Differential Response of Human and Mouse Dendritic Cells to VEGF Determines Interspecies Discrepancies in Tumor-Mediated Th1/Th2 Polarity Shift

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Abstract

Purpose: Metastatic cancer patients exhibit systemic dysregulation of immune polarity and are biased toward Th-2 immune responses. This is due, at least in part, to effects of VEGF on antigen presenting cell (APC) function. We therefore compared immune polarity changes in mouse models of cancer with those seen in human patients.

Experimental Design: We measured plasma levels of vascular growth factors and multiple cytokines via ELISA and multiplex analysis in mice with transplantable and spontaneous tumors. We compared immune cell subsets in naive and vaccinated mice with and without tumors. We assessed cytokine immune responses by multiplex analysis. Finally, we assessed gene expression and receptor surface expression in response to VEGF in mouse and human APCs.

Results: Although human patients have elevated plasma cytokines and altered immune polarity in response to antigen, mice have minimal immune abnormalities. Mouse VEGF does not mediate immune repolarization in vitro. Human but not mouse APCs upregulate VEGFR2 and downregulate interleukin (IL)-12β in response to VEGF.

Conclusions: Whereas humans with metastatic cancer demonstrate dysregulated immune polarity in response to excess plasma VEGF, tumor mice do not. This appears to be due to differences in APC responses to VEGF stimulation. Differential immune effects of VEGF may represent a key species difference in the context of translation of preclinical cancer immunotherapeutics into early clinical testing.

Introduction

As tumors develop and metastasize, they express antigens that have the potential to be recognized by the host immune system and to trigger host antitumor immune responses [reviewed in ref. 1]. Tumor-associated antigens include neoantigens produced by oncogenic mutations in self-proteins as well as differentiation antigens that are overexpressed by tumors relative to normal host tissues. To avoid destruction by the host immune response, tumors employ an array of mechanisms of immune evasion and immune modulation, collectively known as immunoediting (2). The majority of immunoediting mechanisms described to date, including downmodulation of antigen presentation and production of negative regulators of immunity, take place at the tumor itself or in regional lymph nodes. However, in addition to local immune dysregulation, patients with advanced cancer have been found to have evidence of systemic alterations in immunity. For example, DTH (delayed-type hypersensitivity) responses to common recall antigens are impaired in metastatic melanoma patients (3). Influenza and other pathogen vaccine responses in advanced cancer patients have also been reported to be suboptimal (4). In addition to clinical immune alterations, metastatic melanoma patients have been shown to have elevations in numerous plasma cytokine levels including interleukin (IL)-4, IL-5, IL-10, and TGFβ (5). Moreover, peripheral blood T cells from metastatic melanoma patients show skewing from cytotoxic Th1 responses toward chronic inflammatory Th2 responses upon antigen receptor stimulation, as evidenced by elaboration of increased amounts of IL-4 and decreased amounts of IFNγ (5).

As tumors grow, they must initiate vasculogenesis to gain access to adequate blood supply. Central to this process are VEGF, placental growth factor (PIGF in human or PIGF-2 in mouse) and other vascular growth factors (6). In addition to vasculogenesis, VEGF and other vascular growth factors mediate direct effects on tumors, many of which express VEGF receptors (7, 8), as well as on the immune system. VEGF has been reported to block maturation of antigen presenting cells (APC; ref. 9). While we have not observed phenotypic changes in APC upon incubation with VEGF,
we have noted skewing of immune responses from Th1 to Th2 upon incubation of human peripheral blood mononuclear cells (PBMC) with VEGF (5). This effect is not observed with isolated T cells, suggesting that the effects of VEGF require APCs. Nonetheless, these observations indicate VEGF and other vascular growth factors may mediate systemic immune dysregulation in metastatic cancer patients.

Much of what is known regarding in vivo immune responses to tumors is derived from studies involving model systems including laboratory mice. However, successful translation of dramatic preclinical anticancer immunotherapeutic results into clinically relevant therapy has not met expectations. Numerous models in which tumors are transplanted into mice have demonstrated that tumors can be eradicated by immune responses, including passive immune techniques such as adoptive leukocyte transfer (10), and active immune responses including vaccines (11). Unfortunately, despite these promising results, effective antitumor immunotherapy for human cancer patients has been largely unrealized (12).

We have hypothesized that one key difference between murine models of cancer and human cancer is that murine tumors do not elicit dysregulation of systemic immunity to the extent that human cancers do. Here, we compare parameters of systemic immune function (dysregulation) between human and mouse systems and demonstrate that VEGF differentially regulates mouse versus human APC function. To assess for tumor-mediated immune dysregulation, we studied mice inoculated with B16 melanoma cells (13), one of the most commonly used model systems for tumor immunotherapy, as well as mouse mammary tumor (MMT) mice with spontaneous mammary tumors, for which spontaneous and induced antitumor immune responses have been well characterized (14).

**Methods**

**Mice and tumor cell lines**

C57Bl6 and FVB mice were purchased from The Jackson Laboratory. B16 tumor-bearing mice were injected subcutaneously in the right flank with 5 × 10⁶ B16-F1 melanoma cells (American Type Culture Collection, ATCC) that were cultured for less than 2 weeks prior to injection. ANV6 tumor-bearing mice were injected with 1 × 10⁶ to 5 × 10⁶ ANV6 cells. MUC1.Tg and MMT mice were bred as previously described (15). Non–tumor-bearing MUC1.Tg mice were selected as controls for MMT mice because MUC1.Tg and MMT are genetically identical except for an oncogene leading to mammary tumors in MMT (15). Tumor weights were calculated as [length × width²] / 2. All mice were maintained in our animal facility in accordance with IACUC guidelines. Tumor-bearing mice were sacrificed if tumors comprised greater than 10% of body weight or became ulcerated.

**Vaccination**

MMT and C57Bl6 mice were immunized in the right flank with 50 μg ovalbumin (OVA; Sigma-Aldrich Corp.) dissolved in 50 μL PBS and emulsified with 50 μL complete Freund’s adjuvant (CFA; Sigma-Aldrich Corp.). Mice were vaccinated at age 20 weeks and boosted once at age 22 weeks. Vaccinated mice were sacrificed at age 24 weeks and splenocytes were cultured.

**Plasma collection**

Mice were bled via tail vein nicking every 14 days. Blood was collected in heparinized tubes (Milian USA) and was centrifuged at 5,000 rpm for 10 minutes to separate plasma from cells. Plasma was frozen at −80°C.

**Splenocyte and PBMC culture**

For T-cell function assays, mouse splenocytes were cultured at 10⁶ cells/mL with Iscove’s modified Dulbecco’s medium (IMDM; Gibco Invitrogen) with 10% FBS (HyClone Laboratories) and either anti-CD3/anti-CD28–coated magnetic beads (10⁶/mL; Invitrogen) and 20 μg/mL IL-2 (Peprotech) or 1 mg/mL OVA (Sigma-Aldrich Corp.) for 72 to 96 hours. Culture supernatants were frozen at −80°C. Recombinant VEGF (R&D Systems, Inc.) was added to select cultures at 1 to 1,000 ng/mL. For Th1-polarizing conditions, 1 mg/mL anti-IL-4 (BD Biosciences) and 10 μg/mL IL-12 (Peprotech) were added. For Th2-polarizing conditions, 1 mg/mL anti-IL-12 (BD Biosciences) and 10 μg/mL IL-4 (Peprotech) were added. For VEGF expression assays, mouse splenocytes were cultured for 24 or 48 hours with 0.1 μg/mL lipopolysaccharide (LPS), 0.1 μg/mL recombinant VEGF, or IMDM + FBS alone. Similarly, human PBMC were cultured for 24 or 48 hours with 0.1 μg/mL LPS, 0.1 μg/mL recombinant human VEGF (R&D Systems, Inc.), or RPMI (Mediatech, Inc.) plus FBS alone. For culture of dendritic cells (DC) for mRNA expression analysis, mouse bone marrow cells were cultured in IMDM + FBS + 0.001 μg/mL IL-4 + 0.01 μg/mL...
granulocyte-macrophage colony stimulating factor (GM-CSF; Peprotech). Media and IL-4 and GM-CSF were replaced on days 2 and 5, and 0.1 μg/mL LPS and 0.1 μg/mL VEGF (or none) were added on day 5. Similarly, human PMBC were sorted by magnetic bead sorting for CD14+ cells (Invitrogen). CD14+ cells were cultured with RPMI + FBS + recombinant human IL-4 + GM-CSF, which was replaced on days 2 and 5. LPS and recombinant human VEGF (or none) were added on day 5.

**Cytokine analysis**

Protein levels for IL-2, IL-4, IL-5, IL-10, IL-12p70, IFNγ, TNFα, and GM-CSF were measured using the Bio-plex cytokine assay (Bio-Rad) as per the manufacturer’s instructions. Cytokines were detected using the Luminex plate reader (Bio-Rad). Protein concentrations were determined using a standard curve generated using Bio-plex Manager software (Bio-Rad). Alternatively, some cytokines (IL-4, IFNγ, and TGFβ) were assayed by ELISA (R&D Systems, Inc.).

**Flow cytometry**

Immunophenotyping of splenocytes and PBMC was performed by flow cytometry using antibodies to mouse CD3, CD4, CD8, CD11b, CD11c, CD25, CD45R (B220), CD49b (DX5), Ly-6C/C (Gr-1), VEGFR1 (Flt-1), VEGFR2 (Flk-1), VEGFR3 (Flt-4), and neuropilin 1 (Nrp1; BD Biosciences) and to human CD14, CD11c, VEGFR1, VEGFR2, VEGFR3, and Nrp1. Intracellular staining for mouse FoxP3 (eBioscience) was performed according to the manufacturer’s published instructions. Data were processed using Cellquest software (Becton-Dickinson). For splenocyte immunophenotyping, cells were classified as T cells (CD3+DX5-), CD4+ cells (CD3+CD4+DX5-), CD8+ cells (CD3+CD8+DX5-), CD4+ cells (CD3+CD4+DX5-), Ly-6C/C (Gr-1)+ natural killer cells (NK; DX5+CD122+), NKI+ cells (CD3+DX5+DX20+), DC (CD11b+CD11c+), or monocytes (CD11b+CD11c+Gr-1-).

**RNA array analysis**

Total RNA was isolated from mouse cultured DC and human cultured DC using RNeasy kits (Qiagen, Inc.) according to the manufacturer’s instructions. The quality of the RNA was evaluated by obtaining electropherograms on Agilent 2100 Bioanalyzer and RNA integrity number (RIN) using 2100 Expert software (Agilent Technologies, Inc.). cDNA was prepared from a total of 10 μg of RNA. Samples were quantified using standard spectrophotometry using a Tecan spectrophotometer (Tecan US) and considered acceptable if the A260/280 reading was greater than 1.7. The purified cDNA was used as a template for in vitro transcription reaction for the synthesis of biotinylated cRNA using RNA transcript labeling reagent (Affymetrix). Labeled cRNA was then fragmented and hybridized onto the Mouse 430 2.0 array. Appropriate amounts of fragmented cRNA and control oligonucleotide B2 were added along with control cRNA (BioB, BioC, and BioD), herring sperm DNA, and BSA to the hybridization buffer. The hybridization mixture was heated at 99°C for 5 minutes followed by incubation at 45°C for 5 minutes before injecting the sample into the microarray. Then, the hybridization was carried out at 45°C for 16 hours with mixing on a rotisserie at 60 rpm. After hybridization, the solutions were removed and the arrays were washed and then stained with streptavidin–phycoerythrin (Molecular Probes). After washes, arrays were scanned using the GeneChip Scanner 3000 (Affymetrix).

The quality of the fragmented biotin-labeled cRNA in each experiment was evaluated before hybridizing onto the Mouse 430 2.0 expression array by both obtaining electropherograms on Agilent 2100 Bioanalyzer and hybridizing a fraction of the sample onto Test3 Array as a measure of quality control. GCOS 1.3 (Affymetrix) was used to scan and digitize the image. Once the GeneChip has been scanned, the raw data (.CEL files) was uploaded into Partek GS 6.5 software (Partek Inc.), which was used for further data analysis and visualization. Genes whose expression was reported as significantly changed (>2-fold difference) between groups were identified.

**Statistics**

Data were compared using the Mann–Whitney rank-sum test for nonparametric data.

**Results**

**Tumor-bearing mice exhibit normal immune polarity and immune function**

To compare plasma expression of vascular growth factors in tumor-bearing mice with that in human cancer patients, we measured VEGF and PlGF-2 levels in mice with B16 melanoma flank tumors, B16 lung metastases (cells injected i.v.; ref. 13), ANV6 breast adenocarcinoma flank tumors (16), and MMT mice, which develop relatively indolent spontaneous mammary tumors over the course of approximately 24 to 28 weeks (15). When tumor weight reached 10% of body weight, animals were euthanized, and pre-euthanasia plasma VEGF and PlGF-2 levels were measured and compared with non–tumor-bearing controls. VEGF and PlGF-2 levels in tumor-bearing mice were comparable to those seen in human metastatic melanoma patients (ref. 5; Table 1). Although VEGF and PlGF-2 levels were highest at the time the animals were euthanized, VEGF and PlGF-2 levels became elevated in MMT mice approximately 28 days before animals were sacrificed (data not shown). To assess for tumor-mediated immune dysregulation, we measured plasma levels of multiple cytokines in tumor-bearing and non–tumor-bearing mice including VEGF, PlGF-2, IL-2, IL-4, IL-5, IL-10, IL-12p70, IFNγ, TNFα, GM-CSF, and TGFβ (Table 1). Whereas vascular growth factor levels were comparable to those seen in humans,
plasma cytokine levels remained normal or near normal when compared with levels seen in non–tumor-bearing mice (B6, FVB, and MUC1.Tg). The absence of cytokine elevation was noted in mice with either transplantable or spontaneous tumors.

Tumor-bearing mice have previously been reported to have splenomegally and elevated levels of immunosuppressive MDSC (17); these are thought to promote tumor immune evasion through suppression of host immune responses. To further explore whether mice with advanced tumors develop cellular immune dysregulation, we assessed immune cell subset numbers in the spleens of MMT mice, when compared with MUC1.Tg tumor-negative litter mates, develop elevated numbers of total splenic leukocytes, T regulatory cells, B cells, and myeloid suppressor cells (Table 2), whereas numbers of T and NK cells (data not shown) remain relatively constant. Interestingly, when tumor-bearing MMT mice were immunized against an irrelevant xenoantigen (OVA), increases in these cell compartments compared with control mice were not apparent (Table 2).

Whereas levels of plasma Th2 cytokines in transplantable and spontaneous tumor-bearing mice are not elevated, it is possible that tumors might still mediate systemic dysregulation of T-cell immunity by altering T-cell antigen responses. To assess for this, we cultured splenocytes from naive mice with beads containing antibodies to CD3 and CD28. Under these conditions, we compared T-cell production of Th1 and Th2 cytokines by splenocytes from tumor-bearing and control mice. Culture supernatant levels of IL-4, IL-5, IL-10, and IFNγ were not significantly different between tumor-bearing and non–tumor-bearing mice (Table 3). In addition, to assess for differences in endogenous APC function, we compared cytokine production by splenocytes from OVA-immunized mice cultured with OVA. Again, no differences in T-cell polarity, as measured by cytokine production in OVA-immunized mice cultured with OVA. Again, no differences in T-cell polarity, as measured by cytokine production in OVA-immunized mice cultured with OVA. Again, no differences in T-cell polarity, as measured by cytokine production in OVA-immunized mice cultured with OVA.

### Table 1. Vascular growth factor and cytokine plasma levels in tumor-bearing mice

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>B6</th>
<th>B16-sq</th>
<th>B16-iv</th>
<th>FVB</th>
<th>ANV6</th>
<th>MUC1.Tg</th>
<th>MMT</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>26 ± 22</td>
<td>348 ± 549</td>
<td>903 ± 2,085</td>
<td>19 ± 2</td>
<td>45 ± 22</td>
<td>50 ± 59</td>
<td>1,142 ± 934</td>
</tr>
<tr>
<td>PIGF-2</td>
<td>30 ± 40</td>
<td>113 ± 130</td>
<td>113 ± 127</td>
<td>21 ± 6</td>
<td>32 ± 33</td>
<td>2.0 ± 4.8</td>
<td>1,251 ± 364</td>
</tr>
<tr>
<td>IL-2</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>59 ± 52</td>
<td>150 ± 252</td>
</tr>
<tr>
<td>IL-4</td>
<td>13 ± 6</td>
<td>11 ± 6</td>
<td>14 ± 7</td>
<td>5.8 ± 0.5</td>
<td>5.3 ± 0.6</td>
<td>19 ± 8</td>
<td>22 ± 6</td>
</tr>
<tr>
<td>IL-5</td>
<td>33 ± 32</td>
<td>33 ± 45</td>
<td>19 ± 5</td>
<td>33 ± 10</td>
<td>21 ± 8</td>
<td>48 ± 28</td>
<td>84 ± 53</td>
</tr>
<tr>
<td>IL-10</td>
<td>40 ± 22</td>
<td>59 ± 52</td>
<td>83 ± 52</td>
<td>8.2 ± 1.2</td>
<td>59 ± 38</td>
<td>106 ± 41</td>
<td>129 ± 39</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>746 ± 446</td>
<td>749 ± 126</td>
</tr>
<tr>
<td>IL-13</td>
<td>540 ± 281</td>
<td>632 ± 574</td>
<td>951 ± 663</td>
<td>27 ± 10</td>
<td>21 ± 14</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>107 ± 38</td>
<td>178 ± 89</td>
</tr>
<tr>
<td>IFNγ</td>
<td>16 ± 6</td>
<td>15 ± 9</td>
<td>16 ± 6</td>
<td>2.2 ± 1.2</td>
<td>1.8 ± 1.2</td>
<td>413 ± 492</td>
<td>732 ± 1,372</td>
</tr>
<tr>
<td>TGFβ</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>19,298 ± 4,136</td>
<td>17,833 ± 5,198</td>
</tr>
<tr>
<td>TNFα</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>701 ± 633</td>
<td>985 ± 808</td>
</tr>
</tbody>
</table>

**NOTE:** Concentrations given in pg/mL, mean ± SD. Abbreviation: ND, not done.

*B Bold values differ from control plasma with *P* < 0.05.

### Table 2. Immune cell subsets in MMT mice

<table>
<thead>
<tr>
<th>Cell subset</th>
<th>Naive</th>
<th>Immunized</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MUC1.Tg</td>
<td>MMT*</td>
</tr>
<tr>
<td>Splenocytes</td>
<td>125 ± 14</td>
<td>255 ± 78</td>
</tr>
<tr>
<td>CD4 T cells</td>
<td>6.3 ± 1.5</td>
<td>7.1 ± 1.8</td>
</tr>
<tr>
<td>CD8 T cells</td>
<td>2.6 ± 0.5</td>
<td>2.6 ± 0.9</td>
</tr>
<tr>
<td>Treg</td>
<td>0.32 ± 0.09</td>
<td>1.11 ± 0.50</td>
</tr>
<tr>
<td>B cells</td>
<td>15 ± 4</td>
<td>35 ± 9</td>
</tr>
<tr>
<td>MDSC</td>
<td>3.6 ± 0.9</td>
<td>27.4 ± 25.1</td>
</tr>
</tbody>
</table>

**NOTE:** × 10⁸ cells, mean ± SD. *Bold values differ from control plasma with *P* < 0.05.*
In summary, apart from elevations in several immune cell subsets seen in naive (but not immunized) MMT mice, we were unable to detect systemic signs of immune dysregulation or altered T cell function in any of several transplantable or spontaneous mouse models of cancer, whereas these changes are readily appreciable in humans with metastatic cancer.

**Effects of mouse VEGF on T-cell immune polarity**

Human T lymphocytes, when stimulated in *vitro* in the presence of VEGF, exhibit a shift in immune polarity from Th1-biased toward Th2-biased, as evidenced by increased production of IL-4 and decreased production of IFNγ (5); TGFβ production is also increased in the presence of VEGF. To assess whether murine T cells are affected by VEGF in this way, we cultured naive C57Bl6 splenocytes with anti-CD3/anti-CD28 beads in the presence of Th1-polarizing conditions, or increasing concentrations of VEGF. Unlike human cells, mouse splenocytes showed no change in IFNγ, IL-4, or TGFβ production when cultured with VEGF (Fig. 1). Similarly, OVA-recognizing OT-2 CD4 T cells did not show alterations in IFNγ or IL-4 production when cultured in the presence of VEGF (data not shown).

**Effect of VEGF on expression of IL-12β by mouse and human cultured DC**

Human CD4 T cells cultured with bulk PBMC, but not purified CD4 T cells exhibit a Th1 to Th2 immune polarity shift upon incubation with VEGF (5), whereas no shift in immune polarity was seen with mouse bulk splenocytes. Because this effect in humans was seen only with bulk PBMC, we hypothesized that the differential effect of VEGF on immune polarity in mice and humans might be mediated by APCs. To test this, we generated mouse and human DC by culturing BMC and CD14⁺ PBMC with GM-CSF and IL-4. DCs were matured with LPS in the presence or absence of VEGF, and mRNA expression profiles were compared by gene chip analysis. Although the expression of 1,079 human genes was altered at least 2-fold upon incubation with VEGF (range = −430-fold to +39-fold), only 157 murine genes were altered at least 2-fold (range = −6.5-fold to +5-fold), implying that VEGF receptor-mediated signaling differs significantly between mouse and human APCs. In addition, we explored VEGF-mediated changes in expression of immune polarity genes and found that expression of IL-12β, which is critical for Th1 polarization of CD4 T cells (18), is downregulated by VEGF in human cultured DC, but not in mouse DC (Table 4).

**VEGF receptor expression on mouse and human leukocytes**

We then compared surface expression of VEGF receptors on mouse and human APCs. Bulk mouse splenocytes or human PBMC were cultured with LPS, VEGF, or media alone, then assessed by flow cytometry for surface expression of VEGFR1, VEGFR2, VEGFR3, and Nr1 on CD14⁺CD11c⁺ human DC and CD11b⁺CD11c⁺ mouse DC. Freshly isolated cells and cells cultured for 24 to 48 hours in media alone did not express significant levels of any VEGF receptor (data not shown). However, human cells cultured with LPS expressed cell surface Nr1 and VEGFR1, whereas mouse cells expressed only VEGFR1 (Fig. 2, bold histograms). Culture with VEGF induced VEGFR1, VEGFR2, and Nr1 surface expression by human DC, but not mouse DC, which expressed only VEGFR1 (Fig. 2, shaded histograms).

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**Table 3. Cytokine production by MMT splenocytes**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Naïve splenocytes, beads*</th>
<th>Immunized mice, OVA</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFNγ</td>
<td>MUC1.Tg; 1,127 ± 821; MMT; 1,061 ± 718</td>
<td>B6 925 ± 459; MMT 1,705 ± 656</td>
</tr>
<tr>
<td>IL-4</td>
<td>MUC1.Tg; 1,604 ± 3,992; MMT 526 ± 938</td>
<td>B6 87 ± 57; MMT 179 ± 157</td>
</tr>
<tr>
<td>IL-10</td>
<td>MUC1.Tg; 502 ± 457; MMT 194 ± 242</td>
<td>B6 213 ± 202; MMT 304 ± 207</td>
</tr>
</tbody>
</table>

NOTE: Concentrations given in pg/mL, mean ± SD.


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**Figure 1.** Immune polarity in cells stimulated with VEGF in *vitro*. Splenocytes from C57Bl6 mice were incubated with culture medium alone (NS) or anti-CD3/anti-CD28 beads with Th1-inducing conditions (Th1), Th2-inducing conditions, or increasing concentrations of VEGF. Supernatants were assayed by ELISA for IFNγ, IL-4, and TGFβ. Mean cytokine concentrations ± SD are shown, except for IFNγ under Th1 conditions and IL-4 under Th2 conditions (off scale).
Growing evidence shows that patients with metastatic cancer exhibit alterations in immune homeostasis compared with early stage cancer patients and healthy volunteers. Elevated plasma levels of CRP (C reactive protein; ref. 19), IL-1 (20), IL-6 (21), and elevated erythrocyte sedimentation rate (ESR; ref. 22, 23) have all been found in metastatic cancer patients and are associated with poor prognosis. Moreover, metastatic cancer patients exhibit a skewed immune polarity in that CD4 T cells are more prone to produce Th2 cytokines and less prone to produce Th1 cytokines compared with healthy age-matched volunteers (5). This Th2 skewing of immunity in the setting of metastatic cancer appears to be due, at least in part, to elevated levels of VEGF, as VEGF is capable of mediating a Th1 to Th2 immune polarity shift by CD4 T cells in vitro (5).

We have compared plasma cytokine levels and CD4 T-cell function from mice with advanced transplantable and spontaneous tumors. Although splenomegaly and mild increases in B cell, T regulatory cell, and MDSC numbers were seen in tumor-bearing mice, no change in T-cell responses to general stimuli or unrelated antigens were seen, even in mice with very high tumor burdens. This is in distinction to prior reports of T-cell tolerance to tumor-associated antigens (reviewed in ref. 24). Likewise, in sharp contrast to human cancers, in which plasma markers of inflammation and Th2 cytokines are overexpressed, advanced tumor-bearing mice had little or no increase in cytokine levels. Our results indicate a fundamental difference in the systemic interaction between tumors and the host immune system in mice and humans.

Given the role of VEGF in regulating T-cell immune polarity in humans, it is possible that due to smaller tumor volumes and less hypoxia, mice might produce lower levels of VEGF and thereby avoid VEGF-induced, Th2-biased immunity. However, in our spontaneous tumor model, levels of VEGF and PlGF-2 comparable to those seen in metastatic human melanoma patients were seen weeks before the animals were sacrificed; however, no increases in other plasma cytokines were seen. This suggested that a critical difference in VEGF-mediated immune signaling might account for the lack of Th2 bias. Indeed, mouse VEGF does not induce Th2 polarity in mouse CD4 T cells in vitro. This appears to be due to the absence of

**Figure 2.** VEGF receptor expression on mouse and human leukocytes. Splenocytes from C57Bl6 mice and human PBMC were cultured for 48 hours with LPS (bold line) or recombinant VEGF (shaded). Gating on CD11c<sup>+</sup> cells (A and B), we analyzed for surface expression of VEGFR1 (C and D), VEGFR2 (E and F), VEGFR3 (G and H), and Nrp1 (I and J). Isotype controls are shown (dotted line). Results are representative of multiple experiments. MFI = mean fluorescent intensity.

**Table 4.** IL-12 and IL-4 mRNA expression by mouse and human DC

<table>
<thead>
<tr>
<th>Species</th>
<th>Gene</th>
<th>No VEGF</th>
<th>VEGF positive</th>
<th>Relative change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>IL-4</td>
<td>4.89</td>
<td>4.87</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>IL-12α</td>
<td>1,806.38</td>
<td>1,732.09</td>
<td>0.96</td>
</tr>
<tr>
<td></td>
<td>IL-12β</td>
<td>2,652.17</td>
<td>1,937.20</td>
<td>0.73</td>
</tr>
<tr>
<td>Human</td>
<td>IL-4</td>
<td>6.22</td>
<td>6.13</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td>IL-12α</td>
<td>4.97</td>
<td>4.80</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td>IL-12β</td>
<td>1,160.48</td>
<td>77.86</td>
<td>0.07</td>
</tr>
</tbody>
</table>

**Discussion**

Growing evidence shows that patients with metastatic cancer exhibit alterations in immune homeostasis compared with early stage cancer patients and healthy volunteers. Elevated plasma levels of CRP (C reactive protein; ref. 19), IL-1 (20), IL-6 (21), and elevated erythrocyte sedimentation rate (ESR; ref. 22, 23) have all been found in metastatic cancer patients and are associated with poor prognosis. Moreover, metastatic cancer patients exhibit a skewed immune polarity in that CD4 T cells are more prone to produce Th2 cytokines and less prone to produce Th1 cytokines compared with healthy age-matched volunteers (5). This Th2 skewing of immunity in the setting of metastatic cancer appears to be due, at least in part, to elevated levels of VEGF, as VEGF is capable of mediating a Th1 to Th2 immune polarity shift by CD4 T cells in vitro (5).

We have compared plasma cytokine levels and CD4 T-cell function from mice with advanced transplantable and spontaneous tumors. Although splenomegaly and mild increases in B cell, T regulatory cell, and MDSC numbers were seen in tumor-bearing mice, no change in T-cell responses to general stimuli or unrelated antigens were seen, even in mice with very high tumor burdens. This is in distinction to prior reports of T-cell tolerance to tumor-associated antigens (reviewed in ref. 24). Likewise, in sharp contrast to human cancers, in which plasma markers of inflammation and Th2 cytokines are overexpressed, advanced tumor-bearing mice had little or no increase in cytokine levels. Our results indicate a fundamental difference in the systemic interaction between tumors and the host immune system in mice and humans.

Given the role of VEGF in regulating T-cell immune polarity in humans, it is possible that due to smaller tumor volumes and less hypoxia, mice might produce lower levels of VEGF and thereby avoid VEGF-induced, Th2-biased immunity. However, in our spontaneous tumor model, levels of VEGF and PlGF-2 comparable to those seen in metastatic human melanoma patients were seen weeks before the animals were sacrificed; however, no increases in other plasma cytokines were seen. This suggested that a critical difference in VEGF-mediated immune signaling might account for the lack of Th2 bias. Indeed, mouse VEGF does not induce Th2 polarity in mouse CD4 T cells in vitro. This appears to be due to the absence of
VEGFR2—the primary signaling VEGF receptor (25) in mouse APC—and inability of APC to respond to VEGF. In contrast, whereas human APC do not constitutively express high levels of VEGFR2, we found that VEGFR2 expression can be induced in APC upon culture with VEGF. It is unclear whether low levels of VEGF receptors are present on resting human APC or whether some other mechanism is responsible for VEGF-induced VEGF receptor upregulation. Nonetheless, this positive feedback loop provides a mechanistic basis for VEGF to modulate human APC function. Mouse APCs, however, do not appear to upregulate VEGFR2 when VEGF is present, but rather upregulate VEGFR1 with either VEGF or LPS stimulation. VEGFR1 is thought to function as a decoy or negative signaling receptor, as it lacks the primary intracellular signaling residues found on the cytoplasmic tail of VEGFR2 (26); thus, mouse APC are unable to repolarize in response to VEGF. Although LPS induces increased VEGFR2 expression in mouse APC, this ligand is associated with acute bacterial infections and is not likely to cause chronic immune polarity shifts.

Interestingly, human but not mouse APC strongly upregulate Nrp1 upon either VEGF or LPS stimulation. Neutrophils are considered coreceptors for VEGF that do not themselves signal but potentiate signaling by tyrosine kinase containing VEGF receptors (27). Nrp1 upregulation upon VEGF stimulation may further serve to mediate immune polarity changes in human APC.

Cancer immunotherapy strategies including antitumor vaccines have shown promise in numerous murine models of cancer (28). Unfortunately, clinical benefit from vaccines in cancer patients is seen only infrequently (12). This has led some investigators to propose that mouse models of cancer are of limited value in assessing the efficacy of vaccines and other immunomodulatory strategies (29). In this study, we demonstrate a mechanism by which tumor-host interactions differ widely between mice and humans. This highlights a key roadblock in translating vaccine results from mouse models into clinical practice in patients with advanced tumors. Conversely, it is possible that combining therapy targeting VEGF or VEGF pathways with vaccine-based approaches might provide similar benefits in human cancer patients to what has been demonstrated in mouse models of cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Differential Response of Human and Mouse Dendritic Cells to VEGF Determines Interspecies Discrepancies in Tumor-Mediated Th1/Th2 Polarity Shift

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