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Curcumin Prevents Tumor-induced T Cell Apoptosis through Stat-5a-mediated Bcl-2 Induction*

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Patients with advanced cancer exhibit multifaceted defects in their immune capacity, which are likely to contribute to an increased susceptibility to infections and disease progression. We demonstrated earlier that curcumin inhibits tumor growth and prevents immune cell death in tumor-bearing hosts. Here we report that tumor-induced immunodepletion involves apoptosis of thymic $CD4^+/CD8^+$ single/double positive cells as well as loss of circulating $CD4^+/CD8^+$ T cells. Administration of curcumin to tumor-bearing animals resulted in restoration of progenitor, effecter, and circulating T cells. In fact, tumor burden decreased the expression level of the pro-proliferative protein Bcl-2 while increasing the pro-apoptotic protein Bax in T cells. Curcumin down-regulated the Bax level while augmenting Bcl-2 expression in these cells, thereby protecting the immunocytes from tumor-induced apoptosis. A search for the upstream mechanism revealed down-regulation of the common cytokine receptor γ chain (γc) expression in T cells by tumor-secreted prostaglandin E_2. As a result, Jak-3 and Stat-5a phosphorylation and to a lesser extent Stat-5b phosphorylation were also decreased in T cells. These entire phenomena could be reverted back by curcumin, indicating that this phytochemical restored the cytokine-dependent Jak-3/Stat-5a signaling pathway in T cells of tumor bearers. Overexpressed Stat-5a/constitutively active Stat-5a1*6 but not Stat-5b could efficiently elevate Bcl-2 levels and protect T cells from tumor-induced death, whereas C-terminal truncated Stat-$5a_{713}$ overexpression failed to do so, indicating the importance of Stat-5a signaling in T cell survival. Thus, these results raise the possibility of inclusion of curcumin in successful therapeutic regimens against cancer.

The multifaceted defect in the immune capacity of patients with advanced malignancy contributes not only to an increased susceptibility to infection and disease progression but also constitutes a barrier to therapeutic interventions. Both human patients and experimental animals with advanced cancer often exhibit a poorly functioning immune system (1, 2), manifested by anergy to skin test antigens (3), decreased T cell proliferation (4), alteration in signal-transducing molecules (5), reduced $CD4^+$:$CD8^+$ ratios, and deficient production of Th-1 cytokines (6). These alterations correlate with the severity of disease and with poor survival. Removal of tumor burden by surgical resection was associated with normalization of the cytokine production capacity of cancer patients (7).

In order to establish itself, a growing tumor must evade the immune system of the host. This contributes to tumor-induced immune suppression. A growing tumor can evade the proposed immune surveillance by several mechanisms. There is evidence of increased apoptosis among $CD8^+$ T cells in peripheral blood lymphocytes (PBL)[2] from cancer patients and animal models (2, 7). By having evaded the innate immune system, the tumor can progress by turning its genetic instability into an advantage by evading the adaptive immune response in various ways. Increased oxidative stress because of the growing tumor can be another cause of immune disruptions. Recently, several observations indicate that a chronic inflammatory condition develops in patients with advanced cancer, causing oxidative stress that can shut off immune functions, including those of T and NK cells. In addition to that, macrophage-derived nitric oxide as well as soluble mediators from tumors reduce the phosphorylation or down-regulate the expression of Jak-3/Stat-5 (8, 9) thus inhibiting the proliferative responses of T cells to various cytokines, including IL-2 and IL-7 (9). It is acknowledged that targets for Jak-mediated survival signals include the Bcl-2 family of apoptotic regulator proteins (10). Studies with Jak-3-deficient mice showed down-regulation of Bcl-2 in the $CD8^+$ population in thymus (11). Other report demonstrated correlation between loss in Bcl-2 expression and death of thymocytes (12). All these reports indicate the role of Bcl-2 in T cell survival. It is known that the pro-proliferative protein Bcl-2 also plays a significant role in T cell maturation (13). In fact, Bcl-2 expression in the thymus is an indication of both positive selection and survival of $CD4^+$ and $CD8^+$ cells in the organ (14). Depletion of the thymic $CD4^+8^+$ double positive population of tumor-bearing animals coupled with an increase in $CD4^-CD8^-$ double negative immature thymocytes cause a declining $CD4^+$ and $CD8^+$ effecter population in secondary immune compartment as well as in circulation, weakens the

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[2] The abbreviations used are: PBL, peripheral blood lymphocyte; Cox-2, cyclooxygenase-2; EAC, Ehrlich's ascites carcinoma; IL, interleukin; Jak, Janus kinase; PI, propidium iodide; PGE_2, prostaglandin E_2; RCC, renal cell carcinoma; Stat, signal transducer and activator of transcription; FITC, fluorescein isothiocyanate; FACS, fluorescence-activated cell sorter; ELISA, enzyme-linked immunosorbent assay; DTT, dithiothreitol; siRNA, short interfering RNA; TUNEL, TdT-mediated dUTP nick-end labeling; PE, phycoerythrin; TdT, terminal deoxynucleotidyltransferase; PerCP, peridinin chlorophyll protein.

cellular defense mechanism, and induces thymic atrophy (2, 15). Therefore, therapeutic intervention, which can protect the immune system in cancer patients, may enhance the immune competence and increase the survival.

This study was conducted to delineate the mechanisms of tumor-induced immunodepletion and the role of curcumin (the major pigment of ground rhizome *Curcuma longa* and a known antioxidant with proven anti-tumor activity (16)) in prevention of immunosuppression caused by progressing tumor. It was observed that $CD4^+$ and $CD8^+$ effecter T cells as well as progenitor $CD4^+CD8^+$ T cell population was severely depleted in the thymus of tumor-bearing animals, resulting in eventual loss of $CD4^+$and $CD8^+$ effecter T cells in peripheral circulation. The critical role of the Jak/Stat family of signaling proteins as regulators of the Bcl-2 family proteins in cancer-induced immunosuppression was evaluated. Reports of immunomodulatory power of curcumin in the tumor-bearing host were only scantily available, and this is the first time we are reporting the intricate mechanism of curcumin-induced immuno-restoration in tumor-bearing animals. Our study suggests the role of curcumin as a possible therapeutic agent with a strong immunomodulatory effect, which can be used alone or in combination with tumoricidal drugs to treat patients with cancer.

EXPERIMENTAL PROCEDURES

Treatment of Animals—Swiss albino mice (National Clinical and Laboratory Animal Sciences, Hyderabad, India) weighing 25–27 g were maintained in temperature-controlled room with a light-dark cycle. All animal experiments were performed following the "Principles of Laboratory Animal Care" (National Institutes of Health Publication 85-23, revised in 1985) and Indian laws on "Protection of Animals" under the prevision of authorized investigators. Mice were divided into four groups of 10 animals each, including a normal set (non-tumor-bearing), a tumor-bearing set (which were intraperitoneally injected with 1×10^5 exponentially grown ascites carcinoma), a curcumin-treated set (non-tumor-bearing), and a curcumin-treated tumor-bearing set. Curcumin (treatment started 7 days after tumor inoculation) was fed orally (50 mg/kg body weight every other day) (17).

Cell Culture—At day 21 of tumor inoculation, thymus was removed and a single cell suspension was made in RPMI 1640 medium. Macrophages were allowed to adhere at 37 °C for 1 h. Peripheral blood collected from mice and from healthy human volunteers with informed consent (IRB 1382) were centrifuged over Ficoll-Paque density gradient (Amersham Biosciences) to obtain total leukocytes. T cells were purified from total leukocytes and thymocytes by negative magnetic selection using microbeads coated with antibodies to CD14 (macrophages), CD16 (NK cells), CD19 (B cells), CD56 (NK cells), and glycophorin A (erythrocytes) (human T cell enrichment mixture; Stem Cell Technologies, Vancouver, Canada). Viable cell numbers were determined by trypan blue exclusion test. The T cell isolated procedure yielded >97% positive for CD3 cells as defined by immunocytometry. Isolated cells were maintained in complete RPMI 1640 medium (supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 μg/ml sodium pyruvate, 100 μM nonessential amino acids, 100 μg/ml streptomycin, and 50 units/ml penicillin; Sigma) at 37 °C in a humidified incubator containing 5% CO_2.

Tissues from primary lesions of renal cell carcinoma (RCC) were provided by the Cooperative Human Tissue Network. Informed consent was obtained from all patients with localized disease. Tissues were digested and primary RCC cells were allowed to adhere overnight. The adherent cells were maintained in complete RPMI 1640 medium and allowed to reach confluence before use. Ascites fluids were collected from the peritoneal cavity of tumor-bearing mice. Tumor supernatants freed from cellular components were used in a 1:1 ratio with RPMI 1640 medium to study the effect of tumor supernatants on T cells in the absence or presence of 10 μM curcumin, 50 μM celecoxib, and/or recombinant IL-2, 1000 IU/ml (reviewed in Ref. 21). After 48 h of incubation or as described in the figure legends, cells were harvested for further experiments. In separate sets of experiments T cells were incubated for 48 h with 3.5 ng/ml prostaglandin E_2 (PGE_2; Sigma) and/or 1000 IU/ml recombinant IL-2.

Plasmid Constructs, siRNA, and Transfections—FLAG-tagged (N terminus) cDNA encoding full-length Stat-5a and Stat-5b, C-terminal truncated Stat-5a (Stat-$5a_{713}$) and Stat-5b (Stat-$5b_{718}$) (provided by Dr. J. N. Ihle of St. Jude Children's Research Hospital, Memphis, TN), constitutively active mutant Stat-5a1*6 (generous gift from Prof. T. Kitamura, Institute of Medical Science, University of Tokyo), Bcl-2, and Bax (kind gift from Dr. C. S. Tannenbaum of The Cleveland Clinic Foundation, Cleveland, OH) in Prk5 vector (2 μg each/million cells) were introduced separately into isolated T cells using the T cell nucleofector kit (Amaxa, Koein, Germany). Isolation of stably expressing clones was done by limiting dilution and selection with IL-2 (25 units/ml) and hygromycin B (800 mg/ml) for 14 days, and cells surviving this treatment were cloned and assessed for Bcl-2, Bax, and Stat-5 expression by Western blot analysis. Tumor cells were transfected with 300 pmol of Cox-2/control double-stranded siRNA (Santa Cruz Biotechnology) and Lipofectamine 2000 separately for 12 h. mRNA and protein levels of Cox-2 were estimated by reverse transcription-PCR and Western blotting. Transfected cells were cultured for 72 h, and cell-free supernatants were collected for subsequent experiments.

Flow Cytometry—For the determination of cell death, T cells were stained with propidium iodide (PI) and annexin-V-FITC and analyzed on a flow cytometer (FACSCalibur, BD Biosciences), equipped with a 488 nm argon laser light source using CellQuest software. Electronic compensation of the instrument was done to exclude overlapping of the emission spectra. A total of 10,000 events were acquired, and cells were properly gated for analysis (18). The fragmented DNA of apoptotic cells was labeled by catalytically incorporating FITC-dUTP at the 3′-hydroxyl ends of the fragmented DNA by enzyme terminal deoxynucleotidyltransferase (TdT) using the apo-direct kit (Pharmingen) following the principles of TdT-mediated dUTP nick-end labeling (TUNEL). The cells were then analyzed on a flow cytometer. For assaying $CD4^+/CD8^+$ cells, T cells from thymus and PBL were stained with FITC-conjugated CD4 and peridinin chlorophyll protein (PerCP)-conjugated CD8 (Pharmingen) and analyzed in FACS. For the assessment of

apoptotic populations in thymic and peripheral blood, T cells were stained with either FITC-/PerCP-conjugated CD4/CD8 antibodies and then with phycoerythrin (PE)-conjugated annexin-V. The percentage of $CD4^-8^-$, $CD4^+8^+$, $CD4^+8^-$, and $CD4^-8^+$ cells positive for annexin-V was determined using FACSCalibur.

Co-immunoprecipitation and Immunoblotting—Cells were lysed in buffer (10 mM Hepes, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, and 0.5 mM DTT), and nuclei were pelleted by brief centrifugation. The supernatant was spun at 105,000 × *g* to get a cytosolic fraction. The nuclear extract was prepared in buffer containing 20 mM Hepes, pH 7.9, 25% (v/v) glycerol, 420 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, and 0.5 mM phenylmethylsulfonyl fluoride. For whole cell lysates, cells were homogenized in buffer (20 mM Hepes, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM Na-EDTA, 1 mM Na-EGTA, and 1 mM DTT). All the buffers were supplemented with protease and phosphatase inhibitor mixtures (16, 18). For direct Western blot analysis, the cell lysates or the particular fractions containing 50 μg of protein were separated by SDS-PAGE and transferred to nitrocellulose membrane. The protein of interest was visualized by chemiluminescence. For the determination of direct interaction between two proteins, co-immunoprecipitation technique was employed. The immunopurified proteins were then detected by Western blot using specific antibody (Santa Cruz Biotechnology). Equal protein loading was confirmed by reprobing the blots with α-actin/histone H1 antibody (Santa Cruz Biotechnology) (16).

Prostaglandin E_2 Assay—PGE_2 contents in culture supernatants were determined using PGE_2 ELISA bioassay kit (US Biological). For the determination of serum and PGE_2 content of ascites fluid, samples were treated with 0.2 ml of methanol, 1 ml of sample and then applied to C-18 Sep-Pac column (Waters). PGE_2 was eluted with methyl formate. PGE_2 content in eluent was determined according to the manufacturer's instructions.

Statistical Analysis—Values are shown as means ± S.E., except if indicated otherwise. Data were analyzed and, when appropriate, significance of the differences between mean values was determined by a Student's *t* test. Results were considered significant at $p < 0.05$.

RESULTS

Curcumin Prevents Tumor-induced $CD4^+/CD8^+$ T Lymphocyte Depletion—It is known that T cells play a pivotal role in cell-mediated tumor immunity, and tumors induce T cell apoptosis as a mechanism to evade the host defense system. Thymus is the major organ where the T cell maturation process takes place and mature effecter T cells subsequently enter the bloodstream. Closer scrutiny of the thymus using flow cytometry revealed massive loss of $CD4^+CD8^+$ double positive as well as $CD4^+$ or $CD8^+$ single positive effecter populations in tumor-bearing animals, with an apparent increase in the $CD4^-CD8^-$ double negative population (Fig. 1*A*, *upper panels*). Loss of the $CD4^+8^+$ double positive population can cause a decrease in the number of mature effecter cells emerging from the thymus. Similarly, it was also observed that tumor burden severely decreased effecter $CD4^+$ and $CD8^+$ T cell populations in peripheral blood (Fig. 1*A*, *lower panels*), indicating that tumor-induced T cell depletion encompasses both the primary and effecter immune compartments of the host, as decreased thymic output results in disruption of the circulating T cell repertoire. Interestingly, curcumin administration brought back thymic $CD4^+8^+$, $CD4^+$, and $CD8^+$ cells as well as circulatory effecter $CD4^+$ and $CD8^+$ cells to control level (Fig. 1*A*).

Curcumin Protects T Lymphocytes from Tumor-induced Apoptosis—The observations of massive depletion of both thymic and circulatory T cell populations prompted us to investigate the underlying cause. Using a flow cytometric technique, we observed increased annexin-V/PI positivity in $CD3^+$ T cells, which indicates that apoptotic cell death is one of the major causes behind tumor-induced depletion of both thymic and circulatory T cells. Curcumin administration prevents such tumor-induced T cell death. To determine specific subpopulations of T cells most affected by tumor assault, we used three-color flow cytometry (FITC-CD4, PerCP-CD8, and PE-annexin-V). Our results indicated that 5.28% double negative cells, 23.14% double positive cells, 6.21% $CD4^+$, and 8.16% $CD8^+$ cells were apoptotic in the thymus of tumor-bearing animals, whereas the thymus of normal animals showed only 1.66% double negative cells, 4.39% double positive cells, 1.22% $CD4^+$, and 1.81% $CD8^+$ apoptotic cells (Fig. 1*B*, *left panel*). Administration of curcumin reduced the overall percentage of apoptotic cells in thymus (16.64%) from that of its untreated counterpart (42.79%) with marked reduction in both double and single positive populations (Fig. 1*B*, *left panel*).

Similar results were obtained when peripheral T cells were co-cultured with tumor supernatants. When mouse or human T cells were incubated with cell-free EAC or RCC supernatants, both $CD4^+$ and $CD8^+$ cells became susceptible to tumor-induced apoptosis. Interestingly, $CD8^+$ T cells were more vulnerable than $CD4^+$ cells. When both the T cells were preincubated with curcumin, the cells became resistant to killing (Fig. 1*B*, *middle panel*). Similar trends were observed with both mouse and human T cells thereby indicating that curcumin could protect both the $CD4^+$ and $CD8^+$ T cells from tumor-induced apoptosis.

In *in vivo* studies, 13.47% $CD4^+$ and 22.85% $CD8^+$ cells of tumor-bearing mice were apoptotic, and curcumin administration could protect these cells from tumor insult (Fig. 1*B*, *right panel*) indicating that the *in vitro* results are reflected in an *in vivo* system. Similarly, when we compared the percentage of circulatory T cell killing in renal cell carcinoma-bearing patients to that of age- and sex-matched normal individuals, we observed an increase in the percent apoptosis in both $CD4^+$ and $CD8^+$ T cells (Fig. 1*B*, *right panel*). We could not perform a clinical trial with curcumin in RCC patients, but from aforementioned *in vitro* and *in vivo* results, it may be envisaged that curcumin might also protect T cells from a tumor-induced demise in humans. Our results also indicated that tumor-induced depletion of thymic T cells and the eventual loss of peripheral mature T cell pull are both because of increased apoptosis of double positive progenitor T cells and maturation block as suggested by the increased accumulation of double negative immature thymocytes. Curcumin could protect double positive thymocytes from tumor-induced apoptosis thus

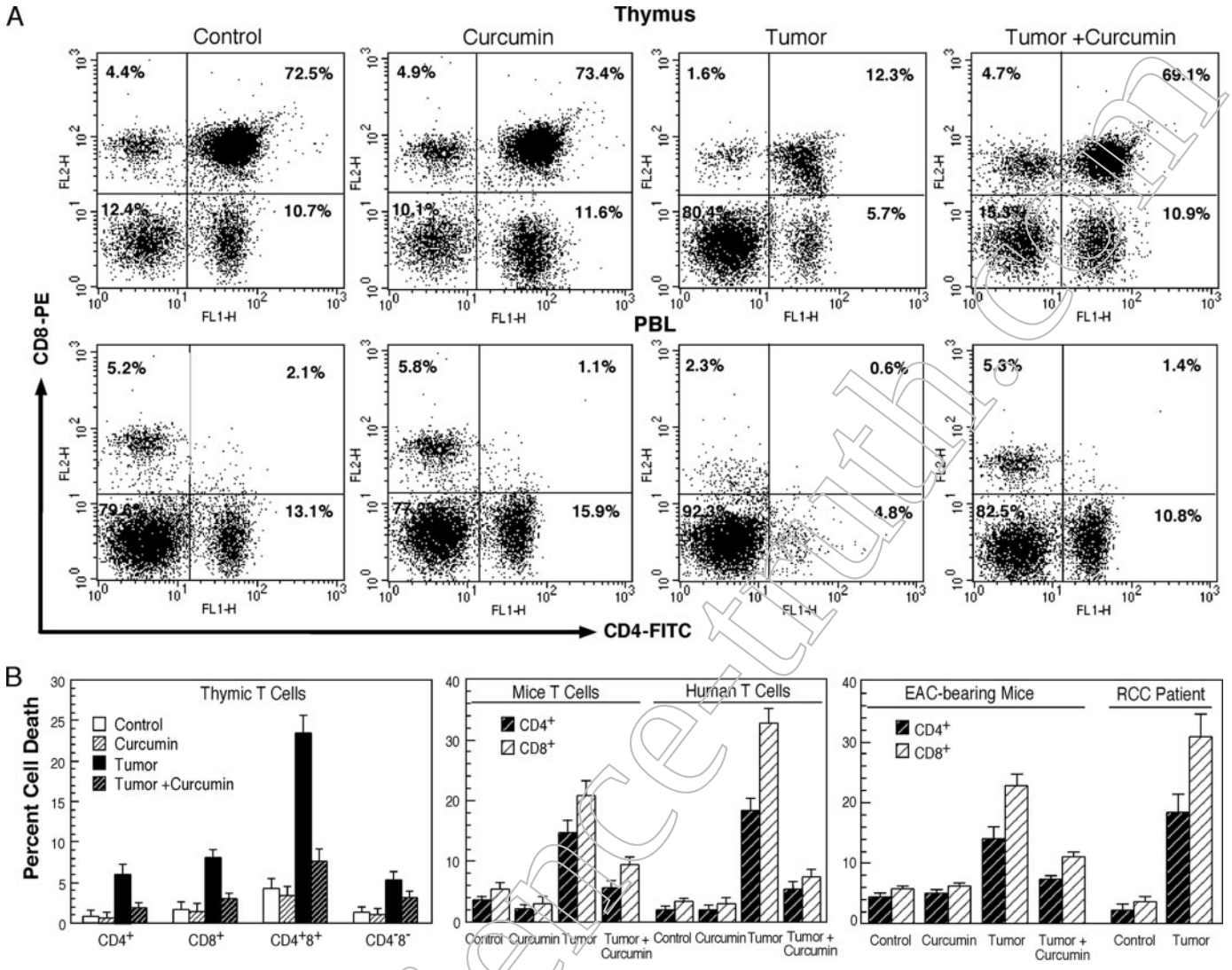

FIGURE 1. **Curcumin prevents tumor-induced $CD4^+/CD8^+$ T lymphocyte apoptosis.** *A,* T cells from thymus (*upper panels*) and PBL (*lower panels*) of untreated or curcumin-treated normal and tumor-bearing mice were stained with FITC-CD4 and PE-CD8 antibodies. Cells were analyzed flow cytometrically, and the dot plot display of FITC fluorescence (FL1-H; *x* axis) *versus* PE fluorescence (FL2-H; *y* axis) has been displayed in logarithmic scale. *Lower right quadrant,* $CD4^+$ cells; *upper left quadrant,* $CD8^+$ cells; *upper right quadrant,* $CD4^+8^+$ cells; and *lower left quadrant,* $CD4^-8^-$ cells. *B,* thymic or peripheral blood T cells were labeled with FITC-/PerCP-conjugated CD4/CD8 antibodies and then with PE-conjugated annexin-V and were analyzed flow cytometrically. Annexin-V-positive cells were regarded as apoptotic cells. *Left panel,* $CD4^+$, $CD8^+$, $CD4^+8^+$, and $CD4^-8^-$ were gated properly, and percent thymic T cell deaths from untreated or curcumin-treated normal and tumor-bearing mice were analyzed flow cytometrically. *Middle panel,* mice/human peripheral blood T cells were incubated with untreated or 10 μM curcumin-treated EAC-/RCC-shed cell-free supernatant for 48 h, and percent $CD4^+$ and $CD8^+$ cell death were analyzed flow cytometrically. *Right panel,* percent $CD4^+$ and $CD8^+$ T cells death from peripheral blood of age- and sex-matched normal and EAC-bearing mice/RCC patients were analyzed flow cytometrically. Values are mean ± S.E. of five independent sets of experiments.

ensuring the continuous export of mature T cells in the peripheral circulation.

Tumor Induces T Cell Apoptosis by Altering Bcl-2/Bax Ratio, Releasing Cytochrome c, and Activating Caspase-3; Protection by Curcumin—To delineate the underlying mechanism, which enables curcumin to prevent tumor-induced T cell apoptosis, we examined the expression level of anti-apoptotic Bcl-2 and pro-apoptotic Bax proteins in T cells. We observed that tumor insult down-regulated Bcl-2 and up-regulated Bax thus lowering the Bcl-2:Bax ratio (Fig. 2, *A* and *B*) creating a pro-apoptotic environment in T cells. We further investigated the release of cytochrome *c* in T cell cytosol as a measure of mitochondrial transmembrane potential loss and initiation of caspase cascades. As shown in Fig. 3*A*, tumor burden significantly increased cytochrome *c* levels in cytosol, whereas in normal T cells cytochrome *c* was minimally detected in the cytosolic fraction. Downstream, the tumor insult activated critical executioner caspase-3, as was evident from the substantial decrease in pro-caspase-3 and increase in caspase-3 bands in these cells (Fig. 2*A*). Curcumin treatment restored the Bcl-2:Bax ratio toward the survival environment, normalized cytochrome *c* release, and inhibited caspase-3 activation thereby protecting thymic T cells from tumor-induced death (Fig. 2, *A* and *B*).

Results obtained so far indicated that tumor-induced immune cell death involves disruption of Bcl-2:Bax ratio and initiation of caspase cascades. Because the decrease in Bcl-2 expression was found to be one of the major causes of T cell death, we overexpressed these anti-apoptotic (Bcl-2) as well as pro-apoptotic (Bax) proteins in T cells in order to alter the Bcl-2:Bax ratios in these cells. Western blot analysis performed

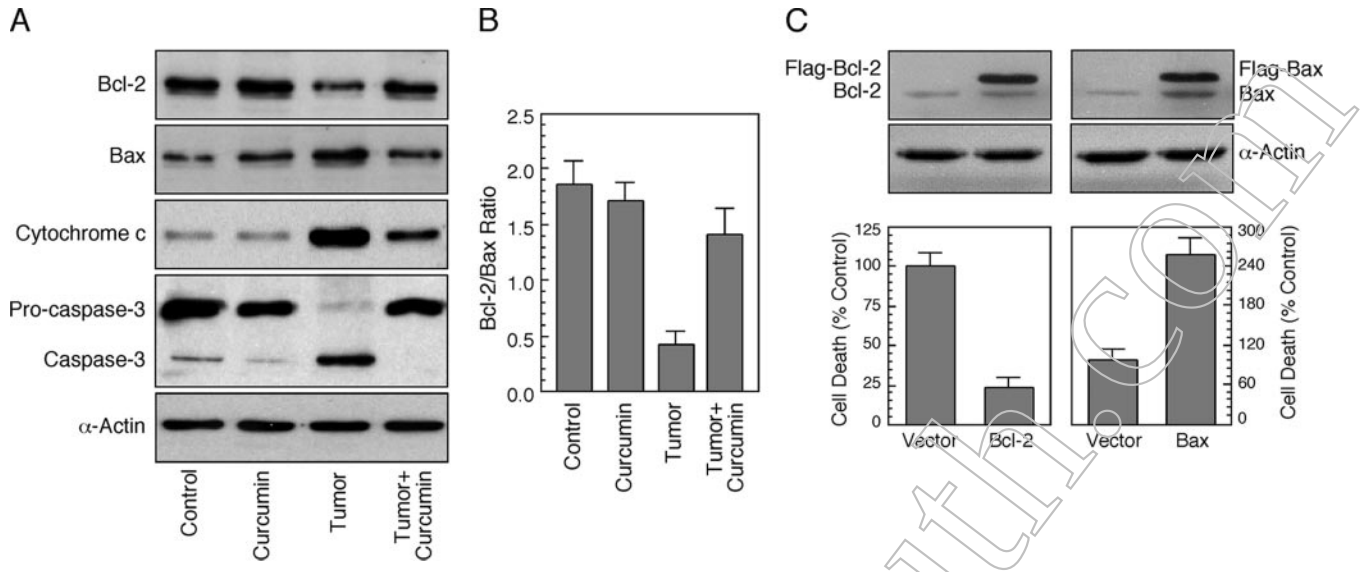

FIGURE 2. **Tumor induces T cell apoptosis by altering Bcl-2:Bax ratio, releasing cytochrome *c*, and activating caspase-3; protection by curcumin.** T cells from normal and tumor-bearing mice (±curcumin) were harvested. *A,* T cell lysates for Bcl-2 and Bax expression at the protein level and cytosolic fraction for the determination of cytochrome *c* release and caspase-3 activation were Western-blotted using specific antibodies. *B,* quantitative chemiluminescence technique was employed to determine Bcl-2:Bax ratios. *C,* control vector or FLAG-tagged *Bcl-2-/Bax*-transfected T cells were incubated with tumor supernatants for 48 h in the presence or absence of 10 μM curcumin. TUNEL method was employed to detect DNA strand break. In a double label system, nuclear DNA was labeled with FITC-dUTP and PI. Percent FITC-dUTP-positive cells were regarded as apoptotic cells (*lower panels*). Values are mean ± S.E. of four independent sets of experiments. Western blots representation of Bcl-2 and Bax expression patterns in transfected T cells (*upper panels*).

on FLAG-tagged Bcl-2/Bax-transfected clones revealed two protein bands recognized by anti-Bcl-2/Bax antibodies as follows: a native molecule also present in lysates from wild-type cells and an additional, approximately higher molecular mass protein specific to transfected cells (Fig. 2*C*). To assess the possibility that Bcl-2 overexpression would confer T cell protection from tumor-shed supernatant-induced apoptosis, control vector and Bcl-2 transfected cells were analyzed for DNA break by TUNEL. Multiple experiments demonstrated that control T cells were highly sensitive to tumor-shed supernatant, with an average of 36% of lymphocytes testing positive for apoptosis. Unlike wild-type cells, however, Bcl-2-transfected cells were only minimally affected, with an average of only 7% of the transfected cells succumbing to a tumor shed-induced apoptotic death (Fig. 2*C*).

The fact that Bcl-2 overexpression provided T cells with significant protection against tumor-mediated apoptosis led us to ask the question whether overexpression of Bax would make T cells more susceptible to tumor-induced death. In fact, when these cells were expressed with the *Bax* gene, more killing was observed (Fig. 2*C*), and curcumin could not rescue them from tumor-induced apoptosis (data not shown). All these data indicate that normalization of the Bcl-2:Bax ratio (which plays an important role in both the selection procedure and survival of T cells) may be the mechanism by which curcumin prevents tumor-induced T cell loss.

Curcumin Prevents Tumor-induced Down-regulation of Jak-3/Stat-5 Activation via Restoration of γ Chain in T Cells—We have already shown that tumor-induced down-regulation of Bcl-2 expression with a moderate increase in Bax level leading to T cell death and depletion of thymic T cells eventually resulted in a diminished peripheral blood T cell repertoire. These results prompted us to investigate the phosphorylation status of Jak and Stat proteins in T cells that are known to be involved in the regulation of both the pro- and anti-apoptotic Bcl-2 family protein expression. It is known that Jak phosphorylation by cytokines or other stimuli leads to Stat activation via phosphorylation and subsequent translocation to the nucleus (19). We observed that out of two Jak proteins (Jak-1 and Jak-3) and three associated Stat proteins (Stat-3, Stat-5, and Stat-6) responsible for Bcl-2 induction, only Jak-3 (Fig. 3*A*) and Stat-5 (Fig. 3*B*) phosphorylations were down-regulated in T cells of tumor-bearing mice. To test whether reduced Jak-3 phosphorylation was associated with decreased Stat-5 phosphorylation, we co-immunoprecipitated Stat-5 with anti-Jak-3 antibody, and the immunopurified proteins were then Western-blotted with anti-phospho-Stat-5 antibody. Fig. 3*C* shows that in tumor-bearing mice there was substantial reduction in phospho-Stat-5 association with Jak-3 as was observed from the chemiluminescence intensities of the immunoprecipitates. Perturbation in phospho-Stat-5 nuclear translocation activity was also observed in tumor bearers in comparison with its normal counterparts (Fig. 3*D*). Curcumin treatment could normalize Jak-3-mediated Stat-5 phosphorylation and nuclear translocation activity to normal levels in T cells of tumor bearer (Fig. 3, *A–D*).

After confirming the Stat-5-mediated pathway as the major pathway in tumor-induced T cell apoptosis, an attempt was then made to identify the isoforms(s) of Stat-5 involved, because both the Stat-5a and Stat-5b isoforms play a critical role in Bcl-2 induction in T cells. Results of Fig. 3*E* depicted that among the two isoforms, the phosphorylation of Stat-5a was inhibited significantly in comparison with that of Stat-5b as a result of tumor insult (Fig. 3*E*). Curcumin was found to restore the phosphorylation status of both the isoforms in tumor-bearing animals (Fig. 3*E*).

To understand the mechanism behind curcumin-induced restoration of Jak-3/Stat-5 phosphorylation, we estimated the

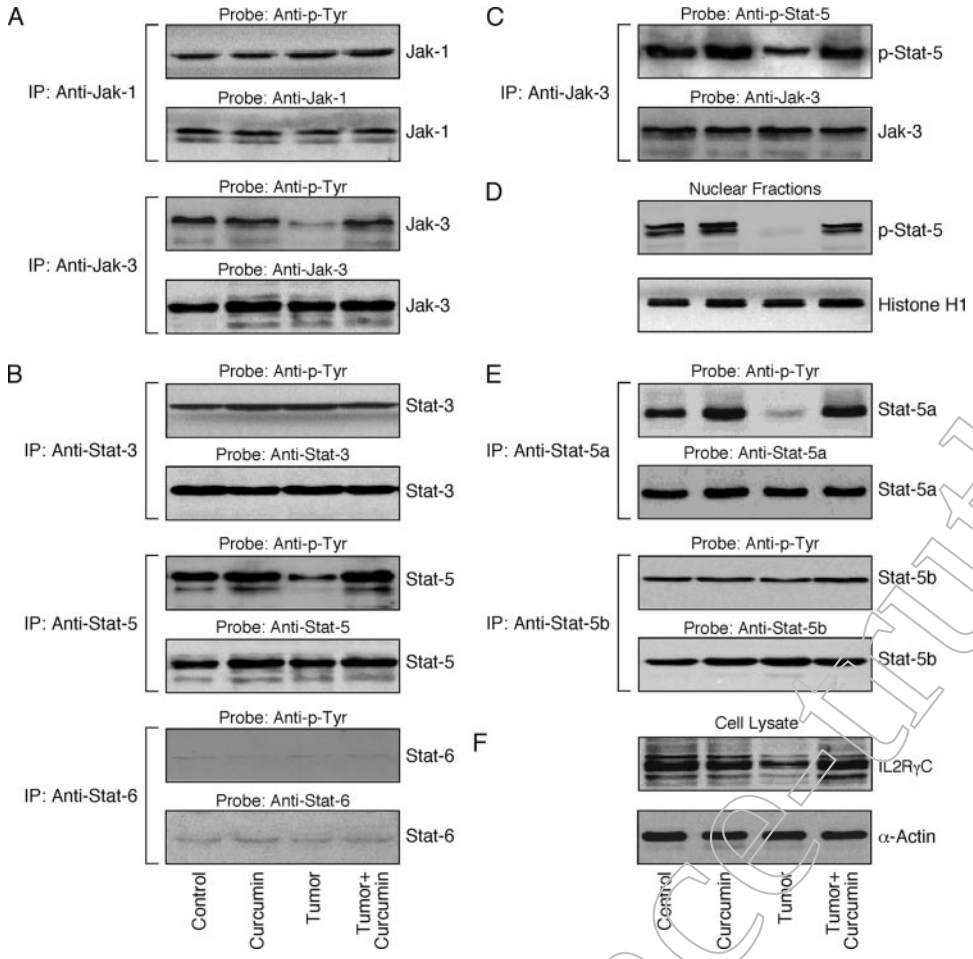

FIGURE 3. **Curcumin prevents tumor-induced down-regulation of Jak-3/Stat-5 activation via restoration of γ chain in T cells.** T cells from untreated or curcumin-treated normal and tumor-bearing mice were lysed. Jak-1/Jak-3 (*A*) and Stat-3/Stat-5/Stat-6 (*B*) were immunoprecipitated (*IP*) using specific antibodies and then Western-blotted with anti-phosphotyrosine or anti-Jak-1/Jak-3/Stat-3/Stat-5/Stat-6 antibodies to determine the phosphorylation status of specific proteins. *C,* Jak-3-Stat-5 complexes were immunopurified with anti-Jak-3 antibody from T cell lysates. The immunopurified proteins were subjected to Western blot analysis to identify the Jak-3-associated phospho-Stat-5. *D,* nuclear fractions from T cells were Western-blotted using anti-phospho-Stat-5 antibodies to determine nuclear translocation as well as activation of Stat-5. Histone H1 was used as internal control. *E,* Stat-5a and Stat-5b isoforms were immunoprecipitated from cell lysates using specific antibodies and then Western-blotted with anti-phosphotyrosine or anti-Stat-5a/Stat-5b antibodies to determine the phosphorylation status of specific proteins. *F,* T cells lysates were Western-blotted with anti-γ chain antibody to determine the expression level of specific protein. α-Actin was used as internal control.

expression level of IL-2Rγ chain (known as common γ chain or γc), as γc is the primary mediator of cytokine signaling and activates Jak-3/Stat-5 signaling cascade (20). In our experimental model, we observed a marked decrease in IL-2R expression in T cells of tumor-bearing mice, which could efficiently be restored to normal levels by curcumin (Fig. 3*F*). Thus, down-regulation of γc expression in tumor-bearers may be the cause behind the hypo-phosphorylation of Jak-3/Stat-5, which ultimately results in the decrease in the Bcl-2:Bax ratio in the T cell micro-environment.

Predominantly Stat-5a Transfection Confers Resistance to T Cells from Tumor-induced Death—Our earlier results suggested that the mechanism of tumor-induced T cell death might include perturbation in Stat-5a activity and decreased Bcl-2 expression. To further confirm the role of Stat-5a inhibition in tumor-induced T cell demise, T cells were overexpressed with wild-type Stat-5a/Stat-5b, C-terminal truncated dominant negative Stat-5 (Stat-$5a_{713}$ and Stat-$5b_{718}$), or constitutively active Stat-5a (Stat-5a1*6) genes (Fig. 4*B*) and then incubated with tumor-shed supernatants. Curcumin was found to augment Bcl-2 induction in wild-type Stat-5a-transfected cells, whereas wild-type Stat-5b-expressing cells showed lesser Bcl-2 increase in comparison with control vector-transfected cells (Fig. 4*A*). On the other hand, in Stat-$5a_{713}$-overexpressing cells, curcumin failed to induce any Bcl-2 protein, although it was able to do so in Stat-$5b_{718}$-expressing cells. In constitutively active Stat-5a1*6-overexpressing cells, Bcl-2 content was very high, and tumor supernatant was unable to down-regulate its expression (Fig. 4*A*). All these observations suggest that activation of Stat-5a plays a crucial role in Bcl-2 induction in T cells.

After establishing the importance of Stat-5a-mediated Bcl-2 induction, the engineered cells were used to assess the possibility that Stat-5a overexpression would confer T cells protection from tumor-shed supernatant-induced apoptosis. Multiple experiments demonstrated that unlike wild-type cells, however, constitutively active Stat-5a1*6-transfected cells were only minimally affected, with an average of only 6–10% of the transfected cells succumbing to a tumor shed-induced apoptotic death, and curcumin was able to protect them further (Fig. 4*C*). Observations that ectopic expression of constitutively active Stat-5a conferred resistance to T cells from tumor-induced death but could not completely abrogate the death suggest that tumor-induced T cell death may be caused also via some other mechanism(s) along with inhibition of Jak-3/Stat-5 signaling pathways. The fact that curcumin administration to constitutively active Stat-5a-expressing T cells confers further protection from tumor-induced T cell death suggests that curcumin can also interfere with those other death pathways directly in T cells. In parallel to Bcl-2 induction, when dominant negative Stat-$5a_{713}$ was introduced into T cells, this protein rendered T cells more susceptible to tumor-induced death that could not be successfully prevented by curcumin administration. In contrast, curcumin was able to protect Stat-$5b_{718}$-transfected T cells from tumor-induced death (Fig. 4*C*). When wild-type Stat-5a was introduced into T cells, this protein could protect T cells from tumor-induced death only in the

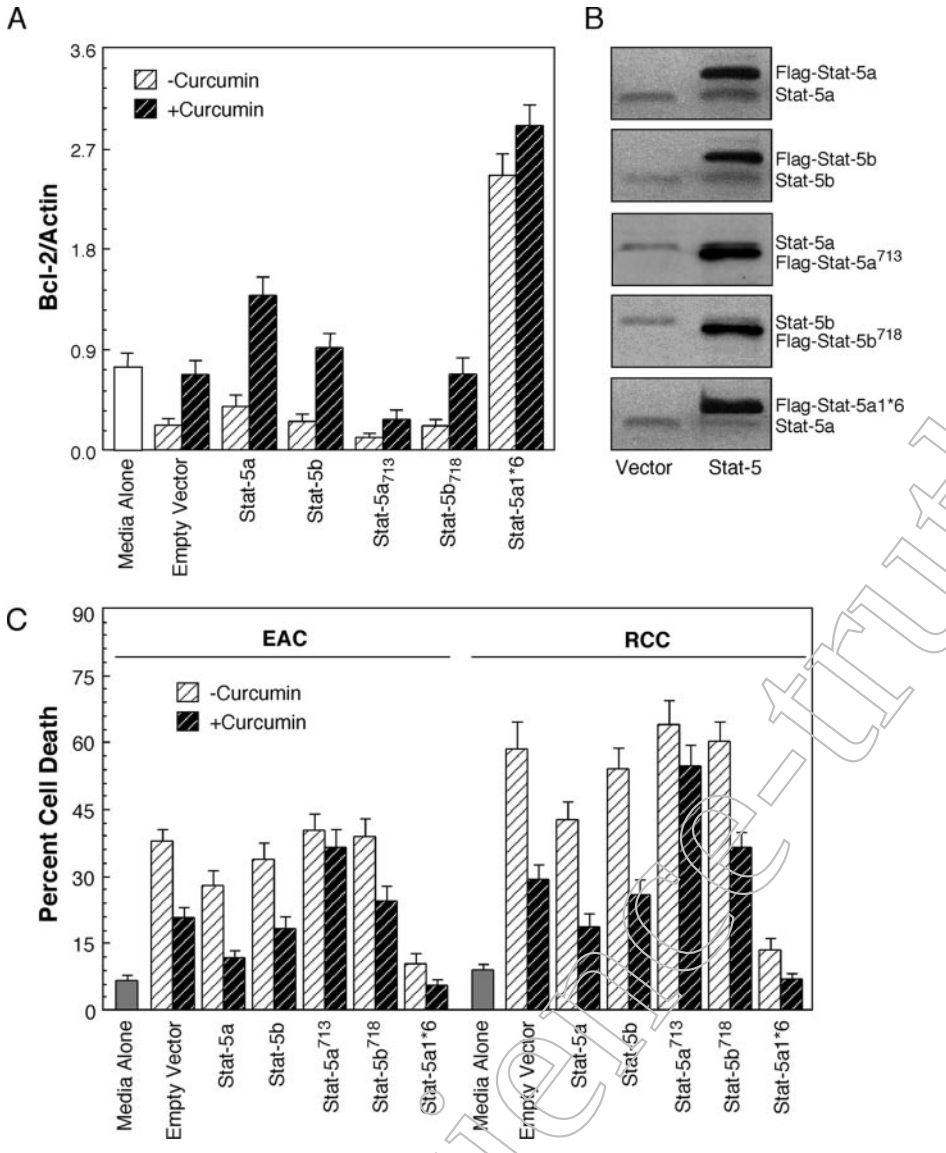

FIGURE 4. **Predominantly Stat-5a transfection confers resistance to T cells from tumor-induced death.** T cells transfected with control vector or wild-type *Stat-5a/Stat-5b*, C-terminal truncated *Stat-5a*$_{713}$/*Stat-5b*$_{718}$, or constitutively active *Stat-5a1*6* genes were incubated with tumor supernatants in the presence or absence of 10 μM curcumin. *A,* Bcl-2 expression level was determined by quantitative chemiluminescence from Bcl-2/α-actin band intensities. *B,* Western blot representations of Stat-5 expression patterns in transfected T cells. *C,* control vector or Stat-5-transfected T cells were incubated with untreated or 10 μM curcumin-treated EAC (*left panels*)/RCC (*right panels*)-shed cell-free supernatants for 48 h. TUNEL method was employed to detect DNA strand break. Percent FITC-dUTP-positive cells were regarded as apoptotic cells. Values are mean ± S.E. of four independent sets of experiments.

presence of curcumin (Fig. 4*C*). All these results strongly reinstate our hypothesis that Stat-5a protects T cells from tumor-induced apoptosis mainly through the induction of the anti-apoptotic protein Bcl-2.

Down-regulation of Tumor PGE$_2$ Production via Cox-2 Inhibition by Curcumin Prevents Tumor-induced Down-regulation of T Cell Survival Signaling—We have observed that growing tumors induced depletion of T cell common γ chain thereby causing the down-regulation of Jak-3/Stat-5a-mediated survival signal eventually leading to the T cell death. All these events indicated the presence of some soluble factor(s) produced by the growing tumor that caused the observed adverse effects on T cells. PGE$_2$ is one such immunosuppressor that many tumor secrete as a part of their immune evasion strategy. In our experimental model, we observed that PGE$_2$ could down-regulate γc expression, Jak-3/Stat-5 phosphorylation, and induce T cell death *in vitro* (Fig. 5*A*). Additionally, in line with the reports from Kolenko *et al.* (21), we observed that PGE$_2$ could also prevent IL-2-induced γc expression and Jak-3/Stat-5 phosphorylation in T cells (Fig. 5*A*). Remarkably similar results were observed when T cells were incubated *in vitro* with tumor-shed supernatants, as incubating T cells with tumor supernatants caused down-regulation of γc expression, Jak-3/Stat-5 phosphorylation, along with the induction of T cell death (Fig. 5*A*). It was also observed that tumor-shed supernatant could efficiently block IL-2-induced up-regulation of γc expression and Jak-3/Stat-5 phosphorylation (Fig. 5*A*). All these observations prompted us to explore the possibility of involvement of PGE$_2$ in tumor-induced T cell death, γc down-regulation, and Jak-3/Stat-5 hypo-phosphorylation. Our results demonstrated that ascites tumor secretes PGE$_2$ that eventually causes the elevation of serum PGE$_2$ level in tumor-bearing animals (Fig. 5*B*). Interestingly, curcumin treatment prevented serum PGE$_2$ elevation in tumor-bearing animals via down-regulation of tumor PGE$_2$ synthesis (Fig. 5*A*, *upper panel*). We observed that culture supernatants from curcumin-treated tumor cells not only contain comparatively low PGE$_2$ (Fig. 5*C*) but also lack the ability to induce severe T cell death (Fig. 5, *A* and *C*) and failed to down-regulate γc expression or Jak-3/Stat-5 phosphorylation in T cells (Fig. 5*A*). Curcumin is a known Cox-2 inhibitor, and in our experimental system, we also observed similar inhibition of *Cox-2* gene expression in tumor cells by this phytochemical (Fig. 5*B*). Down-regulation of Cox-2 by curcumin dampens PGE$_2$ production from tumor cells, thereby offering protection for host T cells from the suppressive effects of PGE$_2$. Further confirmation for our notion came from the results that show treatment of tumor cells with the specific Cox-2 inhibitor celecoxib (50 μM) reduces their ability to produce PGE$_2$ and offers protection for the T cell (Fig. 5, *C* and *A*). To further strengthen our assertion, we transfected tumor cells with Cox-2-siRNA and assessed their ability to produce PGE$_2$. We observed that Cox-2-siRNA-treated tumor-shed superna-

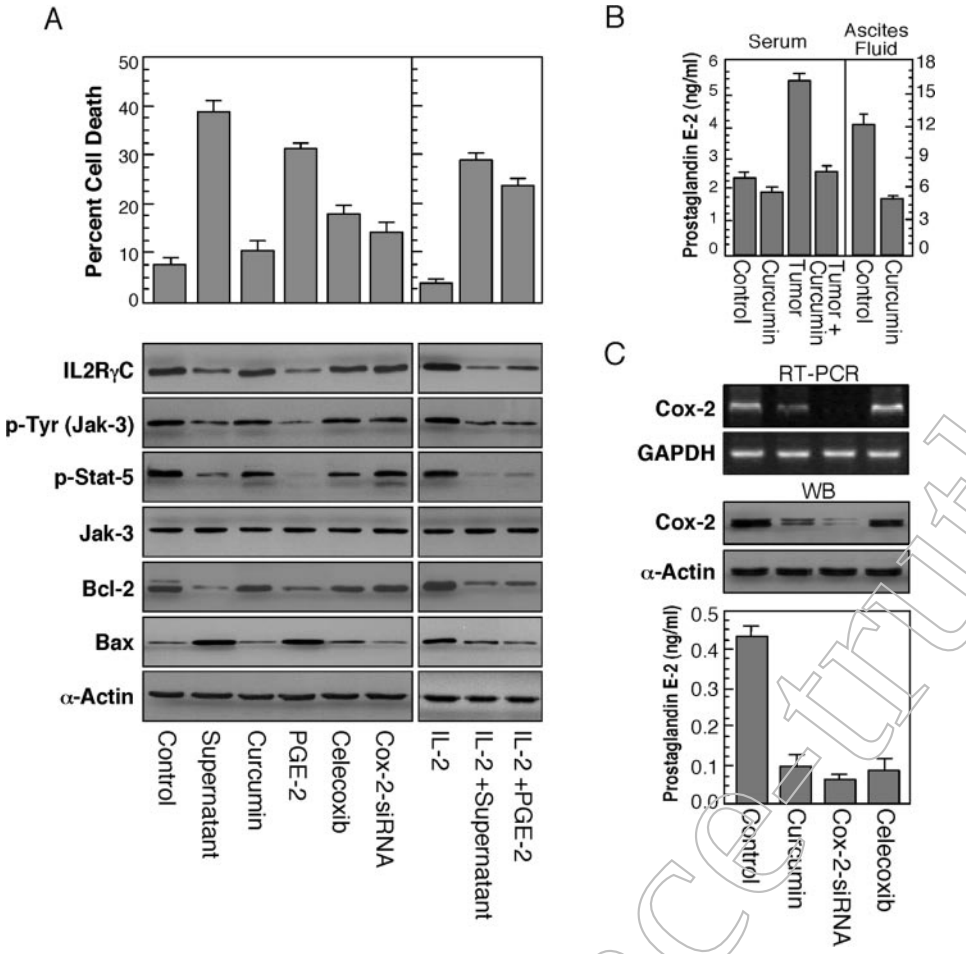

FIGURE 5. **Curcumin inhibits tumor-secreted prostaglandin E_2-mediated perturbation of Jak-3/Stat-5 signaling to restore survival pathway in T cells.** *A*, T cells were cultured with untreated, 10 μM curcumin-treated, PGE_2, celecoxib-treated, or Cox-2-siRNA-transfected tumor-shed supernatants (in some cases in the presence of 1000 IU IL-2) for 48 h, followed by flow cytometric determination of percent cell death (*upper panel*). In parallel experiments, phosphorylation statuses of Jak-3/Stat-5 as well as expression levels of IL2Rγc/Bcl-2/Bax were determined by co-immunoprecipitation and Western blotting. α-Actin was used as internal control (*lower panel*). *B*, PGE_2 levels in serum and ascites fluid from untreated and curcumin-treated mice were determined by ELISA. *C*, ascites carcinoma cells were treated with 10 μM curcumin, 50 μM celecoxib, or transfected with Cox-2-siRNA, and levels of Cox-2 and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (internal control) mRNA were determined by reverse transcription (*RT*)-PCR (*upper panel*). Western blot analysis (*WB*) was performed for the determination of levels of Cox-2 or α-actin (internal control) proteins (*middle panel*). In parallel experiments, the amount of tumor-secreted PGE_2 in the cell-free supernatant was determined by ELISA (*lower panel*). Values are mean ± S.E. of three independent experiments.

tants not only contained relatively less amounts of PGE_2 but also failed to induce T cell death (Fig. 5, *C* and *A*) or down-regulate γc expression and Jak-3/Stat-5 phosphorylation (Fig. 5*A*). Thus our experiments indicate that prevention of PGE_2 secretion by tumor cells via down-regulation of Cox-2 by curcumin can be the cause behind aversion of down-regulation of γc expression and Jak-3/Stat-5 phosphorylation, and that curcumin offered the better protection to T cells from tumor-induced death in comparison with either celecoxib or Cox-2-siRNA (Fig. 5*A*). These results suggest that tumor-induced T cell death might have other mediators along with PGE_2, and overall inhibition of normal cellular function of tumor cells by curcumin can be the cause behind the observed immunoprotection by this compound, thus allowing us to predict a model for curcumin action (Fig. 6).

DISCUSSION

It is well accepted that T lymphocytes play crucial role in the immune response of the host to cancer (22). Modern cancer immunotherapies are therefore designed to induce or enhance T cell reactivity against tumor antigens (23). Evidence indicates that many tumors induce T cell apoptosis as a mechanism of inhibiting anti-tumor activity (24). Recent studies suggest that human carcinoma cells of various origins can activate intrinsic programmed cell death in lymphocytes interacting with the tumor *in vivo* and *in vitro* (25–27). This tumor-induced apoptosis of lymphocytes may have important implications for the success of therapeutic regimens, including vaccination strategies (7). Because tumor-induced apoptosis of lymphocytes may be mediated by an array of death receptors co-expressed on T cells or by tumor-derived soluble factors, it is important to characterize those intracellular events that may be potential targets for therapeutic intervention to minimize T cell apoptosis. Earlier we reported (16–18) that curcumin induces apoptosis in cancer cells. In this study, we demonstrate that curcumin treatment to the tumor-bearing mice improves the status of $CD4^+$ and $CD8^+$ T lymphocytes in peripheral blood and restricts tumor-induced loss of thymic T cells, which is the prime source of effecter $CD4^+/CD8^+$ cells in the circulation.

Our study shows increased apoptosis among the $CD4^+$ and $CD8^+$ effecter cells in the peripheral blood of tumor-bearing mice, which might be the result of increased loss of $CD4^+8^+$ double positive and effecter $CD4^+/CD8^+$ single positive cells in the thymus of tumor-bearing animals. We observed accumulation of $CD4^-CD8^-$ double negative cells with a simultaneous decrease in the number of $CD4^+CD8^+$ double positive cells in the thymus of tumor-bearing animals. Depletion of $CD4^+8^+$ double positive cells in the thymus of tumor-bearing animals along with increased spontaneous apoptosis of circulating T cells resulted in $CD4^+/CD8^+$ effecter population loss in circulation, thereby weakening the cellular defense mechanism and inducing thymic atrophy. These observations are supported by reports indicating a block in thymic maturation (2, 28, 29) and a loss of circulating $CD8^+$ cells because of spontaneous or

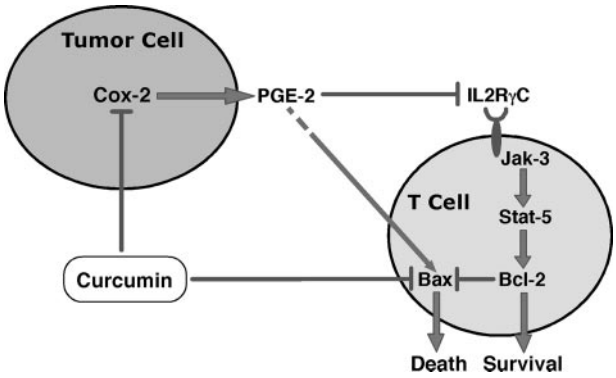

FIGURE 6. **Schematic diagram showing the effect of curcumin on tumor-induced perturbation of the Jak-3/Stat-5 signaling pathway.** Curcumin prevents tumor-induced T cell death by normalizing Jak-3/Stat-5 activity via restoration of IL-2RγC expression through the reduction of PGE_2 synthesis by Cox-2.

induced apoptosis in cancer patients (6, 30). Our study suggests that ascites carcinoma-mediated thymic atrophy and subsequent loss in thymic as well as circulating effecter T cells were because of both increased apoptosis of progenitor and effecter T cells and block in maturation as indicated by accumulation of double negative immature cells in the thymus.

We observed increased cytochrome *c* release and activation of caspase-3 in T cells of tumor-bearing animals indicating activation of apoptotic cascades in those cells. Interestingly, curcumin restored circulating $CD4^+$ and $CD8^+$ effecter populations and prevented apoptosis of the thymic T cells. The mechanism behind curcumin-induced restoration of circulating effecter T cells and thymic $CD4^+/8^+$ single positive and $CD4^+8^+$ double positive cells includes the restoration of the Bcl-2:Bax ratio. Bcl-2 acts as an anti-apoptotic protein and prevents pro-apoptotic Bax to form homodimerization. Loss of *Bcl-2* gene expression causes inefficient lymphopoiesis and death of thymocytes (32). Thymic and peripheral blood T lymphocytes of tumor-bearing animal showed marked decrease in Bcl-2 expression and moderate increase in Bax expression thus disrupting the normal Bcl-2:Bax ratio in T cell microenvironment. Observations of Gratiot-Deans *et al.* (33) and Linette *et al.* (34) suggest the important role of Bcl-2 in T cell maturation and survival both during progenitor and effecter T lymphocyte stage. Bcl-2 is up-regulated during positive selection in double positive T cell progenitors, establishing Bcl-2 as a crucial factor that helps in the survival and selection of progenitors and effecter T cells (33, 34). In T cells of tumor-bearing animals, curcumin restored Bcl-2 expression and normalized the Bcl-2:Bax ratio. Interestingly, we observed that T cells overexpressing Bcl-2 were more resistant to tumor supernatant-induced apoptosis than empty vector-containing cells, whereas Bax-overexpressed cells became more vulnerable to tumor-induced death, and curcumin administration could not protect these cells, indicating that alteration of the Bcl-2:Bax ratio plays a crucial role in tumor-mediated T lymphocyte killing.

Because it is well documented that Jak/Stat signaling molecules are critical regulators of the Bcl-2 family of proteins in T cells, we investigated the status of various Jak/Stat signaling pathways to find out the mechanism by which curcumin restored the Bcl-2:Bax ratio in T lymphocytes. We observed that out of the two Jak proteins (Jak-1 and Jak-3) and three Stat proteins (Stat-3, Stat-5, and Stat-6) responsible for Bcl-2 induction, only Jak-3 and Stat-5a (and to a minimal extent Stat-5b) phosphorylations were down-regulated in the T cells by tumor insult. These observations are consistent with reports that indicate impaired Jak-3/Stat-5 activation in ovarian and renal cell carcinoma supernatant-treated lymphocytes (9, 35). We observed that predominantly Stat-5a overexpression protects T cell from tumor-induced apoptosis, whereas Stat-5b transfection offers only moderate protection. Expression of constitutively active Stat-5a1*6 completely abrogates T cell death induced by tumor supernatant. In contrast, transfection with only C-terminal truncated Stat-5a renders T cells completely susceptible to tumor supernatant-induced apoptosis that could not be protected by curcumin. Studies with Jak-$3^{-/-}$ knock-out mice showing impaired T cell development and severe down-regulation of Bcl-2 protein in T cell populations with a moderate increase in Bax expression (11, 36, 37) strengthen our hypothesis that impaired Jak-3/Stat-5a signaling is responsible for tumor-induced down-regulation of Bcl-2 expression and altered Bcl-2:Bax ratio that eventually causes thymic and circulatory T lymphocyte death. Curcumin could efficiently restore phosphorylation and activation of Jak-3/Stat-5.

It is acknowledged that the cytokine receptor complexes (such as IL-2R, IL-4R, and IL-7R) contain a common γc as a component that plays an important role in mediating signals through the Jak-3/Stat-5 pathway in T cells (38). Tumor burden significantly down-regulated γ chain expression in T cells and that could be efficiently restored back to normal levels by curcumin. Kolenko *et al.* (35) also reported similar phenomena where experiments with renal cell carcinoma culture supernatant showed partial blockage of IL-2R components, including that of common γ chain expression in naive T cells. Our results indicate that impaired IL-2RγC expression leads to hypo-phosphorylation of Stat-5 in T cells of tumor-bearing animals, resulting in disruption of Bcl-2 expression.

Our observation of tumor-induced T cell death along with reduced expression of IL2Rγ chain and subsequent down-regulation of survival signaling prompted us to explore the possibility of some tumor-secreted substance that caused the observed immune depletion. Our data and other reports as well (39, 40) demonstrate that tumor cells produce PGE_2, which is known to be cytocidal for immunocytes (41, 42). Kolenko *et al.* (21) also reported that PGE_2 can down-regulate the expression of γc expression and Jak-3 activity in T cells. In conformity with their results, we observed that PGE_2 can down-regulate T cell survival, γc expression, and Jak/Stat signaling. Our study reveals that the effect of growing tumors on host T cells bears remarkable similarities with the effects of PGE_2 exposure on T cells. Curcumin administration to tumor-bearing animals reduces T cell PGE_2 exposure via down-regulation of Cox-2 in tumor cells. Further support for these results came from the experiments that exploited the use of RNA interference of Cox-2 in tumor cells or treatment of

these cells with the specific Cox-2 inhibitor celecoxib, in both the cases tumor-shed supernatants failed to induce T cell death, Jak-3/Stat-5 hypo-phosphorylation, and reduction in γc expression in T cells. Interestingly, although both celecoxib and Cox-2-siRNA could efficiently block T cell death or restore T cell signaling, treatment of tumor cells with curcumin provides better protection for T cells, indicating the involvement of other factors that can also be down-regulated by curcumin and do not depend on Cox-2 status directly.

Dietary supplements with profound antioxidant property such as vitamin E is known to modulate the immune system of tumor-bearing hosts, and vitamin E can promote IL-2 and IFN-γ production in T cells and favors a Th-1 response (43). Curcumin is a known antioxidant (44, 45) and is known to inhibit Cox-2 activity (46). On the basis of this prior information, our results provide a model for the curcumin action, portraying that the known Cox-2 inhibitory properties of curcumin blunts the PGE_2 production by tumor cells, which eventually prevents the tumor-induced down-regulation of T cell γc expression, Jak-3/Stat-5 hypo-phosphorylation, and subsequent death of T cells, although involvement of NFκB cannot be ruled out completely as it is known to control nearly 150 genes that include cytokine genes (47) and can be inhibited by PGE_2 (31).

Overall our results suggest that curcumin prevents tumor-induced T cell depletion both in primary and effecter immune compartments of the host by normalizing Jak-3/Stat-5 activity via restoration of γc expression through reduction in PGE_2 exposure. From these observations it is suggested that curcumin may be used alone or can be combined with classical anti-tumor drugs in order to sustain the immune capacity of the host, which can be affected by the disease or the treatment or perhaps both.

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Curcumin Prevents Tumor-induced T Cell Apoptosis through Stat-5a-mediated Bcl-2 Induction

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