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Advances in Brief

CXC Chemokine Receptor 3 Expression by Activated CD8$^+$ T cells Is Associated with Survival in Melanoma Patients with Stage III Disease

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Abstract

Despite the presence of tumor Ag-specific CD8$^+$ T cells in the peripheral blood, metastatic melanoma often evades immune-mediated destruction. Even after therapeutic efforts to expand Ag-specific T-cell populations, the correlation between magnitude of response and clinical efficacy has been weak. Because the migratory phenotype of tumor Ag-specific effector T cells may determine their ability for tumor control, we hypothesized that the expression of CC or CXC chemokine receptor (CCR) molecules on activated CD8$^+$ T cells may define phenotypes associated with more effective control of melanoma progression and prolonged survival. In a retrospective evaluation of patient isolates, CCR expression was determined for activated CD8$^+$ T cells derived from the peripheral blood or tumor-involved lymph nodes of 52 patients with stage III or IV metastatic melanoma. In patients with stage III disease, expression of CXCR3 by CD8$^+$CD45RO$^+$ cells was significantly associated with enhanced survival. This was a stage-specific effect, because it was not observed in patients with stage IV disease. In addition, CCR4 and CXCR3 were highly coexpressed and associated with enhanced survival in stage III patients; however, CXCR3 seems to be the dominant receptor associated with clinical outcome. These findings support the hypothesis that the host immune system affects cancer progression and control, and that measures of CCR status of circulating lymphocytes may have prognostic value.

Introduction

T lymphocytes are believed to constitute the main body of effectors in active immune protection against cancer (1). Immunosurveillance of tumors has been shown experimentally in murine models (2), suggesting that newly arising neoplasias frequently induce tumor Ag-specific CD8$^+$ T lymphocytes that may successfully infiltrate and eradicate tumors. Patients with advancing melanoma may likewise develop CD8$^+$ T cell responses to multiple melanoma-associated Ags (3), and immunotherapeutic vaccines have been used to augment these melanoma Ag-specific immune responses (4). Paradoxically, many patients with detectable tumor Ag-specific T cells in their peripheral blood lymphocytes (PBL) continue to present with progressive metastatic melanoma (5, 6); and vaccine-mediated expansion of these antitumor T cell populations often fails to induce clinical tumor regression (7). These observations suggest that the existence alone of peripheral tumor-specific CD8$^+$ effector cells may be insufficient to mediate immunologic control of tumors. One possibility is that many of the activated T cells fail to home to sites of tumor metastasis. This model is supported by the common histologic finding that metastatic melanomas contain far fewer infiltrating lymphocytes than primary, early stage melanomas.

The ability of T cells to access tumor compartments requires that specific interactions take place between lymphocyte cell surface receptors and the selectins and integrins on the vascular epithelium, as well as between lymphocyte-expressed CC or CXC chemokine receptor (CCR) and soluble chemokines derived from the host tissues in proximity to the tumor or the tumor itself. Likewise, successful T cell-mediated antitumor immunotherapy may rely on an understanding of the relationship between lymphocyte CCR expression and clinical efficacy. To date, no study has characterized the role of CCRs on activated T cells in mediating the clinical control of tumors or enhancing patient survival.

We hypothesized that survival in patients with advanced metastatic melanoma may correlate with specific patterns of CCR expression on their activated tumor-specific T cells. We specifically evaluated both the gene expression and surface protein display of select CCRs (CCR1, CCR2, CCR4, CCR5, CCR7, CCR8, CCR11, CXCR3, CXCR4) on CD8$^+$ effector T cells generated from patients with stage III or IV melanoma, and then retrospectively used multivariate statistical analyses to correlate T-cell phenotype with clinical outcome (measured as duration of patient survival). The findings in this study reveal that differential CCR expression by activated tumor-specific CD8$^+$ T cells can be associated with divergent clinical outcomes, suggesting that CCR expression may serve as a biomarker of potential clinical responsiveness to immunotherapy.

Materials and Methods

Patients. Patients presented with advanced metastatic melanoma at the University of Virginia Health System. Primary lesions were, or had previously been, surgically resected with clear margins. Patients were studied with informed consent as approved by the University of Virginia Institutional Human Investigation Committee (HIC protocols 5202, 10598, and 10803). Studies complied with all applicable State and Federal laws and with the Helsinki Declaration. Laboratory screening of patient samples was done in a blinded manner. Twenty-nine of the 52 patients were immunized with HLA-A1, HLA-A2, or HLA-A3-restricted peptide antigens from melanocyte differentiation proteins tyrosinase or gp100, as described previously (4, 8). The remaining patients were conventionally treated.

T-cell Isolation and Expansion. Melanoma Ag-specific T cell lines were derived from PBL or tumor-involved lymph nodes (TIN) of unvaccinated and vaccinated patients, as described previously (4, 8). Regardless of source tissue, lymphocytes were restimulated *in vitro* initially with autologous melanoma cells; lines were subsequently expanded with autologous tumor, HLA-matched allogeneic melanoma cells, or synthetic peptides, as described previously (4, 9). The resultant T-cell lines showed reactivity specific for melanoma-derived epitopes in the context of the appropriate HLA molecule (data not shown).

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T-cell Purification and Separation. *In vitro* restimulated $CD8^+$ cells were enriched by negative immunomagnetic selection (Stem Cell Technologies, Vancouver, BC, Canada), and the resulting T-cell populations were verified to be >95% $CD8^+$ by flow cytometric analysis with antihuman CD8 monoclonal antibody (BD PharMingen, San Diego, CA). Immunomagnetic positive selection (Stem Cell Technologies) was used to enrich $CD8^+$ cells with activated phenotypes to obtain cells expressing CD45RO (BD PharMingen). Final cell preparations were >97% $CD8^+CD4RO^+$ (supplemental Fig. 1*A*).

Gene Expression Analysis. Total RNA from purified $CD8^+CD45RO^+$ cells was obtained with a spin column-based system (Qiagen, Valencia, CA), and RNA integrity and purity was confirmed by absorbance spectrometry and gel electrophoresis (data not shown). cDNA was generated using oligodeoxythymidylic acid primers and TAQ polymerase (Maxim Biotech, South San Francisco, CA) to reverse transcribe the mRNA. Chemokine receptor cDNA was amplified using gene-specific primers in multiplexed PCR reactions (Maxim Biotech; supplemental Fig. 1*B*) and visualized on 2% agarose gels. Gene expression was scored positive when the band for a particular CCR molecule (*a*) migrated at the appropriate molecular weight, as compared with 100 bp ladder, (*b*) migrated to a position consistent with product generated from control transcripts, and (*c*) had a banding intensity that was both 5-fold above background *and* >10% the level of glyceraldehyde-3-phosphate dehydrogenase expression in the same reaction by densitometry (NIHimage).

Flow Cytometric Analyses. Surface expression of CCR protein was assessed by flow cytometry after staining with specific fluorochrome-conjugated mAbs against CCR2 (clone 48607), CCR4 (clone 1G1), CCR7 (clone 3D12), CXCR3 (clone 1C6), and CXCR4 (clone 12G5, BD PharMingen), and CCR8 (clone 191704, RND Systems, Minneapolis, MN; supplemental Fig. 1*C*). Cells were co-stained with antibodies to CD8 and CD45RO (BD PharMingen). Data were captured with a Becton Dickinson FacsCalibur (Beckton Dickinson, Franklin Lakes, NJ). Surface protein expression was scored positive when the number of cells stained with a specific monoclonal antibody-fluorochrome was at least >5-fold background staining, when an isotype control monoclonal antibody was used.

Statistical Analyses. Clinical outcome was quantified as survival (months) post-recurrence of metastatic lesions in patients with resected primary disease. CCR expression was qualitatively scored, as described, with full concordance between gene expression and protein expression, unless noted. Cox proportional modeling was used to investigate whether CCRs expressed singly, or in combination, by activated $CD8^+$ T cells correlated with patient survival time. Kaplan-Meier plots were used to evaluate survival, and the χ^2 test was used to estimate significance.

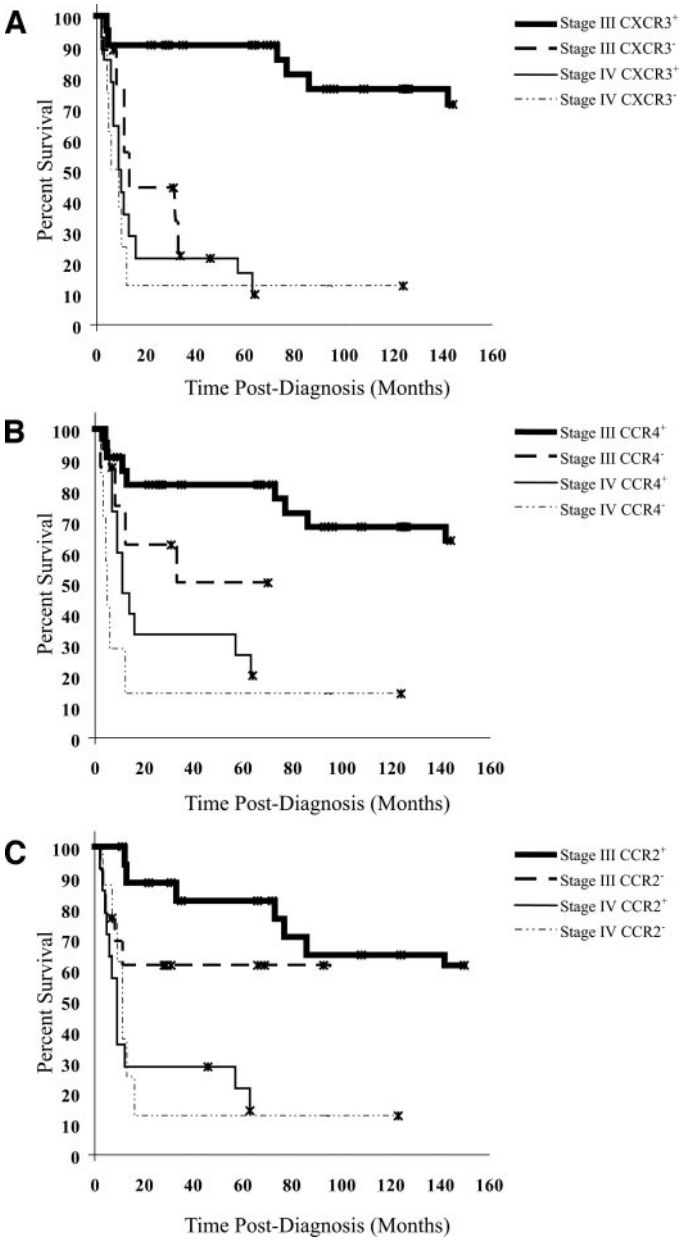

Fig. 1. Association between expression of CCRs by activated T cells and survival. *A*, survival in patients, associated with T cell expression of CXCR3. *B*, survival in patients, associated with T cell expression of CCR4. *C*, survival in patients, associated with T cell expression of CCR2. In all panels, $n = 52$, and * indicates patients surviving at the time of the study.

Results and Discussion

Modeling CCR Expression with Survival. Lymphocyte migration is regulated by a multipart, but coordinated, system of ligands and receptors that include selectins/selectin ligands, integrins/integrin receptors, and chemokines/CCRs. Collectively, these molecules direct T cells to sites of inflammation or disease, including tumors, by enabling lymphocytes to communicate with their vascular and endothelial surroundings. Of particular interest for the immune-mediated control of cancer are the chemokines and CCRs, which orchestrate T-cell tissue-specific homing (10). We hypothesized that effector T-cell CCR expression may contribute to differential clinical outcomes in patients harboring peripheral tumor-reactive lymphocytes. We therefore evaluated the expression of selected CCRs linked to migration and activation (CCR1, CCR2, CCR4, CCR5, CCR7, CCR8, CCR11, CXCR3, and CXCR4) by activated ($CD45RO^+$) tumor-specific $CD8^+$ T cells that were derived from PBL or TIN of 52 patients with metastatic melanoma (stage III or stage IV disease; Table 1) and then generated into short-term lines (hereafter referred to as "activated T cells"). For some patients, lines from both PBL and TIN tissue were evaluated; in both cases, T-cell CCR expression was consistent between sample types, regardless of tissue source. Furthermore, evaluation of primary and *in vitro*-expanded T cells from two patients suggested that short-term culture and expansion did not alter CCR expression (Table 1).

Evaluating the significance of single CCR markers, Cox proportionality modeling indicated an association between the expression of either CXCR3 or CCR4 and survival in patients when disease stage (stage III, $n = 30$; stage IV, $n = 22$) was applied as a stratification variable (Table 2). CCR2 and CCR8 were variably expressed and showed no significant predictive power in association with survival using disease stage as a stratifying variable (Table 2). CXCR4 was expressed by activated T cells in all of isolates evaluated, whereas CCR7 surface protein was absent from all samples; thus, these markers failed to provide discriminatory power and were not analyzed further. Although the study cohort consisted of both vaccinated ($n = 29$) and non-vaccinated ($n = 23$) patients, no single marker evaluated was found to be associated with survival using vaccine status as a stratification variable. Stratification by age, gender, or

Table 1 *Patient staging, vaccination status, T cell source, CCR gene and protein expression scoring of activated CD8$^+$ T cells, and patient survival time*

Study ID	Disease staging *	Vaccine	T-cell Source	CCR1 †	CCR2	CCR4	CCR5	CCR7 ‡	CCR8	CCR11	CXCR3	CXCR4	Survival (months) §
1	3	Yes	PBL/TIN ‖	+	−	+	+	−	+	−	+	+	*28.7*
2	3	Yes	PBL	+	+	−	+	−	+	−	−	+	*31.5*
3	3	Yes	PBL	+	−	−	−	−	+	−	−	+	*7.1*
4	3	Yes	PBL	+	−	+	+	−	+	−	+	+	*93.3*
5	3	Yes	PBL	+	−	+	+	−	+	−	+	+	*66.3*
6	3	Yes	PBL	+	+	+	+	−	+	−	+	+	*35.8*
7	3	Yes	PBL	+	+	+	+	−	−	−	+	+	*66.6*
8	3	Yes	PBL	+	+	+	+	−	+	−	+	+	*9.8*
9	3	Yes	PBL	+	+	+	+	−	+	−	+	+	*22.5*
10	3	Yes	PBL	+	+	+	+	−	+	−	+	+	*72.7*
11	3	No	PBL	+	+	+	+	−	+	−	+	+	*124.4*
12	3	No	PBL	+	−	−	−	−	+	−	+	+	*69.5*
13	3	No	PBL	+	+	+	+	−	+	−	+	+	*125.7*
14	3	No	PBL	+	+	+	+	−	+	−	+	+	*126.0*
15	3	No	PBL	+	+	+	−	−	−	−	+	+	*108.0*
16	3	No	PBL	+	−	+	+	−	+	−	+	+	*95.8*
17	3	Yes	PBL/TIN	+	−	+	+	−	+	−	+	+	*28.6*
18 **	3	Yes	PBL/TIN	+	−	−	+	−	+	−	−	+	1.1
19	3	Yes	PBL	+	+	+	−	−	+	−	+	+	73.7
20	3	Yes	PBL	+	+	+	−	−	−	−	−	+	13.7
21	3	Yes	PBL	+	−	+	+	−	+	−	+	+	3.8
22	3	Yes	PBL	+	−	+	+	−	+	−	−	+	11.4
23	3	Yes	PBL	+	−	−	−	−	+	+	−	+	31.5
24	3	No	PBL	+	+	+	+	−	+	−	+	+	142.5
25	3	No	TIN	+	+	−	+	−	+	−	−	+	33.7
26	3	No	TIN	+	−	+	+	−	−	−	+	+	4.8
27	3	Yes	PBL	−	+	+	−	−	+	+	+	+	77.9
28	3	Yes	PBL	+	+	−	+	−	−	−	−	+	11.8
29	3	Yes	PBL	+	+	+	+	−	+	−	+	+	86.7
30	3	No	PBL	+	−	−	−	−	+	−	−	+	7.8
31	4	Yes	PBL	+	+	+	+	−	+	−	+	+	*46.3*
32	4	No	TIN	−	−	−	+	−	+	−	−	+	*123.5*
33	4	Yes	TIN	+	+	+	+	−	+	−	+	+	7.4
34	4	Yes	PBL	+	+	−	+	−	+	−	+	+	3.5
35	4	Yes	PBL	+	−	+	+	−	+	−	+	+	2.8
36	4	Yes	PBL	+	−	+	+	−	+	−	+	+	11.0
37	4	Yes	PBL	+	−	+	+	−	−	−	−	+	10.7
38	4	Yes	PBL	+	+	−	+	−	+	+	−	+	4.0
39	4	No	PBL	+	−	+	+	−	+	−	+	+	7.4
40	4	No	PBL	+	+	−	+	−	+	+	−	+	5.9
41	4	No	PBL	+	+	+	+	−	+	−	+	+	63.0
42	4	No	TIN	+	−	+	+	−	+	−	+	+	9.0
43 **	4	No	PBL/TIN	+	+	+	+	−	+	−	+	+	57.1
44	4	No	PBL	+	+	−	−	−	+	−	−	+	2.0
45	4	No	PBL	+	−	+	+	−	+	−	+	+	13.9
46	4	No	PBL	+	−	+	+	−	+	−	+	+	16.4
47	4	No	PBL	+	+	+	+	−	+	−	+	+	9.8
48	4	No	PBL	+	+	+	+	−	+	−	−	+	9.4
49	4	Yes	PBL	−	+	−	+	−	−	−	−	+	12.0
50	4	Yes	PBL	+	+	+	+	−	+	−	+	+	9.0
51	4	No	PBL	+	+	+	+	−	+	−	+	+	6.4
52	4	No	PBL	−	+	−	+	−	−	−	−	+	5.7

* By the criteria of American Joint Committee on Cancer Staging (20). Patient cohort consisted of 29 males and 23 females, ranging in age from 32 to 79 years (mean 57.9 ± 15.4 years).

† Expression of gene product by RT-PCR and surface protein by flow cytometry, according to the scoring criteria in the Materials and Methods.

‡ CCR7 was the only marker in which gene expression and surface protein expression analyses yielded discordant results; in *all* samples evaluated for CCR7, gene expression was weakly positive whereas surface expression was negative.

§ Time after recurrence of metastatic lesions following resection of primary disease. Times indicated in italics are censored values for surviving patients at the time of data analysis.

‖ Where more than one tissue is indicated, T cells were generated from both, and the CCR expression profiles were identical in all cases.

** In the indicated patients, CCR gene expression by T cells sorted directly from isolated tissue samples (by immunomagnetic sorting based on binding of Ag-specific HLA-tetramers) were additionally evaluated, and CCR expression profile was identical to T cells from short-term lines generated from the same tissue.

Table 2 *Cox Proportional Modeling prediction of correlation between CCR expression and survival in patients with metastatic melanoma*

Receptor *	Likelihood ratio †	*P* value
CCR2 ‡	0.04	0.840
CCR4	3.54	0.051
CCR6	1.58	0.200
CCR8	0.84	0.340
CXCR3	5.31	0.019
CCR4 + CXCR3 §	5.99	0.013
CCR2 + CXCR3	4.20	0.048
CCR2 + CCR4	3.93	0.054

* Expressed on CD8$^+$CD45RO$^+$ cell line derived from patient PBL.

† Cox proportionality testing, using stage as a stratification variable.

‡ $n = 52$ with 1 degree of freedom for all evaluations of single receptor interactions.

§ $n = 52$ with 2 degrees of freedom for evaluation of double receptor interactions.

anatomic location of primary tumor likewise failed to show any association between CCR expression and survival (data not shown).

Correlation between Expression of Single CCRs and Survival. Kaplan-Meier survival curves were generated for each of the markers deemed to be potentially associated with survival (CCR4 and CXCR3), as well as one comparative marker with a low probability of correlation (CCR2; Fig. 1). Cox proportionality analysis predicted a strong correlation between CXCR3 and survival. Likewise, Kaplan-Meier analysis showed that CXCR3 expression correlated with a significant survival advantage in patients with stage III disease (χ^2 $P = 0.00005$) but failed to correlate with any advantage in stage IV patients ($P = 0.684$, Fig. 1*A*). Cox proportionality analysis also predicted a marginal correlation between CCR4 and survival (Table

2), and Kaplan-Meier analysis suggested that CCR4 expression also correlated with a statistically significant survival advantage in patients with stage III disease ($P = 0.0129$, Fig. 1*B*). Consistent with observations for CXCR3, CCR4 expression failed to correlate with any significant survival advantage in stage IV patients ($P = 0.446$, Fig. 1*B*). Expression of CCR2, which has been associated with migration of T_h1 cells to sites of inflammation (11), was not predicted to be significantly correlated with survival by Cox proportionality modeling. Kaplan-Meier analysis showed that CCR2 expression failed to correlate with enhanced survival (Fig. 1*C*) in patients with either stage III ($P = 0.154$) or stage IV ($P = 0.429$) disease.

Correlation between Expression of Multiple CCRs and Survival. The above data suggest that both CCR4 and CXCR3 expression may individually be associated with survival advantage. Because we performed these analyses using primary data derived from clinical isolates that simultaneously expressed multiple CCRs, we also examined whether multiple CCRs interacted in an additive or cross-cooperative manner to enhance survival. Cox modeling suggested that CCR4 and CXCR3 coexpression would associate with survival (Table 2), and Kaplan-Meier analysis showed a significant survival advantage in stage III patients from which $CCR4^+CXCR3^+$-activated T cells were obtained, as compared with other expression combinations ($CCR4^+CXCR3^-$, $CCR4^-CXCR3^+$, or $CCR4^-CXCR3^-$; $P = 0.00046$, Fig. 2*A*). However, survival in stage III patients with coexpression of CCR4 and CXCR3 (Fig. 2*A*) was not different from that of the single-marker CXCR3 (Fig. 1*A*) or CCR4 (Fig. 1*B*) cohorts, discounting the likelihood that CXCR3 and CCR4 act in an additive or coordinated manner to enhance survival. Further analysis of the CCR phenotype of activated T cells from melanoma patients revealed a highly correlated coexpression of CCR4 and CXCR3 (Pearson's correlation = 0.7326; an average of 88% of cells in $CD45RO^+CD8^+$ cell populations coexpressed CCR4 and CXCR3 by flow cytometry), suggesting that the apparent survival advantage associated with CCR4/CXCR3 coexpression may actually reflect the biological activity of only one of these markers.

To delineate the relative contributions of CCR4 and CXCR3, Kaplan-Meier analysis of these markers, in combination with CCR2, was performed. CCR2 was not significantly associated with survival, and expression of CCR2 was not tightly linked to CCR4 (Pearson's correlation = 0.005) or CXCR3 (Pearson's correlation = 0.0112). A survival advantage was indicated in stage III patients associated with the $CCR2^+CXCR3^+$-activated T cells ($P = 0.0142$ *versus other*, Fig. 2*B*). Because CCR2 is unlikely to impart any survival advantage (Fig. 1*C*) in this model, we conclude that the shift in survival is a result of CXCR3 expression. Kaplan-Meier analysis of CCR2 and CCR4 coexpression showed only a marginal association with survival in stage III patients ($P = 0.042$ *versus other*, Fig. 2*C*), discounting the contribution of CCR4 expression to enhanced survival. Furthermore, many of the $CCR2^+CCR4^+$ activated T cells also expressed CXCR3, suggesting that the modest increase in survival in this model may be attributed to the expression of CXCR3. Collectively, the single receptor that showed a significant association with patient survival in these analyses was CXCR3.

The CXCR3 ligands IP-10 (CXCL10; ref. 12), I-TAC (CXCL11; ref. 13), and Mig (CXCL9; ref. 14) are members of the CXC family that recruit activated T cells, and are all unified in their inducibility by IFN-γ. Previous studies have linked CXCR3 expression to T-cell migration and the induction of autoimmune or DTH reactions (15). Interestingly, the patient cohort for this study exhibited metastatic melanoma lesions, but a survival advantage was associated with the expression by activated T cells of a molecule associated with a skin-homing phenotype (16). However, CXCR3 involvement in T-cell

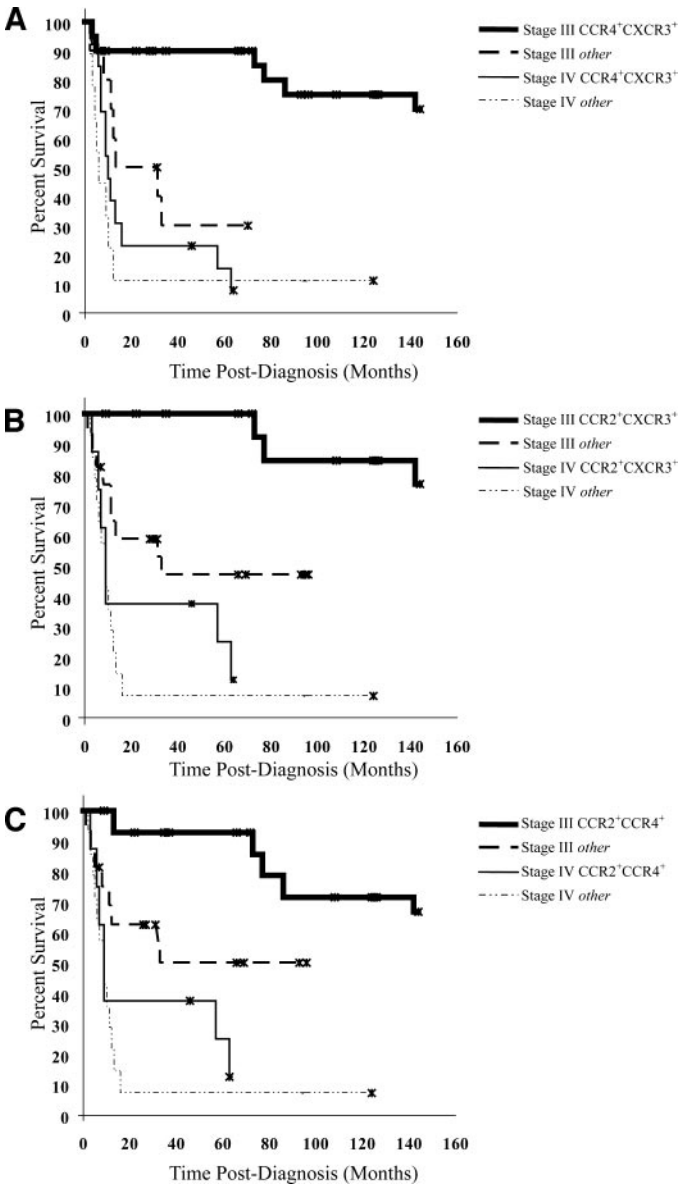

Fig. 2. Association between expression of two CCRs by activated T cells and survival. *A*, survival associated with T cells expressing CCR4 and CXCR3. *B*, survival associated with T cells expressing CCR2 and CXCR3. *C*, survival associated with T cells expressing CCR2 and CCR4. In all panels, $n = 52$, and * indicates patients surviving at the time of the study. *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase

access to non-dermal compartments is not without precedent. For example, blockade of CXCR3 activity prevented immune-mediated bronchial pathology during *bronchiolitis obliterans* syndrome (17), demonstrating a role for CXCR3 in the maintenance of immune responses in lung-associated tissues.

With regard to the present study, we propose several potential roles for CXCR3-expressing activated T cells in extending patient survival. First, metastatic lesions may induce CXCR3 ligands in the vascular microenvironment. Although melanoma cell lines from the patients in the study cohort did not produce measurable IP-10, I-TAC, or Mig protein[6], the tumors may express other cytokines and growth factors that, in turn, redirect chemokine expression by the adjacent vascular endothelial cells or infiltrating immune cells to chemoattract CXCR3-expressing T cells. Alternatively, these data may suggest a more fundamental process of T cell access to tumor compartments, medi-

[6] J. L. Oliver and D. W. Mullins, unpublished observations.

ated through IFN-γ. As a result of the inflammatory processes initiated by the tumor or tumor metastases, early infiltrating immune cells may provide sufficient IFN-γ to induce adjacent epithelial cell production of CXCR3 ligands. The success or failure of this process would likely depend on the ability of early innate responders, such as macrophages, to recruit helper T cells and initiate IFN-γ production in the tumor microenvironment. Finally, enhanced survival may be linked with CXCR3 expression on $CD8^+$ cells through their association with Th1-type $CD4^+$ helper cells, which are known to express CXCR3 (18). Induction and maintenance of a Th1-driven Ag-specific immune response is crucial for the eradication of tumors. Clearly, these potential mechanisms are not mutually exclusive, and animal modeling is under way in our laboratory to mechanistically define the role of CXCR3 in the control of tumor lesions and the association with survival advantage.

From a clinical perspective, two observations from the current study are worthy of further consideration in prospective studies. First, whereas expression of certain CCRs seem to associate with a survival advantage in patients with stage III disease, there was no detectable correlation between CCR expression and survival in patients with stage IV disease. Within the stage IV cohort, in which only 3 of 22 patients survived >5 years after diagnosis, no correlation was detected in CCR expression, even in these long-term survivors. These observations suggest that the host-tumor relationship is categorically different in stage III and stage IV patients, which is supported by other work on the Th1/Th2 balance in patients with differing stages of disease (19). The current study also evaluated patients who had received peptide-based antitumor immunotherapy, but no correlations between vaccination and CCR expression were detected. Therefore, we conclude that immunotherapeutic intervention, in the form of tumor-associated peptide Ags and cytokine support, is successful at expanding tumor Ag-specific $CD8^+$ effector cells and that prevaccination screening of CCR profiles may serve to predict patient responsiveness to immunotherapy. Additionally, new immunologic adjuvants or cytokines that selectively expand cells with CXCR3 expression may be of particular value for improvement of immune therapy strategies.

Collectively, in a retrospective evaluation of patient isolates and outcomes data, we observed that expression of CXCR3 by the activated T cells is strongly associated with survival advantage in patients with stage III disease. This is the first report to show an association between the expression of CXCR3 by $CD45RO^+CD8^+$ T cells and enhanced clinical outcome in patients with metastatic melanoma, suggesting that T-cell activation status and/or migration phenotype is intrinsically linked with immune-mediated antitumor efficacy. Although we cannot discount the potential contributions from other T-cell–expressed molecules (including those with expression that may be tightly linked with CXCR3), these data strongly argue for the central importance of CXCR3 in maintenance of effective antitumor immunity.

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