Pivotal Involvement of the CX3CL1-CX3CR1 Axis for the Recruitment of M2 Tumor-Associated Macrophages in Skin Carcinogenesis

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We previously revealed the crucial roles of a chemokine, CX3CL1, and its receptor, CX3CR1, in skin wound healing. Although repeated wounds frequently develop into skin cancer, the roles of CX3CL1 in skin carcinogenesis remain elusive. Here, we proved that CX3CL1 protein expression and CX3CR1⁺ macrophages were observed in human skin cancer tissues. Similarly, we observed the enhancement of CX3CL1 expression and the abundant accumulation of CX3CR1⁺ tumor-associated macrophages with M2-like phenotypes in the skin carcinogenesis process induced by the combined treatment with 7,12-dimethylbenz[a]anthracene and 12-O-tetradecanoylphorbol-13-acetate. In this mouse skin carcinogenesis process, CX3CR1⁺ tumor-associated macrophages exhibited M2-like phenotypes with the expression of Wnt3a and angiogenic molecules including VEGF and matrix metalloproteinase 9. Compared with wild-type mice, CX3CR1-deficient mice showed fewer numbers of skin tumors with a lower incidence. Concomitantly, M2-macrophage numbers and neovascularization were reduced with the depressed expression of angiogenic factors and Wnt3a. Thus, the CX3CL1-CX3CR1 axis can crucially contribute to skin carcinogenesis by regulating the accumulation and functions of tumor-associated macrophages. Thus, this axis can be a good target for preventing and/or treating skin cancers.

INTRODUCTION

Prolonged inflammation destroys the tissue parenchyma, occasionally resulting in the induction of tumorigenesis, which can be furthered by infiltrating leukocytes (Coussens and Weinberg, 2002; Mantovani et al., 2008). Macrophages are a major cellular component in the tumor microenvironment and play various roles in cancer development in a context-dependent manner (Lewis and Pollard, 2006; Mosser and Edwards, 2008). The depletion of cutaneous macrophages promoted growth of basal cell carcinoma (BCC) in mice, indicating that macrophages had protective roles in BCC of skin (König et al., 2014). On the contrary, the specific depletion of CD11b⁺ macrophages impaired tumor incidence of skin (Weber et al., 2016). Moreover, the polarization of macrophage phenotypes is involved in tumor development (Isidro and Appleyard, 2016; Linde et al., 2012; Tariq et al., 2017).

Chemokines are a family of chemotactic cytokines for the migration of distinct types of immune and nonimmune cells (Rossi and Zlotnik, 2000). Moreover, several chemokines produced by cancer cells can regulate leukocyte trafficking into the tumor microenvironment (Allavena et al., 2011; Balkwill, 2012, 2004; Mantovani et al., 2010). CX3CL1 is a membrane-bound chemokine, in contrast to most other chemokines, which are secreted molecules. The CX3CL1-CX3CR1 axis can be used to augment tumor immunity (Sugaya, 2015). In contrast to its potential antitumor activity, accumulating evidence may indicate the protumorigenic activity of the CX3CL1-CX3CR1 axis, which was overexpressed in various types of cancers (Celesti et al., 2013; Kim et al., 2012; Shulby et al., 2004; Tsang et al., 2013; Yao et al., 2014; Zheng et al., 2013). Similarly, the roles of CX3CL1 in tumor invasion and metastasis are still controversial. CX3CL1 can promote cancer metastasis using various routes (Borsig et al., 2014), whereas CX3CL1 can prevent glioma invasion by promoting tumor cell aggregation and eventually reducing their invasiveness (Sciumé et al., 2010). Thus, the role of the CX3CL1-CX3CR1 axis in carcinogenesis processes remains elusive.

The interaction between CX3CL1- and CX3CR1-expressing cells contributed crucially to the skin wound healing process by promoting the accumulation and function of macrophages (Ishida et al., 2008). Dvorak (2015) proposed that tumors are wounds that do not heal. Moreover, we found the protein expression of CX3CL1 and CX3CR1 in human skin cancer tissues. To address the pathogenic roles of the CX3CL1-CX3CR1 axis in skin carcinogenesis, we utilized 7,12-dimethylbenz[a]anthracene (DMBA)/12-O-tetradecanoylphorbol-13-acetate (TPA)-induced two-stage skin carcinogenesis (Kemp, 2005). Here, we provided definitive evidence to indicate the vital roles of the CX3CL1-CX3CR1 axis in this skin carcinogenesis model.
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RESULTS

CX3CL1 and CX3CR1 expression in human skin cancer tissues of BCC and squamous cell carcinoma (SCC)
The importance of the interaction between CX3CL1 and CX3CR1 has been implicated in many inflammatory diseases (Echigo et al., 2004; Hasegawa et al., 2005; Morimura et al., 2013; Murphy et al., 2008). Hence, initially, we examined the expression of CX3CL1 and CX3CR1 in the BCC and SCC tissues, all of which were histopathologically diagnosed by one of the authors who is specialized in dermatological cancer histopathology (YY) (Supplementary Figure S1). There were few positive cells for CX3CL1 or CX3CR1 in normal human skin samples (Supplementary Figure S2). In comparison with normal skin samples, both CX3CL1 and CX3CR1 proteins were detected robustly in the dermis of all specimens from both BCC and SCC samples that we examined (Figure 1a and b). Subsequently, CD68+ macrophages, but not CD31+ endothelial cells, were the main cellular source of CX3CL1 (Figure 1c and Supplementary Figure S3a). Moreover, CX3CR1 was detected in both CD68+ macrophages and endothelial cells (Figure 1d and e). Most CX3CL1+ cells consistently expressed CX3CR1 (Supplementary Figure S3b). These observations indicated the potential involvement of the CX3CL1-CX3CR1 axis in skin carcinogenesis, probably via its action on tumor-associated macrophages (TAMs), which simultaneously express CX3CL1 and CX3CR1.

CX3CL1 and CX3CR1 expression in the skin after DMBA/TPA treatment

Both CX3cl1 and Cx3cr1 mRNAs were faintly detected in the skin of the untreated wild-type (WT) mice. DMBA/TPA treatment significantly enhanced the Cx3cl1 and Cx3cr1 mRNA expression in the skin later than two weeks after initial TPA treatment (Figure 1f and g). The double-color immunofluorescence analyses further demonstrated that F4/80+ cells were the major cellular source of CX3CL1 (Figure 1h), similar to the observation of the human skin cancer tissues. CX3CR1 protein was mainly detected in F4/80+ macrophages (Figure 1i) and to a lesser degree in endothelial cells (Figure 1j) and CD3+ lymphocytes (Supplementary Figure S4), which is mostly consistent with our data of the human skin cancer tissues. Thus, CX3CL1 and CX3CR1 expression was enhanced in chemical-induced mouse skin carcinogenesis as well as in human skin cancer tissues.

The pathogenic involvement of the CX3CR1-CX3CL1 axis in skin carcinogenesis

To address the roles of the CX3CL1-CX3CR1 axis in skin carcinogenesis, WT and Cx3cr1−/− mice were subjected to DMBA/TPA treatment. There were no apparent differences between the skin structures of the unchallenged WT and Cx3cr1−/− mice (Figure 2a). At 20 weeks after initial TPA treatment, the epidermal layer was apparently thickened in both WT and Cx3cr1−/− mice, and the thickness was more evident in WT mice than in Cx3cr1−/− mice (Figure 2a and b). Consistently, the number of Ki67+ proliferating epidermal cells were increased in both strains, and the increment was significantly larger in WT mice than in Cx3cr1−/− mice (Figure 2c and d). Several lines of evidence demonstrated that the activation of the EGFR signal pathway was closely involved in skin carcinogenesis (Hara et al., 2005; Sibilia et al., 2000; Tardáguila et al., 2013). Although both strains showed EGFR signal pathway activation after DMBA/TPA treatment, the signals were attenuated in Cx3cr1−/− mice compared with WT ones (Figure 2e and f). Although both strains started to develop papillomas later than 10 weeks after initial TPA treatment, the tumor incidence was higher in WT mice than in Cx3cr1−/− mice at all the time points that we examined (Figure 2g and h). Therefore, at 20 weeks after initial TPA treatment, 90% of the WT mice but only 40% of the Cx3cr1−/− mice developed papillomas (Figure 2h). Moreover, the average numbers of papillomas in Cx3cr1−/− mice were significantly lower than those in WT mice (Figure 2i). These observations would imply that the contribution of the CX3CL1-CX3CR1 axis may contribute to chemical-induced skin carcinogenesis by inducing the proliferation of epidermal cells. Next, we explored the contribution of bone marrow (BM)-derived Cx3CR1+ cells to skin carcinogenesis by using BM chimeric mice generated from WT and Cx3cr1−/− mice. Both WT and Cx3cr1−/− mice transplanted with WT mouse-derived BM cells exhibited a higher tumor incidence and developed more papillomas than the recipients of Cx3cr1−/− mice—derived BM cells (Figure 2j and k). Moreover, tumor incidence and average papilloma numbers were similar between Cx3cr1−/− BM cell–receiving WT and Cx3cr1−/− mice (Figure 2j and k). These observations would imply the crucial involvement of radiosensitive BM-derived Cx3CR1+ cells in skin carcinogenesis.

The effects of CX3CR1 deficiency on macrophage recruitment after DMBA/TPA treatment

CX3CR1 deficiency in TAMs prompted us to examine the effects of CX3CR1 deficiency on macrophage infiltration in this carcinogenesis process. There were no significant differences in the number of F4/80+ macrophages, MPO+ neutrophils, and CD3+ lymphocytes between the skin of unchallenged WT and Cx3cr1−/− mice (Supplementary Figure S5). The recruitment of each leukocyte subpopulation was increased in the skin of both mouse strains at one week after initial TPA treatment, whereas the recruitment of F4/80+ macrophages, but not Ly-6G+CD11b+ neutrophils and CD3+ lymphocytes, was significantly reduced in Cx3cr1−/− mice compared with WT mice (Figure 3a and b). Moreover, even at 10 weeks after initial TPA treatment, intradermal macrophage recruitment was attenuated in Cx3cr1−/− mice compared with WT mice, but macrophage recruitment was decreased to a similar extent in both WT and Cx3cr1−/− mice 20 weeks after initial TPA treatment (Figure 3c and d). Given the important role of macrophage polarization to M2-like phenotype in carcinogenesis (Isidro and Appleyard, 2016; Tariq et al., 2017), we determined the expression of M2-related molecules in dermal macrophages isolated from DMBA/TPA-treated WT or Cx3cr1−/− mice. The numbers of the isolated whole macrophages were significantly reduced in Cx3cr1−/− mice compared with the WT mice, both at 48 and 72 hours after initial TPA treatment (Figure 3e). Moreover, the Cx3cr1−/−–derived dermal macrophages exhibited a reduced expression of M2-macrophage markers, such as Mrc1/Cd206, Cd163, Il10, Ccl17, and...
arginase1, compared with the WT-derived dermal macrophages (Figure 3f–j). Consistently, even at five days after initial TPA treatment, CD68⁺CD206⁺ M2-like macrophage numbers were reduced in Cx3cr1⁺⁻/⁻ mice compared with WT mice (Figure 3k and l). Furthermore, nearly 75% of the CX3CR1⁺ macrophages expressed CD206, an M2-macrophage marker (Figure 3m). A triple-color immunofluorescence analysis demonstrated that a large number of F4/80⁺CD206⁺ M2 macrophages expressed CX3CR1 in DMBA/TPA-induced carcinogenesis (Figure 3n). Consistently, the expression of CD163, a human M2 marker, was conspicuous in human skin cancer lesions, particularly SCC ones, compared with that of HLA class II, an M1 marker (Figure 4a–c). A triple-color immunofluorescence analysis
Figure 2. The evaluation of tumor incidence in WT and Cx3cr1<sup>−/−</sup> mice after DMBA/TPA treatment. (a) Histologic observations on the epidermal layers. Bar = 100 μm. (b) The average of the epidermal thickness. All values represent mean ± SEM (n = 6). **P < 0.01 versus WT. (c) Ki67 was immunohistochemically detected. Bar = 100 μm. (d) The number of Ki67<sup>+</sup> cells. (e) Western blotting analysis. (f) The ratio of p-EGFR to EGFR. (g) Macroscopic pictures of skin papillomas. (h) The percentage of tumor-free mice (n = 20). **P < 0.01 versus WT. (i) The average number of papillomas per mouse (n = 20). (j) The percentage of tumor-free mice (n = 5). *P < 0.05 versus WT-BM to WT. (k) The average number of papillomas per mouse (n = 5). BM, bone marrow; cont, control; DMBA, 7,12-dimethylbenz[a]anthracene; ITT, initial 12-O-tetradecanoylphorbol-13-acetate treatment; p-EGFR, phosphorylated EGFR; TPA, 12-O-tetradecanoylphorbol-13-acetate; WT, wild-type.
demonstrated that a large number of CD68<sup>+</sup>CD163<sup>+</sup> M2 macrophages expressed CX3CR1 in human SCC lesions (Figure 4d). Thus, M2 macrophage infiltration could be a prominent feature of skin carcinogenesis in mice and humans. Finally, DMBA/TPA-treated WT and Cx3cr<sup>1−/−</sup> mice displayed similar levels of gene expression of Ccl2 and Ccl3, which exhibit potent chemotactic activities for macrophages but utilize distinct receptors from CX3CR1 (Supplementary Figure S6). These observations imply that DMBA/TPA treatment induced the infiltration of macrophages and their...
subsequent polarization into the M2 phenotype by inducing CX3CL1, which can act on CX3CR1 expressed on macrophages.

Reduced neovascularization in mice lacking CX3CR1 after DMBA/TPA treatment

CX3CR1 expression by CD31-positive cells incited us to examine the effects of CX3CR1 deficiency on angiogenesis during the course of skin carcinogenesis. DMBA/TPA treatment increased the intradermal vessel density with the upregulation of Vegf and Mmp9 (two potent angiogenic molecules) in WT and Cx3cr1⁻/− mice (Figure 5a–d). The increments of the vessel density, however, were significantly reduced in Cx3cr1⁻/− mice (Figure 5b). Consistently, the enhanced upregulation of Vegf and Mmp9 was significantly suppressed in Cx3cr1⁻/− mice compared with WT ones (Figure 5c and d). Moreover, the VEGF and matrix metalloproteinase (MMP) 9 proteins were detected mainly in CX3CR1⁺ macrophages (Figure 5e and f), suggesting that CX3CR1⁺ M2-like macrophages could contribute to angiogenesis in the development of chemical-induced skin carcinogenesis. Thus, the absence of the CX3CR1 axis reduced neovascularization directly and indirectly by reducing the expression of the potent angiogenic factors, VEGF and MMP9, during the course of DMBA/TPA-induced skin carcinogenesis.

Reduced expression of Wnt3a in DMBA/TPA-treated Cx3cr1⁻/⁻ mice

The crucial roles of β-catenin–mediated signaling in a variety of skin cancers (Gat et al., 1998; Malanchi et al., 2008) provoked us to determine the gene expression of Wnt3a. There were no significant differences in Wnt3a mRNA expression and Wnt3a⁺ cell numbers between unchallenged skin tissues of WT and Cx3cr1⁻/⁻ mice (Figure 6a–c). Indeed, DMBA/TPA treatment enhanced the intradermal Wnt3a mRNA expression in WT mice later than six weeks after the treatment to a larger extent than Cx3cr1⁻/⁻ mice (Figure 6a). Consistently, the Wnt3a⁺ cell numbers were significantly lower in Cx3cr1⁻/⁻ mice than in WT mice 10 weeks after initial TPA treatment (Figure 6c). A double-color immunofluorescence analysis detected Wnt3a in CX3CR1⁺ and F4/80⁺ cells, indicating that CX3CR1⁺ M2-like macrophages could produce Wnt3a, eventually contributing to chemical-induced skin carcinogenesis (Figure 6d and e). Moreover, CX3CL1 augmented the Wnt3a expression in a mouse
Immunohistochemical analyses using anti-CD31 mAb. Bar = 50 μm. (b) The number of vessels in the skin tissue. All values represent mean ± SEM (n = 6). *P < 0.05, WT versus Cx3cr1−/− mice. (c) Intradermal gene expression of Vegf in WT and Cx3cr1−/− mice before and after DMBA/TPA treatment. Values represent mean ± SEM (n = 6). (d) Intradermal gene expression of Mmp9 in WT and Cx3cr1−/− mice before and after DMBA/TPA treatment. Values represent mean ± SEM (n = 6). (e) Cell types expressing VEGF in the skin of DMBA/TPA-treated WT mice. (f) Cell types expressing MMP-9 in the skin of DMBA/TPA-treated WT mice. Bar = 20 μm. Cont, control; DMBA, 7,12-dimethylbenz[a]anthracene; ITT, initial 12-O-tetradecanoylphorbol-13-acetate treatment; MMP, matrix metalloproteinase; TPA, 12-O-tetradecanoylphorbol-13-acetate; WT, wild-type.

macrophage cell line, RAW264.7 (Figure 6f). Immunohistochemical analysis demonstrated that the nuclear accumulation of β-catenin in the epidermal cells was markedly decreased in Cx3cr1−/− mice compared with WT mice at 10 weeks after initial TPA treatment (Figure 6g). The amount of unphosphorylated (active) β-catenin protein was increased in WT mice at 10 weeks after initial TPA treatment, but this increase was significantly lowered in Cx3cr1−/− mice (Figure 6h and i). Moreover, DMBA/TPA treatment increased Cox2 expression in the skin of both WT and Cx3cr1−/− mice, but these increments were significantly depressed in Cx3cr1−/− mice at two weeks and later after initial TPA treatment (Figure 6). A double-color immunofluorescence analysis detected COX-2 in F4/80+ and Cx3cr1+ cells (Figure 6k and l). Given the observation that COX-2 and prostaglandin E2 can exert pro-oncogenic actions through β-catenin signaling (Castellone et al., 2005; Shao et al., 2005). Thus, locally produced CX3CL1 can recruit Wnt3a- and COX-2—expressing macrophages and can further enhance their Wnt3a expression, thereby activating the β-catenin signaling pathway and eventually skin carcinogenesis.

DISCUSSION

The crucial involvement of the CXCL1-CX3CR1 axis in skin wound healing incited us to examine the CX3CL1 and CX3CR1 expression in human skin cancer tissues. We detected abundant CX3CL1 expression together with CX3CR1+ cell infiltration in human skin cancer tissues. To investigate its role in skin carcinogenesis, we utilized DMBA/TPA-induced two-step skin carcinogenesis, where a single DMBA application induces DNA mutations in epidermal cells and subsequent repeated exposure to TPA induces chronic inflammation, to accelerate tumorigenesis (DiGiovanni, 1992). Indeed, similar CX3L1 and CX3CR1 expression patterns were observed in the skin of mice after DMBA/TPA treatment. Both CX3CL1 and CX3CR1 were mainly expressed on macrophages in human skin cancer tissues and in DMBA/TPA-treated mouse skin tissues. Moreover, given the exclusive use of CX3CR1 by its ligand, CX3CL1, reduced DMBA/TPA-induced skin carcinogenesis in Cx3cr1−/− mice indicates the crucial involvement of the CX3CL1-CX3CR1 axis on macrophages in this carcinogenesis model.

TAMs are frequently a major cell component of cancer tissues and usually lack cytotoxic activity but exhibit M2-like phenotypes with the expression of angiogenic factors (Mantovani et al., 2017). Indeed, CX3CR1+ TAMs in this mouse model exhibited M2-like phenotypes and abundantly expressed two potent angiogenic factors, VEGF and MMP-9. CX3CR1 deficiency reduced macrophage infiltration, with depressed M2-like macrophage numbers, and reduced the expression of VEGF and MMP-9. Although TAMs are presumed to be derived mostly from circulating monocytes that are attracted toward tumor sites by locally produced chemokines, including CCL2 and CCL3 (Singh et al., 2009; Teicher and Fricker, 2010), DMBA/TPA treatment induced CCL2 and CCL3 expression in WT and Cx3cr1−/− mice to similar extents. Thus, it is likely that macrophage-derived CX3CL1 directly recruited CX3CR1+ M2-like macrophages to tumor tissues via using an amplifying autocrine loop. Moreover, mice lacking MMP-9 showed impaired macrophage recruitment (Bradley et al., 2012; Kluger et al., 2013), probably due to the ability of MMP-9 to degrade matrix
proteins in the basement membrane of vessels and to eventually promote leukocyte migration. Thus, the reduced macrophage recruitment would arise also partially from attenuated expression of Mmp9 gene in Cx3cr1−/− mice.

In this carcinogenesis model, Cx3cr1−/− mice exhibited reduced neovascularization with depressed tumor formation compared with WT mice. Indeed, CX3CR1+ TAMs expressed several angiogenic molecules, including VEGF and MMP-9,
and the expression of these two molecules was reduced in Cx3cr1−/− mice compared with WT mice. Thus, neovascularization can be ascribed at least partially to TAM-derived VEGF and MMP-9. Moreover, CX3CR1 expression by CD31+ endothelial cells may suggest the direct involvement of the CX3CL1-CX3CR1 axis in neovascularization at tumor sites, similar to the observations in case of skin wound healing (Ishida et al., 2008). Nevertheless, in this skin carcinogenesis model, the CX3CL1-CX3CR1 axis could promote neovascularization directly by acting on endothelial cells and/or indirectly by inducing the production of angiogenic factors by TAMs.

Accumulating evidence indicates the crucial involvement of proinflammatory cytokines in DMBA/TPA-induced skin carcinogenesis. TPA-induced AP-1 activation in the epidermis is indispensable for tumor development and requires TNF-α-mediated TNF receptor signals, particularly at the early phase, in this carcinogenesis process (Arnott et al., 2004; Moore et al., 1999). IL-1 receptor—MyD88 signaling additionally contributes to keratinocyte transformation and carcinogenesis by further activating the NF-kB pathway (Cataisson et al., 2012). Moreover, TNF-α-dependent MMP-9 expression promoted epithelial cell migration during tumor promotion (Scott et al., 2004). Thus, these molecules can directly regulate the migration, proliferation, and transformation of keratinocytes, eventually resulting in carcinogenesis. We observed that the gene expression of Il1a, Il1b, and Tnfα was upregulated in skin carcinogenesis of WT and Cx3cr1−/− mice and that their protein expression was mainly detected in F4/80+ macrophages (Supplementary Figure S7).

Moreover, their enhanced gene expression was attenuated in Cx3cr1−/− mice together with reduced F4/80+ macrophage recruitment compared with WT mice. Thus, locally produced CX3CL1 recruits CX3CR1+ macrophages, a rich source of these keratinocyte activators, thereby causing skin carcinogenesis.

Several lines of evidence have implied that CD11b+ Gr1+ myeloid cells had tumor-promoting roles (Kowanetz et al., 2010; Qian et al., 2011). Di Piazza et al. (2012) have demonstrated that these cells could exert this effect by augmenting Wnt/β-catenin signaling in neighboring epithelial cells via the secretion of Wnt ligands. Consistent with this observation, we also observed that TAMs were a major source of Wnt3a. Moreover, CX3CL1-CX3CR1 signaling augmented Wnt3a expression in a mouse macrophage cell line, and Cx3cr1−/− mice exhibited depressed Wnt3a expression. Furthermore, we observed enhanced COX-2 expression in F4/80+ macrophages. Given the capacity of prostaglandin E2 to trigger the Wnt/β-catenin pathway (Castellone et al., 2005; Shao et al., 2005), macrophage-derived COX-2 can activate this pathway. Nevertheless, CX3CL1 can activate the Wnt/β-catenin pathway, which is crucially involved in skin carcinogenesis, by attracting CX3CR1+ macrophages with a capacity to express Wnt3a and/or COX-2.

The activation of the EGFR signal pathway was crucially involved in tumor proliferation including skin cancer (Hara et al., 2005; Sibilia et al., 2000; Tardáguila et al., 2013). In line with this, we found that the absence of CX3CR1 suppressed skin carcinogenesis with reduced TAM recruitment and attenuated activation of the EGFR signal pathway. TAM, particularly M2-type TAM, produced EGF, which can directly act on EGFR-expressing epidermal cells (Quail and Joyce, 2013; Yin et al., 2016). Moreover, the CX3CL1-CX3CR1 axis can induce the transactivation of EGFR signals in several types of cells (Tardáguila et al., 2013; White et al., 2010). Thus, the attenuated EGFR signal pathway can be explained by reduced TAM recruitment as well as defective EGFR transactivation in Cx3cr1−/− mice.

Collectively, these observations reveal that the CX3CL1-CX3CR1 axis can regulate infiltration and polarization of macrophages and eventually provide VEGF, Wnt3a, IL-1, TNF-α, and macrophage-derived factors, which can promote chemical-induced skin carcinogenesis, particularly at its early phase. Moreover, abundant CX3CL1 expression and the presence of CX3CR1+ macrophages in human skin cancer tissues will support the notion that the CX3CL1-CX3CR1 axis can be a novel target for the prevention and/or treatment of human skin cancer. This assumption may require validation by examining the protective and/or therapeutic effects of a CX3CR1 or CX3CL1 inhibitor on skin carcinogenesis using this mouse model.

MATERIALS AND METHODS

Reagents, antibodies, and samples
Reagents, antibodies, mice, and human skin cancer samples used in this study were described in the Supplementary Materials and Methods (Ishida et al., 2008).

Skin carcinogenesis
Skin tumors were induced as described in the Supplementary Materials and Methods (Wang et al., 2010) (Supplementary Figure S8).

Generation of BM chimeric mice
BM chimeric mice were prepared as described in the Supplementary Materials and Methods.

Histopathologic, immunohistochemical, and double-color immunofluorescence analyses
Histopathologic, immunohistochemical analyses, and double-color immunofluorescence analyses were performed as described in the Supplementary Materials and Methods (Inui et al., 2011; Ishida et al., 2012).

Quantitative RT-PCR analysis
Quantitative RT-PCR analysis was performed as described in the Supplementary Materials and Methods (Inui et al., 2011) (Supplementary Table S2).

Flow cytometry analysis
Flow cytometry analysis was performed as described in the Supplementary Materials and Methods (Ishida et al., 2012).

Separation of F4/80+ cells by magnetic-activated cell sorting
F4/80+ cells were separated as described in the Supplementary Materials and Methods (Ishida et al., 2012).

Western blotting analysis
Skin samples were subjected to Western blotting analysis as described in the Supplementary Materials and Methods.
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In vitro assay
RAW264.7 cells were cultured and stimulated as described in the Supplementary Materials and Methods.

Statistical analysis
Statistical analyses were performed as described in the Supplementary Materials and Methods.

Study approval
Human samples were obtained under the approval of the Institutional Review Boards of Wakayama Medical University. Written informed consent was obtained from the participants before inclusion in the study. All animal experiments were approved by the Committee on Animal Care and Use at Wakayama Medical University. All methods were performed in accordance with the relevant guidelines and regulations.

Data availability statement
No data sets were generated or analyzed during this study.

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CONFLICT OF INTEREST
The authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS
Conceptualization: YI, TK; Formal Analysis: YI, YK; Funding Acquisition: YI, MN, TK; Investigation: YI, MN, AK, FF; Project Administration: TK; Validation: MN, AK; Writing - Original Draft Preparation: YI; Writing - Review and Editing: NM, TK

SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2020.02.023.

REFERENCES


SUPPLEMENTARY MATERIALS AND METHODS

Reagents and antibodies (Abs)
7,12-dimethylbenz[a]anthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate were purchased from Sigma (St. Louis, MO). Recombinant murine CX3CL1 was obtained from R&D Systems (Minneapolis, MN). The following mAbs and polyclonal Abs (pAbs) were used for immunohistochemical and immunofluorescence analyses: goat anti-mouse CX3CL1 pAbs, which cross-react with human CX3CL1; goat anti-mouse COX2 pAbs; goat anti-mouse VEGF pAbs; goat anti-mouse matrix metalloproteinase-9 pAbs (Santa Cruz Biotechnology, Dallas, TX); goat anti-human TNF-α pAbs, which cross-react with mouse TNF-α; goat anti-human Wnt3a pAbs, which cross-react with mouse Wnt3a; goat anti-mouse CD31 pAbs, which cross-react with human CD31; rabbit anti-mouse IL-1α pAbs (Abcam, Cambridge, United Kingdom); rabbit anti-human IL-1β pAbs, which cross-react with mouse IL-1β (Santa Cruz Biotechnology); rabbit anti-human CX3CR1 pAbs, which cross-react with mouse CX3CR1 (Abnova, Walnut, CA); rat anti-mouse F4/80 mAb (clone, BM8; BMA Biomedicals, Augst, Switzerland); rabbit anti-human CD3 pAbs, which cross-react with mouse CD3 (Dako Cytomation, Kyoto, Japan); rat anti-mouse F4/80 mAb (clone, A3-1; AbD Serotec, Oxford, United Kingdom); mouse anti-human CD68 mAb (clone, S14H12; PIERCE, Sunnyvale, CA); rabbit anti-mouse Ki67 mAb (clone, D3B5; Cell Signaling, Danvers, MA); mouse anti-human HLA-DR mAb (clone, TAL.1B5; Dako Cytomation); mouse anti-human CD163 mAb (clone, 10D6; Leica Biosystems, Buffalo Grove, IL); rabbit anti-mouse β-catenin pAbs (Proteintech, Rosemont, IL); rabbit anti-mouse Keratin1 pAbs (BioLegend, San Diego, CA); Cy3-conjugated donkey anti-rat IgG pAbs; FITC-conjugated donkey anti-goat IgG pAbs; FITC-conjugated donkey anti-rabbit IgG pAbs (Jackson Immunoresearch Laboratories, West Grove, PA); rabbit anti-human Wnt3a pAbs, which cross-react with mouse Wnt3a; and rabbit anti-IL-1α pAbs, which react with mouse IL-1α (Abcam). For flow cytometric analyses, the following Abs were commercially obtained: phycoerythrin-conjugated rat anti-mouse Ly-6G mAb (clone, 1A8, BD Bioscience, San Jose, CA); violet-Fluor450-conjugated rat anti-mouse CD11b mAb (clone, M1/70, TONBO, San Diego, CA); allopheophycocyanin-conjugated rat anti-mouse F4/80 mAb (clone BM8.1, TONBO); FITC-conjugated rat anti-mouse CD3 mAb (clone, 17A2, TONBO); PerCP/Cy5.5-conjugated rat anti-mouse CD45 mAb (clone, 30-F11, BioLegend); FITC-conjugated rat anti-mouse CD68 mAb (clone, FA-11, Bio-Rad, Hercules, CA); phycoerythrin-conjugated rat anti-mouse CD206 mAb (clone, MR6F3, Thermo Fisher Scientific, Waltham, MA); allopheophycocyanin-conjugated rat anti-mouse CD86 mAb (clone, GL-1, TONBO); and PerCP-conjugated goat anti-mouse CX3CR1 pAbs (R&D Systems). For Western blotting analyses, the following Abs were used: rabbit anti-mouse EGFR mAb (clone, D38B1, #4267); rabbit anti-mouse phosphorylated EGFR mAb (clone, D7A5, #3777); rabbit anti-mouse β-catenin (active) mAb (clone D13A1, #8814); and rabbit anti-mouse GAPDH mAb (clone, D16H11, #5174, Cell Signaling).

Animals
Pathogen-free 8-week-old male C57BL/6 mice were obtained from Sankyo Laboratories (Tokyo, Japan) and designated as wild-type (WT) mice. CX3CR1-deficient (Cx3cr1−/−) mice with the C57BL/6 genetic background were a generous gift from Drs. P. M. Murphy and J. L. Gao (National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD) (Ishida et al., 2008). All animals were housed individually in cages under specific pathogen-free conditions during the experiments. Age- and sex-matched mice were used for the experiments. All animal experiments complied with the standards set by the Guidelines for the Care and Use of Laboratory Animals at the Wakayama Medical University.

Human skin cancer tissues
Skin cancer tissue specimens (basal cell carcinoma, n = 5, cases 1–5; squamous cell carcinoma, n = 5, cases 6–10) were obtained by biopsy from the patients in the Wakayama Medical University Hospital after obtaining their written informed consent for diagnosis (Supplementary Table S1). Using the densitometric tool of Photoshop, the extents of HLA-DR positivity were measured in the human basal cell carcinoma and squamous cell carcinoma specimens and were expressed as the pixel number per field (×200). The study design was approved by the Local Ethical Committee of the Wakayama Medical University Hospital.

Skin carcinogenesis
Skin tumors were induced by two-step application of DMBA and 12-O-tetradecanoylphorbol-13-acetate as described in a previous study (Wang et al., 2010) (Supplementary Figure S8). First, 25 µg of DMBA in 100 µl of acetone was applied onto the shaved dorsal skin of the mice on day −7 (~1 week). On day 0, topical application of 30 µg of 12-O-tetradecanoylphorbol-13-acetate in 100 µl of acetone was initiated and was continued for 20 weeks with a frequency of twice a week. Tumor development was monitored on a weekly basis and lesions greater than 2 mm in length were counted as positive.

Generation of bone marrow (BM) chimeric mice
The following BM chimeric mice were prepared: male Cx3cr1−/− BM to female WT mice, male WT-BM to female WT mice, male WT-BM to female Cx3cr1−/− mice, and male Cx3cr1−/− BM to female Cx3cr1−/− mice. BM cells were collected from the femurs of donor mice by aspiration and flushing. Recipient mice were irradiated with a radiation dose of 12 Gy using an RX-650 irradiator (Faxitron X-ray Inc., Wheeling, IL). Then, the animals intravenously received 5 × 106 BM cells from the donor mice in a volume of 200 µl of sterile PBS under anesthesia. Thereafter, mice were housed in sterilized microisolator cages and were fed normal chow and autoclaved hyperchlorinated water for 60 days. To verify successful engraftment and reconstitution of the BM in the transplanted mice, genomic DNA was isolated from the peripheral blood and tail tissues of each chimeric mouse 30 days after BM transfer using a NucleoSpin tissue kit (Macherey-Nagel, Duren, Germany). Then, we performed PCR to detect the Sry gene contained in the
Y chromosome (F, 5'-TTGCCCTCAACAAAA-3'; R, 5'-AAACTGCTTCTGCTGGT-3'). The amplified PCR products were fractionated on a 2% agarose gel and visualized by ethidium bromide staining. After durable BM engraftment was confirmed, the mice were treated with DMBA/12-O-tetradecanoylphorbol-13-acetate as described previously.

**Histopathologic, immunohistochemical, and double-color immunofluorescence analyses**

At the indicated time intervals before and after DMBA application, skin tissues were removed, fixed in 10% formalin buffered with PBS (pH 7.2), and embedded in paraffin. Sections 6 μm thick were prepared and stained with H&E. Epidermal thickness was measured using Photoshop (at ×40 magnifications). Immunohistochemical analyses were also performed using anti-F4/80, anti-CD31 mAb, anti-Ki67, anti-VEGF, anti-β-catenin, or anti-Wnt3a Abs as described in a previous report. The number of positive cells or CD31-positive tube-like vessels were counted on five randomly chosen visual fields at 200-fold magnifications, and the average of the five selected microscopic fields was calculated. All measurements were performed by an examiner without prior knowledge regarding the experimental procedures. A double- or triple-color immunofluorescence analysis was also conducted to identify the types of CX3CL1-, CX3CR1-, VEGF-, MMP-9-, COX-2- or Wnt3-expressing cells in the skin, as described in a previous report.

**Quantitative RT-PCR analysis**

Total RNA was extracted from skin tissue using ISOGEN (Nippon Gene, Toyama, Japan), according to the manufacturer's instructions. Next, 3 μg of total RNA was reverse transcribed to cDNA with Oligo(dT)$_{15}$ primers using PrimeScript Reverse Transcriptase (Takara Bio, Shiga, Japan). The resultant cDNA was subjected to real-time PCR by using SYBR Premix Ex Taq II (Takara Bio) and specific primer sets (Takara Bio), as described in a previous report (Inui et al., 2011) (Supplementary Table S2). Amplification and detection of mRNA were conducted by using the Thermal Cycler Dice Real Time System (Takara Bio, TP800), according to the manufacturer's instructions. To standardize the mRNA concentrations, transcript levels of β-actin were determined in parallel for each sample, and relative transcript levels were normalized to the β-actin transcript levels.

**Flow cytometry analysis**

Single-cell suspensions were prepared from wound tissue homogenates, as described in a previous report. Contaminated red blood cells were hemolyzed using ammonium chloride solution (IMGENEX). The resulting single-cell suspensions were incubated with the Abs for 20 minutes on ice. Isotype-matched control Igs were used to detect the nonspecific binding of Ig in the samples. The stained cells were analyzed on a CytoFLEX S system (Beckman Coulter, Brea, CA), and the obtained data were analyzed using the CytExpert 2.2 software (Beckman Coulter).

**Separation of F4/80$^+$ cells by magnetic-activated cell sorting**

Skin cells were harvested, prepared into single-cell suspensions, and counted as described in a previous study. All the incubations were conducted at 4 °C for 20 minutes. To isolate the F4/80-positive cell population, the resultant single-cell preparation was stained with anti-F4/80 MicroBeads Ultra-Pure mouse Abs (Miltenyi Biotec, Bergisch Gladbach, Germany). The sorting column was fixed on a magnetic-activated cell sorting stand (Miltenyi Biotec) and equilibrated by using 500 μl of PBS containing 0.5% BSA. After the cell suspension was passed through a magnetic-activated cell sorting mass spectrometry separation column that was placed in mini magnetic-activated cell sorting, the obtained F4/80$^+$ cell fraction exhibited a purity of more than 95%, as determined using a flow cytometer.

**Western blotting analysis**

Skin samples were homogenized and the resultant lysates (30 μg) were electrophoresed on a 7.5% SDS-PAGE gel and transferred onto a nitrocellulose membrane. The membrane was then incubated with 1,000-fold diluted Abs against β-catenin (active), EGFR, phosphorylated EGFR, or GAPDH. After the incubation of the membrane with horseradish peroxidase-conjugated secondary Abs, the immune complexes were visualized using the ECL Plus System (Amersham Biosciences Corp., Piscataway, NJ), according to the manufacturer's instructions.

**In vitro assay**

RAW264.7 cells (the mouse macrophage cell line) were cultured in DMEM medium containing 10% fetal bovine serum, seeded at a density of 1 × 10$^6$ cells/well into six-well plates, and cultured overnight. After the cells were stimulated further with various concentrations of recombinant mouse CX3CL1 for 2 hours at 37 °C, the supernatants were collected, and the Wnt3a protein levels in these supernatants were determined using a commercially available ELISA kit (My Biosource, San Diego, CA), according to the manufacturer's instructions. The detection limits of Wnt3a was >23.5 ng/ml.

**Statistical analysis**

Data were expressed as the mean ± SEM. For the comparison between WT and Cx3cr1$^{+/−}$ mice at multiple time points, two-way ANOVA, followed by Dunnett's post hoc test, was used. To compare the values between two groups, unpaired Student's t-test was performed. In case of the series of CCL3 stimulations of RAW264.7 cells for the in vitro and the flow cytometric analysis, one-way ANOVA, followed by Dunnett's post hoc test, was used. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using the Statcel3 software under the supervision of a medical statistician.
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Roles of the CX3CL1-CX3CR1 in Skin Carcinogenesis

Supplementary Figure S1. Histopathologic images of the human BCC (Case 1–5) and SCC tissues (Case 6–10). The samples were diagnosed by our dermatologist (a specialist of skin cancer). Representative results are shown here. Bar = 50 μm. BCC, basal cell carcinoma; SCC, squamous cell carcinoma.

Supplementary Figure S2. CX3CL1 and CX3CR1 expression in normal human skin. The samples were processed to immunohistochemical analysis using anti-CX3CL1 or anti-CX3CR1 antibodies. Representative results are shown in this figure. Bar = 50 μm.
Supplementary Figure S3. Cell types expressing CX3CL1 and CX3CR1 in the skin of DMBA/TPA-treated WT mice. (a) CD31⁺ endothelial cells did not express CX3CL1 in the skin. Bar = 20 μm. (b) Almost all CX3CL1-expressing monocytes also expressed CX3CR1 in the skin. Representative results from six individual animals are shown. Signals were merged digitally. Bar = 20 μm. DMBA, 7,12-dimethylbenz[a]anthracene; TPA, 12-O-tetradecanoylphorbol-13-acetate; WT, wild-type.

Supplementary Figure S4. Double-color immunofluorescence analyses for CD3⁺ T cells and CX3CR1. Representative results are shown here. Signals were merged digitally. Bar = 20 μm. SCC, squamous cell carcinoma.

Supplementary Figure S5. The presence of MPO⁺ neutrophils and CD3⁺ T cells in the skin samples of unchallenged WT and Cx3cr1⁻/⁻ mice. The samples were processed to immunohistochemical analysis using anti-MPO or anti-CD3 antibodies. Representative results are shown here. Bar = 50 μm. Cont, control; WT, wild-type.
Supplementary Figure S6. Intradermal mRNA expression of Ccl2 and Ccl3 in WT and Cx3cr1<sup>-/-</sup> mice before and after DMBA/TPA treatment. (a) Ccl2. (b) Ccl3. Quantitative RT-PCR analyses were carried out. Values represent mean ± SEM (n = 4). DMBA, 7,12-dimethylbenz[a]anthracene; ITT, initial 12-O-tetradecanoylphorbol-13-acetate treatment; TPA, 12-O-tetradecanoylphorbol-13-acetate; WT, wild-type.

Supplementary Figure S7. Evaluation of intradermal expression of Il1a, Il1b, and Tnf in WT and Cx3cr1<sup>-/-</sup> mice. (a) Intradermal gene expression of Il1a, (b) Il1b, and (c) Tnf in WT and Cx3cr1<sup>-/-</sup> mice before and after DMBA/TPA treatment. Values represent mean ± SEM (n = 4). *P < 0.05, **P < 0.01, WT versus Cx3cr1<sup>-/-</sup> mice, by 2-way ANOVA followed by Dunnett's post hoc test. (d) Cell types expressing IL-1α, (e) IL-1β, and (f) TNF-α in the skin of DMBA/TPA-treated WT mice. Representative results from six individual animals are shown here. Signals were merged digitally. Bar = 20 μm. Cont, control; DMBA, 7,12-dimethylbenz[a]anthracene; ITT, initial 12-O-tetradecanoylphorbol-13-acetate treatment; TPA, 12-O-tetradecanoylphorbol-13-acetate; WT, wild-type.

Supplementary Figure S8. Schematic representation of the DMBA/TPA-induced skin carcinogenesis. DMBA, 7,12-dimethylbenz[a]anthracene; TPA, 12-O-tetradecanoylphorbol-13-acetate; w, week.
### Supplementary Table S1. Profile of Each Patient with Skin Cancer

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<td>M</td>
<td>Face</td>
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Abbreviations: BCC, basal cell carcinoma; F, female; M, male; SCC, squamous cell carcinoma.

### Supplementary Table S2. Sequences of Primers Used for Real-Time RT-PCR

<table>
<thead>
<tr>
<th>Transcript</th>
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| Cx3cr1     | F 5'-CCGGTCTCTATTTGAGGCTTA-3'  
|            | R 5'-TGGAATTGAACTTTGAGCCT-3' |
| Cx3cl1     | F 5'-ACCTATGGGCCGACATATAC-3'  
|            | R 5'-CTTGGGAGCCCTGACATAC-3'  |
| Ccl2       | F 5'-GCATCCGGCTACTGAGGACT-3'  
|            | R 5'-CTCCAGGCCTACTTGGGAGGAT-3' |
| Ccl3       | F 5'-CATGACACTCTGCAACCAAGTTC-3'  
|            | R 5'-GAGCAAAGGCTGCTGGTTTCA-3'  |
| Vegf       | F 5'-TCCAAACATCACATGACAGAT-3'  
|            | R 5'-CATCTGCAAGTCAGTTTTCA-3'  |
| Mmp9       | F 5'-GCCCTGGAAACCTCACAGCACA-3'  
|            | R 5'-TTGGAACACCTCACGACGGAAG-3'  |
| Mrc        | F 5'-ACCTCTCTTCCGAGGCTTTG-3'  
|            | R 5'-GTTGACCCATTCTGGACTTTAG-3'  |
| Ccl17      | F 5'-ACTCTCAATCTAAGAGAAGAAG-3'  
|            | R 5'-GAGGGTCTCCTAAATGCTTCA-3'  |
| II10       | F 5'-GCCAGAGCAGCAGTCTCTCA-3'  
|            | R 5'-GATAAGGCTTTGGCAACCCAGAAGTAA-3'  |
| Ccl17      | F 5'-TCAGTGAGGTGTCAGCAAG-3'  
|            | R 5'-GGGCTCTTCAAAATGTCTTCA-3'  |
| Arg        | F 5'-ACGCCCTGGGAACATCGCTTGAAG-3'  
|            | R 5'-ATGGAACAGACGTTCAGCTAGTA-3'  |
| Wnt3a      | F 5'-CCATGAACCGTCACCAATGAG-3'  
|            | R 5'-GCGGTTCCTGAATGTCTACAG-3'  |
| Cox2       | F 5'-GCCAGGGCTGAACTCCGAGACA-3'  
|            | R 5'-GTCACACAGGCGACTGACT-3'  |
| IIIa       | F 5'-TGATTGGAATACGAGACAAAGA-3'  
|            | R 5'-AGTCTGGTCATACGTATGAG-3'  |
| IIIb       | F 5'-TGGCAGGATGAGCAATGAG-3'  
|            | R 5'-GAACGCACACACAAAGCAGTGA-3'  |
| Tnf        | F 5'-ACCAGAGTTGGCAGGTTGCT-3'  
|            | R 5'-GGCCACACTGTGGGCTTCTT-3'  |
| Actb       | F 5'-CATCCGTAAGACACTCTT-3'  
|            | R 5'-ATGGAAGCAGCCGCTACACA-3'  |

Abbreviations: F, forward primer; R, reverse primer.