Immune Evasion by Murine Melanoma Mediated through CC Chemokine Receptor-10

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Abstract

Human melanoma cells frequently express CC chemokine receptor (CCR)10, a receptor whose ligand (CCL27) is constitutively produced by keratinocytes. Compared with B16 murine melanoma, cells rendered more immunogenic via overexpression of luciferase, B16 cells that overexpressed both luciferase and CCR10 resisted host immune responses and readily formed tumors. In vitro, exposure of tumor cells to CCL27 led to rapid activation of Akt, resistance to cell death induced by melanoma antigen-specific cytotoxic T cells, and phosphatidylinositol-3-kinase (PI3K)–dependent protection from apoptosis induced by Fas cross-linking. In vivo, cutaneous injection of neutralizing antibodies to endogenous CCL27 blocked growth of CCR10-expressing melanoma cells. We propose that CCR10 engagement by locally produced CCL27 allows melanoma cells to escape host immune antitumor killing mechanisms (possibly through activation of PI3K/Akt), thereby providing a means for tumor progression.

Key words: metastasis • chemokine receptor • cancer • cell signaling

Introduction

Malignant melanoma is a potentially fatal skin cancer that is increasing in incidence (1). The ability of tumor cells to avoid immune surveillance is likely to be central to the progression of melanoma and other cancers, and many mechanisms have been described which, in theory, enable cancers to escape immune-mediated cell death (2).

Because engagement of Fas on tumor cells by Fas ligand (FasL)-expressing NK cells and CTLs constitutes a major mechanism by which immune cells kill tumors, tumor cells have evolved several strategies to escape Fas-mediated cell death. Malignant cells may down-regulate surface Fas (3) to avoid engagement by FasL or begin to express FasL themselves in order to kill attacking immune cells (4). Some tumors, including melanoma, up-regulate FLICE-like inhibitory protein (FLIP), which blocks the action of caspase-8 in the Fas death pathway (5). Thus, antagonism of tumor-associated signaling pathways that interfere with Fas-mediated cell death might enhance the effectiveness of cancer therapy.

In this paper, we explore the role of chemokine receptors in the context of tumor escape from immune cell clearance mechanisms. Chemokine receptors are comprised of a family of G protein–coupled, seven-transmembrane–spanning proteins that bind primarily to four groups of homologous chemokine ligands (6). Although cancer cells produce a variety of chemokine ligands that may be involved in neoangiogenesis, the attraction and retention of inflammatory cells, and proliferation (7), tumor cells express a limited repertoire of chemokine receptors (7, 8). These receptors play distinct roles in distant organ metastasis (via CXC chemokine receptor [CXCR]4) (8, 9) and nodal metastasis (via CC chemokine receptor [CCR]7) (10, 11).

Interestingly, many melanoma cell lines express the chemokine receptor CCR10 in conjunction with CCR7 and CXCR4 (8). CCR10 (12, 13) is expressed by a population of skin-homing memory T cells, but it can also be detected on endothelial cells and cytokine-stimulated melanocytes (13). CCR10 has two known chemokine ligands: CCL27

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Abbreviations used in this paper: CCR, CC chemokine receptor; CXCR, CXC chemokine receptor; IHC, immunohistochemistry; p-Akt, phospho-Akt; PI3K, phosphatidylinositol-3-kinase.

(14) and CCL28 (15). CCL27 is unique among chemokines because it is selectively and constitutively produced in skin by epidermal keratinocytes (14), and it has been shown to play a role in T cell homing to inflamed skin (16, 17). CCL27's role in melanoma pathogenesis has not been delineated.

Whereas B16 cells are normally poorly immunogenic and aggressively form tumors in syngeneic mice, we observed that transduction with luciferase rendered tumor cells less aggressive, which allowed the recipient mice to generate effective anti–tumor responses after implantation of these cells into ear skin. Using this model, we assessed the effects of CCR10 expression on the growth and metastatic characteristics of CCR10-positive tumor cells. Collectively, our results suggest a novel mechanism that may enable melanoma cells to evade the host immune response through the expression of specific chemokine receptors.

Materials and Methods

Animals, Reagents, and Cell Lines. Female C57BL/6 mice (9) (8–12 wk old) were used in all experiments. 6-wk-old FasL^{gld} mice on the C57BL/6 background were purchased from Jackson Laboratories and used at 8 wk of age (i.e., before the development of characteristic lymphoproliferative disease [18]). All animal experiments were conducted with the approval of the NCI Animal Use and Care Committee. Syngeneic B16/F1 melanoma cells (19) were grown in DME (GIBCO BRL) with 10% heat-inactivated FCS and supplements (10). Wortmannin, LY294002, and PD98059 were purchased from Sigma-Aldrich.

Retroviral Transduction of B16/F1 Melanoma Cells. Mouse CCR10 cDNA (12) (a gift from Dr. Gerald Graham, Beatson Institute, Glasgow, Scotland) was subcloned into the pLNCX2 retroviral vector (CLONTECH Laboratories). B16/F1 melanoma cells were transduced with cDNA for CCR10 as described previously with G418-based selection (9, 10). The pLNCX2- and CCR10-transduced tumor lines were transduced again with firefly (*Photinus pyralis*) luciferase cDNA and maintained with puromycin. For convenience, the CCR10-luciferase B16 cell line is hereby referred to as CCR10-B16. The CCR10-B16 cell line expressed a 42-kD protein band by Western blotting for CCR10 and showed specific calcium flux with the addition of CCL27 (Fig. S1, A and B, available at http://www.jem.org/cgi/content/full/jem.20030593/DC1). The human CXCR4-luciferase–transduced cell line, CXCR4-B16, and the control vector-luciferase–transduced line, pLNCX2-B16, were maintained in DME with 10% FCS, G418, and puromycin (9). Luciferase activity per cell was comparable in all cell lines (pLNCX2-, CXCR4-, and CCR10-B10). Similar in vivo phenotypes (see Results) were observed using CCR10- and pLNCX2-transduced cells derived from three or more separate transductions with each retroviral construct.

Immunohistochemistry and Western Blotting. Immunohistochemistry (IHC) and Western blotting for CCR10 were performed using goat anti–human CCR10 (CI0126) and anti–mouse CCR10 (CI0127) polyclonal antibodies, respectively, from Capralogics. For detection of phospho-Akt (p-Akt) at serine 473, serum-starved CCR10-B16 cells (5×10^4/well) were preincubated for 2 h at 37°C with or without various kinase inhibitors (LY294002, wortmannin, PD98059; Sigma-Aldrich), and pertussis toxin (Calbiochem) before addition of CCL27. Cells were lysed and analyzed by Western blotting using anti–p-Akt (Ser 473) and anti-total Akt antibodies (Cell Signaling Technology). A rabbit anti–p-Akt antibody (9277; Cell Signaling Technology) was used for IHC of formalin-fixed tumor sections as described (20).

Quantitative RT-PCR. Quantitative RT-PCR was performed as described previously (10) using the following primer pairs: human CCR10, forward, 5′-GGGGATGAAGAGGACGCATACT-3′ and reverse, 5′-CCTGGACATCGGCCTTGT-3′; CCL27 (mouse), forward, 5′-CTGCTGAGGAGGATTGTCCAC-3′ and reverse, 5′-CACGACAGCCTGGAGGTGA-3′; CCL28 (mouse), forward, 5′-CAGGGCTCACACTCATGGCT-3′ and reverse, 5′-CCATGGGAAGTATGGCTTCTG-3′. Results were normalized to expression of GAPDH: forward, 5′-ACCCACTCCTCCACCTTTGA-3′ and reverse, 5′-CATACCAGGAAATGAGCTTGACAA-3′) and then multiplied by 10^7.

S.c. Inoculation of Transduced Cell Lines. CCR10- and pLNCX2-B16 cells in exponential growth phase were harvested by trypsinization and washed twice in PBS before injection. For s.c. footpad injections, cells (4×10^5 cells in ~20 μl PBS) were injected into the left hind footpads of C57BL/6 mice. For ear injections, 10^5 cells in ~20 μl PBS were injected into the subcutaneous space under the central dorsal surface of the left ear immediately above the cartilage. Tumor growth was monitored by measurement of the two maximum perpendicular tumor diameters. Experiments were performed two to four times each with similar results.

In vivo Luciferase Imaging. Mice were anesthetized with a mixture of ketamine and xylazine, injected with D-luciferin (Biotium) (2 mg/mouse) via the intraperitoneal route and imaged for 1–3 min in a pitch-black chamber with a cooled CCD camera (Xenogen). Images were analyzed with the LivingImage software package system (Xenogen). In vitro luciferase quantification was performed as described (9).

Apoptosis and Cytotoxicity Assays. To increase Fas expression, CCR10-B16 cells were treated with recombinant murine IFN-γ (50 ng/ml; Peprotech) for 12–16 h in DME/0.5% FCS. To confirm Fas expression, cells were harvested and washed, and then stained with PE-conjugated anti–mouse Fas mAb (BD Biosciences) (see Fig. 5 A) for flow cytometric analysis using FloJo analysis software (Tree Star). For induction of apoptosis, IFN-γ–treated CCR10-B16 cells were exposed to 10 ng/ml recombinant human FLAG-tagged FasL (Apotech) in combination with 1 μg/ml anti-FLAG M2 mAb (Sigma-Aldrich) for 16 h plus 1 μg/ml chemokine (when indicated) at 37°C in the presence of 0.5% FCS as described (21). VAD-fmk (CLONTECH Laboratories) was used at 1 μM as an inhibitor of Fas-mediated cell death. IFN-γ–treated CCR10-B16 cells that had not been exposed to FasL were stained with annexin V for baseline assessment of apoptosis. After exposure of B16 cells to apoptosis-inducing conditions for 16 h, attached (and detached) cells were collected from tissue culture plates for annexin V staining according to manufacturer's instructions (BD Biosciences). Analysis was performed by flow cytometry with FloJo software. P values were based on two-sided, nonparametric Student's *t* tests (unless otherwise specified).

For in vitro CTL assays, T cells (PMEL) from transgenic mice that expressed T cell receptors specific for a H-2D^b–restricted, mouse gp100 (mgp100)–specific peptide (22) were activated by incubation in culture medium (CM) (consisting of RPMI 1640 with 10% heat inactivated FCS) with 30 IU/ml recombinant human IL-2 in the presence of 1 μM human $gp100_{25–33}$ peptide and used on day 5–10 after the start of culture (23). CCR10-B16 target cells were treated overnight with IFN-γ (as above) and labeled with calcein-AM (Molecular Probes) at 1 μM final concen-

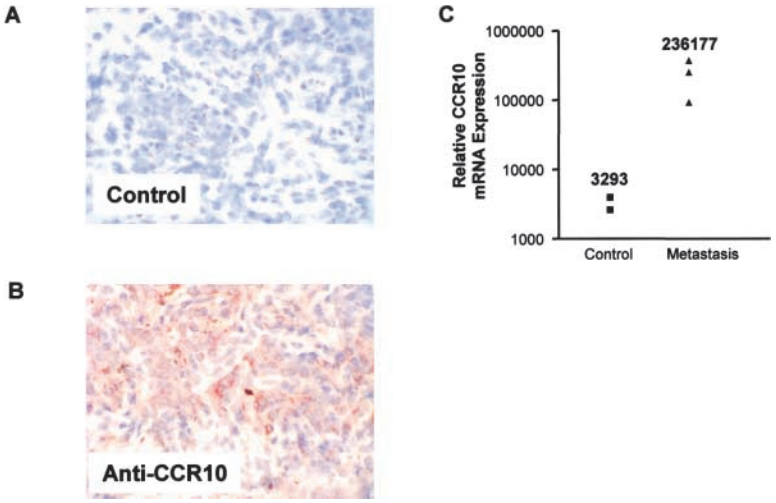

Figure 1. Human primary and metastatic melanomas express CCR10 protein and mRNA. (A and B) Primary cutaneous melanoma was probed with anti–human CCR10 antibodies (B) or species-matched control (A). Magnification is ×400. The red color (amino-9-ethycarbazole) indicates positive staining. (C) Cutaneous melanoma metastases ($n = 3$) and normal human skin ($n = 2$) were analyzed for CCR10 mRNA expression. The means of the samples are shown above individual sample results.

tration, washed, and added to round-bottom microtiter plates (1.5×10^4/well) with specific ratios of effector PMEL cells for 2.5 h at 37°C. The supernatants were recovered, and calcein release was measured using a CytoFluor 2350 plate reader (Millipore). Specific lysis = ([experimental − spontaneous]/[maximal − spontaneous]) × 100. Maximal lysis was achieved with 0.1% Triton X-100, whereas spontaneous lysis was obtained by incubating target cells alone. The percentage of cytotoxicity was calculated as the average of triplicate assays.

Online Supplemental Material. Fig. S1 shows functional expression of transduced CCR10 in B16 cells by Western blot analysis (Fig. S1 A) and calcium flux assay (Fig. S1 B). Fig. S2 indicates in vivo luciferase imaging of tumor progression. Fig. S3 shows Fax expression in pLNCX2-B16 footpad tumor. Fig. S4 shows the fate of pLNCX2-B16 tumor in FasL-deficient mice by luciferase assay (Fig. S4 A). Fig. S5 shows phospho-Akt staining of pLNCX2-B16 footpad tumor. Fig. S6 indicates mouse CCL27 and CCL26 mRNA expression in various organs. Figs. S1–S6 are available at http://www.jem.org/cgi/content/full/jem.20030593/DC1.

Results

Human Melanomas Express CCR10. Whereas CCR10 mRNA expression has been reported in melanoma cell lines (8), protein expression of CCR10 in primary human melanoma has not been investigated. We stained primary cutaneous melanomas from 13 patients with anti–human CCR10 antibody and observed seven tumors with nearly 100% expression of CCR10 (see Fig. 1 B for a representative sample), three tumors with 10–50% CCR10 expression, and three tumors without apparent CCR10 expression. We also analyzed three samples of cutaneous metastatic melanoma tissue by quantitative RT-PCR and observed ~800-fold increases in CCR10 mRNA compared with skin from healthy volunteers (Fig. 1 C). Thus, both primary and metastatic human melanomas frequently express CCR10 in situ.

Enhanced Regional LN Metastasis and Local Growth of CCR10-B16 Cells. To characterize the role of CCR10 in a murine model of melanoma growth and metastasis, we transduced B16/F1 cells with the full-length cDNA-encoding murine CCR10 (12). As noted previously (10), B16 cells show negligible levels of mRNA expression for several chemokine receptors tested, including CCR10. Whereas CCR10 was not detectable by RT-PCR (not depicted) and Western blotting in lysates of B16 cells transduced with the vector alone (pLNCX2-B16) or with CXCR4 cDNA (Fig. S1 A, lanes 1 and 2), CCR10-transduced B16 cells (CCR10-B16) expressed a 42-kD protein that was recognized by a specific anti-CCR10 antibody. CCR10-B16 cells underwent a calcium flux in response to CCL27 but not to CXCL12 (Fig. S1 B). Footpad tumors of CCR10-B16–injected mice were moderately increased in size ($P = 0.003$) compared with the tumors from pLNCX2-B16–injected mice (Fig. 2 A). Strikingly, the popliteal LN from CCR10-B16–injected mice showed frequent gross metastases (Fig. 2 B). By contrast, pLNCX2-B16 cells rarely metastasized to the draining LN (Fig. 2 B), a phenotype of these cells that we had reported previously (10). Luciferase quantification revealed a mean luciferase activity of 13988 relative light units in the popliteal LN from CCR10-B16–injected mice versus 21 relative light units for pLNCX2-B16–injected mice ($P = 0.003$, $n = 7$, Mann-Whitney test). Thus, in addition to demonstrating a moderate advantage in growth of the primary tumor compared with control cells, CCR10-B16 cells display a striking increase in metastasis to the draining LN after injection into the footpads of mice.

CCR10-B16 Cell Tumor Progression in the Ear Skin. In a separate set of experiments, we injected CCR10-B16 and pLNCX2-B16 cells into the skin superficial to the ear cartilage. In contrast to the growth pattern observed with pLNCX2-B16 cells in the footpad, tumors did not form in 13 of 15 pLNCX2-B16–injected ears (Fig. 2 C). However, some pLNCX2-B16–injected mice exhibited small (<1 mm) residual, dark macules at the initial inoculation sites (Fig. 2 D). Interestingly, parental (untransfected) B16/F1 cells (not depicted) and CCR10-transduced B16 cells (Fig. 2 E) grew progressively in the ear skin (Fig. 2, C compared with E). 13 of 15 (87%) mice injected in the ear skin with

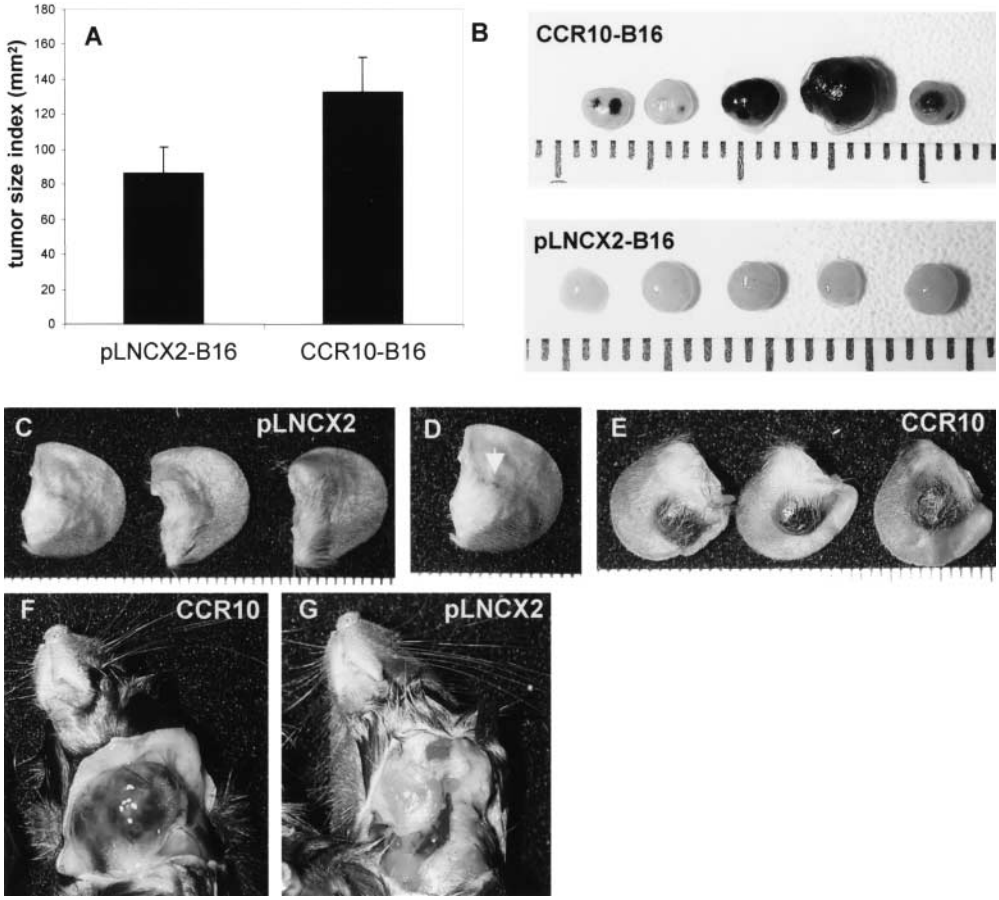

Figure 2. Growth and regional LN metastasis of CCR10- and pLNCX2-B16 tumor cells. CCR10- or pLNCX2-B16 cells were injected into the footpads of mice. (A) After 21 d, animals were killed and tumor sizes in the footpads were compared ($P = 0.003$, $n = 5$ per group). (B) Draining popliteal LN from injected footpads. (C–E) Representative ears from mice injected in the dermis of the ear with pLNCX2-B16 cells (C) showing a residual tumor macule indicated by the white arrow (D) and from mice injected at analogous sites with CCR10-B16 cells (E) are shown 20 d after inoculation. Dissection of the cervical region ipsilateral to the tumor injection sites reveals a large cervical LN metastasis in a CCR10-B16–injected mouse (F), but not in a pLNCX2-B16–injected mouse (G; representative animals are shown in F and G). Scale bar in B–E is calibrated in mm.

CCR10-B16 cells developed tumors that were >4 mm in diameter ($P < 0.001$ compared with pLNCX2-B16–injected mice, Fisher's exact test). To determine whether another chemokine receptor, CXCR4, had similar effects in tumor formation, we injected CXCR4-B16 cells (9) into mouse ears and found that only 1 of 10 mice developed tumors (unpublished data).

After inoculation of CCR10-B16 cells into ear skin, mice developed pronounced ipsilateral cervical LN enlargement (Fig. 2 F). 14 of 15 (93%) of CCR10-B16–injected animals developed gross LN metastases, whereas gross cervical LN metastasis was observed in only 20% (3 out of 15) of pLNCX2-B16–injected mice ($P < 0.001$, Fisher's exact test; Fig. 2 F compared with G). Although CXCR4-B16 cells did not display an enhanced ability to form tumors in ears, we did observe enhanced nodal metastasis in 8 of 10 mice injected with these cells, which was consistent with a postulated role for CXCR4 in nodal metastasis (8).

In vivo imaging based on the presence of luciferase in both types of tumor cells was also used to detect the presence of metastases. Light emanating from the primary tumor and ipsilateral cervical LN from CCR10-B16–injected mice was readily detected, whereas no light signal could be detected in the cervical region, abdominal cavity, or cranium of pLNCX2-B16–injected mice 20 d after inoculation (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20030593/DC1). Thus, expression of CCR10 was associated with the capacity of CCR10-B16 cells (in contrast to pLNCX2-B16 cells) to grow in the ear skin and metastasize to regional draining LN.

CCR10 Protects B16 Cells From Host Immune Responses. When injected into ear skin, ~90% of pLNCX2-B16–injected skin sites did not show overt tumors. Microscopically, these injected sites contained predominantly inflammatory cells and rare tumor cells (Fig. 3, A, C, and D) in sharp contrast to CCR10-B16 cell–injected sites, which were composed of highly atypical cells that filled the dermis (Fig. 3 B). To determine if pLNCX2-B16 cell–injected mice were protected from rechallenge with tumor cells, mice that did not develop ear tumors after initial challenge with pLNCX2-B16 cells were rechallenged 30 d later with pLNCX2-B16 cells in the footpads. No evidence of growth of pLNCX2-B16 cells was observed 17 d after inoculation in rechallenged mice (Fig. 3 E, middle column). The same lot of pLNCX2-B16 cells, however, formed tumors in the footpads of nonimmunized, naive mice (left column). Interestingly, injection of CCR10-B16 cells into vaccinated mice resulted in tumors similar in size ($P > 0.3$) to those arising from pLNCX2-B16 cells injected into naive mice (Fig. 3 E, right column). Thus, injection of pLNCX2-B16 cells into the ear of mice leads to an effective host response that resists tumor formation upon rechallenge with the same cells. The expression of CCR10,

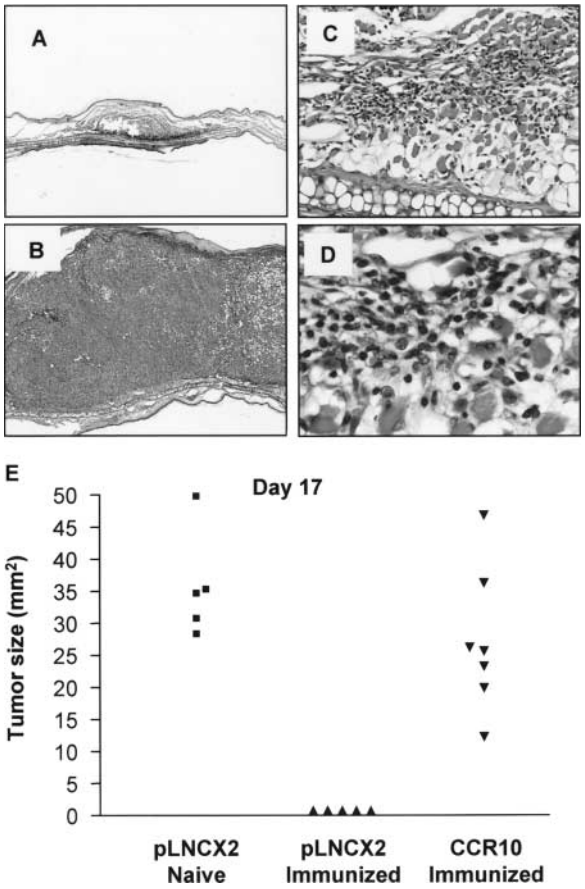

Figure 3. Antitumor responses in pLNCX2-B16–injected mice. Ear skin from pLNCX2-B16 (A, C, and D) and CCR10-B16–injected (B) mice was removed 18 d after inoculation, formalin fixed, and stained with hematoxylin and eosin. (A and B) Low power (×25), (C) medium power (×200), and (D) high power (×400) views. (E) Tumor-free mice that had been injected previously with pLNCX2-B16 cells in the ear were rechallenged in the hind footpad with either pLNCX2-B16 cells ($n = 5$, middle column) or CCR10-B16 cells ($n = 7$ mice, right column). $P < 0.001$, CCR10 versus pLNCX2-B16 injected into immunized mice. Note no tumor growth was observed in the pLNCX2-B16–rechallenged mice. As a control, pLNCX2-B16 cells were injected into footpads of age/sex-matched naive mice ($n = 5$ mice, left column). $P > 0.3$, naive control versus CCR10-B16–rechallenged group.

however, allows B16 cells to escape this host response and form progressive tumors.

CCR10 Mediates Resistance to Fas-mediated Apoptosis. The ability of the CCR10-B16 cells (in contrast to the pLNCX2-B16 cells) to resist immune clearance (Figs. 2 and 3) and our observation of clear Fas protein expression by pLNCX2-B16 tumor cells in vivo (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20030593/DC1) prompted us to determine if CCR10 activation could regulate the Fas death pathway. Resting CCR10-B16 melanoma cells in vitro expressed low levels of surface Fas protein (Fig. 4 A). Because interferon (IFN)-γ (either in recombinant form or as produced by cytotoxic T cells [24–26]) is known to increase Fas expression by melanoma cells, we treated CCR10-B16 cells with IFN-γ, which resulted in uniform expression of Fas (>92% expression; Fig. 4 A).

We then exposed IFN-γ–treated CCR10-B16 cells to multimerized FasL in the presence or absence of CCL27 to determine if CCR10 engagement could protect B16 cells from FasL-triggered apoptosis, which was measured by annexin V staining. In the absence of CCL27, CCR10-B16 cells readily underwent apoptotic cell death after Fas cross-linking (Fig. 4 B, far left column). The magnitude of annexin V staining was nearly identical to that achieved by treatment of melanoma cells with the drug camptothecin (not depicted). In the presence of CCL27, however, CCR10-B16 cells were more resistant to Fas-mediated apoptosis, whereas CCL21 (a chemokine that activates CCR7) could not reduce cell death (Fig. 4 B). Anti-FLAG antibody alone did not induce apoptosis, and a general caspase inhibitor (VAD-fmk) effectively blocked Fas-mediated apoptosis, demonstrating the specificity of the assay (Fig. 4 B). While pLNCX2-B16 cells underwent apoptosis in the presence of multimerized FasL, these cells were not protected from apoptosis by exposure to CCL27 (Fig. 4 B).

To determine if CCR10 ligation could protect CCR10-B16 cells from cytotoxicity induced by melanoma antigen-specific CTLs, we exposed CCR10-B16 (target) cells to in vitro–activated $CD8^+$ T cells from a transgenic mouse (PMEL-1), which expressed the Vα2Vβ13 T cell (TCR) from the $H\text{-}2D^b$ restricted, murine gp100–specific clone #9 T cell (22). PMEL-1 $CD8^+$ T cells are capable of eradicating B16 tumors in vivo after adoptive transfer and restimulation (23). PMEL-1 T cells (PMEL) demonstrated highly efficient killing of IFN-γ–stimulated CCR10-B16 cells, even at low effector:target (E:T) ratios (Fig. 4 C). The caspase inhibitor, VAD-fmk (Fig. 4 C), and the addition of anti-FasL antibodies (not depicted) both effectively reduced CCR10-B16 cell killing by PMEL-1 T cells by >80%. The addition of CCL27 also resulted in markedly decreased killing of target cells that was comparable to target killing in the presence of VAD-fmk. CCL27, however, did not affect killing of pLNCX2-B16 cells by PMEL T cells, suggesting that CCL27 was not reducing apoptosis in the CCR10-B16 cells via effects on T cells. Moreover, by quantitative RT-PCR, CCR10 expression was largely absent in PMEL T cells (not depicted). Thus, engagement of CCR10 on the surface of melanoma cells conferred resistance to Fas-mediated cell apoptosis induced by recombinant Fas ligand and by CTLs specific for a bona fide tumor antigen.

Fas and FasL in the Host Response to pLNCX2-B16 Tumor Cells. To determine if Fas–FasL interactions were critical for the destruction of pLNCX2-B16 tumors, we injected pLNCX2-B16 cells into the ear skin of WT C57BL/6 mice and C57BL/6 mice (*Fasl*^{gld}; gld) that had a homozygous inactivating point mutation in the COOH-terminal region of FasL (27). Strikingly, pLNCX2-B16 cells formed progressive tumors in the FasL-deficient (*gld*) mice but not WT mice, whereas CCR10-B16 cells formed tumors in both strains (Fig. 4 D, quantified by luciferase assay in Fig. S4 A, available at http://www.jem.org/cgi/content/full/jem.20030593/DC1). Moreover, large cervical LN metastases were observed in *gld* but not WT mice (Fig. S4 B). The difference in the ability of pLNCX2-B16 cells to form

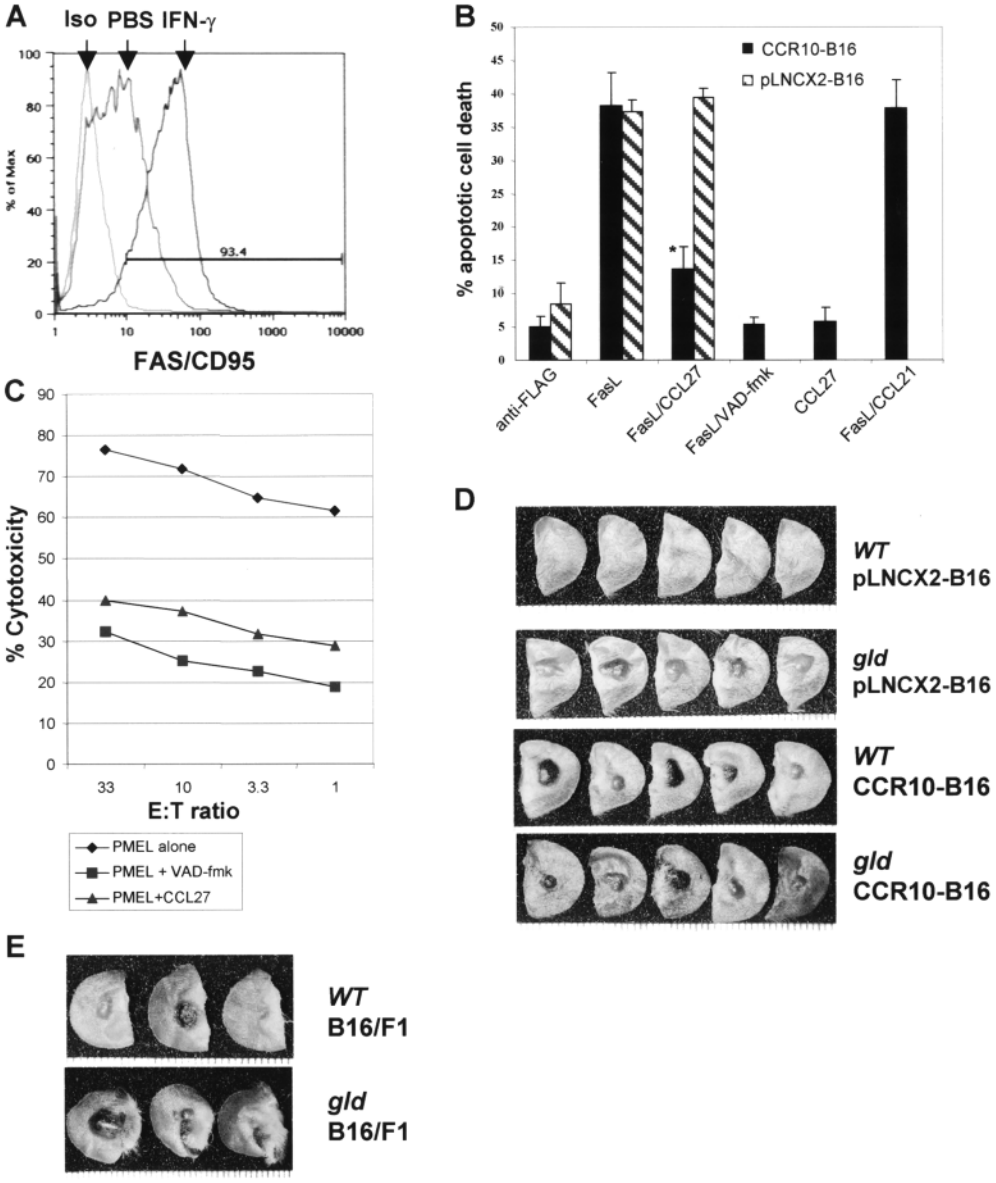

Figure 4. Protection of CCR10-B16 cells from Fas-mediated apoptotic cell death via exposure to CCL27. (A) CCR10-B16 cells were either treated with IFN-γ or with PBS before staining with anti-CD95 (Fas) antibody. Iso: isotype control antibody. (B) IFN-γ–treated pLNCX2- and CCR10-B16 cells were exposed to recombinant FLAG-tagged FasL and multimerized with anti-FLAG antibodies. Note that both tumor lines showed equivalent low levels of Fas-staining before treatment, which were similarly up-regulated after IFN-γ treatment. Cells were then collected and stained with propidium iodide (PI) and annexin V–FITC. Data from PI-negative B16 cells were quantified using FloJo software and are presented as percentage of annexin V–positive cells. pLNCX2-B16 cells were not used under the three right-most conditions. *P = 0.0016 versus FasL (n = 3 per condition). (C) IFN-γ–treated CCR10-B16 cells, as in B, were incubated with antigen-stimulated PMEL T cells at the indicated E:T ratios and assessed for cytotoxicity. (D) pLNCX2-, CCR10-B16 (100×10^3 per ear), (E) parental B16/F1 cells (100×10^3 cells/ear) were injected into the ear skin of *gld* mice or age- and gender-matched WT C57BL/6 mice (14-d tumor assay) as indicated.

tumors in WT versus *gld* mice was specific since parental B16/F1 cells (Fig. 4 E) and RMA murine lymphoma cells (28; not depicted) readily formed skin tumors in both WT and *gld* mice. Thus, Fas antigen is induced in pLNCX2-B16 tumor cells in vivo, and FasL appears to be critical for the host antitumor response that prevents pLNCX2-B16 cells from forming tumors in ear skin.

Involvement of Akt and Phosphatidylinositol-3-kinase in CCR10-mediated Protection from Apoptosis. After engagement with appropriate ligands, chemokine receptors trigger a complex cascade of intracellular signaling events (29) that activate MAP kinase family members and the phosphatidylinositol-3-kinase (PI3K)/Akt pathway. Because Akt activation can inhibit apoptosis caused by a variety of cellular stresses including administration of Fas (30), we hypothesized that Akt activation contributed to chemokine receptor-mediated protection from apoptosis.

Within 5 min after administration of CCL27 to CCR10-B16 cells, we observed a strong increase in Akt phosphorylation at serine 473, a site known to be necessary for the cell survival activity of Akt (Fig. 5 A). Phosphorylation of this critical serine residue was nearly completely inhibited by selective PI3K inhibitors (wortmannin and LY294002), and by PTX, but not by the MEK inhibitor, PD98059 (Fig. 5 A). To detect activation of CCR10-B16 cells by CCL27 in vivo, we stained tissue sections from ears of mice injected with CCR10- or pLNCX2-B16 cells with activation state-specific Akt antibodies. Tumors comprised of CCR10-B16 cells showed increased levels of active Akt compared with residual pLNCX2-B16 cells found in the ear (Fig. 5 B). Finally, to demonstrate the significance of PI3K/Akt signaling in CCL27-mediated protection of CCR10-B16 cells, IFN-γ–treated CCR10-B16 cells were exposed to multimerized FasL in the absence or presence of

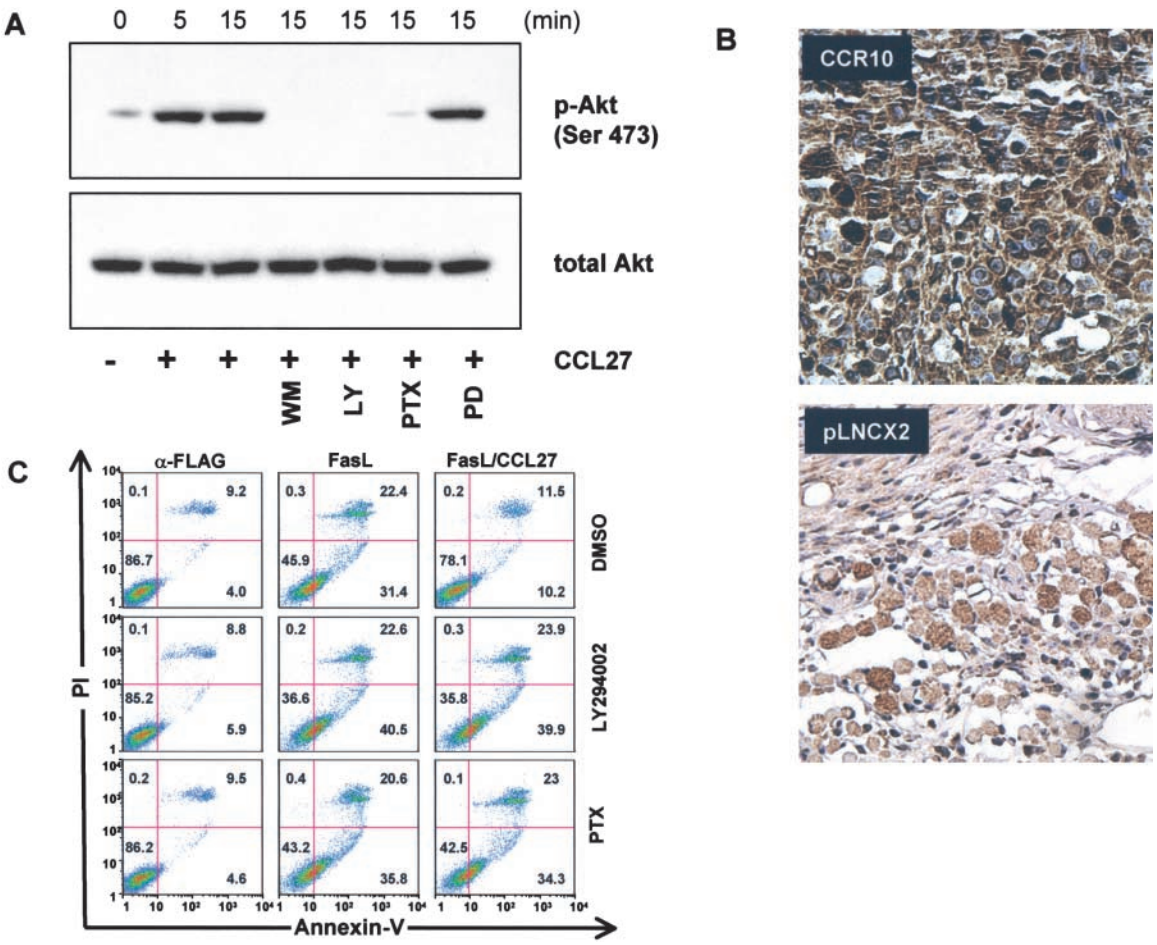

Figure 5. Involvement of PI3K and Akt in CCR10-mediated protection from apoptosis. (A) CCR10-B16 cells were exposed to PBS or CCL27 (1 μg/ml) for the indicated time in the presence of cell signaling inhibitors (wortmannin (200 nM, WM), LY294002 (10 μM, LY), pertussis toxin (200 ng/ml, PTX), and PD98059 (20 μM, PD), lysed, and analyzed by Western blot for p-Akt and total Akt. (B) 14 d after inoculation into the dermis, CCR10-B16 and residual pLNCX2 tumors in the ears were probed for p-Akt expression by IHC. (C) CCR10-B16 cells that had been pretreated for 18 h with the PI3K inhibitor, LY294002 (10 μM), PTX (200 ng/ml), or DMSO alone were exposed to multimerized FasL in the presence and absence of CCL27 (1 μg/ml) for 16 h at 37°C. As a negative control, cells were exposed to the anti-FLAG epitope antibody without prior exposure to FasL (α-FLAG). One of three experiments with similar results.

CCL27 and/or a PI3K inhibitor. As before, administration of multimerized FasL alone increased apoptosis (Fig. 5 C, top row, middle dot plot). When CCL27 was added in conjunction with multimerized FasL, the increase in annexin V staining was completely blocked (Fig. 5 C, top row, right dot plot). Pretreating CCR10-B16 cells with LY294002, however, abrogated protection conferred by CCL27 (Fig. 5 C, middle row, right dot plot). However, LY294002 alone did not induce significant cell death (Fig. 5 C, middle row, left dot plot). Treatment with PTX also rendered CCR10-B16 cells susceptible to Fas-mediated apoptosis even in the presence of CCL27 (Fig. 5 C, bottom row, right histogram). Therefore, CCL27 protects CCR10-B16 cells from FasL-induced apoptosis in a PI3K- and G_i protein–dependent manner.

Anti-CCL27 Neutralizing mAb Prevent Formation of CCR10-B16 Tumors in Ear Skin. CCL27 is constitutively produced at the protein level by epidermal keratinocytes (14, 16). Thus, we postulated that skin-derived CCL27 protected CCR10-B16 cells in the skin from host immune responses. To test this hypothesis, we injected CCR10-B16 cells into ear skin either with anti-CCL27 neutralizing mAb (16) or with isotype-matched control antibodies. This anti-CCL27 mAb has been shown by others to inhibit the influx of T cells into skin in vivo (16) and completely inhibited downstream p-Akt activation in CCR10-B16 cells in vitro at a concentration of 100 μg/ml (unpublished data). The anti-CCL27 mAb treatment effectively prevented CCR10-B16 tumor formation (Fig. 6). However, parental B16/F1 cells that were treated with the same reagents under similar conditions showed no differences in tumor progression, demonstrating that the effect of the anti-CCL27 mAb on CCR10-B16 cells was specific (Fig. 6). In two independent experiments, a total of zero out of six animals injected with anti-CCL27 mAb developed gross CCR10-B16 tumors, whereas six out of six animals injected with the control antibody developed tumors ($P = 0.0022$, Fisher's exact test).

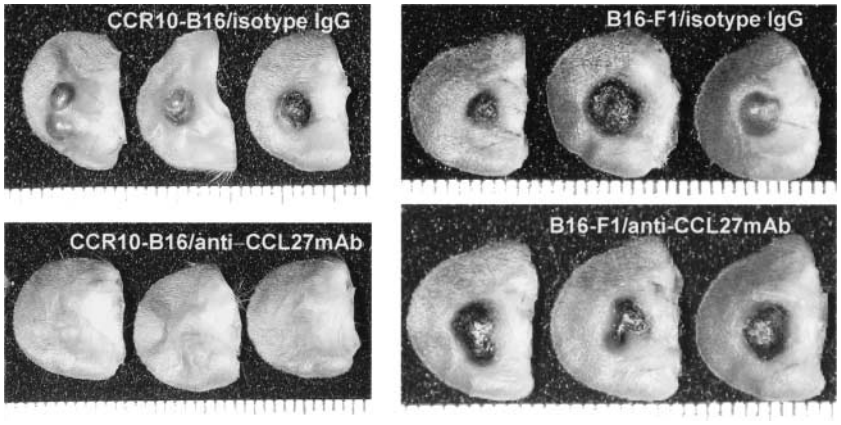

Figure 6. Prevention of CCR10-B16 cell tumor formation with anti-CCL27 treatment. CCR10-B16 or B16/F1 cells were injected into the ear skin of mice in an 18-d tumor formation assay. At the time of injection, the cells were suspended in injection buffer containing either 100 μg/ml of neutralizing rat anti–mouse CCL27 IgG (16) (clone 68623; R&D Systems) or 100 μg/ml of rat IgG, which was coinjected with tumor cells in a volume of 20 μl. One of two independent experiments with similar results.

Discussion

Herein, we report that CCR10 is frequently expressed in primary cutaneous human melanomas but not in murine (B16) melanoma. To explore the possible function of CCR10, we transduced B16 melanoma cells with CCR10 cDNA and found three remarkable features of the resulting cells: (1) they formed tumors at skin sites where luciferase-expressing B16 cells could not, (2) they resisted Fas-mediated apoptosis in vitro in a PI3K-dependent fashion, and (3) they metastasized with high frequency to draining regional LN.

Unlike control B16 cells, CCR10-B16 cells formed tumors after ear skin injection. Because of the intense inflammatory reaction surrounding residual pLNCX2-B16 cells in the skin, we suspected that a host immune response was responsible for the destruction of these cells. After observing Fas expression in pLNCX2-B16 cells injected into the footpads, we explored the effect of CCR10 on Fas-mediated cell death because the Fas pathway can be critical to the efficacy of antitumor responses by CTLs and natural killer cells and to the efficacy of some antitumor drugs (31). Our results clearly suggested that (1) Fas–FasL is required for the eradication of pLNCX2-B16 cells in the ear (Fig. 4 D and Fig. S4) and (2) CCR10-mediated signaling protects B16 tumor cells from Fas-triggered apoptosis in vitro.

Nontransduced parental B16/F1 melanoma cells that lacked CCR10 expression also formed progressive tumors in ear skin, which was not surprising since unmanipulated B16 cells are poorly immunogenic (32). Because these cells lacked expression of luciferase, it is possible that part of the host response to the pLNCX2-B16 cells was directed at this neo-antigen. However, the immune response that develops after ear injection of pLNCX2-B16 cells is not solely directed at luciferase. We have observed that pLNCX2-B16–immunized mice (Fig. 3) are also partially protected from tumor formation resulting from footpad injection of parental B16/F1 tumor cells (unpublished data), suggesting that ear immunization also leads to responses directed at endogenous melanoma antigens. Furthermore, CCR10-B16 cells were resistant to Fas-dependent cell death (Fig. 4) mediated by CTL that are specific for a bona fide melanoma tumor antigen, gp100 (22, 33, 34). Thus, CCR10 activation protects tumor cells from immune responses directed at endogenous as well as non-endogenous targets.

To explain the potent immune response against pLNCX2-B16 cells injected in the ear (as opposed to the footpad), we believe that anatomical factors in the ear permitted rapid movement of tumor cells from the skin to draining cervical LN that resulted in a potent immunological response against pLNCX2-B16 cells (Fig. 3). Indeed, by in vivo luciferase imaging we have observed the presence of both pLNCX2- and CCR10-B16 cells in the draining cervical LN within 5 min of injection into ear skin, whereas neither cell line accumulated in the popliteal LN shortly after footpad injections (unpublished data). This differential site-specific accumulation of tumor cells in regional LN may be the reason we previously observed no difference in the ability of CXCR4-B16 cells and pLNCX2-B16 cells to metastasize to the popliteal LN after footpad injection (9), whereas in the current work, cervical LN involvement was observed after injection of CXCR4-B16 cells into ear skin. Interestingly, others have shown that early migration of cancer cells to secondary lymphoid organs may be a factor in enhanced host anti-tumor responses (35), and direct injection of DCs loaded with melanoma tumor antigen into the LN can lead to clinically effective antimelanoma responses (36).

Although chemokine receptor ligation leads to activation of multiple intracellular signaling pathways involving Jak-Stat proteins, MAP kinases, focal adhesion kinases, and PI3K (29), we focused on the PI3K/Akt pathway because Akt regulates cellular survival, motility, and protects against Fas-mediated apoptosis (37). Akt was activated in CCR10-B16 cells after in vitro exposure to CCL27 and was also easily detectable in CCR10-B16 tumors in vivo (Fig. 5, A and B). Of note, exposure of CCR10-B16 cells to LY294002 reestablished the susceptibility of these cells to Fas-mediated cell death (Fig. 5 C) in the presence of CCL27, suggesting that the PI3K/Akt signaling pathway is critical for chemokine receptor–mediated protection from apoptosis in vitro. Although these data raise the possibility that chemokine-mediated up-regulation of PI3K/Akt may

be an important factor for tumor progression in vivo, additional studies will be necessary to validate this proposal. Substantial staining for active Akt was also observed in footpad tumors arising from pLNCX2-B16 cells (Fig. S5, available at http://www.jem.org/cgi/content/full/jem.20030593/DC1), suggesting CCR10-independent activation of Akt. Although precise quantification of pAkt by IHC has limitations, this may reflect the action of other regulators of cancer growth, including the epidermal growth factor receptor, that are upstream activators of PI3K/Akt (38).

Our observation that CCR10 strongly enhanced LN metastasis was intriguing but unexpected. Although the mechanism underlying this result is unclear, it may be related to enhanced survival of a small number of CCR10-B16 cells that arrive in the LN after cutaneous injection. Because neither lymphatics nor LN are likely to express high levels of CCR10 ligand (8), direct attraction of the melanoma cells to the lymphatics via CCR10 is an unlikely possibility. The only other known ligand for CCR10, CCL28, was also found at low levels in the LN and in the skin (Fig. S6, available at http://www.jem.org/cgi/content/full/jem.20030593/DC1). However, CCL27 from the skin may actually enter the lymphatics for transport to the LN as has been shown for other chemokines that were injected into skin (39). Alternatively, CCL27 has been reported to enhance cell migration via unique nuclear signaling (40), and this property may contribute to enhanced metastatic potential.

The ability of chemokine receptor–mediated signaling to inhibit apoptosis may not be unique to CCR10. For example, activation of CCR9 partially protects lymphoid cells from both Fas- and cycloheximide-induced apoptosis in vitro (41), and engagement of CX3CR1 has antiapoptotic effects in brain microglia (42). Whereas prior studies have implicated chemokine receptors in the survival of other types of cells (41, 43, 44), the present study is the first in vivo demonstration of chemokine receptor–mediated immune evasion by tumor cells.

Although CCL27 is constitutively synthesized in the epidermis (14), CCL27 protein (presumably coming from epidermal cells) is also present in the dermis (16). Thus, CCL27 may have effects on tumor cells implanted into dermis because of its ability to cross the basement membrane after synthesis by keratinocytes. The presence of CCL27 in the skin may potentially explain why CCR10 expression could be advantageous to melanoma, which arises in the environs of skin and frequently metastasizes to skin. Interestingly, CXCR4-B16 cells did not form tumors in ear skin, perhaps because levels of CXCL12 (versus CCL27) are relatively low in the skin compared with lung and LN (8).

Our results demonstrate that chemokine receptors may play critical roles in tumor progression apart from their ability to affect organ-selective metastasis. Ligation of tumor-associated chemokine receptors by locally produced chemokines may be a mechanism that promotes cancer cell survival. Based on our results showing protection of CCR10-B16 from Fas-triggered apoptosis, we predicted that inhibition of the CCL27-CCR10 pathway should make CCR10-B16 cells more susceptible to the host antitumor response. Indeed, CCR10-B16 tumor formation was markedly reduced by treatment with neutralizing antibodies to CCL27 (Fig. 6). These findings demonstrate that therapy directed at specific chemokine receptor pathways may enhance antitumor immunity and may be useful in a clinical setting.

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References

1. Dreiling, L., S. Hoffman, and W.A. Robinson. 1996. Melanoma: epidemiology, pathogenesis, and new modes of treatment. *Adv. Intern. Med.* 41:553–604.
2. Khong, H.T., and N.P. Restifo. 2002. Natural selection of tumor variants in the generation of "tumor escape" phenotypes. *Nat. Immunol.* 3:999–1005.
3. Maecker, H.L., Z. Yun, H.T. Maecker, and A.J. Giaccia. 2002. Epigenetic changes in tumor Fas levels determine immune escape and response to therapy. *Cancer Cell.* 2:139–148.
4. Strand, S., W.J. Hofmann, H. Hug, M. Muller, G. Otto, D. Strand, S.M. Mariani, W. Stremmel, P.H. Krammer, and P.R. Galle. 1996. Lymphocyte apoptosis induced by CD95 (APO-1/Fas) ligand-expressing tumor cells—a mechanism of immune evasion? *Nat. Med.* 2:1361–1366.
5. Bullani, R.R., B. Huard, I. Viard-Leveugle, H.R. Byers, M. Irmler, J.H. Saurat, J. Tschopp, and L.E. French. 2001. Selective expression of FLIP in malignant melanocytic skin lesions. *J. Invest. Dermatol.* 117:360–364.
6. Rossi, D., and A. Zlotnik. 2000. The biology of chemokines and their receptors. *Annu. Rev. Immunol.* 18:217–242.
7. Balkwill, F. 2003. Chemokine biology in cancer. *Semin. Immunol.* 15:49–55.
8. Müller, A., B. Homey, H. Soto, N. Ge, D. Catron, M.E. Buchanan, T. McClanahan, E. Murphy, W. Yuan, S.N. Wagner, et al. 2001. Involvement of chemokine receptors in breast cancer metastasis. *Nature.* 410:50–56.
9. Murakami, T., W. Maki, A.R. Cardones, H. Fang, A.T. Kyi, F. Nestle, and S.T. Hwang. 2002. Expression of CXC chemokine receptor (CXCR)-4 enhances the pulmonary metastatic potential of murine B16 melanoma cells. *Cancer Res.* 62:7328–7334.
10. Wiley, H., E.B. Gonzalez, W. Maki, M. Wu, and S.T. Hwang. 2001. Expression of CC chemokine receptor-7 (CCR7) and regional lymph node metastasis of B16 murine

melanoma. *J. Natl. Cancer Inst.* 93:1638–1643.
11. Mashino, K., N. Sadanaga, H. Yamaguchi, F. Tanaka, M. Ohta, K. Shibuta, H. Inoue, and M. Mori. 2002. Expression of chemokine receptor CCR7 is associated with lymph node metastasis of gastric carcinoma. *Cancer Res.* 62:2937–2941.
12. Jarmin, D.I., M. Rits, D. Bota, N. Gerard, G.J. Graham, I.C. Lewis, and C. Gerard. 2000. Cutting edge: Identification of the orphan receptor G-protein-coupled receptor 2 as CCR10, a specific receptor for the chemokine ESkine. *J. Immunol.* 164:3460–3464.
13. Homey, B., W. Wang, H. Soto, M. Buchanan, A. Wiesenborn, D. Catron, A. Muller, T. McClanahan, M.C.D. Nosjean, R. Orozco, et al. 2000. The orphan chemokine receptor G protein-coupled receptor-2 (GPR-2, CCR10) binds the skin-associated chemokine CCL27 (CTACK/ALP/ILC). *J. Immunol.* 164:3465–3470.
14. Morales, J., B. Homey, A.P. Vicari, S. Hudak, E. Oldham, J. Hedrick, R. Orozco, N.G. Copeland, N.A. Jenkins, L.M. McEvoy, et al. 1999. CTACK, a skin-associated chemokine that preferentially attracts skin-homing memory T cells. *Proc. Natl. Acad. Sci. USA.* 96:14470–14475.
15. Wang, W., H. Soto, E.R. Oldham, M.E. Buchanan, B. Homey, D. Catron, N. Jenkins, N.G. Copeland, D.J. Gilbert, N. Nguyen, et al. 2000. Identification of a novel chemokine (CCL28), which binds CCR10 (GPR2). *J. Biol. Chem.* 275:22313–22323.
16. Homey, B., H. Alenius, A. Muller, H. Soto, E.P. Bowman, W. Yuan, L. McEvoy, A.I. Lauerma, T. Assmann, E. Bunemann, et al. 2002. CCL27-CCR10 interactions regulate T cell-mediated skin inflammation. *Nat. Med.* 8:157–165.
17. Reiss, Y., A.E. Proudfoot, C.A. Power, J.J. Campbell, and E.C. Butcher. 2001. CC chemokine receptor (CCR)4 and the CCR10 ligand cutaneous T cell- attracting chemokine (CTACK) in lymphocyte trafficking to inflamed skin. *J. Exp. Med.* 194:1541–1547.
18. Takahashi, T., M. Tanaka, C.I. Brannan, N.A. Jenkins, N.G. Copeland, T. Suda, and S. Nagata. 1994. Generalized lymphoproliferative disease in mice, caused by a point mutation in the Fas ligand. *Cell.* 76:969–976.
19. Fidler, I.J. 1973. Selection of successive tumour lines for metastasis. *Nature (New Biol.).* 242:148–149.
20. West, K.A., J. Brognard, A.S. Clark, I.R. Linnoila, X. Yang, S.M. Swain, C. Harris, S. Belinsky, and P.A. Dennis. 2003. Rapid Akt activation by nicotine and a tobacco carcinogen modulates the phenotype of normal human airway epithelial cells. *J. Clin. Invest.* 111:81–90.
21. Schneider, P., N. Holler, J.L. Bodmer, M. Hahne, K. Frei, A. Fontana, and J. Tschopp. 1998. Conversion of membrane-bound Fas(CD95) ligand to its soluble form is associated with downregulation of its proapoptotic activity and loss of liver toxicity. *J. Exp. Med.* 187:1205–1213.
22. Overwijk, W.W., A. Tsung, K.R. Irvine, M.R. Parkhurst, T.J. Goletz, K. Tsung, M.W. Carroll, C. Liu, B. Moss, S.A. Rosenberg, and N.P. Restifo. 1998. gp100/pmel 17 is a murine tumor rejection antigen: induction of "self"-reactive, tumoricidal T cells using high-affinity, altered peptide ligand. *J. Exp. Med.* 188:277–286.
23. Overwijk, W.W., M.R. Theoret, S.E. Finkelstein, D.R. Surman, L.A. de Jong, F.A. Vyth-Dreese, T.A. Dellemijn, P.A. Antony, P.J. Spiess, D.C. Palmer, et al. 2003. Tumor regression and autoimmunity after reversal of a functionally tolerant state of self-reactive $CD8^+$ T cells. *J. Exp. Med.* 198:569–580.
24. Peter, I., A. Mezzacasa, P. LeDonne, R. Dummer, and S. Hemmi. 2001. Comparative analysis of immunocritical melanoma markers in the mouse melanoma cell lines B16, K1735 and S91-M3. *Melanoma Res.* 11:21–30.
25. Bohm, W., S. Thoma, F. Leithauser, P. Moller, R. Schirmbeck, and J. Reimann. 1998. T cell-mediated, IFN-gamma-facilitated rejection of murine B16 melanomas. *J. Immunol.* 161:897–908.
26. Mullbacher, A., M. Lobigs, R.T. Hla, T. Tran, T. Stehle, and M.M. Simon. 2002. Antigen-dependent release of IFN-gamma by cytotoxic T cells up-regulates Fas on target cells and facilitates exocytosis-independent specific target cell lysis. *J. Immunol.* 169:145–150.
27. van den Brink, M.R., E. Moore, K.J. Horndasch, J.M. Crawford, G.F. Murphy, and S.J. Burakoff. 2000. Fas ligand-deficient gld mice are more susceptible to graft-versus-host-disease. *Transplantation.* 70:184–191.
28. Schumacher, T.N., M.T. Heemels, J.J. Neefjes, W.M. Kast, C.J. Melief, and H.L. Ploegh. 1990. Direct binding of peptide to empty MHC class I molecules on intact cells and in vitro. *Cell.* 62:563–567.
29. Wong, M.M., and E.N. Fish. 2003. Chemokines: attractive mediators of the immune response. *Semin. Immunol.* 15:5–14.
30. Gibson, S., S. Tu, R. Oyer, S.M. Anderson, and G.L. Johnson. 1999. Epidermal growth factor protects epithelial cells against Fas-induced apoptosis. Requirement for Akt activation. *J. Biol. Chem.* 274:17612–17618.
31. Kalechman, Y., G. Strassmann, M. Albeck, and B. Sredni. 1998. Up-regulation by ammonium trichloro(dioxoethylene-0,0′) tellurate (AS101) of Fas/Apo-1 expression on B16 melanoma cells: implications for the antitumor effects of AS101. *J. Immunol.* 161:3536–3542.
32. Avent, J., C. Vervaert, and H.F. Seigler. 1979. Non-specific and specific active immunotherapy in a B16 murine melanoma system. *J. Surg. Oncol.* 12:87–96.
33. Tsai, V., S. Southwood, J. Sidney, K. Sakaguchi, Y. Kawakami, E. Appella, A. Sette, and E. Celis. 1997. Identification of subdominant CTL epitopes of the GP100 melanoma-associated tumor antigen by primary in vitro immunization with peptide-pulsed dendritic cells. *J. Immunol.* 158: 1796–1802.
34. Yee, C., J.A. Thompson, D. Byrd, S.R. Riddell, P. Roche, E. Celis, and P.D. Greenberg. 2002. Adoptive T cell therapy using antigen-specific CD8+ T cell clones for the treatment of patients with metastatic melanoma: in vivo persistence, migration, and antitumor effect of transferred T cells. *Proc. Natl. Acad. Sci. USA.* 99:16168–16173.
35. Ochsenbein, A.F., S. Sierro, B. Odermatt, M. Pericin, U. Karrer, J. Hermans, S. Hemmi, H. Hengartner, and R.M. Zinkernagel. 2001. Roles of tumour localization, second signals and cross priming in cytotoxic T-cell induction. *Nature.* 411:1058–1064.
36. Nestle, F.O., S. Alijagic, M. Gilliet, Y. Sun, S. Grabbe, R. Dummer, G. Burg, and D. Schadenforf. 1998. Vaccination of melanoma patients with peptide- or tumor lysate-pulsed dendritic cells. *Nat. Med.* 4:328–332.
37. Vivanco, I., and C.L. Sawyers. 2002. The phosphatidylinositol 3-kinase AKT pathway in human cancer. *Nat. Rev. Cancer.* 2:489–501.
38. Bunn, P.A., Jr., and W. Franklin. 2002. Epidermal growth factor receptor expression, signal pathway, and inhibitors in non-small cell lung cancer. *Semin. Oncol.* 29:38–44.
39. Stein, J.V., A. Rot, Y. Luo, M. Narasimhaswamy, H. Na-

kano, M.D. Gunn, A. Matsuzawa, E.J.Q.M.E. Dorf, and U.H. von Andrian. 2000. The CC chemokine thymus-derived chemotactic agent 4 (TCA-4, secondary lymphoid tissue chemokine, 6Ckine, Exodus-2) triggers lymphocyte function–associated antigen 1–mediated arrest of rolling T lymphocytes in peripheral lymph node high endothelial venules. *J. Exp. Med.* 191:61–75.

40. Gortz, A., R.J. Nibbs, P. McLean, D. Jarmin, W. Lambie, J.W. Baird, and G.J. Graham. 2002. The chemokine ESkine/CCL27 displays novel modes of intracrine and paracrine function. *J. Immunol.* 169:1387–1394.
41. Youn, B.S., K.Y. Yu, J. Oh, J. Lee, T.H. Lee, and H.E. Broxmeyer. 2002. Role of the CC Chemokine receptor 9/TECK interaction in apoptosis. *Apoptosis*. 7:271–276.
42. Boehme, S.A., F.M. Lio, D. Maciejewski-Lenoir, K.B. Bacon, and P.J. Conlon. 2000. The chemokine fractalkine inhibits Fas-mediated cell death of brain microglia. *J. Immunol.* 165:397–403.
43. Peled, A., I. Petit, O. Kollet, M. Magid, T. Ponomaryov, T. Byk, A. Nagler, H. Ben-Hur, A. Many, L. Shultz, et al. 1999. Dependence of human stem cell engraftment and repopulation of NOD/SCID mice on CXCR4. *Science.* 283: 845–848.
44. Vlahakis, S.R., A. Villasis-Keever, T. Gomez, M. Vanegas, N. Vlahakis, and C.V. Paya. 2002. G protein-coupled chemokine receptors induce both survival and apoptotic signaling pathways. *J. Immunol.* 169:5546–5554.