Cargo from Tumor-Expressed Albumin Inhibits T-Cell Activation and Responses

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ABSTRACT

In this study, we show that rodent albumin is expressed by and cell surface localized on at least some murine tumor cells. We have been able to purify this tumor-expressed albumin from in vivo grown tumor masses. The tumor-expressed albumin, unlike normal serum albumin purified from blood, is capable of inhibiting T-cell activation, proliferation, and function in both in vitro and in vivo settings. Tumor-expressed albumin does not appear to affect antigen processing or presentation by professional antigen-presenting cells. The activity appears to lie in relatively small, lipid-like moieties that are presumably cargo for tumor-expressed albumin, and that activity can be removed from the albumin by lipid removal or treatment with lipase. Thus, we herein report of a novel form of tumor-induced immune suppression attributable to lipid-like entities, cloaked by albumin produced by tumors.

INTRODUCTION

Tumor-induced immune suppression is one of the many causes whereby anticancer immunotherapeutic modalities may fail (1–4). Tumors may escape circulating cytotoxic leukocytes by numerous mechanisms; down-regulating cell surface expression of MHC class I molecules (5); insufficient antigen display via selection for cellular variants that no longer express a particular antigen (6–8); antigen-processing defects (9, 10); and tumor-induced immune suppression by secretion of cytokines/factors, such as transforming growth factor-β (TGF-β), interleukin-10 (IL-10), granulocyte macrophage-colony stimulating factor, and vascular endothelial growth factor (11–18). Identifying and characterizing tumor-induced immunosuppression is necessary to enhance responses to immunotherapy.

We have successfully used tumor-derived chaperone protein vaccines against aggressive murine tumors using chaperone-rich cell lysates. Chaperone-rich cell lysate vaccines consist of a conglomerate of chaperone/heat shock proteins enriched from cell lysates that form a high molecular weight complex after free solution-isoelectric focusing. Among the known immunogenic chaperones, chaperone-rich cell lysate contains GRP94/gp96, heat shock protein 90, heat shock protein 70, and calreticulin (19–21), as well as numerous other chaperones and unidentified polypeptides. Consistent with the efficacy of such chaperone vaccines as peptide traffickers, chaperone-rich cell lysate contains many peptides,1 and proteomic results indicate that heat shock proteins and albumin constitute a portion of the vaccine.4 Chaperone-rich cell lysate vaccine dose escalation studies demonstrated an abrogation of the protective effects of vaccines at doses >20 μg (19). We speculated that this immune suppression might be attributable to the presence of an inhibitor reaching active concentrations at higher vaccine doses; our modified vaccine preparations functionally exclude this presumed inhibitory factor, as demonstrated by recent chaperone-rich cell lysate dose escalation experiments (21). One of the prominent (and tenaciously copurifying) proteins in our early vaccine preparations (22) was albumin, which we believed was actually expressed by the tumors themselves. Thus, we had encountered, purified, and identified albumin previously during chaperone purifications. We considered that this tumor-expressed albumin might harbor a TGF-β-like activity that could exhibit immune inhibition (23–26), thus facilitating tumor progression. Western blots of purified tumor-expressed albumin demonstrated reactivity of that protein with anti-TGF-β antibodies. We tested the ability of tumor-expressed albumin to inhibit immune function in numerous immunologic assays, and it was indeed capable of suppressing T-cell activation and function. We also discovered that normal (commercially available) murine serum albumin displayed anti-TGF-β immunoreactivity on Western blots; however, normal murine serum albumin had no suppressive effects in our immunologic assays. Thus, the inhibition did not track with the TGF-β immunoreactivity, but there was nonetheless an immunosuppression exerted by tumor-expressed albumin. Therefore, we examined other aspects of tumor-expressed albumin, such as structure and cargo, in search of the immune inhibitory activity of tumor-expressed albumin, and were able to use murine serum albumin as a control. Why these albumins react with anti-TGF-β antibodies and the biological relevance of that observation remains a mystery.

Here, we report the purification of tumor-expressed albumin from A20 lymphoma and 12B1 leukemia cells, its identification as albumin, and its demonstration of immune inhibitory effects both in vitro and in vivo. Commercially available, “normal” murine serum albumin had none of these inhibitory effects. We additionally refined the immune inhibitory activity of tumor-expressed albumin to a relatively small, lipophilic entity that appears to be noncovalently associated with tumor-expressed albumin. This activity is not associated with normal murine serum albumin, leading us to speculate that tumors may use the transport capacity of albumin as a means of delivering immune inhibitory molecules into the tumor microenvironment to protect the tumor from responding lymphocytes. To our knowledge, this represents a novel form of tumor-induced immune suppression via what would ordinarily be seen as an ordinary and unobtrusive molecule, albumin.

MATERIALS AND METHODS

Mouse Strains and Cell Culture. BALB/c and C57BL/6 mice (4–8 weeks) were obtained from The Jackson Laboratory (Bar Harbor, ME) or the National Cancer Institute (Frederick, MD). Mice were housed in microisolation in a dedicated, pathogen-free facility at the University of Arizona, and all of the animal experimentation was conducted under protocols approved by the University of Arizona Institutional Animal Care and Use Committee. Mure

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chronic myelogenous leukemia model (12B1) and murine B-cell lymphoma (A20) cells have been grown in vitro and in vivo, used, and described by us previously (19, 20, 22, 27, 28). CTL-2 (American Type Culture Collection, Rockville, MD) is an interleukin (IL) 2-dependent T-cell line that was routinely grown in complete RPMI (22, 27), supplemented with 300 units/mL IL-2 (Peprotech, Rocky Hill, NJ). DO-11.10 (obtained from Dr. Ken Rock, University of Massachusetts Medical Center, Worcester, MA) is a T-cell hybridoma that recognizes the chicken ovalbumin epitope (OVA23–323).

Analytical Biochemical Methods. SDS-PAGE and Western blotting were performed as described (22). Murine serum albumin antibody (UCB-249/RSH) was purchased from Accurate Chemical and Scientific (Westbury, NY). TGF-β antibodies (sc-146, sc-90, and sc-83 against TGF-β 1, 2, and 3, respectively) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Matrix-assisted laser desorption-ionization/time-of-flight mass spectrometry was performed on tumor-derived, purified albumin and on commercially available murine serum albumin (Sigma, St. Louis, MO) at the University of Arizona’s Chemistry Department Mass Spectrometry Facility.

For NH₃-terminal amino acid sequencing, purified protein was electrophoresed on 10% SDS-PAGE and electrotransferred to a polyvinylidene difluoride membrane (Sequi-Blot polyvinylidene difluoride, Bio-Rad, Hercules, CA) in 10 mmol/L 3-[cyclohexylamino]-1-propanesulfonic acid and 10% methanol (pH 11.0). The protein was visualized with Ponceau Red, excised, and submitted to the University of Arizona Biotechnology Facility for automated NH₃-terminal amino acid sequencing.

Tumor-Expressed Albumin Purification. Tumor-expressed albumin was purified from 12B1 and A20 tumor tissue, and from normal BALB/c murine liver, as follows: frozen tumor was ground to a fine powder in a liquid nitrogen-chilled mortar, and the powder was extracted overnight at 4°C in homogenization buffer as described (19, 22) at a ratio of 5 mL buffer/g tissue. The homogenate was centrifuged at 10,000 × g, 4°C, for 30 minutes. Alternatively, high-speed supernatants were prepared as described previously (19, 22), but this resulted in reduced yields. The supernatant was made to 50% and then 90% ammonium sulfate saturation. The precipitated materials from the 90% cut were harvested by centrifugation; the pellet was resuspended in water and dialyzed extensively against water. After dialysis, insoluble materials were removed by centrifugation, and the supernatant was quantified (BCA assay, Pierce Endogen, Rockford, IL.).

Precipitated protein (10 mg) was prepared for isoelectric focusing in a Rotofor device (Bio-Rad) by combining the sample with a mixture of pH gradient buffer pairs followed by isofocusing as described previously (20, 21). Fractions of interest were identified by SDS-PAGE and Western blotting as described above.

For strong anion exchange chromatography, after isofocusing, fractions were pooled and dialyzed into 10 mmol/L Tris acetate/10 mmol/L NaCl (pH 7.1), 1 mmol/L EDTA. The protein pool was chromatographed over a Hi Trap Q column (Amersham Biosciences, Piscataway, NJ) in 20 mmol/L Tris-acetate/20 mmol/L NaCl (pH 7.1), 1 mmol/L EDTA (Buffer 20/20), and eluted in a linear salt gradient. Fractions were analyzed by SDS-PAGE and Western blotting. Fractions containing pure protein were dialyzed against water and concentrated either with Centricron devices (Millipore, Bedford, MA) or by vacuum centrifugation (Speed Vac, Thermo Savant, Farmingdale, NY).

In some cases, tumor-expressed albumin was purified after isoelectric focusing by Cibacron Blue 3GA chromatography. Proteins were dialedyzed into Buffer 20/20 and chromatographed over the affinity ligand column. Proteins were eluted by stepwise increases in NaCl concentrations, followed by a stepwise increase in NaSCN. Fractions were analyzed as described above. To verify that our purification procedure did not engender immunosuppression in normal albumin, commercially available mouse serum albumin was repurified, starting with the Rotofor-free solution-isoelectric focusing by Cibacron Blue 3GA chromatography. Proteins were dialyzed into 10 mmol/L Tris acetate/10 mmol/L NaCl, placed on ice for 15 minutes, and then microcentrifuged (15,000 × g, 15 minutes, 4°C). The clarified cell lysates were precleared with Protein A/G agarose beads (Pierce Endogen). Anti-serum albumin antibody, specific for murine serum albumin, was added to the cleared lysate (1:500). Negative control antibody (rabbit antimonuse IgG, Sigma) was added to other reactions. Antibody incubations were performed for 1 hour on ice, after which washed Protein A/G agarose beads were added for 2 hours. Antibody/antigen/Protein A/G complexes were recovered by centrifugation as described above. Pellets were resuspended in SDS-PAGE sample buffer and heated to 100°C for 5 minutes. Electrophoresis and Western blotting were conducted as described above.

In vitro Immune Response Assays. Mixed lymphocyte reactions were performed as described (21), using BALB/c splenocytes as stimulators. Splenocytes were cocultured in the presence of increasing quantities of tumor-expressed albumin, and 1 μCi [3H]thymidine (ICN Pharmaceuticals, Costa Mesa, CA) was added for an additional 18 hours. Proliferation was measured as described (21).

Antigen presentation by dendritic cells to T cells in the presence of tumor-expressed albumin or murine serum albumin was assessed by IL-2 secretion from DO-11.10 T cells after incubation with dendritic cells pulsed with ovalbumin protein. Dendritic cells were prepared as described (21); dendritic cells were pulsed with chicken ovalbumin (Sigma) at 1 mg/mL overnight and then coincubated with DO-11.10 cells for 24 hours. Supernatants were harvested, and IL-2 concentrations were determined by CTTL-2 biosassay as described (27). For experiments involving tumor-expressed albumin inactivation of T cells, CTTL-2 cells were grown in varying amounts of IL-2 and/or varying amounts of tumor-expressed albumin or murine serum albumin. Proliferation was measured by [3H]thymidine incorporation as described (27).

Murine (BALB/c) splenocytes were harvested, washed, and plated (100,000–200,000 cells/well) on 96-well culture plates that were coated with anti-(murine) CD3+ antibodies (Becton Dickinson, Bedford, MA) or on control plates (no antibody) in the presence of increasing quantities of tumor-expressed albumin or murine serum albumin. Proliferation was measured as described (28) or by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2–9-sulfophenyl)-2H-tetrazolium assay (CellTiter 96 Aqueous One Solution, Promega Corp., Madison, WI) per the manufacturer’s instructions.

In vivo Immune Response Assays. ELISPOTs measured IFN-γ production from restimulated splenocytes derived from vaccinated mice as described (20, 21). Splenocytes harvested from vaccinated mice (dendritic cells pulsed with 12B1 chaperone-rich cell lysates) were restimulated with 12B1 chaperone-rich cell lysates in the presence or absence of tumor-expressed albumin or murine serum albumin. After washing, detection antibody was added and incubated, and spots were detected with colored substrate deposition, followed by microscope-aided counting.

For in vivo tumor rejection assays, chaperone-rich cell lysate vaccines were prepared from 12B1 tumor as described previously (20, 21). Groups of 4 mice were vaccinated on days −14 and −7 with 20 μg of 12B1-derived chaperone-rich cell lysate vaccine, with 12B1 vaccine +20 μg of tumor-expressed albumin (injected 15 minutes later near the initial site of vaccination), with 12B1 vaccine +20 μg murine serum albumin (performed as with tumor-expressed albumin), murine serum albumin alone, tumor-expressed albumin alone, or with saline. 12B1 tumor challenge (10,000 viable cells) was given subcutaneously on day 0. Tumor volumes were monitored as described (19).

Enzymatic Treatments of Albumins. Albumins were exhaustively digested with Protease K (PCR-Grade, Roche) at a 1:10 (wt/wt) enzyme to substrate ratio. Samples were incubated in 50 mmol/L Tris-Cl (pH 7.7) at 37°C overnight. Albumins or Protease K fragments of albumins were treated with lipase (EC 3.1.1.3, Sigma) at a ratio of 7.5 μg of protein per unit of lipase under the same conditions.
Reactions were spun through $M_i$ 3,000 Centricon membranes, and the flow-through materials were used in T-cell proliferation assays as described above.

**Extraction of Lipid Fractions from Albumin.** Albumin-bound lipids were extracted from tumor-expressed albumin and murine serum albumin using a Lipidex column (hydroxyalkoxypropyl dextran, Sigma) as described (29). The lipid-depleted serum albumin was collected in the column flow-through, concentrated to dryness, washed in sterile water, and again dried. The protein was then resuspended in media for use in splenocyte assays using plated anti-CD3+ antibodies as described above. The lipid fraction was eluted in high-performance liquid chromatography-grade methanol (IC Baker, VWR, Tempe, AZ) and treated identically to the protein; however, a contaminant coeluting from the column with the lipids was found to be cytotoxic (even in the absence of applied protein).

**Statistical Analyses.** Differences between groups were assessed statistically using the Student t test, with significance defined as $P < 0.05$. All of the experiments were performed at least twice.

**RESULTS**

**Biochemical Purification and Identification of Tumor-Expressed Albumin.** Our early dose-titration studies using tumor-derived chaperone-rich cell lysate vaccines resulted in a loss of vaccine activity at higher doses (19). Those results prompted us to consider that our vaccine preparations might harbor an immune inhibitor of which the activity became evident at higher vaccine doses.

One logical candidate was TGF-β, a multipotent growth factor with immunosuppressive properties (14–16), which had also been demonstrated to be transported by serum immunoglobulins (30, 31). We separated A20 lymphoma tumor cell lysate via isoelectric focusing as if preparing chaperone-rich cell lysate vaccines (19–21), and we probed Western blots of those fractions with antibodies against TGF-β isoforms 1, 2, and 3. We saw no reactivity in the molecular weight range expected for active TGF-β under reducing SDS-PAGE conditions (i.e., $M_i$ 12,500; data not shown). However, antibodies to TGF-β3 reacted with a $M_i$ 66,000 protein in those blots (Fig. 1A) in the fractions we would use as chaperone-rich cell lysate vaccines. Using TGF-β3 immunoreactivity as our guide, we purified the protein from in vivo grown tumors (Fig. 1, B and C). We obtained soluble proteins (see Materials and Methods for details) and separated them via isoelectric focusing (Fig. 1B). Additional chromatographic purification on the affinity resin Cibacron Blue 3GA (Fig. 1C) led us to believe that the protein was an albumin, as verified by Western blotting with antibodies specific for murine serum albumin (Fig. 1C, bottom panel). NH$_2$-terminal amino acid sequencing of pooled, purified protein confirmed the identity of the protein (Fig. 1D). Both the signal peptide and pro-albumin sequences were absent, implying that the protein was fully processed to its secretable form by the time it was purified. Mass spectrometry of the intact protein also revealed a...
nearly identical mass to that of commercially available murine serum albumin (65,929 mw to 65,805 mw respectively; data not shown). We have also obtained the same results with albumin purified from 12B1 leukemia tumor cells. Because of its relative abundance (~100 μg/g tumor), we speculated that this albumin was expressed by the tumor (henceforth referred to as tumor-expressed albumin). We speculated that the protein could be transporting an immunologically active fragment of TGF-β as a form of tumor-induced immune suppression.

12B1 and A20 Tumors Express Albumin. The amount of purified albumin seemed unusually large if the protein came solely from the blood supply of the tumor. We thus speculated that albumin was being expressed by tumor cells themselves and demonstrated that by analyzing 12B1 and A20 cells grown in tissue culture in fetal calf serum (i.e., no blood supply, no exogenous source for murine albumin) for albumin content. We initially tested for the presence of murine albumin by immunoprecipitation of 12B1 cells (and A20 cells; data not shown) and the spent media from the culture, with a murine albumin-specific antibody. As seen in Fig. 2A, Western blots of the immunoprecipitated tissue culture cell lysates indicated that large quantities of albumin were obtained from those cells, as seen in the large smear of albumin in the right lane (Fig. 2A, IP Cells) of the top panel, which was also immunoreactive with antibodies to TGF-β3 (Fig. 2A, middle panel; the IP Cells Lane contains 10-fold less material than in the top panel). 12B1 lysate (Fig. 2A, 12B1 Lys, left lane) served as a positive Western blotting control. Immunoprecipitation of the spent media showed relatively little murine albumin (Fig. 2A, IP Media), which was also reactive with the TGF-β3 antibody (Fig. 2A, middle panel). The antibody is specific for murine albumin, and it neither immunoprecipitates nor cross-reacts with the bovine serum albumin present in the fetal calf serum in the tissue culture media (Fig. 2A, bottom panel). The paucity of murine albumin in the media implied that the tumor-derived albumin was either not readily secreted into the media or was being taken back up by the tumor cells. We examined the second possibility by flow cytometry of in vitro grown cells. As seen in Fig. 2B (middle panel), A20 cells (and 12B1 cells; data not shown) have murine albumin bound to their cell surfaces (Fig. 2B, top panel, a negative control immunostaining; bottom panel, a positive control for cell surface MHC class II). We suggest that the albumin must come from the tumor cells themselves, and we feel justified in referring to it as tumor-expressed albumin. As controls, we used DO-11.10 cells grown in cell culture; we could immunoprecipitate only minuscule amounts of albumin, and the cells displayed little albumin on their surfaces (data not shown).

Tumor-Expressed Albumin Inhibits T-Cell Activities in In vitro Assays. We used tumor-expressed albumin as an additive to various immune response proliferation/activation assays to determine whether tumor-expressed albumin had immunosuppressive effects at the T-cell level. By this time, we realized that tumor-expressed albumin and normal murine serum albumin both reacted with anti-TGF-β3 antibodies, and we used commercially available murine serum albumin in the same assays at the same concentrations. Mixed lymphocyte reactions, in which responder C57BL/6 splenocytes demonstrated the expected proliferation when cocultured with mitomycin C-treated BALB/c stimulator spleen cells, displayed an increase in proliferation at low levels of exogenous tumor-expressed albumin. However, at higher tumor-expressed albumin concentrations, responder cell proliferation was dramatically reduced to background levels. Proliferation of responder splenocytes remained unaffected by exogenously added murine serum albumin (Fig. 3A). The decreased proliferation was not attributable to cytotoxicity of the tumor-expressed albumin preparation, as measured by trypan blue exclusion (data not shown). The lack of suppressive activity of murine serum albumin led us to question the hypothesis that TGF-β was playing a role in the immune response in this assay; however, there clearly was immune suppression exerted by tumor-expressed albumin, so we continued to examine this property of tumor-expressed albumin in other immune assays, using murine serum albumin as a control.

To determine whether tumor-expressed albumin interfered with antigen presentation, we incubated bone marrow-derived dendritic cells in the presence of increasing concentrations of tumor-expressed albumin or murine serum albumin along with ovalbumin protein. Dendritic cells were then washed before coincubation with OVA peptide-responsive DO-11.10 cells, and IL-2 secretion from the T cells was measured by CTLL-2 bioassay, in which CTLL-2 cell proliferation in response to IL-2 was measured by tritiated thymidine incorporation (see below). As seen in Fig. 3B, there was no significant decrease in DO-11.10 IL-2 output after those cells had been stimulated by dendritic cells pulsed with OVA protein while being exposed to tumor-expressed albumin (that was washed out before incubation with the T cells). Thus, tumor-expressed albumin appeared to have no effect on antigen presentation at least by dendritic cells, for that particular antigen/T-cell combination, and with washout of the tumor-expressed albumin before coincubation of the dendritic cells and T cells. Murine serum albumin also had no effects on dendritic cell-stimulated IL-2 release by DO-11.10 cells.

We next studied whether tumor-expressed albumin affected stimulation of CTLL-2 cell proliferation by IL-2. As shown in Fig. 4A,
tumor-expressed albumin in a dose-dependent manner inhibited proliferation of CTLL-2 cells grown in saturating concentrations of IL-2. To examine whether T cells harvested from naïve animals were similarly prevented from activation, we harvested splenocytes from normal, naïve mice and applied them to plates coated with anti-CD3 + antibody. Fig. 4B shows that tumor-expressed albumin addition inhibited anti-CD3-stimulated proliferation of naïve splenocytes and that murine serum albumin had no such inhibitory effect. Cultured murine fibroblasts proliferate normally in the presence of exogenous murine serum albumin or tumor-expressed albumin (data not shown).

Tumor-Expressed Albumin Inhibits Immune Responses Generated In vivo. We next asked whether tumor-expressed albumin could influence immune responses that occurred in vivo in a vaccine setting. In one set of experiments, mice were vaccinated with chaperone-rich cell lysate anticancer vaccines derived from 12B1 tumors, under conditions we have described previously (19–21). Seven days after the second weekly vaccination, splenocytes were harvested from the immunized mice and restimulated with 12B1 tumor-derived chaperone-rich cell lysate +/− tumor-expressed albumin. ELISPot spots (20, 21) measured IFN-γ production. Adding tumor-expressed albumin at the restimulation phase greatly reduced the number of IFN-γ spots, indicating that in vivo primed T cells could be inhibited from producing cytokine on restimulation with antigen (Fig. 5A). Murine serum albumin had no such inhibitory effect (Fig. 5A).

In addition, we examined the effect of exogenous tumor-expressed albumin on chaperone-rich cell lysate vaccine efficacy in a tumor rejection assay. Groups of mice were vaccinated with 12B1-derived chaperone-rich cell lysate as described previously (20, 21). Mice were immunized with 20 μg of chaperone-rich cell lysate at weekly intervals; some mice were also given 20 μg of 12B1-derived tumor-expressed albumin (or murine serum albumin). Control groups received PBS, chaperone-rich cell lysate alone, or tumor-expressed albumin or murine serum album alone. All of the mice received equal numbers of injections, using PBS as a vehicle control. One week after the second immunization, mice were inoculated with 10,000 12B1 tumor cells (a 10-fold lethal dose), and tumor volumes were monitored thereafter. As seen in Fig. 5B, mice that were unimmunized or mock treated with either of the albumins alone grew tumors at similar rates; tumor growth was significantly delayed in mice vaccinated with chaperone-rich cell lysate, as expected, and injections of murine serum albumin did not compromise the vaccine effect. However, in mice that were vaccinated with chaperone-rich cell lysate but that were also injected with tumor-expressed albumin, tumors grew at rates comparable with control (PBS injected) mice, indicating that the tumor-expressed albumin was capable of abrogating the potent chaperone-rich cell lysate antitumor effect. We have demonstrated previ-
Fig. 5. TEA inhibits in vivo activated T-cell responses. In A, TEA reduces IFN-γ production by in vivo activated T cells. Mice were vaccinated twice at weekly intervals with 12B1 tumor-derived CRCL vaccines (or mock vaccinated with saline, PBS), and their spleens were harvested 1 wk later. Splenocytes were plated and restimulated with 12B1 CRCL vaccine material with or without exogenous TEA or MSA added (50 µg/mL). Enzyme-linked immunospot assays were performed as described in Materials and Methods. TEA reduced IFN-γ output from vaccinated/restimulated splenocytes significantly compared with MSA (P < 0.0001). In B, exogenous TEA abrogates vaccine effects of 12B1 CRCL anticancer vaccines. Mice (4/group) were vaccinated at weekly intervals (i.e., day −14 and day −7) with 20 µg of 12B1 CRCL vaccines (or mock vaccinated with saline, PBS), followed by injections of equal amounts of TEA or MSA or equal volumes of PBS. On day 0, mice were challenged with 10,000 viable 12B1 leukemia cells, and tumor volumes were monitored thereafter. Tumor volumes in mice immunized with CRCL alone versus mice immunized with TEA plus CRCL differed significantly from day 16 onward (P < 0.03). (MSA, murine serum albumin; TEA, tumor-expressed albumin).

Figure 5: TEA inhibits in vivo activated T-cell responses. In A, TEA reduces IFN-γ production by in vivo activated T cells. Mice were vaccinated twice at weekly intervals with 12B1 tumor-derived CRCL vaccines (or mock vaccinated with saline, PBS), and their spleens were harvested 1 wk later. Splenocytes were plated and restimulated with 12B1 CRCL vaccine material with or without exogenous TEA or MSA added (50 µg/mL). Enzyme-linked immunospot assays were performed as described in Materials and Methods. TEA reduced IFN-γ output from vaccinated/restimulated splenocytes significantly compared with MSA (P < 0.0001). In B, exogenous TEA abrogates vaccine effects of 12B1 CRCL anticancer vaccines. Mice (4/group) were vaccinated at weekly intervals (i.e., day −14 and day −7) with 20 µg of 12B1 CRCL vaccines (or mock vaccinated with saline, PBS), followed by injections of equal amounts of TEA or MSA or equal volumes of PBS. On day 0, mice were challenged with 10,000 viable 12B1 leukemia cells, and tumor volumes were monitored thereafter. Tumor volumes in mice immunized with CRCL alone versus mice immunized with TEA plus CRCL differed significantly from day 16 onward (P < 0.03). (MSA, murine serum albumin; TEA, tumor-expressed albumin).

DISCUSSION

We have demonstrated herein that certain murine tumors express a mature form of albumin, which carries lipophilic moieties capable of inhibiting T-cell responses in vitro and in vivo assays. This tumor-expressed albumin assumes a cell surface localization in tissue culture cells; purified tumor-expressed albumin prevents proliferation of naïve splenocytes in mixed lymphocyte reactions and anti-CD3 activation assays but has no effect on proliferation of normal cell types (data not shown). Tumor-expressed albumin seemingly does not adversely affect the processes leading to antigen presentation to T cells, and tumor-expressed albumin overcomes saturating IL-2 conditions to prevent proliferation of CTL-L-2 cells. Tumor-expressed albumin can prevent the antigen restimulated secretion of IFN-γ from splenocytes primed by in vivo immunization, and tumor-expressed albumin can abrogate the antitumor effects of anticancer vaccines. Normal murine serum albumin, whether directly commercially obtained or repurified by our procedures, had none of these immune inhibitory effects, nor did that procured from normal mouse liver. The inhibitory effects of tumor-expressed albumin are protease insensitive but lipase sensitive,
and delipidation of tumor-expressed albumin also diminishes its immunosuppressive activity. It is conceivable that this lipid-like entity contributes to tumor escape from the immune response by driving quiescence in those T cells that come in contact with tumor-expressed albumin carrying the lipid as cargo. As such, this is a unique form of tumor-induced immune suppression in terms of the cloaking of the inhibitor in a generally innocuous protein.

We had encountered albumin as a component of tumor lysate previously during purification of individual chaperone proteins as anticancer vaccines (22). Thus, the presence of albumin in our tumor-derived chaperone-rich cell lysate vaccines was not entirely surprising but presumably bland. However, in vivo dosage escalation experiments with chaperone-rich cell lysate vaccines demonstrated loss of vaccinating potential at quantities >20 μg (19). We postulated the existence of an immune inhibitor within our vaccine and probed the protein content for TGF-β, discovering that murine serum albumin (both normal and tumor derived) reacts with anti-TGF-β3 antibodies. Additional experimentation led us to conclude that there was no clear link between the immune inhibition activity of tumor-expressed albumin and reactivity of albumin with anti-TGF-β antibodies. Nonetheless, that suppressive activity was present, as demonstrated in this study. We wish to point out that alterations in our isolectric focusing protocol (20, 21) have since eliminated the dose-limiting immune inhibition, making the immune suppressive contributions of excess GRP94 (33, 34) seem unlikely. The reactivity of the albums with TGF-β3 antibodies remains enigmatic; albumin may indeed carry a fragment of TGF-β3 (35), but the physiologic consequences are unclear. It does highlight the possibility that (unexpected) entities undetectable by common assays may contribute to the immunosuppression; e.g., minute quantities of IL-10 or prostaglandin E2 could be present with sufficient activity to affect the outcome of the T cell-based assays used here, but such molecules might be imperceivable via electrophoretic or chromatographic analyses.

Extrahepatic expression of albumin is rare and usually in the context of early vertebrate development (36–39). We do not know how extensive this phenomenon is in tumor tissues, but we have been able to purify appreciable amounts of albumin from three different types of in vivo grown murine tumors, including B16 melanoma.5 There have been reports of tumor uptake of serum albumin (40–42), as well as its use as an immunologically inert shroud to avoid immune detection (43). We assert the possibility that tumors may have co-opted serum albumin as a means of delivering T cell-inactivating substances into the tumor microenvironment as an active form of immunosuppression. Albumins purified from plasma and ascites fluid of cancer patients also display this immunosuppressive phenomenon.6 Thus, human tumors may have similar means of altering the immunologic landscape.

Although we do not yet know the identity of the albumin-carried substance, we believe it is a lipid moiety, possibly an acylglycerol, which would be a feasible cargo for serum albumin (32) and account for the lipase sensitivity of the immune inhibitory activity. The sum effect of the data from the Protease K treatments of tumor-expressed albumin, (Fig. 6A), delipidation of tumor-expressed albumin via Lipidex chromatography (Fig. 6B), and combined Protease K/lipase treatments of tumor-expressed albumin (Fig. 6C) point to the existence of a small, protease-insensitive/lipase-sensitive entity that can be removed from serum albumin by a matrix known to delipidate serum albumin. Recently, we have extracted lipids from albumins purified from plasma and ascites fluid of cancer patients, a far more abundant

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5 A. Likhacheva and M. Gruner, unpublished observations.
albumin source than murine tumors. The TLC lipid profiles from these albumins were quite complex compared with normal albumin (extensive amounts of cholesterol/cholesterol esters, mono-, di-, and triacylglycerols, relatively reduced amount of free fatty acids). These TLC-separated lipid components were harvested and reconstituted with lipid-free albumin; some of those complexes were antiproliferative in T-cell stimulation assays. The full characterization of these potentially immunosuppressive lipids is under way in our laboratory. We do not know how albumin is retained at the tumor cell surface nor in vivo how much cell surface-bound albumin is derived from tumor expression versus that taken up from the bloodstream. There may be specific receptors for albumin on tumor cell surfaces (44), which could bind the albumin secreted by the tumor, thus concentrating the T-cell inhibiting factor as a protective barrier against immune assault. Additionally, there may be albumin receptors on lymphocytes (45), and the release of tumor-expressed albumin into the blood stream of tumor-bearing animals may result in the systemic dissemination of the immunosuppressive agent. Experiments comparing the immune-suppressive effects of sera from animals with advanced tumor burden versus sera from healthy animals have demonstrated such immune inhibition.

In summary, we have identified albumin as a protein expressed by at least some murine hematologic malignancies; this protein harbors a relatively small, lipid-like immunosuppressive substance that can prevent the activation of naïve T cells, and also it can disengage activated T cells both in vitro and in vivo. Although the binding and transport of lipid payload is expected of serum albumin, tumors appear to have usurped this role of albumin for a defensive posture. It will be a challenge of anticancer therapies, particularly immunotherapy, to find ways to bypass, overcome, or control these mechanisms by which tumors fend off the immune system.

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