Chemotherapy induces intratumoral expression of chemokines in cutaneous melanoma, favoring T cell infiltration and tumor control

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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 intra-tumoral expression of these chemokines and favored T cell infiltration into cutaneous tumors. In melanoma patients, these chemokines were also up-regulated 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 intra-tumoral expression of chemokines as a novel cell-extrinsic mechanism of action of chemotherapy that results in the recruitment of immune cells with anti-tumor 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.
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 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 anti-tumor 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, intra-tumoral chemokines are likely to have a major impact (11-12). Gene expression profiling studies have shown that intra-tumoral 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 anti-tumor 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). We therefore 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 which metastasize to the skin and visceral organs (18). Tumor-bearing animals mount a strong anti-tumor T cell response, but while this response controls the outgrowth of visceral metastases, they have no effect on cutaneous tumors. We suspected that differential T cell trafficking might explain this apparent paradox. Using global transcriptome analysis, we also analyzed cutaneous metastases resected from melanoma patients before and after chemotherapy, and found increased T cell infiltration into the chemotherapy-sensitive tumors (19).

The aim of the present 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 melanoma patients 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 ATCC. The Melan-ret cell line was previously described (20). The four 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 (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). ΔCt 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 RT² profiler PCR array system (SABiosciences) and data analysis was performed according to the manufacturer’s instructions.

Immunofluorescence
Formalin-fixed paraffin-embedded sections (5μm) 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 (1mg/ml) and DNase I (0.1mg/ml) (Roche) in RPMI for 20min. After red blood cell lysis, Fc receptors were blocked with anti-mouse CD16/CD32 for 30min before incubation with antigen-specific antibodies at 1:50 dilution for 20min. 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 Figure 3. Briefly, RNA from LPS-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 GCCCTCTAGACTCGAGCTCTTGTAGTCTTCTTG). PCR products were cloned into pcDNA3.1 Hygro(+) (Invitrogen) at BamHI/XbaI sites (for Ccl5) and at BamHI/XhoI 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 30-50 weeks) were injected subcutaneously in the right flank with 2x10^5 B16-F10luc cells (Xenogen, Alameda, CA). Mice were euthanized when the tumor diameter reached 1cm. 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 5µg of pcDNA3.1(+)CCL5 or -CXCL9 or empty plasmid using in vivo JetPEI transfecting reagent (Polyplus Transfection). Three injections were performed on alternate days for 5 days. On day 6, the mice were euthanized and the tumors excised, weighed and divided into two parts. One part was used for intra-tumoral 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 co-injection studies, 2.5µg of pcDNA3.1(+)CCL5, -CXCL9, empty plasmid or a combination of 5µg of pcDNA3.1(+)CCL5 and -CXCL9 were injected into cutaneous RETAAD tumors using the protocol described above. T cell infiltration and intra-tumoral chemokine expression were measured as previously described.

Chemotherapy and adoptive transfer

Rag1−/− mice were injected subcutaneously with 10⁶ Melan-ret cells. When tumors became palpable, mice were treated for 2 consecutive days with 2mg Temozolomide or vehicle (DMSO) only. On the following day, mice were injected i.v. with 10⁷ in vitro activated T cells purified from C57BL/6 with or without 2µg anti-CXCR3 antibody. T cell infiltration and intra-tumoral expression of chemokines were measured 24h after transfer.

Chemotherapeutic drug treatment and chemokine gene expression
Human melanoma cells were seeded into 12-well plates (4x10^5/well). 48h later, drugs were added at indicated concentrations. RNA extraction was performed 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 Biorad and according to the manufacturer’s protocol.

**Statistical analyses**

Normalised gene expression data were log-transformed and initially compared using a manova multivariate analysis. This was followed by post-hoc testing using Student’s t-test or ANOVA. P-values for multiple comparisons were adjusted using Bonferroni correction. Other parameters (tumor weight, tumor area, percentage of tumor-infiltrating cells) were compared using non-parametric tests (Mann-Whitney or Kruskal-Wallis). Patient survival was analyzed by the Kaplan-Meier method using log-rank (Mantel-Cox) test. Statistical analyses were performed 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 et al., 2008 and Supplementary Figure 1). 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 Figure 1A, the median percentage of CD3\(^+\) T cells was 2.7 times higher in visceral metastases than in cutaneous tumors (two-tailed Mann-Whitney, p=0.04). Immunofluorescent staining of tumor sections confirmed that T cells were more abundant in visceral tumors (14.5 vs 4.5 cells/field of view; two-tailed Mann-Whitney, p=0.0007) (Figure 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 intra-tumoral expression of \(Cd3g\) faithfully reflected T cell infiltration (Pearson’s \(r=0.75\), \(p<0.0001\), linear regression slope=0.927; Supplementary Figure 6A). 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 to visceral metastases (\(n=10\); one-tailed t-test, \(p=0.001\); Figure 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; -0.64, \(p=0.009\), 0.02, 0.01, respectively), suggesting that the few infiltrating T cells were able to control tumor growth (Figure 1D).
Taken together these data demonstrate 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 Figure 2A, CD3+, CD4+ and CD8+ T cells were respectively 11-fold, 9-fold and 11-fold more abundant in B16 than in RETAAD tumors (two-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. We therefore 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 Figure 2B, 14 out of the 26 chemokines analyzed were differentially expressed in transplanted B16 compared to autochthonous RETAAD tumors (open squares; p<0.05, fold-change>2), with the vast majority of these chemokines (12 out 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. Four 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 expression was analyzed by flow cytometry on naïve, central memory and effector memory T cells. The chemokine receptor CXCR3 was highly expressed by several subsets of circulating T cells while CCR1, CCR3 and CCR5 were only expressed at marginal levels (Figure 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 Figure 2). Within the CD4⁺ T cells, 4±1.9% of the central memory and 58±10% of the effector memory cells expressed CXCR3 (Figure 2D) while 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 three 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 demonstrate 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=60mg) were selected for this experiment. Three intra-tumoral injections were performed on alternate days. In each mouse, one or two tumors were injected with the CXCL9-encoding plasmid and one or two 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 Figure 4A, injection of the CXCL9-encoding plasmid induced a 60-fold increase in intra-tumoral 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 (one-tailed Mann-Whitney, p=0.0026 and 0.0025, respectively; Figure 3A). Injection of the control plasmid did not significantly alter the T cell infiltrate (Supplementary Figure 4B).

To further 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 to control tumors by 7-, 5- and 5-fold, respectively (one-tailed t-test, p=0.006, 0.01, and 0.02, respectively; Figure 3B). Moreover, using flow cytometry, we detected a 43% increase in tumor cell death (one-tailed Mann-Whitney, p=0.03) in treated tumors compared to control tumors (Figure 3C).

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

**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 2-3 days for 16 days. Cxcl9-expressing tumor cells showed severely impaired growth compared to control cells; tumor formation was abolished in 2 out of 5 mice (Figure 3D). Mice were euthanized at day 16, tumors were collected and weighed, and T cell infiltration was analyzed by qRT-PCR. Cxcl9-expressing Melan-ret tumors were on average 10 times smaller (one-tailed Mann-Whitney, p =0.004; mean weight=0.14g vs 0.014g) than control tumors (Figure 3D), and this was associated with a 4-fold increase in Cxcl9.
expression and a 3-fold increase in \( Cd3g \) expression (Figure 3E). When the same experiment was repeated in Rag1\(^{-/-}\) mice, there was no significant difference in the growth of \( Cxcl9 \)-transfected and control tumors (Figure 3F). These data demonstrate 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 \( r=0.65, p=4.75\times10^{-6} \); Supplementary Figure 5). Surprisingly, RETAAD circulating T cells express very low levels of the known receptors of CCL5, namely CCR1, -3 and -5 (Figure 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. Three injections were performed 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 Figure 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 (one-way Kruskal-Wallis, \( p=0.03 \)) and \( Cd3g \) expression within the tumors (one-way ANOVA, \( p<0.0001 \)). This synergistic effect was observed for both CD4\(^+\) and CD8\(^+\) T cells (Supplementary Figure 6B).
This increased infiltration of T cells in tumors co-injected 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. We therefore compared CCR5 expression on 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 to blood lymphocytes (Figure 3H). Altogether, these data demonstrate a synergy between CXCL9 and CCL5 for T cell recruitment and suggest that CCR5 up-regulation 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). We therefore tested three 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 72h after drug treatment. As shown in Figure 4A, each drug and each cell line displayed a unique pattern of chemokine expression; at least two T cell-attracting chemokines were actively transcribed in all cell lines after treatment with at least one drug. Temozolomide strongly induced all three chemokines in M102 and M134, and CXCL10 in HTB-71 and M131 (>10-fold increase). Cisplatin induced all three chemokines in M102 but only the CXCR3 ligands in M134 and CXCL10 in M88. Dacarbazine induced all three chemokines in HTB-71. Dose-response relationships were observed for temozolomide and cisplatin (Figure 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 (MTIC), which relies on spontaneous catalytic reaction \textit{in vitro} while liver enzymatic activity carries out this step \textit{in vivo}. We therefore 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 100µg/ml temozolomide. Transcription of $CXCL10$ was detectable as soon as 24h after treatment, while $CXCL9$ and $CCL5$ transcription was only detected at 48h and 72h. Under these experimental conditions, cell death was not observed before 48h, 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 72h after treatment with temozolomide (100µg/ml) or cisplatin (10µg/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 Figure 4D, temozolomide induced a more than 9-fold increase in the secretion of five chemokines ($CCL2$, $CCL5$, $CXCL8$, $CXCL9$ and $CXCL10$) and one chemokine-like factor (MIF), while cisplatin induced CXCL8, CXCL10 and MIF. Importantly, none of the other factors were significantly induced (Table 1).

Altogether, these data demonstrate that three 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 intra-tumoral T cell infiltration by inducing chemokine expression in cutaneous tumors. Rag1\(^{-/-}\) mice bearing subcutaneous Melan-aet tumors were first treated with temozolomide and then adoptively transferred with \textit{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 Figure 5A, temozolomide treatment induced significant up-regulation of \textit{Ccl5, Cxcl9} and \textit{Cxcl10} (one-tailed t-test, \(p=0.01, 0.02, 0.0003, \) 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.

\textbf{Enhanced expression of CCL5, CXCL9 and CXCL10 after chemotherapy is associated with tumor control and superior survival of melanoma patients.}

We previously showed increased T cell infiltration in chemotherapy-sensitive tumors of melanoma patients treated with dacarbazine (19). In the previous study, we performed global transcriptome analysis of 33 cutaneous metastases resected before or after chemotherapy. We now re-analyzed these data, asking whether there were any chemokine whose expression correlated with T cell infiltration. Among all the genes coding for chemokines, \textit{CCL5, CXCL9} and \textit{CXCL10} were the most significantly correlated with \textit{CD3Z} and \textit{CD8A} expression, and among the most significantly correlated with \textit{CD4} (Figure 6A). We next compared \textit{CD4} and \textit{CD8A} expression in three categories of tumor samples: those that expressed low levels of the three chemokines, those that expressed high levels of \textit{CCL5} or high levels of CXCR3 ligands, and those that expressed high levels of \textit{CCL5} and high levels of at least one CXCR3 ligand. This comparison revealed a clear synergy between \textit{CCL5} and CXCR3 ligands for T cell infiltration (Figure 6B). To determine whether chemotherapy had any impact on chemokine expression, we
compared *CCL5*, *CXCL9* and *CXCL10* expression in tumors before and after therapy. As shown in Figure 6C, while dacarbazine did not induce any significant changes in chemotherapy-resistant tumors, i.e. progressing lesions, an increase in *CCL5*, *CXCL9* and *CXCL10* expression was observed after treatment in chemotherapy-sensitive tumors (one-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; Figure 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; Figure 6E).

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 the present 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.

While anti-tumor T cells are often detected in the blood of melanoma patients, 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 cancer patients undergoing therapeutic vaccination have enhanced anti-tumor 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 (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 \textit{ex vivo} by anti-melanoma T cells (17); moreover, CD8$^+$ T cells retain their functionality \textit{in vivo} and control visceral metastases (18). We now show that very few T cells infiltrate RETAAD skin tumors in comparison to the RETAAD visceral tumors or transplanted B16 skin tumors. The few T cells that infiltrate RETAAD skin tumors apparently retain their functionality since 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 melanoma patients, 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 melanoma patients treated by chemotherapy. In these patients, tumor control and prolonged survival are positively associated with increased T cell infiltration and intra-tumoral 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 et al. 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 (stage IIb-III) cutaneous melanoma (28-29). Based on our data, it is tempting to speculate that induction of CXCR3 ligands participates in the anti-melanoma activity of IFN-α. Expression of CXCL10 by melanoma patients’ PBMC 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 the present study suggests that cancer cells expressing CXCR3 might escape the immune response by competing for chemokines able to attract T cells. Expression of a non-functional allele of CCR5 results in decreased survival of melanoma patients 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 accounting for this synergy is unclear since 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 up-regulate CCR5. We propose that intra-tumoral 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 melanoma patients, but we did find that intra-tumoral expression of CXCR3 ligands and CCL5 in chemotherapy-treated tumors does lead to a synergistic increase in T cell infiltration.

The present 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, since out of 48 soluble factors analyzed only 5 chemokines and 1 chemokine-like factor were significantly induced by temozolomide; while only 2 chemokines and 1 chemokine-like factor were induced by cisplatin. Treatment of tumor-bearing mice with temozolomide also induced intra-tumoral chemokine expression and CXCR3-dependent T cell infiltration. Chemotherapies are known to alter the cancer cell transcriptome [reviewed in (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 et al 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 which favorably impacts immune cell trafficking to the tumor, the present study reveals a novel mechanism of action of common chemotherapeutic agents. Chemotherapy induces clinical responses in about 20% of melanoma patients (16). In our limited cohort, tumor regression or stabilization was observed in 6 out of 13 patients and 11 out 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, the present 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.
ACKNOWLEDGEMENTS

The authors thank Jo Keeble, Jeremy Wang and Muly Tham for help with animal studies, Benjamin Toh for help in 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 proof-reading the manuscript.
REFERENCES


FIGURE LEGENDS

Figure 1. Low T cell infiltration of RETAAD cutaneous metastases. (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, two-tailed.* (B) Immunofluorescence labeling of CD3 (pink) in RETAAD cutaneous (left panel) and reproductive tract (right panel) tumors. Scale bar=50µm. 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, two-tailed.* (C) Expression of T cell markers (Cd3g, Cd4, Cd8a) and effector molecules (Ifng, Prf1b, Gzmb) was measured in cutaneous (n=20) and visceral (n=10) tumors. *Unpaired t-test, two-tailed.* (D) Inverse correlation between the percentages of CD3+, CD4+ and CD8+ T cells and cutaneous tumor weight (n=12). *Pearson correlation, one-tailed.* Statistical significance between groups is represented by *p<0.05; **p<0.01; ***p<0.001. Cut – Cutaneous; Vis – Visceral.

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 (DAPI). *Mann-Whitney U test, two-tailed.* Statistical significance between groups is represented by **p<0.01. (B) Intra-tumoral 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 to autochthonous RETAAD skin tumors (n=5). Open squares represent chemokine genes with >2-fold differential expression and p<0.05. Horizontal line shows p=0.05. Vertical line represents 10-fold increase in B16 compared to 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 – grey filled histograms; Naïve (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.

Figure 3. Chemokine induces T cell infiltration in RETAAD cutaneous tumors and inhibits tumor growth. Cxcl9-encoding or control plasmids (5µg) were injected into established RETAAD cutaneous tumors three 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, one-tailed. (B) Relative expression of several effector molecules, i.e. Ifng, Gzma, and Gzmb in Cxcl9-treated (n=8) and control tumors (n=5). Unpaired t-test, one-tailed. (C) Percentage of dead tumor cells (CD45-DAPI+/CD45−) in Cxcl9-treated (n=5) and control (n=5) tumors. Mann-Whitney U test, one-tailed. (D to 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 2-3 days. Statistical analysis was carried out using two-way ANOVA, two-tailed. At necropsy on day 16, Cxcl9-transfected tumors (n=3) and control tumors (n=5) were collected and weighed. Mann-Whitney U test, one-tailed. (E) The expression of Cxcl9 and Cd3g was measured by qRT-PCR in Cxcl9-transfected and control tumors. Unpaired t-test,
Two-tailed. (F) Tumor growth curves and tumor weights of Cxcl9-transfected (n=5) and control (n=5) tumors in Rag1−/− mice. Two-way ANOVA, two-tailed and Mann-Whitney U test, one-tailed. (G-H) Ccl5 synergizes with Cxcl9 to recruit T cells. RETAAD cutaneous tumors were injected with 2.5µg 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’s multiple comparison post test. (H) Surface expression of CCR5 on CD4+ and CD8+ T cells in peripheral blood (n=6) and TIL (n=16). Mann-Whitney U test, one-tailed. All data are representative of three independent experiments performed. Statistical differences between groups are represented by ***p<0.001; **p<0.01; *p<0.05; ns=non-significant. C – Control plasmid; Tr – Tumors injected with the Cxcl9 plasmid; Tf – Melan-ret transfected with Cxcl9 plasmid.

Figure 4. Chemotherapeutic drugs induce chemokine expression in human melanoma cells in vitro. Temozolomide, cisplatin, dacarbazine were tested on five different human melanoma cell lines (HTB-71, M88, M102, M131, 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 (100µg/ml), cisplatin (10µg/ml), or dacarbazine (100µg/ml) for 72h. Data shows 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, 100µg/ml) or cisplatin (0, 1.3, 2.5, 5, 10µg/ml) for 72h and the expression of CCL5, CXCL9 and CXCL10 was measured by qRT-PCR. Comparisons were performed using two-way ANOVA. (C) Kinetics of CCL5, CXCL9 and CXCL10 expression in M102 cells at 6, 12, 24, 48, and 72h after temozolomide (100µg/ml) and cisplatin (10µg/ml) treatment. Comparisons were performed using
two-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 to control cells. Only cytokines/chemokines with ≥9-fold increase in secretion after drug treatment and p-value<0.05 are represented from temozolomide treatment group. Mann-Whitney U test, one-tailed.

Figure 5. Temozolomide induces intra-tumoral expression of chemokines and promotes T cell infiltration. Tumor-bearing Rag1−/− mice were treated intraperitoneally with 2mg temozolomide (TMZ; n=10) or DMSO (n=8) for 2 consecutive days and then injected i.v. with 10⁷ in vitro activated T cells. (A) Intra-tumoral expression of chemokines was analyzed by qRT-PCR. Unpaired t-test, one-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, one-tailed. Temozolomide enhanced intra-tumoral T cell infiltration. Anti-CXCR3 blocking antibody (TMZ + α-CXCR3) abolished chemotherapy-induced infiltration of T cells.

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, one-tailed with Bonferroni’s 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 – CCL5loo CXCR3 ligandslob; 1 – CCL5hi or CXCR3 ligandshi; 2/3 – CCL5hi and CXCR3 ligandshi. One-way ANOVA. (C and D) Intra-tumoral 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 one-tailed t-test on log-transformed expression values. (E) Kaplan-Meier analysis of patient survival with high or low intra-tumoral chemokine expression after chemotherapy. Statistical significance between groups is presented as *p<0.05; **p<0.01 and ***p<0.001.
Table 1

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<td>VEGF</td>
<td>36847 ± 6117</td>
<td>44477 ± 33965</td>
<td>0.83</td>
<td>0.35</td>
<td>16688 ± 5206</td>
<td>55394 ± 46310</td>
<td>0.3</td>
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Multiplex analysis of soluble factors secreted by M102 cell line after chemotherapeutic drug treatment. Human melanoma cell line M102 was cultured with temozolomide (100µg/ml) or cisplatin (10µg/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 to control cells. Results are representative of 3 independent samples analyzed. Secreted factors that are significantly upregulated after drug-treatment are indicated in bold. ND = Not Detected; NA = Not Available. Mann-Whiney U test, one-tailed.
Figure 5

A

CCL5

CXCL9

CXCL10

B

CD3+ T cells

% CD3+ T cells

CCL5 expression (log 2)

CXCL9 expression (log 2)

CXCL10 expression (log 2)

DMSO	TMZ

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Figure 6

(A) Gene expression (log2) vs. CD4 (log2) for CCL5, CXCL9, and CXCL10.

(B) CD4 and CD8A expression (log2) vs. number of factors.

(C) Box plots showing CCL5, CXCL9, and CXCL10 expression before (pre) and after (CT-R-CT-S) treatment.

(D) CD4 and CD8A expression (log2) after treatment.

(E) Percent survival for CC high and CC low groups with an HR of 31 and p = 0.0002.
Chemotherapy induces intratumoral expression of chemokines in cutaneous melanoma, favoring T cell infiltration and tumor control

Michelle Hong, Anne-Laure Puaux, Caleb Huang, et al.

Cancer Res  Published OnlineFirst September 26, 2011.