Maximal T Cell–Mediated Antitumor Responses Rely upon CCR5 Expression in Both CD4⁺ and CD8⁺ T Cells

Alicia González-Martín¹, Lucio Gómez², Joseph Lustgarten²,†, Emilia Mira¹, and Santos Mañes¹

Abstract

Immune responses against cancer rely upon leukocyte trafficking patterns that are coordinated by chemokines. CCR5, the receptor for chemotactic chemokines MIP1alpha, MIP1beta, and RANTES (CCL3, CCL4, CCL5), exerts major regulatory effects on CD4⁺- and CD8⁺ T cell-mediated immunity. Although CCR5 and its ligands participate in the response to various pathogens, its relevance to tumoral immune control has been debated. Here, we report that CCR5 has a specific, ligand-dependent role in optimizing antitumor responses. In adoptive transfer studies, efficient tumor rejection required CCR5 expression by both CD4⁺ and CD8⁺ T cells. CCR5 activation in CD4⁺ cells resulted in CD40L upregulation, leading to full maturation of antigen-presenting cells and enhanced CD8⁺ T-cell crosspriming and tumor infiltration. CCR5 reduced chemical-induced fibrosarcoma incidence and growth, but did not affect the onset or progression of spontaneous breast cancers in tolerogenic Tg(MMTV-neu) mice. However, CCR5 was required for TLR9-mediated reactivation of antineu responses in these mice. Our results indicate that CCR5 boosts T-cell responses to tumors by modulating helper-dependent CD8⁺ T-cell activation. Cancer Res; 71(16): 1–12. ©2011 AACR.

Introduction

For decades, immunologists have tried to exploit the specificity of the immune system to discriminate between normal and malignant tissue for cancer therapy. Success with certain immunotherapeutic approaches shows that the immune system can restrict the onset and/or progression of some malignancies (1–3). Complete, durable antitumor responses are nonetheless rare, due largely to the induction of a regulatory environment in which immune tolerance dominates over activation (4). The challenge for immunotherapy is thus to identify cell networks and molecular factors that enhance the expansion and activation of tumor-reactive effector T cells.

Immune responses are multistep processes that entail antigen presentation, optimal triggering of specific T cells, and localization of immune effectors to appropriate sites. Completion of these steps requires intricate leukocyte trafficking patterns coordinated by chemokines and their receptors (5). CCR5 and its ligands CCL3, CCL4, and CCL5 have emerged as key regulators of T-cell function. CCR5 is a central element in modulating helper-dependent CD8⁺ T-cell responses, by guiding these cells to productive CD4⁺/antigen-presenting cell (APC) complexes (6). CCL5 produced by tumor-infiltrating CD4⁺ T cells is also involved in recruiting APC to the tumor site where, after CD40-mediated activation, they become competent to crosspresent and trigger CD8⁺ T cells in the tumor parenchyma (7). In vitro studies indicated that CCR5 might also have migration-independent effects on T-cell activation, by participating in the costimulation of CD4⁺ lymphocytes in cooperation with the TCR (8–10). It remains unclear whether CCR5 effects on T-cell function are relevant in physiopathologic situations, or are restricted to certain conditions of immune activation. Moreover, to our knowledge, no studies have addressed the precise role of CCR5 in vivo. CD4⁺ T-cell function. For full comprehension of the role of CCR5 in T-cell-mediated responses, it is particularly important to determine whether its expression in CD4⁺ lymphocytes is necessary for optimal dendritic cell (DC) activation and antigen crosspresentation.

Although CCR5 participates in the response to various pathogens in mice and humans (11, 12), the relevance of this receptor and its ligands in the immune control of tumors is debated. Elevated serum CCL5 levels are associated with breast and cervical cancer progression (13). Evidence also implicates CCR5 in the induction of proliferation, metastasis, and angiogenesis (14, 15), supporting its protumorigenic role. Some of these activities have been linked to the ability of CCR5 to recruit macrophages and other immune suppressor cells to the tumor site, creating an inflammatory environment that promotes tumor progression and immune evasion (16–19). In contrast, increased levels of CCR5 ligands improved antitumor responses in mice (20–25) and in humans (26). Furthermore,
the ccr5Δ32 polymorphism, which causes defective CCR5 expression in humans, is associated with poor prognosis in colorectal (27) and breast (28) cancers, and with reduced efficacy of immunotherapy in melanoma (29).

Here, we provide genetic evidence for a CCR5 requirement to maximize the immune response to tumors. Our data show a previously unreported role for CCR5 in optimizing CD4⁺ T-cell help in vivo, with clinical implications in the design of therapeutic intervention for cancer patients.

Materials and Methods

**Mice**

C57BL/6J wild-type (WT), CCR5⁻/⁻, CCR1⁻/⁻, PL-Thy1a/CyJ (Thy1.1), Tg(TcraTcrb)425Cbn/J (OT-II), and FVB/N-Tg(MMTVneu)202Mul/J (MMTV-neu) mice were from The Jackson Laboratory and C57BL/6-Rag2⁻/⁻ mice from Taconic. OT-I and OT-II mice were crossed with CCR5⁻/⁻ mice and bred to homozygosis for CCR5. CCR5⁺/⁺ mice were crossed with mouse mammary tumor virus (MMTV)-neu mice to generate MMTV-neu-CCR5/crossbacked for 10 generations on the FVB background [≤99.6% FVB, determined by speed congenics analysis by using 220 DNA microsatellite markers (Bionostra)] maintaining CCR5 in heterozygosis. Live animal experiments were supervised by the Centro Nacional de Biotecnología Ethics Committee according to national and European Union guidelines.

**Tumor cell lines**

The C57BL/6 syngeneic tumor cell lines EG7, LLC [both from the American Type Culture Collection (ATCC)] and Panc02 were cultured in RPMI or Dulbecco’s modified Eagle’s medium with 10% FBS, 2 mmol/L L-glutamine and antibiotics; β-mercaptoethanol (50 μmol/L) was added to EG7 cultures. Panc02-luc cells were generated by transduction with pR-IRE5-LUC retroviruses. Firefly luciferase was amplified from the pGL3-basic vector (Promega) using 5'-CGATTATTAAATCCACC-ATGGAGACGCC-3' and 5'-GCGGCGGCGCTTACACGGGCATCTCCG-3', and subcloned in Sspl/NotI-digested pRV-IRE5 (Genetrix). Retroviruses were generated by cotransfection of 293T cells with pR-IRE5-LUC, pM-LAG-POL, and pSVG-G plasmids (Genetrix) and used to infect Panc02 in the presence of polybrene (4 μg/μL). Transduced cells were subcloned by limiting dilution and selected by Luc expression.

LLC-CCL5 cells were obtained by transduction with retroviruses generated by cloning CCL5 CDNA into BamHI/Xhol-digested pR-IRE5-LUC plasmid. Short hairpin RNA (shRNA) for murine CCL5, shRNA-CCL5 pSM2026-2-C (clone V2MM_73846, Open Biosystems), was packaged in retroviruses and used to generate LLC-si-CCL5 cells. Stable LLC-CCL5 cells were obtained by limiting dilution, and LLC-si-CCL5 cells were selected with puromycin (2 μg/mL).

Concentrations of endogenous or ectopically expressed CCL3, CCL4, and CCL5 were determined by ELISA (R&D; Biosource).

**Quantitative RT-PCR analyses**

cDNA was synthesized by reverse transcription (Promega) of total RNA (1 μg). Quantitative reverse transcriptase PCR (qRT-PCR) reactions were done with the Power SYBR Green PCR Master Mix System (Applied Biosystems) with specific primers for mouse CCR1, CCR3, and CCR5 using β-actin to normalize data.

**Tumor induction**

Syngeneic tumors were induced by injection of Panc02-Luc (5 x 10⁶), LLC sublines (5 x 10⁷), or EG7 (5 x 10⁶) cells in indicated hosts. For chemical carcinogenesis, mice were inoculated (s.c.) with a single dose of 3-methylcholanthrene (MCA; 100 μg Sigma) dissolved in corn oil (Sigma). Breast tumors in MMTV-neu mice were detected by weekly palpation.

In all cases, tumors were measured periodically with calipers, and volume calculated (length x width²/2). Tumor growth was analyzed by bioluminescence after inoculation (intraperitoneally) of mice with β-luciferin (15 mg/g) images were recorded with a DCC camera (Hamamatsu) and signal emission quantified with Wasabi software. At the endpoint, tumors were disaggregated to obtain single cell suspensions and analyzed for immune cell infiltration by staining with anti-CD4, -CD45, -Gr1 (Beckman Coulter), -CD11c, -CD19, -Mac3, -NK1.1, -CD3, -CD8, and -CD11b (eBioscience) for FACS (FACS Calibur, Beckton Dickinson).

**Adoptive transfer experiments**

WT and CCR5-deficient OT-I and OT-II cells were purified by negative selection [Dynal Mouse CD8 and CD4 Negative Isolation Kits (Invitrogen), respectively]. Combinations of these cells (5 x 10⁶ each) were adoptively transferred into CCR5⁻/⁻ or Thy1.1 mice bearing EG7 tumors [a thymic lymphoma stably expressing ovalbumin (OVA) protein; ATCC] generated 3 days earlier by s.c. inoculation (5 x 10⁶ cells) in the right flank. Tumor volume was calculated as above.

**Homing assays**

Thy1.1 mice bearing EG7 tumors received OT-I and OT-II cells by adoptive transfer. After 4 and 7 days, cell suspensions from tumors, draining lymph nodes (dLN), and non-dLN were stained with anti-Thy1.2 (clone 53-2.1), -Vα₂ (B20.1), -Vβ₅-biotin (MR9-4; BD-Pharimingen), and -CD8 (53-6.7) or -CD4 (RM4-5; eBioscience), followed by streptavidin-APC (Beckman Coulter), and were analyzed by FACS (BD LSRII System; Becton Dickinson). OT-I and OT-II cells were identified as Thy1.2⁺Vα₂⁻Vβ₅⁺CD8⁻ and Thy1.2⁺Vα₂⁺Vβ₅⁻CD4⁺.

**Activation assays**

For in vivo assays, Thy1.1 mice bearing EG7 tumors received OT-I-OT-II cells. After 6 days, cell suspensions from tumors and dLN were assayed for IFNγ production. Tumor lymphocytes were partially purified by Ficoll-Hypaque from a pool of 4 tumors per condition. Lymphocytes (5 x 10⁶) from each compartment were cocultured (5 hours, 37°C) with OVA (257-264) + OVA (323-339) and 10 μg/mL, respectively)-pulsed Thy1.1-splenocytes (2 x 10⁶; 1 hour, 37°C) in the presence of brefeldin A (Sigma). IFNγ production in OT-I cells was determined by fluorescence-activated cell sorting (FACS); cells were...
stained with anti-Thy1.2-FITC (53-2.1) and -CD8-PE (53-5.8; BD-Pharminingen); then permeabilized (Cytofix/Cytoperm kit; BD-Pharminingen) and stained with anti-IFNγ (XMG1.2; BD-Pharminingen).

For CD69 determination, dLN cell suspensions were stained with anti-Vα2 (B20.1), -CD4 (RM4-5), -Vβ5-biotin (MR9-4; BD-Pharminingen), -CD69 (HI.2-F3), and -CD8 (53-6.7; eBioscience), followed by streptavidin-APC and analyzed by FACS (BD LSRII).

For CD40L determination, bone marrow–derived dendritic cells (BMDC) were prepared from bone marrow cells cultured with murine granulocyte-macrophage colony-stimulating factor (15 ng/mL) and IL-4 (10 ng/mL); at day 7 these cells expressed CD11c, CD80, CD86, and MHC-II, as determined by FACS (not shown). BMDC were pulsed with OVA (323–339: 10 μg/mL) or medium (unpulsed) in the presence of LPS (20 ng/mL; 1 hour, 37°C), and then cocultured (48 hours) with purified OT-II WT or OT-II KO cells. CD40 and CD40L were detected by staining with anti-CD40 (3/23), -CD11c (RM4-5; eBioscience), followed by streptavidin-eFluor450 (eBioscience). CD11c+ cells were stained with anti-I-Ab/E (2G9; MHC-II), -CD80 (16-10A1), -CD86-biotin (GL1; BD-Pharminingen) and -CD11c (N418; eBioscience), followed by streptavidin-eFluor450. IFNγ production in OT-II cells was determined by staining with anti-CD3 (145-2C11), -CD4 (GK1.5; Beckman Coulter) and -IFNγ (XMG1.2; BD-Pharminingen) in brefeldin A-treated cells. Samples were analyzed by FACS (GALLIOS; Beckman Coulter).

For in vitro assays, OT-I and OT-II cells (2 × 10⁵ each) were cocultured (20 hours, 37°C) with OVA peptide-pulsed splenocytes (10⁵) as above; brefeldin A was added for the last 5 hours of incubation. IFNγ production in OT-I cells was assessed by FACS as above.

**Immunization assays**

CCR5−/− mice received adoptive transfers of OT-I and OT-II cells (3 × 10⁶ each); after 24 hours, they were immunized by s. c. injection in the hind foot with alum mixed with OVA (323–339; 10 μg) and CpG (GCTAGACGTTAGGT and TCAACGTTGA; 20 μg total). OT-I cells were labeled with CFSE (5 μmol/L, 10 minutes, 37°C; Invitrogen) prior to transfer. OT-I cells in the dLN and the contralateral popliteal LN were quantified by FACS after 40 hours.

**Chemokine production in dLN**

CCR5−/− mice transferred with OT-II WT and OT-II KO cells (3 × 10⁶) were immunized with OVA (323–339) peptide as above. dLN were extracted after 30 hours and incubated (12 hours) in RPMI medium (50 μL) containing 10% FCS, CCL3, CCL4, and CCL5 in conditioned media were quantified by ELISA (R&D Systems).

**CpG treatment of tumors in Tg-neu mice**

Spontaneous breast tumors in MMTV-neu and MMTV-neu-CCR5−/− mice were detected by palpation. Tumor-free mice were inoculated s.c. in the right flank with 5 × 10⁵ N202.1A cells (30). These cells express high Her-2/neu levels and form tumors histologically similar to spontaneous tumors in MMTV-neu but not in conventional FVB mice. Intratumor injections of CpG (ODN-1826, InvivoGen; 30 μg/mouse, 3 times/week) were initiated 16 days after tumor inoculation (tumor volume ~100 mm³); tumor volume was measured twice weekly. After sacrifice of mice, cell suspensions from tumors and dLN were stained for neu-specific T cells with H2Dβ/-neu (420-129) tetramer (National Institute of Allergy and Infectious Diseases, Tetramer Core Facility, Emory University, Atlanta, GA). Cells from tumors and dLN were stained with anti-CD4 (RM4-5; BD-Pharminingen), -FoxP3 (FJK-16s), and -CD8 (53-6.7; eBioscience) to detect regulatory T (Treg) cells. Splenocytes (5 × 10⁶) were restimulated ex vivo (22 hours) with rat-neu (420–429) peptide (10 μg/mL), and secreted IFNγ was quantified by Luminex (Biosource).

**Statistical analysis**

Comparison of 2 data groups was done with the 2-tailed Student’s t test, using logit transformation for percentages. Dunnett or Kruskal–Wallis tests were used for multiple comparisons. The log-rank test was used to compare Kaplan–Meier curves.

**Results**

**CCR5 expression in the host delays tumor growth**

We determined the growth of s.c. grafted tumors in syngeneic, immunocompetent WT, and CCR5−/− mice. To avoid possible cell-autonomous effects, we used the Lewis lung adenocarcinoma (LLC) and a luciferase-expressing variant of the pancreatic adenocarcinoma Panc02 (Panc02-Luc), as no mRNA encoding CCR5 was detected in these cell lines (Supplementary Fig. S1A). LLC and Panc02-Luc cells expressed high levels of CCR1, which shares binding with CCR5 to CCL3 and CCL5, and marginal levels of CCR3, which also binds CCL5 (Supplementary Fig. S1A). LLC produced low levels of CCL5, whereas Panc02-Luc cells produced CCL4 and CCL5 (Supplementary Fig. S1B).

Tumors formed by LLC cells grew more rapidly (Fig. 1A) and were ultimately larger (Fig. 1B) in CCR5−/− than in WT recipients. These CCR5-dependent differences were exacerbated for tumors induced with Panc02-Luc cells (Fig. 1C). Reduced Panc02-Luc tumor growth in WT mice was confirmed by bioluminescence (Fig. 1D and E). These results indicate that host CCR5 expression restricts tumor growth.

The differences in tumor growth were not associated with changes in the frequency of proliferating (phospho-histone H3+) or apoptotic (TUNEL−) cells between WT and CCR5−/− hosts (not shown). There were nonetheless statistically significant changes in the pattern of leukocytes infiltrating Panc02-Luc–derived tumors. CCR5 is expressed in CD4+ and CD8+ lymphocytes, NK and NKT cells, monocytes, macrophages, and immature DC. FACS analyses showed greater infiltration by DC and T cells, particularly CD8+ cells, in tumors growing in WT than in CCR5−/− hosts (Fig. 1F). Infiltrating myeloid cells showed the opposite pattern. We
detected no CCR5-dependent changes in natural T regulatory (Treg) cells infiltrating LLC or Panc02-Luc tumors (Fig. 1G). NK cells could not be identified reliably in these tumors. CCR5, but not CCR1, restricts tumor growth in an agonist-dependent manner in immunocompetent hosts

Host-associated differences in LLC- and Panc02-Luc tumor growth might be explained by the distinct CCR5 agonist levels in these cell lines (Supplementary Fig. S1B). We generated LLC cell lines in which CCL5 secretion was enhanced approximately 20-fold (LLC-CCL5) or decreased approximately 4-fold (LLC-siCCL5) compared with the parental cell line (LLC-mock; Supplementary Fig. S2). LLC-mock, -siCCL5, and -CCL5 cells proliferated in vitro at similar rates (not shown). LLC-siCCL5 formed larger tumors than LLC-mock cells, whereas LLC-CCL5 cells formed the smallest tumors when injected into WT but not into CCR5−/− mice (Fig. 2A). We found no changes in the frequency of proliferating or apoptotic tumor cells, or in blood vessel number and diameter as a result of CCL5 overexpression or silencing (Supplementary Fig. S3).

Because CCL5 is also a CCR1 agonist, we analyzed the growth of tumors formed by these LLC sublines in CCR1−/− mice. LLC-siCCL5 cells again formed the largest and LLC-CCL5 cells formed the smallest tumors in CCR1−/− recipients (Fig. 2B). There were no differences in the final volume of tumors formed by LLC-mock, -CCL5, or -siCCL5 cells inoculated in parallel in WT and CCR1−/− mice (Fig. 2C). The results indicate that CCR5 specifically restricts tumor growth in an agonist-dependent manner.

Because CCR5 expression influences T-cell infiltration into Panc02-Luc tumors (Fig. 1F), we tested whether CCL5...
we used the EG7 thymoma, which does not express CCR5 mRNA (not shown), but secretes the CCR5 ligands CCL3 (~120 pg/mL/24 hours) and CCL4 (~15 pg/mL/24 hours). Initial experiments showed more rapid EG7 tumor growth in CCR5−/− than in WT mice (Supplementary Fig. S4), suggesting a CCR5-mediated polyclonal response in WT mice. Because polyclonal CD8+ T cells enhance the response of antigen (Ag)-specific cytotoxic T lymphocytes (CTL), a CCR5-dependent phenomenon termed CD8+ T-cell help (31), we used CCR5−/− mice as tumor hosts in subsequent experiments.

Adoptive transfer of single WT (OT-IWT or OT-IIWT) or CCR5-deficient CD4+ (OT-IKO) or CD8+ (OT-IKO) T cells poorly restricted growth of EG7-induced tumors (Supplementary Fig. S5A and B). Optimal tumoricidal activity usually involves cooperation between CD4+ and CD8+ T cells (32); this cooperation was evident in mice cojected with OT-IWT plus OT-IIWT cells, which led to greater restriction of tumor growth than in mice cojected with OT-IWT+OT-IIKO mixtures (Fig. 3A). Tumor rejection was complete in 9/13 OT-IWT+OT-IIWT cell recipients but in only 2/15 OT-IKO+OT-IIKO recipients. These results suggest CCR5-mediated, CD4+/CD8+ cooperation for optimal T cell-mediated elimination of EG7 tumors.

CCR5 enhances tumor-specific CD8+ T lymphocyte infiltration and effector function

To determine the effect of CCR5 expression on T-cell infiltration into the tumor parenchyma, we injected OT-IWT+OT-IIWT or OT-IKO+OT-IIKO cell mixtures into EG7-bearing mice, and used FACS to identify transferred cells infiltrating the tumor days 4 and 7. At day 4, transferred OT-I or OT-II cells were undetectable. At day 7, we observed an increase in tumor-infiltrating OT-IWT versus OT-IKO cells (Fig. 3B); OT-IIWT and OT-IIKO cells were undetectable in tumors. There was no difference in OT-I and OT-II cell numbers in dLN (Supplementary Fig. S6) at day 7, indicating that CCR5 does not affect T-cell trafficking to LN.

The activation state of CD8+ T cells, which can be monitored by IFNγ production, influences their infiltration into tumors (33, 34). The percentage of IFNγ-producing OT-I cells and mean fluorescence intensity (MFI) of IFNγ staining were increased in tumors (Fig. 3C and D) and in dLN (Fig. 3E and F) from OT-IWT+OT-IIWT compared with OT-IKO+OT-IIKO cell recipients. OT-IWT cells also tended to express higher levels of the CD69 activation marker than OT-IKO cells (Fig. 3G), suggesting that CCR5 expression in CD8+ T cells is needed for maximal antitumor activity

CCR5 expression in CD4+ T cells is needed for maximal CD8+ T-cell tumoricidal activity

CD4+/CD8+ T-cell help requires CCR5 expression by CD8+ T cells (6). To test whether this cooperation also requires CCR5 expression in CD4+ cells, we analyzed the efficiency of EG7 tumor rejection following OT-IWT+OT-IIWT or OT-IKO+OT-IIWT cell transfer. Lack of CCR5 expression in either OT-I or OT-II cells resulted in inefficient restriction of tumor growth compared with control OT-IWT+OT-IIWT cell recipients (Fig. 4A). Growth kinetics correlated with the number of

overexpression restricted tumor growth in RAG2−/− mice, which lack mature B and T lymphocytes. Growth kinetics was similar for LLC-mock, -CCL5, and -siCCL5 tumors induced in RAG2−/− mice (Fig. 2D). These findings implicate the adaptive immune system as a mechanism by which CCR5 restricts tumor growth.

CCR5 expression in T cells is necessary for optimal antitumor responses

To determine how CCR5 regulates T-cell antitumor responses, we crossed CCR5−/− mice with OVA-specific OT-I (CD8+) or OT-II (CD4+) transgenic mice. As tumor cells,
tumor-infiltrating OT-I cells, which was maximal in OT-IWT+OT-IIWT cell recipients (Fig. 4B). These data suggested that CCR5 expression in OT-II cells regulates OT-I cell trafficking into the tumor mass.

To test whether CCR5 expression in OT-II cells influenced OT-I cell activation, we stained CTL isolated from a pool of 4 EG7 tumors from OT-IWT+OT-IIWT or OT-IWT+OT-IKO cell recipients with anti-IFNg antibodies. The percentage of IFNg+OT-I cells and MFI values were increased in tumors from OT-IWT+OT-IIWT compared with OT-IWT+OT-IKO cell recipients (Fig. 4C). OT-I cell activation was likewise higher in dLN from OT-IIWT than from OT-IIKO cell-transferred mice (Fig. 4D and E). CCR5 expression on CD4+ cells could thus be an important factor for CD4+ T-cell help in CD8+ T-cell activation.

**CCR5 enhances CD40L levels in antigen-stimulated CD4+ T cells and APC maturation**

Ag-specific interaction of CD4+ cells with APC in LN induces secretion of the proinflammatory chemokines CCL2, CCL3, and CCL4 (6, 35). CCL3 and CCL4 produced by CD4+ T cells and APC attract CD8+ T cells to productive crosspriming (6). We observed CCL3, CCL4, and CCL5 production in dLN of OT-IIWT or OT-IIKO cell-transferred mice immunized in the hind foot with the OVA (323–339) peptide, the cognate antigen (Ag for OT-II cells). CCL3 and CCL5 levels were significantly higher in dLN from OT-IIWT compared with OT-IIKO cell recipients (Fig. 5A); in vitro analyses showed that these chemokines were produced by APC and CD4+ T cells (Supplementary Fig. S7). Despite the increase in chemokine production, there was no difference in OT-I cell recruitment in dLN from OVA (323–339)-immunized OT-IWT+OT-IIKO or OT-IKO+OT-IIWT cell recipients compared with controls (Fig. 5B); there was nevertheless a significant reduction in CD8+ T-cell recruitment to dLN from OT-IKO+OT-IIKO cell-transferred mice (Fig. 5B). These findings confirm a role for CCR5 in active CD8+ T-cell recruitment to dLN, although CCR5 dependency was apparent only when the receptor was absent in both CD4+ and CD8+ T cells.

The CD4+ T-cell help mechanism for CD8+ T-cell activation also involves APC conditioning via binding of CD40 on the APC to CD40L on the CD4+ T cell (34). CD40L is upregulated by TCR engagement, which also induces autocrine secretion of CCR5 ligands (Refs. 8, 9; and not shown). We found that CD40L levels were higher in WT than in CCR5-deficient CD4+ T cells following anti-CD3-mediated activation (Fig. 5C). CD40L upregulation was also higher in OT-IIWT than in OT-IIKO cells cocultured with OVA (323–339)-loaded BMDC (Fig. 5D); as predicted (8), CCR5 expression also enhanced...
OT-II activation, as determined by IFNγ production (Fig. 5E). Direct CCL4 stimulation of CD4⁺ T cells nonetheless did not induce CD40L expression (not shown), suggesting that CCR5 acts as a costimulator for TCR-mediated CD40L induction.

We tested whether increased CD40L levels in CCR5-expressing CD4⁺ T cells affect DC maturation and subsequent CD8⁺ T-cell activation. Levels of MHC class II (Fig. 5F), CD80 (Fig. 5G), and CD86 markers (Fig. 5H) were higher in BMDC cocultured with OT-IWT than with OT-IIKO cells; CD40 expression on the BMDC was similar and independent of the CCR5 genotype of CD4⁺ T cells (Supplementary Fig. S8).

Finally, we analyzed whether CCR5 expression on CD4⁺ T cells affects CD8⁺ T-cell crosspriming. APC were incubated with class I- and class II-restricted OVA peptides and then cocultured with OT-IIWT than with OT-IIKO cells; IFNγ production was significantly increased by OT-I cells when cocultured with OT-IIWT-APC compared with APC-OT-IIKO cell complexes (Fig. 5I). The results suggest that CCR5 expression in CD4⁺ T cells improves APC maturation and subsequent CD8⁺ T-cell activation.

**CCR5 deficiency accelerates the onset of some primary tumors in the mouse**

The evidence from this study with transplantable tumors suggests that CCR5 plays a role in the immune control of tumor outgrowth. We next analyzed whether CCR5 affects MCA-induced fibrosarcoma, used to study innate and adaptive immune surveillance in cancer (36). Sarcoma incidence was higher in CCR5⁻/⁻ mice than in WT mice (Fig. 6A); the median time for tumor detection in 50% of the animals was 22.5 and 15 weeks for WT and CCR5⁻/⁻ mice, respectively. Fibrosarcomas also tended to grow more rapidly in CCR5⁻/⁻ mice than in WT mice (Fig. 6B).

To evaluate whether CCR5 affects the onset of other primary tumors, we crossed CCR5⁻/⁻ mice on the FVB/N background with transgenic FVB/N-Tg(MMTV-neu)202Mul/J (MMTV-neu) mice, which overexpress the rat neu protooncogene in mammary tissue and develop spontaneous breast tumors (37). We observed no differences in tumor incidence (median time: 268 and 276 days for MMTV-neu and MMTV-neu-CCR5⁻/⁻ mice, respectively; Fig. 6C), in tumor progression as determined by the growth kinetics slope (Fig. 6D), or in final tumor weight (Fig. 6E). The results concur with the lack of association between the ccr5D32 polymorphism, which renders a nonfunctional CCR5 receptor, and the incidence of breast cancer in humans (28).

**CCR5 in Antitumor Immunity**
spontaneous tumors induce suppression of tumor-experienced T cells by central and/or peripheral tolerance mechanisms (38). There is nonetheless a residual low avidity, tumor-associated antigen (TAA)-specific T cell repertoire that, when stimulated, can elicit an effective antitumor response (39). MMTV-neu mice are functionally tolerant to neu antigens (40), but local or systemic administration of TLR agonists can reactivate the antineu T-cell repertoire to restrict tumor growth (41).

We studied the reactivation of neu-specific T cells by the TLR9 agonist CpG (ODN-1826) in WT and MMTV-neu-CCR5−/− mice. To avoid ambiguity due to asynchronous appearance of spontaneous tumors, we generated grafts with the H2Dq/H2Lq-restricted N202.1A mammary cell line derived from an MMTV-neu mouse tumor. N202.1A cells grew similarly in untreated MMTV-neu and MMTV-neu-CCR5−/− mice (Supplementary Fig. S9). Intratumor injection of CpG nonetheless induced a significant reduction in tumor growth in MMTV-neu but not in MMTV-neu-CCR5−/− mice (Fig. 7A), suggesting that CCR5 expression is necessary for TLR9-mediated stimulation of antitumor responses.

Although CpG initially targets the innate immune system, CpG-induced responses are CD4+ and CD8+ T-cell dependent (41). We determined the number and activation state of neu-specific CD8+ T cells using a tetramer loaded with the rat-neu (420–429) peptide. Expansion of H2Dq/rat-neu (420–429) tetramer-positive cells was greater in dLN of CpG-injected than in control-injected MMTV-neu mice; this enhancement was not observed in CpG-treated MMTV-neu-CCR5−/− mice (Fig. 7A).
CCR5 in Antitumor Immunity

**Figure 7.** CCR5 is required for TLR9-mediated reactivation of antitumor responses. A, growth of untreated and CpG-treated N202.1A tumors in MMTV-neu and MMTV-neu-CCR5–/– mice (n = 6 mice/group). Arrow indicates CpG injection. One representative experiment is shown of 2 conducted experiments (*, P < 0.03). B, number of rat-neu (420–429)–CD8+ T cells per 2 × 10⁶ cells in dLN of control or CpG-injected mice (*, P = 0.04). C, IFNγ levels secreted by splenocytes from control or CpG-treated mice after ex vivo restimulation with rat-neu (420–429) peptide. D–E, infiltration of rat-neu (420–429)–CD8+–specific (D) and polyclonal CD8+ T cells (E) into tumors, expressed as the ratio of CpG-treated:untreated mice. B–E, data show mean ± SEM from 1 representative experiment of 2 (n = 6/group).

**Discussion**

Reactivation of antitumor adaptive immune responses could be of clinical benefit, alone or combined with conventional cancer therapies (1). We used a number of transplantable and primary tumor models to provide genetic evidence that (i) CCR5 agonists, such as CCL5, in the tumor environment can inhibit tumor growth in immunocompetent hosts, (ii) this effect seems to be CCR5 specific, (iii) CCR5 is a central element in maximizing T cell-mediated antitumor responses after adoptive transfer of TAA-specific T cells or after immunostimulation with a TLR9 agonist in tolerized mice, and (iv) the maximal tumoricidal T cell-mediated response requires CCR5 expression in both CD4+ and CD8+ T cells.

Like other inflammatory mediators, the chemokines and their receptors can either promote or restrict the onset and/or progression of established tumors (4), CCR5 and its ligands epitomize this paradox. We analyzed the pro- and antitumor effects of CCR5 by comparing the growth of subcutaneous grafts and the incidence of carcinogen-induced and spontaneous tumors in WT and CCR5-deficient mice. Graft experiments with LLC, Panc02, and EG7 tumor cell lines indicated that CCR5 expression in host cells restricts progression of these subcutaneous tumors. Using the LLC cell line, we show that the CCR5 antitumor effects are dependent on CCL5 levels at the tumor site and on the adaptive immune system. Our results thus concur with other reports showing that CCR5 activation is not a positive determinant for tumor progression (42).

One striking observation was that inhibition of tumor grafts by forced CCL5 expression was CCR5 dependent but CCR1 independent, although CCR1 and CCR5 are both expressed on effector and helper T cells. Differential in vivo roles for CCR1 and CCR5 were also described in models for atherosclerosis (43) and renal fibrosis (44), among others. Functional specificity of CCR1 and CCR5 receptors were reported for CCL3-mediated potentiation of the immune response after radiofrequency ablation of murine hepatoma (22); in this case, however, CCL3 antitumor activity was CCR1 dependent but CCR5 independent. These results highlight the functional specificity of CCR1/CCR5, which might be linked to tissue context or ligand abundance. Moreover, CCR1 and CCR5 have distinct roles in T-cell transmigration, probably associated to the differential recycling of these receptors (45). Independently of the specific mechanism involved, our data reinforce the concept that the functional redundancy observed in vitro in the chemokine system is even more complex in a pathologic context.

In support of a CCR5 antitumor effect, we found enhanced incidence and accelerated onset of MCA-induced sarcomas in CCR5–/– compared with WT mice. CCR5 showed neither a protective nor a detrimental effect in the onset of spontaneous breast cancers in MMTV-neu mice. These differences can be explained by distinct tumor immunogenicity in each model; whereas mice show potent innate and adaptive immune responses to MCA-induced tumors (36), MMTV-neu mice are functionally tolerant to neu antigens (40). Forced CCL5 expression in fibrosarcoma cells inhibits tumor growth in a CD8+ T cell-dependent manner (42), and we found that MCA-induced sarcoma grew more rapidly in CCR5–/– than in WT mice.

A major conclusion of our study is that, by modulating helper-dependent CD8+ T-cell responses, CCR5 is an important factor in optimizing antitumor immune responses. Effective CD8+ T-cell responses are achieved through CD4+ T-cell help (33, 34). Although there is a consensus that CD4+/CD8+ cooperation requires interaction of both cell types with the same APC, the temporal regulation of these interactions is debated. One model suggests that CD4+...
and CD8+ cells interact sequentially with the APC (34), whereas in a second model, the CD4+ and the CD8+ cells would interact simultaneously with a single mature APC that presents MHC I- and MHC II peptides (46). On the basis of a peritoneal ovarian cancer model, it was suggested that CCL5 produced by CD4+ cells at the tumor site steers CCR5+ DC for in situ CD40L-mediated licensing (7); although CCR5 was not formally implicated in this process, the results of this study support a role for CCR5 agonists in sequential CD4/CD8 cooperation. In contrast, an intravitral 2-photon study showed that CCL3 and CCL4, produced by DC-CD4+ T cell conjugates at the dLN, guide preactivated naive CD8+ cells to these complexes and promote simultaneous interaction of the 3 cells (6).

Our data for CCR5-deficient OT-I and OT-II cells support the second model. Activated OT-I cells preceded OT-II cells to the tumor site; in our model, DC licensing and crosspriming thus seem not to occur at the tumor site. We also observed a significant reduction in the number of OVA-specific CD8+ cells at the dLN of immunized mice receiving OT-IKO+OT-IIKO compared with OT-IWT+OT-IIWT cell recipients; reduced recruitment to the dLN correlated with the lower capacity of transferred OT-IKO+OT-IIKO cells to restrict tumor growth compared with CCR5-expressing mixtures. The finding that CCR5 agonist levels are higher in OT-IIWT-dLN than in OT-IIKO-dLN complexes suggests a positive feedback effect, which reinforces the guidance function of CCR5 when expressed in both cell types. Nevertheless, lack of CCR5 expression in either OT-I or OT-II cells did not affect recruitment to the dLN, although it impaired OT-I-mediated rejection of EG7 tumors. One interpretation of these data is that CCR5 is not only a cell guidance system for CD8+ T cells, which can be partially replaced by other chemokines/receptor pairs (35), but is also directly involved in activating the APC, CD4+, and/or CD8+ cells implicated in clustering.

In support of this hypothesis, we identify the requirement for CCR5 expression on CD4+ cells to achieve maximal CD40L upregulation after Ag engagement. In addition, CCR5-expressing CD4+ cells induced more complete DC maturation than CCR5-deficient counterparts, resulting in enhanced crosspresentation and activation of CD8+ cells. Our results thus indicate an in vivo function of CCR5 in modulating helper-dependent CD8+ T-cell responses. CCR5 not only steers CD8+ T cells (6) but also delivers an early activation signal in CD4+ cells that enhances CD40L/CD40-mediated APC maturation and CD8+ T-cell activation. This model would explain the reduced effectiveness of transferred OT-IWT+OT-IIKO cells in EG7 tumor rejection, although OT-I cell trafficking to dLN after immunization is unaffected. The observation that CCR5 signaling in T cells induces transactivation of NFAT (10), a major transcriptional regulator of CD40L (47), argues in favor of direct CCR5 regulation of CD40L levels in CD4+ T cells.

The most notable implication of these results is that lack of CCR5 can influence the efficiency of immune-based cancer therapies, as seen in the reduced response of MMTV-neu-CCR5-/- mice to the TLR9 agonist. Defective CCR5 expression in ccr5Δ32 individuals also reduces cell-mediated immunity to pathogens such as HIV-1 and West Nile virus (11, 12). In the latter case, CCR5 deficiency is not a risk factor for infection, but negatively affects disease outcome (12). Although susceptibility to tolerogenic cancers is independent of CCR5 expression, the progression of some tumors has been associated to CCR5 levels (27, 28).

The CCR5 effect on induction of antitumor immune responses might depend on the inflammatory environment. CCR5 expression is reported to counteract the antitumor response elicited by a combination of a TLR3 agonist and chemotherapy (48). This differential role of CCR5 in TLR9- and TLR3-mediated immunostimulation suggests integration of local and systemic signaling pathways that affect CCR5 and the TLR. The mechanism underlying this specificity is unknown; some reports pinpoint differences in tumor response to TLR9- and TLR3-mediated signaling to explain the specific counter regulation of TLR9-mediated inflammatory responses by the glucocorticoid receptor (49) and the different effects of TLR9 and TLR3 agonists on age-associated antitumor responses (41) or on arachidonic acid mobilization (50). TLR9 and TLR3 are thus not wholly equivalent in reactivating the immune system and suggest future studies to understand the combinatorial control of CCR5 responses by TLR.

In summary, our study shows that CCR5 is necessary for optimal activation of adaptive immune responses to tumors. This function might be relevant for cancer therapy and explain the decreased survival of stage IV melanoma ccr5Δ32 patients receiving immunotherapy compared with patients without the polymorphism (29). Moreover, because the adaptive immune system is implicated in the success of some radio and chemotherapy protocols (22, 23), appropriate activation of CCR5, rather than its inhibition, might have broader applications for cancer therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank C. Martínez-A. for critical reading of the manuscript, J. Hernández and C. Ardavín for helpful advice and reagents, V. Bronte and R. Brekken for CCL5 cDNA, F. Ortego for statistical analysis, and C. Mark for editorial assistance.

Grant Support

This work was supported in part by the Spanish Ministry of Science and Innovation (grant SAF2008-00790), the Carlos III Health Institute RIER Network (RD08/0075), and the Comunidad de Madrid grant IMMUNOTHERCAN (to S. Mañes). A. González-Martín was partially supported by a predoctoral FPI fellowship from the Comunidad de Madrid.

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 17, 2011; revised June 24, 2011; accepted June 24, 2011; published OnlineFirst June 29, 2011.
References


Maximal T Cell–Mediated Antitumor Responses Rely upon CCR5 Expression in Both CD4+ and CD8+ T Cells

Alicia González-Martín, Lucio Gómez, Joseph Lustgarten, et al.

Cancer Res  Published OnlineFirst June 29, 2011.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-11-1687

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2011/06/29/0008-5472.CAN-11-1687.DC1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.