Tumor-Derived Vascular Pericytes Anergize Th Cells

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**Supplementary Material**

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Tumor-Derived Vascular Pericytes Anergize Th Cells

Anamika Bose,* Subhasis Barik,† Saptak Banerjee,‡ Tithi Ghosh,‡ Atanu Mallick,‡ Suchandra Bhattacharyya Majumdar,* Kuntal Kanti Goswami,‡ Aviskeh Bhuniya,‡ Sayantan Banerjee,* Rathindranath Barai,† Walter J. Storkus,‡ Partha Sarathi Dasgupta,† and Subrata Majumdar*

Immune evasion within the tumor microenvironment supports malignant growth and is also a major obstacle for successful immunotherapy. Multiple cellular components and soluble factors coordinate to disrupt protective immune responses. Although stromal cells are well-known for their parenchymal supportive roles in cancer establishment and progression, we demonstrate for the first time, to our knowledge, that tumor-derived vascular pericytes negatively influence CD4+ T cell activation and proliferation, and promote anergy in recall response to Ag by CD4+CD44+ T cells via regulator of G protein signaling 5– and IL-6–dependent pathways. Our data support a new specific role for tumor-derived pericytes in the immune evasion paradigm within the tumor microenvironment and suggest the targeting of these cell populations in the context of successful immunotherapeutics for the treatment of cancer. The Journal of Immunology, 2013, 191: 971–981.

Solid tumor microenvironment (TME) is composed of tumor cells and heterogeneous population of stromal cells, including cells of the immune system (1, 2). Interactions between malignant cells and immune cells or with surrounding stromal cells have been well studied (3–6). However, the interactions of immune cells with stromal cells and their corollary impact on tumor growth remain understudied. Investigators have long viewed stromal cells as providing parenchymal support of tumor cell survival and metastasis in addition to pathological angiogenesis (7–9). The ability of stromal cell populations to regulate immune responses has gained appreciation in the context of myeloid-derived suppressor cells (MDSCs) and infiltrating regulatory T cells (Tregs) (10). However, it has become increasingly evident that alternate, nonhematopoietic stromal cells, such as lymphatic endothelial cells, vascular endothelial cells, and reticular fibroblasts, may also shape evolving immune responses (11–15).

Pericytes or perivascular cells are an important class of stromal cells that can conditionally exhibit APC-associated function (14–16). The significance of pericytes in the regulation of T cell responses is further strengthened from observations that immunomodulatory cytokines/growth factors and adhesion molecules may be secreted or elaborated from pericytes under certain conditions (17–19). Recently, elegant work by Stark et al. (20) established the instructive role of NG2+ pericytes on innate myeloid leukocytes for their homing into sites of inflammation as a consequence of upregulating ICAM-1 expression and macrophage migration inhibitory factor–mediated signaling. Alon and Nourshargh (21) commented on this work and argued whether this inflammation sensor (NG2+ pericytes) instructs other adaptive leukocytes such as NK cells and effector T cells infiltrating sites of inflammation? However, no evidence is yet available to support a critical role being played by pericytes in modulating immune function at the vascular (blood)–tumor interface.

This study reveals for the first time, to our knowledge, that tumor-derived pericytes induce CD4+ T cell dysfunction or anergy and hence might participate in the subversion of immune surveillance associated with progressive tumor growth. Furthermore, the immunoregulatory phenotype/function associated with pericytes in the TME appears to be reinforced by IL-6 and to require intrinsic expression of regulator of G protein signaling 5 (RGS5).

Materials and Methods

Abs

Primary Abs were as follows: anti-CD31, -CD45, -CD140b, -VCAM-1, -LFA-1, and -ICAM-1 procured from BioLegend (San Diego, CA); and anti-CD4, -CD8, -CD25, -MHC class II (MHC-II), -CD40, -CD69, -CD80, -CD86, -CD44, –programmed death ligand 1, –T-bet, –GATA3, –Kif67, –IL-4, and –IFN-γ purchased from BD Biosciences (San Jose, CA). Abs against NG2 and β-actin were obtained from Abcam (Cambridge, MA). Abs against CD31, RGS5, and platelet-derived growth factor receptor-β (PDGFRβ; applicable for Western blotting analysis) were purchased from Santa Cruz Biotechnology. All neutralizing Abs were obtained from BD Biosciences (San Jose, CA). Recombinant cytokines were purchased from either PeproTech (Rocky Hill, NJ) or Invitrogen (Camarillo, CA).

Mice

Female C57BL/6 and BALB/c wild-type mice (age, 4–6 wk, body weight, 25 g average) were procured from Animal Facilities of National Institute of Nutrition (Hyderabad, India). Autoclaved dry pellet diet (Epic Laboratory Feed, Kalyani, India) and water were provided ad libitum. The care and treatment of animals conformed to guidelines established by the Institutional Animal Care and Ethics Committee.
Isolation of pericytes
To isolate mouse pericytes, we sacrificed C57BL/6 and BALB/c mice bearing established s.c. day 21 B16 and CT26 tumors, respectively, to harvest tumors and tumor-uninvolved kidneys. Tissues were minced and enzymatically digested as described by Crisan et al. (22) using collagenase IA, II, and IV, and DNAse I (Sigma-Aldrich, St. Louis, MO) for 15–20 min at 37°C, with gentle shaking. Cells were then being passed through a 70-μm cell strainer (BD Biosciences, San Jose, CA) and washed with PBS, and single-cell suspensions were stained with anti-mouse CD45-allophycocyanin, CD31-ITC, NG2-PE, and CD140b-PerCP. After washing with PBS, cells were sorted into enriched populations containing pericytes (CD45- CD31+ NG2+CD140b+) using a multicolor fluorescence-activated cell sorter (FACSaria; Becton Dickinson, Mountain View, CA). In all cases, cells were >95% pure for the stated phenotypes.

Culture and differentiation of C3H/101T1/2 cells
The cell line C3H/101T1/2 (ATCC #CCL-226) was purchased from the American Type Culture Collection (Manassas, VA). These cells isolated from a line of C3H mouse embryo cells (23) show pericyte/smooth muscle cell–like properties and express markers of the pericyte/smooth muscle cell lineage such as anti-SMA, PDGFRβ, and NG2 (as shown by flow cytometry and RT-PCR). C3H/101T1/2 cells were cultured at subconfluent density in DMEM supplemented with 10% FCS, 233.6 pg/ml glutamine, 25 mM glucose, and 80 U/ml penicillin/streptomycin according to the supplier’s protocol. Before using with PBS, cells were sorted into enriched populations containing pericytes (CD45- CD31+ NG2+CD140b+) using a multicolor fluorescence-activated cell sorter (FACSaria; Becton Dickinson, Mountain View, CA). In all cases, cells were >95% pure for the stated phenotypes.

CFSE labeling and inoculation of pericytes into tumors in vivo
C3H/101T1/2 differentiated pericytes were labeled with 0.5 mM CFSE (Sigma-Aldrich) for 15 min at 37°C, after which the cells were washed once with medium and incubated in fresh, prewarmed complete medium for an additional 30 min at 37°C. Cells were then washed three times with medium, checked for viability, and inoculated in situ into B16 melanoma tumors (tumor volume, 1500–2000 mm3). After 48 h, CFSE+ cells were sorted using flow sorting (from tumor) and used for subsequent experiments.

Generation of bone marrow–derived dendritic cells
Dendritic cells (DCs) were generated from bone marrow (BM) precursors isolated from the tibias/femurs of mice, as previously described (25). At days 7–10 of culture, BM-derived DCs were stimulated to mature by ad- (i.e., IFN-γ, IL-4, T-bet, and GATA3) were stained with anti-mouse fluorescence-labeled Abs using Cytofix/Cytoperm reagents per the manufacturer’s instructions (BD Biosciences, San Diego, CA). For Ki67 staining, cells were pelleted by centrifugation, and 70–80% chilled ethanol was added to fix the pellet (1–5 × 107 cells) with vortexing, before a subsequent incubation at −20°C for 2 h. The cells were then washed twice with staining buffer and centrifuged for 10 min at 200 × g, before being diluted to a concentration of 1 × 107 cells/ml for staining and corollary flow cytometry analysis. For all immunofluorescence analyses, cells were fixed with 1% paraformaldehyde in PBS and screening was performed using a FACSCalibur (Becton Dickinson) and with Cell Quest software. Suitable negative isotype controls were used to establish background staining profiles. The percentage of positively stained populations and their mean fluorescence intensity (MFI) values were determined using quadrant statistics established using FlowJo software (Tree Star, Ashland, OR).

Lymphocyte proliferation
Isolated CD4+ T cells (5 × 105/well) were cocultured with mitomycin C (80 μg/ml for 1 h)-treated pericytes (2 × 105/well) in the presence or absence of OVA protein (1 mg/ml) for 3 d in 96-well flat-bottom plates, with each test performed in triplicate. Assay wells containing no OVA or DCs along with OVA were used as negative and positive proliferation controls, respectively. After 72-h incubation at 37°C, 20 μl (0.5 μCi) [3H]thymidine was added to each well and plates were incubated for an additional 24 h. The cells were then harvested with a cell harvester (PerkinElmer, Waltham, MA) and analyzed for uptake of radioactivity on a beta-scintillation counter (27).

Adhesion assay
OVA-primed CD4+ T cells were isolated and in vitro stimulated with CD3/ CD28 Ab for 18 h. Resting and activated T cells were then preincubated for 20 min at 37°C with anti–LFA-1 mAb (1 μg/ml) or isotype-matched control Ab IgG. Differentially treated T cells were added to confluent OVA-pulsed pericytes or tumor-derived pericytes, which were preincubated for 20 min with ICAM-1 (1 μg/ml) and washed extensively before initiation of coculture. Plates were centrifuged briefly and incubated for 15 min. Cells were observed in five high-powered fields per well, and supernatants were discarded. Afterward, pericyte-adhered T cells were counted and images acquired.

Fluorescence imaging of tumor sections
Tumor tissue samples were prepared and 6-μm sections were stained as previously reported (28). The following Abs were used: rabbit anti-mouse (First Strand cDNA Synthesis kit; Fermentas, Hanover, MD). Amplification was performed using 2 × Bio Mix Red (Bioline; Taunton, MA) with the following program: 94°C for 5 min; 35 cycles of 94°C for 30 s, 58–60°C for 30 s, and 72°C for 1 min; and 72°C for 5 min. PCR products were identified by image analysis software for gel documentation (VersadoC; BioRad Laboratories) after electrophoresis on 1.5% agarose gels and staining with ethidium bromide (Sigma-Aldrich). RT-PCR primers were designed and purchased from MWG-Biotech (Bangalore, India). The primer sequences are listed in Table I.

Western blot
Western blotting was performed as previously described (26). In brief, harvested cells were incubated with lysis buffer (1% Triton X-100, 10 mmol/l Tris-HCl [pH 7.4], 1 mM EDTA, 0.2 mM sodium orthovanadate, 0.5% Nonidet P-40 in PBS; all reagents from Sigma-Aldrich containing a protease inhibitor mixture [complete mini; Roche Diagnostics, Indianapolis, IN]) for 30 min on ice. After centrifugation at 15,300 × g for 30 min, the supernatant was mixed 1:1 with sample buffer, and proteins were separated on 10–12% PAGE gels. Expression of CD31, PDGFRβ, and RGS5 were analyzed using specific Abs (Santa Cruz Biotechnology). β-Actin was analyzed as a control protein using a specific mAb purchased from Abcam (Cambridge, MA). HRP-conjugated goat anti-rabbit or rat anti-goat Abs (Santa Cruz Biotechnology) were used as a secondary Ab to probe blots. Probed proteins were visualized by Western Lightning chemiluminescence detection kit (Pierce, Rockford, IL, and Santa Cruz Biotechnology) and exposed to X-Omat film (Eastman Kodak, Rochester, NY) for 30 s to 1 min.
NG2 (Millipore, Bedford, MA) and FITC-conjugated anti-CD4 or matching isotype controls (all from BD Biosciences, San Jose, CA). Imaging was performed under fluorescence microscope (Leica DM4000B; Leica, Wetzlar, Germany).

Statistical analysis
All reported results represent the mean ± SD of data obtained in either three (for in vivo analyses) or four or five (for in vitro assays) independent experiments. Statistical significance was established by unpaired t test using INSTAT 3 Software (GraphPad Software), with differences between groups attaining a p value <0.05 considered as significant.

Results
Tumor-derived pericytes exhibit an aberrant phenotype
Immunofluorescence staining of day 21 B16 melanoma tumor section revealed colocalization of CD4+ T cells with NG2+ pericytes in multiple places (Fig. 1A). Considering this observation, we sought to study the influences of tumor-derived pericytes on CD4+ T cells. Flow cytometric, RT-PCR, and Western blot analyses revealed that CD45−CD31−CD140b+NG2+ pericytes isolated from progressively growing (day 21) s.c. B16 melanomas or CT26 colon carcinomas show significant upregulation of RGS5 versus (normal) pericytes isolated from the tumor-uninvolved kidneys of these same animals. However, both pericyte populations strongly express PDGFRβ, desmin, anti-SMA, and NG2, and neither population of pericytes expressed CD31 or von Willebrand factor (Fig. 1B, Table I), consistent with our previous published studies (26, 28). Surprisingly, we also observed that these tumor-derived pericytes express significantly higher amounts of various immunologically relevant cell-surface markers including MHC-II and costimulatory, as well as coinhibitory, molecules. Normal kidney-derived pericytes express only a low level of CD86 and moderate levels of the CD40 and MHC-I markers, but they fail to express MHC-II and CD80 molecules. In contrast, tumor-derived pericytes express both MHC-I and MHC-II molecules, and moderate-to-high levels of the CD40, CD80, and CD86 costimulatory molecules (Fig. 1C). Both populations of pericytes expressed significant levels of the PDL-1, coinhibitory molecule, and interestingly, PDL-1 expression was further upregulated on

FIGURE 1. Phenotypic characterization of tumor-derived pericytes. (A) Six-micrometer tissue sections, prepared from day 21 B16 melanoma tumors, were costained with anti-CD4 (green) and anti-NG2 (red) Abs, and imaged by fluorescence microscopy. Blue signal, nuclear counterstain using DAPI. Representative four figures are presented. Original magnification ×200. (B) Day 21 B16 or CT26 tumor and tumor uninvolved kidney were removed from C57BL/6 or BALB/c mice, and tissues were processed (manually and enzymatically) into single-cell suspensions, with CD45−CD31−CD140b+NG2+ pericytes sorted to uniformity by flow cytometry. The percentage of RGS5+ and PDGFRβ+ cells (and their intensity of staining for these markers) was determined by flow cytometry and is presented in a bar diagram. Sorted cells were also analyzed by RT-PCR for expression of cd31, rgs5, pdgfrb, ng2, sma, desmin, and β-actin mRNAs and by Western blotting for CD31, RGS5, PDGFRβ, and β-actin proteins, as described in Materials and Methods. All data are reflective of four independent experiments performed for each tumor type. Cells were also studied for the expression of von Willebrand factor by flow cytometry. A representative dot plot of each case is presented. *p < 0.001 versus kidney-derived pericytes. (C) Cells sorted from tumor and kidney were analyzed for the expression of MHC-I, MHC-II, CD80, CD86, CD40, and PDL-1 by flow cytometry. A representative histogram (CI) and mean ± SD as a bar diagram (CII) from four independent experiments are presented. *p < 0.001, **p < 0.05 versus kidney-derived pericytes.
normal kidney pericytes after their exposure to tumor supernatant in vitro (Fig. 2C).

To further validate the correlation between upregulated expression of RGS5 and immunophenotypic markers by pericytes in response to tumor-associated factors, we performed in vitro assays using differentiated pericytes derived from C3H10T1/2 mesenchymal stem cells. More than 75% differentiated C3H10T1/2 pericytes expressed anti-SMA after 24–48 h of culture in the presence of rTGF-β. Differentiated cells exhibited a slender, bipolar morphology with extensive cellular processes (Fig. 2A). To mimic the conditions inherent in the TME, we further cultured pericytes in vitro in the presence of B16 melanoma–derived supernatant (Supplemental Fig. 1A) along with osteosclerotic fragmented tumor cells. Pericytes cultured under such conditions expressed significantly higher levels of RGS5 when compared with control pericyte populations (Fig. 2A). Flow cytometric analyses of pericyte culture in the presence of tumor-derived products also result in the upregulation of MHC-II, co-stimulatory (CD40, CD80, CD86), as well as coinhibitory (PD-L1) molecule expression (Fig. 2B, 2C). The PDL-1/CD86 expression ratio (based on MFI) on pericytes was increased from 1.7 to 2.5 after tumor conditioning in vitro (Fig. 2C).

Again, to confirm that the TME conditions may help pericytes to assume an RGS5+ hypoinnune stimulatory phenotype, we injected CFSE-labeled C3H10T1/2-differentiated pericytes directly into established day 21 B16 tumors and assessed their Ag profile 48 h later. The expression of RGS5 on CFSE+ cells sorted from TME was significantly upregulated at both the mRNA and protein levels versus control noninjected cells (Fig. 2D).

### Table I. RT-PCR primers used in this study

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<th>Target</th>
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<td>CD31</td>
<td>Forward: 5′-ATGAGGAATTGTCTGGGTAC-3′</td>
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</tr>
<tr>
<td>RGS5</td>
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<td>NG2</td>
<td>Forward: 5′-CAGAACACCCTTTTGTTCAGT-3′</td>
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<tr>
<td>PDGFRβ</td>
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<td>Desmin</td>
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<td>TNF-αR</td>
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*Tumor-derived pericytes downregulate the activation, proliferation, and cytokine secretion of Ag-primed CD4+ T cells*

We next examined whether tumor-derived pericytes exhibit regulatory APC functions. Mice were immunized with OVA protein in CFA/IFA adjuvant a total of four times (on a weekly schedule), and CD4+ T splenocytes were isolated 1 wk after the final vaccination. Purified CD4+ T cells were stimulated with plate-bound CD3 and soluble CD28 Ab in vitro to activate cells before initiation of coculture to simulate the in vivo TME where lymph node Ag-primed activated T cells entered into TME and assume to interact with pericytes in the tumor microvessel walls (19, 20), whereas migrating into interstitial tissue spaces because of surrounding inflammatory factors. In contrast, normal and tumor-derived pericytes were pulsed with OVA for 24 h before initiation of coculture with activated or resting CD4+ T cells. Our results suggest that only tumor-derived pericytes downregulate responder CD4+ T cell expression of early activation markers such as CD69 and CD25 selectively on Ag (OVA)-experienced CD4+CD44+ T cells (Fig. 3A) and reduced the...
ability of these responder T cells to proliferate (Fig. 3B). Normal pericytes isolated from the kidneys of tumor-bearing mice did not exhibit such regulatory effects on CD4⁺ T cells (Fig. 3B).

Given the ability of tumor-derived pericytes to affect CD4⁺ T cell proliferation in vitro, we next evaluated whether alternate T cell functions, such as cytokine profile/production, were also affected by coculture with these pericytes. Although normal pericytes were unable to influence IFN-γ or IL-4 secretion from OVA-pulsed CD4⁺ T cells, tumor-derived pericytes coordinately suppressed both IL-4 and IFN-γ secretion, and GATA3 (type 2) and T-bet (type 1) transactivator protein expression in OVA-reactive CD4⁺CD44⁺ T cells in vitro in an Ag-specific manner (Fig. 3C, 3D). Similar results were observed when tumor-derived pericytes cocultured with OVA-pulsed CD4⁺CD44⁺ T cells without having CD3/CD28 stimulation; however, the extent or magnitude of immune reaction was less (Supplemental Fig. 2).

Upregulated expression of MHC-II on tumor-derived pericytes is associated with the ability of these cells to downregulate CD4⁺ T cell functions in vitro

To evaluate the significance of upregulated MHC molecule of tumor-derived pericytes on CD4⁺ T cell functional responsiveness, we used inhibitors of the Ag processing pathway. CD4⁺ T cells isolated from OVA-immunized mice were incubated with pericytes in the presence of OVA protein. Normal unstimulated pericytes (MHC-II⁻) fail to induce proliferative or cytokine responses from anti-OVA CD4⁺ T cells, whereas tumor-derived pericytes (MHC-II⁺) promoted downregulation in expression of Ki67 by responding CD4⁺ T cells along with reductions in production of both IL-4 and IFN-γ by these T effector cells. Pretreatment of tumor-derived pericytes with inhibitors (chloroquine, brefeldin A) of the MHC-II Ag processing/presentation pathway blocked the ability of these cells to negatively impact OVA-specific CD4⁺ T cell responses in vitro (Fig. 4B).

Tumor-derived pericytes induce CD4⁺ T cell anergy

Given the hyporesponsiveness of OVA-specific CD4⁺ T cells stimulated by tumor-derived pericytes, we next sought to determine whether these tumor-derived pericytes were indeed promoting T cell anergy. To do so, we examined expression of anergy-related factors (diacylglycerol kinase α [dgkα], transcription factors (early growth response 2 [egr2] and 3 [egr3]), and genes related to anergy in lymphocytes (grail) (29, 30) in CD4⁺ T cells cocultured with OVA-pulsed kidney- or tumor-derived pericytes in vitro for overnight. We observed that the expression of dgkα in CD4⁺ T cells was significantly upregulated at the mRNA level and was accompanied by increased expression of additional negative regulators, including egr2, egr3, and the E3 ubiquitin ligase grail selectively after stimulation of CD4⁺ T cells with tumor-derived pericytes (Fig. 5A, Table I). These results suggest that tumor-derived pericytes, but not normal tissue-derived pericytes, can induce an anergy-like T cell dysfunctional state.

Silencing of RGS5 expression in tumor-derived pericytes ablates their immunoregulatory APC function

We and others have demonstrated differential (over)expression of the RGS5 gene product in tumor-derived pericytes, as well as in...
tumor-conditioned 10T1/2-differentiated pericyte cell populations (Figs. 1, 2) (27–29). Indeed, Hamzah et al. (31) showed that tumor pericytes in RGS5\(^2\)/2 recipient mice appear “normal” in their vascular supportive functions in association with dramatic T cell infiltration into the TME and slowed tumor growth (31). Therefore, we next tested whether RGS5 also serves to reinforce regulatory APC function in tumor-derived pericytes. We noted that the immunophenotypic characteristics of tumor-derived pericytes did not change significantly after RGS5 silencing (Fig. 6A, Supplemental Fig. 1); however, this manipulation markedly ablated the ability of these pericytes to blunt anti-OVA CD4\(^+\) T cell activation and cytokine secretion (Fig. 6B) without affecting activation-induced T cell death (Fig. 6C) and to instigate a state of T cell anergy (based on T cell expression of \(dgk\alpha\), \(grail\), \(egr2\), and \(egr3\); Fig. 6C). Hence, RGS5 expression appears clearly tied to the ability of tumor-derived pericytes to extinguish CD4\(^+\) T effector cell function(s).

IL-6 plays a crucial role in the immunophenotypic alteration of tumor-derived pericytes

The regulatory immunophenotypes of tumor-derived pericytes were characterized by elevated expression of MHC-II, as well as a range of costimulatory and coinhibitory molecules. Many of these are known to be IFN-\(\gamma\)-inducible proteins (16), yet the TME of progressor tumors was observed to be largely devoid of this cytokine (25) (Supplemental Fig. 1). In contrast, the progressor TME is rich in expression of regulatory cytokine such as IL-6, IL-10, and TGF-\(\beta\). As a consequence, we directly evaluated the impact of culturing normal (kidney) pericytes in the presence of rIL-6, rIL-10, and rTGF-\(\beta\), and determined that only rIL-6 was competent to...
upregulate MHC-II, CD80/CD86, as well as PDL-1 expression on these pericytes (Fig. 7C). We also found that Ab neutralization of IL-6 within tumor supernatants significantly abrogated their ability to condition normal pericytes to assume the immunophenotype of tumor-derived pericytes (Fig. 7B). Interestingly, we also observed that IFN-γ, a type 1 antitumor cytokine, also induces the expression of MHC-II and is efficient in shifting the balance of costimulatory over coinhibitory molecules (Fig. 7C). Therefore, it was speculated that pericytes’ nature might be modulated under several immunotherapeutic regimens that upregulate IFN-γ in tumor-bearing hosts.

**IL-6- and RGS5-dependent pathways coordinate support pericyte immunoregulatory capacity**

Our results reveal that IL-6 plays an important role in upregulation of signal 1 (MHC-II) and signal 2 (costimulatory/coinhibitory molecules), required for tumor-derived pericyte–CD4+ T interactions, and upregulated RGS5 was shown to correlate with tumor-derived pericyte-induced T cell anergy. This suggested that combined inhibition of both pathways might lead to a more complete eradication of the ability of tumor-derived pericytes to promote CD4+ T cell anergy in vitro. Indeed, we found that combined RGS5 knockdown and IL-6 neutralization dramatically suppressed the capacity of tumor-derived pericytes to anergize OVA-specific CD4+ T cells in culture (Fig. 7DII).

**FIGURE 4.** MHC-II molecules on tumor-derived pericytes crucially impact CD4+ T cell response. Kidney- and tumor-derived pericytes were pretreated with chloroquine (10 μM) or brefeldin A (1 μg/ml) and then cocultured with preactivated OVA-primed CD4+ T cells along with OVA protein. Expression of Ki67 (A) and IFN-γ/IL-4 (B) on CD4+ T cells was analyzed by flow cytometry, and mean ± SD of data reported as a bar diagram from three independent experiments performed. *p < 0.05 (for Ki67), **p < 0.01 (for IFN-γ/IL-4) versus activated CD4+ T cells in presence of tumor-derived pericytes ± OVA.

**FIGURE 5.** Tumor-derived pericytes induce CD4+ T cell anergy. CD4+ T cells were isolated from OVA protein-immunized mice and in vitro cocultured with kidney- or tumor-derived pericytes in the absence or presence of OVA protein. Expression of T cell anergy-related molecules, dgkα, grail, egr2, egr3, and β-actin was then analyzed by RT-PCR. Representative figures (A) from three independent experiments and mean ± SD of the relative expression of target genes normalized against β-actin is provided as a bar diagram (B). *p < 0.005 (for dgkα), **p < 0.001 (for grail), **p < 0.05 (for egr2 and egr3) versus CD4+ T cells stimulated in presence of kidney-derived pericytes ± OVA or tumor-derived pericytes only.

**RGS5- and IL-6-dependent upregulated ICAM-1 on tumor-derived pericytes interact with LFA-1 of CD4+ T cells to induce T cell anergy**

In addition to expressing MHCs and costimulatory/coinhibitory molecules, tumor-derived pericytes express a significant amount of adhesion molecules, ICAM-1 and VCAM-1. These expressions on normal kidney-derived pericytes or unstimulated pericytes were upregulated after their treatment with tumor supernatant and fragmented tumor (Fig. 7E). Furthermore, such upregulated expression of ICAM-1 is RGS5 and IL-6 dependent, because RGS5 small interfering RNA (siRNA) and anti–IL-6 Ab almost abrogated tumor-induced ICAM-1 expression. However, this neutralization remains ineffective for VCAM-1. Considering the importance of adhesion molecules on cell–cell physiological interaction, next we sought to study the relative contribution of either molecule in regulating the tumor-derived pericytes–CD4+ T cell interaction, and thereby the influence of tumor-derived pericytes on T cell functions. The inhibition of either LFA-1 or ICAM-1 resulted in significant downregulation of cell–cell adhesion and tumor-derived, pericyte-induced CD4+ T cell anergy (Fig. 7E, Table I).

**Discussion**

Pericytes represent an important stromal cell component that has been studied extensively in tumor neovascularization (32). Our results for the first time, to our knowledge, suggested a new and novel role of pericytes as a negative regulator of immune responses within TME. In the TME, NK cells, CD8+ effector cells, and CD4+ Th cells constitute the principal cellular framework of immune surveillance (33, 34). Optimal type 1 CD4+ T cell responses help in amplifying and sustaining protective CD8+ cytotoxic T cell accumulation, functionality, and survival within TME (35, 36). Hung et al. (33) clearly showed that depletion of CD4+ and CD8+ T cells almost completely abrogate vaccine-induced antitumor effect. However, in the face of tumor progression, CD4+ Th and/or CD8+ T effector cells may fail to become activated or they may become functionally exhausted or anergized (37–39). Although the regulatory role of MDSCs and Tregs in antagonizing protective type 1 T cell–mediated immunity in the TME (10) has well been recognized, additional stromal cell constituents of the TME may also serve to stunt the effectiveness of immune surveillance in this tissue compartment (11–13, 40). Furthermore, recent work by Stark et al. (20) suggested that NG2+ pericytes during sterile inflammatory conditions can interact with macrophages and neutrophils of innate immune compartment and...
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Thereby regulate their migratory functions, which further fuel the possibility of interactions between NG2+ pericytes and other immune cells within inflammatory TME. Nevertheless, the role of pericytes in ongoing immune response and particularly on CD4+ T cells within TME remains undefined. Considering these lacunae, we attempted to explore the regulation of CD4+ T cell functions by tumor-derived pericytes. Our data suggest that pericytes and T cells not only colocalize in the TME (Fig. 1), but there may be functional consequences to this interaction in vivo.

Following our previous studies (26, 28), we assessed the regulatory role of tumor-derived (day 21 B16F10) pericytes on the functional activity of Ag-primed CD4+ T cells. Pericytes were flow-sorted for the exclusion of CD45 and CD31, and positively selected for PDGFRβ and NG2. Cells were enriched to purity >95%. This type of enrichment minimizes the chance of contamination by cells of the hematopoietic lineage (such as NKT cells, Tregs, and MDSCs), endothelial cells, or fibroblasts. These tumor-derived pericytes were assessed for their phenotypic and immunophenotypic characteristics, and showed considerable difference from their counterparts isolated from normal kidney tissue.

Consistent with our earlier observations (26, 28), we found significant overexpression of RGS5 on tumor-derived pericytes, along with upregulation of CD80 and PDL-1 expression. Higher MHC-II expression and moderate expression of CD86/CD40 was also demonstrated in normal pericytes after the culture of these derived pericytes were assessed for their phenotypic and immunophenotypic characteristics, and showed considerable difference from their counterparts isolated from normal kidney tissue. Consistent with our earlier observations (26, 28), we found significant overexpression of RGS5 on tumor-derived pericytes, along with upregulation of CD80 and PDL-1 expression. Higher MHC-II expression and moderate expression of CD86/CD40 was also demonstrated in normal pericytes after the culture of these cells with tumor-derived products. Interestingly, in contrast with professional APCs, such as DCs, the ratio of coinhibitory to costimulatory molecules in presenting Ag, the overexpression of these surface molecules in tumor-derived pericytes prompted us to evaluate the role of tumor-derived pericytes in regulating Ag-primed CD4+ T cell function(s) in vitro. It is evident from this study that Ag-experienced CD4+CD44+ T cells proliferated poorly and failed to produce type 1 or 2 cytokines even in the presence of costimulation, a feature associated with anergic T cells. Several mechanisms have been proposed to account for the anergic phenotype of tumor Ag-specific T cells. Ligation of inhibitory receptors and defective activation of APCs, among others, appear to contribute to the establishment of a hyporesponsive state in tumor-specific T cells, which ultimately promote tumor growth (42, 43). Indeed, that tumor-derived pericytes induced T cell anergy was strongly supported by significant upregulation of key anergy target gene dgka and central transcription factor egr2 in mRNA level (30, 44), along with other associated molecules such as grail, egr3, and so on. DGKs phosphorylate diacylglycerol into phosphatidic acid, thereby regulating their migratory functions, which further fuel the possibility of interactions between NG2+ pericytes and other immune cells within inflammatory TME. Nevertheless, the role of pericytes in ongoing immune response and particularly on CD4+ T cells within TME remains undefined. Considering these lacunae, we attempted to explore the regulation of CD4+ T cell functions by tumor-derived pericytes. Our data suggest that pericytes and T cells not only colocalize in the TME (Fig. 1), but there may be functional consequences to this interaction in vivo.

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There are limitations in using the transplantable B16 melanoma model rather than using spontaneous and autochthonous tumors to study the immunological interactions between tumor stromal cells and immune effector cells. However, this B16-F10 mouse model of melanoma is the most widely used model to study many aspects of cancer biology and immunotherapy. Culp et al. (41) performed proteomic analysis of various TME components and concluded from their data that melanomas aggressively progress within a dynamic microenvironment containing stromal cells, such as fibroblasts, immune cells, and vascular cells, all of which significantly influence tumor growth. Furthermore, in our previous study, we showed stroma-targeted (pericytes and vascular endothelial cells) immunotherapy causes tumor growth restriction in B16-HLA A-2 transgenic mouse model (26, 28).

Considering the significance of MHCs and costimulatory molecules in presenting Ag, the overexpression of these surface molecules in tumor-derived pericytes prompted us to evaluate the role of tumor-derived pericytes in regulating Ag-primed CD4+ T cell function(s) in vitro. It is evident from this study that Ag-experienced CD4+CD44+ T cells proliferated poorly and failed to produce type 1 or 2 cytokines even in the presence of costimulation, a feature associated with anergic T cells. Several mechanisms have been proposed to account for the anergic phenotype of tumor Ag-specific T cells. Ligation of inhibitory receptors and defective activation of APCs, among others, appear to contribute to the establishment of a hyporesponsive state in tumor-specific T cells, which ultimately promote tumor growth (42, 43). Indeed, that tumor-derived pericytes induced T cell anergy was strongly supported by significant upregulation of key anergy target gene dgka and central transcription factor egr2 in mRNA level (30, 44), along with other associated molecules such as grail, egr3, and so on. DGKs phosphorylate diacylglycerol into phosphatidic acid,
FIGURE 7. IL-6 and RGS5 act together to foster the ability of tumor-derived pericytes to induce CD4+ T cell anergy. (A) Pericytes differentiated from C3H/10T1/2 cells were treated in vitro with either tumor supernatant or osmotically fragmented tumor, or both, or pericytes isolated from resected kidneys or tumors. CFSE-labeled pericytes were injected intratumorally and CFSE+ cells sorted from tumors 48 h after injection and analyzed for expression of the indicated cytokine receptors using RT-PCR. Representative figures (right) and bar diagram for mean ± SD of relative expression of target genes normalized against β-actin is presented. *p < 0.01 versus untreated and kidney-derived pericytes. **p < 0.05 versus CFSE-labeled tumor conditioned pericytes. (B) Pericytes differentiated from C3H/10T1/2 cells were treated in vitro with tumor supernatant, along with neutralizing Abs (5 μg) against IL-6, IL-10, and TGF-β. Expression of MHC-II and costimulatory/coinhibitory molecules was analyzed by flow cytometry. Representative figures are presented from three independent experiments performed. (C) Differentiated pericytes treated with various cytokines (dose ranges from 200 to 500 ng) for 24 h, and expression of MHC-II and costimulatory/coinhibitory molecules were analyzed by flow cytometry and representative figures are provided from three independent experiments performed. (Di) Pericytes isolated from resected kidney and tumors were in vitro treated with RGS5 siRNA and anti–IL-6, with expression of MHC-II studied using flow cytometry (top row). CD4+ T cells isolated from OVA-immunized mice were in vitro cocultured with earlier mentioned pericytes, and expression of CD69 on CD4+CD44+ T cells was studied by flow cytometry. A representative figure from three independent experiments performed is depicted (bottom row). (Di) Expression of anergy-related genes in CD4+ T cells was analyzed using RT-PCR. Representative figures and bar diagram of mean ± SD of adhered CD4+ T cells (counts based on five fields) are presented. Expression of anergy-related genes in CD4+ T cells from earlier mentioned coculture after ICAM-1 and LFA-1 neutralization was analyzed using RT-PCR and representative figures are presented. *p < 0.01 versus ICAM-1 neutralized tumor-derived pericytes and LFA-1 neutralized CD4+ T cells.
thus depleting the amount of diacylglycerol that otherwise could activate RasGRP1. Because RasGRP is the dominant form of RasGEF-activating Ras in T cells, its failed activation blunts the Ras–MAPK–AP-1 pathway blocking T cell activation (in this study, blocking of T cell activation was supported by decreased expression of CD69 and CD25) even in the presence of costimulation (44).

In corollary mechanistic studies, we determined regulatory APC-like activity of tumor-derived pericytes was attributed to IL-6 (tumor pericytes express IL-6R; Fig. 7A), promoting expression of MHC-II and a high coinhibitory/costimulatory molecule ratio, along with (over)expression of RGS5 (a regulator of G protein signaling) in tumor pericytes. RGS molecules, which are biochemically well characterized, are expressed constitutively in pericytes of brain, heart, kidney, skeletal muscle, and so on, but are upregulated during embryonic development and again during disease progression in several tumors (45, 46). Functionally, it inhibits signaling from G protein–coupled receptors by stimulating the intrinsic GTPase activity of activated G proteins (31). Obtained results clearly indicate that RGS5 silencing in tumor-derived pericytes in coculture experiments restored the activation and cytokine production of CD4+ T cells from an anergy state. However, the molecular mechanism(s) of RGS5 upregulation in tumor-derived pericytes and the underlying mechanisms of its influence on immune function remain unclear. This will be a major subject for future investigations.

Our data also suggest that IL-6 plays a major role in influencing pericyte immunophenotypes. Although this result is not in line with the observation showing STAT3 signaling pathway–generated IL-6 led to loss of MHC-II expression by DCs, and thereby attenuates T cell activation (47). However, our results are consistent with the observations of Vassiliadis and Papadopoulos (48), who demonstrated a 5-fold increase in MHC-II expression on RIN-5AH insulinoma cells after treatment with IL-6 in vitro. However, additional factors in the TME may be involved in the promotion of MHC-II expression by pericytes because IL-6 neutralization only partially abrogates the immunoregulatory phenotype of tumor-derived perivascular cells. Interestingly, IFN-γ also possess ability to upregulate MHC-II expression (16) whereas maintaining a costimulatory axis, and immunotherapeutics promoting IFN-γ expression might be effective in antagonizing the effect of IL-6 and other immunosuppressive cytokines. This IL-6, along with RGS5, also positively modulates the expression of adhesion molecule ICAM-1, but not VCAM-1, on tumor-derived pericytes as observed in this experiment. And these adhesion molecules crucially regulate the differential homing of immune cells within TME. Stark et al. (20) in recent studies have shown the importance of pericytes in homing of innate immune cells. However, their roles in homing of adaptive immune cells are yet to be known. Although contradictory reports are available regarding the expression of adhesion molecules on pericytes (might be because of tissue specificity), our results suggest that only NG2*ICAM-1+ tumor-derived pericytes can interact with LFA1*CD44*CD4+ T cells in a RGS5/IL-6-dependent manner leading to T cell anergy. Although the observed immunosuppressive effects of tumor-derived pericytes may differ between transplanted and autochthonous tumor models, as well as between in vivo and in vitro situations, the present in vitro observation generates new information on the energizing ability of an important tumor–stromal cell component, namely, pericytes on T cells. In the present experiments, considering the disparity and differences of response magnitude between in vivo and in vitro results, we have tried to simulate the in vivo TME to our limit in vitro. Therefore, the present in vitro observation on the immunosuppressive behavior of tumor-derived pericytes on T cell function may be considered with sufficient optimism to be replicated in vivo because pericytes have been collected from TME directly.

Notably, pericytes share many similarities with MSCs, which show strong immune regulatory functions (49) on T and B cells, NK cells, as well as DCs by secreting IDO, IL-10, TGF-β, and PGE2 (50–52). In addition, BM MSCs use galec tin 1 and 3 as immunosuppressive weapons to suppress T cell functions (52). Although in some of our present experiments, we used differentiated pericytes from C3H10T1/2, mesenchymal stem cells. Observations from our laboratory demonstrated that pericytes after differentiation from MSCs appeared morphologically different in FSC/SSC plot on flow cytometric analysis and also exhibited some degree of differences in expression of immunophenotypic markers, as well as RGS5 (A. Bose, A. Bhuniya, T. Ghosh, and R. Baral, unpublished observations). Although pericytes express some MSC markers as observed in our study, like CD105, vimentin, among others, evidences are still lacking on Ag-presenting functions of MSCs (maybe because of lack of MHC-II) (53, 54). Therefore, the immunosuppressive effect of MSCs and pericytes might be differentially operated.

In summary, our data suggest the involvement of a dynamic TME in the transformation of immunologically inert pericytes into potent negative regulators of CD4+ T effector cells. Intrinsic RGS5 expression in pericytes and IL-6, which is pervasive in the TME, are suggested to play important roles in shaping the immunophenotype of tumor-derived pericytes, leading to the development of a novel “suppressor” cell population. Additional experiments will be required to determine molecular mechanisms underlying regulatory pericyte development and therapeutic means by which to antagonize this biology in vivo. Furthermore, tumor-derived pericytes not only affect CD4+ T cells but simultaneously alter host-protecting CD8+ T cell functions (A. Bose, A. Bhuniya, S. Barik, Saptak Banerjee, Sayantant Banerjee, and R. Baral, unpublished observations). Other immune cell activities might also be further influenced by tumor-derived pericytes for tumor progression. All these undefined facts clearly need to be determined to understand better the biology of the progressor TME and to define improved therapeutics for cancer management.

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Disclosures
The authors have no financial conflicts of interest.

References


Figure S1. Analysis of cytokines in confluent B16 melanoma culture supernatant and in lysate prepared from day 21 B16 melanoma tumors (A). RT-PCR and Western blot analysis after knockdown of RGS5 in pericytes (B).
Figure S2. CD4+ T cells were isolated from OVA protein-immunized mice and co-cultured with kidney- and tumor-derived particles + OVA protein as described in Material and Methods. (A) Expression of CD69 (A). (B) CD28 (B). (C) IFN-γ (C). (D) GATA3 (D). (E) IL-4 (E) and IL-17 (F) was analyzed on CD4+CD44+ T cells by flow cytometry.