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Tumors induce the formation of suppressor endothelial cells in vivo

Jennifer Konopa Mulligan and

Research Service, Ralph H. Johnson VA Medical Center, 109 Bee Street, Charleston, SC 29401, USA

Department of Otolaryngology, Medical University of South Carolina, Charleston, SC, USA

M. Rita I. Young

Research Service, Ralph H. Johnson VA Medical Center, 109 Bee Street, Charleston, SC 29401, USA

Department of Otolaryngology, Medical University of South Carolina, Charleston, SC, USA

Department of Medicine, Medical University of South Carolina, Charleston, SC, USA

Jennifer Konopa Mulligan: konopa@musc.edu; M. Rita I. Young: rita.young@va.gov

Abstract

Patients with solid tumors have defects in immune effector cells, which have been associated with a poorer prognosis. Previous studies by our laboratory have shown that exposure to Lewis lung carcinoma (LLC)-secreted products induces the formation of suppressor endothelial cells in vitro. The current studies examined if tumors could induce the formation of suppressor endothelial cells in vivo. Endothelial cells were immunomagnetically isolated from the lungs of tumor-bearing mice or normal controls and examined for their ability to modulate NK cell, T-cell and macrophage functions. Compared to normal controls, supernatants from endothelial cells isolated from tumor-bearing lungs had elevated secretion of PGE₂, IL-6, IL-10 and VEGF. Conditioned media from endothelial cells isolated from normal lungs increased CD8⁺ T-cell IFN- γ and CD4⁺ T-cell IL-2 production in response to anti-CD3 stimulation, while media conditioned by endothelial cells from tumor-bearing lungs had a diminished stimulatory capacity. Examination of NK cell functions showed that supernatants from endothelial cells isolated from normal lungs were potent activators of NK cells, as indicated by their secretion of TNF- α and IFN- γ . Endothelial cells isolated from tumor-bearing lungs had a significantly diminished capacity to activate NK cells. Finally, supernatants from endothelial cells of tumor-bearing lungs diminished macrophage phagocytosis compared to either treatment with supernatants of normal endothelial cells or treatment with media alone. The results of these studies demonstrate that tumors induce the formation of suppressor endothelial cells in vivo and provide support for the role of endothelial cells in tumor-induced immune suppression.

Keywords

Endothelial cell; Tumor; Immune suppression; Lewis lung carcinoma

Introduction

The immune system serves as the final natural defense against tumor development. While the immune system has the potential to destroy tumor cells, tumor cells possess numerous mechanisms by which they can suppress anti-tumor immune functions. This can be observed in patients with various types of solid tumors by the defects in T-cell, NK cell and macrophage populations, which is associated with worsened prognosis and increased rate of recurrence [4, 9, 10, 27]. Tumors possess both direct and indirect mechanisms by which they suppress immune functions. An example of direct immune suppression by tumors includes the secretion of immune suppressive products such as PGE₂, VEGF, IL-10 and TGF- β [28]. One mechanism by which tumors indirectly suppress immune functions is by recruiting normal cells to aid in suppressing anti-tumor immune responses. These suppressive cells include tumor-associated macrophages, tumor-associated fibroblasts, Tregs, myeloid derived suppressor cells (MDSC) and CD34⁺ progenitor cells [21, 24, 28]. For example, tumor-associated fibroblasts secrete elevated levels of the immune suppressant and pro-angiogenic factor TGF- β [24]. While macrophages have the ability to destroy tumor cells and support other arms of tumor antigen-specific immunity, tumor-associated macrophages have reduced IL-12 secretion and elevated production of IL-10 and TGF- β [13, 16, 21]. Tregs are recruited into the tumor microenvironment where they inhibit CD4⁺ and CD8⁺ T-cell proliferation and suppress anti-tumor immunity [19]. MDSC are also recruited into the tumor microenvironment where they can inhibit tumor-specific T-cell functions [26]. CD34⁺ progenitor cells represent a unique group of immune suppressive cells that can be differentiated to become antigen-presenting dendritic cells or endothelial cells composing the tumor vasculature [29, 30].

Endothelial cells are a critical structural component of the blood and lymphatic vasculature. In addition to their structural functions, endothelial cells have the potential to serve as stimulators or suppressors of immune functions [5]. Co-culture experiments with T-cells and endothelial cells have demonstrated that endothelial cells stimulate IFN- γ production by CD8⁺ T-cells and induce CD4⁺ T-cells to increase production of IL-2, IL-4 and IFN- γ in response to PHA stimulation [3, 20]. Conversely, in the liver, sinusoidal endothelial cells are capable of inducing T-cell tolerance [23]. Endothelial cells can also aid in suppressing T-cell functions through the induction of Treg cells [14].

While endothelial cells have the potential to regulate immune functions, their role as modulators in the tumor environment remains unclear. Endothelial cells serve as ideal targets for utilization by tumors to suppress immune functions due to their capacity to secrete numerous immune suppressive products, including VEGF, PGE₂, TGF- β , IL-6 and IL-10 [17, 18, 25]. Previous studies by our laboratory have demonstrated in vitro that tumor-secreted factors skew endothelial cells to disrupt T-cell, NK cell and macrophage functions [22]. The purpose of the present study was to test the hypothesis that tumors induce the formation of suppressor endothelial cells in vivo. Utilizing a metastatic murine LLC model, CD31⁺ endothelial cells were isolated from normal or tumor-bearing lungs. Compared to products secreted by endothelial cells from normal lungs, conditioned media from endothelial cells isolated from tumor-bearing lungs disrupted T-cell cytokine production in response to anti-CD3 stimulation, had a diminished ability to activate NK cells, and reduced macrophage phagocytosis. The results of these studies demonstrate that tumors can induce the formation of suppressor endothelial cells in vivo and provide support for a novel role of endothelial cells in tumor-induced immune suppression.

Materials and methods

Mice and tumor inoculation

C57BL/6 mice (8–12-week-old females) were obtained from Charles River (Wilmington, MA, USA). Mice were housed five to a cage with a 12/12 h light/dark cycle and fed ad libitum. C57BL/6 mice were injected i.v. via the tail vein with 1×10^6 LLC tumor cells or PBS, and tumors were allowed to develop for 21 days. This time point was selected based on the recommendations of the Institutional Animal Care and Use Committee (IACUC) to minimize discomfort to the mice as mice on average succumb to tumor by day 25. Mice were humanely euthanized by CO₂ asphyxiation followed by cervical dislocation. Lungs were then collected and used as the source of endothelial cells. All procedures were conducted with IACUC approval.

Endothelial cell isolation

Endothelial cells were immunomagnetically isolated from the lungs of normal and LLC tumor-bearing mice following the protocol provided by the microbead manufacturer (Miltenyi Biotech Inc., Auburn, CA, USA). Lungs were removed, rinsed twice in HBSS and finely minced. Lung fragments were then enzymatically digested for 90 min in an HBSS enzyme solution containing collagenase (type IV), DNase and hyaluronidase (Sigma-Aldrich, St. Louis, MO, USA). Cells were then passed through a 70- μ m strainer and washed twice. Red blood cells were lysed with ACK lysis buffer and washed twice more. Fc γ II/III receptors were then blocked with anti-CD16/CD32 monoclonal antibody to prevent non-specific antibody binding (BD Biosciences, San Jose, CA, USA). Endothelial cells were then labeled with biotin-labeled anti-CD31 antibody as per the manufacturer's instructions (eBioscience, San Diego, CA, USA). Following washing to remove excess anti-CD31 antibody, cells were resuspended in separation buffer, streptavidin-labeled microbeads were added, and cells were immunomagnetically separated per the manufacturer's instructions (Miltenyi Biotech Inc.). Endothelial cell purity was confirmed by immunostaining for endothelial cell-selective adhesion molecule (ESAM) (eBioscience) and was determined to be at minimum 91%. Additional confirmation of endothelial cell purity was conducted by immunostaining for endoglin (CD105). Following isolation, endothelial cells were washed, counted and plated in 96-well round-bottom tissue culture plates at a concentration of 5×10^4 cells/well in phenol-free DMEM with 10% fetal bovine serum, 200 U/ml penicillin G, 200 μ g/ml streptomycin sulfate, 500 μ g/ml amphotericin B and 5×10^{-5} M 2-mercaptoethanol. Cells were incubated for 24 h at 37°C with 5% CO₂. Conditioned media from isolated endothelial cells was collected and stored at -80°C until it could be assayed by ELISA or used to treat isolated immune cell populations.

Immune cell isolation

T-cells were immunomagnetically isolated from the spleens of normal C57BL/6 mice as described previously [22]. Briefly, spleens were homogenized using a Stomacher 80 Biomaster to obtain a single cell suspension. After lysing red blood cells with ACK lysis buffer, spleen cells were washed three times and resuspended in separation buffer. Labeling antibody was added per the manufacturer's instructions (Miltenyi Biotech Inc.). T-cells were isolated using a CD90⁺ (Thy1.2⁺) immunomagnetic positive selection column. After isolation, T-cells were washed and resuspended in RPMI culture medium with 10% heat-inactivated FBS, 200 U/ml penicillin G, 200 μ g/ml streptomycin sulfate, 500 μ g/ml amphotericin B and 5×10^{-5} M 2-mercaptoethanol. Ten units/ml of recombinant mouse IL-2 (R&D Systems, Minneapolis, MN, USA) was added to maintain cell viability. T-cells were plated at a density of 2.5×10^5 cells per well on immobilized anti-CD3 antibody-coated plates. T-cell purity was confirmed by flow cytometric analysis of CD3 expression and was determined to be 94% or higher.

NK cells were isolated from single cell spleen suspensions of healthy C57BL/6 mice using a negative selection magnetic bead column per the manufacturer's instructions (Miltenyi Biotech Inc.). After isolation, NK cells were washed twice and plated at a density of 2.5×10^5 cells per well. Isolated NK cells were maintained in phenol-free RPMI 1640 tissue culture medium and 10 units/ml recombinant mouse IL-2. NK cell purity was determined to be 92% or higher as confirmed by immunostaining for the NK cell marker DX5.

Macrophages were isolated from C57BL/6 mice by peritoneal lavage using 8 ml of ice-cold phosphate buffered saline (PBS). Once collected, peritoneal cells were washed, resuspended in phenol-free DMEM tissue culture medium and plated in 24-well plates at a density of 5×10^5 cells per well. Macrophages were cultured in DMEM due to its higher glucose content that better supports macrophage functions. After allowing the macrophages to adhere for 90 min, they were washed twice to remove non-adherent cells. Macrophage purity was confirmed by examination of cell morphology, adherence and phagocytic ability.

Immune function assays

T-cells, NK cells and macrophages were treated for 24 h with either medium alone or 40% conditioned media from endothelial cells isolated from lungs of normal or tumor-bearing mice. Immune cells were then washed, new medium was added, and cells were incubated for an additional 24 h. After this time, supernatants from immune cells were collected for measurement of secreted immune regulatory products or immune cells were used for functional assessments. Secretion of immune modulatory products was assessed by ELISA and included measurement of TNF- α (eBioscience), PGE₂, VEGF, TGF- β , IL-6, IL-10, IL-12 (R&D Systems), IFN- γ and IL-2 (BD Biosciences). Culture media alone was assayed as an additional control. Supernatants used for measurement of TGF- β levels were first acid activated in accordance to the manufacturer's instructions. Since detectable quantities of TGF- β and PGE₂ were present in FBS, endothelial cell culture medium alone was assayed for PGE₂ alongside endothelial cell conditioned medium. The average amount of TGF- β and PGE₂ detected in the medium alone control was subtracted from the levels detected in the endothelial cell conditioned medium to calculate the quantity of mean quantity secreted by endothelial cells. All ELISA's were performed according to the manufacturers' instructions.

Intracellular T-cell cytokine production was measured by immunostaining followed by flow cytometric analysis. Prior to surface and intracellular staining, monensin (GolgiStop) was added to T-cells for 2 h according to the CytoStain Kit protocol. Fc γ II/III receptors were blocked with anti-CD16/CD32 monoclonal antibody. Cell surface staining was performed using anti-CD4 and anti-CD8 monoclonal antibodies. After staining, cells were washed twice, then fixed and permeabilized with Cytofix/Cytoperm. Intracellular cytokine staining was performed by adding anti-IL-2 and anti-IFN- γ antibodies. Marker channels were set using the antibody isotype controls specific to each antibody. Four-color flow cytometric analyses were performed on a BD FACSCanto™ using FACS Diva flow cytometry analysis software. All reagents for immunostaining and subsequent flow cytometric analysis were obtained from BD Bioscience. T-cell apoptosis and necrosis were measured by staining with anti-Annexin V and propidium iodide followed by flow cytometric analysis.

To determine the effects of endothelial cell-secreted products on macrophage phagocytosis, a fluorescent microbead uptake assay was used. Bead uptake was quantified by flow cytometric analysis as described previously [22]. To measure macrophage phagocytosis, 10 μ l of 1:100 diluted FITC polymer microspheres (Thermo Fisher Scientific, Waltham, MA, USA) were added to macrophages for 4 h at 37°C. To confirm that bead uptake was a result of phagocytosis, control cells were incubated with beads at 0°C and less than 1% of total cells were shown to phagocytose any beads. Following treatment with the microbeads, macrophages were washed three times, resuspended in PBS, detached from the plates by

gentle scraping, and bead phagocytosis was immediately quantified by flow cytometric analysis.

Statistical analysis

Statistical analyses were conducted using GraphPad Prism 5.01 software. Student's *t* test was used to determine statistically significant differences in the secretion of immune modulatory products between endothelial cells isolated from lungs of normal and tumor-bearing mice. Data points shown in scatter plots represent results from treatments using endothelial cells isolated from individual animals. In bar graphs, error bars represent standard deviation or standard error of the mean, as indicated in each figure legend. Histograms of macrophage bead phagocytosis are representative results of multiple experiments.

Results

Tumors alter endothelial cell secretion of immune regulatory products

First examined was the ability of tumors to alter endothelial cell production of immune modulatory products. Media conditioned for 24 h by endothelial cells isolated from normal or tumor-bearing lungs were examined by ELISA for levels of immune modulatory products (Fig. 1a–e). When compared to endothelial cells isolated from normal lungs, those isolated from tumor-bearing lungs had increased secretion of IL-6 ($P < 0.0001$), VEGF ($P = 0.001$), PGE₂ ($P = 0.0047$) and TGF- β ($P = 0.002$) (Fig. 1a–d). Interestingly, endothelial cell production of the immune stimulatory factor IL-12 (Fig. 1e) was diminished when endothelial cells were isolated from tumor-bearing lungs as compared to when endothelial cells were isolated from normal lungs ($P < 0.0001$). Endothelial cell production of IL-4 and IL-10 were also examined, although there were no statistically significant differences between the levels produced by endothelial cells isolated from normal lungs or tumor-bearing lungs. These results demonstrate the ability of tumors to alter endothelial cell production of immune modulatory products and support the potential for tumor-derived endothelial cells to disrupt immune functions.

Supernatants from endothelial cells isolated from the lungs of tumor-bearing mice disrupt T-cell responses to anti-CD3 stimulation

Next examined was the ability of endothelial cell supernatants to alter T-cell responses to anti-CD3 stimulation. T-cell responses were measured by immunostaining followed by flow cytometric analysis for IFN- γ and IL-2 production by CD4⁺ and CD8⁺ T-cells. In comparison to T-cells treated with endothelial cell supernatant from normal lungs, supernatants from endothelial cells isolated from tumor-bearing lungs had reduced CD8⁺IFN- γ ⁺ staining ($P < 0.0001$) (Fig. 2a). CD4⁺ T-cell production of IL-2 was also examined (Fig. 2b). Treatment of T-cells with supernatants from endothelial cells of normal lungs significantly increased CD4⁺ cell staining for IL-2 compared to treatment with media alone ($P < 0.0001$). Conditioned media from endothelial cells isolated from tumor-bearing lungs had a diminished capacity to stimulate CD4⁺ T-cell IL-2 production compared to conditioned media from normal lung endothelial cells ($P < 0.0001$). CD4⁺ T-cells were also examined for expression of IFN- γ , with no significant differences being seen between treatment groups (data not shown).

In addition to intracellular expression, T-cell cytokine secretion was also examined to determine if endothelial cells were inducing T-cell Th1/Th2/Th17 skewing. Consistent with the immunostaining results, T-cell secretion of IFN- γ (Fig. 3a) was reduced upon exposure to endothelial cell supernatant from tumor-bearing lungs, as compared to normal lungs ($P = 0.02$). Measurement of the Th2 cytokines IL-4, IL-6 and IL-10 revealed further alterations in

T-cell functions by endothelial cell-secreted products (Fig. 3b–d). Treatment of T-cells with normal endothelial cell conditioned media slightly heightened T-cell IL-4 secretion ($P < 0.0001$). T-cell treatment with conditioned media from endothelial cells of tumor-bearing lungs further stimulated the production of IL-4 ($P < 0.0001$ compared to treatment with media or treatment with conditioned media from endothelial cells of normal lungs). Compared to treatment with media alone, T-cells treated with normal endothelial cell conditioned media exhibited increased secretion of IL-6 ($P = 0.002$). Treatment of T-cells with conditioned media from endothelial cells isolated from tumor-bearing lungs further heightened this secretion of IL-6 ($P < 0.001$ compared to treatment with media and $P = 0.01$ compared to treatment with normal endothelial cell conditioned media). Examination of T-cell IL-10 secretion demonstrated that T-cells treated with normal endothelial cell conditioned media did not increase their production of IL-10 compared to T-cells treated with media alone. However, when treated with conditioned media from endothelial cells from tumor-bearing lungs, T-cell secretion of IL-10 was significantly increased ($P = 0.004$). Finally, the ability of tumor-derived endothelial cells to induce T-cell production of IL-17 was examined (Fig. 3e). Treatment of T-cells with normal endothelial cell conditioned media induced a small, but statistically significant decrease in T-cell IL-17 production. In comparison to treatment with normal endothelial cell conditioned media, treatment of T-cells with tumor-isolated endothelial cell conditioned media stimulated T-cells to double their secretion of IL-17 ($P = 0.005$). These results suggest that endothelial cells isolated from tumor-bearing lungs have the ability to partially skew T-cells toward a Th2 and Th17 cytokine secretion profile.

Tumors disrupt endothelial cells' ability to stimulate NK cells

While NK cells were originally identified as killers of infected cells and tumors, they also serve as potent immune regulators [6]. Therefore, the ability of endothelial cells to modulate NK cells secretion of the immune regulatory products TNF- α , IFN- γ and IL-10 was examined. NK cells that were treated with media alone secreted little to no detectable TNF- α , IFN- γ or IL-10 (Fig. 4). Figure 4a, b demonstrates that treatment of NK cells with normal endothelial cell-conditioned media stimulated NK cells to significantly increase their secretion of TNF- α and IFN- γ compared to treatment with media alone ($P < 0.0001$ for both cytokines). Supernatants from endothelial cells isolated from tumor-bearing lungs had a diminished ability to stimulate NK cell secretion of IFN- γ and TNF- α compared to the stimulation induced by normal lung endothelial cell supernatants ($P = 0.002$ and $P = 0.0046$, respectively).

NK cell secretion of the Th2 cytokine IL-10 in response to treatment with isolated endothelial cell supernatant was also examined (Fig. 4c). Compared to treatment with media alone, conditioned media from normal lung endothelial cells increased NK cell IL-10 secretion ($P = 0.008$). Treatment of NK cells with conditioned media from endothelial cells isolated from tumor-bearing lungs further heightened NK cell IL-10 secretion ($P = 0.009$). These results demonstrate that tumors disrupt endothelial cell secretion of products that activate NK cell secretion of TNF- α and IFN- γ , and further heighten their secretion of the immune inhibitory mediator IL-10.

Tumor-derived endothelial cell products diminish macrophage phagocytosis and alter macrophage secretion of select immune modulatory products

The effects of media conditioned by endothelial cells from normal or tumor-bearing lungs on macrophage functions were also examined. First examined were the effects of endothelial cell supernatants on macrophage phagocytosis (Fig. 5a–d). Macrophage treatment with conditioned media from endothelial cells isolated from normal lungs mildly increased the percent of macrophages that were highly phagocytic, as indicated by macrophage

phagocytosis of four or more beads (summary in Fig. 5d; $P = 0.04$ compared to media alone). Treatment with conditioned media from endothelial cells isolated from tumor-bearing lungs significantly suppressed macrophage phagocytosis of four or more beads compared to treatment with normal endothelial cell supernatants ($P = 0.0005$) or media alone ($P = 0.0012$). Total bead uptake and uptake of two or three beads was also reduced with treatment of supernatant of endothelial cells from tumor-bearing versus normal lungs (summary of data not shown).

In addition to phagocytosis, macrophage secretion of TNF- α , IL-10, latent TGF- β and PGE₂ in response to exposure to endothelial cell secreted products was examined (Fig. 6). Compared to treatment with media alone, conditioned media from normal endothelial cells stimulated macrophages to produce IL-10 ($P = 0.01$) and TNF- α ($P < 0.0001$), and to increase their production of PGE₂ ($P < 0.0001$). Media conditioned by normal endothelial cells increased macrophage production of TGF- β , although these changes were not statistically significant. Figure 6a, b demonstrates that, compared to supernatants from normal lung endothelial cells, conditioned media from endothelial cells from tumor-bearing lungs had a diminished ability to stimulate macrophage TNF- α and IL-10 secretion ($P = 0.02$ and $P = 0.001$, respectively). However, supernatants from endothelial cells isolated from tumor-bearing lungs did not further alter macrophage secretion of TGF- β or PGE₂ when compared to supernatants from normal endothelial cells (Fig. 6c, d). These results demonstrate that tumors induce endothelial cells to diminish macrophage phagocytosis and alter their ability to stimulate production of select immune modulatory mediators.

Discussion

Prior studies by our laboratory identified that, when cultured with tumor-secreted products, a normal endothelial cell line was induced to become immune inhibitory [22]. The purpose of the current study was to expand these findings by examining the ability of tumors to induce the formation of suppressor endothelial cells in vivo. Overall, the results of the current study with endothelial cells isolated from tumor-bearing lungs were strikingly similar to those of our prior studies that employed a murine endothelial cell line and showed induction of suppressor endothelial cells in vitro. Studies utilizing both in vitro and in vivo-induced suppressor endothelial cells demonstrated that endothelial cell-secreted products activate NK cells and that tumors disrupt this endothelial cell-stimulatory capacity. Both sets of studies also demonstrated that the tumor can disrupt endothelial cells' ability to heighten T-cell IFN- γ production. Finally, the results of these studies and our prior work demonstrated that tumors induce endothelial cells to diminish macrophage phagocytosis and selectively alter macrophage production of immune regulatory products. Of note is that the effects of suppressor endothelial cells on each immune cell population varied. Products secreted by endothelial cells isolated from tumor-bearing lungs were able to impact almost every aspect of T-cell and NK cell function examined. Alterations in NK cytokine production may have implications in altering NK cells' ability to regulate other aspect of innate and adaptive immunity [1]. In contrast, macrophage phagocytosis was reduced, but production of mediators such as PGE₂ and TGF- β was unaffected by whether endothelial cells were from normal or tumor-bearing lungs. The applicability of these results is that these alterations in immune functions may support tumor progression by diminishing numerous arms of anti-tumor immune competence, though the impact of the immune regulatory endothelial cells on tumor progression remains to be investigated.

While many of the results of these studies with endothelial cells isolated from normal and tumor-bearing lungs confirmed our prior results with endothelial cells treated in vitro with tumor-conditioned media, one difference between the in vitro and in vivo-induced suppressor endothelial cell model was identified. The prior studies utilizing the endothelial

cell line showed that endothelial cell conditioned media failed to induce CD4⁺ T-cell expression of IL-2. Therefore, it was not possible in the in vitro model to determine if tumors could induce endothelial cells to suppress the induction of IL-2 expression. These differences in endothelial cell stimulation of CD4⁺ T-cell functions could be attributed to differences in using in vitro-maintained endothelial cells versus endothelial cells that were isolated from tumor-bearing lungs and which are exposed to signals from other cells in the lung microenvironment.

Several studies have examined the ability of endothelial cells to regulate T-cell functions, though most have focused on contact-dependent regulation and little work has focused on endothelial cell regulation of immune functions in the context of tumor immunology. Briscoe et al. [3] demonstrated that co-culture of T-cells with endothelial cells enhances CD4⁺ T-cell IFN- γ production in vitro, while Oneo et al. [23] have shown that they can induce T-cell tolerance. Prior studies by other laboratories have also shown that endothelial cells may be involved in tumor escape from immune detection. For example, Flati et al. [7] demonstrated that tumor secretion of basic FGF altered leukocyte adhesion in tumor vessels by decreasing endothelial cell ICAM-1 expression. Furthermore, liver sinusoidal endothelial cells can present antigen and induce CD8⁺ T-cell tolerance, providing an additional model of tumor escape [2]. The novelty of the studies presented here is that they are the first to demonstrate in vivo the ability of tumors to induce endothelial cells in vivo to become suppressive toward T-cell, NK cell and macrophage functions.

Many of the immune alterations shown in the present study to be induced by conditioned media from tumor-bearing lungs have previously been observed in patients with solid tumors. For example, in patients with oral squamous cell carcinomas, plasma levels of the Th1 cytokine IFN- γ were decreased while the Th2 cytokines IL-4, IL-6 and IL-10 were increased [15]. These results were similar to those of our previous studies demonstrating that conditioned media from endothelial cells from tumor bearing-lungs had on T-cell secretion of Th1/Th2 cytokines. Furthermore, it was observed that conditioned media from tumor-derived endothelial cells downregulated macrophage phagocytosis, which has been previously reported as being downregulated in tumor-associated macrophages [11]. Reductions in macrophage phagocytosis provide not only a means of immune escape and decreased tumor antigen presentation but also alter the pro-inflammatory response to cancer cells during tumor growth [12]. Furthermore, studies showed that the use of metronomic delivery of cyclophosphamide to disrupt tumor vasculature was able to improve T-cell and NK cell functions [8]. Correlations between what is observed clinically and the results presented in these studies warrant further investigation into the contribution of suppressor endothelial cells to tumor-induced immune suppression.

While these studies demonstrated the ability of tumors to induce suppressor endothelial cells in vivo, numerous questions remain to be answered. The ability of murine and human tumors of other tissue origins to induce suppressor endothelial cell activities remains to be examined. Not yet determined in these studies is the mechanism by which tumors induce the formation of suppressor endothelial cells or the mechanism by which suppressor endothelial cells inhibit immune cell functions. In vitro studies have been conducted demonstrating that antibody neutralization of endothelial cell derived IL-6 failed to prevent suppressor endothelial cells from diminishing CD8 IFN- γ production. Furthermore, additional studies have determined that suppressor endothelial cell production of TGF- β is not responsible for the disruption of T-cell functions.

Also to be determined is suppressor endothelial cell regulation of immune cell trafficking by chemokines and the extent to which endothelial cells contribute to tumor-induced immune suppression. By further understanding how suppressor endothelial cells are being induced

and how they are suppressing immune functions, it may be possible to identify therapeutic targets that can block the immune suppressive effects of these cells. Blocking immune suppressive endothelial cells may also aid in improving the efficacy of existing immunotherapies, particularly those consisting of T-cell or NK cells as these cells must pass through the tumor vasculature to infiltrate tumors. Of interest would be to examine in clinical studies the ability of vascular targeting therapies to serve additional functions as immunotherapies by disrupting the tumor vasculature and, in turn, inhibiting the effects of suppressor endothelial cells.

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References

1. Andoniou CE, Coudert JD, Degli-Esposti MA. Killers and beyond: NK-cell-mediated control of immune responses. *Eur J Immunol*. 2008; 38:2938–2942. [PubMed: 18979519]
2. Berg M, Wingender G, Djandji D, Hegenbarth S, Momburg F, Hammerling G, Limmer A, Knolle P. Cross-presentation of antigens from apoptotic tumor cells by liver sinusoidal endothelial cells leads to tumor-specific CD8⁺ T cell tolerance. *Eur J Immunol*. 2006; 36:2960–2970. [PubMed: 17039564]
3. Briscoe DM, Henault LE, Geehan C, Alexander SI, Lichtman AH. Human endothelial cell costimulation of T cell IFN- γ production. *J Immunol*. 1997; 159:3247–3256. [PubMed: 9317123]
4. Correa MR, Ochoa AC, Ghosh P, Mizoguchi H, Harvey L, Longo DL. Sequential development of structural and functional alterations in T-cells from tumor-bearing mice. *J Immunol*. 1997; 158:5292–5296. [PubMed: 9164948]
5. Danese S, Dejana E, Fiocchi C. Immune regulation by microvascular endothelial cells: directing innate and adaptive immunity, coagulation and inflammation. *J Immunol*. 2007; 178:6017–6022. [PubMed: 17475823]
6. Erten G, Aktas E, Deniz G. Natural killer cells in allergic inflammation. *Chem Immunol Allergy*. 2008; 94:48–57. [PubMed: 18802336]
7. Flati V, Pastore LI, Griffioen AW, Satijn S, Toniato E, D'Alimonte I, Laglia E, Marchetti P, Gulino A, Martinotti S. Endothelial cell anergy is mediated by bFGF through the sustained activation of p38-MAPK and NF- κ B inhibition. *Int J Immunopathol Pharmacol*. 2006; 19:761–773. [PubMed: 17166398]
8. Ghiringhelli F, Menard C, Puig PE, Ladoire S, Roux S, Martin F, Solary E, Le Cesne A, Zitvogel L, Chauffert B. Metronomic cyclophosphamide regimen selectively depletes CD4⁺CD25⁺ regulatory T cells and restores T and NK effector functions in end stage cancer patients. *Cancer Immunol Immunother*. 2007; 56:641–648. [PubMed: 16960692]
9. Heimdal JH, Aarstad HJ, Olofsson J. Peripheral blood T-lymphocyte and monocyte function and survival in patients with head and neck carcinoma. *Laryngoscope*. 2000; 110:402–407. [PubMed: 10718427]
10. Katz A. Update on immunology of head and neck cancer. *Med Clin North Am*. 1993; 77:625–631. [PubMed: 8492614]
11. Kim R, Emi M, Tanabe K. Cancer cell immune escape and tumor progression by exploitation of anti-inflammatory and pro-inflammatory responses. *Cancer Biol Ther*. 2005; 4:924–933. [PubMed: 16177562]
12. Kim R, Emi M, Tanabe K. Cancer cell immune escape and tumor progression by exploitation of anti-inflammatory and pro-inflammatory responses. *Cancer Biol Ther*. 2005; 4:924–933. [PubMed: 16177562]
13. Konur A, Kreutz M, Knuchel R, Krause SW, Andreesen R. Three-dimensional co-culture of human monocytes and macrophages with tumor cells: analysis of macrophage differentiation and activation. *Int J Cancer*. 1996; 66:645–652. [PubMed: 8647627]

14. Krupnick AS, Gelman AE, Barchet W, Richardson S, Kreisel FH, Turka LA, Colonna M, Patterson GA, Kreisel D. Murine vascular endothelium activates and induces the generation of allogeneic CD4⁺25⁺Foxp3⁺ regulatory T-cells. *J Immunol.* 2005; 175:6265–6270. [PubMed: 16272276]
15. Lathers DM, Achille NJ, Young MR. Incomplete Th2 skewing of cytokines in plasma of patients with squamous cell carcinoma of the head and neck. *Hum Immunol.* 2003; 64:1160–1166. [PubMed: 14630398]
16. Leek RD, Harris AL. Tumor-associated macrophages in breast cancer. *J Mammary Gland Biol Neoplasia.* 2002; 7:177–189. [PubMed: 12463738]
17. Lefkowitz DL, Roberts E, Grattendick K, Schwab C, Stuart R, Lincoln J, Allen RC, Moguilevsky N, Bollen A, Lefkowitz SS. The endothelium and cytokine secretion: the role of peroxidases as immunoregulators. *Cell Immunol.* 2000; 202:23–30. [PubMed: 10873303]
18. Lienenluke B, Germann T, Kroczeck RA, Hecker M. CD154 stimulation of interleukin-12 synthesis in human endothelial cells. *Eur J Immunol.* 2000; 30:2864–2870. [PubMed: 11069068]
19. Lizee G, Radvanyi LG, Overwijk WW, Hwu P. Improving antitumor immune responses by circumventing immunoregulatory cells and mechanisms. *Clin Cancer Res.* 2006; 12:4794–4803. [PubMed: 16914564]
20. Ma W, Pober JS. Human endothelial cells effectively costimulate cytokine production by, but not differentiation of, naive CD4⁺ T cells. *J Immunol.* 1998; 161:2158–2167. [PubMed: 9725207]
21. Mantovani A, Schioppa T, Porta C, Allavena P, Sica A. Role of tumor-associated macrophages in tumor progression and invasion. *Cancer Metastasis Rev.* 2006; 25:315–322. [PubMed: 16967326]
22. Mulligan J, Lathers D, Young M. Tumors skew endothelial cells to disrupt NK cell, T-cell and macrophage functions. *Cancer Immunol Immunother.* 2008; 57:951–961. [PubMed: 18058097]
23. Onoe T, Ohdan H, Tokita D, Shishida M, Tanaka Y, Hara H, Zhou W, Ishiyama K, Mitsuta H, Ide K, Asahara T. Liver sinusoidal endothelial cells tolerate T-cells across MHC barriers in mice. *J Immunol.* 2005; 175:139–146. [PubMed: 15972640]
24. Orimo A, Gupta PB, Sgroi DC, Arenzana-Seisdedos F, Delaunay T, Naeem R, Carey VJ, Richardson AL, Weinberg RA. Stromal fibroblasts present in invasive human breast carcinomas promote tumor growth and angiogenesis through elevated SDF-1/CXCL12 secretion. *Cell.* 2005; 121:335–348. [PubMed: 15882617]
25. Pirtskhalaishvili GNJ. Endothelium-derived factors as paracrine mediators of prostate cancer progression. *Prostate.* 2000; 44:77–87. [PubMed: 10861760]
26. Serafini P, Borrello I, Bronte V. Myeloid suppressor cells in cancer: recruitment, phenotype, properties, and mechanisms of immune suppression. *Semin Cancer Biol.* 2006; 16:53–65. [PubMed: 16168663]
27. Vlock DR. Immunobiologic aspects of head and neck cancer. *Hematol Oncol Clin North Am.* 1991; 5:797–820. [PubMed: 1716250]
28. Young MR. Protective mechanisms of head and neck squamous cell carcinomas from immune assault. *Head Neck.* 2006; 28:462–470. [PubMed: 16284974]
29. Young MR, Cigal M. Tumor skewing of CD34⁺ cell differentiation from a dendritic cell pathway into endothelial cells. *Cancer Immunol Immunother.* 2006; 55:558–568. [PubMed: 16025265]
30. Young MR, Wright MA, Vellody K, Lathers DM. Skewed differentiation of bone marrow CD34⁺ cells of tumor bearers from dendritic toward monocytic cells, and the redirection of differentiation toward dendritic cells by 1 α , 25-dihydroxyvitamin D3. *Int J Immunopharmacol.* 1999; 21:675–688. [PubMed: 12609462]

Fig. 1. Secretion of immune regulatory factors by endothelial cells isolated from the lungs of normal and tumor-bearing mice. Supernatants from endothelial cells isolated from normal and tumor-bearing lungs were examined by ELISA for secretion of immune regulatory products. Data points represent results from individual mice. Statistics shown are the results of Student's *t* test analyses between groups

Fig. 2.

Effects of media conditioned by endothelial cells from normal and tumor-bearing lungs on T-cell IFN- γ and IL-2 expression. Shown are the mean fluorescent intensities of T-cells that immunostained double positive for **a** IFN- γ ⁺ and CD8⁺ or **b** IL-2⁺ and CD4⁺. Data shown are means $\pm$ SEM, with $n = 4$ for media-treated-T-cells and $n = 7$ for T-cells treated with media conditioned by endothelial cells from either normal or tumor-bearing lungs and are representative of four separate experiments. Statistics shown are the results of Student's t test analyses between indicated treatment groups. NS indicates no statistically significant difference between treatments

Fig. 3.

T-cell secretion of Th1/Th2/Th17 cytokines in response to treatment with supernatants from endothelial cells of normal and tumor-bearing lungs. T-cell secretion of the Th1 cytokine, IFN- γ , the Th2 cytokines, IL-4, IL-6 and IL-10, and Th17 cytokine, IL-17, in response to anti-CD3 in the presence of conditioned media from endothelial cells was measured by ELISA. Data shown are means $\pm$ SEM, with $n = 4$ for each treatment group and representative of data for three or more separate experiments. Statistics shown are the results of Student's t test analyses between indicated treatment groups. NS indicates no statistically significant difference between treatments.

Fig. 4. Effects of media conditioned by endothelial cells isolated from normal and tumor-bearing lungs on NK cell secretion of immune modulatory products. NK cell secretion of TNF- α , IFN- γ and IL-10 was assessed by ELISA as a measure of NK activation in response to treatment with media alone or conditioned media from endothelial cells isolated from normal or tumor-bearing lungs. *Data points* represent results from individual mice combined from two separate experiments. Statistics shown are the results of Student's *t* test analyses between indicated treatment groups

Fig. 5. Conditioned media from endothelial cells isolated from tumor-bearing lungs diminish macrophage phagocytosis. Macrophage phagocytosis was assessed by uptake of FITC polymer microbeads. Bead phagocytosis was assessed by flow cytometric analysis. **a–c** Representative histograms of flow cytometric analyses of microbead phagocytosis. Macrophages phagocytosing no beads are indicated in the *far left black peak* of each panel. Proceeding toward the right of that peak are macrophages phagocytosing 1, 2, 3 or 4 or more beads. **d** Percent cells phagocytosing four or more beads, with each *data point* showing effects of media conditioned by endothelial cells from individual mice and are representative of results from three experiments. Statistics shown are the results of Student's *t* test analyses between indicated treatment groups

Fig. 6.

Assessment of cytokine secretion by macrophages in response to treatments with conditioned media from endothelial cells isolated from normal or tumor-bearing mice. Macrophage-conditioned media was assessed by ELISA for the presence of TNF- α , PGE₂, IL-10 and latent TGF- β following treatment with endothelial cell supernatants. Data shown are means $\pm$ SEM with $n = 4$ for each treatment group and are combined results from two separate experiments. Statistics shown are Student's t test results between indicated treatment groups. NS indicates no statistically significant difference between treatments