-green fluorescence that is monitored with a fluorometer (with excitation at 360 nm and emission at 460 nm).

Results

Contact with glioma cells induced the expression of IL-10 in Jurkat and peripheral circulating T cells

Jurkat and Molt-4 T cells and T cells isolated from peripheral blood had low basal levels of IL-10 transcripts. The transcriptional activity of the IL-10 gene in those T cells was significantly augmented by coculture with the glioma cell lines, U-118MG and U-373MG (Fig. 1, lanes 2 and 3, respectively). The transcriptional activity of the IL-10 gene in primary T cells was also augmented by coculture with glioma cells. U-118MG cells were relatively more potent in inducing IL-10 in Jurkat cells, Molt-4 cells, and peripheral primary T cells than were U-373MG cells. When a semipermeable membrane separated Jurkat cells from U-118MG in a Transwell plate, the induction of IL-10 was diminished (Fig. 1B). Moreover, there was no significant IL-10 induction in Jurkat cells seeded in the upper chamber of a coculture set in the Transwell plate in which Jurkat cells and U-118MG were being grown together in the lower chamber; our intention was to determine whether a potential soluble factor(s) mediating IL-10 induction was transferred from the glioma cells to the T cells.
FIGURE 1. Enhancement of the expression of IL-10 in T cell lines upon contact with glioma cells. A, Jurkat cells; Molt-4 T cells; or T cells isolated from peripheral blood were cultured alone (lane 1) or in the presence of U-118MG (lane 2) or U-373MG (lane 3) for 24 h. Transcripts of IL-10 and β-actin genes were determined by RT-PCR. B, Jurkat and glioma U-118MG cells were either cultured together in a chamber or separated by a 0.4-μm pore size membrane insert for 24 h. Lane 1, Jurkat cells alone; lane 2, Jurkat cells in the upper chamber and U-118MG in the lower chamber; lane 3, Jurkat cells in the upper chamber and Jurkat cells plus U-118MG in the lower chamber (in this set, Jurkat cells in the upper chamber were analyzed); lane 4, Jurkat cells cocultured with U-118MG.

Enhanced transcription of the IL-10 gene in Jurkat cells is mediated through the Fas signal and caspase activation, but is not dissociated with apoptosis.

Approximately fifty to 80% of Jurkat cells became apoptotic within 24 h after co-culture with glioma cells and increased gradually with time (Fig. 2).

Enhanced transcription of the IL-10 gene in Jurkat cells is mediated through the Fas signal and caspase activation, but is not dissociated with apoptosis.

The finding that Fas cross-linking induces the expression of IL-10 in murine lymphoid cells (10, 11) prompted us to investigate whether a similar pathway operates in Jurkat cells. The transcription of IL-10 in Jurkat cells was induced by CH-11 in a dose-dependent manner (Fig. 4). In addition, the extent of IL-10 expression closely paralleled the formation of apoptotic cells. By taking advantage of the fact that U118(R) and U373(R) express reduced levels of Fas-L (25), we used those cells to explore the effect of tumor Fas-L on glioma-associated IL-10 induction. Fig. 5 shows that U118(V) and U373(V), expressing higher levels of Fas-L, significantly induced the transcription of IL-10 in Jurkat cells (Fig. 5A, lanes 2 and 4). However, U118(R) and U373(R), expressing lower levels of Fas-L, did not stimulate IL-10 in Jurkat cells (Fig. 5A, lanes 3 and 5). Importantly, blocking the Fas/Fas-L engagement by a 1.5-h preincubation of Jurkat cells with ZB4 completely diminished glioma-associated IL-10 induction (Fig. 5B). This finding is consistent with the results obtained from coculture experiments using Fas-L-ribozyme-carrying cells and establishes the requirement of the Fas/Fas-L interaction for IL-10 induction in T cells upon contact with glioma cells.

Human peripheral T cells expressed a small amount of IL-10 transcripts, which could be stimulated by PHA treatment (Fig. 5C). CH-11 treatment did not affect the transcription of IL-10 in peripheral T cells, either fresh or PHA-treated ones (compare lanes 1 and 2 in Fig. 5C). A 24-h co-culture with glioma cells stimulated transcription of the IL-10 gene in fresh T cells, which was more significant in coculture with glioma cells with high Fas-L levels (compare lanes 3 and 4 in Fig. 5C for U118(V) vs U118(R), respectively). PHA-activated T cells in coculture with U118(V) had a slightly higher amount of Fas-L transcripts than those with U118(R).

We further evaluated the caspase activity of Jurkat cells in coculture with glioma cells by focusing on caspase-3, caspase-8, and caspase-9. The finding that Fas cross-linking induces the expression of IL-10 in murine lymphoid cells (10, 11) prompted us to investigate whether a similar pathway operates in Jurkat cells. The transcription of IL-10 in Jurkat cells was induced by CH-11 in a dose-dependent manner (Fig. 4). In addition, the extent of IL-10 expression closely paralleled the formation of apoptotic cells. By taking advantage of the fact that U118(R) and U373(R) express reduced levels of Fas-L (25), we used those cells to explore the effect of tumor Fas-L on glioma-associated IL-10 induction. Fig. 5 shows that U118(V) and U373(V), expressing higher levels of Fas-L, significantly induced the transcription of IL-10 in Jurkat cells (Fig. 5A, lanes 2 and 4). However, U118(R) and U373(R), expressing lower levels of Fas-L, did not stimulate IL-10 in Jurkat cells (Fig. 5A, lanes 3 and 5). Importantly, blocking the Fas/Fas-L engagement by a 1.5-h preincubation of Jurkat cells with ZB4 completely diminished glioma-associated IL-10 induction (Fig. 5B). This finding is consistent with the results obtained from coculture experiments using Fas-L-ribozyme-carrying cells and establishes the requirement of the Fas/Fas-L interaction for IL-10 induction in T cells upon contact with glioma cells.
caspase-9, which are major protease components in the cascade of cellular responses to the Fas signal. Induction of apoptosis by CH-11 activated the activities of caspase-3, caspase-8, and caspase-9 in Jurkat cells (Fig. 6, A–C). However, only caspase-8, not caspase-3 or caspase-9, were slightly activated in Jurkat cells after coculture with glioma cells for 4 h. Moreover, Western blot analysis of Jurkat cells in coculture with glioma cells did not detect cleaved intermediate p43/41 and the active subunit p18 of caspase-8 (Fig. 6D). Activation of the caspase cascade is responsible for many cellular phenotypes observed during the course of Fas-mediated apoptosis (30, 31). Probably due to a detection limit but was suppressed by dexamethasone (Fig. 9, lane 2). Cyclosporin A had no effect on the expression of IL-10. A surrogate approach to evaluate the PKA-associated pathway is to assess the phosphorylation of CREB family members, CREB and ATF-1, transcription factors belonging to the CREB family, become phosphorylated upon PKA activation and bind to CRE. In this study we analyzed the phosphorylation of CREB and ATF-1 in Jurkat cells by immunoblotting (Fig. 10). Forskolin could potently induce phosphorylation of CREB and ATF-1 in Jurkat cells in 30 min, while the application of KT5720 (2 μM) reduced it. In contrast, neither cross-linking of Fas by CH-11 nor coculture with glioma cells stimulated the phosphorylation of CREB or ATF-1 in Jurkat cells.

To exclude the glioma-associated IL-10 induction through a possible CREB-independent pathway, which is, however, still mediated by PKA activity, we measured the IL-10 transcripts in PKA pathway and induction of IL-10

There is a putative cAMP response element (CRE) located at −420 bp of the IL-10 promoter region (32). In addition, the expression of IL-10 in T cells and monocytes can be induced by activation of PKA with forskolin, a cAMP agonist (33–35). Thus, we examined the role of the PKA pathway in this glioma-Fas signal-associated induction of IL-10. The basal transcription of IL-10 in Jurkat cells was stimulated by forskolin (Fig. 9, lane 3), but was suppressed by dexamethasone (Fig. 9, lane 2). Cyclo-

Detection of intracellular IL-10 protein in Jurkat and peripheral circulating T cells

Peripheral T cells had few IL-10 proteins that were not stimulated by CH-11. The amount of intracellular IL-10 protein in fresh T cells was elevated upon coculture with glioma cells. PHA stimulated the expression of IL-10 protein in peripheral T cells. Neither treatment with CH-11 nor coculture with glioma cells affected the level of IL-10 protein in PHA-activated T cells (Fig. 8B). CH-11 dose-dependently elevated the intracellular IL-10 protein level in Jurkat cells (Fig. 8C). Nevertheless, coculture with glioma cells did not enhance the amount of IL-10 protein measured by intracellular staining (Fig. 8C), although the transcription of IL-10 in those cells was stimulated (compare Fig. 5A).

PKA pathway and induction of IL-10

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**FIGURE 4.** Fas cross-linking enhancement of the expression of IL-10 in Jurkat cells. To induce the IL-10 gene, Jurkat cells were treated with CH-11 at 0, 0.1, 0.5, or 1 ng/ml (lanes 1, 2, 3, and 4, respectively) for 24 h. RT-PCR was used to detect the transcripts of IL-10 and β-actin. Apoptotic cells were detected by PI staining. The percentage of the sub-G₀ cell population shown in the lower panel was deduced from a representative experiment.

**FIGURE 5.** Association of the expression of Fas-L in glioma cells with induction of IL-10 in T cells. A, Jurkat and Molt-4 cells were cultured alone (lane 1) or in the presence of U118(V), U118(R), U373(V), or U373(R) (lanes 2, 3, 4, and 5, respectively) for 24 h. B, The Fas/Fas-L interaction was interrupted by the antagonistic Ab, ZB-4. Jurkat cells were cultured alone (lanes 1, 2, and 3) or cocultured with U-118MG (lanes 4, 5, and 6) in the presence of ZB4 (at 125 ng/ml for lanes 2 and 4 and at 200 ng/ml for lanes 3 and 5) or mouse isotype IgG1 control (125 ng/ml; lane 6). RT-PCR was used to analyze the transcripts for IL-10 and β-actin genes in Jurkat cells.

**FIGURE 6.** A, Simultaneously, Z-VAD at concentrations of 8 or 40 μM effectively inhibited CH-11-triggered apoptosis in Jurkat cells to lower panel

**FIGURE 7.** A, Activation of the caspase cascade is responsible for many cellular phenotypes observed during the course of Fas-mediated apoptosis (30, 31). Probably due to a detection limit but was suppressed by dexamethasone (Fig. 9, lane 2). Cyclosporin A had no effect on the expression of IL-10. A surrogate approach to evaluate the PKA-associated pathway is to assess the phosphorylation of CREB family members, CREB and ATF-1, transcription factors belonging to the CREB family, become phosphorylated upon PKA activation and bind to CRE. In this study we analyzed the phosphorylation of CREB and ATF-1 in Jurkat cells by immunoblotting (Fig. 10). Forskolin could potently induce phosphorylation of CREB and ATF-1 in Jurkat cells in 30 min, while the application of KT5720 (2 μM) reduced it. In contrast, neither cross-linking of Fas by CH-11 nor coculture with glioma cells stimulated the phosphorylation of CREB or ATF-1 in Jurkat cells.

To exclude the glioma-associated IL-10 induction through a possible CREB-independent pathway, which is, however, still mediated by PKA activity, we measured the IL-10 transcripts in PKA pathway and induction of IL-10

**FIGURE 8.** A, IL-10 induction is prevented when the application of KT5720 (2 μM) reduced it. In contrast, neither cross-linking of Fas by CH-11 nor coculture with glioma cells stimulated the phosphorylation of CREB or ATF-1 in Jurkat cells.

To exclude the glioma-associated IL-10 induction through a possible CREB-independent pathway, which is, however, still mediated by PKA activity, we measured the IL-10 transcripts in PHA-activated T cells (Fig. 11). Pretreatment with KT5720 for 1 h completely prevented the transcription of IL-10 in Jurkat cells activated by forskolin. In contrast, application of KT5720 did not alter the induction of IL-10 in Jurkat cells upon coculture with glioma cells.

**Discussion**

Based on several lines of evidence, we conclude that the Fas signal mediates the transcription of IL-10 in Jurkat cells upon contact with glioma cells. First, IL-10 induction is prevented when the
Fas/Fas-L interaction is blocked by various treatments, including interruption of the Fas/Fas-L interaction with the antagonistic Ab, ZB4, reduction of Fas-L expression in glioma cells using Fas-Lribozyme, or avoidance of cell-to-cell contact in a Transwell system. Second, direct stimulation of the Fas signal by the agonistic Ab, CH-11, led to IL-10 production in Jurkat cells. In addition, IL-10 transcripts and intracellular IL-10 protein could be detected in PHA-activated T cells and in Jurkat cells upon the cross-linking of Fas by CH-11. Glioma cells could also stimulate allogenic primary T cells to express IL-10 mRNA and protein. Our results partially agree with those of a previous report that Fas-mediated apoptosis of lymphoid cells leads to rapid production of IL-10 in those cells (21) and provides direct evidence for the effect of tumor Fas-L on IL-10 production. There are some distinct features of our findings. Although Zou and co-workers (24) reported that soluble glioma-generated factors stimulate the IL-10 expression of PB-MCs, our data do not support such factors for T cell lines. Glioma cells express abundant matrix metalloproteinases that cause shedding of membrane proteins, including Fas-L (Ref. 36 and our unpublished observations). When it is retained on matrix substances, soluble Fas-L can trigger the Fas signal as effectively as does membrane Fas-L (37). The reported glioma-generated soluble factors exert a significant IL-10 stimulatory effect only when they have been concentrated by 20-fold (24). These findings corroborate the possibility that the minute effect of soluble Fas-L produced by glioma cells is amplified after extensive purification processes, either by the elevated concentration or by chemical/physical association with matrices. In addition, the divergent molecular weights observed for the reported glioma-generated factors (24) point toward the existence of a heterogeneous complex of soluble Fas-L and matrices. In our coculture setting, amplification of soluble Fas-L did not occur, and we saw no effective factors secreted by glioma cells. Moreover, IL-10 induction in Jurkat cells required direct cell-to-cell contact with glioma cells, which further excludes...
A

Representative histograms for peripheral T cells

B

T cells coculture for 24 h

C

Jurkat coculture for 24 h
RT-PCR was used to analyze the transcripts of IL-10 and Fas-L for 24 h. Intracellular IL-10 was stained with an Ab specific for human IL-10 protein and analyzed by flow cytometry.

**FIGURE 8.** Intracellular staining of IL-10. Jurkat cells or peripheral T cells were either treated with CH-11 or cocultured with U-118MG-derived cells. Expression of IL-10 in primary T cells was significantly augmented by coculture with glioma cells. It is noteworthy that coculture with Fas-L− tumor cells did not stimulate the transcription of IL-10, but enhanced the level of intracellular IL-10 protein in primary T cells. Apparently a mechanism exists that leads to enhanced translation of IL-10 protein or delayed protein degradation in primary T cells. These results together suggest that different levels, transcriptional or translational, can control the IL-10 gene of T cells in sophisticated ways.

Fas-L− glioma cells were not cytotoxic to Jurkat cells in coculture. This is a surprising finding, because Jurkat cells are commonly used to show the cytotoxic effect of several tumors expressing ectopic or endogenous Fas-L. When we incorporated additional agonistic Fas Abs in coculture experiments, few Jurkat cells became apoptotic as long as glioma cells were present (data not shown). Signaling through MHC class II molecules blocks Fas-induced apoptosis in B cells by directly inhibiting caspase-8 activation (38). On the other hand, elevation of cAMP protects some cells against apoptosis, not only from Fas-induced morphological changes and dephosphorylation, but also from functional deterioration (39). PKA is required for caspase-3/p21 complex formation, which may lead to resistance against Fas-mediated cell death (8). However, Jurkat cells in coculture with glioma cells showed little phosphorylation of CREB and ATF-1, and served as a positive control. The expression of IL-10 in Jurkat cells either treated with CH-11 (10 or 100 ng/ml) or cocultured with U-118MG-derived cells, represented IL-10-positive cells. The expression of Fas-L in modulating immunity by driving T cells to express IL-10 without killing them. Thus, understanding the cross-talk between tumors and immune cells through the Fas/Fas-L system may provide insight into the complicated pathophysiology of tumor microenvironments. It will be important to determine the nonapoptotic pathway of Fas, which provides targets to switch off tumor Fas-L-associated immunosuppression.

**FIGURE 9.** Induction of the IL-10 gene by PKA agonists. Jurkat cells were treated with dexamethasone (10 μM; lane 2), forskolin (50 μM; lane 3), or cyclosporin A (5 μM; lane 4) or were mock-treated (lane 1) for 24 h. RT-PCR was used to analyze the transcripts of IL-10 and β-actin genes.

**FIGURE 10.** Phosphorylation of CREB/ATF-1 in Jurkat cells. Jurkat cells were cocultured with U-118MG or were treated with CH-11 (1 ng/ml) for 2 h in the presence or the absence of the PKA inhibitor, KT5720 (2 μM). Proteins of treated Jurkat cells were extracted and subjected to Western blot analysis on CREB, pCREB, and pATF-1. Jurkat cells treated with forskolin at a concentration of 50 μM for 30 min showed an increase in the phosphorylation of CREB and ATF-1 and served as a positive control.

A major contribution of soluble factors in an autocrine or paracrine manner.

Although coculture with glioma cells stimulated transcription of the IL-10 gene in Jurkat cells, few IL-10 proteins were detected. Furthermore, we detected no IL-10 protein in the medium of the coculture sets using glioma and Jurkat cells by ELISA (data not shown). The lack of detectable IL-10 protein in the culture medium may be due to the blocked secretion of newly synthesized IL-10. We speculate that intracellular IL-10 will be released to the environment when those tumor-infiltrating T cells die. Alternatively, the secreted IL-10 was too low and beyond the sensitivity of the methodology, which is consistent with the idea that IL-10 acts at the secreted IL-10 was too low and beyond the sensitivity of the methodology, which is consistent with the idea that IL-10 acts at

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FIGURE 11. There were no changes in glioma-associated induction of IL-10 with a PKA inhibitor. Jurkat cells were cocultured with U-118MG or were treated with CH-11 (1 ng/ml) for 24 h in the presence or the absence of KT5720 (2 μM) and then harvested for RNA isolation. Transcripts of IL-10 and β-actin genes were analyzed by RT-PCR. The bands of IL-10 and β-actin PCR products were quantitated by a densitometer. IL-10/β-actin, The ratio of IL-10 over β-actin represents the relative transcription of IL-10 in treated Jurkat cells.