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Human Glioma-Induced Immunosuppression Involves Soluble Factor(s) That Alters Monocyte Cytokine Profile and Surface Markers[1]

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Patients with gliomas exhibit deficient in vitro and in vivo T cell immune activity, and human glioblastoma culture supernatants (GCS) inhibit in vitro T lymphocyte responses. Because APC are essential for initiating and regulating T cell responses, we investigated whether GCS would affect cytokines produced by monocytes and T cells from healthy donors of PBMC. Incubation of PBMC with GCS decreased production of IL-12, IFN-γ, and TNF-α, and increased production of IL-6 and IL-10. The GCS-induced changes in IL-12 and IL-10 occurred in monocytes, and involved changes in IL-12 p40 and IL-10 mRNA expression. Incubation with GCS also resulted in reduced expression of MHC class II and of CD80/86 costimulatory molecules on monocytes. The immunosuppressive effects were not the result of IL-6 or TGF-β1 that was detected in GCS. However, it was due to a factor(s) that is resistant to pH extremes, differentially susceptible to temperature, susceptible to trypsin, and has a minimum molecular mass of 40 kDa. Our findings show that glioblastoma-generated factors that are known to suppress T cell responses alter the cytokine profiles of monocytic APC that, in turn, inhibit T cell function. This model indicates that monocytes can serve as an intermediate between tumor-generated immune-suppressive factors and the T cell responses that are suppressed in gliomas. ***The Journal of Immunology,*** **1999, 162: 4882–4892.**

It has long been recognized that an impaired cellular immune response is a characteristic of many tumors in both animal models and human patients (1, 2). This diminished cellular immunity is not necessarily limited to reactivity against tumor-specific Ags, but can include unresponsiveness to nontumor Ags and T cell mitogens (3, 4). Cytokine dysfunction appears to contribute to tumor-associated immune dysregulation, with decreases of in vitro IL-2 and/or IFN-γ production and increases in IL-4, IL-5, IL-6, and/or IL-10 production. Human tumors in which one or more of these cytokine changes have been reported include Hodgkin's lymphomas (4), cervical (5, 6) and ovarian carcinomas (7), melanomas (8), basal and squamous cell carcinomas (9), renal cell carcinomas (10), non-small cell lung cancer (11), and gliomas (12). Tumor-associated immune dysregulation can also be reflected in T cells at the level of signal transduction, as defects in STAT5 have been reported in a murine breast tumor model (13), and Janus kinase 3 (Jak3) expression is down-regulated by a soluble factor from a human renal cell carcinoma (14).

Human gliomas provide an interesting example of tumor-associated immune dysfunction. The in vitro responses of T cells from patients who present with primary gliomas are impaired in their ability to respond in vitro to Ags and T cell mitogens by proliferation and IL-2 production (3, 15, 16). Surgical removal of the primary tumor can result in restoration of systemic in vitro responses to T cell mitogens, which again declines with recurrence of the tumor (17). Glioma patients also frequently fail to elicit delayed skin reactions (15), and patients' T cells express reduced numbers of high affinity IL-2R (18, 19). One of our laboratories recently reported that T cells from glioma patients exhibit defects in tyrosine phosphorylation of several proteins, reduced levels of phospholipase Cγ1 and p56^{lck}, as well as reduced mobilization of calcium (20). Other studies demonstrated that cultures of glioblastoma cell lines produce a factor(s) that inhibits Ag- and mitogen-stimulated proliferation and IL-2 production by T cells from healthy individuals (21, 22). These findings suggest that one or more factors contained in glioma culture supernatant (GCS)[3] exerts immunoregulatory effects on systemic cellular immunity, as well as at the site of the primary tumor.

Although Th cell proliferation and IL-2 production have been demonstrated to be defective in glioma patients and in cultures of PBMC exposed to GCS (21), the possibility that these T cell defects have their origin in APC has not been addressed. The production of IL-12 and/or IL-10 and the stimulatory and costimulatory molecules that are important for T cell activation are altered in certain infectious diseases such as leprosy (23) and AIDS (24, 25). Increased IL-10 production and mRNA expression have also been reported in several tumors including gliomas (5, 8–11, 26–29), and IL-12 has been used to inhibit the growth of murine

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[3] Abbreviations used in this paper: GCS, glioblastoma culture supernatant; CASTA, *Candida albicans* Ag; CGRP, calcitonin gene-related peptide; CNS, central nervous system; EIA, enzyme immunoassay; FLU, influenza A virus; SAC, *Staphylococcus aureus* Cowan strain 1; TT, tetanus toxoid.

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tumors (30, 31). These findings raise the possibility that the depressed cellular immune condition associated with certain tumors contributes to their neoplastic disease and is linked to cytokine dysregulation (2).

In the present study, we investigated whether exposure of healthy human blood donors' PBMC and monocytes to GCS would result in decreased IL-12 and increased IL-10 production and mRNA expression. Because MHC class II and CD80/86 expression have been shown to be down-regulated by IL-10 in human and murine in vitro models (32, 33), we also tested for reduced expression of MHC class II and CD80/86. Finally, we tested whether in vitro Th response would be generated by a mixture of autologous T cells and GCS-exposed monocytes. Our results indicate that GCS induces down-regulation of IL-12, MHC class II, and CD80/86, and concomitant up-regulation of IL-10 in monocytes. We also observed that proliferative responses to recall Ags were abrogated when monocytes were exposed to GCS before mixing with autologous T cells. These findings suggest that the defects seen in the Th of patients with gliomas originated as defective APC function that, in turn, resulted in aberrant signaling of T cells and subsequent down-regulation of IL-2 and IFN-γ production and gene expression. No single cytokine that we detected in GCS has been demonstrated to induce all of these changes in monocytes. The GCS activity was attributed to a factor(s) that is resistant to pH extremes, differentially susceptible to temperature, susceptible to trypsin, with a minimum molecular mass of approximately 40 kDa.

Materials and Methods

Isolation of PBMC

Samples of whole blood were provided for in vitro laboratory studies by the Transfusion Medicine Department (National Institutes of Health, Bethesda, MD), under a National Institute of Health Institutional Review Board-approved protocol. The PBMC were separated on Lymphocyte Separation Media (Organon Teknika, Rockville, MD), and resuspended at 1.5×10^6 cells/ml in RPMI 1640 (Life Technologies, Rockville, MD), supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 5 μM HEPES buffer, and 2 μM glutamine (National Institutes of Health Media Unit, Bethesda, MD), and 5% human AB^+ serum (Sigma, St. Louis, MO).

Production of GCS from different glioblastoma cell lines

The SNB-19 and U251 glioma cell lines were used for production of GCS. Two independently carried SNB-19 lines were studied: Both originated from Dr. Paul Kornblith (University of Pittsburgh, Pittsburgh, PA). One has been carried for several years by the University of Kentucky laboratory (Lexington, KY); the other was recently obtained as a cryopreserved sample from American Type Culture Collection (ATCC, Manassas, VA). The U251 line (34) is carried in National Cancer Institute laboratory. These cells were maintained in RPMI 1640 culture media containing 5% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 μM HEPES buffer in a humidified, 37°C, 5% CO_2 incubator. Cells were passaged at 4- to 7-day intervals using 0.25% trypsin (Life Technologies, Grand Island, NY) in PBS (pH 7.3–7.4). Supernatants were harvested from all of the glioblastoma lines after 4 to 7 days of culture in 5% FCS/RPMI 1640 medium. GCS was also generated by culturing the U251 cell line for 3 days in conditioned Cellgro Complete Serum Free Media (Mediatech, Herndon, VA) or FCS-free RPMI 1640 to obtain GCS samples for factor purification studies. Culture supernatants of these glioblastoma lines were tested directly for factor activity by production of IL-12, IL-10, IFN-γ, and T cell proliferation (see below). Supernatants from one of the SNB-19 cell lines grown to confluency were concentrated (38–89-fold) on a Minitan tangential flow concentrator using 100-kDa molecular mass cutoff, low protein binding, and regenerated cellulose filters to collect GCS. Following concentration, the GCS was filtered through 0.22-mm filters (Costar, Cambridge, MA) and stored at −80°C until needed. In most experiments, GCS preparations were used at a final 1/20 dilution. Supernatants from the other SNB-19 and the U251 cell lines were either concentrated 20–40-fold using a differential molecular mass cutoff Centricon Plus-80 Centrifugal Filter Device (Millipore, Bedford, MA), or were tested without concentration. Culture supernatants exhibited activity, irrespective of whether they had been concentrated. We verified that different preparations of GCS do not exert a toxic effect on PBMC cultured for 7 days.

As controls for the glioblastoma lines, we tested supernatants of ovarian carcinoma A2780, A2780/CP (35), and National Institute of Health-ovcar-3; the T2, U937, and K562 lymphoma lines; the prostate carcinoma PC-3 (National Institutes of Health-ovcar-3, T2, U973, K562, and PC-3 were obtained from ATCC); and two EBV-transformed lymphoma cell lines generated in our laboratory.

T cell function assay

Different preparations of GCS were tested for inhibition of T cell function by culturing 1.5×10^6 PBMC/ml, or 1×10^6 T cells/ml with 0.5×10^6 autologous monocytes/ml in 200 μl of culture media in 96-well flat-bottom culture plates (Costar) in a humidified, 37°C, 7% CO_2 incubator. The cultures were either unstimulated, or were stimulated with PHA-M (1/80 dilution) (Life Technologies) or a pool of recall Ags consisting of: influenza A virus (FLU) (A/Bangkok/RX173, H3N2) (final dilution of 1/800); tetanus toxoid (TT) (Connaught Laboratories, Swiftwater, PA) (final dilution of 1/800); and *Candida albicans* Ag (CASTA) (Greer Laboratories, Lenoir, NC) (10 μg/ml). The cultures were pulsed with [^{3}H]thymidine on day 2 for PHA and day 6 for recall Ags, harvested 20 h later using a Basic 96 Harvester (Skatron Instruments, Sterling, VA), and counted in a β-spectrometer (Wallac, Gaithersburg, MD).

Enrichment of monocytes and T cells

Enriched monocytes and T cells were obtained from elutriated lymphocyte-depleted and monocyte-depleted populations isolated from PBMC of healthy blood donors. Remixing experiments were performed using autologous depleted and enriched cell populations.

To obtain enriched monocytes, lymphocyte-depleted PBMC were incubated on ice for 30 min with an Ab mixture consisting of mouse anti-human CD3, CD16, and CD19 mAb (IgG) (PharMingen, San Diego, CA), at 5 μg of each mAb per 10×10^6 cells in 100 μl PBS containing 10% FBS (PBS/FBS). The cells were washed three times in PBS/FBS, the cell pellet was resuspended in PBS/FBS in the presence of Dynabeads M280 sheep anti-mouse IgG (Dynal, Oslo, Norway) (10 beads/cell), and the mixture was incubated on ice for 30 min. The cell-bead mixture was exposed to a magnet through three cycles of magnetic separation and washing of the unattached cells. This procedure resulted in enrichment of monocytes to greater than 90% $CD14^+$ cells, determined by flow cytometry. The enriched monocytes were tested by flow cytometry for the presence of $CD83^+$ cells, a marker of mature dendritic cells (36), and none were detected.

To obtain enriched T cells, monocyte-depleted cells were incubated with the Lympho-Quik-T Isolation Reagent (One Lambda, Canoga Park, CA), which depletes of all cell types except T cells by Ab-mediated, complement-dependent lysis (37).

Cytokine production and detection

The production of cytokines by PBMC, enriched monocytes, or monocytes plus autologous T cells was assessed by culturing 3×10^6 PBMC, 1×10^6 monocytes, or 1×10^6 monocytes plus 2×10^6 T cells in 2 ml of culture media in 24-well plates (Costar), respectively, in a humidified, 37°C, 7% CO_2 incubator. Cells were either unstimulated or were stimulated with *Staphylococcus aureus* Cowan strain 1 (SAC) (0.01%) (Pansorbin, Calbiochem-Behring, La Jolla, CA). Culture supernatants were harvested after 24 h and stored at −80°C.

The IL-12 p70 heterodimer production was assessed by ELISA from R&D (Minneapolis, MN). Total IL-12 p40, IL-2, IL-4, and IL-6 productions were detected by ELISA from Genzyme (Cambridge, MA). IL-10, IL-5, IFN-γ, and TNF-α productions were assessed in the supernatants of 24-h SAC-stimulated cultures, using PharMingen capture and detection Abs, as previously described (37). The limit for detection of these cytokines was in the range of 5–20 pg/ml.

Detection of IL-12 and IL-10 mRNA

Expression of hypoxanthine phosphoribosyltransferase (HPRT), IL-10, and IL-12 p40 mRNA was assessed on PBMC stimulated with SAC for 6 h, using a semiquantitative RT-PCR protocol, as previously described (37).

Detection of intracellular cytokines

PBMC were cultured for 6 h with or without stimulation in Teflon vials (Pierce Chemicals, Rockford, IL) in a 37°C, 7% CO_2 incubator; then Brefeldin A (Sigma) (5 μg/ml) was added for an additional 18 h of incubation. In some experiments, PBMC were cultured without or with 10 μg/ml Brefeldin A in 5% human AB^+ serum, RPMI 1640 medium for 1

day. Cell viability was tested by trypan blue exclusion or propidium iodide staining. The cells also were analyzed by FACS with anti-CD3 and CD14 staining. No differences were obtained in cell viability (>90%) in either the $CD3^+$ or $CD14^+$ populations in the presence or absence of Brefeldin A. Cells were harvested and washed in a staining buffer (PBS containing 1% FBS and 0.1% w/v sodium azide), preincubated with human IgG at 4°C for 30 min to block FcR. The cells were then stained at 4°C for 30 min with fluorochrome-conjugated mAb specific for a cell surface Ag such as CD14 and CD3 (PharMingen, San Diego, CA). The cells were subsequently washed with staining buffer, pelleted by centrifugation, and fixed in 500 μl of fixing buffer (4% w/v paraformaldehyde in PBS) at 4°C for 30 min or overnight. The cells were washed in the staining buffer, pelleted by centrifugation, and resuspended in 100 μl of permeabilization buffer (PBS containing 1% FBS, 0.1% w/v sodium azide, 0.1% w/v saponin). The cells were incubated for 30 min at 4°C with 0.5 μg fluorochrome-conjugated anti-cytokine Abs (anti-IL-12 p40 and p70, IL-10, IL-6, IFN-γ, and TNF-α from PharMingen). The cells were then washed twice in permeabilization buffer, resuspended in staining buffer, and analyzed by flow cytometry using a FACScan (Becton Dickinson, San Jose, CA). The cells were gated on monocytes or lymphocytes based on forward and side light scatter. In some experiments, the binding of fluorochrome-conjugated anti-cytokine mAb was blocked by preincubation of the conjugated mAb with excess recombinant cytokine (IL-12 p40 and IL-10; PharMingen).

Immunoprecipitation

To remove IL-6, TGF-β, and CGRP, GCS was diluted sevenfold in PBS. Anti-IL-6 (clone MQ2-13A5 rat IgG1, final concentration of 5 μg/ml), anti-TGF-β mAb (mouse IgG1, final concentration of 10 μg/ml), and rabbit anti-human CGRP serum (final 1/120 dilution) were added singly or in combination. The GCS and Ab mixture was incubated overnight at 4°C under rotating conditions. An excess of GammaBind G Sepharose (Pharmacia Biotech, Piscataway, NJ) was added for 10 h, and the mixture was centrifuged for 10 min at 2000 $\times$ *g*. An excess of protein A-Sepharose (Pharmacia) was added to the supernatant, which was incubated overnight under rotating conditions at 4°C. The mixture was centrifuged again, and the supernatant was sterilized by passing through a 0.22-μm filter, then tested for ability to suppress the Th function and induce the cytokine changes seen with the original GCS. We verified by specific ELISA or enzyme immunoassay (EIA) that the immunoprecipitation removed all detectable IL-6, TGF-β, and CGRP.

Ion exchange and gel filtration columns

The supernatants were harvested from the U251 glioblastoma line after 3 days of culture in conditioned FCS-free RPMI 1640 media. The GCS samples for factor purification studies were tested for binding to CM, Q, SP, and DEAE Sepharose Fast Flow Columns (Pharmacia Biotech). The unbound fraction and eluted fractions were tested for GCS activity. The bound fractions that contained GCS activity were fractionated on the Superdex 75 and Superdex 200 columns (Pharmacia Biotech). To determine the molecular mass of the active factor(s), 25–50 fractions were each tested for GCS activity by analysis of IL-12, IL-10, IFN-γ production, and PHA-stimulation response of PBMC, and compared with the unfractionated GCS, as described above.

Reagents

The additional following reagents were used in this study: anti-human IL-10 neutralizing mAb (clone JES 319 F11; DNAX, Palo Alto, CA); anti-human IL-10R mAb (clone 37607.11; R&D); anti-human IL-6 neutralizing mAb (clone MQ2-13A5; PharMingen); paraformaldehyde and saponin (Sigma); TGF-β1 human ELISA kit and PGE_2 EIA kit (Biotrak, Amersham, Arlington Heights, IL); Ultrapure natural TGF-β1, human rTGF-β2, and mouse monoclonal anti-human TGF-β1, TGF-β2, TGF-β3 neutralizing Ab (Genzyme); human CGRP, rabbit anti-human CGRP serum, and high sensitivity EIA kit (Pennisula Laboratories Europe, Belmont, CA); and insoluble trypsin (Sigma).

Results

Inhibition of T cell proliferative responses by GCS

To test whether the preparations of GCS generated by the SNB-19 glioblastoma cell lines inhibited in vitro T cell responses to a mitogen and recall Ags, PBMC from healthy individuals were stimulated with PHA (Fig. 1*A*) or with a mixture of FLU, TT, and CASTA (Fig. 1*B*) in the absence or presence of GCS. The results indicate that GCS inhibited proliferative responses to both stimuli

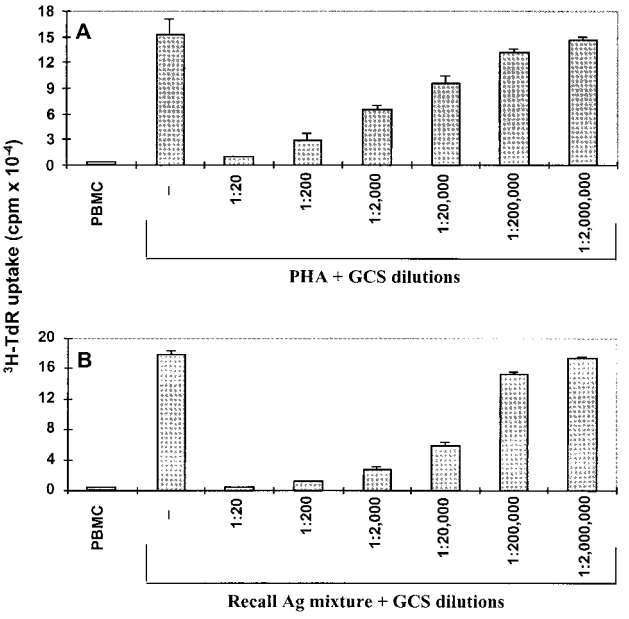

FIGURE 1. GCS inhibits PHA and recall Ag-induced proliferation of PBMC in a dose-dependent manner. Effect of different dilutions of GCS on T lymphocyte proliferative responses to PHA (*A*) and a pool of recall Ags (*B*) consisting of FLU, TT, and CASTA. The PHA-stimulated cultures were pulsed with [^{3}H]thymidine after 2 days, and the recall Ag-stimulated cultures were pulsed after 6 days of culture. Four experiments were performed with PBMC from four donors each (n = 3, cpm mean per minute ± SD), and the results shown are from one representative experiment.

in a dose-dependent manner at dilution ranging from 1/20 to 1/20,000. Therefore, the GCS produced by the tumor cell line strongly inhibited T lymphocyte responses to a T cell mitogen and to Th-dependent recall Ags that require intact APC function. As negative controls, we found that culture supernatants from three of seven tumor lines and the two laboratory-generated EBV-transformed cell lines did not inhibit T cell proliferation or induce changes in IL-12 and IL-10 production when added to PBMC (data not shown).

Effect of GCS on cytokine production

Because SAC is a strong stimulator of IL-12 and IL-10 production by monocytes, we tested different dilutions of GCS on SAC-stimulated IL-12 and IL-10 production in 24-h cultures of PBMC. The data in Fig. 2 demonstrate that GCS decreased IL-12 and increased IL-10 in a dose-dependent way.

To determine the kinetics of cytokine production, GCS was added to PBMC at the time of SAC stimulation, and the cultures were carried for 3, 6, 12, and 24 h. In addition to IL-12 and IL-10, we tested for other SAC-stimulated monokines and cytokines, including IFN-γ, IL-6, and TNF-α. PBMC were also preincubated with GCS for 1 h, the GCS was washed out, and the treated PBMC were stimulated with SAC for 3, 6, 12, and 24 h (Fig. 3). The kinetics of the response of control cultures, either unstimulated or stimulated but not incubated with GCS, was also followed. The production of IL-12 p70 and p40, as well as IFN-γ was greatly reduced by addition of GCS to SAC-stimulated PBMC (Fig. 3, *A*, *B*, and *D*). In contrast, GCS increased SAC-stimulated IL-10 production (Fig. 3*C*). IL-6 production was appreciably increased by GCS or SAC alone, and the combination of GCS and SAC induced an additional increase (Fig. 3*E*). SAC-stimulated TNF-α production was reduced approximately twofold by GCS (Fig. 3*F*). Data similar to those shown in Fig. 3 were obtained in six independent

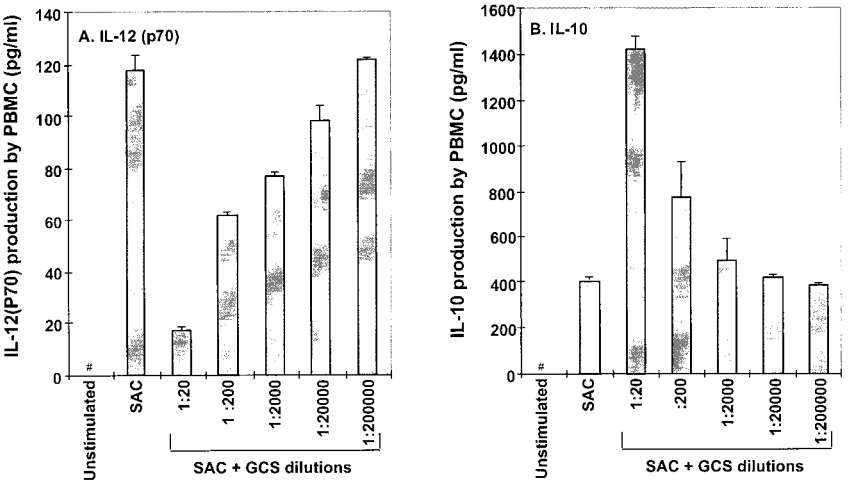

FIGURE 2. GCS affects SAC-stimulated IL-12 and IL-10 production by PBMC in a dose-dependent manner. Effect of 10-fold changes in concentration of GCS on IL-12 (*A*) and IL-10 (*B*) production by SAC-stimulated PBMC for 24 h. Production of cytokines was measured by ELISA. Two experiments were performed with PBMC from two donors each ($n = 2$), and the results shown are from one representative experiment. #, Below detectable level.

experiments. We also observed that 48-h cultures of GCS and SAC yielded results indistinguishable for the 24-h cultures (data not shown). These results indicate that GCS can rapidly induce a decrease in the production of IL-12 and IFN-γ and a concomitant increase in IL-6 and IL-10 production. The 1-h preincubation of PBMC with GCS before SAC stimulation induced changes in cytokine profiles that were similar to those observed when PBMC were exposed to GCS and SAC simultaneously.

Culture supernatants generated by the two SNB-19 glioblastoma lines as well as by the U251 glioblastoma line all induced decreased IL-12 and IFN-γ, and increased IL-10 production, and also abolished PHA-stimulated T cell proliferation (Table I). Culture supernatants from four of seven control tumor cell lines exhibited weak GCS-like activity (A2780, National Institute of Health-ovcar-3, T2, PC-3); as noted above, supernatants from the three other tumor lines did not show any GCS-like activity (A2780/CP, U937, K562).

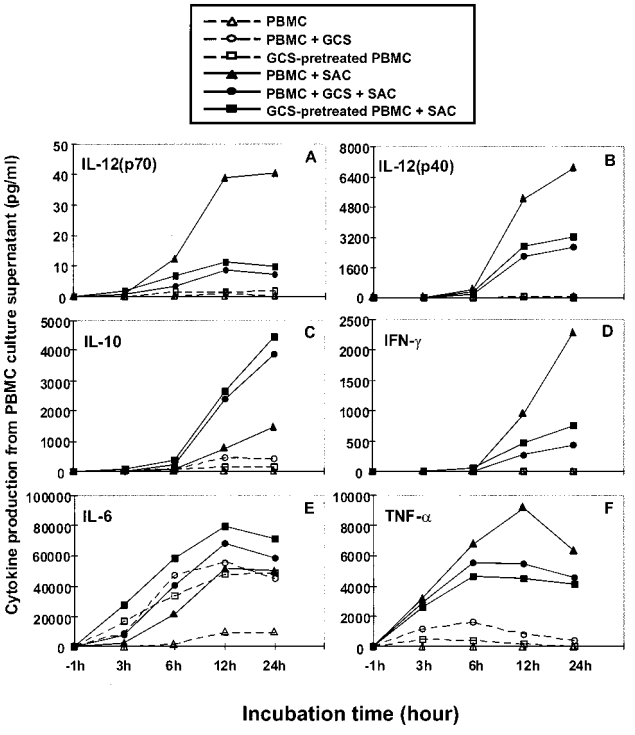

FIGURE 3. GCS can rapidly induce cytokine production changes in SAC-stimulated PBMC. Effect of SAC and/or GCS on the kinetics of production of the cytokines indicated in the panels. See key for details of materials contained in the cultures. −1 h (hour) indicates the group that was preincubated with GCS for 1 h before washout and stimulation with SAC. GCS were used as final 1/20 dilution. Two experiments were performed with PBMC from two donors each ($n = 2$), and the results shown are from one representative experiment.

The results of additional kinetic experiments in which GCS was preincubated with PBMC for different time intervals before SAC stimulation are summarized in Table II. Incubation of GCS with PBMC for as little as 3 min resulted in SAC-stimulated decreased IL-12 p40 and IFN-γ and increased IL-10 production. Preincubation with GCS for 1 h was as effective as maintaining GCS in the cultures with SAC for 24 h.

Immunoregulatory factors contained in GCS

To identify immunoregulatory factors that might be contained in GCS, we tested six different lots of supernatants collected from one of the SNB-19 glioblastoma cell lines that had been shown to inhibit in vitro T cell proliferation. The supernatants were found to contain IL-6, TGF-β1, CGRP (38), and very low levels (below 7 pg/ml) of PGE_2 (Table III), but not detectable levels of IL-4, IL-10, IL-12, TNF-α, or IFN-γ (data not shown). The levels of PGE_2 detected were below those reported to reduce IL-12 production by human PBMC or dendritic cells (39, 40). Based on these data, three types of experiments were performed to determine whether the changes in cytokine production induced by GCS could be attributed to any of these immunosuppressive factors.

First, we tested whether the addition of exogenous IL-6, TGF-β1, TGF-β2, or CGRP to PBMC, either singly or in combination, would decrease IL-12 and/or increase IL-10 production. We added IL-6 (10 ng/ml to 10 pg/ml), TGF-β1 or TGF-β2 (10 ng/ml to 10 pg/ml), or CGRP (1 μg to 10 pg) to SAC-stimulated PBMC, as well as the combination of IL-6 (5 ng/ml), TGF-β1 (1 ng/ml), TGF-β2 (1 ng/ml), and CGRP (50 ng/ml). Under none of the above conditions were changes detected in IL-12 or IL-10 production (data not shown). Second, treatment of GCS with neutralizing Abs against IL-6, CGRP, TGF-β1, TGF-β2, and TGF-β3 had no effect on its ability to decrease IL-12 and IFN-γ production and to increase IL-10 production by PBMC. Third, we immunoprecipitated IL-6, TGF-β, and CGRP, either singly or in combination, with the same Abs used for inhibition. The GCS treated in this way did not lose any of its ability to decrease IL-12 production. Only slight reductions were observed in ability to increase IL-10 and decrease IFN-γ by combination immunoprecipitation of IL-6 and

Table I. *Comparison of effects of culture supernatants from different glioblastoma lines on cytokine production and T cell proliferation*

Culture Conditions		Cytokine Production of SAC-Stimulated PBMC (pg/ml)			PHA-Stimulated PBMC [^{3}H]TdR uptake (cpm)
		IL-12 (p70)	IL-10	IFN-γ	
PBMC	No GCS	64.9	540.4	9649.0	63917.0
PBMC	SNB-19[b]	1.4	1337.2	483.4	2683.0
PBMC	SNB-19[c]	14.3	1724.8	3514.8	16321.2
PBMC	U251[d]	6.2	1497.5	2292.6	6549.8

[a] A representative experiment from 10 or more experiments performed.
[b] Concentrated SNB-19 supernatant from the University of Kentucky laboratory; final dilution of 1/20.
[c] Unconcentrated SNB-19 supernatant from the NCI laboratory; final dilution of 1/2.
[d] Unconcentrated U251 supernatant; final dilution of 1/2.

TGF-β (Table IV). Taken together, these results do not support the conclusion that the cytokine dysregulation induced by GCS is the result of IL-6, TGF-β, or CGRP, acting singly or in combination.

Effect of GCS on IL-12 p40 and IL-10 mRNA expression

To determine whether GCS also affected IL-12 and IL-10 mRNA, expression of IL-12 p40 and IL-10 mRNA was analyzed in PBMC that were incubated with SAC, GCS, and SAC + GCS. The results of one representative of four independent experiments are shown in Fig. 4 for IL-12 p40 and IL-10. Incubation with GCS resulted in a modest increase in IL-12 p40 mRNA, and in a large increase in IL-10 mRNA. As previously reported (25), SAC stimulation alone resulted in expression of IL-12 p40 and of IL-10 mRNA. Incubation with GCS + SAC increased expression of IL-10 message above SAC alone, and decreased IL-12 p40 expression compared with SAC alone. These data are in agreement with the regulation of IL-12 and IL-10 production by GCS.

GCS induced changes in monocyte production of IL-12 and IL-10

To determine whether monocytes contained in the PBMC were responsible for the production of IL-12 and IL-10, 24-h SAC-stimulated PBMC were gated for $CD14^+$ and $CD3^+$ cells and stained with anti-IL-12 and anti-IL-10 Abs. IL-12 and IL-10 were detected only in the $CD14^+$ population. PBMC incubated alone or with GCS, SAC, or SAC + GCS were used for isotype control staining (data not shown). The data presented in Fig. 5 illustrate the IL-12 and IL-10 intracellular staining patterns using the same conditions. Incubation of PBMC with GCS indicated that 3.4% of the monocytes stained for intracellular IL-10, but only 0.3% stained for IL-12. Stimulation of PBMC with SAC alone resulted in the staining of 8.1% for IL-10, 5.7% for IL-12, and 1.5% for both. The PBMC incubated with SAC + GCS resulted in a skewing toward IL-10-producing cells, as 13% stained for IL-10, 1.9% stained for IL-12, and 1.6% stained for both cytokines. Similar results were obtained in four repetitive experiments. In contrast to the cells gated for lymphocytes, no T cells were found that produced IL-10 or IL-12 after a 24-h incubation with GCS, SAC, or SAC + GCS (data not shown).

Effect of GCS on enriched SAC-stimulated monocytes

To determine whether incubation with GCS also affected IL-12 p70, p40, and IL-10 produced by enriched monocytes, the following experiments were performed. The monocytes were enriched from PBMC by elutriation, followed by negative selection of T, B, and NK cells. The enriched monocytes (shown >90% $CD14^+$, but undetectable <1% $CD3^+$, $CD19^+$, $CD1a^+$, and $CD16^+$) were stimulated with SAC for 24 h in the absence or presence of GCS. The results indicate that GCS abrogated IL-12 production (Fig. 6*A*) and increased IL-10 production (Fig. 6*B*). GCS also decreased SAC-stimulated IL-12 p40 production by twofold (data not shown). These results indicate that GCS can also affect IL-12 and IL-10 production by enriched monocytes in the absence of other cell types.

To determine whether T cells would affect SAC- and GCS-regulated monokine production, we added purified autologous T cells (>95% $CD3^+$) to SAC-stimulated monocytes. The T cells enhanced IL-12 fourfold, but addition of GCS greatly reduced IL-12 production (Fig. 6*A*). Addition of autologous T cells increased IL-10 production by twofold above monocytes alone, and addition of GCS further increased IL-10 production (Fig. 6*B*). We also assessed SAC-stimulated IFN-γ production in the same cultures (Fig. 6*C*), and demonstrated that without T cells, this cytokine was not produced. Addition of T cells resulted in IFN-γ production, which was reduced approximately fourfold by GCS.

To determine whether the observed GCS-induced changes in monokine production would be reflected in Th cell function, a mixture of autologous monocytes and T cells was stimulated with the recall Ag mixture of FLU, TT, and CASTA, and thymidine incorporation measured 6 days later. Similar to the data obtained for IL-12 and IL-10 production, addition of GCS to cultures of monocytes during Ag stimulation, or preincubation of monocytes

Table II. *Effect of incubation of PBMC with GCS for different time intervals on SAC-stimulated IL-12, IL-10, and IFN-γ production*

PBMC Were Preincubated in 37°C for 1 h	Content of 24 h Cultured	Cytokine Determination by ELISA (pg/ml)			
		IL-12 (p70)	IL-12 (p40)	IL-10	IFN-γ
PBMC	SAC	34.3 ± 8.6	4870 ± 476	932 ± 189	3327 ± 719
PBMC	SAC+GCS	↓[a] > 10[c]	↓3.4 ± 0.3	↑[b]2.2 ± 0.2	↓4.2 ± 0.5
Preincubation with GCS for 1 h	SAC	↓ > 10[c]	↓3.6 ± 0.2	↑3.5 ± 0.6	↓5.9 ± 1.1
Preincubation with GCS for 15–30 min	SAC	↓ > 10[c]	↓3.4 ± 0.3	↑3.0 ± 0.4	↓3.3 ± 0.5
Preincubation with GCS for 3–10 min	SAC	↓6.3 ± 1.1	↓1.8 ± 0.1	↑2.5 ± 0.2	↓2.8 ± 0.4

[a,b] The fold increase (↑) or fold decrease (↓) change from SAC-stimulated PBMC based on the mean of four independent experiments (SEM).
[c] Below detectable level. GCS was used at final dilution of 1/20 in the culture and preincubation system.

Table III. *Immunosuppressive molecules detected in six different preparations of undiluted GCS*[a]

GCS Lot.	Activity Estimate[b]	IL-6 (pg/ml)	TGF-β1 (pg/ml) (active form)	CGRP (pg/ml)	PGE_2 (pg/ml)
A	++++[c]	1367	ND	ND	ND
B	+	1689	706	5700	<7
C	++++	533	850	8300	<7
D	+	626	812	4600	0
E	+++	253	564	15500	<7
F	++++	1392	ND	27600	<7

[a] Cytokines that were not detected in GCS were IL-2, IL-4, IL-5, IL-10, IL-12, TNF-α, and IFN-γ.

[b] Activities of different GCS preparations were compared for modulation of IL-12, IL-10, and IFN-γ production by PBMC.

[c] ++++ and + indicate GCS preparations that had the greatest and least effects, respectively, on changing IL-12, IL-10, and IFN-γ.

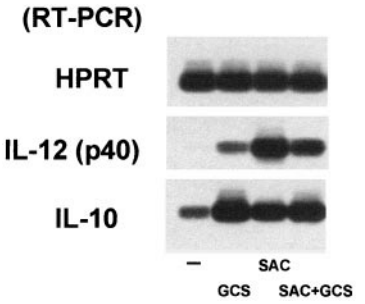

FIGURE 4. GCS up-regulated IL-10 and down-regulated IL-12 p40 mRNA expression. mRNA for hypoxanthine phosphoribosyltransferase (HPRT) (control), IL-12 p40, and IL-10 detected by reverse-transcriptase PCR. −, PBMC cultured alone. PBMC cultured for 6 h with GCS, SAC, or GCS + SAC. Four experiments were performed with PBMC from four donors, and the results shown are from one representative experiment.

with GCS for 1 h before mixing with T cells and Ag stimulation, abrogated the Ag-stimulated proliferative response (Fig. 7).

Effect of anti-IL-10, rIFN-γ, and anti-IL-10R on IL-12, IL-10, and IFN-γ production

Enriched monocytes were incubated with anti-IL-10 mAb (10 μg/ml) or with rIFN-γ (10 U/ml) during the 24-h incubation with GCS and/or SAC. SAC-stimulated IL-12 p40 production was appreciably increased by anti-IL-10 and even more by IFN-γ. In the combination, GCS partially decreased the enhancing effects of anti-IL-10 mAb and rIFN-γ on SAC-stimulated IL-12 p40 production (Fig. 8). Our finding that anti-IL-10 did not completely block the GCS-induced reduction of IL-12 p40 suggests that GCS affects IL-12 production by a partly IL-10-independent mechanism(s).

We also tested whether GCS would influence the cross-regulatory effect of IL-10 through the IL-10R (Fig. 9). Incubation of PBMC with anti-IL-10R raised IL-12 p70, p40, and IFN-γ production, but had no effect on IL-10 production. In the presence of anti-IL-10R mAb (20 μg/ml), GCS still partially reduced p70, p40, and IFN-γ production, confirming that the inhibitory effect of GCS on IL-12 production is not mediated exclusively via IL-10 production.

Effect of GCS on monocyte surface MHC class II and CD80/86 expression

To determine whether exposure to GCS would affect expression of MHC class II and CD80/86 (B7.1 and B7.2) expression, PBMC were preincubated for 1 h with GCS and then incubated with medium in the absence or presence of rIFN-γ for 24 h (Table V). The cultured cells were analyzed by flow cytometry by gating on $CD14^+$ population of monocytes to determine class I, class II, CD80, and CD86 expression. The results indicate that MHC class II, but not class I expression was reduced by approximately twofold. Expression of CD80 and CD86 was similarly reduced. Although rIFN-γ enhanced class I, class II, and CD80 expression on monocytes, GCS reduced the mean channel fluorescence increases by approximately twofold for class II and CD80, but not for class I.

IL-12 and IL-10 production by PBMC from primary glioma patients

The mitogen and Ag-specific responses of T cells obtained from patients with glioma are severely impaired (15). To determine whether PBMC from glioma patients would also exhibit changes in IL-12 and IL-10 production similar to that shown above using GCS in vitro, PBMC from primary glioma patients who had not received steroids or phenytoin and healthy control blood donors were stimulated with SAC to initiate IL-12 and IL-10 synthesis. The production of IL-10 was increased, whereas production of IL-12 p70 and p40 as well as IFN-γ were all decreased compared with controls, as shown for one patient in Fig. 10. Similar data were obtained using PBMC from three additional glioma patients. These preliminary results resemble the IL-12/IL-10 shifts generated in our in vitro model of GCS-exposed PBMC and monocytes from healthy blood donors.

Partial characterization of factor in GCS

Studies are in progress to isolate, identify, and purify the factor responsible for the biologic activity contained in GCS. To characterize the factor, GCS was generated using the U251 glioblastoma cultured in protein-free conditioned RPMI 1640 medium. We observed that heating GCS to 56°C for 30 min did not affect its ability to modulate cytokine production (IL-12, IL-10, or IFN-γ),

Table IV. *Removal of IL-6, TGF-β, and CGRP by immunoprecipitation had no effect on the ability of GCS to alter IL-12 and IL-10 cytokine profiles of PBMC*

PBMC	Culture Without or With Immunoprecipitated GCS[a] (final 1:40)	Cytokine Determination by ELISA (pg/ml)		
		IL-12 (p40)	IL-10	IFN-γ
Unstimulated	—	0	5	0
SAC	—	3372	1339	1120
SAC	GCS (none Ab)	1263	3104	78
SAC	GCS (anti-IL-6)	1283	2920	73
SAC	GCS (anti-TGF-β)	1092	3072	137
SAC	GCS (anti-CGRP)	1075	3017	98
SAC	GCS (anti-IL-6 and TGF-β)	1256	2678	225
SAC	GCS (anti-IL-6, TGF-β, and CGRP)	1296	2496	209

[a] The experimental procedure is shown in *Materials and Methods*.

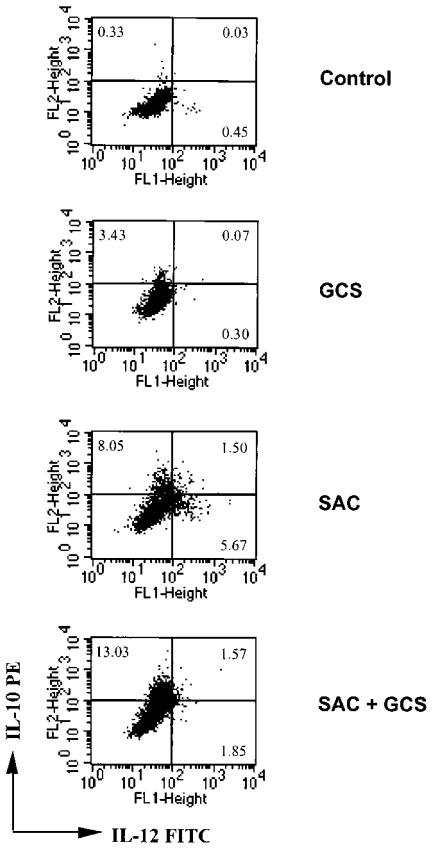

FIGURE 5. GCS increased IL-10-producing monocytes and decreased IL-12-producing monocytes. Two-color cytometric analysis of intracellular staining of monocytes for IL-12 and IL-10. PBMC were gated for monocytes and stained with anti-IL-12 FITC and anti-IL-10 phycoerythrin. PBMC were incubated alone (control) or with GCS, SAC, or SAC + GCS. Four experiments were performed with PBMC from different donors, and the results shown are from one representative experiment. Statistical analysis of the four experiments indicates IL-12-producing cells (mean ± STD, %) in unstimulated control (0.49 ± 0.33) and with GCS (0.37 ± 0.43), $p = 0.0848$; with SAC (8.53 ± 3.06) and with SAC + GCS (2.49 ± 1.36), $p = 0.0072$; IL-10-producing cells in unstimulated control (0.61 ± 0.60) and with GCS (3.60 ± 0.38), $p = 0.0002$; with SAC (8.03 ± 2.53) and with SAC + GCS (12.32 ± 3.87), $p = 0.0188$.

but did reduce its ability to abrogate PHA-stimulated T cell proliferation (Table VI). Heating GCS to 100°C for 30 min abrogated the activity responsible for modulating cytokine production. Exposure to pH extremes of 2 and 11 for 15 min resulted in retention of 40–60% of the GCS activity for altering cytokine production, but had no affect on its ability to abolish PHA-stimulated T cell proliferation. All functional activity of GCS was lost by exposure to insoluble trypsin for 30 min at room temperature. The activity of GCS for all of the above parameters was retained by anion-exchange columns (Q and DEAE Sepharose), but not by cation-exchange columns (SP and CM Sepharose). Preliminary characterization by Superdex 200 gel filtration column experiments of the anion-exchange-retained fractions indicates a minimum molecular mass of approximately 40 kDa, although GCS functional activity for changes in all three cytokines and T cell proliferation was observed over a wide molecular mass range up to approximately 150 kDa (Table VI). This range of activity suggests various states of aggregate formation, and is consistent with molecular sizing filter experiments in which functional activity was present, but reduced by a 100-kDa filter (data not shown). Each condition that decreased IL-12 also decreased IFN-γ and increased IL-10 (Table VI).

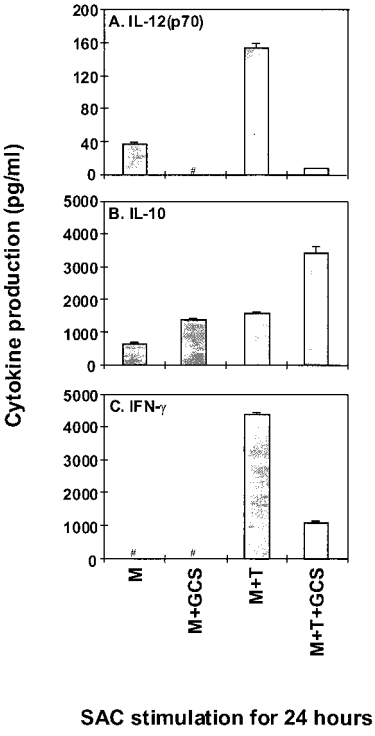

FIGURE 6. The presence of T cells affects IL-12 and IL-10 production by monocytes. Production of IL-12 p70 (*A*), IL-10 (*B*), and IFN-γ (*C*) by SAC-stimulated monocytes with/without T cells: monocytes (M); monocytes + GCS (M + GCS); monocytes + T cells (M + T); and monocytes + T cells + GCS (M + T + GCS). Four experiments were performed with PBMC from four donors each ($n = 2$), and the results shown are from one representative experiment. #, Below detectable level.

Discussion

The results of the present study suggest that the T cell defect(s) reported in glioma patients, and in PBMC cultured with GCS is the result of negative response signals passed to the T cells by monocytes that have been modified upon exposure to this factor(s). Our

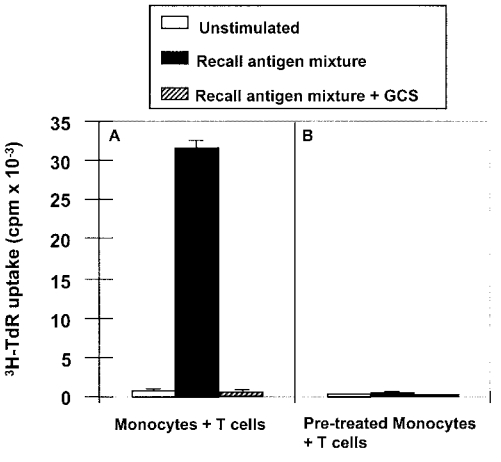

FIGURE 7. GCS-pretreated monocytes lose APC function in recall Ag-induced T cell proliferative response. Recall Ag pool-stimulated T cell proliferation with autologous monocytes in the absence or presence of GCS (*A*); T cells with GCS-pretreated monocytes (after preincubation with GCS for 1 h) in the absence or presence of GCS (*B*). Three experiments were performed with purified T cells and autologous purified monocytes from four donors each ($n = 3$), and the results shown are from one representative experiment.

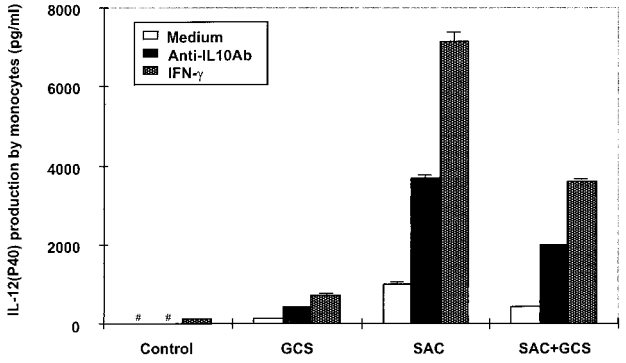

FIGURE 8. Effect of anti-IL-10 mAb and rIFN-γ on IL-12 production by monocytes. Monocytes were incubated alone (control), with GCS, or SAC, or SAC + GCS, and with medium, or anti-IL-10 mAb (10 μg/ml), or rIFN-γ (10 U/ml). Using anti-IL-10 mAb (JES 319 F11), 10 μg/ml in above culture condition shown completely neutralized IL-10 in the culture sample (data not shown). Two experiments were performed with PBMC from two donors each (n = 2), and the results shown are from one representative experiment. #, Below detectable level.

findings also suggest that these monocytes with increased IL-10 expression and decreased IL-12, class II, and CD80/86 expression represent a primary cellular defect in glioma-associated immune dysfunction. Thus, these monocytes may serve as the intermediary between the immunosuppressive factor(s) produced by the tumor cells and the T cells that are affected. The GCS-induced changes that we observed in the monocytes are those that initiate signals essential for T cell activation. Using flow cytometry and intracellular staining, we demonstrated that the IL-12 and IL-10 detected were produced by monocytes and not by T cells, and we verified these GCS-induced changes in IL-12 and IL-10 using purified monocytes. Other cytokines that were affected by GCS were IL-6 (increased), IFN-γ (decreased), and TNF-α (decreased). The demonstration that exposure of monocytes to GCS before mixing with autologous T cells and Ag abolished proliferative responses indicated that the changes in monocyte IL-12, IL-10, MHC class II, and/or CD80/86 expression affected Th function. Importantly, the

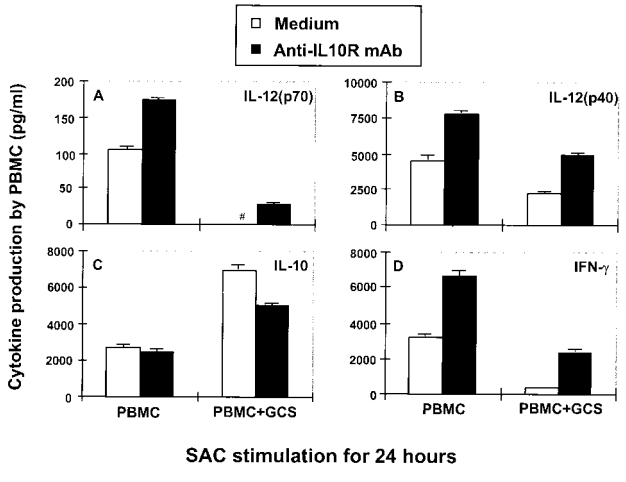

FIGURE 9. Anti-IL-10R mAb did not block GCS-induced changes in cytokine production of PBMC. SAC-stimulated PBMC without/with anti-IL-10R mAb (20 μg/ml) on the production of IL-12 (p70) (*A*), IL-12 (p40) (*B*), IL-10 (*C*), and IFN-γ (*D*) in the absence or presence of GCS. Two experiments were performed with PBMC from two donors each (n = 2), and the results shown are from one representative experiment. #, Below detectable level.

data also show that PBMC from glioma patients exhibit a similar shift in IL-12 and IL-10 production.

It has been suggested that the immunosuppressive effect of glioma culture supernatant is the result from the synthesis of TGF-β (41, 42). However, our mAb inhibition and immunoprecipitation data do not support the hypothesis that the factor responsible for rapidly inducing shifts in IL-12 and IL-10 expression/production and down-regulation of MHC class II and CD80/86 was TGF-β, IL-6, CGRP, or a combination of these immunoregulatory molecules. It remains to be determined whether this glioma-generated factor, which appears to be a protein that has a minimum molecular mass of approximately 40 kDa, is a known immunoregulatory molecule or represents a new immune modulator that affects monokine expression. Other tumor-generated molecules have been reported to inhibit in vitro T cell function. For example, adenocarcinoma-associated MUC1 mucin inhibits T cell proliferation that is reversible by IL-2 (43). In contrast, addition of rIL-2 to our GCS-treated culture did not restore T cell function (data not shown). As controls for the GCS, we tested the supernatants of seven other tumor lines of different origins, and found that three had no effect when cultured with PBMC. In contrast, supernatants from four other tumor lines moderately decreased IL-12 and IFN-γ production, increased IL-10 production, and reduced PHA-stimulated T cell proliferation. However, these effects were not as dramatic as those induced by GCS. Therefore, factors that exert immunologic effects on monocytes and T lymphocytes similar to those we report in this work for GCS may be produced by some but not all cell lines from other types of tumor.

Our observations that anti-IL-10 and anti-IL-10R Abs did not completely neutralize the GCS-induced reduction in IL-12 indicate that part of the GCS effect on IL-12 was independent of IL-10. Although glioma cell lines have been reported to produce IL-10 (44), the SNB-19 glioblastomas that we studied did not produce IL-10. Instead, our experiments demonstrate the induction of IL-10 gene expression and production in monocytes exposed to factor(s) contained in the SNB-19 culture supernatants.

Particularly noteworthy is our finding that GCS decreased both MHC class II and CD80/86 costimulatory molecule expression, but did not affect MHC class I. Earlier reports indicated that IL-10 reduced Ag-stimulated human T cell proliferation by decreasing MHC class II expression on monocytes and induced T cell anergy (32). In a murine model, IL-10 inhibited Con A-induced T cell proliferation when APC function was provided by macrophages, but not when provided by B cells (33). This inhibitory effect was associated with reduced costimulatory activity and B7 expression (33). Thus, in the human Ag-stimulated model, down-regulation of class II was adequate for loss of APC function leading to T cell anergy; in the murine mitogen-stimulated model, down-regulation of costimulatory molecules was sufficient for loss of APC function required for T cell activation. It is also known that IL-10 can down-regulate IL-12 (25, 45), and therefore is likely to contribute to the decreased IL-12 production observed in the GCS-exposed monocytes.

Astrocytes and microglial cells can be induced to become macrophage-like cells in the CNS. MHC molecules detected on microglial cells are down-regulated under normal conditions, but can be induced to express MHC class II Ags under pathologic conditions or upon exposure to IFN-γ (46). Up-regulation of MHC molecules can transform astrocytes into APC (47). Furthermore, increased expression of IL-12 and CD80/86 has been reported in multiple sclerosis lesions (48). Although external stimuli can increase MHC expression in potential APC in the CNS, reduced or absent expression of MHC is often considered to be the normal

Table V. *GCS down-regulates MHC class II and B7, but does not affect MHC class I expression on monocytes*[a]

Cells	Culture	Mean Channel Fluorescence (MCF) HLA-DR (MHC class II)	CD80 (B7.1)	CD86 (B7.2)	HLA-A, B, C (MHC class I)
PBMC	Media	572.6 (4.5)[b]	5.2 (3.4)	818.0 (4.8)	1557.1 (5.5)
GCS-treated PBMC	Media	283.9 (4.8)	5.2 (4.2)	414.3 (4.4)	1544.7 (5.5)
PBMC	Media + IFN-γ	2011.8 (18.9)	30.2 (5.4)	876.8 (5.1)	2212.7 (7.1)
GCS-treated PBMC	Media + IFN-γ	895.9 (12.8)	13.1 (6.1)	351.6 (6.5)	2445.3 (7.8)

[a] PBMC were preincubated with or without GCS (final 1:20) at 37°C for 1 h and washed twice with medium. PBMC (1.5×10^6/ml) were then cultured with or without human IFN-γ (100 U/ml) for 24 h. Cells were stained with anti-CD14-phycoerythrin/FITC and gated on $CD14^+$ monocytes to show the difference in the MCF of anti-HLA-DR, CD80, CD86, HLA-A, -B, -C-FITC/phycoerythrin staining, respectively. The data shown are representative of one of four experiments performed on PBMC from six donors.
[b] Numbers in parentheses indicate the MCF of the isotype control.

situation. Our finding that GCS contains a factor(s) that down-regulates class II expression and counters the up-regulatory effects of IFN-γ on class II expression raises the possibility that reduced or absent MHC expression by astrocytes and glial cells is an active process. This process could involve localized autocrine and/or paracrine production of a GCS-like factor that would prevent damaging CNS inflammation and neuroimmunologic reactions. The potential for functional APC activity in the CNS would be further inhibited by factor-initiated reduced expression of the CD80/86 costimulatory molecule. Thus, there could be two mechanistically distinct but related sites at which a glioma-generated factor prevents APC in the CNS from stimulating T cells: one interfering with T cell activation via APC class II ~ TCR interaction, and the other reducing costimulatory function via CD80/86 ~ CD28 interaction.

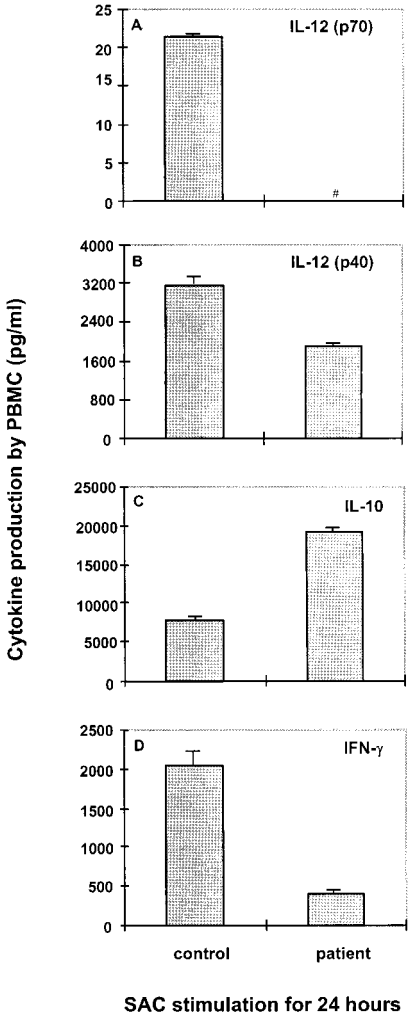

FIGURE 10. The primary glioma patient shows lower IL-12 and higher IL-10 production than the healthy donor. SAC-stimulated production of IL-12 p70 (*A*), IL-12 p40 (*B*), IL-10 (*C*), and IFN-γ (*D*) by PBMC from a healthy control donor and a patient with a primary glioma. Three experiments were performed with PBMC from five healthy donors and four primary glioma patients each ($n = 2$), and the results shown are from one representative experiment. #, Below detectable level.

Glioma patients exhibit a number of immunologic abnormalities that resemble those seen in AIDS patients (49), including decreases in the absolute number and percentage of $CD3^+$ and $CD4^+$, but not of $CD8^+$ T lymphocytes (50). Furthermore, the monocyte cytokine and surface molecule expression pattern seen in AIDS and in our GCS-exposed monocytes are similar (24, 25, 51–53). It is possible that HIV-infected and/or exposure to HIV-1 protein (for example, Tat, *nef*, gp120, or gp41) or to tumor-derived factors such as GCS induce mechanistically similar changes in IL-12, IL-10, MHC class II, and CD80/86 expression by monocytes (54). These modified APC could then initiate anergic rather than stimulatory signals to Th cells that would contribute to apoptotic-mediated depletion of the T cell repertoire reported in AIDS patients (55, 56), as well as to the loss of tumor-specific T cells in cancer patients. Such factor-induced aberrant APC may activate common immunologic dysregulatory signals in the Th of patients with AIDS or immunosuppressive tumors. It is noteworthy that escape of malignant melanoma from T cell surveillance has been suggested to be due to absence of expression of CD80/86 on

Table VI. *Summary of partial characterization of activity contained in GCS*[a]

Treatment of GCS	Test for GCS Functional Activity: Changes in IL-12, IL-10, and IFN-γ	Test for GCS Functional Activity: Inhibition of PHA proliferation
56°C for 30 min	+++	−
100°C for 30 min	−	+
pH 2 for 15 min	++	+++
pH 11 for 15 min	+	+++
Trypsin for 30 min	−	−
Bind to anion exchange column	Yes	Yes
Bind to cation exchange column	No	No
Gel filtration fraction ~45 kDa	++++	++
Gel filtration fraction ~68 kDa	++++	++
Gel filtration fraction ~150 kDa	++++	++

[a] GCS generated by U251 glioblastoma line grown in protein-free media. Percent of effect compared with untreated GCS control: ++++, 80–100%; +++, 60–79%; ++, 40–59%; +, 10–39%; −; activity completely lost.

the tumor cells (57). Tumor cell lines that are inoculated into cancer patients for increasing tumor-specific immunity (58) should be tested for expression of IL-10, IL-12, class II, and CD80/86. If these cells function to present tumor Ags, but exhibit an APC profile similar to that described in this work after GCS exposure, negative rather than positive signals could be transmitted to the tumor-specific T cells, resulting in anergy and clonal depletion. Thus, an appreciation and understanding of the negative immunoregulatory signals initiated by suppressive factor-altered monocytic APC could be important in designing optimal therapeutic strategies aimed at enhancing tumor-specific immunity by dendritic cell immunization (59, 60). It will be important to determine whether dendritic cells are more resistant than monocytes to tumor-derived immune dysregulatory-inducing factors such as GCS, and whether Ag presentation via dendritic cells can effectively counteract and/or circumvent the potential negative signals induced by monocytes that express increased IL-10 and decreased IL-12, MHC class II, and CD80/86.

References

1. Fujiwara, H., and T. Hamaoka. 1995. Regulatory mechanisms of antitumor T cell responses in the tumor-bearing state. *Immunol. Res. 14:271.*
2. Clerici, M., G. M. Shearer, and E. Clerici. 1998. Cytokine dysregulation in invasive cervical carcinoma and other human neoplasias: time to consider the TH1/TH2 paradigm. *J. Natl. Cancer Inst. 90:261.*
3. Mahaley, M. S., W. H. Brooks, T. L. Roszman, D. D. Binger, L. Duka, and S. Richardson. 1977. Immunobiology of primary intracranial tumors. *J. Neurosurg. 46:463.*
4. Gruss, H.-J., A. Pinto, J. Duyster, S. Poppema, and F. Herrmann. 1997. Hodgkin's disease: a tumor with disturbed immunological pathways. *Immunol. Today 18:156.*
5. Clerici, M., M. Merola, E. Ferrario, D. Trabattoni, M. L. Villa, B. Stefanon, D. J. Venzon, G. M. Shearer, G. De Palo, and E. Clerici. 1996. Cytokine production patterns in cervical intraepithelial neoplasia: association with human papillomavirus infection. *J. Natl. Cancer Inst. 89:245.*
6. Tartour, E., A. Gey, X. Sastre-Garau, I. L. Surin, V. Mosseri, and W. H. Fridman. 1998. Prognostic value of intratumoral interferon γ messenger RNA expression in invasive cervical carcinomas. *J. Natl. Cancer Inst. 90:287.*
7. Merogi, A. J., A. J. Marrogi, R. Ramesh, W. R. Robinson, C. D. Fermin, and S. M. Freeman. 1997. Tumor-host interaction: analysis of cytokines, growth factors, and tumor-infiltrating lymphocytes in ovarian carcinomas. *Hum. Pathol. 28:321.*
8. Chen, Q., V. Daniel, D. W. Maher, and P. Hersey. 1994. Production of IL-10 by melanoma cells: examination of its role in immunosuppression mediated by melanoma. *Int. J. Cancer 56:755.*
9. Kim, J., R. L. Modlin, R. L. Moy, S. M. Dubinett, T. McHugh, B. J. Nickoloff, and K. Uyemura. 1995. IL-10 production in cutaneous basal and squamous cell carcinomas: a mechanism for evading the local T cell immune response. *J. Immunol. 155:2240.*
10. Nakagomi, H., P. Pisa, I. K. Pisa, Y. Yamamoto, E. Halapi, K. Backlin, C. Juhlin, and R. Kiessling. 1995. Lack of interleukin-2 (IL-2) expression and selective expression of IL-10 mRNA in human renal cell carcinoma. *Int. J. Cancer 63:366.*
11. Huang, M., J. Wang, P. Lee, S. Sharma, J. T. Mao, H. Meissner, K. Uyemura, and Modlin. 1995. Human non-small cell lung cancer expresses a type 2 cytokine pattern. *Cancer Res. 55:3847.*
12. Urbani, F., A. Maleci, A. LaSala, R. Lande, and C. M. Ausiello. 1995. Defective expression of interferon-γ, granulocyte-macrophage colony-stimulating factor, tumor necrosis factor α, and interleukin-6 in activated peripheral blood lymphocytes from glioma patients. *J. Interferon Cytokine Res. 15:421.*
13. Pericle, F., R. A. Kirken, V. Bronte, G. Sconocchia, L. DaSilva, and D. M. Segal. 1997. Immunocompromised tumor-bearing mice show a selective loss of STAT5a/b expression in T and B lymphocytes. *J. Immunol. 159:2580.*
14. Kolenko, V., Q. Wang, M. C. Riedy, J. O'Shea, J. Ritz, M. K. Cathcart, P. Rayman, R. Tubbs, M. Edinger, A. Novick, R. Bukowski, and J. Finke. 1997. Tumor-induced suppression of T lymphocyte proliferation coincides with inhibition of Jak3 expression and IL-2 receptor signaling. *J. Immunol. 159:3057.*
15. Brooks, W. H., M. G. Netsky, D. E. Normansell, and E. A. Horwitz. 1972. Depressed cell-mediated immunity in patients with primary intracranial tumors. *J. Exp. Med. 136:1631.*
16. Elliott, L. H., W. H. Brooks, and T. L. Roszman. 1984. Cytokinetic basis for the impaired activation of lymphocytes from patients with primary intracranial tumors. *J. Immunol. 132:1208.*
17. Brooks, W. H., R. B. Latta, M. S. Mahaley, T. L. Roszman, L. Dudka, and C. Skaggs. 1981. Immunology of primary intracranial tumors. Part V. Correlation of a lymphocyte index and clinical status. *J. Neurosurg. 54:331.*
18. Elliott. L. H., W. Brooks, and T. L. Roszman. 1990. Role of interleukin-2 and IL-2 receptor expression in the proliferative defect observed in mitogen stimulated lymphocytes from patients with gliomas. *J. Natl. Cancer Inst. 75:919.*
19. Elliott, L. H., W. H. Brooks, and T. L. Roszman. 1990. Inability of mitogen-activated lymphocytes obtained from patients with malignant primary intracranial tumors to express high affinity interleukin 2 receptors. *J. Clin. Invest. 86:80.*
20. Morford, L. A., L. H. Elliott, S. L. Carlson, W. H. Brooks, and T. L. Roszman. 1997. T cell receptor-mediated signaling is defective in T cells obtained from patients with primary intracranial tumors. *J. Immunol. 159:4415.*
21. Roszman, T. L., W. H. Brooks, and L. H. Elliott. 1987. Inhibition of lymphocyte responsiveness by a glial tumor cell-derived suppressive factor. *J. Neurosurg. 67:874.*
22. Elliott, L. H., W. H. Brooks, and T. L. Roszman. 1992. Suppression of high affinity IL-2 receptors on mitogen activated lymphocytes by glioma-derived suppressor factor. *J. Neurooncol. 14:1.*
23. Libraty, D. H., L. E. Airan, K. Uyemura, D. Jullien, B. Spellberg, T. H. Rea, and R. L. Modlin. 1997. Interferon-γ differentially regulates interleukin-12 and interleukin-10 production in leprosy. *J. Clin. Invest. 99:336.*
24. Denis, M., and E. Ghadirian. 1994. Dysregulation of interleukin-8, interleukin-10, and interleukin-12 release by alveolar macrophages from HIV type 1-infected subjects. *AIDS Res. Hum. Retroviruses 10:1619.*
25. Chougnet, C., T. A. Wynn, M. Clerici, A. L. Landay, H. A. Kessler, J. Rusnak, G. P. Melcher, A. Sher, and G. M. Shearer. 1996. Molecular analysis of decreased IL-12 production in HIV-infected individuals and in vitro reciprocal regulation of IL-10 and IL-12. *J. Infect. Dis. 174:46.*
26. Bost, K. L., S. C. Bieligk, and B. M. Jaffe. 1995. Lymphokine mRNA expression by transplantable murine B lymphocytic malignancies: tumor-derived IL-10 as a possible mechanism for modulating the anti-tumor response. *J. Immunol. 154: 718.*
27. Huettner, C., W. Paulus, and W. Roggendorf. 1995. Messenger RNA expression of the immunosuppressive cytokine IL-10 in human gliomas. *Am. J. Pathol. 146:317.*
28. Kruger-Krasagakes, S., K. Krasagakis, C. Garbe, E. Schmitt, C. Huls, T. Blankenstein, and T. Diamantstein. 1994. Expression of interleukin-10 in human melanoma. *Br. J. Cancer 70:1182.*
29. Smith, D. R., S. L. Kunkel, M. D. Burdick, C. A. Wilke, M. B. Orringer, R. I. Whyte, and R. M. Strieter. 1994. Production of interleukin-10 by human bronchogenic carcinoma. *Am. J. Pathol. 145:18.*
30. Zou, J.-P., N. Yamamoto, T. Fujii, H. Takenaka, M. Kobayashi, S. H. Herrmann, S. F. Wolf, H. Fujiwara, and T. Hamaoka. 1995. Systemic administration of rIL-12 induces complete tumor regression and protective immunity: response is correlated with a striking reversal of suppressed IFN-γ production by anti-tumor T cells. *Int. Immunol. 7:1135.*
31. Fujiwara, H., J.-P. Zou, S. Herrmann, and T. Hamaoka. 1995. A sequence of cellular and molecular events involved in IL 12-induced tumor regression. *Res. Immunol. 146:638.*
32. De Waal Malefyt, R., J. Haanen, H. Spits, M.-G. Roncarolo, A. te Velde, C. Figdor, K. Johnson, R. Kastelein, H. Yssel, and J. E. de Vries. 1991. Interleukin 10 (IL-10) and viral IL-10 strongly reduce antigen-specific human T cell proliferation by diminishing the antigen-presenting capacity of monocytes via down-regulation of class II major histocompatibility complex expression. *J. Exp. Med. 174:915.*
33. Ding, L., P. S. Linsley, L.-Y. Huang, R. N. Germain, and E. M. Shevach. 1993. IL-10 inhibits macrophage costimulatory activity by selectively inhibiting the up-regulation of B7 expression. *J. Immunol. 151:1224.*
34. De Ridder, L. I., O. D. Laerum, S. J. Mork, and D. D. Bigner. 1987. Invasiveness of human glioma cell lines in vitro: relation to tumorigenicity in athymic mice. *Acta Neuropathol. (Berl.) 72:207.*
35. Behrens, B. C., T. C. Hamilton, H. Masuda, K. R. Grotzinger, J. Whang-Peng, K. G. Louie, T. Knutsen, W. M. McKoy, R. C. Young, and R. F. Ozols. 1987. Characterization of a *cis*-diaminedichloroplatinum(II)-resistant human ovarian cancer cell line and its use in evaluation of platinum analogues. *Cancer Res. 47:414.*
36. Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature 392:245.*
37. Clerici, M., T. A. Wynn, J. A. Berzofsky, S. P. Blatt, C. W. Hendrix, A. Sher, R. L. Coffman, and G. M. Shearer. 1994. Role of interleukin-10 in T cell helper cell dysfunction in asymptomatic individuals infected with the human immunodeficiency virus. *J. Clin. Invest. 93:768.*
38. Fox, F. E., M. Kubin, M. Cassin, Z. Niu, J. Hosoi, H. Torii, R. D. Granstein, G. Trinchieri, and A. H. Rook. 1997. Calcitonin gene-related peptide inhibits proliferation and antigen presentation by human peripheral blood mononuclear cells: effects on B7, interleukin 10, and interleukin 12. *J. Invest. Dermatol. 108: 43.*
39. Van der Pouw Kraan, T. C. T. M., L. C. M. Boeije, R. J. T. Smeenk, J. Wijdenes, and L. A. Aarden. 1995. Prostaglandin-E_2 is a potent inhibitor of human interleukin 12 production. *J. Exp. Med. 181:775.*
40. Kalinski, P., C. M. U. Hilkens, A. Snijders, F. G. M. Snijdewint, and M. L. Kapsenberg. 1997. IL-12-deficient dendritic cells, generated in the presence of prostaglandin E_2, promote type 2 cytokine production in maturing human naive T helper cells. *J. Immunol. 159:28.*
41. Wrann, M., W. Bodmer, R. de Martin, C. Siepl, R. Hofer-Warbinek, K. Frei, E. Hofer, and A. Fontana. 1987. T-cell suppressor factor from human glioblastoma cells is a 12.5kd protein closely related to transforming growth factor-β. *EMBO J. 6:1633.*
42. Bodmer, S., K. Strommer, K. Frei, C. Siepl, N. DeTribolet, I. Heid, and A. Fontana. 1989. Immunosuppression and transforming growth factor-β in glioblastoma: preferential production of transforming growth factor-β2. *J. Immunol. 143:3222.*

43. Agrawal, B., M. J. Karntz, M. A. Reddish, and B. M. Longenecker. 1998. Cancer-associated MUC1 mucin inhibits human T-cell proliferation, which is reversible by IL-2. *Nat. Med. 4:43.*
44. Hishii, M., T. Nitta, H. Ishida, M. Ebato, A. Kurosu, H. Yagita, K. Sato, and K. Okumura. 1995. Human glioma-derived interleukin-10 inhibits antitumor immune responses in vitro. *Neurosurgery 37:1160.*
45. Chougnet, C., M. Clerici, and G. M. Shearer. 1996. Immunoregulation by interleukin-12: role of IL12 in HIV disease/AIDS. *Res. Immunol. 147:615.*
46. Frei, K., C. Siepl, P. Groscurth, S. Bodmer, C. Schwerdel, and A. Fontana. 1987. Antigen presentation and tumor cytotoxicity by interferon-γ-treated microglial cells. *Eur. J. Immunol. 17:1271.*
47. Fontana, A., W. Fierz, and H. Wekerle. 1984. Astrocytes present myelin basic protein to encephalitogenic T-cell lines. *Nature 307:273.*
48. Windhagen, A., J. Newcombe, F. Dangond, C. Strand, M. N. Woodroofe, M. L. Cuzner, and D. A. Hafler. 1995. Expression of costimulatory molecules B7-1 (CD80), B7-2 (CD86), and interleukin 12 cytokine in multiple sclerosis lesions. *J. Exp. Med. 182:1985.*
49. Dix, A. R., J.-P. Zou, C. Chougnet, G. M. Shearer, L. A. Morford, W. H. Brooks, and T. L. Roszman. 1998. T cell-monocyte interactions in patients with glioblastomas [abstract]. In *Keystone Symposium on Molecular and Cellular Biology: T Lymphocyte Activation, Differentiation, and Death.* (Abstr. 3026) Keystone, CO, p. 95.
50. Bhondeley, M. K., R. D. Mehra, N. K. Mehra, A. K. Mohapatra, P. N. Tandon, S. Roy, and V. Bijlani. 1988. Imbalances in T cell subpopulations in human gliomas. *J. Neurosurg. 68:589.*
51. Clerici, M., A. L. Landay, H. A. Kessler, R. A. Zajac, R. N. Boswell, S. C. Muluk, and G. M. Shearer. 1991. Multiple patterns of alloantigen presenting/stimulating cell dysfunction in patients with AIDS. *J. Immunol. 146: 2207.*
52. Zwilling, B. S., J. Salkowitz, H. Laufman, and D. Pearl. 1991. Differences in the expression of histocompatibility antigen-DR and in anti-mycobacterial activity of monocytes from HIV-infected individuals. *AIDS 5:1327.*
53. Dudhane, A., B. Conti, T. Orlikowsky, Z. Q. Wang, N. Mangla, A. Gupta, G. P. Wormser, and M. K. Hoffmann. 1996. Monocytes in HIV type 1-infected individuals lose expression of costimulatory B7 molecules and acquire cytotoxic activity. *AIDS Res. Hum. Retroviruses 12:885.*
54. Shearer, G. M. 1998. HIV-induced immunopathogenesis. *Immunity 9:587.*
55. Ameisen, J. C. 1992. Programmed cell death and AIDS: from hypothesis to experiment. *Immunol. Today 13:388.*
56. Imberti, L., A. Sottini, A. Bettinardi, M. Puoti, and D. Primi. 1991. Selective depletion in HIV infection of T cells that bear specific T cell receptor Vβ sequences. *Science 254:860.*
57. Denfeld, R. W., A. Dietrich, C. Wuttig, E. Tanczos, J. M. Weiss, W. Vanscheidt, E. Schopf, and J. C. Simon. 1995. In situ expression of B7 and CD28 receptor families in human malignant melanoma: relevance for T-cell-mediated anti-tumor immunity. *Int. J. Cancer 62:259.*
58. Morton, D. L., L. J. Foshag, D. S. B. Hoon, J. A. Nizze, L. A. Wanek, C. Chang, D. G. Davtyan, R. K. Gupta, R. Elashoff, and R. F. Irie. 1992. Prolongation of survival in metastatic melanoma after active specific immunotherapy with a new polyvalent melanoma vaccine. *Am. Surg. 216:463.*
59. Rosenberg, S. A., J. C. Yang, D. J. Schwarzentruber, P. Hwu, F. M. Marincola, S. L. Topalian, N. P. Restifo, M. E. Dudley, S. L. Schwarz, P. J. Spiess et al. 1998. Immunologic and therapeutic evaluation of a synthetic peptide vaccine for the treatment of patients with metastatic melanoma. *Nat. Med. 4:321.*
60. Nestle, F. O., S. Alijagic, M. Gilliet, Y. Sun, S. Grabbe, R. Dummer, G. Burg, and D. Schandendorf. 1998. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. *Nat. Med. 4:328.*