Microenvironment and Immunology

CXCR3 Enhances a T-Cell–Dependent Epidermal Proliferative Response and Promotes Skin Tumorigenesis

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Abstract

The chemokine receptor CXCR3 has been proposed to play a critical role in host antitumor responses. In this study, we defined CXCR3-expressing immune cell infiltration in human skin squamous cell carcinomas and then used CXCR3-deficient mice to assess the contribution of CXCR3 to skin tumorigenesis. Our studies employed two established protocols for chemical skin carcinogenesis [methylcholanthrene (MCA) or 7,12-dimethylbenz(a)anthracene (DMBA)/12-O-tetradecanoylphorbol-13-acetate (TPA) models]. CXCR3 deletion did not affect tumor development in the MCA model; however, CXCR3 was important in the DMBA/TPA model where gene deletion reduced the incidence of skin tumors. This decreased incidence of skin tumors did not reflect differences in epidermal development but rather was associated with reduced epidermal thickness and proliferation in CXCR3–/– mice, implicating the CXCR3 pathway in DMBA/TPA-induced epidermal inflammation and proliferation. Notably, CXCR3 expressed in CD4+ and CD8+ T cells was found to be important for enhanced epidermal proliferation. Specifically, CXCR3-deficient mice reconstituted with T cells isolated from wild-type mice treated with DMBA/TPA restored wild-type levels of epidermal proliferation in the mutant mice. Taken together, our findings establish that CXCR3 promotes epidermal tumorigenesis likely through a T-cell–dependent induction of keratinocyte proliferation. Cancer Res; 71(17); 5707–16. ©2011 AACR.

Introduction

The chemokine receptor CXCR3 and its 3 ligands CXCL9, CXCL10, and CXCL11 are part of a large family of chemotactic cytokines whose major function is the chemotractation of specific hematopoietic cellular subsets during homeostatic or inflammatory conditions. In addition, chemokines function in diverse processes such as T-cell proliferation, tissue remodeling, and angiogenesis (1–3). The majority of studies assessing the role of CXCR3 in tumor growth and rejection have relied on enforced expression of ligands in syngeneic tumors, xenograft, or therapeutic models. For example, enforced CXCL10 expression in melanoma xenografts or CXCL9/10 in lymphoma xenografts reduced, but did not abrogate, growth of tumors in immunodeficient mice (4–6). CXCL10 was also implicated as the critical mediator of actions of interleukin 12 (IL12) in a therapeutic model of tumor rejection via its effects on angiogenesis (7). Thus, limited studies to date have explored the role of endogenously produced CXCR3 ligands or their receptor in the host antitumor response in models of primary tumor development.

To address the role of CXCR3 in skin tumorigenesis, we first analyzed human cutaneous squamous cell carcinomas (CSCC) and identified infiltration of CXCR3-expressing cells. We then utilized CXCR3 null mice in 2 of the most well-characterized chemical carcinogen models of primary tumor formation—the methylcholanthrene (MCA) fibrosarcoma and the 7,12-dimethylbenz(a)anthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate (TPA) skin tumor systems. Over the last 15 years, Schreiber (8), Smyth (9), and other groups used the MCA model to show that the impact of the immune system on nascently transformed cells results in successful control of neoplastic growth consistent with the predictions of Burnet and Thomas and which is now more broadly described as cancer immunoediting (10–12). Dissection of this dynamic process has revealed the critical role of lymphocytes and immunologically relevant cytokines in protecting the murine host from primary tumor development. For example, mice deficient in the IFNγ receptor α-chain (IFNGR1) display an increased MCA-induced tumor burden compared with wild-type (WT) counterparts (13). Importantly, the downstream mediators of the actions of IFNγ have been incompletely defined but may include CXCR3 ligands, which are directly induced by this cytokine. Although MCA carcinogenesis has
been utilized predominantly to reveal the host-protective actions of the immune system, it is surprisingly also influenced by inflammation (14). A second commonly used model, based on DMBA/TPA application, revealed the essential role of epithelial restricted γδ T cells in immune surveillance (15). However, this model has also been utilized to show the contribution of cancer-promoting inflammation. Mice deficient in tumor-promoting CD8+ T cells (16), inflammatory molecules, such as COX-2 (17), or inflammatory environment regulators such as RAGE (18), all show a decreased incidence of tumors compared with WT mice. Thus, depending on the model, the specific polarization of the immune response to one that is either inflammatory or host protective leads to 1 of 2 diametrically opposed outcomes for the host (19).

Herein, we detail our findings using both of these classical carcinogenesis models in CXCR3−/− mice where (i) DMBA/TPA treatment revealed decreased tumor development, suggesting that CXCR3 contributes to the inflammatory environment and (ii) no role was found for CXCR3 in protecting mice from carcinogenesis in the MCA system. Although there were no developmental differences between WT and CXCR3−/− mice in epidermal differentiation markers, analysis of epidermal proliferation in response to DMBA/TPA showed a decreased proliferative response in CXCR3−/− mice. We then determined that both CD4+ and CD8+ T cells, known CXCR3-expressing cells, were the critical immune cells regulating epidermal proliferation in WT mice and were able to reconstitute the epidermal proliferation deficit in CXCR3−/− mice. Whereas CXCR3 does not affect immune surveillance in MCA carcinogenesis, it does promote DMBA/TPA tumor development and enhances DMBA/TPA T-cell–dependent keratinocyte proliferation.

Materials and Methods

Animals

Studies were conducted under approved protocols of the Animal Studies Committees of Washington University and the Department of Veteran’s Affairs. C57BL/6 and RAG2−/− C57BL/6 (both from Taconic Farms) and CXCR3−/− mice were used (20). Mice were maintained in a specific pathogen-free environment and were sex and age matched for experiments.

Antibodies

Antibodies to mouse CD3 (145-2C11), CD4 (RM4-5), CD8 (Ly-2, 53-6.7), CD16/CD32 (2.4G2), CD45 (30-F11), Gr-1 (RB6/8C5), CD11b (M1/70), γδ TCR (GL3), Vγ5 (536), human CXCR3 (1C6), and isotype controls were from BioLegend and BD Biosciences. For in vivo studies, control and antibodies targeting CD4, CD8, and NK1.1 were from Bio X Cell. Anti-keratin antibodies were from Covance.

Cutaneous carcinogenesis

The protocol of Roberts and colleagues (21) was followed with 100 μg DMBA for initiation and 25 μg TPA for promotion (both from Sigma). Tumor development was monitored weekly, and lesions greater than 2 mm in size were counted as positive. A short-term experiment was also adapted where back skin was shaved, treated with a single dose of DMBA followed by 3 doses of TPA, and harvested 16 to 20 hours after the final TPA application. For MCA carcinogenesis, tumors were induced and monitored as described (13).

Immunohistochemistry

Skin samples were fixed in formalin, paraffin embedded, and sectioned. After antigen retrieval using an IHC-Tek epitope retrieval set (IHC World), incubations were carried out with the primary (4°C overnight) and secondary biotinylated IgG (room temperature: 30 min). The Vectastain Elite ABC and Peroxidase Substrate Kit were used for detection (Vector Laboratories). Immunohistochemical sections were counterstained with hematoxylin (Fisher Scientific). Human CSCC samples were obtained under a Washington University Institutional Review Board–approved protocol and were immunostained after citrate-based antigen retrieval (Santa Cruz Biotechnology) with an antibody to human CXCR3 (1C6; BD Pharmingen). They were evaluated by a single study pathologist (M.E. Pittman) for the presence of staining in both lymphocytes and tumor cells. Staining was subjectively graded on a scale of 1 to 3, with 1 representing scattered cell staining, 2 clusters, and 3 large aggregates or bands.

ELISA

CXCL9 and CXCL10 kits (R&D Systems) were used to detect protein in total lysates from mouse skin.

Proliferation analysis

DMBA/TPA-treated mice (16–20 hours after last TPA dosing) were injected with 100 mg/kg of bromodeoxyuridine (BrdUrd; Sigma) either 1 or 24 hours before harvest. BrdUrd staining on fixed sections was carried out using the Labeling and Detection Kit II (Roche) according to the manufacturer’s protocol with eosin counterstain (Fisher Scientific). All BrdUrd+ cells and total basal keratinocytes were counted for each section. A proliferation index was calculated using the formula [(total number BrdUrd+ cells/total number of basal keratinocytes) × 1,000] to compare between sections.

Fetal liver chimeric mice

Fetal liver cells from E13.5 embryos were harvested and transferred into sublethally irradiated (1,100 rads) recipient mice. Fetal liver cells have been successfully used to reconstitute epidermal T-cell responses (22). Mice were allowed to reconstitute for 12 weeks and analyzed for appropriate reconstitution by fluorescence-activated cell sorting (FACS) of splenocytes at the end of the DMBA/TPA experiments.

Skin-infiltrating lymphocyte analysis

Epidermal preparations of DMBA/TPA-treated skin were made by treating with Dispase II (2.4 units/mL; Roche) at 37°C for 2 to 4 hours. Single-cell suspensions were generated by treatment with 0.25% trypsin (Hyclone), filtered, washed, and plated overnight in complete RPMI medium to allow antigen reexpression (21). Staining and FACS were carried out as described (8, 23).
Hyperplasia

Hematoxylin and eosin (H&E)-stained DMBA/TPA-treated mouse skin was analyzed for the thickness of the epidermis using a calibrated ocular micrometer (at 40×).

Adoptive T-cell transfers

WT or CXCR3−/− spleen and lymph nodes were used to isolate CD3+ T cells using magnetic bead–based negative selection (Miltenyi Biotec). Purity of cells was between 85% and 95% by FACS and 15 × 10⁶ purified CD3+ cells were transferred into the indicated recipients 1 week after DMBA application, followed by short-course TPA (3 doses) treatment and analysis for BrdUrd incorporation.

Statistics

All statistical analysis was conducted in Prism (GraphPad Software). Tumor incidence in the MCA groups was analyzed by survival curve analysis and the log-rank (Mantel–Cox) test. The paired, 2-tailed Student’s t test was used for the DMBA/TPA experiments and an unpaired, 2-tailed Student’s t test was used to analyze all other data. A P < 0.05 value was considered significant and all error bars represent SEM.

Results

CXCR3 is expressed on infiltrating cells in human CSCC

To assess the association of CXCR3 with human CSCC, we examined 24 individual patient samples by immunohistochemistry for CXCR3-expressing cells (Fig. 1A). The key findings of these analyses were that (i) CXCR3 expression was limited to infiltrating cells and was not present on tumor cells, (ii) the majority of tumors (20 of 24) had strong (grade 3) peritumoral CXCR3+ cells, (3) intratumoral CXCR3+ staining was more variable (Fig. 1C; grade 1 = 9, grade 2 = 8, and grade 3 = 6), and (4) no CXCR3 was detected on adjacent normal epidermis (Fig. 1B). This analysis showed that infiltrating cells expressing CXCR3 were present in human CSCC and that neither tumor nor other adjacent normal cells express CXCR3. Because of a limited number of samples, patient outcomes relative to CXCR3 expression could not be determined.

CXCR3 promotes DMBA/TPA tumorigenesis

To assess the role of CXCR3 in tumor development, we first compared the susceptibilities of WT and CXCR3−/− mice to DMBA/TPA tumorigenesis. Groups of male WT C57BL/6 and CXCR3−/− mice were treated with DMBA (100 µg) followed by twice weekly TPA (25 µg) and monitored for tumor development (Fig. 1D). Note the background of the CXCR3−/− mice was confirmed via genome-wide analysis of informative polymorphic markers between the 129Sv/Ev and C57BL/6 strains, which showed that the gene-deficient mice were 99.3% (145 of 146 markers) C57BL/6 strain (data not shown). These experiments showed that, as a group, the CXCR3−/− mice developed 2-fold fewer tumors per mouse compared with WT mice. Similar results were obtained in independent, repeat experiments with male and female mice (combined cohort totals for all experiments were 59 WT and 45 CXCR3−/− mice). The difference in tumor incidence between WT and CXCR3−/− mice was more robust in males than in females, which led us to use males for the remainder of this study (Supplementary Fig. S1A and B). In separate experiments, directly comparing male and female cohorts, we noted a significant gender difference in that WT males were more sensitive than females to DMBA/TPA, whereas male and female CXCR3−/− mice were uniformly resistant to tumor development (data not shown). C57BL/6 mice develop mostly papillomas in response to DMBA/TPA with rare progression to SCCs (24). In these experiments, there were no differences in tumor sizes or progression to carcinomas between WT and CXCR3−/− mice (data not shown). Thus, CXCR3 has a tumor-promoting role in the inflammation-induced DMBA/TPA model of cutaneous tumorigenesis.

Similar incidence of MCA-induced tumors in CXCR3−/− and WT mice

Although the MCA model has been critical for delineating the central tenets of cancer immunoeediting, it also relies on an inflammatory contribution for tumor development (14). To assess the role of CXCR3 deficiency in fibrosarcoma development, large cohorts of WT (n = 60), RAG2−/− (n = 29), and CXCR3−/− (n = 67) mice were injected with 2 different doses of MCA and monitored. Consistent with the known role for lymphocytes in immunosurveillance, all 14 RAG2−/− mice developed tumors when treated with 25 µg of MCA compared with 20 of 29 WT mice (P = 0.0067, data not shown). In contrast, CXCR3−/− mice displayed tumor incidences that were similar to that of WT mice both at the 25-µg dose where 24 of 34 mice developed tumors (Fig. 1E) and at a 6.25-µg dose (Supplementary Fig. S1C). In addition, CXCR3−/− mice were transplanted with 21 different RAG regressor tumors, which are RAG2−/−-derived tumor cell lines that are rejected in WT mice and grow progressively in RAG2−/− mice (8, 23). These experiments revealed that the vast majority (20 of 21) of transplanted tumor lines underwent rejection in CXCR3−/− mice similar to WT mice (Supplementary Fig. S1D). Thus, CXCR3 did not contribute to immune surveillance or tumor promotion in MCA carcinogenesis and was very rarely required to reject transplanted tumors.

No differences in epidermal differentiation in CXCR3−/− versus WT mice

Two possible explanations for the effects of CXCR3 on skin tumor development were that there was an inherent developmental defect in CXCR3−/− skin or that there was dysregulated chemokine biology influencing the developing tumor or microenvironment. To address the former possibility, we first examined standard H&E-stained WT and CXCR3−/− skin and saw no differences (data not shown). To compare developmental markers in the epidermis, immunohistochemistry was used to assess keratin expression in WT and CXCR3−/− mice. In untreated mice, the epidermis is thin and no keratin differences were observed. To better examine keratin distribution, DMBA/TPA-treated skin was stained and showed no
differences in the distribution of keratin 5, which is expressed in all layers of epidermal keratinocytes (data not shown) and of keratin 1, which is expressed only in developing but not basal keratinocytes (Fig. 2A and B). Therefore, CXCR3 deficiency has no effect on epidermal development and thus, the decreased tumor development must be because of altered chemokine biology.

**DMBA/TPA treatment induces CXCL9 and CXCL10**

To establish that CXCR3 ligands are induced in DMBA/TPA-treated mouse skin thus serving as a target for CXCR3 expressing cells, we assessed ligand levels by ELISA. Importantly, we focused only on CXCL9 and CXCL10 because C57BL/6 mice do not express CXCL11 due to a 2-bp insertion in the coding sequence that leads to a premature stop codon (25). Untreated or vehicle-treated skin (no differences between these 2 were noted) had low levels of both CXCL9 and CXCL10 protein in both WT and CXCR3−/− mice (Fig. 2C and D). Upon DMBA/TPA treatment, significant induction of CXCL9 and CXCL10 was detected in both WT and CXCR3−/− mice. Interestingly, compared with WT mice, CXCR3−/− mice had significantly lower levels of both CXCL9 and CXCL10, suggesting that CXCR3-expressing cells potentially induce expression of CXCR3 ligands in a feed-forward pathway. Thus, consistent with the effects of CXCR3 in tumor development, DMBA/TPA led to the induction of CXCR3 ligands in the skin.

**CXCR3−/− mice have decreased hyperplasia in response to DMBA/TPA**

As there were no differences in epidermal cell differentiation, we then asked whether there was a differential response to DMBA/TPA between WT and CXCR3−/− mice. Epidermal hyperplasia was analyzed in mouse skin treated with DMBA and then 3 doses of TPA (Fig. 3A, short term) or DMBA and then biweekly TPA for 30 weeks (Fig. 3B, long term) by measuring the thickness from the basement membrane to the stratum corneum. In the short-term protocol, there was a decrease in the mean measured thickness from 73.7 ± 4.9 μm in WT mice to 53.9 ± 1.3 μm in CXCR3−/− mice, and in the long-term protocol, the mean measured thickness decreased from 71.2 ± 3 μm in WT mice to 51.8 ± 2.7 μm in CXCR3−/− mice. These data suggest that WT mice have a CXCR3-dependent epidermal proliferative response to DMBA/TPA. In addition, the short-term assay replicates the findings of the long-term assay at the level of epidermal hyperplasia and provides a surrogate assay to dissect the components contributing to CXCR3 related epidermal proliferation.

**Decreased epidermal proliferation in CXCR3−/− mice**

As the epidermal hyperplasia assessment provides a "snapshot" end result view of DMBA/TPA treatment, we next asked whether BrdUrd incorporation in proliferating keratinocytes would also reveal differences between WT and
CXR3−/− mice, as this is a more dynamic assay revealing specific cellular behavior. Using both DMBA/TPA application protocols (short and long), mice were pulsed for 1 hour with BrdUrd followed by immediate harvest of skin. Incorporated BrdUrd was detected after antigen recovery and a proliferation index was calculated for each stained section by counting BrdUrd-positive cells and normalizing this number relative to total numbers of basal keratinocytes. As expected, keratinocyte proliferation was increased relative to untreated mice (data not shown). However, there was a marked contrast in the proliferative response upon DMBA/TPA application between WT and CXCR3−/− mice (Fig. 3C–E). In WT mice, more basal keratinocytes incorporated BrdUrd both in the short-term (proliferation index of 522.6 ± 27 WT vs. 165.7 ± 9.5 in CXCR3−/− mice) and in the long-term protocols (proliferation index of 482 ± 19.8 WT vs. 310.1 ± 30.3 in CXCR3−/− mice). When BrdUrd was injected 24 hours prior to harvest of skin, the differences between WT and CXCR3−/− mice continued and extended into the upper layers of the epidermis (Fig. 3F). Whereas WT mice showed incorporation of BrdUrd into proliferating basal keratinocytes and migration of these cells, CXCR3−/− mice continued to have both poor basal proliferation and decreased migration. We also examined these sections by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining and saw no differences between WT and CXCR3−/− mice (data not shown). These data show that CXCR3 deficiency leads to impaired DMBA/TPA-induced proliferation in the epidermis.

**CXCR3-expressing hematopoietic cells drive proliferation**

Having shown a quantitative difference in the response of WT versus CXCR3−/− mice to DMBA/TPA treatment, the specific compartment (hematopoietic or nonhematopoietic) where CXCR3 acts to promote this phenotype was next addressed. Although most data show that CXCR3 expression is limited to immune cells and we found no CXCR3 expression in human epidermis (Fig. 1B), some groups suggest that it is also expressed in keratinocytes (e.g., see ref. 26). To address this issue, we generated chimeric mice by sublethal radiation of recipient mice and subsequent transfer of fetal liver cells to reconstitute the hematopoietic system. Using this technique, we generated 4 different groups of mice (i) WT → WT, (ii) WT → CXCR3−/−, (iii) WT → RAG2−/−, and (iv) CXCR3−/− → RAG2−/−. RAG2−/− mice were used as recipients to avoid any contribution of radio-resistant CXCR3-expressing B or T cells. At the end of the experiments, all mice were analyzed by counting total splenocytes and FACs for CXCR3-expressing lymphocyte populations (Supplementary Fig. S2). Reconstitution restored splenocyte...
populations to levels seen in WT mice and CXCR3 expression in T and natural killer (NK) cells was similar to our previous work (27).

Using these mice, we then assessed epidermal proliferation after DMBA/TPA using the BrdUrd incorporation assay. As shown in Figure 4, we found that all mice expressing CXCR3 in the hematopoietic compartment displayed proliferation rates similar to WT mice. These groups and proliferation indexes were (i) WT→WT, 481.6 ± 27.9; (ii) WT→RAG2−/−, 495.2 ± 35.4; and (iii) WT→CXCR3−/−, 478.7 ± 10.4. In contrast, mice that lacked CXCR3 in the hematopoietic compartment (the CXCR3−/−→RAG2−/− mice) had significantly decreased epidermal proliferation (index = 249.3 ± 28.2). The proliferation index from CXCR3−/−→RAG2−/− was similar to that seen in DMB/TPA-treated CXCR3−/− mice (data not shown). These findings show that CXCR3 is necessary and sufficient in hematopoietic cells to induce epidermal proliferation by DMBA/TPA treatment.

**T cells promote epidermal proliferation**

Having shown that CXCR3 is important in the hematopoietic compartment for DMBA/TPA-induced responses, we next addressed which subset(s) of the CXCR3-expressing immune cell(s) were involved. CXCR3 was originally associated with CD4+ Th1 differentiation (28, 29), and in our previous work, we established that the major CXCR3-expressing cells in naive mice included populations of CD4+ and CD8+ T cells, a subset of NK and all NK-T cells (27). To compare possible epidermal infiltration differences between WT and CXCR3−/− mice, we used FACS to characterize the immune infiltrate in epidermal preparations of untreated or DMBA/TPA-treated mice. We found minimal infiltration of NK or NK-T cells into treated skin in either set of mice (data not shown). As expected, there was an overall increase in inflammatory cell infiltrate consisting of CD11b+/Gr1− and T-cell subsets in both WT and CXCR3−/− mice after DMBA/TPA treatment. However, there were no differences in infiltration of CD11b+ or CD11b+/Gr1−, or importantly epidermal Vγ5+ γδ T cells in WT mice compared with CXCR3−/− mice (either untreated or DMBA/TPA treated, Supplementary Fig. S3). The most significant difference noted was a modest but statistically significant decrease of both CD4+ and CD8+ T cells in DMBA/TPA-treated CXCR3−/− mice compared with WT mice (Fig. 5A).

Having shown a decreased infiltration of T cells into treated CXCR3−/− mouse epidermis, we next assessed their contribution to CXCR3-dependent epidermal proliferation. WT mice were treated with control or depleting monoclonal antibodies (mAb) that ablated CD4+, CD8+, or NK1.1-expressing populations. By FACS analysis of mouse spleens at the end of the experiment, the specific cell populations were reduced by more than 95% (data not shown). After mAb treatment, mice were treated with DMBA/TPA and analyzed with the BrdUrd proliferation assay. In these experiments, the proliferation index for WT and CXCR3−/− mice was 550 ± 37 and 187 ± 15, respectively. Treatment of WT mice with a rat IgG control resulted in a proliferation index of 517 ± 20, which was significantly reduced in mice treated with αCD8 mAb (255 ± 19) or αCD4 mAb (374 ± 14.9). When we depleted mice with αNK1.1 mAb, no decrease in the proliferation index was apparent compared with mice treated with control mouse mAb (Fig. 5B). These experiments revealed that CD4+ or CD8+ T cells had the capacity to induce epidermal proliferation in response to short-coures DMB/TPA treatment.

**CXCR3+ T cells reconstitute epidermal proliferation in CXCR3−/− mice**

Finally, having identified that T cells could promote DMBA/TPA-induced epidermal proliferation in WT mice, we next asked whether CXCR3−/− mice could be reconstituted with WT total CD3+ T cells to enhance epidermal proliferation. This was tested using the short-term DMB/TPA protocol and by transferring purified CD3+ T cells from WT mice into CXCR3−/− mice 1 week after DMB/TPA treatment. T cells were transferred after DMB/TPA treatment due to previous reports of
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Figure 4. Fetal liver chimeric mice reveal that CXCR3 expression in the hematopoietic system promotes DMBA/TPA-induced epidermal proliferation. A, the indicated mice were generated with fetal liver transplantation of irradiated recipients and treated with short-course DMBA/TPA. **, $P < 0.01; n = 3$ for WT→RAG2−/−, n = 4 for WT→CXCR3−/−, and n = 4 for CXCR3−/−→RAG2−/−; combined from 2 different experiments. BrdUrd incorporation was then determined and all mice (representative section in B of WT→CXCR3−/−) displayed WT levels of proliferation, except the CXCR3−/−→RAG2−/− (representative section in C).

Figure 5. T-cell infiltration is reduced in CXCR3−/− compared with WT mice and T-cell depletion in WT mice attenuates epidermal proliferation. A, FACS analysis of DMBA/TPA-treated skin reveals reduced CD4+ and CD8+ T cells in CXCR3−/− compared with WT mice. Epidermal preparations were generated from untreated WT and CXCR3−/− mice or short-course DMBA/TPA-treated skin (designated as D/T), and FACS analysis was conducted for CD4+ and CD8+ T cells (data shown) and CD11b−, Gr1−CD11b+, and γδ/νγ5+ cells (data in Supplementary Fig. S4). Each point represents an individual mouse and data are expressed as percentage of cells relative to total CD45+/propidium iodide (PI)− cells and revealed significant reductions in CD4+ and CD8+ T cells (*, $P < 0.05$). B, WT C57BL/6 mice were treated with the indicated mAbs and DMBA/TPA-treated skin was assessed for proliferation. ***, $P < 0.001$ for WT (n = 4) versus CXCR3−/− (n = 3); ***, $P < 0.001$ for control IgG (n = 2) versus anti-CD8 (n = 7); and **, $P < 0.01$ for control IgG (n = 2) versus anti-CD4 (n = 6); combined data from 3 experiments.

Discussion

Previous studies have implicated CXCR3 and its ligands as mediators of tumor growth inhibition via effects both on protective host immune infiltration and tumor vasculature. All of these studies relied on therapeutic manipulation of transplanted tumors (e.g., see refs. 7, 30–32) and many of these studies were xenografts in the setting of immunodeficient mice, which ignores the actions of CXCR3 and its ligands on the immune system (e.g., see refs. 4–6). One study showed that CXCR3 deficiency in the TRAMP prostate cancer model enhanced tumor growth; however, the genetic background of these mice and CXCR3 expression on prostate cancer cells themselves makes the interpretation of the immune impact on tumor development unclear (33, 34). Importantly, all these studies shed light on the capacity of CXCR3 and its ligands to contribute to angiostasis and immune infiltration but the question of the role of endogenously produced CXCR3 chemokines in primary tumor development in immunocompetent hosts remained similar successful reconstitution (21) and as the proinflammatory effects in this model are due to TPA application (18). In addition, separate CXCR3−/− mice receiving cells from CXCR3−/− donors served as controls. When tested in these experiments, CXCR3−/− mice again displayed a reduction in BrdUrd incorporation with the proliferation index falling from 487 ± 15 to 255 ± 54.3 (Fig. 6A). When CXCR3−/− mice were reconstituted with WT CD3+ T cells, the proliferation index increased to 428 ± 29 (representative image Fig. 6B). In contrast, control CXCR3−/− mice that received T cells purified from CXCR3−/− mice displayed a proliferation index of 299 ± 38, which was significantly lower than mice that received WT T cells (representative image, Fig. 6C). Thus, WT T cells, but not CXCR3−/− T cells, are able to complement the DMBA/TPA-induced epidermal proliferation deficiency in CXCR3−/− mice.

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unanswered. We chose to address this question by assessing
the contribution of CXCR3 in 2 commonly utilized chemical
carcinogen-induced tumor models. Both the MCA and
DMBA/TPA models have been used to dissect the contribu-
tion of immune system to tumor development in host-
protective and tumor-promoting modes. We found that
CXCR3 promoted tumor development in the DMBA/TPA
model, whereas it had no role in MCA carcinogenesis or in
rejection of transplantable, immunogenic tumor cell lines.
CXCR3 was important for epidermal proliferation in
response to DMBA/TPA, and this effect was likely due to
CXCR3 activity in the CD3\(^+\) T cells of the hematopoietic
system. As has been documented in a number of studies, we
used epidermal proliferation and thickness as surrogate
markers for tumor development (reviewed in ref. 24) and
we will focus our future studies on translating this finding to
the causation of tumorigenesis by specific subsets of T cells.
These studies support a model where CXCR3 chemokines
are induced in response to DMBA/TPA treatment and
recruit CXCR3-expressing CD4\(^+\) and CD8\(^+\) cells that induce
inflammatory epidermal proliferation, which, in the context
of DMBA-induced Ras mutations, then promotes tumor
development. Consistent with this, CXCR3\(^{-/-}\) mice would
recruit fewer CD4\(^+\) and CD8\(^+\) T cells relative to WT mice
thus attenuating the inflammatory environment, resulting
in decreased tumorigenesis. WT T-cell transfers into
CXCR3\(^{-/-}\) mice followed by tumorigenesis will delineate
the specific T-cell requirements and further clarify this model—
these experiments will be the subject of a follow-up study. In
contrast, CXCR3 is not involved in promoting or preventing
MCA-induced sarcoma development. This minimal effect may
reflect a redundancy in the chemokine system that allows
lymphocyte recruitment in the absence of CXCR3. In FACS
analysis of some of the RAG regressor tumors transplanted into
WT or CXCR3\(^{-/-}\) mice, where the vast majority of tumors
transplanted into CXCR3\(^{-/-}\) mice were rejected at the same
kinetics as WT mice (20 of 21 tumors; Supplementary Fig, S1D),
minimal differences were noted in lymphocyte recruitment,
suggesting that CXCR3 did not contribute to recruiting the
critical lymphocytes needed for tumor rejection (data not
shown). These results, along with our previous work defini-
tively showing that IFN\(\gamma\) is required for MCA tumor surveil-
ance, suggest that IFN\(\gamma\)-mediated surveillance of MCA
sarcomas is not dependent on CXCR3.

Our initial analysis of human CSCC showed an association
of CXCR3-expressing infiltrating cells especially in peritum-
oral regions. The impact of this infiltrate in clinically
evident tumors is unclear, as opposed to our findings in
the mouse system where CXCR3-expressing cells enhance
tumor development. Several studies on inflammatory lesions
such as those found in psoriasis and in CSCC have repe-
tedly shown an association with CXCR3 \(^{3}\) lymphocytes. For
example, CXCR3-expressing CD8\(^+\) T cells have been found at
the basal epidermal layer in lichenoid graft-versus-host
disease and in lichen planus lesions (35) and dermal
CXCR3\(^{3}\)-expressing CD3\(^{+}\) T cells were strongly associated
with psoriatic plaques (36). In CSCC, a strong IFN\(\gamma\)-associated
transcriptional signature, including CXCL9, and immuno-
histochemical evidence of CXCR3\(^{3}\) cells, CD3\(^{+}\) cells, and
granzyne B \(^{3}\) cells correlating with the signature have been
described (37). No studies have correlated patient outcomes
with CXCR3 receptor or ligand expression. Clearly, the

Figure 6. CXCR3-expressing
cD4\(^+\) and CD8\(^+\) cells are critical
for DMBA/TPA-induced epidermal
proliferation. A, control WT and
CXCR3\(^{-/-}\) mice showed
significant proliferation
differences with DMBA/TPA
application. **, \(P < 0.01; n = 3\) for
each genotype. CXCR3\(^{-/-}\) mice
were reconstituted with purified
WT or CXCR3\(^{-/-}\) CD3\(^{+}\) T cells
and assessed for BrdUrd incorporation, revealing that WT
but not CXCR3\(^{-/-}\) cells are able to
enhance epidermal proliferation.
*, \(P < 0.05; n = 8\) for
WT—CXCR3\(^{-/-}\) and
n = 6 for
CXCR3\(^{-/-}\)—CXCR3\(^{-/-}\);
combined from 3 separate
adoptive transfers. Representative
images of (B) WT—CXCR3\(^{-/-}\)
and (C) CXCR3\(^{-/-}\)—CXCR3\(^{-/-}\).
Comparison of WT and
WT—CXCR3\(^{-/-}\) groups showed
no statistical significance showing
reconstitution to WT levels. n.s.,
not significant.
association of CXCR3-expressing cells in human CSCC—either correlatively or causally—deserves further study.

Relevant to our findings are 3 other investigations on the role of other chemokines and receptors in DMBA/TPA skin tumorigenesis. In their findings on the contribution of TNFα to DMBA/TPA tumorigenesis, Moore and colleagues also examined the role of CCL2 (MCP-1 or monocyte chemoattractant protein 1) and found a 50% reduction in tumor development in CCL2−/− mice (38). Although these investigators did not pursue the associated mechanisms in these mice, further work by Nibbs and colleagues on the decoy chemokine receptor D6 revealed a critical role for chemokine-mediated inflammation in tumor development (39). D6 acts as a sink to remove the inflammatory CC (iCC) class of chemokines, which includes CCL2, CCL3, CCL4, CCL5, CCL7, CCL8, CCL11, CCL17, and CCL22. Thus, D6−/− mice, which have a reduced clearance of iCC, develop a T-cell-dependent exaggerated inflammatory epidermal response which is associated with enhanced tumor development in response to DMBA/TPA application. Finally, CXCR2 expression on keratinocytes was found to be critical for epidermal migration and tumorigenesis (40).

The broad range of chemokines addressed by the decoy receptor D6 did not reveal the specific contributing molecules for controlling tumorigenesis but highlighted the effects of T-cell trafficking to epidermal inflammation. Other skin conditions, such as allergic contact dermatitis, psoriasis, and atopic dermatitis, are mediated by an inflammatory infiltrate that includes neutrophils and T cells and is, in a large part, dependent on specific chemokine production in the epidermal compartment (reviewed in ref. 41). The involvement of CXCR3 in skin wound healing has been the focus of several studies and the specific mechanism is under investigation (26, 42, 43). In skin tumorigenesis induced by DMBA/TPA, T-cell contributions are variable depending on the genetic background of the mouse and the dose of TPA used to promote cutaneous lesions. For example, in the FVB/n strain, CD4+ and CD8+ T cells have a protective role when promoted with low-dose TPA. However, with high-dose TPA, a population of IL-17−producing RORγT+ CD8+ T cells promoted malignant degeneration of benign lesions (16, 21). In contrast, in C3H/HeN, where allergic contact hypersensitivity to DMBA plays a key role in tumor development, CD8+ T cells are host protective and CD4+ T cells promoted tumor development (44). Our data on epidermal hyperplasia in the C57BL/6 background revealed a contribution of both CD8+ and CD4+ T cells—the characterization of how CXCR3 and these cells work together in this model constitutes our current focus. Candidates for the cytokines emanating from these T cells to promote DMBA/TPA tumorigenesis include IL-17 (45) and IFNγ (46). In addition, we noted a gender difference in tumor development with WT male mice developing more tumors (data not shown). To our knowledge, this has not been described previously for the DMBA/TPA model but gender differences in UVB-induced skin tumorigenesis (47) and DEN-induced hepatocellular carcinomas have been reported (48). Interestingly, the latter study involves differential secretion of IL-6, which has been shown to be a growth factor for keratinocytes and may potentially promote tumor formation (49). Finally, the CXCR3 ligand(s) regulating lymphocyte recruitment to the skin are undefined. Notably, C57BL/6 mice do not express CXCL11 (25), thus identifying either CXCL9 or CXCL10 (or both) as the chemoattractant signal. Analysis of DMBA/TPA-treated skin showed induction of both chemokines but the levels were reduced in CXCR3−/− mice, suggesting the lack of an inducing signal. Although speculative, one possible scenario is that the reduced immune infiltrate in CXCR3−/− mice results in a decrease of a chemokine-inducing cytokine. Our current work is aimed at defining the specific cytokine(s) and other chemokines contributing to tumor development. In conclusion, our work highlights the influence of CXCR3-expressing T cells in epidermal proliferation and likely tumorigenesis and lays the foundation for further delineation of specific chemokine activity and tumorigenic pathways in cutaneous tumorigenesis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References


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