Cancer Research

Chemotherapy Induces Intratumoral Expression of Chemokines in Cutaneous Melanoma, Favoring T-cell Infiltration and Tumor Control

Michelle Hong[1], Anne-Laure Puaux[1], Caleb Huang[1], Laure Loumagne[1], Charlene Tow[1], Charles Mackay[2], Masashi Kato[3], Armelle Prévost-Blondel[4,6], Marie-Françoise Avril[4,5,6], Alessandra Nardin[1], and Jean-Pierre Abastado[1]

Abstract

T-cell infiltration is known to impact tumor growth and is associated with cancer patient survival. However, the molecular cues that favor T-cell infiltration remain largely undefined. Here, using a genetically engineered mouse model of melanoma, we show that CXCR3 ligands and CCL5 synergize to attract effector T cells into cutaneous metastases, and their expression inhibits tumor growth. Treatment of tumor-bearing mice with chemotherapy induced intratumoral expression of these chemokines and favored T-cell infiltration into cutaneous tumors. In patients with melanoma, these chemokines were also upregulated in chemotherapy-sensitive lesions following chemotherapy, and correlated with T-cell infiltration, tumor control, and patient survival. We found that dacarbazine, temozolomide, and cisplatin induced expression of T-cell–attracting chemokines in several human melanoma cell lines *in vitro*. These data identify the induction of intratumoral expression of chemokines as a novel cell-extrinsic mechanism of action of chemotherapy that results in the recruitment of immune cells with antitumor activity. Therefore, identifying chemotherapeutic drugs able to induce the expression of T-cell–attracting chemokines in cancer cells may represent a novel strategy to improve the efficacy of cancer immunotherapy. *Cancer Res; 71(22); 6997–7009. ©2011 AACR.*

Introduction

T-cell infiltration is a known predictor of patient survival in several cancers, including colorectal and ovarian cancers, non–small cell lung carcinomas, and melanomas (1–5). T-cell–adoptive therapies represent an attractive approach to treating cancers and have yielded some promising results in melanoma, even though complete clinical responses are only observed in a minority of patients (6). Recently, the U.S. Food and Drug Administration approved the first therapeutic cancer vaccine (sipuleucel-T) for advanced prostate cancer (7). However, even when cancer vaccines induce immune responses in the majority of patients, only a few benefit from the treatment (8, 9). This continuing growth of tumors in the presence of functional antitumor immune responses, whether spontaneous or induced, is the most disturbing paradox of tumor immunology (10). The so far limited success of therapeutic cancer vaccines is largely due to our incomplete understanding of the mechanisms preventing the action of T cells locally. T-cell recruitment to the tumor is one of the potential rate-limiting steps in immunotherapy, and thus, intratumoral chemokines are likely to have a major impact (11, 12). Gene expression profiling studies have shown that intratumoral expression of chemokines, indeed, correlate with T-cell infiltration (13). Therefore, identifying drugs that favor T-cell trafficking to the tumors is essential to improve cancer vaccines.

Chemotherapy acts, in part, by direct induction of apoptosis in cancer cells. In addition, selected chemotherapies may promote immunogenic cell death, which stimulates the antitumor immune response (14). Chemotherapy can also induce stress signals leading to increased susceptibility of cancer cells to immune attack (15). In melanoma, mixed responses to chemotherapy are frequently observed (16). Therefore, we sought to understand the signals responsible for T-cell trafficking to cutaneous tumors in melanoma and to determine whether they are altered by chemotherapy.

We recently described the RETAAD model of melanoma (17). RETAAD mice develop spontaneous uveal melanomas that metastasize to the skin and visceral organs (18). Tumor-bearing animals mount a strong antitumor T-cell response, but although this response controls the outgrowth of visceral metastases, they have no effect on cutaneous tumors. We

Authors' Affiliations: [1]Singapore Immunology Network, BMSI, A-STAR, Singapore, Singapore; [2]Faculty of Medicine, Monash University, Clayton, Victoria, Australia; [3]College of Life and Health Sciences, Chubu University, Aichi, Japan; [4]Institut Cochin, Université Paris Descartes, CNRS UMR 8104; [5]Dermatology Department, Cochin Hospital, AP-HP, University Paris Descartes; and [6]INSERM, U567, Paris, France

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Corresponding Author: Jean-Pierre Abastado, Laboratory of Tumour Immunology, Singapore Immunology Network, 8A Biomedical Grove, #04-06 Immunos, Singapore 138648. Phone: 65 64070003; Fax: 65 6464 2057; E-mail: abastado@immunol.a-star.edu.sg

doi: 10.1158/0008-5472.CAN-11-1466

suspected that differential T-cell trafficking might explain this apparent paradox. Using global transcriptome analysis, we also analyzed cutaneous metastases resected from patients with melanoma before and after chemotherapy and found increased T-cell infiltration into the chemotherapy-sensitive tumors (19).

The aim of this study is to identify molecular cues controlling T-cell infiltration into cutaneous tumors and to find treatments promoting T-cell infiltration. We address this question in the RETAAD model and in patients with melanoma treated with chemotherapy. Our findings indicate that chemotherapy works, in part, through the induction of chemokine expression in cancer cells and the subsequent recruitment of T cells into the tumors.

Materials and Methods

Cell lines and antibodies

The B16-F10 (Cat Nr CRL-6475) and HTB-71 cell lines were from American Type Culture Collection. The Melan-ret cell line was previously described (20). The 4 human melanoma cell lines (M88, M102, M131, and M134) were kindly provided by J-B Latouche and P. Musette. The anti-CXCR3 antibody was produced by immunizing CXCR3-deficient mice with L1.2 cells expressing mouse CXCR3, as described previously (21).

Mice and patients

Animal care and experimental procedures were approved by the Institutional Animal Care and Use Committee of the Biological Resource Center, A*STAR, Singapore. Human tumor samples, patient demographics, and clinical characteristics can be found in Nardin and colleagues (19).

Gene expression analysis

Expression of immune genes relative to *Gapdh* (for mouse) and *ACTB* (for human) was measured by quantitative real-time PCR (qRT-PCR) as previously described (18). ΔC_t and primer efficiency were used to calculate the relative expression.

Expression of chemokine and chemokine receptor genes was determined by qRT-PCR with the Mouse Inflammatory Cytokines and Receptors RT^2 profiler PCR array system (SABiosciences), and data analysis was conducted according to the manufacturer's instructions.

Immunofluorescence

Formalin-fixed paraffin-embedded sections (5 μm) were immunolabeled for CD3 (Acris SM1754P; 1:50) using the protocol previously described (18).

Flow cytometry analyses

Single-cell suspensions were obtained by digestion with Collagenase A (1 mg/mL) and DNase I (0.1 mg/mL; Roche) in RPMI for 20 minutes. After red blood cell lysis, Fc receptors were blocked with anti-mouse CD16/CD32 for 30 minutes before incubation with antigen-specific antibodies at 1:50 dilution for 20 minutes. All antibodies were from Biolegend, except those specific for CCR1, CCR2, and CCR5 which were from R&D Systems.

Construction of expression plasmids

CCL5- and CXCL9-expressing plasmids are described in Supplementary Fig. S3. Briefly, RNA from lipopolysaccharide-activated macrophages was reverse transcribed, PCR amplified using specific primers for *Ccl5* (5′-primer TACCGAGCTCGGATCCATGAAGATCTCTGCAGCTG; 3′-primer AAACGGGCCCTCTAGAGCAGGGTCAGAATCAAGAAACC), and *Cxcl9* (5′-primer TACCGAGCTCGGATCCGCCACCATGAAGTCCGCTGTTC; 3′-primer GCCCTCTAGACTCGAGCTCTTATGTAGTCTTCCTTG). PCR products were cloned into pcDNA3.1 Hygro(+; Invitrogen) at *Bam*HI/*Xba*I sites (for *Ccl5*) and at *Bam*HI/*Xho*I sites (for *Cxcl9*). Chemokine-expressing and control plasmid preparations were tested for endotoxin using Limulus amebocyte lysate assay (Lonza, 50-647U).

In vivo experiments

RETAAD T-cell migration to skin tumors. Tumor-bearing RETAAD mice (aged between 30 and 50 weeks) were injected subcutaneously in the right flank with 2×10^5 B16-F10luc cells (Xenogen). Mice were euthanized when the tumor diameter reached 1 cm. The B16 transplanted skin tumor and 1 or 2 RETAAD skin tumors were collected from the same mouse. T-cell infiltration into tumors was measured by flow cytometry.

In vivo transfection of cutaneous tumors

Tumor-bearing RETAAD mice (30–50 weeks) were used. For each mouse, 2 to 7 cutaneous tumors were transfected with 5 μg of pcDNA3.1(+)-CCL5 or pcDNA3.1(+)-CXCL9 or empty plasmid using *in vivo* JetPEI transfecting reagent (Polyplus Transfection). The mice were injected 3 times on alternate days for 5 days. On day 6, the mice were euthanized and the tumors excised, weighed, and divided into 2 parts. One part was used for intratumoral mRNA expression of *Ccl5*, *Cxcl9*, *Cxcl10*, *Ifng*, *Gzma*, and *Gzmb* by qRT-PCR; the other was dissociated and analyzed for immune cell infiltration by flow cytometry. For *Ccl5* and *Cxcl9* coinjection studies, 2.5 μg of pcDNA3.1(+)-CCL5, pcDNA3.1(+)-CXCL9, empty plasmid, or a combination of 5 μg of pcDNA3.1(+)-CCL5 and pcDNA3.1(+)-CXCL9 were injected into cutaneous RETAAD tumors using the protocol described previously. T-cell infiltration and intratumoral chemokine expression were measured as previously described.

Chemotherapy and adoptive transfer

Rag1$^{-/-}$ mice were injected subcutaneously with 10^6 Melan-ret cells. When tumors became palpable, mice were treated for 2 consecutive days with 2 mg temozolomide or vehicle [dimethyl sulfoxide (DMSO)] only. On the following day, mice were injected intravenously with 10^7 *in vitro* activated T cells purified from C57BL/6 with or without 2 μg anti-CXCR3 antibody. T-cell infiltration and intratumoral expression of chemokines were measured 24 hours after transfer.

Chemotherapeutic drug treatment and chemokine gene expression

Human melanoma cells were seeded into 12-well plates (4×10^5 per well). Forty-eight hours later, drugs were added

at indicated concentrations. RNA extraction was conducted as described previously, and the expression of *CCL5*, *CXCL9*, and *CXCL10* was analyzed by qRT-PCR.

Multiplex analysis of chemokine/cytokine production by tumor cells

Concentrations of various cytokines/chemokines in the supernatant of drug-treated cells were analyzed using multiplex bead-based assays based on xMAP technology, combining both the 21-plex (MF0-005KMII) and 27-plex (M50-0KCAF0Y) kits from Bio-Rad and according to the manufacturer's protocol.

Statistical analyses

Normalized gene expression data were log-transformed and initially compared using a manova multivariate analysis. This was followed by post-hoc testing using Student *t* test or ANOVA. *P* values for multiple comparisons were adjusted using Bonferroni correction. Other parameters (tumor weight, tumor area, and percentage of tumor-infiltrating cells) were compared using nonparametric tests (Mann–Whitney or Kruskal–Wallis). Patient survival was analyzed by the Kaplan–Meier method using log-rank (Mantel–Cox) test. Statistical analyses were done using Graphpad or Bioconductor/R.

Results

Limited T-cell infiltration of cutaneous tumors

In RETAAD mice, $CD8^+$ T cells control the outgrowth of visceral metastases but have no effect on cutaneous tumors (Lengagne and colleagues, 2008 and Supplementary Fig. S1). We hypothesized that the lack of control of skin tumors by T cells results from the absence of T-cell infiltration or from the loss of function of infiltrating T cells. To measure T-cell infiltration, we collected 22 cutaneous and 4 visceral metastases from RETAAD mice and compared the density of $CD3^+$ T cells by flow cytometry. As shown in Fig. 1A, the median percentage of $CD3^+$ T cells was 2.7 times higher in visceral metastases than in cutaneous tumors (2-tailed Mann–Whitney, $P = 0.04$). Immunofluorescent staining of tumor sections confirmed that T cells were more abundant in visceral tumors

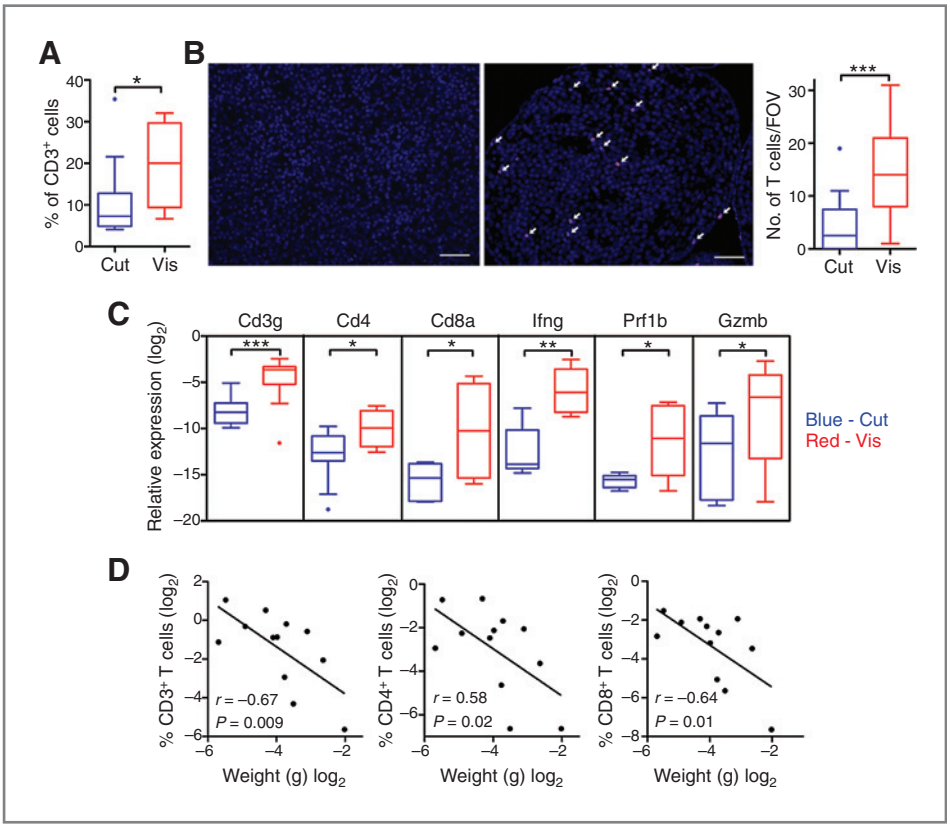

Figure 1. Low T-cell infiltration of RETAAD cutaneous metastasis. A, cutaneous ($n = 22$) and visceral ($n = 4$) tumors were collected from 9 RETAAD mice and analyzed for the presence of $CD3^+$ cells by flow cytometry. Data represent the percentage of $CD3^+$ cells among live $CD45^+$ cells. Mann–Whitney *U* test, 2-tailed. B, immunofluorescence labeling of CD3 (pink) in RETAAD cutaneous (left) and reproductive tract (right) tumors. Scale bar, 50 μm. Quantification of tumor-infiltrating T cells detected by immunofluorescence in cutaneous ($n = 4$) and visceral ($n = 6$) tumors. Data represent the number of T cells per field of view (FOV). Mann–Whitney *U* test, 2-tailed. C, expression of T-cell markers (*Cd3g*, *Cd4*, and *Cd8a*) and effector molecules (*Ifng*, *Prf1b*, and *Gzmb*) was measured in cutaneous ($n = 20$) and visceral ($n = 10$) tumors. Unpaired *t* test, 2-tailed. D, inverse correlation between the percentages of $CD3^+$, $CD4^+$, and $CD8^+$ T cells and cutaneous tumor weight ($n = 12$). Pearson correlation, 1-tailed. Statistical significance between groups is represented by *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Cut, cutaneous; Vis, visceral.

(14.5 vs. 4.5 cells/field of view; 2-tailed Mann–Whitney, $P = 0.0007$; Fig. 1B, right). Using qRT-PCR, we were able to analyze a greater number of tumors, especially smaller ones. In a preliminary experiment, we verified that intratumoral expression of *Cd3g* faithfully reflected T-cell infiltration (Pearson's $r = 0.75$, $P < 0.0001$, linear regression slope = 0.927; Supplementary Fig. S6A). qRT-PCR analysis revealed that markers of T cells (*Cd3g, Cd4, Cd8a, Ifng, Prf1b,* and *Gzmb*) were less expressed in cutaneous metastases ($n = 20$), confirming that there were fewer infiltrating T cells compared with visceral metastases ($n = 10$; 1-tailed t test, $P = 0.001$; Fig. 1C). Expression of both *Cd4* and *Cd8a* was lower in cutaneous tumors, suggesting that both compartments were affected ($P = 0.026$ and 0.023, respectively). Tumor weights were inversely correlated with the percentages of $CD3^+$, $CD4^+$, and $CD8^+$ infiltrating cells (Pearson's $r = -0.67$, -0.58, and -0.64 and $P = 0.009$, 0.02, and 0.01, respectively), suggesting that the few infiltrating T cells were able to control tumor growth (Fig. 1D).

Taken together, these data show that both $CD4^+$ and $CD8^+$ T cells poorly infiltrate cutaneous tumors in RETAAD mice. This observation is likely to explain the lack of T cell control over cutaneous tumors.

T cells infiltrate exogenous skin tumors

To determine whether the poor infiltration of T cells into cutaneous tumors was due to an intrinsic defect in T cells from RETAAD mice or a feature of the tumors themselves, we compared autochthonous RETAAD and transplanted B16 cutaneous tumors. B16 cells were injected subcutaneously into tumor-bearing RETAAD mice. Fourteen days after injection, the transplanted B16 and autochthonous RETAAD tumors were excised and T-cell infiltration was measured. As shown in Fig. 2A, $CD3^+$, $CD4^+$, and $CD8^+$ T cells were, respectively, 11-fold, 9-fold, and 11-fold more abundant in B16 than in RETAAD tumors (2-tailed Mann–Whitney, $P < 0.01$).

These data show that T cells from RETAAD mice have the capacity to infiltrate cutaneous tumors. Therefore, the paucity of T cells in RETAAD cutaneous tumors is most likely due to the tumor environment being poorly permissive to T-cell infiltration.

T-cell infiltration of exogenous skin tumors correlates with high chemokine expression

Chemokines present in tumors are likely to play a major role in T-cell recruitment. Therefore, we compared the repertoire of chemokines expressed in autochthonous RETAAD tumors and transplanted B16 tumors grown in RETAAD mice using low-density PCR arrays. Thirty-nine genes, including 26 chemokines and 13 chemokine receptors, involved in inflammation were analyzed by qRT-PCR. As shown in Fig. 2B, 14 of the 26 chemokines analyzed were differentially expressed in transplanted B16 compared with autochthonous RETAAD tumors (open squares; $P < 0.05$, fold change > 2), with the vast majority of these chemokines (12 of 14) being more highly expressed in B16 tumors. *Cxcl9* and *Cxcl10* were the two most differentially expressed chemokines, with 105- and 42-fold greater expression in B16 tumors, respectively. Five additional chemokines, *Ccl2*, *Ccl3*, *Ccl4*, *Ccl7*, and *Cxcl5* were at least 10-fold more highly expressed in B16 tumors than in autochthonous RETAAD tumors.

To gain further insight into the possible role of these differentially expressed chemokines in T-cell recruitment, we analyzed the expression of the corresponding chemokine receptors on peripheral blood T cells from RETAAD mice. CCR1, CCR2, CCR3, CCR5, and CXCR3 expressions were analyzed by flow cytometry on naive, central memory, and effector memory T cells. The chemokine receptor CXCR3 was highly expressed by several subsets of circulating T cells, whereas CCR1, CCR3, and CCR5 were only expressed at marginal levels (Fig. 2C). CCR2 was expressed by 25% ± 2% of effector memory $CD4^+$ T cells. CCR1, CCR2, CCR3, and CCR5 were highly expressed on various subsets of myeloid cells (Supplementary Fig. S2). Within the $CD4^+$ T cells, 4% ± 1.9% of the central memory and 58% ± 10% of the effector memory cells expressed CXCR3 (Fig. 2D), whereas among the $CD8^+$ T-cell population, 93% ± 3.6% of the central memory and 75% ± 5.7% of the effector memory cells expressed CXCR3.

CXCR3 has only 3 known ligands: CXCL9, CXCL10, and CXCL11. Therefore, our data strongly suggest a role for CXCL9 and CXCL10 in T-cell recruitment into cutaneous tumors.

Transfection of RETAAD skin tumors with *Cxcl9* induces T-cell infiltration

To directly show the role of CXCR3 ligands in T-cell recruitment, we transfected established RETAAD autochthonous tumors with a plasmid encoding CXCL9. RETAAD mice bearing multiple cutaneous metastases (median weight = 60 mg) were selected for this experiment. The mice received three intratumoral injections on alternate days. In each mouse, 1 or 2 tumors were injected with the CXCL9-encoding plasmid and 1 or 2 tumors were injected with a control plasmid. On day 6, tumors were excised, weighed, and T-cell infiltration was assessed by flow cytometry. As shown in Supplementary Fig. S4A, injection of the CXCL9-encoding plasmid induced a 60-fold increase in intratumoral expression of *Cxcl9* measured by qRT-PCR at day 6. Expression of *Cxcl10* was not affected. Importantly, CXCL9 transfection resulted in a 6-fold increase in both $CD4^+$ and $CD8^+$ T-cell infiltration into RETAAD cutaneous tumors (1-tailed Mann–Whitney, $P = 0.0026$ and 0.0025, respectively; Fig. 3A). Injection of the control plasmid did not significantly alter the T-cell infiltrate (Supplementary Fig. S4B).

To characterize CXCL9-recruited T cells, we analyzed the expression of several effector molecules by qRT-PCR. The expression of *Ifng*, *Gzma*, and *Gzmb* was enhanced in CXCL9-treated tumors compared with control tumors by 7-, 5-, and 5-fold, respectively (1-tailed t test, $P = 0.006$, 0.01, and 0.02, respectively; Fig. 3B). Moreover, using flow cytometry, we detected a 43% increase in tumor cell death (1-tailed Mann–Whitney, $P = 0.03$) in treated tumors compared with control tumors (Fig. 3C).

Taken together, these observations show that CXCL9 attracts effector T cells exhibiting type 1 polarization and cytotoxic potential.

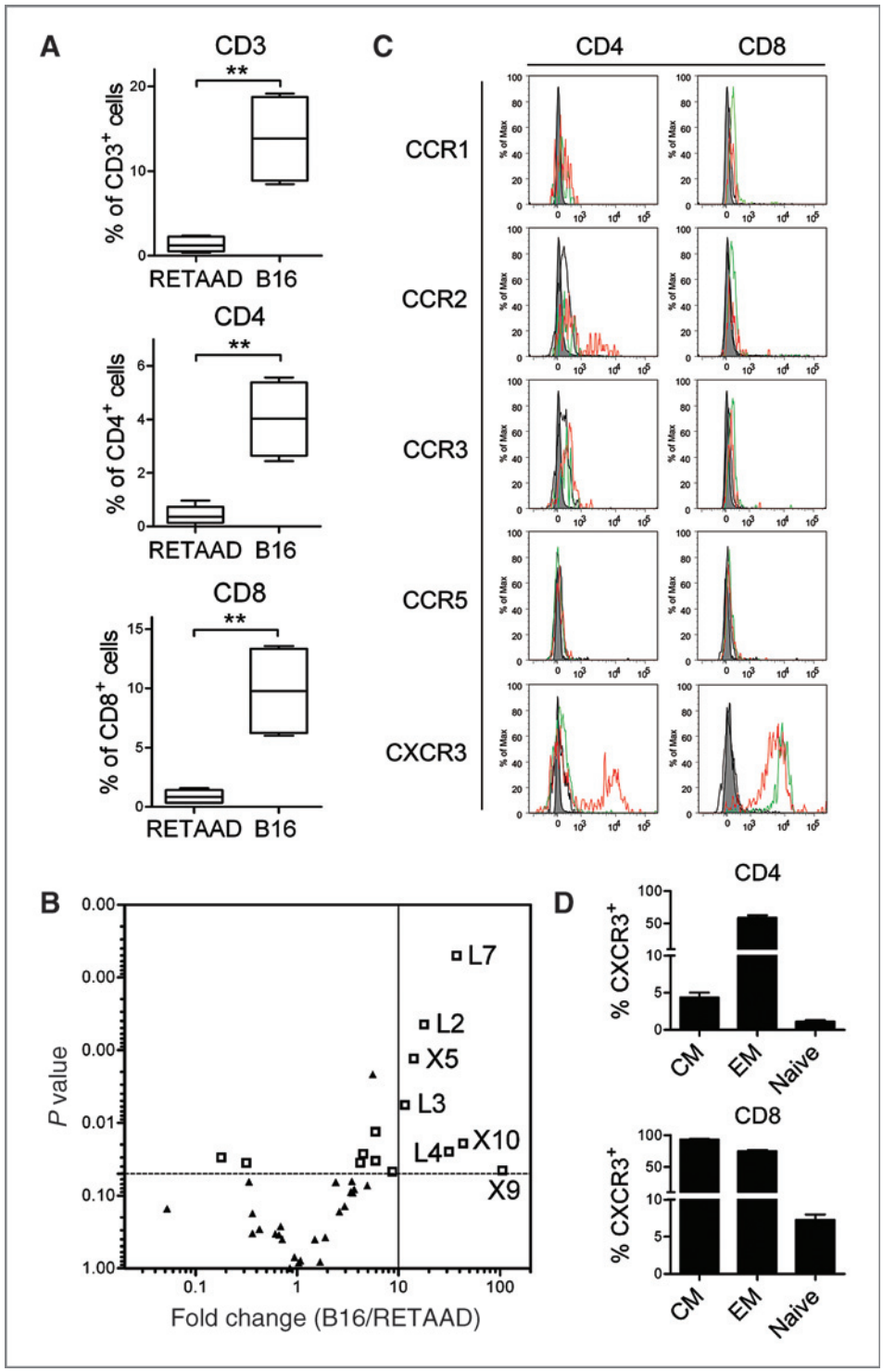

Figure 2. RETAAD T cells infiltrate exogenous B16 skin tumors. B16 cells were injected subcutaneously into the right flank of tumor-bearing RETAAD mice. Fourteen days after injection, transplanted B16 and autochthonous RETAAD skin tumors from the same mice were analyzed for T-cell infiltration. A, flow cytometric comparison of T-cell infiltrates in transplanted B16 ($n = 4$) and autochthonous RETAAD ($n = 6$) skin tumors from the same mice. Data show the percentages of $CD3^+$, $CD4^+$, and $CD8^+$ T cells among total live cells [4′,6-diamidino-2-phenylindole (DAPI)-]. Mann–Whitney *U* test, 2-tailed. Statistical significance between groups is represented by **, $P < 0.01$. B, intratumoral expression of chemokine and chemokine receptor genes. Volcano plot shows fold change in gene expression in B16 skin tumors grown in RETAAD mice ($n = 3$) compared with autochthonous RETAAD skin tumors ($n = 5$). Open squares represent chemokine genes with greater than 2-fold differential expression and $P < 0.05$. Horizontal line shows $P = 0.05$. Vertical line represents 10-fold increase in B16 compared with RETAAD tumors. L2, L3, L4, L7, X5, X9, and X10 indicate the chemokine genes *Ccl2*, *Ccl3, Ccl4, Ccl7*, *Cxcl5*, *Cxcl9*, and *Cxcl10*, respectively. C, surface expression of chemokine receptors on $CD4^+$ and $CD8^+$ T cells was analyzed in peripheral blood collected from RETAAD mice. Data are representative of 8 mice analyzed. Isotype control, gray filled histograms; naive ($CD44^-$ $CD62L^+$), black solid line; effector memory ($CD44^+$ $CD62L^-$), red solid line; central memory ($CD44^+$ $CD62L^+$), green solid line. D, percentages of $CXCR3^+$ cells within $CD4^+$ and $CD8^+$ T cell subsets of RETAAD mice ($n = 8$). CM, central memory; EM, effector memory.

Cxcl9 expression inhibits exogenous tumor growth in a T-cell–dependent manner

We next investigated whether ectopic expression of *Cxcl9* could control tumor growth *in vivo*. Therefore, we transfected *Cxcl9* into Melan-ret cells, a cell line derived from a RETAAD cutaneous tumor. *Cxcl9*-transfected cells were injected subcutaneously into syngeneic C57BL/6 mice. Tumor growth was monitored every 2 to 3 days for 16 days. *Cxcl9*-expressing tumor cells showed severely impaired growth compared with control cells; tumor formation was abolished in 2 of 5 mice (Fig. 3D). Mice were euthanized at day 16, tumors were collected and weighed, and T-cell infiltration was analyzed by qRT-PCR.

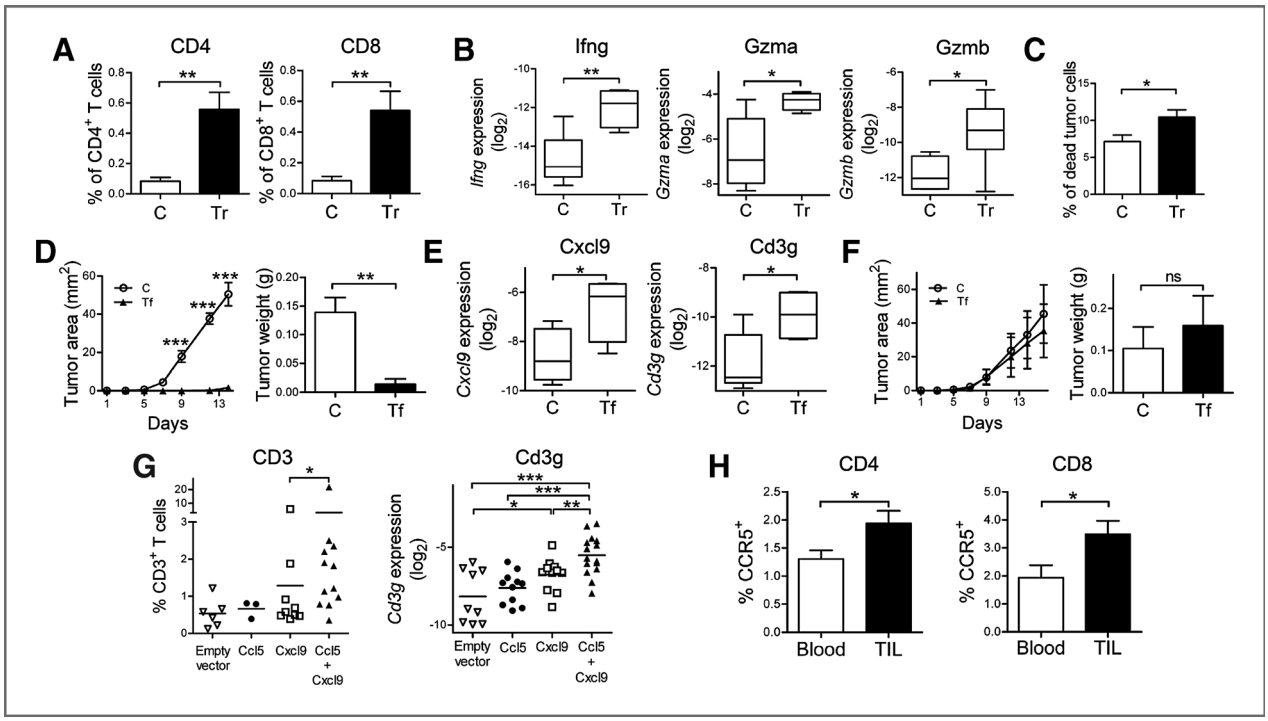

Figure 3. Chemokine induces T-cell infiltration in RETAAD cutaneous tumors and inhibits tumor growth. *Cxcl9*-encoding or control plasmids (5 μg) were injected into established RETAAD cutaneous tumors 3 times on alternate days. On day 6, tumors were analyzed for T-cell infiltration. A, the percentages of $CD4^+$ and $CD8^+$ T cells among total live cells in *Cxcl9*-treated tumors ($n = 5$) and control tumors ($n = 7$) were determined using flow cytometry. Mann–Whitney *U* test, 1-tailed. B, relative expression of several effector molecules, that is, *Ifng*, *Gzma*, and *Gzmb* in *Cxcl9*-treated ($n = 8$) and control tumors ($n = 5$). Unpaired *t* test, 1-tailed. C, percentage of dead tumor cells ($CD45^-DAPI^+/CD45^-$) in *Cxcl9*-treated ($n = 5$) and control ($n = 5$) tumors. Mann–Whitney *U* test, 1-tailed. D–F, Melan-ret cells were transfected with a CXCL9-encoding plasmid and injected subcutaneously into both flanks of C57BL/6 mice ($n = 5$). D, tumor size was measured every 2 to 3 days. Statistical analysis was carried out using 2-way ANOVA, 2-tailed. At necropsy on day 16, *Cxcl9*-transfected tumors ($n = 3$) and control tumors ($n = 5$) were collected and weighed. Mann–Whitney *U* test, 1-tailed. E, the expression of *Cxcl9* and *Cd3g* was measured by qRT-PCR in *Cxcl9*-transfected and control tumors. Unpaired *t* test, 2-tailed. F, tumor growth curves and tumor weights of *Cxcl9*-transfected ($n = 5$) and control ($n = 5$) tumors in *Rag1*$^{-/-}$ mice. Two-way ANOVA, 2-tailed and Mann–Whitney *U* test, 1-tailed. G and H, *Ccl5* synergizes with *Cxcl9* to recruit T cells. RETAAD cutaneous tumors were injected with 2.5 μg plasmid DNA encoding *Ccl5* ($n = 10$) or *Cxcl9* ($n = 11$), a combination of both plasmids ($n = 15$), or control plasmid ($n = 9$) on alternate days for 5 days. On day 6, tumors were analyzed for T-cell infiltration. G, the percentages of $CD3^+$ T cells measured by flow cytometry were compared using Kruskal–Wallis test. The expression of *Cd3g* was compared by ANOVA and Bonferroni multiple comparison posttest. H, surface expression of CCR5 on $CD4^+$ and $CD8^+$ T cells in peripheral blood ($n = 6$) and TIL ($n = 16$). Mann–Whitney *U* test, 1-tailed. All data are representative of 3 independent experiments conducted. Statistical differences between groups are represented by ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$; ns, nonsignificant. C, control plasmid; Tr, tumors injected with the *Cxcl9* plasmid; Tf, Melan-ret transfected with *Cxcl9* plasmid.

Cxcl9-expressing Melan-ret tumors were on average 10 times smaller (1-tailed Mann–Whitney, $P = 0.004$; mean weight = 0.14 g vs. 0.014 g) than control tumors (Fig. 3D), and this was associated with a 4-fold increase in *Cxcl9* expression and a 3-fold increase in *Cd3g* expression (Fig. 3E). When the same experiment was repeated in *Rag1*$^{-/-}$ mice, there was no significant difference in the growth of *Cxcl9*-transfected and control tumors (Fig. 3F). These data show that *Cxcl9* expression inhibits tumor growth and that this inhibition is dependent on a lymphoid cell population.

CXCL9 synergizes with CCL5 to recruit T cells

Knowing that CXCL9 promotes T-cell recruitment and that chemokines often act in concert, we then asked whether other chemokines expressed in RETAAD tumors were also involved in T-cell infiltration. By comparing a set of 37 cutaneous or visceral tumors, we found that *Ccl5* expression was highly correlated with *Cd3g* expression (Pearson's $r = 0.65$, $P = 4.75e\text{-}6$; Supplementary Fig. S5). Surprisingly, RETAAD circulating T cells express very low levels of the known receptors of CCL5, namely, CCR1, CCR3, and CCR5 (Fig. 2C). Therefore, secretion of CCL5 by the T cells may account for the observed correlation. Alternatively, CCL5 might play a role in T-cell attraction, for example, by synergizing with CXCR3 ligands. To test the latter hypothesis, RETAAD cutaneous tumors were transfected with a plasmid encoding CCL5 alone, CXCL9 alone, or a combination of both plasmids. Suboptimal doses of the CXCL9 plasmid were used. The mice were injected 3 times on alternate days for 5 days. On day 6, the tumors were harvested, weighed, and analyzed for $CD3^+$ T-cell infiltration by cytometry and qRT-PCR. As shown in Fig. 3G, treatment with CCL5 plasmid alone did not lead to enhanced T-cell infiltration. Similarly, treatment with suboptimal doses of CXCL9 plasmid had only a marginal effect. However, the combination of both CCL5 and CXCL9 resulted in a synergistic increase in T-cell infiltration (1-way Kruskal–Wallis, $P = 0.03$) and *Cd3g*

expression within the tumors (1-way ANOVA, $P < 0.0001$). This synergistic effect was observed for both $CD4^+$ and $CD8^+$ T cells (Supplementary Fig. S6B).

This increased infiltration of T cells in tumors coinjected with CCL5 and CXCL9 plasmids was not due to CCL5-mediated increase in CXCL9 production (data not shown). Conversely, T cells may undergo changes in chemokine receptor expression upon infiltration into the tumor. Therefore, we compared CCR5 expression on tumor-infiltrating lymphocytes (TIL; $n = 16$) and blood lymphocytes ($n = 6$). Interestingly, we detected a small but reproducible increase in the percentage of CCR5-expressing $CD4^+$ and $CD8^+$ (40% and 80% increase, respectively) in TIL compared with blood lymphocytes (Fig. 3H).

Altogether, these data show a synergy between CXCL9 and CCL5 for T-cell recruitment and suggest that CCR5 upregulation in the tumor microenvironment may favor T-cell retention in CCL5-expressing tumors.

Chemotherapy induces chemokine production in human melanoma cell lines

Chemotherapy is known to alter the transcriptome of cancer cells and has been reported to modify their profile of chemokine secretion (22). Therefore, we tested 3 chemotherapeutic drugs commonly used to treat stage IV melanoma (temozolomide, cisplatin, and dacarbazine), for their ability to induce T-cell–attracting chemokines *in vitro*. Five human melanoma cell lines (HTB-71, M88, M102, M131, and M134) were included in the study. Transcription of the *CCL5*, *CXCL9*, and *CXCL10* genes was measured by qRT-PCR 72 hours after drug treatment. As shown in Fig. 4A, each drug and each cell line displayed a unique pattern of chemokine expression; at least 2 T-cell–attracting chemokines were actively transcribed in all cell lines after treatment with at least one drug. Temozolomide strongly induced all 3 chemokines in M102 and M134, and *CXCL10* in HTB-71 and M131 (>10-fold increase). Cisplatin induced all 3 chemokines in M102 but only the CXCR3 ligands in M134 and *CXCL10* in M88. Dacarbazine induced all 3 chemokines in HTB-71. Dose–response relationships were observed for temozolomide and cisplatin (Fig. 4B), but no reproducible dose–response relationships were observed for dacarbazine. This is likely due to the fact that dacarbazine requires conversion into the active metabolite 5-(3-methyltriazen-1-yl)imidazole-4-carboxamide, which relies on spontaneous catalytic reaction *in vitro* whereas liver enzymatic activity carries out this step *in vivo*. Therefore, we focused on temozolomide (which shares the same active metabolite as dacarbazine) and cisplatin for the rest of the study. Figure 4C shows the kinetics of *CCL5*, *CXCL9*, and *CXCL10* induction in M102 cells following treatment with 100 μg/mL temozolomide. Transcription of *CXCL10* was detectable as soon as 24 hours after treatment, whereas *CXCL9* and *CCL5* transcription was only detected at 48 and 72 hours. Under these experimental conditions, cell death was not observed before 48 hours, showing that *CXCL10* transcription preceded cell death. In fact, for lower concentrations of temozolomide, *CXCL9* and *CCL5* expression also preceded cell death (data not shown).

We confirmed that *CCL5*, *CXCL9*, and *CXCL10* mRNA were translated into proteins by analyzing supernatants of M102 cells 72 hours after treatment with temozolomide (100 μg/mL) or cisplatin (10 μg/mL). Using ELISA and Luminex-based Bio-Plex suspension arrays, culture supernatants were tested for a variety of secreted proteins, including 28 cytokines, 7 additional chemokines, and 10 angiogenic and growth factors. As shown in Fig. 4D, temozolomide induced more than 9-fold increase in the secretion of 5 chemokines (CCL2, CCL5, CXCL8, CXCL9, and CXCL10) and 1 chemokine-like factor (MIF), whereas cisplatin induced CXCL8, CXCL10, and MIF. Importantly, none of the other factors was significantly induced (Table 1).

Altogether, these data show that 3 chemotherapeutic drugs commonly used for the treatment of human metastatic melanoma induce specific expression of chemokines involved in T-cell attraction.

Enhanced chemokine expression and T-cell infiltration in cutaneous tumors from mice treated with temozolomide

We next determined whether chemotherapy could facilitate intratumoral T-cell infiltration by inducing chemokine expression in cutaneous tumors. $Rag1^{-/-}$ mice bearing subcutaneous Melan-ret tumors were first treated with temozolomide and then adoptively transferred with *in vitro* activated T cells. Tumors were collected and analyzed for chemokine expression by qRT-PCR and T-cell infiltration by cytometry. As shown in Fig. 5A, temozolomide treatment induced significant upregulation of *Ccl5*, *Cxcl9*, and *Cxcl10* (1-tailed t test, $P = 0.01$, 0.02, and 0.0003, respectively). Importantly, this enhanced chemokine expression was associated with a more than 50% increase in $CD3^+$ T-cell infiltration. Moreover, this increase was completely suppressed by anti-CXCR3 blocking antibody. This indicates that temozolomide-induced chemokines are functional and are able to attract T cells into cutaneous tumors.

Enhanced expression of *CCL5*, *CXCL9*, and *CXCL10* after chemotherapy is associated with tumor control and superior survival of patients with melanoma

We previously showed increased T-cell infiltration in chemotherapy-sensitive tumors of patients with melanoma treated with dacarbazine (19). In the previous study, we carried out global transcriptome analysis of 33 cutaneous metastases resected before or after chemotherapy. We now reanalyzed these data, asking whether there were any chemokine whose expression correlated with T-cell infiltration. Among all the genes coding for chemokines, *CCL5*, *CXCL9*, and *CXCL10* were the most significantly correlated with *CD3Z* and *CD8A* expression, and among the most significantly correlated with *CD4* (Fig. 6A). We next compared *CD4* and *CD8A* expression in 3 categories of tumor samples: those that expressed low levels of the 3 chemokines, those that expressed high levels of *CCL5* or high levels of CXCR3 ligands, and those that expressed high levels of *CCL5* and high levels of at least 1 CXCR3 ligand. This comparison revealed a clear synergy between *CCL5* and CXCR3 ligands for T-cell infiltration (Fig. 6B). To determine whether chemotherapy had any impact on chemokine expression, we

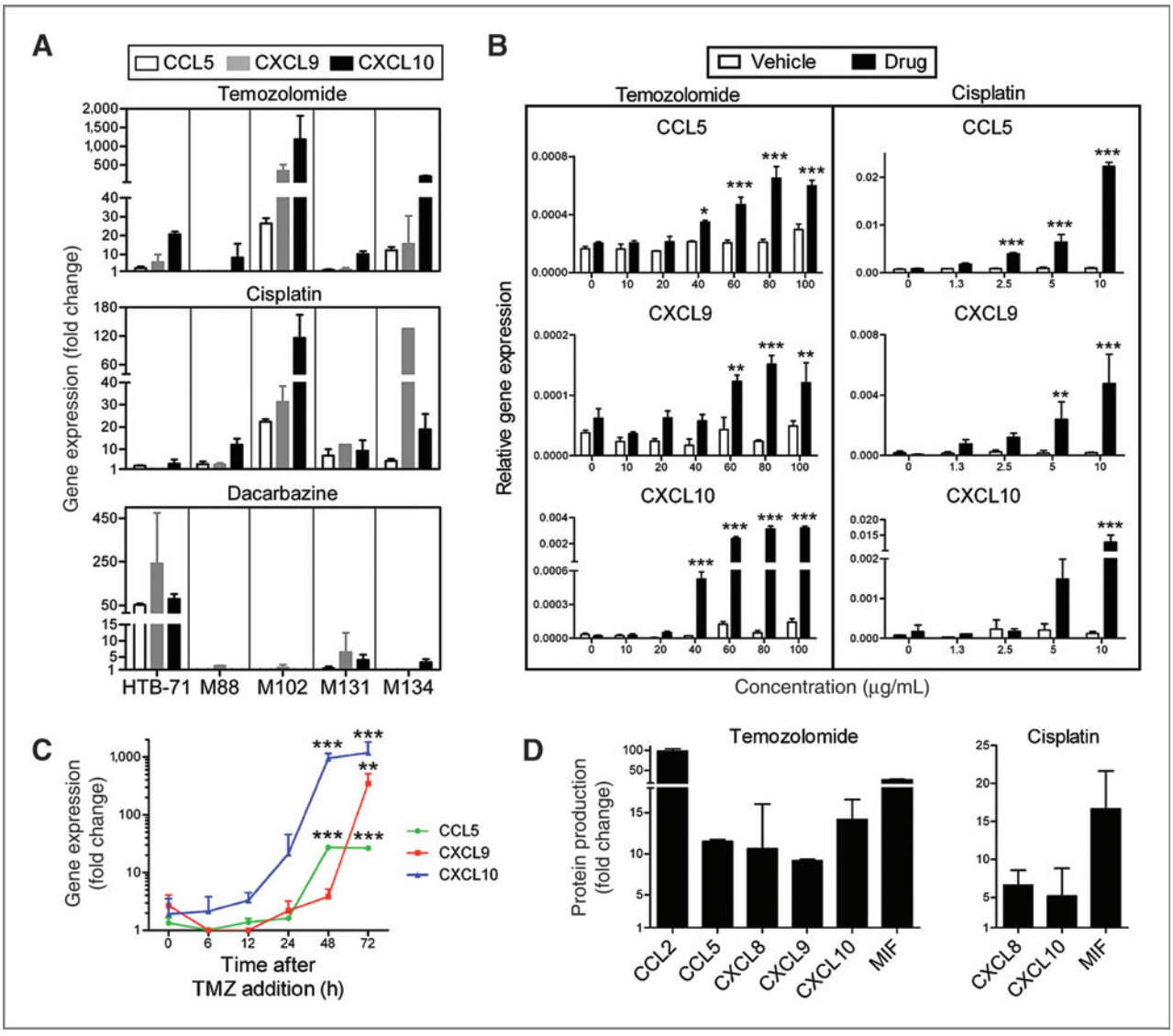

Figure 4. Chemotherapeutic drugs induce chemokine expression in human melanoma cells *in vitro*. Temozolomide, cisplatin, and dacarbazine were tested on 5 different human melanoma cell lines (HTB-71, M88, M102, M131, and M134) for their effect on CCL5, CXCL9, and CXCL10 production. A, *CCL5*, *CXCL9*, and *CXCL10* expression was measured by qRT-PCR in cells treated with either temozolomide (100 μg/mL), cisplatin (10 μg/mL), or dacarbazine (100 μg/mL) for 72 hours. Data show the fold change in chemokine expression in drug-treated cells over control cells. B, M102 cells were treated with increasing dose of temozolomide (0, 10, 20, 40, 60, 80, and 100 μg/mL) or cisplatin (0, 1.3, 2.5, 5, and 10 μg/mL) for 72 hours and the expression of *CCL5*, *CXCL9*, and *CXCL10* was measured by qRT-PCR. Comparisons were done using 2-way ANOVA. C, kinetics of *CCL5*, *CXCL9*, and *CXCL10* expression in M102 cells at 6, 12, 24, 48, and 72 hours after temozolomide (100 μg/mL) and cisplatin (10 μg/mL) treatment. Comparisons were done using 2-way ANOVA. D, concentrations of 40 different cytokines, chemokines, angiogenic factors, and growth factors in the supernatants of drug-treated and control cells were determined using multiplex immunobeads technology. Data are presented as fold change in protein production from drug-treated cells compared with control cells. Only cytokines/chemokines with 9-fold or more increase in secretion after drug treatment and *P* value lesser than 0.05 are represented from temozolomide treatment group. Mann–Whitney *U* test, 1-tailed.

compared *CCL5, CXCL9,* and *CXCL10* expression in tumors before and after therapy. As shown in Fig. 6C, whereas dacarbazine did not induce any significant changes in chemotherapy-resistant tumors, that is, progressing lesions, an increase in *CCL5*, *CXCL9*, and *CXCL10* expression was observed after treatment in chemotherapy-sensitive tumors (1-tailed *t* test, $P = 0.006$, 0.017, and 0.041, respectively). *CD4* and *CD8A* expression was also significantly increased in chemotherapy-sensitive tumors ($P = 0.0004$ and 0.0009, respectively; Fig. 6D). Manova multivariate analysis showed that chemokines and T-cell markers were significant predictors of the response to chemotherapy (Wilks test, $P = 0.0014$). Post-hoc tests showed that expression levels of *CXCL9*, *CXCL10, CCL5, CD4,* and *CD8A* were all significant predictors of tumor response ($P = 0.00022$, 0.00024, 3.2e-5, 4.1e-5, and 2.7e-5, respectively). Finally, Kaplan–Meier analysis showed that patients exhibiting higher chemokine expression after chemotherapy survived longer ($P = 0.0002$; HR = 35; Fig. 6E).

Table 1. Multiplex analysis of soluble factors secreted by M102 cell line after chemotherapeutic drug treatment

Analyte	Mean ± SD (pg/mL; temozolomide)	Mean ± SD (pg/mL; DMSO)	Fold increase	*P*	Mean ± SD (pg/mL; cisplatin)	Mean ± SD (pg/mL; DMF)	Fold increase	*P*
b-NGF	ND	ND	NA	NA	ND	ND	NA	NA
CTACK	ND	ND	NA	NA	ND	ND	NA	NA
Eotaxin	42.1 ± 16.4	97.3 ± 89.8	0.43	0.25	84.5 ± 101	119.3 ± 133.6	0.71	0.35
FGF basic	41.5 ± 12.7	22.3 ± 18.7	1.86	0.10	44.8 ± 16.7	42.9 ± 45	1.04	0.35
G-CSF	ND	ND	NA	NA	ND	ND	NA	NA
GM-CSF	100.1 ± 73.8	75 ± 81.5	1.33	0.20	58.5 ± 58.7	124.6 ± 166.8	0.47	0.50
GROα	3,776 ± 977.8	1,009 ± 377.1	3.74	0.05	309.8 ± 87.4	293.9 ± 198.7	1.05	0.35
HGF	ND	ND	NA	NA	ND	ND	NA	NA
IFN-α2	ND	ND	NA	NA	ND	ND	NA	NA
IFNγ	254 ± 199.3	319.6 ± 276.4	0.79	0.35	83.1 ± 42.4	700.5 ± 776.8	0.12	0.10
IL-1α	ND	ND	NA	NA	ND	ND	NA	NA
IL-1β	ND	ND	NA	NA	ND	ND	NA	NA
IL-1ra	200.2 ± 162.1	221.4 ± 184.1	0.9	0.41	131.3 ± 118.1	298 ± 256.4	0.46	0.35
IL-2	ND	ND	NA	NA	ND	ND	NA	NA
IL-2ra	ND	ND	NA	NA	ND	ND	NA	NA
IL-3	ND	ND	NA	NA	ND	ND	NA	NA
IL-4	ND	ND	NA	NA	ND	ND	NA	NA
IL-5	ND	ND	NA	NA	ND	ND	NA	NA
IL-6	ND	ND	NA	NA	ND	ND	NA	NA
IL-7	ND	ND	NA	NA	ND	ND	NA	NA
IL-8/CXCL8	**83,229 ± 67,810**	**7,869 ± 2,354**	**10.58**	**0.05**	**11,343 ± 1,856**	**2,144 ± 1,243**	**5.29**	**0.05**
IL-9	75.3 ± 50.9	71.5 ± 55.5	0.92	0.50	10.2 ± 1.5	113 ± 117.1	0.09	0.05
IL-10	19.4 ± 16.2	48.1 ± 29.4	0.4	0.10	10.3 ± 7	65.8 ± 71	0.16	0.10
IL-12p40	ND	ND	NA	NA	ND	ND	NA	NA
IL-12p70	88 ± 87.8	91.6 ± 77.6	1.13	0.41	23.4 ± 19.4	143.6 ± 162.1	0.16	0.10
IL-13	ND	ND	NA	NA	ND	ND	NA	NA
IL-15	ND	ND	NA	NA	ND	ND	NA	NA
IL-16	40.8 ± 34.7	56.1 ± 62.3	0.73	0.50	41.8 ± 35.1	103 ± 123.3	0.41	0.35
IL-17	103.8 ± 97	130.1 ± 147.4	0.8	0.50	114.4 ± 125.8	125.6 ± 109.8	0.91	0.50
IL-18	ND	ND	NA	NA	ND	ND	NA	NA
IP-10/CXCL10	**3,810 ± 1,122**	**268.7 ± 268.8**	**14.18**	**0.05**	**667.3 ± 215.3**	**115.8 ± 100.2**	**5.76**	**0.05**
LIF	13,478 ± 11,843	6,288 ± 5,508	2.14	0.25	188.1 ± 16.4	2,258 ± 383.4	0.08	0.05
MCP-1/CCL2	**522.8 ± 46.8**	**5.3 ± 1.6**	**98.29**	**0.04**	ND	ND	NA	NA
MCP-3/CCL7	ND	ND	NA	NA	ND	ND	NA	NA
M-CSF	86.7 ± 75.7	30.7 ± 35.7	2.92	0.17	121.4 ± 103.6	27.7 ± 30.5	4.38	0.17
MIF	**6,475 ± 522.8**	**246.7 ± 54.9**	**26.25**	**0.05**	**13,356 ± 3,597**	**874.3 ± 236.8**	**15.28**	**0.05**
MIG/CXCL9	**47 ± 1.4**	**5.1 ± 5.1**	**9.17**	**0.05**	ND	ND	NA	NA
MIP-1α/CCL3	ND	ND	NA	NA	ND	ND	NA	NA
MIP-1β/CCL4	ND	ND	NA	NA	ND	ND	NA	NA
PDGF-bb	65.7 ± 44.1	57 ± 67	1.15	0.35	22.9 ± 14.4	93.3 ± 82.6	0.25	0.10
RANTES/CCL5	**330.4 ± 12.1**	**28.7 ± 11.6**	**11.5**	**0.05**	29 ± 10.4	27.3 ± 22.7	1.06	0.35
SCF	40.6 ± 35.6	44.4 ± 40.1	0.91	0.50	77.2 ± 66.9	20 ± 28.2	3.87	0.27
SCGF-β	1,025 ± 405.7	1,290 ± 431.5	0.79	0.20	832.9 ± 226.9	2,916 ± 2,790	0.29	0.05
SDF-1α	ND	ND	NA	NA	ND	ND	NA	NA
TNF-α	ND	ND	NA	NA	ND	ND	NA	NA
TNF-β	ND	ND	NA	NA	ND	ND	NA	NA
TRAIL	ND	ND	NA	NA	ND	ND	NA	NA
VEGF	36,847 ± 6,117	44,477 ± 33,965	0.83	0.35	16,688 ± 5,206	55,394 ± 46,310	0.3	0.35

NOTE: Human melanoma cell line M102 was cultured with temozolomide (100 μg/mL) or cisplatin (10 μg/mL) for 3 days. Supernatants were collected and the concentrations of various soluble factors were analyzed using xMAP multiplex technology. Data show the production of cytokines, chemokines, angiogenic, and growth factors in pg/mL ± SD from the supernatant of drug-treated cells compared with control cells. Results are representative of 3 independent samples analyzed. Secreted factors that are significantly upregulated after drug treatment are indicated in bold.

Abbreviations: ND, not detected; NA, not available. Mann–Whiney *U* test, 1-tailed.

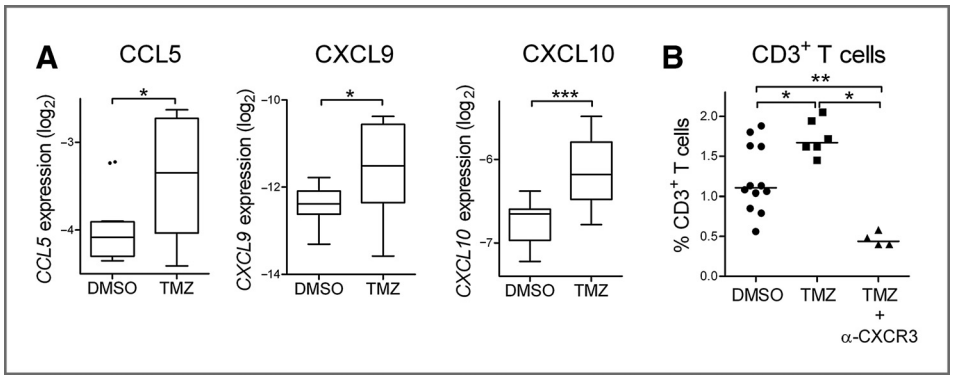

Figure 5. Temozolomide induces intratumoral expression of chemokines and promotes T-cell infiltration. Tumor-bearing *Rag1*$^{-/-}$ mice were treated intraperitoneally with 2 mg temozolomide (TMZ; $n = 10$) or DMSO ($n = 8$) for 2 consecutive days and then injected intravenously with 10^7 *in vitro* activated T cells. A, intratumoral expression of chemokines was analyzed by qRT-PCR. Unpaired *t* test, 1-tailed. B, T-cell infiltration was measured in individual tumors by flow cytometry. Data show the percentage of CD3$^+$ cells among live CD45$^+$ cells. Mann–Whitney *U* test, 1-tailed. Temozolomide enhanced intratumoral T-cell infiltration. Anti-CXCR3 blocking antibody (TMZ + α-CXCR3) abolished chemotherapy-induced infiltration of T cells. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Taken together, these data show that chemotherapy induces enhanced expression of CXCR3 ligands and *CCL5* in chemotherapy-sensitive tumors. Furthermore, increased expression of these chemokines translates into enhanced T-cell infiltration, improved tumor control, and prolonged overall survival.

Discussion

In this study, we show that CXCR3 ligands and CCL5 are the main determinants of T-cell infiltration into cutaneous melanoma tumors. Moreover, we show that chemotherapy induces expression of these chemokines in human melanoma cell lines and that chemotherapy-induced chemokines correlate with T-cell infiltration in mouse and human melanoma tumors. Finally, chemotherapy-induced chemokines is associated with tumor control and prolonged patient survival.

Although antitumor T cells are often detected in the blood of patients with melanoma, their presence only rarely translates into favorable clinical outcome (23). This applies to spontaneous T-cell responses as well as those induced by cancer vaccines; the vast majority of patients with cancer undergoing therapeutic vaccination have enhanced antitumor T-cell responses, but only a small percentage control their tumors. Several explanations have been proposed for this apparent paradox, including local immune suppression, tumor immune editing, and escape from immune attack (reviewed in ref. 24). In RETAAD mice, we found that the main reason why T cells do not control cutaneous tumors is because they fail to traffic to these tumors. We previously showed that cutaneous tumors are not immune-edited: resected cutaneous tumors are recognized *ex vivo* by antimelanoma T cells (17); moreover, CD8$^+$ T cells retain their functionality *in vivo* and control visceral metastases (18). We now show that very few T cells infiltrate RETAAD skin tumors in comparison with the RETAAD visceral tumors or transplanted B16 skin tumors. The few T cells that infiltrate RETAAD skin tumors apparently retain their functionality because their density correlates inversely with tumor size. Moreover, transduction of CXCR3 ligands and CCL5 into the cutaneous tumors results in increased T-cell infiltration and reduced tumor growth. In patients with melanoma, tumor regressions have been observed with similar frequencies of tumor-infiltrating T cells (25, 26). Therefore in this model, differential T-cell trafficking is the main reason for tissue-specific control of metastases. Importantly, we extended this finding to cutaneous tumors from patients with melanoma treated by chemotherapy. In these patients, tumor control and prolonged survival are positively associated with increased T-cell infiltration and intratumoral expression of CXCR3 ligands and CCL5.

Several correlative studies have highlighted the importance of CCL5 and CXCR3 ligands in the progression of cutaneous melanoma. Harlin and colleagues identified several chemokines whose expression is associated with CD8$^+$ T-cell infiltration; however, this study did not distinguish between chemokines that attract T cells and those that are produced by T cells (13). In patients with stage III disease, expression of CXCR3 by circulating memory T cells correlates with prolonged survival (27). IFN-α, a known inducer of CXCL9 and CXCL10, is approved for adjuvant treatment of high-risk (stages IIb and III) cutaneous melanoma (28, 29). On the basis of our data, it is tempting to speculate that induction of CXCR3 ligands participates in the antimelanoma activity of IFN-α. Expression of CXCL10 by peripheral blood mononuclear cell of patients with melanoma has been reported to correlate with tumor control (30). Interestingly, CXCR3 expression in cutaneous melanoma correlates negatively with lymphocyte infiltration (31). The mechanism underlying this correlation is unclear but this study suggests that cancer cells expressing CXCR3 might escape the immune response by competing for chemokines able to attract T cells. Expression of a nonfunctional allele of CCR5 results in decreased survival of patients with melanoma receiving immunotherapy (32). CXCR3 ligands and CCL5 have also been involved in other types of cancer, including colorectal and Ewing's sarcoma (33, 34).

Our study also reveals a striking synergism between CXCR3 ligands and CCL5 in attracting T cells into melanoma tumors of both humans and mice. At this stage, the exact mechanism

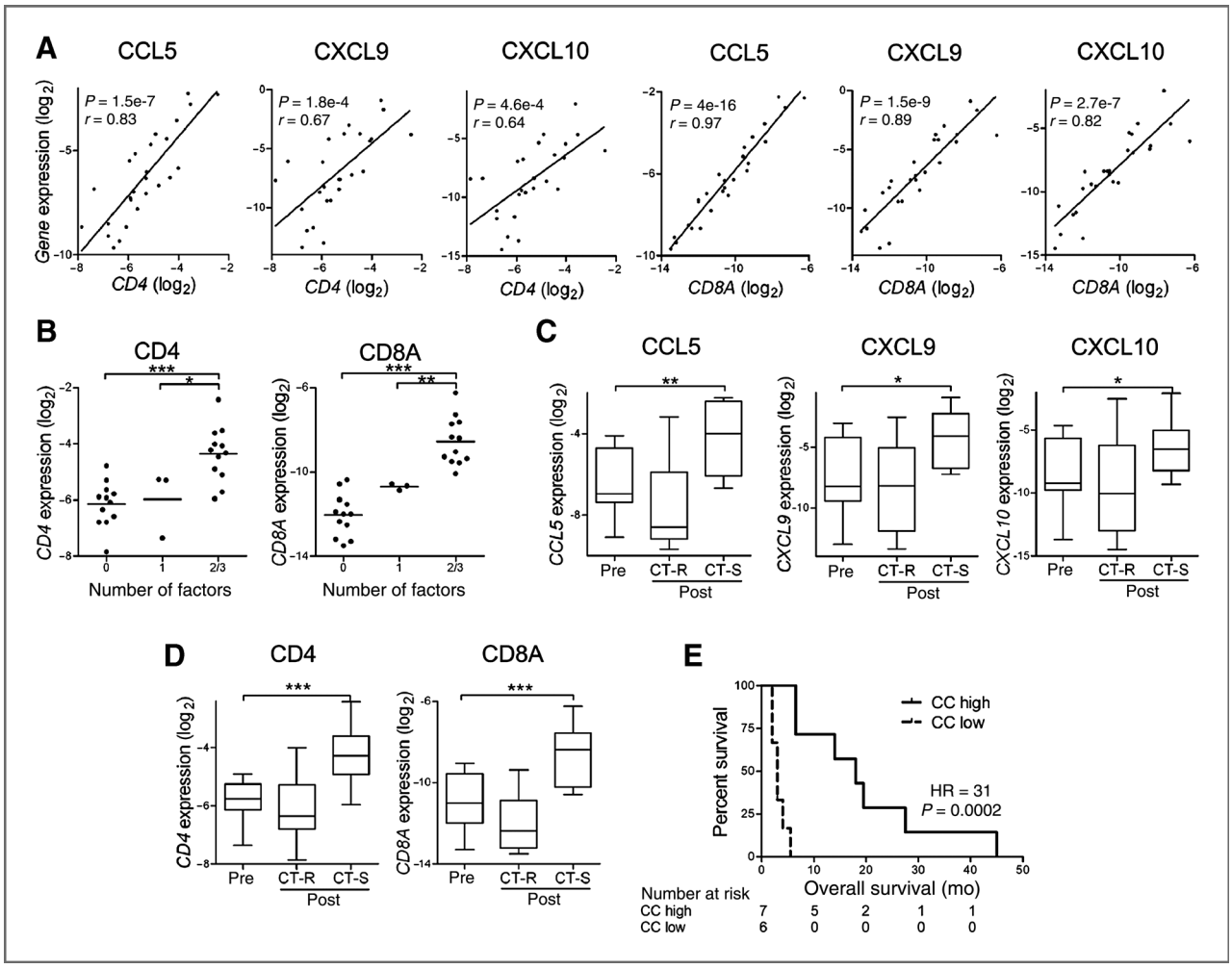

Figure 6. Enhanced chemokine expression in human melanoma skin tumors after chemotherapy correlates with increased T-cell infiltration, tumor control, and patient survival. A total of 33 cutaneous melanoma tumors from 13 stage III or IV patients were collected before and after chemotherapy. Gene expression was measured by qRT-PCR. A, *CD4* or *CD8A* expression correlates with *CCL5*, *CXCL9*, and *CXCL10* expression in the tumors. Spearman correlation, 1-tailed with Bonferroni correction for multiple testing. B, the expression of *CD4* and *CD8A* was compared between tumors with high or low expression of *CCL5* and CXCR3 ligands. 0, $CCL5^{lo}$ CXCR3 $ligands^{lo}$; 1, $CCL5^{hi}$ or CXCR3 $ligands^{hi}$; 2/3, $CCL5^{hi}$ and CXCR3 $ligands^{hi}$. One-way ANOVA. C and D, intratumoral expression of *CCL5*, *CXCL9*, and *CXCR3* as well as *CD4* and *CD8A* was compared between cutaneous tumors before (pre; $n = 13$) and after chemotherapy (post; $n = 22$). Tumors collected after treatment were divided into chemotherapy resistant (CT-R; $n = 12$) or chemotherapy sensitive (CT-S; $n = 10$). Differential gene expression between tumor samples collected before treatment and chemotherapy-sensitive tumors was assessed by 1-tailed *t* test on log-transformed expression values. E, Kaplan–Meier analysis of patient survival with high- or low- intratumoral chemokine expression after chemotherapy. Statistical significance between groups is presented as *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

accounting for this synergy is unclear because circulating T cells only express very low levels of CCL5 receptors. In fact, less than 2% of $CD8^+$ T cells in the RETAAD mice express CCL5 receptors. CCL5 transfection did not enhance CXCL9 expression in the tumor and transfection of CCL5 alone has no effect on T-cell attraction. However, we did observe an increased percentage of $CD4^+$ and $CD8^+$ T cells expressing CCR5 among the TIL, suggesting either that CCR5-expressing T cells are preferentially attracted into the tumor or that upon infiltration, T cells upregulate CCR5. We propose that intratumoral expression of CCL5 might facilitate the retention of CCR5-expressing T cells, thereby augmenting the percentage of $CCR5^+$ TIL. We could not directly test this hypothesis in patients with melanoma, but we did find that intratumoral expression of CXCR3 ligands and CCL5 in chemotherapy-treated tumors does lead to a synergistic increase in T-cell infiltration.

This study provides evidence for a novel mode of action of chemotherapeutic drugs. First, we show that dacarbazine, cisplatin, and temozolomide induce expression of T-cell–attracting chemokines in several melanoma cell lines. Transcription of CCL5, CXCL9, and/or CXCL10 was induced in all tested cell lines, even though each cell line displayed a unique profile of response to the chemotherapeutic drugs. Time-course experiments showed that under most experimental conditions tested, transcription of the chemokine genes preceded cell death. Secretion of the chemokines was confirmed for one of the cell lines. Chemokine induction was highly

specific because of 48 soluble factors analyzed, only 5 chemokines and 1 chemokine-like factor were significantly induced by temozolomide; whereas only 2 chemokines and 1 chemokine-like factor were induced by cisplatin. Treatment of tumor-bearing mice with temozolomide also induced intratumoral chemokine expression and CXCR3-dependent T-cell infiltration. Chemotherapies are known to alter the cancer cell transcriptome (reviewed in ref. 35). By damaging the tumor and stimulating a wound healing response, chemotherapy may also induce epithelial–mesenchymal transition and favor the outgrowth of cells with higher motility and invasiveness (36); it may also select drug-resistant cells with stem cell and metastasis-initiating properties (37). Levina and colleagues previously reported that *in vitro* treatment of some human cancer cell lines with doxorubicin or cisplatin induces expression of several cytokines, chemokines, and growth factors, which protect the cancer cells from drug-induced apoptosis (22). These previous reports identified cancer cell alterations that limit chemotherapy-induced cell death. By showing that chemotherapy can alter cancer cell phenotype in a way that favorably impacts immune cell trafficking to the tumor, this study reveals a novel mechanism of action of common chemotherapeutic agents. Chemotherapy induces clinical responses in about 20% of patients with melanoma (16). In our limited cohort, tumor regression or stabilization was observed in 6 of 13 patients and 11 of 20 tumors resected after chemotherapy. Remarkably, enhanced expression of CCL5, CXCL9, and CXCL10 after chemotherapy correlated with tumor response. Importantly, prolonged patient survival was associated with enhanced chemokine expression after chemotherapy.

In conclusion, this study identifies CXCR3 ligands and CCL5 as the main determinants of T-cell infiltration into cutaneous metastases and shows that chemotherapy, by inducing expression of these chemokines within human tumors, may trigger T-cell infiltration and tumor control, resulting in prolonged patient survival. Therefore, screening for chemotherapeutic products able to induce the expression of T-cell–attracting chemokines in cancer cells may identify drugs that improve the efficacy of immunotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors thank Jo Keeble, Jeremy Wang, and Muly Tham for help with animal studies, Benjamin Toh for help with flow cytometry experiments, Shen Yi for help with *in vivo* transfection experiments, Cindy Phua for mouse husbandry, Michael Poidinger for help with statistical analyses, and Lucy Robinson for proofreading the manuscript.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received May 2, 2011; revised September 10, 2011; accepted September 14, 2011; published OnlineFirst September 26, 2011.

References

1. Galon J, Costes A, Sanchez-Cabo F, Kirilovsky A, Mlecnik B, Lagorce-Pages C, et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. Science 2006;313:1960–4.
2. Pages F, Galon J, Dieu-Nosjean MC, Tartour E, Sautes-Fridman C, Fridman WH. Immune infiltration in human tumors: a prognostic factor that should not be ignored. Oncogene 2010;29:1093–102.
3. Clemente CG, Mihm MC Jr, Bufalino R, Zurrida S, Collini P, Cascinelli N. Prognostic value of tumor infiltrating lymphocytes in the vertical growth phase of primary cutaneous melanoma. Cancer 1996;77:1303–10.
4. Sato E, Olson SH, Ahn J, Bundy B, Nishikawa H, Qian F, et al. Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. Proc Natl Acad Sci U S A 2005;102:18538–43.
5. Kawai O, Ishii G, Kubota K, Murata Y, Naito Y, Mizuno T, et al. Predominant infiltration of macrophages and CD8(+) T Cells in cancer nests is a significant predictor of survival in stage IV nonsmall cell lung cancer. Cancer 2008;113:1387–95.
6. Rosenberg SA, Dudley ME. Adoptive cell therapy for the treatment of patients with metastatic melanoma. Curr Opin Immunol 2009;21:233–40.
7. Kantoff PW, Higano CS, Shore ND, Berger ER, Small EJ, Penson DF, et al. Sipuleucel-T immunotherapy for castration-resistant prostate cancer. N Engl J Med 2010;363:411–22.
8. Speiser DE, Romero P. Molecularly defined vaccines for cancer immunotherapy, and protective T cell immunity. Semin Immunol 2010;22:144–54.
9. Rosenberg SA, Yang JC, Restifo NP. Cancer immunotherapy: moving beyond current vaccines. Nat Med 2004;10:909–15.
10. Zitvogel L, Tesniere A, Kroemer G. Cancer despite immunosurveillance: immunoselection and immunosubversion. Nat Rev Immunol 2006;6:715–27.
11. Mantovani A, Allavena P, Sozzani S, Vecchi A, Locati M, Sica A. Chemokines in the recruitment and shaping of the leukocyte infiltrate of tumors. Semin Cancer Biol 2004;14:155–60.
12. Balkwill F. Chemokine biology in cancer. Semin Immunol 2003;15:49–55.
13. Harlin H, Meng Y, Peterson AC, Zha Y, Tretiakova M, Slingluff C, et al. Chemokine expression in melanoma metastases associated with CD8+ T-cell recruitment. Cancer Res 2009;69:3077–85.
14. Zitvogel L, Kepp O, Kroemer G. Immune parameters affecting the efficacy of chemotherapeutic regimens. Nat Rev Clin Oncol 2011;8:151–60.
15. Fine JH, Chen P, Mesci A, Allan DS, Gasser S, Raulet DH, et al. Chemotherapy-induced genotoxic stress promotes sensitivity to natural killer cell cytotoxicity by enabling missing-self recognition. Cancer Res 2010;70:7102–13.
16. Bajetta E, Del Vecchio M, Bernard-Marty C, Vitali M, Buzzoni R, Rixe O, et al. Metastatic melanoma: chemotherapy. Semin Oncol 2002;29:427–45.
17. Lengagne R, Graff-Dubois S, Garcette M, Renia L, Kato M, Guillet JG, et al. Distinct role for CD8 T cells toward cutaneous tumors and visceral metastases. J Immunol 2008;180:130–7.
18. Eyles J, Puaux AL, Wang X, Toh B, Prakash C, Hong M, et al. Tumor cells disseminate early, but immunosurveillance limits metastatic outgrowth, in a mouse model of melanoma. J Clin Invest 2010;120:2030–9.
19. Nardin A, Wong WC, Tow C, Molina TJ, Tissier F, Audebourg A, et al. Dacarbazine promotes stromal remodeling and lymphocyte infiltration in cutaneous melanoma lesions. J Invest Dermatol 2011;131:1896–905.

20. Lengagne R, Le Gal FA, Garcette M, Fiette L, Ave P, Kato M, et al. Spontaneous vitiligo in an animal model for human melanoma: role of tumor-specific CD8+ T cells. Cancer Res 2004;64:1496–501.
21. Heath H, Qin S, Rao P, Wu L, LaRosa G, Kassam N, et al. Chemokine receptor usage by human eosinophils. The importance of CCR3 demonstrated using an antagonistic monoclonal antibody. J Clin Invest 1997;99:178–84.
22. Levina V, Su Y, Nolen B, Liu X, Gordin Y, Lee M, et al. Chemotherapeutic drugs and human tumor cells cytokine network. Int J Cancer 2008;123:2031–40.
23. Salcedo M, Bercovici N, Taylor R, Vereecken P, Massicard S, Duriau D, et al. Vaccination of melanoma patients using dendritic cells loaded with an allogeneic tumor cell lysate. Cancer Immunol Immunother 2006;55:819–29.
24. Rabinovich GA, Gabrilovich D, Sotomayor EM. Immunosuppressive strategies that are mediated by tumor cells. Annu Rev Immunol 2007;25:267–96.
25. Lurquin C, Lethe B, De Plaen E, Corbiere V, Theate I, van Baren N, et al. Contrasting frequencies of antitumor and anti-vaccine T cells in metastases of a melanoma patient vaccinated with a MAGE tumor antigen. J Exp Med 2005;201:249–57.
26. Carrasco J, Van Pel A, Neyns B, Lethe B, Brasseur F, Renkvist N, et al. Vaccination of a melanoma patient with mature dendritic cells pulsed with MAGE-3 peptides triggers the activity of nonvaccine anti-tumor cells. J Immunol 2008;180:3585–93.
27. Mullins IM, Slingluff CL, Lee JK, Garbee CF, Shu J, Anderson SG, et al. CXC chemokine receptor 3 expression by activated CD8+ T cells is associated with survival in melanoma patients with stage III disease. Cancer Res 2004;64:7697–701.
28. Hauschild A, Gogas H, Tarhini A, Middleton MR, Testori A, Dreno B, et al. Practical guidelines for the management of interferon-alpha-2b side effects in patients receiving adjuvant treatment for melanoma: expert opinion. Cancer 2008;112:982–94.
29. Stadler R, Luger T, Bieber T, Kohler U, Linse R, Technau K, et al. Long-term survival benefit after adjuvant treatment of cutaneous melanoma with dacarbazine and low dose natural interferon alpha: a controlled, randomised multicentre trial. Acta Oncol 2006;45:389–99.
30. Antonicelli F, Lorin J, Kurdykowski S, Gangloff SC, Le Naour R, Sallenave JM, et al. Cxcl10 reduces melanoma proliferation and invasivity *in vitro* and *in vivo*. Br J Dermatol 2011;164:720–8.
31. Monteagudo C, Martin JM, Jorda E, Llombart-Bosch A. CXCR3 chemokine receptor immunoreactivity in primary cutaneous malignant melanoma: correlation with clinicopathological prognostic factors. J Clin Pathol 2007;60:596–9.
32. Ugurel S, Schrama D, Keller G, Schadendorf D, Brocker EB, Houben R, et al. Impact of the CCR5 gene polymorphism on the survival of metastatic melanoma patients receiving immunotherapy. Cancer Immunol Immunother 2008;57:685–91.
33. Mlecnik B, Tosolini M, Charoentong P, Kirilovsky A, Bindea G, Berger A, et al. Biomolecular network reconstruction identifies T-cell homing factors associated with survival in colorectal cancer. Gastroenterology 2010;138:1429–40.
34. Berghuis D, Santos SJ, Baelde HJ, Taminiau AH, Egeler RM, Schilham MW, et al. Pro-inflammatory chemokine-chemokine receptor interactions within the Ewing sarcoma microenvironment determine CD8(+) T-lymphocyte infiltration and affect tumour progression. J Pathol 2011;223:347–57.
35. Harless WW. Cancer treatments transform residual cancer cell phenotype. Cancer Cell Int 2011;11:1.
36. Rosano L, Cianfrocca R, Spinella F, Di Castro V, Nicotra MR, Lucidi A, et al. Acquisition of chemoresistance and EMT phenotype is linked with activation of the endothelin A receptor pathway in ovarian carcinoma cells. Clin Cancer Res 2011;17:2350–60.
37. Ahmed N, Abubaker K, Findlay J, Quinn M. Epithelial mesenchymal transition and cancer stem cell-like phenotypes facilitate chemoresistance in recurrent ovarian cancer. Curr Cancer Drug Targets 2010;10:268–78.