Cancer
Research

Active Immunotherapy Induces Antibody Responses That Target Tumor Angiogenesis

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Abstract

The inhibition of VEGF signaling with antibodies or small molecules achieves clinical benefits in diverse solid malignancies. Nonetheless, therapeutic effects are usually not sustained, and most patients eventually succumb to progressive disease, indicating that antiangiogenic strategies require additional optimization. Vaccination with lethally irradiated, autologous tumor cells engineered to secrete granulocyte-macrophage colony stimulating factor (GM-CSF) and antibody blockade of cytotoxic T lymphocyte–associated antigen-4 (CTLA-4) trigger a tumor vasculopathy in some long-term responding subjects. These reactions are characterized by disrupted tumor blood vessels in association with lymphocyte and granulocyte infiltrates and zonal areas of ischemic tumor necrosis. However, the mechanisms underlying this immune-mediated destruction of the tumor vasculature remain to be clarified. Here, we show that GM-CSF–secreting tumor cell vaccines and CTLA-4 blockade elicit a functionally important humoral reaction against multiple angiogenic cytokines. Antibodies to angiopoietin-1 and angiopoietin-2 block Tie-2 binding, downstream signaling, endothelial cell tube formation, and macrophage chemotaxis. Antibodies to macrophage inhibitory factor (MIF) attenuate macrophage Tie-2 expression and matrix metalloproteinase-9 (MMP-9) production. Together, these results delineate an immunotherapy-induced host response that broadly targets the angiogenic network in the tumor microenvironment.
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Introduction

Substantial evidence indicates that the angiogenic switch plays a decisive role during tumor development (1). Because oxygen and other essential metabolites diffuse from the existing vasculature for only a limited distance, progressive tumor growth and systemic dissemination require the acquisition of additional blood supplies. Whereas several mechanisms may contribute to the angiogenic switch, the generation of new blood vessels from pre-existing vascular structures is the most intensively studied (2). Angiogenesis is now understood to reflect the integration of multiple pro- and antiangiogenic factors and to involve the concerted activities of not only vascular elements, but also myeloid cell populations (3).

Among the components of the angiogenic network, VEGF-A was the first to be validated as a target for cancer therapy (4). Antibodies and small molecule inhibitors of VEGF function mediate antitumor activity alone or in combination with chemotherapy in carcinomas of the colon, kidney, lung, breast, and liver, but the overall magnitude of the benefit is modest, and most patients still succumb to progressive disease (5, 6). Several factors might limit the efficacy of VEGF-A–targeted treatments, including the activation of other soluble or cellular angiogenic factors and a switch to nonangiogenic modes of accessing a vascular supply (7). The relative importance of these pathways to therapeutic resistance in patients, however, remains to be determined.

Tumor pathogenesis may involve an impaired wound healing response (8). Because tissue damage normally elicits a coordinated immune and vascular reaction, immunologic mechanisms might be able to modulate tumor angiogenesis. Indeed, Coley's toxins, one of the first immunotherapies to be developed, evoke hemorrhagic tumor necrosis through a cascade of cytokines and cells that perturb the tumor vasculature (9, 10). More recently, vaccination against VEGF, VEGFR, and tumor-associated macrophage gene products was shown to elicit protective tumor immunity in several murine models (11–13).

In this context, we reported that vaccination with lethally irradiated, autologous tumor cells engineered to secrete

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GM-CSF and antibody blockade of cytotoxic T lymphocyte associated antigen-4 (CTLA-4) engendered a coordinated cellular and humoral response that effectuated clinically significant tumor destruction in some patients with advanced solid malignancies (14–18). Metastases resected following therapy revealed the stimulation of dense intratumoral infiltrates composed of $CD4^+$ and $CD8^+$ T cells and antibody-producing B cells in long-term responding patients. Disrupted tumor blood vessels were also observed in association with lymphocyte and granulocyte infiltrates and zonal areas of ischemic tumor necrosis. These findings suggested that multiple immune effector mechanisms might participate in tumor destruction.

Through antibody-based screening of tumor-derived cDNA expression libraries, we previously characterized several tumor-associated gene products that were recognized by high-titer antibodies and cytotoxic T cells and were linked with therapy-induced tumor necrosis (14). Here, we employed a similar approach to uncover a potent humoral reaction against multiple angiogenic cytokines.

Materials and Methods

Clinical protocols

The phase I trials of vaccination with lethally irradiated, autologous tumor cells engineered to secrete GM-CSF in advanced melanoma and non–small cell lung carcinoma patients have been described (15, 16, 19). The phase I trials of the fully human anti-CTLA-4 blocking monoclonal antibody (Ipilimumab) in previously vaccinated melanoma and ovarian carcinoma patients have also been reported (17, 18). All clinical protocols received approval from the Dana-Farber/Harvard Cancer Center Institutional Review Board, the Food and Drug Administration, and the Recombinant DNA Advisory Committee.

cDNA library construction and screening

A cDNA expression library was generated from B16 cells employing previously described methods (20). In brief, total RNA was isolated using guanidine isothiacyanate, mRNA purified over oligo-dT cellulose columns, and cDNA synthesized with Superscript II Reverse Transcriptase (RT, Invitrogen). The cDNA was cloned into the Lambda Zap vector and the library screened according to the manufacturer's instructions (ZAP-cDNA Gigapack III Gold cloning and picoBlue Immunoscreening kits, Stratagene). K008 serum diluted 1:500 in Tris-buffered saline with Tween (TBST) was precleared against a negative control phage expression library overnight and then used for screening. Positive clones were developed with mouse horseradish peroxidase (HRP)-conjugated anti-human antibodies (Zymed) at a 1:2,000 dilution and then isolated through 3 rounds of purification. Phagemids were excised, purified (Qiagen kit) and sequenced by the Dana-Farber Cancer Institute Core Facility. Inserts were compared using BLAST search protocols to the Entrez Nucleotide database maintained by the NCBI.

Anticytokine antibodies

For immunoblotting, 250 ng of recombinant VEGF-A and basic fibroblast growth factor (bFGF; R&D Systems) were run on a 10% reducing SDS polyacrylamide gel, transferred to a nylon membrane, probed with patient sera (1:100 dilution in 2% milk), followed by an HRP-conjugated goat anti-human IgG (Zymed) at a 1:2,000 dilution, and developed with a Chemiluminescence reagent (Perkin-Elmer). Control anti-VEGF-A and bFGF antibodies (R&D Systems) were used at a 1:2,000 dilution.

Anticytokine antibodies were measured by coating ELISA plates (Nunc) overnight at 4°C with recombinant human angiopoietin-1, angiopoietin-2, MIF (macrophage inhibitory factor), bFGF, placental growth factor (PLGF), VEGF-A, VEGF-B, VEGF-C, and VEGF-D protein (R&D Systems) dissolved in a carbonate buffer (pH = 9.6). Next, the wells were blocked overnight at 4°C with 2% milk, washed, and then incubated in duplicate or triplicate with 100 μL of patient or donor sera diluted 1:100 in 2% milk overnight at 4°C. A goat anti-human IgG conjugated to alkaline phosphatase (Jackson Laboratories) was added at room temperature, the plate developed with pNPP substrate (Sigma-Aldrich), and the absorbance at 450 nm determined.

In some experiments, immunoglobulins were enriched from plasma through an initial clearing on glass beads (Sigma) followed by treatment with recombinant protein G agarose (Invitrogen), according to the manufacturer's protocol. For absorption studies, the clarified plasma was incubated with plate-bound angiopoietin-1 or angiopoietin-2 overnight at 4ºC and then purified with protein G agarose. Absorption was repeated until the antiangiopoietin titer became undetectable in ELISA.

Angiopoietin/Tie-2 binding

ELISA plates were coated with 20 ng per well of angiopoietin-1 or angiopoietin-2 in a carbonate buffer overnight at 4°C. After washing and blocking overnight with milk, sera diluted 1:7 in 2% milk were added for 8 hours at room temperature, and then 7.5 ng of recombinant Tie-2-Fc (R&D Systems) in 2% milk was added overnight at room temperature (final sera dilution 1:10). After washing, Tie-2 specific binding was determined with an anti-Tie-2 mAb (R&D Systems), an HRP-conjugated anti-mouse IgG, and developing reagents. Absorbance at 450 nm was measured. In some experiments, Tie-2-Fc was first coated on the plate and the specific binding of angiopoietin-1 and angiopoietin-2 was determined using comparable procedures.

Tube-forming assays

Human umbilical vein endothelial cells (HUVEC) were obtained from Cambrex and early passage cells (<12) were used for all experiments. Tube-forming assays were conducted using 10,000 cells per well maintained in Ham F12 media containing 15% FBS in 96-well cell culture plates and a Fibrin Gel In Vitro Angiogenesis Assay Kit (Chemicon International) according to the manufacturer's instructions. At least 2 replicate wells were used for each experimental condition. In some experiments, 250 ng of recombinant angiopoietin-1, angiopoietin-2, or MFG-E8 were added to the culture media, and patient sera were used at a 1:10 dilution. Tube formation was assayed by pattern recognition according to manufacturer's

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instructions, using at least 3 random view-fields per well by an observer who was blinded to the treatment conditions. Values were assigned as follows: 0, cells isolated or in a sheet-like monolayer; 1, cells begin to migrate and align themselves; 2, capillary tubes visible, no sprouting; 3, sprouting of new capillary tubes visible; 4, closed polygons begin to form; 5, complex mesh like structures develop.

Angiopoietin-induced migration

Migration assays were performed with transwells containing 16-μm pore size inserts (Corning) coated with Basement Membrane Extract Cultrex (BD Biosciences). Monocytes were isolated from peripheral blood mononuclear cells using CD14 microbeads (Miltenyi Biotech) and cultured for 5 days with M-CSF (20 μg/mL added on days 0, 2, and 4). Recombinant angiopoietin-1 or angiopoietin-2 (100 ng/mL) and patient sera diluted 1:100 were placed in serum-free RPMI medium (500 μL) in the bottom compartment of the chamber, and 100 μL of monocytes (10^4 cells/mL) was added to the top compartment. The chambers were incubated at 37°C in humidified air with 5% CO_2 for 24 hours. Migrated cells were labeled with 5 μg/mL of calcein-AM (Molecular Probes) in RPMI at 37°C for 1 hour and counted under a fluorescence microscope. The number of cells migrated in the absence of angiopoietin-1/2 (−) was used as a reference value and set to 1. Results are expressed as a migration index relative to the reference value. Spontaneous migration was typically 1% to 6% of input cell number.

Phospho-ERK-1/2 activation

Subconfluent HUVECs were serum starved in 1% bovine serum albumin (BSA) supplemented Ham's F-12 medium for 6 hours, treated with angiopoietins and/or patient sera as indicated, washed twice in ice-cold PBS, and lysed in Cell Signaling Technology buffer supplemented with 1 mmol/L of phenylmethylsulfonyl fluoride. Immunoblotting was performed as above using antibodies to phospho-p44/42 extracellular signal-regulated kinase (ERK)-1/2 (Cell Signaling). The PathScan(R) Phospho-p44/42 MAPK (Thr202/Tyr204) Sandwich ELISA Kit (Cell Signaling Technology) was also used according to the manufacturer's instruction.

MIF-induced responses

Human monocytes obtained as above were incubated for 48 hours (2.5×10^5 per well in 12-well plates) with recombinant MIF (200 ng/mL; R&D Systems) with or without MEL15 sera (10%) in serum-free RPMI medium. The cells were stained with PE-conjugated anti-Tie-2 mAb (R&D Systems) and analyzed with a FW501 flow cytometer (Beckman-Coulter) and FlowJo software (Tree Star). In some experiments, monocytes were treated for 1 day with recombinant MIF in the presence of anti-MIF neutralizing antibodies (20 μg/mL: R&D Systems), control IgG, or MEL15 sera diluted 1:100. MMP-9 (matrix metalloproteinase-9) levels were measured using ELISA systems according to the manufacture's instruction (R&D Systems).

Statistics

The exact Wilcoxon rank-sum test was used to compare the vaccinated and control subjects for antibodies to angiopoietin-1 and angiopoietin-2, and Tie-2/angiopoietin binding. The *t* test was used to compare MMP-9 levels in response to MIF.

Results

Vaccination stimulated blocking antibodies to angiogenic cytokines

In earlier work aimed at identifying antigens associated with immune-mediated tumor destruction, we screened cDNA expression libraries prepared from densely infiltrated metastases with sera from patients who achieved durable clinical responses to vaccination with lethally irradiated, autologous tumor cells engineered to secrete GM-CSF alone or with subsequent CTLA-4 antibody blockade. These experiments established the immunogenicity of multiple tumor-associated gene products, including ATP6S1, melanoma inhibitor of apoptosis protein, opioid growth factor receptor, MHC class I chain-related protein A, focal adhesion kinase, AKT-2, upstream binding factor (UBF), and HIDA (20–24). Interestingly, ATP6S1 was also found to elicit high-titer antibodies in mice immunized with irradiated, GM-CSF–secreting B16 melanoma cells, suggesting that some tumor antigens might be recognized in both human and murine systems (20). Consistent with this possibility, a screen of a B16 cDNA expression library with sera from mice vaccinated with GM-CSF secreting B16 cells yielded MIDA and UBF-2, the murine orthologs of the human antigens HIDA and UBF (unpublished findings).

To explore further the idea that vaccine targets might show conserved immunogenicity, we screened a B16 murine melanoma cDNA expression library with sera from long-term responding melanoma patient K008. This subject underwent harvest of a pulmonary lesion for vaccine manufacture and harbored a soft tissue metastasis at the time of initiating therapy with irradiated, autologous, GM-CSF secreting tumor cells. After the fifth immunization, a subcutaneous nodule in K008 was noted to become hemorrhagic, which reflected the induction of a tumor vasculopathy with brisk lymphocyte and granulocyte infiltrates, as revealed through pathologic examination of the resected lesion (16). Without any additional treatment, K008 remained disease free for 14 years following receipt of the 6 vaccinations.

Sera obtained from K008 after completion of immunization detected 13 gene products in the B16 library, and these included proteins linked to transcription, translation, DNA repair, protein homeostasis, the cytoskeleton, and the surface membrane (Supplemental Table 1). Functionally, these targets were similar to antigens identified in previous studies using the human cDNA expression libraries. However, the B16 screen also yielded VEGF-A, a potent angiogenic factor with a key role in tumor pathogenesis (25), and we elected to investigate this target further. Consistent with the high-sequence conservation of the cytokine between mice and humans, K008 sera also recognized recombinant human VEGF-A protein in immunoblotting (Fig. 1A). Antibodies to VEGF-A were not observed in several healthy donors, whereas K008 sera failed to react with bFGF, underscoring the specificity of the anti-VEGF-A response. K008 sera did not detect VEGF-A in an ELISA format, precluding more detailed quantitative

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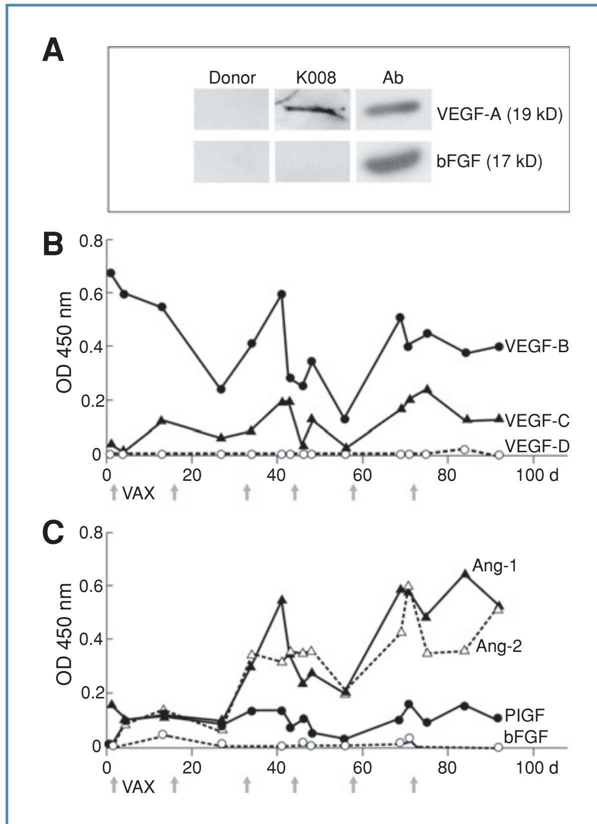

Figure 1. Vaccinated melanoma patient K008 developed antibodies to multiple angiogenic cytokines. A, sera from K008, but not healthy donors, ($n = 3$) recognize VEGF-A, but not bFGF by immunoblotting (sera at 1:100). Control anticytokine antibodies are shown for comparison. B, longitudinal analysis of antibodies to VEGF family members in K008. Antigen-specific IgG levels were determined with an ELISA (sera at 1:100). Arrows denote vaccinations. Day 0 is prevaccination. C, vaccination-stimulated potent humoral reactions to angiopoietin (Ang)-1 and Ang-2 in K008. A subcutaneous nodule developed hemorrhagic necrosis after the fifth immunization. Day 0 is prevaccination.

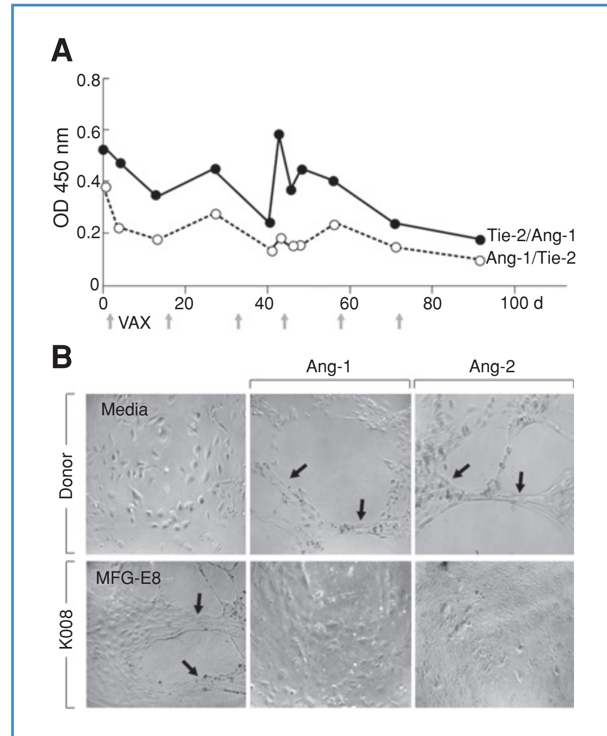

Figure 2. Vaccine-induced antibodies to Ang-1 and Ang-2 manifest blocking activity. A, postvaccination K008 sera inhibit the binding of Tie-2 and Ang-1 as measured with an ELISA (sera at 1:10). Day 0 is prevaccination. Full time-course studies were performed twice with similar results, whereas high- and low-blocking samples were analyzed 2 additional times, with equivalent findings. B, sera from K008, but not healthy donors, inhibit Ang-1 and Ang-2–induced tube formation with HUVECs. K008 sera failed to block MFG-E8–stimulated tube formation. This experiment was performed 2 times with similar results. Prevaccination sera from K008 were not available for this assay. Representative high-power fields are shown. Tube-forming scores (averages of 6 replicates) were: donor (media) 0, (angiopoetin-1) 3.5, (Ang-2) 3; K008 (MFG-E8) 2.67, (Ang-1) 0.83, and (Ang-2) 0.33.

analysis. The reasons for this discordance in reactivity remain to be clarified, but might include differences in the recognition of denatured linear versus conformation epitopes.

As VEGF-A is closely related to several other proteins that contribute to angiogenesis, we investigated whether K008 sera recognized other human VEGF family members (Fig. 1B and C). An ELISA revealed that K008 harbored antibodies to VEGF-B at the time of study enrollment. While these varied somewhat during the course of immunization, a clear association with therapy was not evident. Antibodies to VEGF-C and PLGF were of a relatively low titer, whereas no reactivity against VEGF-D was observed. Together, these humoral reactions did not appear sufficient to account for the therapy-induced tumor vasculopathy.

Because angiogenesis involves the coordinated activities of several soluble proteins, we expanded our survey to include angiopoietin-1 and angiopoietin-2. These growth factors cooperate with VEGF during tumor progression, but their precise functions in endothelial cell and pericyte biology, and the signaling pathways triggered after cognate binding to the Tie-2 receptor tyrosine kinase remain under active study (26, 27). Intriguingly, vaccination increased antibodies to both angiopoietin-1 and angiopoietin-2 in K008, and these peaked concurrently with the development of hemorrhage in the subcutaneous nodule (Fig. 1C). The maximal reactions were detectable in sera dilutions of 1:10,000.

To explore whether the antiangiopoietin antibodies modulated cytokine activity, we established an ELISA to measure ligand–receptor interactions *in vitro*. Recombinant Tie-2 protein was coated on the plate and the ability of K008 sera to inhibit cognate angiopoietin-1 binding was then determined (Fig. 2A). The vaccine-stimulated increase in antibody titer was associated with diminished angiopoietin-1/Tie-2 binding. Similar results were obtained when angiopoietin-1 was initially applied to the dish and the binding of Tie-2 was subsequently evaluated. Additional studies showed that K008 sera mediated comparable inhibition of angiopoietin-2/Tie-2 interactions as a function of immunization (not shown).

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To examine whether the ability of the antiangiopoietin antibodies to block Tie-2 binding *in vitro* attenuated cytokine biological activity, we established tube formation assays using HUVECs. While minimal tube formation was elicited in the absence of endothelial growth factor supplementation, both angiopoietin-1 and angiopoietin-2 stimulated responses, which were not affected by the addition of sera from healthy donors lacking antiangiopoietin antibodies (Fig. 2B). Sera obtained from K008 after vaccination markedly inhibited angiopoietin-1 and angiopoietin-2–induced tube formation, but did not influence tube formation elicited with milk fat globule EGF-8, a protein highly expressed in melanomas that promotes angiogenesis through binding $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins on endothelial cells (28). Unfortunately, insufficient sera from K008 prior to vaccination were available for evaluation in the tube formation assays. Nonetheless, because previous work in murine models showed that antagonizing angiopoietin function stimulated tumor destruction (29, 30), our findings raise the possibility that antiangiopoietin-1 and angiopoietin-2 antibodies in K008 might contribute to the tumor vasculopathy.

Antiangiopoietin antibodies in vaccinated cancer patients

To determine whether other patients mounted humoral reactions to angiopoietins, we tested a panel of stage IV melanoma, non–small cell lung carcinoma, and ovarian carcinoma patients that were enrolled on our phase I clinical trials of vaccination with lethally irradiated, autologous tumor cells engineered to secrete GM-CSF (Fig. 3A–C, Supplemental Table 2). Immunized subjects harbored higher titers of antibodies to angiopoietin-1 and -2 compared to healthy donors (antiangiopoietin-1 antibodies, vaccinated vs. donors, $P = 0.0014$; antiangiopoietin-2 antibodies, vaccinated vs. donors, $P = 0.00007$). These humoral reactions were evident in sera dilutions ranging from 1:400 to 1:12,500 (Supplemental Table 3). The sera from immunized patients also manifested greater blockade of angiopoietin-1/Tie-2 interactions *in vitro* compared to the donors (vaccinated vs. donors, $P = 0.003$). Although angiopoietin-1 and angiopoietin-2 share 55% sequence identity, 1 subject (MEL15) mounted humoral responses to angiopoietin-2 alone, indicating that unique determinants may be targeted in some patients. Further studies are required, however, to delineate the specific epitopes evoking immune recognition in the vaccinated cohort.

Longitudinal analysis revealed that immunization enhanced antibody titers in 11 patients, and these ranged from 3- to 30-fold increases (Supplemental Table 4). Eight of the subjects with enhanced antibody responses achieved prolonged survival as a function of treatment, which ranged from a minimum of 4.5 through 14 years. On the basis of these favorable clinical courses, we wondered whether the humoral reactions to angiopoietins might be functionally active.

Two patients with sufficient sera available were selected for more detailed characterization. M30 is an advanced melanoma patient with visceral and brain metastases who was treated with a combination of vaccination, brain irradiation, and dimethyl triazeno imidazole carboxamide (DTIC) therapy and achieved a complete response that is ongoing 9 years following vaccination. L19 is a stage IV non–small cell lung carcinoma patient who was surgically rendered without evidence of disease following vaccination and remains disease-free 10 years after study enrollment.

Both M30 and L19 displayed increases in antibodies to angiopoietin-1 and angiopoietin-2 as a consequence of immunization (Fig. 4A and B). Late in the treatment course, however, antibody titers declined slightly; this might reflect a reduction in tumor burden, consistent with the clinical course, or less likely a waning of the immune response. Postvaccination sera from M30-attenuated angiopoietin-1 and angiopoietin-2 driven endothelial cell tube formation (Fig. 4C), consistent with the results obtained for K008, whereas pretreatment sera failed to. Moreover, the blocking activity was attenuated by absorption of the sera with recombinant cytokine, demonstrating that antiangiopoietin antibodies, and not other factors present in the sera, mediated the inhibition. In contrast, sera obtained from L19 after vaccination elicited tube formation in the absence of supplemental growth factors

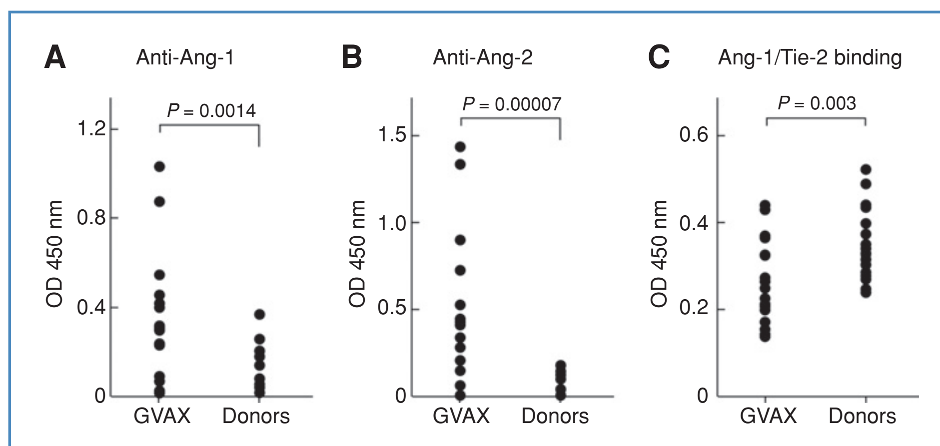

Figure 3. Vaccinated patients harbor neutralizing antibodies to Ang-1 and Ang-2. A and B, anti–Ang-1 and –Ang-2 IgG antibodies were measured with an ELISA (sera 1:100) in normal donors ($n = 16$) and vaccinated patients ($n = 16$). C, antibodies in vaccinated patients block the binding of Ang-1 and Tie-2.

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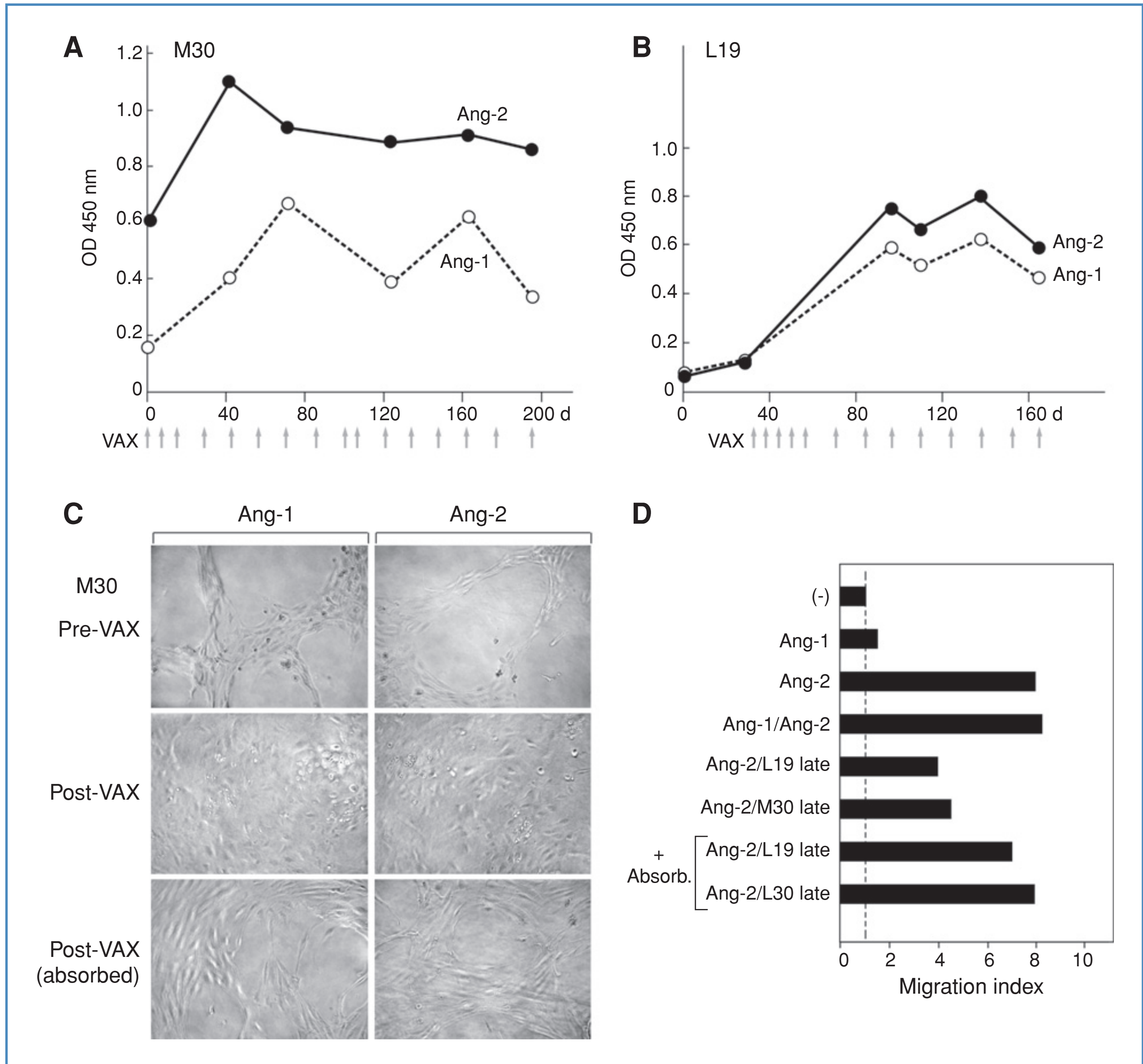

Figure 4. Vaccination stimulated functional antibodies to Ang-1 and Ang-2. A, longitudinal analysis of metastatic melanoma patient M30 (sera 1:100). Day 0 is prevaccination. B. longitudinal analysis of metastatic non–small cell lung carcinoma patient L19. Day 0 is prevaccination. C, postvaccination sera from M30 specifically blocks Ang-1 and Ang-2–induced tube formation. Serum was absorbed against recombinant cytokine as indicated. This experiment was performed twice with similar results. Tube forming scoring (average of 2 replicates): pre-VAX (Ang-1 and Ang-2) 3.5, post-VAX (Ang-1 and Ang-2) 0, post-VAX absorbed (Ang-1 and Ang-2) 1.5. D, postvaccination sera from M30 and L19 specifically block Ang-2–stimulated migration of Tie-2–expressing monocytes. Cell migration in response to cytokine as compared with media is depicted. Serum was absorbed against recombinant cytokine as indicated. Similar results were observed in a second experiment.

(not shown), which precluded application of this assay to monitor functional activity.

However, recent studies have highlighted a key role for Tie-2 expressing macrophages in tumor angiogenesis and demonstrated that these cells migrate toward angiopoietin-2 *in vitro* (31–33). Consistent with this work, we found that angiopoietin-2, but not angiopoietin-1 displayed chemotactic activity toward Tie-2 expressing monocytes obtained from normal donor peripheral blood (Fig. 4D). Furthermore, postvaccination sera obtained from both L19 and M30 diminished angiopoietin-2–induced monocyte chemotaxis, and this inhibition was decreased with angiopoietin-2 absorption.

To examine the mechanisms underlying the ability of L19 and M30 sera to decrease angiopoietin-1 and angiopoietin-2 responses, we characterized downstream Tie-2 signaling. HUVECs treated with recombinant angiopoietin-1 and angiopoietin-2 showed rapid ERK phosphorylation, which was maximal at 10 minutes (Fig. 5A). Peak levels were observed

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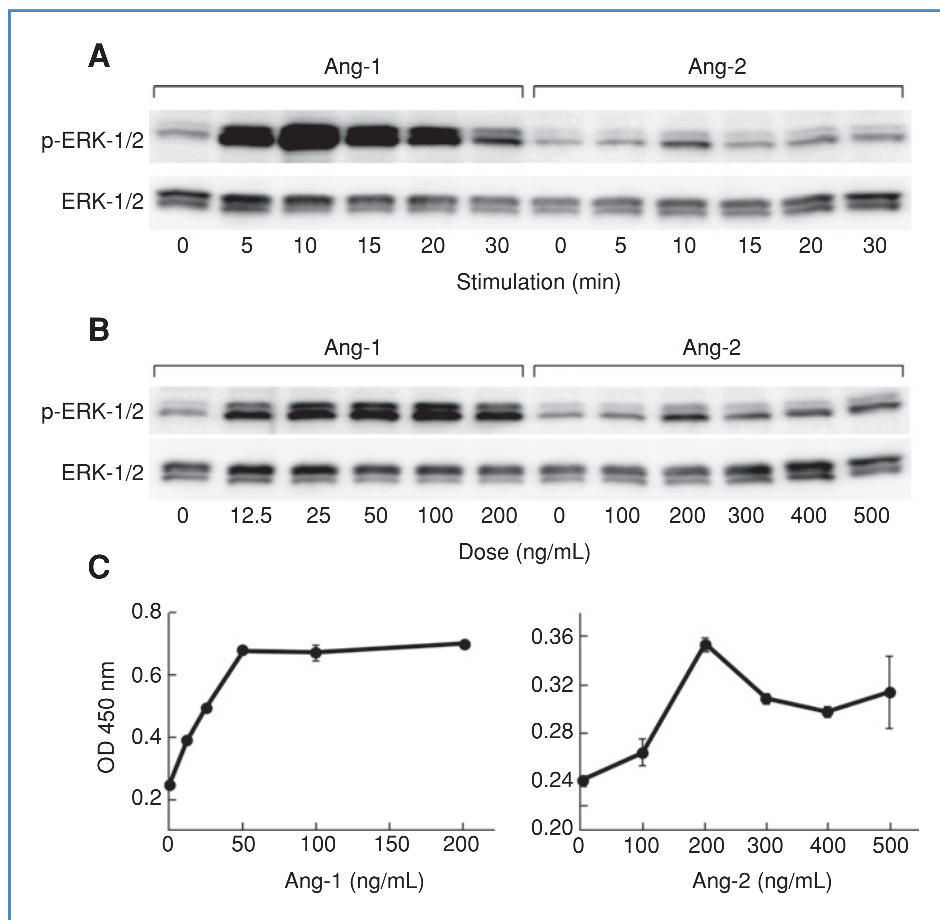

Figure 5. Angiopoetin-1 and -2 trigger ERK phosphorylation. A, HUVECs were treated with 200 ng/mL of Ang-1 or 500 ng/mL of Ang-2 for varying times. Cell lysates were analyzed for phospho-ERK with immunoblotting. B, HUVECs were treated for 10 minutes with varying concentrations of Ang-1 and Ang-2, and then phospho-ERK was assayed with immunoblotting. C, phospho-ERK levels from B were analyzed with an ELISA.

with 50 ng/mL of angiopoietin-1 and 200 ng/mL of angiopoietin-2 (Fig. 5B and C). Using these optimized conditions, we found that postvaccination, but not prevaccination sera from both L19 and M30 inhibited angiopoietin-1 and angiopoietin-2–triggered ERK phosphorylation (Fig. 6A and B). Moreover, the blocking activity was decreased with cytokine absorption, underscoring the specificity of the response.

Therapy-induced antibodies to MIF antagonize angiogenesis

Some patients who manifested prolonged survival after immunization did not generate antiangiopoietin reactions, suggesting that other pathways may contribute to therapeutic activity. Moreover, 3 patients who developed increased anti-angiopoietin antibody titers as a function of vaccination did not show clinical benefits, indicating that these humoral responses may not be sufficient for tumor destruction (Supplemental Table 2). In this context, our earlier investigations of MEL15, a stage IV melanoma patient who achieved an ongoing partial response (50+ months) to irradiated, autologous GM-CSF secreting tumor cell vaccines followed by CTLA-4 blockade identified MIF as a target of high-titer antibodies (23). This cytokine also contributes to tumor blood vessel formation, as illustrated by the reduction in intestinal polyp-associated angiogenesis following the introgression of a null MIF allele into mice harboring a mutant adenomatous polyposis coli gene (34). Longitudinal analysis of MEL15 revealed that immunotherapy engendered an increase in anti-MIF antibody titers (Fig. 7A). Furthermore, OV65, an advanced ovarian carcinoma patient who manifested a striking clinical response to the combination treatment, similarly showed augmented anti-MIF humoral reactions (Fig. 7B). The anti-MIF antibody titers in both of these subjects fluctuated in association with the periodic infusion of CTLA-4 blockade, which raises the possibility of a dynamic interplay of antitumor humoral reactions and immune regulation. Three additional melanoma, myeloid leukemia, and lung cancer patients also generated antibodies to MIF as a function of treatment (not shown).

Because MEL15 and OV65 manifested coordinated humoral reactions to MIF and the angiopoietins, we wondered whether these cytokines might functionally interact in an angiogenic network. Indeed, MIF enhanced the expression of Tie-2 on peripheral blood monocytes cultured with CSF-1, whereas this activity was blocked with sera obtained from MEL15 after immunotherapy (Fig. 7C). MEL-15 late sera also diminished

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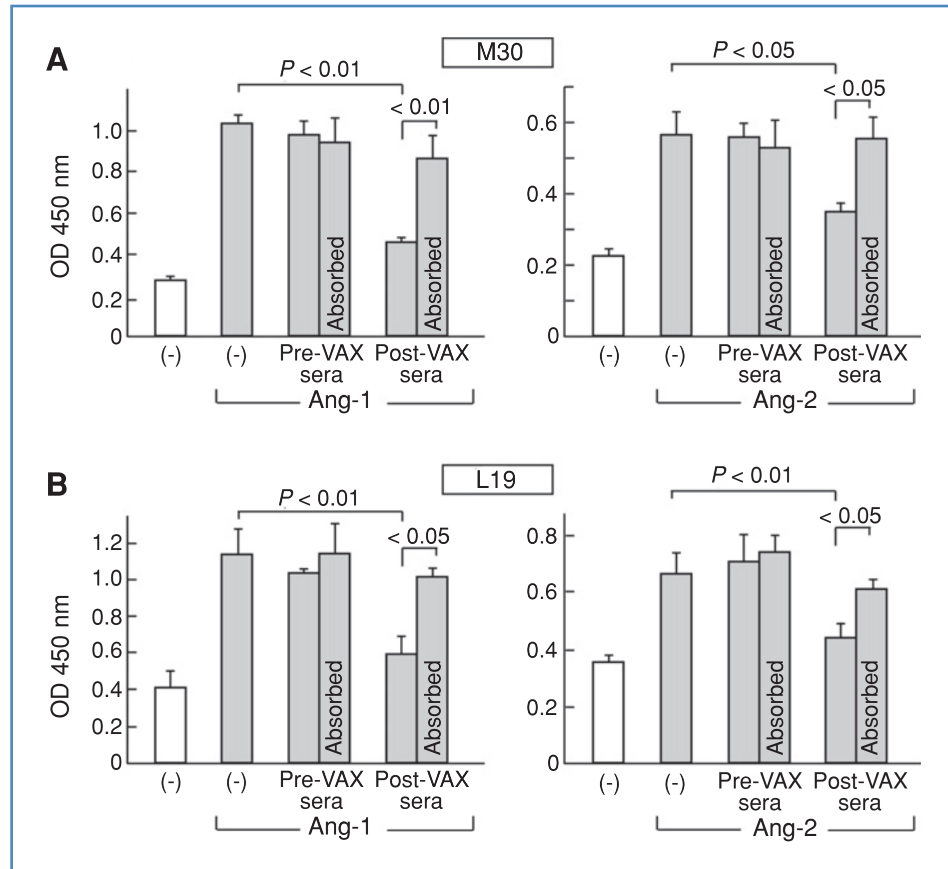

Figure 6. Vaccine-induced antibodies block angiopioetin-1/2 signaling. HUVECs were treated for 10 minutes with 50 ng/mL of Ang-1 or 200 ng/mL of Ang-2 in the presence of PBS or 1 mg/mL of purified immunoglobulins from pre- or postvaccination sera (with and without absorption with recombinant cytokine). Cell lysates were assayed for phospho-ERK with an ELISA. A, stage IV melanoma patient M30. B, stage IV lung carcinoma patient L19. Similar results were observed in 2 experiments.

the ability of MIF to stimulate MMP-9 production from monocytes (35), at levels comparable to the addition of an anti-MIF monoclonal antibody (Fig. 7D). Together, these results raise the possibility that the therapy induced anti-MIF humoral responses might cooperate with the antiangiopoietin antibodies to perturb tumor angiogenesis.

Discussion

The detailed analysis of patients achieving sustained clinical benefits from lethally irradiated, autologous GM-CSF–secreting tumor cell vaccines and CTLA-4 antibody blockade affords a rich opportunity to delineate mechanisms of therapeutic immunity and identify antigens associated with immune-mediated tumor destruction. Previous investigations revealed the development of dense intratumoral T- and B-cell infiltrates that accomplished extensive tumor necrosis, and uncovered several tumor-associated gene products that served as targets for these reactions (14). Nonetheless, a tumor vasculopathy linked with zonal areas of ischemic tumor necrosis was also observed, yet the basis for this immune targeting of tumor blood vessels was not evident.

Becuase many key principles of irradiated, GM-CSF secreting tumor cell vaccines are conserved between murine and human systems (36), we screened a B16 murine melanoma cDNA expression library with sera from a long-term surviving melanoma patient who manifested hemorrhagic tumor necrosis as a function of vaccination. Among the gene products identified through this approach was VEGF-A, a validated target for antiangiogenic therapies (4). Further studies established that vaccination stimulated a coordinated humoral reaction to angiopoietin-1 and angiopoietin-2, and MIF, whereas these antibodies blocked the ability of the cytokines to promote angiogenesis in several *in vitro* assays. Together, these results support the idea that humoral immunity might contribute to disruption of the angiogenic network *in vivo* and provoke ischemic tumor necrosis. Interestingly, long-term follow-up of the patients has not revealed any toxicities that might be related to the antiangiogenic responses, suggesting that a favorable therapeutic index may have been achieved.

Our finding that sustained responses to immunotherapy may involve neutralizing antibodies to diverse angiogenic cytokines suggests that concurrently targeting multiple arms of the angiogenic network might enhance clinical efficacy. In this context, therapeutic agents that target the angiopoietins demonstrate considerable promise in model systems and early clinical trials (29, 37, 38), whereas combined angiopoietin and VEGF inhibition displays synergistic effects in some of the experimental settings (39). Moreover, we showed here that

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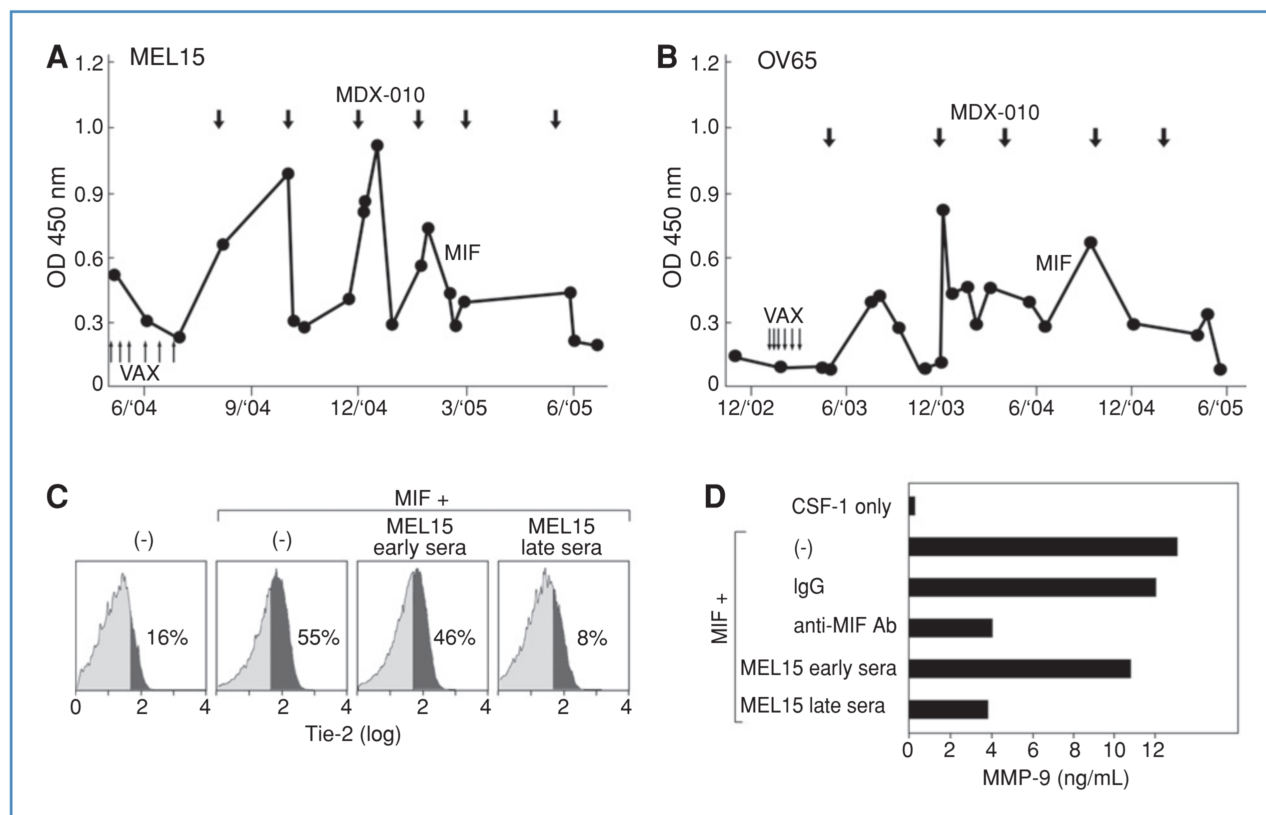

Figure 7. Immunotherapy stimulated blocking antibodies to MIF. A, longitudinal analysis of anti-MIF antibodies in metastatic melanoma patient MEL 15 (sera 1:500). Upward arrows denote vaccinations, downward arrows indicate anti–CTLA-4 mAb (MDX-010) infusions. B, longitudinal analysis of stage IV ovarian carcinoma patient OV65. C, MEL15 sera obtained after immunotherapy inhibited MIF-induced Tie-2 expression on monocytes. D, late MEL15 sera attenuated MIF-stimulated MMP-9 production. Similar results were observed in 3 experiments; $P = 0.0314$ for MEL15 early versus late sera.

blockade of the angiopoietins and MIF inhibited not only the direct stimulation of endothelial cells, but also the recruitment and activation of proangiogeneic myeloid elements.

The pathways underlying the generation of antibodies that antagonize the function of soluble angiogenic factors remain to be clarified. Analysis of sera samples obtained prior to vaccination revealed the presence of nascent antibody responses, but these manifested limited blocking activity. Thus, although tumor development may provoke immune recognition, the endogenous reactions appear to be insufficient to impede disease progression. The autologous tumor cell vaccines employed in our clinical trials derive from single cell suspensions of metastatic lesions, and thus include a mixture of vascular components, stromal cells, and cancer cells. The engineered local production of GM-CSF enhances the capture and presentation of these elements by recruited dendritic cells, which likely contributes to the breach of tolerance to some self-antigens. Consistent with this idea, experiments in murine models illustrate that vaccination can engender protective immunity against angiogenic moieties (11–13). Nevertheless, the selection for antibodies with blocking activity in cancer patients is surprising. Whether humoral immunity plays a more general role in tissue homeostasis, perhaps during wound healing, is an intriguing issue that warrants further investigation.

In addition to the impact on tumor blood vessel development, the generation of neutralizing antibodies to angiogenic cytokines might potentiate tumor immunity. Accumulating evidence indicates that VEGF, angiopoietins, and MIF attenuate antitumor lymphocyte cytotoxicity through modulating dendritic cells, macrophages, NK cells, and FoxP3-expressing regulatory T cells (40). An inhibition of immunosuppressive circuits with therapy-induced anticytokine antibodies might allow for enhanced intratumoral infiltration with activated $CD4^+$ and $CD8^+$ effector T cells. The increase in tumor cell killing might in turn establish a more immunogenic environment for the presentation of angiogenic factors, thereby creating a feed-forward amplification loop that coordinates cellular and humoral pathways for further tumor destruction. Such interplay of host reactivity and angiogenesis suggests that immunotherapies might be effectively combined with antiangiogenic treatments to improve patient outcomes.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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