Evidence of Systemic Th2-Driven Chronic Inflammation in Patients with Metastatic Melanoma

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Abstract

Purpose: Immunotherapeutic modalities are commonly used for treatment of patients with melanoma. The therapeutic success in preclinical models has not yielded the expected clinical results. To understand this discrepancy, we attempted to define immune homeostasis of 209 patients with melanoma across stages of disease relative to normal controls.

Experimental Design: Peripheral blood mononuclear cells (PBMC) and plasma were collected from patients and healthy donors. PBMC were analyzed for frequencies of natural killer, dendritic, and T cells and their functional status. Matched plasma samples were analyzed for the concentrations of 27 cytokines, chemokines, and growth factors. RNA was isolated from 24 metastatic melanoma tumor biopsies and profiled by microarray analysis.

Results: The frequency of natural killer, T, and dendritic cells in patients does not significantly change across stages of melanoma. However, plasma concentrations of Th2 cytokines [interleukin (IL)-4, IL-5, IL-10, and IL-13] in tumor-bearing patients were significantly higher than those with resected melanoma. Expression array analysis of metastatic melanoma revealed that the malignant melanocytes were not the source of the Th2 cytokines but did highly up-regulate vascular endothelial growth factor (VEGF) transcripts, consistent with plasma VEGF concentrations. In vitro VEGF exposure of normal PBMC lead to repolarization from Th1 to Th2 emulating the state of metastatic melanoma.

Conclusions: Patients with metastatic melanoma exist in a state of Th2-mediated “chronic inflammation” as a result of at least VEGF overproduction by malignant tumors. These data support prior observations regarding the effect of VEGF on immune cell function and suggests consideration of VEGF inhibitors in future cancer immunotherapy clinical studies in metastatic melanoma.

Immunotherapeutic approaches for the treatment of melanoma have been successfully tested in preclinical models (1). Similar therapeutic success in clinical testing remains unrealized (2). In particular, this has been the experience with melanoma cancer vaccines. Although immunization with tumor-specific vaccines can frequently induce a measurable CTL response in patients with metastatic melanoma (increased frequencies of tumor antigen-specific CTL determined by tetramer or ELISPOT assay), clinical benefit remains anecdotal (3). This has not only been the experience of peptide-based melanoma vaccines but also other vaccination strategies: (a) tumor cell lysates (4), (b) irradiated whole-cell tumor vaccines (5), and (c) dendritic cell-based vaccines (6, 7). The increasingly accepted reason for the lack of therapeutic success of these interventions has been the demonstration of the immunosuppressive properties of the tumor microenvironment (8). In this model, fully functional, vaccine-induced, antitumor immune cells lose their tumoricidal capacity on entry into the tumor microenvironment, thereby yielding tumor progression. Thus, most of the recent clinical efforts have focused on overcoming this immunosuppressive barrier by one of the following approaches: (a) generating greater numbers of tumor-specific CTL using in vitro expansion strategies (9, 10), (b) nonspecifically activating all endogenous CTL (anti-CTLA4) thereby expanding naturally developed tumor-specific CTL (11), or (c) blocking immunosuppressive signals at the tumor site by depletion of regulatory T cells (12). These efforts are ongoing and have shown anecdotal clinical success (13).

Faced with the discrepancy between effective antitumor immunization described with cancer vaccines (increased numbers of peripheral blood tumor-specific CTL following cancer vaccination) and the associated lack of clinical benefit, we hypothesized that the quality of the vaccine-generated
antitumor immune response in patients with advanced cancer may not be adequate despite seemingly appropriate quantity, and that this may be a function not only of the therapy (poor cancer vaccine immunogenicity) but also of the patient’s immune system’s ability to respond to the vaccine, that is, the state of immune homeostasis. As most therapies in these clinical studies were done in patients with advanced (metastatic) melanoma, we hypothesized that the increasingly recognized immunosuppressive properties of the tumor microenvironment may be affecting (“spilling over”) global immune homeostasis, thereby hindering systemic immune responsiveness to cancer vaccination. Several groups have already suggested the existence of abnormalities in systemic immune competence (homeostasis) in patients with metastatic melanoma: (a) exhausted phenotype of tumor-specific CTL (14), (b) circulating myeloid derived suppressor cells (15), (c) increased frequency of circulating regulatory T cells and Th2 cells in patients with advanced cancer versus normal controls (16, 17), and (d) diminished capacity of circulating dendritic cells to present antigens (18). This would suggest that the barriers to the therapeutic success of cancer vaccines (or other immunotherapeutic strategies that rely on endogenous immune cells to achieve a therapeutic goal) may not be limited to the tumor microenvironment but also might include systemic immune dysfunction. To address this issue, we systematically analyzed a broad range of variables of immune homeostasis in 209 patients across all clinical stages of melanoma, patients with atypical/dysplastic nevi, and normal volunteers. To define immune homeostasis in these patients, we compared the following immune variables: (a) peripheral blood frequencies of immune cell subsets and their functional status, (b) frequencies and functional status of tumor and recall antigen (e.g., CMV)-specific CTL, (c) plasma concentrations of 27 cytokines, chemokines, and growth factors in matched samples, and (d) microarray analysis of RNA isolated from matched tumor biopsies of metastatic melanoma. Herein, we describe the results of these comparisons.

Translational Relevance

The study presented in this article is a comprehensive look at immune homeostasis among individuals with progressive stages of melanoma (benign, atypical, dysplastic, in situ, and stage I-IV melanoma). In summary, we found that elevated levels of Th2 cytokines exist in plasma of stage IV patients. Additionally, using RNA microarrays, we found that the tumors express soluble factor(s), including vascular endothelial growth factor-A, which mediate the production of Th2 cytokines by T helper cells, and thereby render the immune system incapable of preventing tumor growth. This is important in the clinical setting because it potentially explains the limited success of immunotherapeutic strategies (cancer vaccines, dendritic cells, and adoptive therapy) employed to date. These data suggest that a multimodal treatment option including immunoablative chemotherapy to reset the immune system in conjunction with inhibitors of angiogenesis (anti-vascular endothelial growth factor) followed by immune reconstitution with anti-CTL4 or adoptive transfer, for example, may improve clinical outcomes of these patients.

Patients and Methods

Patient population. Blood samples collected from patients with early-stage melanoma (melanoma in situ and melanoma stages I-III) and benign nevi (atypical/dysplastic nevi) were newly diagnosed patients with no previous treatment. All patients were tumor-free at the time of peripheral blood collection. Samples from patients with metastatic melanoma (newly diagnosed, previously untreated) as well as healthy volunteers/controls were collected under a separate melanoma blood and tissue banking protocol. Both protocols were reviewed and approved by the Mayo Clinic Institutional Review Board for use in these studies. All biospecimens were collected, processed, and stored in uniform fashion following established standard operating procedures in our laboratory. All patients signed an informed consent document approved by the Institutional Review Board at Mayo Clinic. Patient samples were collected between 2000 and 2007. The presented study describes data obtained from 113 men and 96 women ranging in age from 21 to 85 years (Table 1).

Collection of plasma and peripheral blood mononuclear cells. Peripheral venous blood (50-90 mL) was drawn into heparinized Vacutainer tubes that were processed and separated into plasma and peripheral blood mononuclear cells (PBMC) following gradient centrifugation using Ficoll-Paque (GE Healthcare). Plasma was collected and immediately frozen at -80°C (1 mL aliquots). PBMC were collected, washed in PBS, counted, diluted to 1 × 10^7/mL, and viably frozen in 90% cosmic calf serum (HyClone) and 10% DMSO (Sigma). All assays were batch analyzed at the end of the study.

Immunophenotyping. The following anti-human monoclonal antibodies were used in PBMC immunophenotyping for flow cytometry: anti-CD3 antigen-presenting cells (APC), anti-CD3 FITC, anti-CD3 phycoerythrin (PE), anti-CD4 FITC, anti-CD8 PE, anti-CD16 phycoerythrin-Cy7 (BD Biosciences). The following antibodies were used in PBMC immunophenotyping for intracellular cytokine staining: anti-CD3 phycoerythrin (PE), anti-CD4 PE, anti-CD8 FITC, anti-CD16/FITC, and anti-CD69 PE (BD Biosciences). The following antibodies were used in PBMC immunophenotyping for tetrameric analysis: anti-CD3 PE-Cy5 (BD Biosciences). The following antibodies were used in PBMC immunophenotyping for RNA array analysis: anti-CD3 phycoerythrin (PE), anti-CD4 FITC, anti-CD8 PE, anti-CD16 phycoerythrin-Cy7 (BD Biosciences).

Table 1. Study patient population distributed by clinical category, age, sex, and assayed immune variables

<table>
<thead>
<tr>
<th>Clinical category</th>
<th>Total patients</th>
<th>Age, mean ± SD (range)</th>
<th>% Female</th>
<th>Assayed immune variables</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Cell subset</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>Plasma cytokines</td>
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<td></td>
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<td>Tetramer</td>
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<td></td>
<td>T-cell function assay</td>
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<td></td>
<td></td>
<td>RNA array</td>
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<tr>
<td>Benign nevi</td>
<td>34</td>
<td>51 ± 12 (21-71)</td>
<td>68</td>
<td>26</td>
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<tr>
<td>Atypical/dysplastic</td>
<td>25</td>
<td>52 ± 16 (25-84)</td>
<td>44</td>
<td>22</td>
</tr>
<tr>
<td>In situ melanoma</td>
<td>36</td>
<td>61 ± 16 (26-84)</td>
<td>36</td>
<td>30</td>
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<tr>
<td>Stage I</td>
<td>45</td>
<td>54 ± 17 (21-82)</td>
<td>44</td>
<td>36</td>
</tr>
<tr>
<td>Stage II</td>
<td>16</td>
<td>55 ± 17 (22-81)</td>
<td>44</td>
<td>11</td>
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<tr>
<td>Stage III</td>
<td>16</td>
<td>53 ± 19 (23-83)</td>
<td>44</td>
<td>14</td>
</tr>
<tr>
<td>Stage IV</td>
<td>37</td>
<td>56 ± 14 (28-85)</td>
<td>43</td>
<td>32</td>
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PE, anti-CD56 PE, anti-CD62L APC, anti-CD69 FITC, anti-CD14 FITC, anti-CD16 FITC, anti-CD19 FITC, anti-CD11c APC, anti-CD80 PE, anti-CD83 PE, anti-CD86 PE, anti-CD40 APC, anti-HLA-DR PC5, and anti-CD1-P1 (BD Pharmingen). The human monoclonal antibodies anti-CD45 PC5 and anti-CD25 PE were purchased from Biolegend and used in conjunction with anti-human FoxP3 for the enumeration of regulatory T cells. The following anti-human monoclonal antibodies were used for intracellular staining for flow cytometry: anti-IFN-γ FITC, anti-IL-13 PE, and anti-IL-4 PE (R&D Systems) and anti-FoxP3 Alexa Fluor 488 (Biolegend).

Previously frozen PBMC (0.5 × 10^6-1.0 × 10^6/mL) were thawed and aliquoted into 96-well round-bottomed plates (100 μL/well). The desired antibody or antibody pool was added at 5 μL/well. The cells and antibodies were incubated for 30 min at 4°C and washed twice with 1× PBS (Cellgro), 0.1% bovine serum albumin, and 0.05% sodium azide (Sigma). Four-color flow cytometry was done on a LSRII flow cytometer (Becton Dickinson) and CellQuest software (Becton Dickinson) was used for data analysis.

To determine the effect of VEGF on Th1 and Th2 polarity, PBMC from healthy donors were stimulated from 10 to 1,000 pg/mL. Four-color flow cytometry was done with a FACSCalibur and CellQuest software (Becton Dickinson) was used for data analysis.

**Plasma cytokine, chemokines, and growth factor concentrations.** Protein levels for 27 cytokines, chemokines, and growth factors, including IL-1β, IL-1α, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-15, IL-17, Eotaxin, basic fibroblast growth factor, granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, IFN-γ, IP-10, MCP-1, MIP-1α, MIP-1β, platelet-derived growth factor, RANTES, tumor necrosis factor-α, and vascular endothelial growth factor (VEGF), were measured using the Bio-plex cytokine assay (Bio-Rad) as per manufacturer's instructions. Plasma samples were diluted 1:4 in dilution buffer and 50 μL were added to washed, fluorescently dyed microspheres (beads) to which biomolecules of interest are bound. The beads and diluted patient plasma were incubated for 30 min at room temperature with agitation. After incubation, the beads were washed in Bio-plex wash buffer and placed in 25 μL detection antibody and incubated for 30 min as described above. After washing, the beads were placed in streptavidin-PE, incubated, and washed a final time. The bound beads were resuspended in 125 μL Bio-plex assay buffer and read with the LumineX plate reader (Bio-Rad). Protein concentrations were determined using a standard curve generated using the high PMT concentrations with sensitivity from 10 to 1,000 pg/mL.

**VEGF-mediated Th1/Th2 polarity.** To determine the effect of VEGF on Th1 and Th2 polarity, PBMC from healthy donors were stimulated for 3 days with CD3/CD28 expander beads (Invitrogen) with and without increasing doses of recombinant VEGF (1-16 pg/mL). Cells were also cultured with 10 μg/mL recombinant human IL-12 (R&D Systems) or 8 μg/mL of a monoclonal anti-human IL-12 (R&D Systems; clone 24910). After the culture, the cells were harvested and restimulated with 50 ng/mL phorbol 12-myristate 13-acetate (Sigma) and 1 μg/mL ionomycin (Sigma) in the presence of 10 μL/bottle brefeldin A for 4 h. The cells were then stained with anti-human CD4, anti-human IFN-γ, and anti-human IL-4 flow cytometry (see above).
**Tumor tissue RNA extraction and microarray.** Frozen tissue sections of melanoma biopsies were examined. Regions of pure tumor with little/no evidence of necrosis or stromal infiltration were outlined, scraped off the slides, and used for RNA extraction. Total RNA was isolated from the excised tumor tissue using the Qiagen RNA extraction kit. The quality of the RNA was evaluated by obtaining electropherograms on Agilent 2100 Bioanalyzer and RNA integrity number using 2100 Expert software (Agilent Technologies). cDNA was prepared from a total of 10 μg RNA. Samples were quantified using standard spectrophotometry using a Tecan spectrophotometer (Tecan US) and considered acceptable if the A260/A280 reading was >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 U133 Plus 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 bovine serum albumin to the hybridization buffer. The hybridization mixture was heated at 99°C for 5 min followed by incubation at 45°C for 5 min before injecting the sample into the microarray. Then, the hybridization was carried out at 45°C for 16 h with mixing on a rotisserie at 60 rpm. After hybridization, the solutions were removed and the arrays were washed and then stained with streptavidin-PE (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 U133A expression array by both obtaining electropherograms on Agilent 2100 Bioanalyzer and hybridizing a fraction of the sample onto test-3 array as a measure of quality control. GeneSpring GX 7.3 (Agilent Technologies) data analysis software was used to analyze the results of the microarray experiment. Gene expression values were normalized by the GCRMA algorithm (21).

**Statistical analysis.** The majority of samples analyzed in this report were randomly assigned to batches for each laboratory assay due to the fact that all samples were not collected/processed at the same time. The randomization was stratified to assure an even distribution across the stages of disease for each batch. The distributions of the results of each run were examined and those that did not appear to be normally distributed were transformed using either logarithmic or square root transformations. To look at differences in various variables between stages of disease, analysis was done using analysis of covariance, adjusting for age, gender, and batch effects. Results of this analysis are summarized by least square means and 95% confidence intervals for each stage of disease. P values presented are those from the overall using analysis of covariance, which compares the mean levels of each variable across all stages of disease. P values < 0.05 were considered to be statistically significant. Due to the magnitude of the cytokine data from the multiplex assay, data were processed using Partek 6.3 software and analyzed using a principal component analysis approach. We used principal component analysis in an effort to vector space transform a multidimensional data set representing 27 variables for each individual patient and group patients based on similar cytokine concentrations revealing the internal relationships of cytokines within patient groups (e.g., per stage of melanoma) in an unbiased way.

**Results**

**Plasma cytokine concentrations across stages of melanoma.** Frozen aliquots of plasma collected from patients with benign/atypical nevi as well as all stages of melanoma (in situ and stages I-IV) were analyzed for the concentration of 27 cytokines/chemokines/growth factors. The large volume of data was analyzed per patient cohort looking for data groupings within clinical categories (stage of disease) using principal component analysis (Fig. 1). The data suggested that the cytokine profiles across stages of disease differed significantly only in patients with stage IV melanoma versus all other cohorts. Closer analysis comparing each of the 27 cytokines/chemokines/growth factors between patients with stage IV melanoma and all others revealed that the greatest difference of plasma cytokine concentrations in patients with stage IV melanoma was attributed to Th2 cytokines (Table 2). This was consistent with previous reports of increased numbers of peripheral blood Th2 cells in patients (and laboratory animals) with metastatic cancer (22, 23).

**Immune cell subsets across stages of melanoma.** PBMC isolated from patients with benign nevi, atypical (including dysplastic) nevi, and patients with in situ and stages I to IV melanoma were analyzed by flow cytometry to determine the frequencies of T, natural killer (NK), and dendritic cell subsets. There were not significant differences in frequencies of T cells among stages of melanoma as determined by numbers of CD3+, CD4+, or CD8+ T cells (Supplementary Table S1A). Similarly, no significant differences were found in activated T cells (CD3/CD69), total NK cells (CD16/56+, CD3+), or most dendritic cell subset variables. As patients with stage IV melanoma appeared to differ significantly from all others with regard to plasma cytokine profiles, we proceeded to compare the cell subset analysis of patients with stage IV melanoma relative to all others. The analysis revealed no significant differences among most variables with the following exceptions: (a) the frequency of naive T cells (CD3/CD62L+) as well as activated dendritic cells (CD11c/CD83+) were significantly less in patients with stage IV melanoma and (b) the frequency of tetramer-positive CTL for gp100 and tyrosinase (but not MART-1 or CMV and EBV) were increased in patients with stage IV melanoma. Due to lack of available biospecimens, Th1 and Th2 enumeration could not be done. These data suggested that there appeared to be some level of “immune activation” in patients with metastatic melanoma that was different from all other cohorts and this was consistent with a state of Th2-mediated “chronic inflammation.”

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>P (stage IV vs all other)</th>
</tr>
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<tbody>
<tr>
<td>IL-4</td>
<td>1.73 × 10^−12</td>
</tr>
<tr>
<td>RANTES (CCL5)</td>
<td>6.17 × 10^−6</td>
</tr>
<tr>
<td>IL-10</td>
<td>5.29 × 10^−5</td>
</tr>
<tr>
<td>Eotaxin (CCL11)</td>
<td>8.31 × 10^−5</td>
</tr>
<tr>
<td>IP-10 (CXCL10)</td>
<td>0.0007</td>
</tr>
<tr>
<td>IL-13</td>
<td>0.002</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>0.005</td>
</tr>
<tr>
<td>IL-7, IL-9</td>
<td>0.009</td>
</tr>
<tr>
<td>VEGF, MIB-1b (CCL4)</td>
<td>0.02</td>
</tr>
<tr>
<td>Granulocyte-macrophage colony-stimulating factor</td>
<td>0.03</td>
</tr>
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</table>
Systemic Chronic Inflammation in Metastatic Melanoma

Assessment of functional immunity in patients with stage IV melanoma. The emerging data seemed to suggest that patients with stage IV melanoma, unlike all other patients with earlier stages of melanoma (or healthy controls), existed in a state of systemic Th2 dominance with some evidence of cellular immune activation in peripheral blood (increased frequencies of tumor-specific CTL and decreased frequencies of naive T cells). This immune homeostasis profile resembled a state of Th2 dominant “chronic inflammation,” similar to chronic viral infection (24). A reflection of the chronic inflammatory state of chronic viral infection as well as metastatic melanoma is an increase in peripheral blood PD-1+ (exhausted) T cells (25). We found the same to be true in our patient cohort of stage IV melanoma patients compared with healthy controls (Fig. 2A). This was further supported by functional assessment of antigen-specific CTL (20), revealing a significant reduction in the frequency of functional recall antigen (CMV<sub>495-503</sub>)-specific CTL in patients with stage IV melanoma versus healthy volunteers (Fig. 2B). Less than 5% of tumor antigen-specific, CTL in patients with stage IV melanoma versus healthy melanoma (stage IV melanoma). In all other subgroups of stages of malignant melanoma were detected in patients that had extensive tumor burden as evidenced by metastatic (stage IV) melanoma. The greatest differences in systemic immune homeostasis in patients across the spectrum of different clinical stages of malignant melanoma were detected in patients that had extensive tumor burden as evidenced by metastatic melanoma (stage IV melanoma). In all other subgroups of melanoma (in situ and stages I-III), disease burden was minimal (stage I or II melanoma) or nonexistent (blood collected after complete surgical resection). Therefore, the presence of visible tumor bulk could be responsible for the plasma profile of Th2 cytokine dominance, that is, the visible tumor may be the source of the Th2 cytokine overproduction. In an effort to understand this relationship and determine if the presence of tumor was responsible for the predominance of Th2 cytokines in plasma of patients with metastatic melanoma, we isolated RNA from 24 clinical specimens of metastatic melanoma (19 specimens were matched to our patient cohort of stage IV melanoma). Microscopically dissected fragments of nonneoplastic, noninfiltrated tumor tissue (as determined by H&E stain) were excised from slide-mounted frozen core biopsies, and RNA was isolated and analyzed on Affymetrix GeneChip microarray. We evaluated the expression of 23 cytokines (45 probes): IL-1α, IL-1β, IL-1ra, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12, IL-13, IL-15, IL-17, IFN-γ, CCL5, CCL11, CSF-2, MCP-1, tumor necrosis factor-α, and VEGF. The objective of the experiment was to determine whether the malignancy itself was the source of Th2 cytokines that were detected in plasma. The data revealed that many of the probed cytokines, chemokines, and growth factors are up-regulated in tumor tissue (Fig. 3A). However, there were no differences in expression of Th1 versus Th2 cytokines (IFN-γ versus IL-4, IL-5, IL-10, and IL-13; Fig. 3B) in tumor tissues, suggesting that the observed Th2 cytokine predominance in plasma was not derived from the tumor. However, of the tested cytokines, the most highly/frequently up-regulated transcript in the tumor samples was VEGF (Fig. 3B). Plasma VEGF levels were significantly higher in metastatic melanoma patients relative to healthy donors (Fig. 3C), consistent with published reports (26). Considering the described immune modulatory (down-regulatory) properties of VEGF (27), we postulated that tumor-derived VEGF could be responsible for the Th2 polarization in patients with stage IV melanoma (away from the normal state of Th1 dominance).

VEGF mediates Th2 bias. VEGF has been associated with Th2 bias in human disease (lung inflammation; ref. 28). We hypothesized that elevated levels of plasma VEGF, derived at least in part from malignant melanocytes in patients with stage IV melanoma, could be responsible for the predominance of Th2 plasma cytokines. To test this hypothesis, we stimulated healthy donor PBMC with CD3+CD28+ expander microbeads for 3 days with increasing concentrations (1-16 pg/mL) of recombinant VEGF and assessed intracellular cytokine production of IL-4 (Th2 cytokine) and IFN-γ (Th1 cytokine) in CD4<sup>+</sup> T cells at the end of in vitro culture (Fig. 4A). The data clearly showed that increasing concentrations of VEGF resulted in a dose-dependent reversal of the relative ratio of Th1 to Th2 cells in favor of Th2. Increased concentrations of VEGF were associated with a decrease in the number of Th1 cells (CD4<sup>+</sup>/IFN-γ<sup>+</sup>) with an associated reciprocal increase in Th2 cells (CD4<sup>+</sup>/IL-4<sup>+</sup>).
polarizing effects of VEGF were lost if the assay was done with purified CD4 cells only (Fig. 4B), suggesting that the observed Th polarization effect of VEGF is indirect, likely mediated by other PBMC. The addition of 10 μg/mL IL-12 to the culture containing 16 pg/mL VEGF prevented the shift in T-helper polarity from Th1 to Th2; addition of anti-human IL-12 antibody to the stimulated PBMC mimicked the effect of VEGF (Fig. 4A). These data suggest a possible role for monocyte/macrophages in the PBMC preparation as the mediators of the VEGF-induced Th polarization.

Fig. 3. VEGF levels in patients with metastatic melanoma. A, RNA expression of cytokines in human metastatic melanoma tissue. Twenty-four frozen biopsies of metastatic melanoma tumor tissues were used to extract RNA for expression array analysis. RNA expression intensity profiles of 45 probes for 24 cytokines. B, comparison of expression intensities between genes coding for Th1 (IFN-γ and IL-2), Th2 (IL-4, IL-5, IL-10, and IL-13), cytokines and VEGF. There were no statistically significant differences when comparing Th1 versus Th2 cytokine expression levels (P = 0.04); there was a statistically significant difference when comparing VEGF expression with Th1 or Th2 cytokines (P < 0.001). Levels of significance were determined using the Wilcoxon signed-rank test. C, ELISA (mean ± SD concentration) for VEGF-A was done on plasma samples from healthy donors (n = 30) and stage IV melanoma patients (n = 40).
Discussion

The presented study is a description of the state of immune homeostasis of select variables among 209 patients (and healthy volunteers) across the clinical spectrum of malignant melanoma from patients with benign nevi arousing clinical suspicion of melanoma to patients with widely disseminated metastatic melanoma. Our data showed (a) Th2 cytokine dominant systemic environment in patients with metastatic (stage IV) melanoma, not present in any other clinical categories; (b) no significant differences in the relative concentrations of peripheral blood T, NK, and dendritic cell subsets (and selected functional subsets) across clinical categories (with few noted exceptions correlating with systemic Th2 bias); and (c) possible role for tumor-derived VEGF as a relevant mediator of the Th2 bias of systemic immune homeostasis.

The main observation from the presented data is that patients with visible metastatic melanoma (stage IV) exist in a state of “chronic inflammation” that could at least in part be the result of tumor-derived VEGF. This is different from healthy controls or patients with completely surgically resected melanoma exhibiting Th1-biased immune homeostasis (acute inflammation). Our data suggest that the malignancy may play an active role in “reprogramming” systemic immunity toward Th2 dominance/chronic inflammation that may be permissive to tumor progression/metastases. These data are consistent with clinical reports of associations of global variables of chronic inflammation and outcomes in patients with advanced cancer (e.g., C-reactive protein elevation in the setting of metastatic cancer; ref. 29) as well as specific tumor antigen-associated Th2 cell bias (e.g., MAGE-6) identified in peripheral blood of patients with metastatic melanoma and renal cell carcinoma (16). In the latter example, Tatsumi et al. describe the dominance of MAGE-6 peptide-specific Th2 cells in patients with clinically evident metastatic melanoma/renal cell carcinoma and absent in healthy volunteers and patients with no detectable cancer. The authors conclude that future immunotherapeutic interventions will likely have to overcome systemic Th2-dominated, tumor-reactive CD4+ T-cell responses to achieve optimal clinical benefit. It is noteworthy that the Th2 bias was described in both metastatic melanoma and renal cell
carcinoma, two malignancies characterized by high levels of VEGF in plasma.

The notion that inflammation plays a role in tumor progression is not new. The association of inflammation and neoplastic tissue progression was originally suggested by Virchow during the latter part of the 19th century (30). Progression of several malignancies (including melanoma) has been associated with “chronic inflammation” at the level of the tumor microenvironment (30–33). Tumor microenvironment-associated immune dysfunction (suppression) has clearly been associated with poor clinical outcomes revealing several potential, clinically relevant, mechanisms of immune escape (18, 34–36). One such example is that of Th2 cytokine-producing tumor-associated macrophages that recognize tumor antigens but cannot properly mature in the tumor microenvironment, thereby yielding a state of T-cell immune tolerance (36). Additionally, tumor-associated macrophages down-regulate T-cell receptor expression on tumor-infiltrating lymphocytes (37) and produce IL-4 and IL-5 (Th2) rather than IFN-γ (Th1; ref. 38). Tumor infiltration by regulatory T cells has also been associated with poor prognosis in patients with ovarian cancer (39), non-Hodgkin's lymphoma (40), basal cell carcinoma (41), and squamous cell carcinoma of the head and neck (42). Thus, dysfunctional (suppressed) immunity in the tumor microenvironment appears clinically relevant, is associated with Th2 bias, and leads to poor clinical outcome. Therefore, evidence of systemic reprogramming of Th2 immunity from a normal state of Th1 dominance to a state of Th2-biased (cytokine and cellular repolarization) systemic immune homeostasis may be an extension of the immunologic changes in the tumor microenvironment and contribute to the observed poor clinical results of immunotherapy (specifically cancer vaccines) in patients with stage IV melanoma.

Systemic Th2 immune bias of chronic inflammation is a state of immune homeostasis present in both pathologic and physiologic states in humans. Similar pathologic states of Th2-driven systemic chronic inflammation have already been described in the setting of chronic viral infections (43, 44). Additionally, there are also examples of physiologic (normal) Th2-mediated “chronic inflammation” that are organ specific (the mucosa of the gut and lungs; ref. 45) as well as systemic (normal pregnancy; ref. 46). In both settings, local/systemic Th2 polarization seems to allow a symbiotic coexistence of the immunocompetent “host” with another organism (bacteria in the gut and lung; fetus in the uterus) by dampening robust immune Th1 acute inflammatory responses in the microenvironment (gut/lung) or both microenvironment and systemic immunity (pregnancy). Maternal systemic Th2 polarization of pregnancy may explain the existence of peripheral blood microchimerism (small numbers of persisting fetal blood cells surviving in maternal blood) in parous women. Thus, similar to the placental microenvironment leading to systemic Th2 polarization (at least in part mediated by placental growth factor), the immunosuppressive tumor microenvironment may lead to systemic Th2 polarization via VEGF overproduction (a placental growth factor analogue; ref. 47). High plasma VEGF levels have been associated with poor clinical outcomes in patients with metastatic melanoma (48). Thus, in the context of the immunomodulatory properties of VEGF and its structural homology to placental growth factor, we postulated that systemic VEGF overproduction by tumors could be responsible for systemic Th2 immune polarization favoring tumor progression. As illustrated in Fig. 4, nonspecific stimulation of CD4+ Th cells from healthy PBMC donors in the presence of recombinant VEGF results in a dose-dependent increase in the numbers of Th2 cells with a reciprocal decrease in Th1 cells emulating the findings in peripheral blood of patients with stage IV melanoma. The specific mechanisms of this reprogramming remain under ongoing investigation.

In summary, the presented data suggest that the state of systemic immune homeostasis in patients with metastatic melanoma resembles Th2-mediated chronic inflammation and appears at least in part mediated by tumor-derived VEGF. These data expand on published reports of the increased frequency of Th2 (over Th1) tumor antigen-specific CD4+ T cells in the setting of advanced melanoma/renal cell carcinoma (16) and in part support the observed improved efficacy of human adoptive transfer experiments of tumor-specific CTL delivered following “lymphodepletion” (depletion of systemic Th2 immune homeostasis; ref. 49) when compared with adoptive transfer alone (50). These data may also offer some insight into the unrealized therapeutic benefit of nonspecific systemic T-cell activation (e.g., high-dose IL-2, or anti-CTLA-4), which may have augmented a preexisting “dysfunctional” systemic immune response. Perhaps, therapeutic combinations adding inhibitors of angiogenesis (anti-VEGF) to systemic lymphodepletion (removing established Th2 bias) followed by attempts at immune reconstitution (anti-CTLA4, adoptive transfer, etc.) may yield desirable immune recovery and beneficial clinical outcomes.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank LuRaye Eischens for administrative support in the completion of this article.

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