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Non-platelet-derived CXCL4 differentially regulates cytotoxic and regulatory T cells through CXCR3 to suppress the immune response to colon cancer

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ABSTRACT

CXCL4 is mainly produced by activated platelets, and certain somatic cells and cancer cells also express CXCL4. However, the physiological function of non-platelet-derived CXCL4 is unclear. Previously, we reported that CXCL4 produced by cancer cells accelerated tumor growth by suppressing the antitumor activities of cytotoxic T lymphocytes (CTLs). To elucidate the mechanism of CXCL4 in tumor immunity, we compared the CTLs and regulatory T cells (Tregs) from CXCL4$^{-/-}$, CXCR3$^{-/-}$ and C57BL/6 mice overexpressing CXCL4 via intramuscular electroporation. CXCL4 accelerated tumor growth in CXCL4$^{-/-}$ and C57BL/6 mice but not in CXCR3$^{-/-}$ mice. Furthermore, CXCL4 decreased CTLs proliferation and IFN-γ production and enhanced CTLs apoptosis and programmed death 1 (PD-1) expression. Conversely, CXCL4 promoted Tregs proliferation and TGF-β production and downregulated PD-1 expression in Tregs. Notably, these effects of CXCL4 were both observed in the splenic and tumor-infiltrating CTLs and Tregs from wild-type but not CXCR3$^{-/-}$ mice. Thus, we revealed a negative immune regulatory function for non-platelet-derived CXCL4 through CXCR3 that cancer cells could hijack to evade the host immune system, suggesting that the CXCL4/CXCR3 axis may serve as a novel target for colorectal cancer immunotherapy.

1. Introduction

CXCL4, also known as platelet factor-4 [1], has two distinct sources. Platelet-derived CXCL4 is stored in platelets and released in high amounts at μM levels upon platelet activation to regulate blood coagulation [2,3]. Non-platelet-derived CXCL4, which is at a low nM level in plasma, is produced and secreted by certain somatic cells, such as lymphocytes [2,4]. At high concentrations (μM), CXCL4 was reported to suppress tumor growth through its angiostatic activity [5–7]. In contrast, a recent study found that non-platelet-derived CXCL4 promoted lung cancer progression in mice [8]. Previously, we reported that CXCL4 produced by colon cancer cells promoted tumor growth in mice through suppression of cytotoxic T lymphocytes (CTLs) [9]. Because the μM level of CXCL4 is only present in pathological conditions involving bleeding when the platelets are activated [10], we propose that non-platelet-derived CXCL4 plays an important physiological function in the immune response to cancer.

Indeed, previous studies have shown several immunological functions of CXCL4. Exogenous CXCL4 protein in cultures of human T cells stimulated with CD3 and CD28 antibodies or recall bacterial antigens suppressed their proliferation through downregulation of paracrine IL-2 production [11]. Furthermore, CXCL4 differentially regulated a subset of human T cells in culture by inhibiting the proliferation of and the cytokine release from CD4$^+$CD25$^-$ T cells while promoting the expansion of and the cytokine release from CD4$^+$CD25$^+$ T cells [12]. However, the role of non-platelet-derived CXCL4 in the regulation of cancer immunity in vivo is unknown.

In this study, we found that overexpression of CXCL4 promoted MC38 colon cancer growth in CXCL4$^{-/-}$ mice. This growth was strongly associated with the suppression of a CD8$^+$ T cell-mediated immune response. More specifically, CXCL4 inhibited the proliferation and IFN-γ secretion from CTLs and promoted the apoptosis of CTLs in association with elevated programmed death 1 (PD-1) expression on CTLs. Conversely, CXCL4 promoted proliferation and TGF-β secretion

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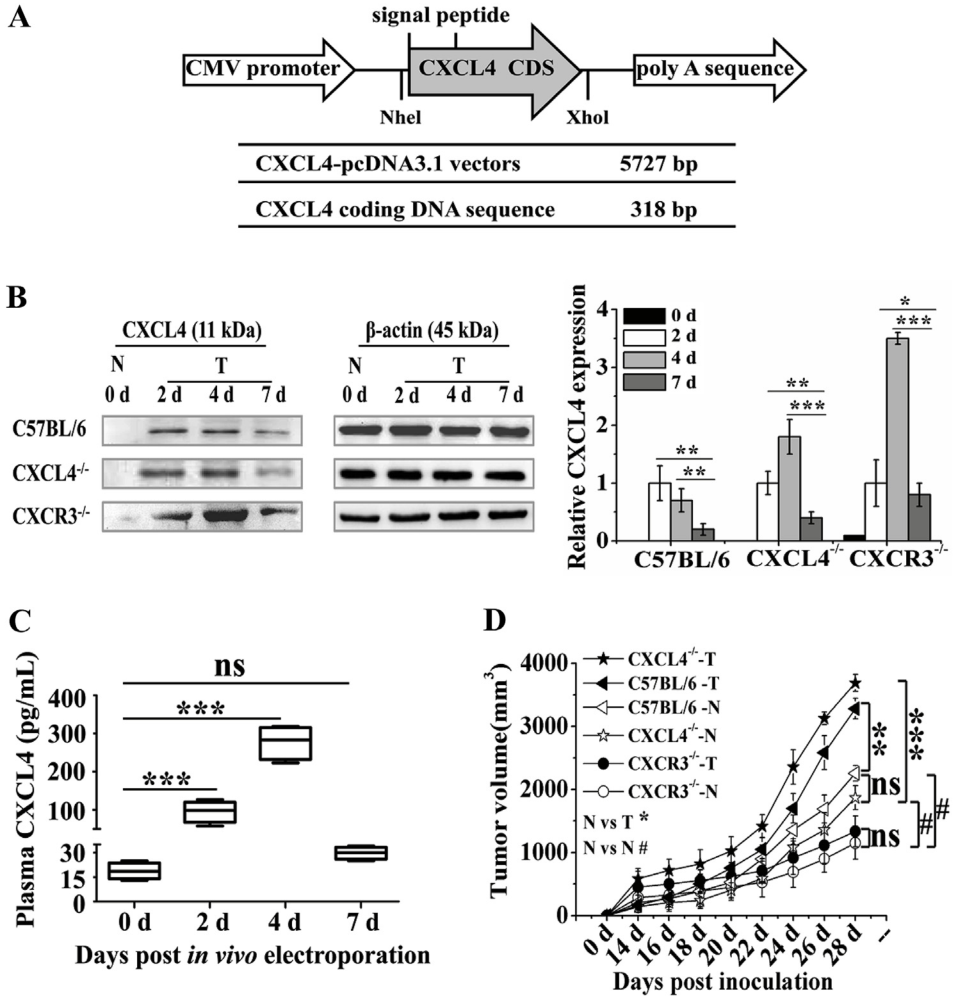

Fig. 1. Non-platelet-derived CXCL4 accelerates tumor growth. C57BL/6, CXCL4$^{-/-}$ and CXCR3$^{-/-}$ mice were transfected with mCXCL4-pcDNA3.1 (T) or pcDNA3.1 plasmids (N) into the mouse skeletal muscle by electroporation once a week for four weeks. Three days after the first electroporation, the mice were inoculated with MC38 colon cancer cells. (**A**) Plasmid construct of mCXCL4-pcDNA3.1 contains the CMV promoter and the mCXCL4 coding sequence (CDS) with its native secretory signal peptide. (**B**) Western blot analysis of CXCL4 expression in the muscle of C57BL/6, CXCL4$^{-/-}$ and CXCR3$^{-/-}$ mice at the indicated times. Data are shown as the mean ± SD ($n = 6$ mice per group). The following symbols represent the corresponding *P* values determined by two-sided unpaired *t* tests: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$. (**C**) Plasma concentration of CXCL4 measured by ELISA post-electroporation in CXCL4$^{-/-}$ mice at the indicated times. Data are shown as the mean ± SD ($n = 4$ mice per group). The following symbol represents the corresponding *P* values determined by two-sided unpaired *t* tests: ***, $P \leq 0.001$. (**D**) Tumor volume after inoculation of MC38 cells. Data are shown as the mean ± SD ($n = 10$ mice per group). The following symbols present the corresponding *P* values determined by repeated measures ANOVA between N and T (*), N and N ($^{\#}$): $^{*/\#}$, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ns, not significant.

from regulatory T cells (Tregs) and decreased PD-1 expression on Tregs. These immunosuppressive activities were not observed in CXCR3$^{-/-}$ mice overexpressing CXCL4, suggesting that CXCR3 mediates the function of CXCL4. Our findings demonstrate a novel T cell regulatory function of non-platelet-derived CXCL4 that cancer cells may hijack to evade the host immune system.

2. Materials and methods

2.1. Mice

C57BL/6 and BALB/c mice were from SLAC Lab Animal Co., Ltd. (Shanghai, China). CXCL4$^{-/-}$ mice [3] and CXCR3$^{-/-}$ mice [13] with a C57BL/6 background were kindly provided by MU-MMRRC (The University of Missouri Mutant Mouse Regional Resource Center) and Dr. Bao Lu (Harvard Medical School). All male mice were used at 6–8 weeks for experimentation. The Animal Care and Use Committee of Shanghai Jiao tong University authorized the animal experiments.

2.2. Cell culture

CT26 and CTLL-2 cells were obtained from ATCC (Rockville, MD, USA) and maintained in 10% FBS-1640 medium (Gibco, Grand Island, USA). The MFC and MC38 cells were purchased from Biovector NTCC, Ltd., (Beijing, China) and cultured in 10% FBS-DMEM and 10% FBS-1640 medium, respectively.

2.3. Overexpression of non-platelet-derived CXCL4 in mice

CXCL4-pcDNA3.1 and pcDNA3.1 plasmids were introduced at 50 μg/mouse through electroporation [9]. Briefly, we injected the plasmids into the skeletal muscle of mice and applied electric pulses directly to the skeletal muscle via “clothespin” electrodes using a square-wave BTX T820 electropulser (BTX, San Diego, CA). Moreover, to achieve durable expression of CXCL4 over a month, we performed plasmid electroporation once a week for four weeks.

2.4. Enzyme linked immunosorbent (ELISA) assays

The supernatant (platelet-poor plasma) of the murine orbital blood was collected [14]. Then, the plasma CXCL4 was measured using a mouse CXCL4/PF4 DuoSet ELISA kit (R&D Systems, Minneapolis, MN).

2.5. Mouse tumor models

The mCXCL4-pcDNA3.1 and pcDNA3.1 plasmids were transferred into C57BL/6, CXCL4$^{-/-}$, and CXCR3$^{-/-}$ mice through intramuscular electroporation once a week for four weeks. Three days after the first electroporation, we injected the mice subcutaneously with 3×10^6 MC38 and monitored the tumor size (mm^3) every 2 days for 4 weeks. In tumor re-challenge experiments, we inoculated BALB/c mice subcutaneously with 1×10^7 CT26 cells. At 2 or 4 weeks post-inoculation, the tumors were resected. Then, a second inoculation of 1×10^7 CT26 or MFC cells was carried out three days after the tumor resection.

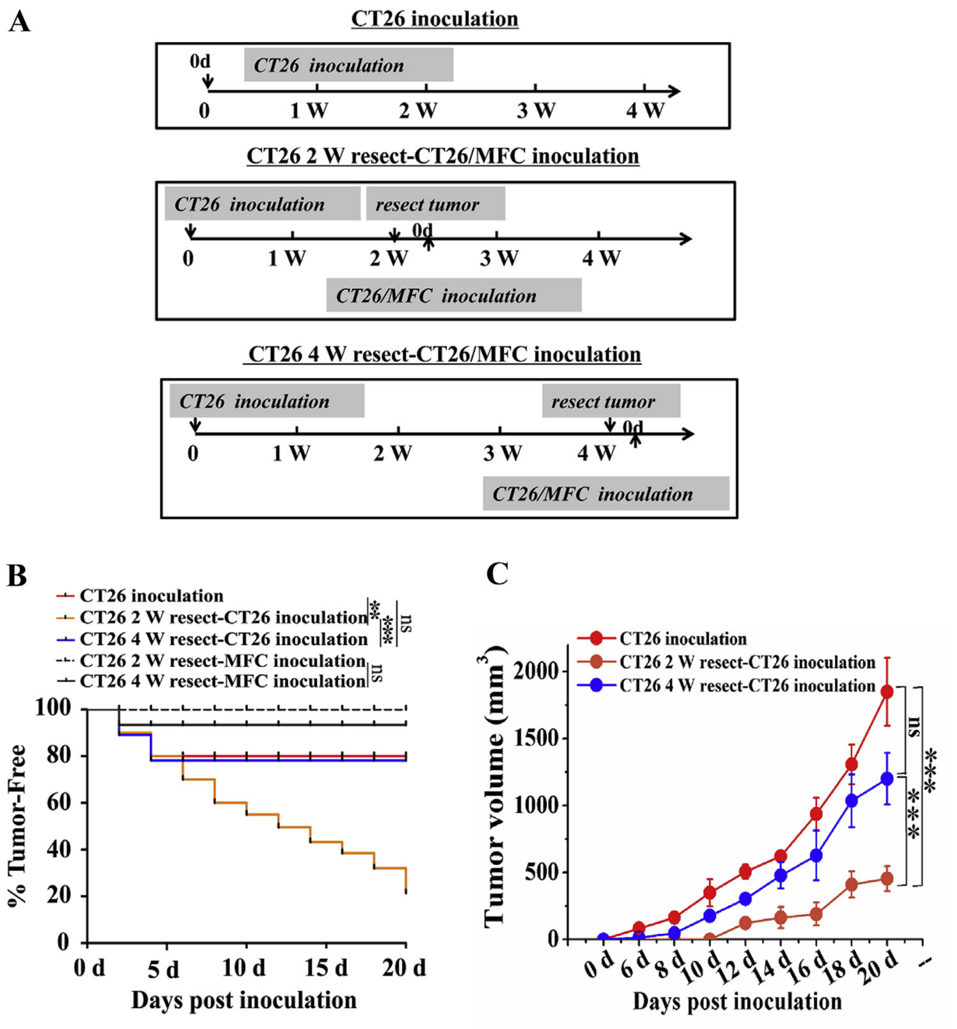

Fig. 2. Inhibition of CT26 colon-cancer growth on second challenge. **(A)** Experimental design. For *CT26 inoculation*, BALB/c mice were challenged with 1×10^7 colon cancer CT26 under one armpit. For *resected tumor*, the CT26 tumor was resected. For *CT26/MFC inoculation*, 1×10^7 CT26 or MFC gastric cancer cells were injected on the opposite side of the armpit three days after the resection. Arrow indicates the time in weeks (w) or days (d) when the operation was performed. **(B, C)** Percentages of tumor-free mice and tumor volumes. Data are shown as the mean ± SD ($n = 6$ mice per group). The following symbols represent the corresponding P values determined by log rank tests (**B**) and repeated measures ANOVA (**C**): **, $P \le 0.01$; ***, $P \le 0.001$.

2.6. Isolation of tumor-infiltrating and splenic lymphocytes

Tumor-infiltrating lymphocytes were isolated according to a previous report [15]. The lymphocyte fraction was isolated after gradient-centrifugation of the cell suspension using Percoll (GE Healthcare Europe, Uppsala, Sweden). T lymphocytes from the spleen were isolated after removing red blood cells using ammonium chloride.

2.7. Antibodies and flow cytometry

Fluorescent dye-conjugated anti-mouse antibodies against CD8 (53–6.7), CD4 (GK1.5), CD25 (PC61.5), Foxp3 (FJK-165), CXCR3 (CXCR3-173), Ki67 (B56), CD69 (H1·2F3), IFN-γ (XMG1.2), TGF-β (TWT-16B4), IL-10 (JES5-16E3), active caspase 3 (C92-605), and PD-1 (J43) and their matched isotype control were from eBioscience (MA, USA) and BD Biosciences (CA, USA). The stained cells were analyzed by an LSRFortessa™ flow cytometer (BD Bioscience, San Diego, CA, USA) and FlowJo version 7.6 software (Tree Star Inc., Ashland, Oregon, USA). A gating strategy for flow cytometry analysis of Tregs is shown in Fig. S1. Tregs were defined as CD4$^+$ cells that were CD25$^+$Foxp3$^+$. Tregs (CD4$^+$CD25$^+$Foxp3$^+$) were used to determine the percentages of Tregs expressing Ki67, CD69, TGF-β, IL-10 and CFSE$^{\text{low}}$. The CD8$^+$ lymphocytes were further analyzed for Ki67, CD69, IFN-γ and CFSE$^{\text{low}}$. The isotype control was used to set the gates for all plots.

2.8. Carboxy-fluorescein succinimdyl ester (CFSE) proliferation assay

For proliferation analysis by the CFSE method [16], 1×10^6 spleen lymphocytes were washed and resuspended in PBS with 0.1% BSA. Then, 5 mM CFSE (Molecular Probes, Eugene, Oregon) was used to label the cells for 10 min in a 37 °C water bath. For stimulation of the labeled cells, CT26 cells were cocultured with them for 5 days.

2.9. Immunoblotting

The CTLL-2 cells were reconstituted in ice-cold RIPA buffer. Then, the protein sample was subjected to SDS-PAGE and blotted with the primary and secondary antibodies. Lastly, the membranes were detected using an ECL detection system (Beyotime Biotechnology, Jiangsu, China). Antibodies against β-actin, p53, Bax, phosphorylated p38 MAPK, and the cleaved forms of caspase-3 were obtained from Cell Signaling Technology (Beverly, MA).

2.10. Statistical analysis

Data are presented as the mean ± SD. Student's unpaired *t*-test and one-way ANOVA were used for comparison of means between two groups or multiple groups, respectively. Repeated measures ANOVA was used to compare the tumor volume between the two groups. Tumor incidence was analyzed using a log-rank test and is expressed as Kaplan–Meier survival curves. A P value of less than 0.05 was considered statistically significant.

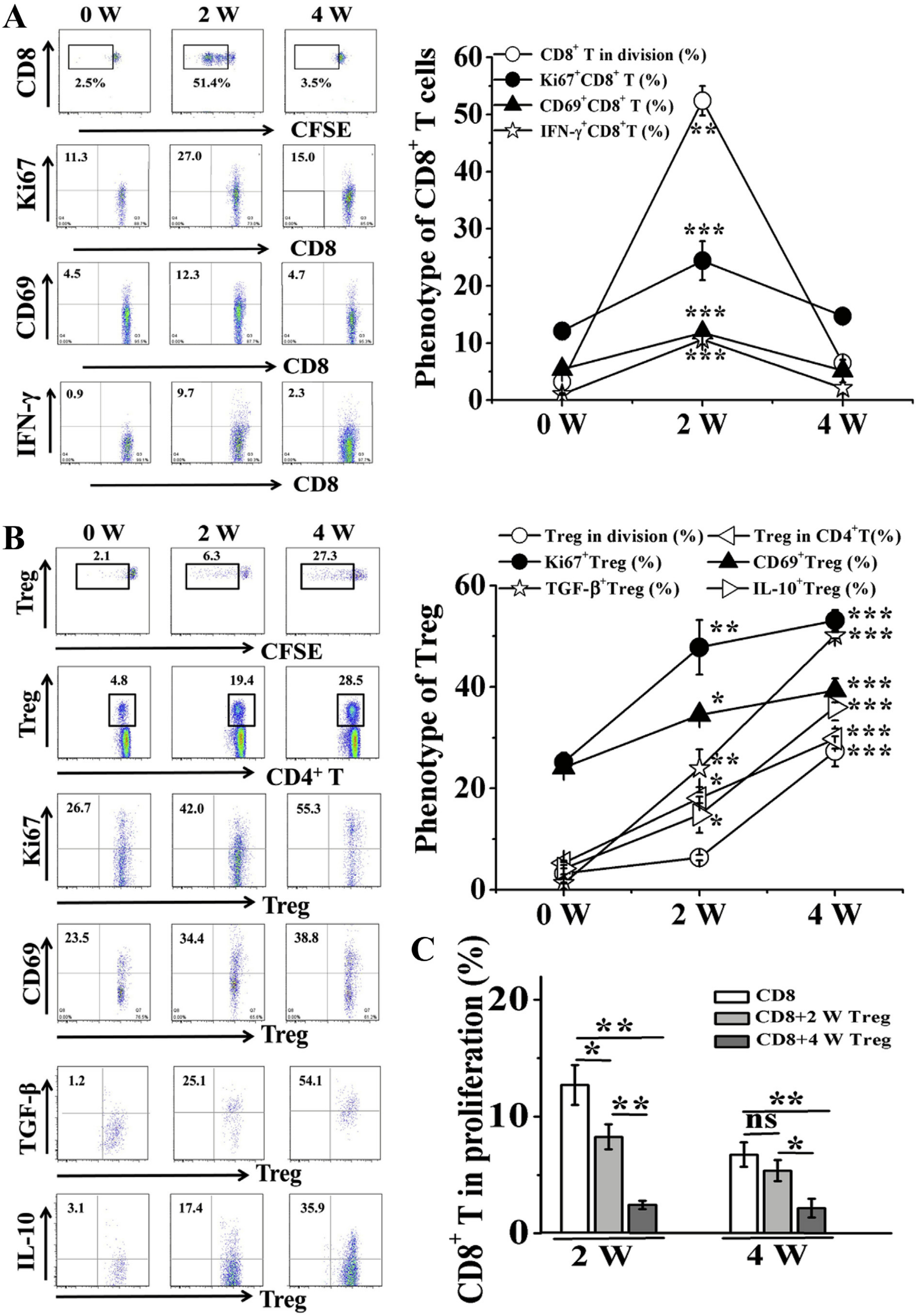

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Fig. 3. Activation of CD8$^+$ T and Treg cells in CT26 tumor-bearing mice. Splenocytes of BALB/c mice challenged with 1×10^6 CT26 cells were collected at 0, 2, and 4 weeks (w) after the tumor cell injection. **(A, B)** The splenocytes restimulated with CT26 cells in vitro were analyzed by flow cytometry for the percentages of CFSE^{low} and IFN-γ$^+$ in CD8$^+$ T cells and CFSE^{low}, TGF-β$^+$ and IL-10$^+$ in Tregs (CD4$^+$CD25$^+$Foxp3$^+$). The Ki67$^+$, CD69$^+$ and CD8$^+$ T cells and Tregs were analyzed directly without culture. Representative dot plots (left) and summary graphs (right) show the functional status of CD8$^+$ T cells and Tregs. Data are shown as the mean ± SD ($n = 12$ mice per group). The following symbols represent the corresponding P values determined by one-way ANOVA, 2 w and 4 w versus 0 w: *, $P \leq 0.0$; **, $P \leq 0.01$; ***, $P \leq 0.001$. **(C)** The CD8$^+$ T cells and Tregs isolated from the splenocytes at 2 w and 4 w were cocultured in the presence of CT26 cells and IL-2. The bar graph shows the percentage of CFSE^{low}CD8$^+$ T cells in total CD8$^+$ T cells. Data are shown as the mean ± SD ($n = 6$ mice per group). The following symbols represent the corresponding P values determined by one-way ANOVA: *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ns:, not significant.

3. Results

3.1. Non-platelet-derived CXCL4 accelerates colon cancer growth in mice

Many previous studies analyzed xenografts of immunodeficient mice or performed intratumoral injection of CXCL4 at high concentrations to demonstrate that CXCL4 suppressed tumor growth [17–20]. To clarify the conflicting results, we overexpressed CXCL4 in immunocompetent MC38 tumor-bearing mice.

The mCXCL4-pcDNA3.1 and control pcDNA3.1 plasmids were constructed (Fig. 1A) and transferred into C57BL/6, CXCL4$^{-/-}$, and CXCR3$^{-/-}$ mice through intramuscular electroporation. We named the mice administered the control plasmid N (non-transferred CXCL4) and the mice administered the mCXCL4 plasmid T (transferred CXCL4). CXCL4 expression in the muscles reached the highest levels at days 2 and 4 and was detected for 7 days (Fig. 1B). The CXCL4 plasma level in CXCL4$^{-/-}$ mice reached sub-nanomolar concentrations in a similar temporal pattern as the expression in muscles (Fig. 1C), suggesting successful secretion of CXCL4 protein into circulation. To sustain this CXCL4 level over 4 weeks, we repeatedly transferred the CXCL4 plasmid and empty plasmid four times at a weekly interval.

Tumor growth was significantly accelerated in C57BL/6-T mice and CXCL4$^{-/-}$-T mice compared with the empty-vector controls (Fig. 1D). However, the tumor growth in CXCR3$^{-/-}$-T mice was not affected (Fig. 1D). Furthermore, the tumor growth in CXCR3$^{-/-}$−N mice was slower than that in C57BL/6-N and CXCL4$^{-/-}$−N mice (Fig. 1D). Taken together, our data suggest that non-platelet-derived CXCL4 accelerates colon cancer growth in a CXCR3-dependent manner.

3.2. Suppression of CT26 colon cancer upon re-challenge in immunocompetent mice

The host immune response to colorectal cancer (CRC) is generally low, and clinical immunotherapy is not successful [21]. Thus, we carried out tumor re-challenge experiments to verify whether host immune surveillance against colon cancer exists in mice [22]. CT26 colon cancer or MFC gastric cancer [23,24] was used to address the cell specificity of the host response. We found that the tumor incidence of CT26, but not MFC, re-challenged at 2 weeks was significantly lower than that at 4 weeks (Fig. 2B). Consistently, the CT26 re-challenged at 2 weeks grew significantly slower than that re-challenged at 4 weeks (Fig. 2C). The data suggest that the immunocompetent mice are able to limit CT26 tumor growth upon a second challenge. We speculate that tumor-bearing mice have developed a time-limited immune response to CT26.

3.3. Activation of CD8$^+$ T cells and Tregs in mice with colon cancer

To confirm the time-limited immune response to CT26, we analyzed the activation status of CD8$^+$ T and Tregs (CD4$^+$CD25$^+$Foxp3$^+$) in the spleen of tumor-bearing mice, where high levels of circulating T cells frequently pass through to regulate innate and adaptive immunity [25]. The CT26-specific proliferation of CTLs and Tregs was analyzed by CFSE label-retaining assays [16] and nuclear staining of Ki67 [26]. Their activation was revealed by detection of CD69 [27].

We observed that the proliferation, activation, and IFN-γ production of CTLs were at the highest levels at 2 weeks and returned back to baseline at 4 weeks (Fig. 3A). We previously confirmed that CTLs from the isolated splenocytes of CT26 tumor-bearing mice at 2 weeks showed effective cytolytic effects on CT26 cells in vitro [9]. In contrast, the proliferation, activation, and TGF-β and IL-10 production of Tregs were continuously enhanced from 0 to 4 weeks (Fig. 3B). Next, the enhanced function of Tregs in suppressing proliferation of CTLs was confirmed. The proliferation of CD8$^+$ T cells (2 weeks) was inhibited by Tregs from 2 to 4 weeks. However, the proliferation of CD8$^+$ T cells (4 weeks) was suppressed by the Tregs only after 4 weeks (Fig. 3C) and not after 2 weeks.

Collectively, these data demonstrate that CT26 can indeed induce the activation of CD8$^+$ T cells and immunosuppressive Tregs in mice. However, activation of Tregs that lasted for 4 weeks compared to 2 weeks for the CD8$^+$ T cells is detrimental to the host. These findings characterize a mouse model that can be used for further analysis of colon cancer immunity and its regulation.

3.4. Non-platelet-derived CXCL4 suppresses the immune response to colon cancer through CXCR3

Previously, we reported that CXCL4 did not affect the proliferation of CT26 cells in vitro [9]. Thus, we hypothesized that CXCL4 suppresses immune function and thereby promotes colon cancer growth in vivo.

First, we observed the opposite expression change of CXCR3 on CTLs and Tregs in C57BL/6-N/T tumor-bearing mice. That is, CXCR3 was elevated on CD8$^+$ T cells at 2–4 weeks (Fig. 4A) but was decreased on Tregs at 2–4 weeks compared with 0 weeks (Fig. 4B). Furthermore, CXCL4 had no effect on CXCR3 expression in C57BL/6-N/T mice (Fig. 4A and B).

Next, we found that CXCL4 reduced CD69$^+$CD8$^+$ and Ki67$^+$CD8$^+$ T cells in CXCL4$^{-/-}$-T mice, but not in CXCR3$^{-/-}$-T mice, compared with those in empty-vector controls at 2 weeks (Figs. S2, A, C) (Figs. S2, B, D). Strikingly, its effect on Tregs was completely opposite to that on CD8$^+$ T cells (Fig. S3). The high ratio of CD8$^+$ T/Tregs at 2 weeks decreased to baseline (0 weeks) (Fig. 4C and D), suggesting that the immune response to colon cancer is completely abolished in mice overexpressing CXCL4. We also observed that CXCL4 significantly reduced IFN-γ$^+$CD8$^+$ T cells (Fig. 4E) but increased TGF-β$^+$Tregs in the CXCL4$^{-/-}$-T mice (Fig. 4F) compared with the CXCL4$^{-/-}$−N mice at 2 weeks.

Taken together, our data demonstrate that CXCL4 inhibits the activation of CD8$^+$ T cells but promotes the activation of Tregs in MC38-bearing mice in a CXCR3-dependent manner.

3.5. Non-platelet-derived CXCL4 accelerates apoptosis of CD8$^+$ T cells through CXCR3

The CD8$^+$ T cells activated at 2 weeks were not detectable at 4 weeks even though the tumor antigen was present (Fig. 3). To determine whether CXCL4 was involved in the hypofunction of CD8$^+$ T cells, we evaluated the influence of CXCL4 on caspase 3-mediated apoptosis and PD-1 expression in the CD8$^+$ T cells of MC38 tumor-bearing mice. At 2 weeks, the PD-1 expression and apoptosis of CD8$^+$ T cells increased over the baseline at 0 weeks in C57BL/6-N and CXCR3$^{-/-}$−N mice (Fig. 5A and B). Then, PD-1 expression and apoptosis were further elevated at 4 weeks compared to those at 2 weeks in C57BL/6-N

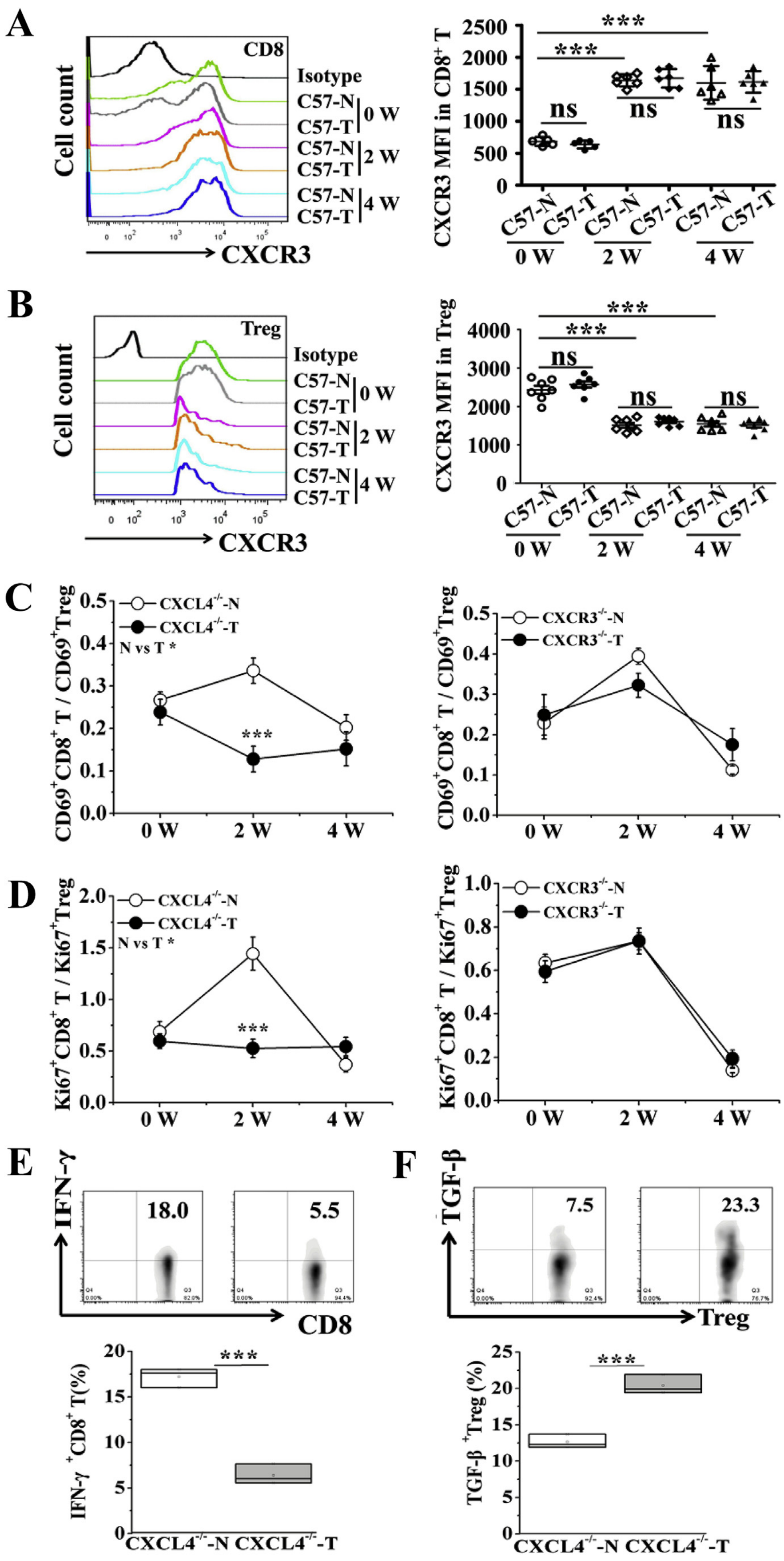

Fig. 4. CXCL4 reduces the ratio of activated CD8$^+$ T to Tregs through CXCR3. C57BL/6 (**C57**), CXCL4$^{-/-}$ and CXCR3$^{-/-}$ mice were transfected by electroporation with mCXCL4-pcDNA3.1 (**T**) or empty vector (**N**) once a week for four weeks. The mice were inoculated with syngeneic MC38 colon cancer cells three days after the first electroporation. Mouse splenocytes were analyzed by flow cytometry at 0, 2 and 4 weeks (**w**) after the tumor inoculation. (**A, B**) Histograms (right) and scatter plots (left) show the mean fluorescence intensity (MFI) of CXCR3 in CD8$^+$ T and Tregs at the indicated times. (**C, D**) Line graphs show the ratio of CD69$^+$CD8$^+$ per CD69$^+$Tregs and Ki67$^+$CD8$^+$ per Ki67$^+$Tregs in CXCL4$^{-/-}$ (right) and CXCR3$^{-/-}$ mice (left). **E, F**) Representative density plots (top) and boxplots (bottom) show percentages of IFN-γ$^+$CD8$^+$ T in total CD8$^+$ T cells (left) and TGF- β$^+$ Tregs in total Tregs (right) at 2 weeks. Data are shown as the mean ± SD (n = 6–7 mice per group). The following symbol represents the corresponding P values determined by two-sided unpaired t tests between N and T: ***, $P \leq 0.001$.

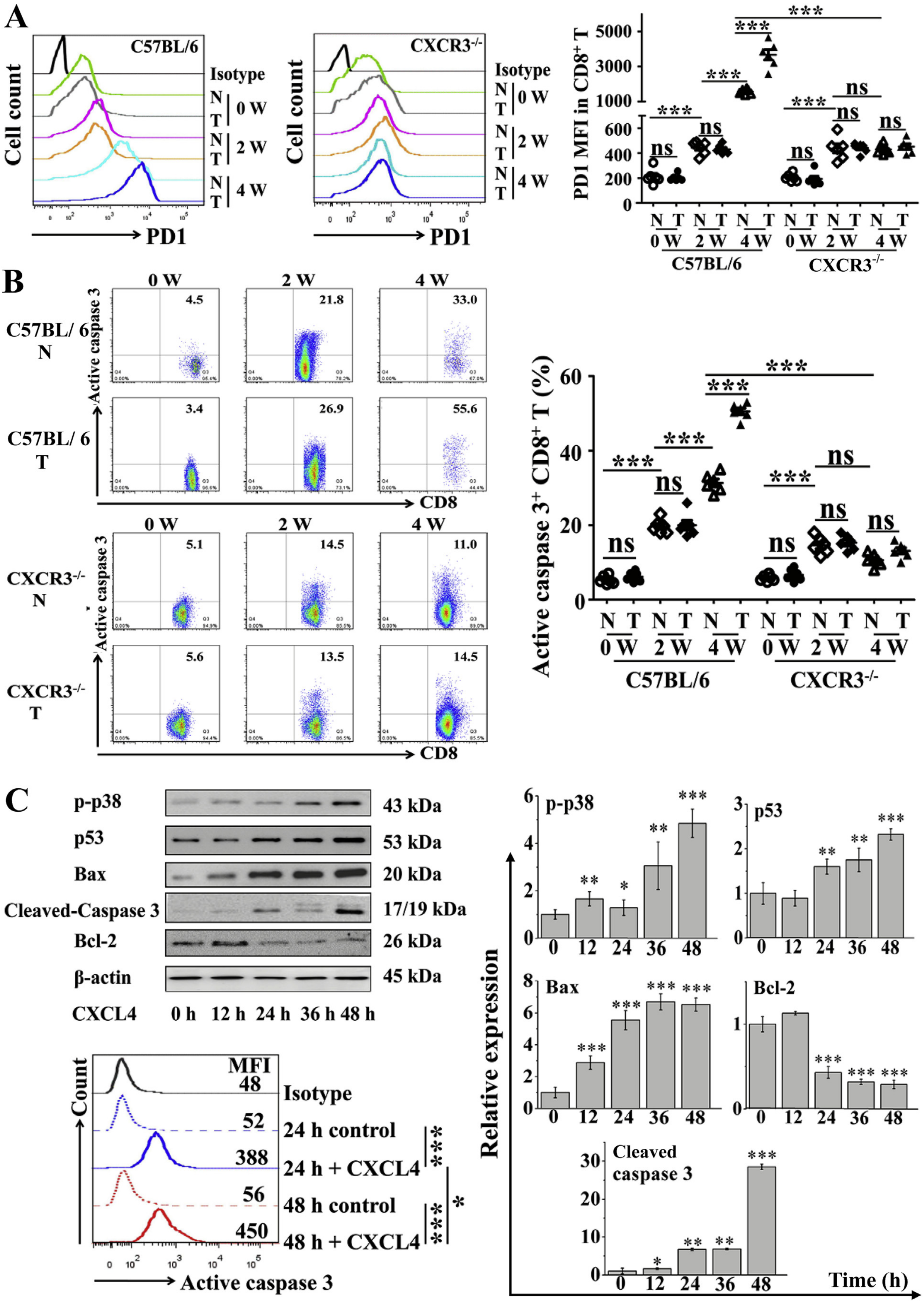

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Fig. 5. CXCL4 promotes apoptosis of $CD8^+$ CTLs through CXCR3. **(A, B)** C57BL/6 (**C57**) and $CXCR3^{-/-}$ mice were transfected by electroporation with mCXCL4-pcDNA3.1 (T) or empty vector (N) once a week for four weeks. The mice were inoculated with syngeneic MC38 colon cancer cells three days after the first electroporation. Data are shown as the mean ± SD ($n = 6–8$ mice per group). **(A)** Histograms and scatter plots show the mean fluorescence intensity (MFI) of PD-1 in $CD8^+$ T cells. The following symbols represent the corresponding P values determined by one-way ANOVA: ***, $P \le 0.001$; ns, not significant. **(B)** Dot plots and summary graph (right) show the percentage of active caspase-3^+CD8^+ T cells. The following symbol represents the corresponding P values determined by two-sided unpaired t tests: ***, $P \le 0.001$. **(C)** A mouse CTL cell line (CTLL-2) was cultured with recombinant human CXCL4 (rhCXCL4) at 15 μg/mL for 48 h. The cells were analyzed using flow cytometry with antibodies against cleaved caspase-3. The cell extract was analyzed using Western blots with antibodies against p-p38, p53, Bax, cleaved caspase-3, and Bcl-2 for the indicated times after initiation of the culture. β-actin was the loading control in the Western blot. Western blots (top) and their quantifications (right) show the expression levels of the detected proteins at the indicated times relative to those at time 0. Histograms show MFI of active caspase 3 in CTLL-2 by flow cytometry (bottom). Data are shown as the mean ± SD ($n = 3$ per group). The following symbols represent the corresponding P values determined by one-way ANOVA: *, $P \le 0.05$; **, $P \le 0.01$; ***, $P \le 0.001$.

mice only (Fig. 5A and B), suggesting that the apoptosis of $CD8^+$ T cells is associated with PD-1 expression [28]. In addition, at 4 weeks, CXCL4 increased the PD-1 level and apoptosis of $CD8^+$ T cells in C57BL/6-T mice, but not $CXCR3^{-/-}$-T mice, compared with the empty-vector controls (Fig. 5A and B). The data suggest that non-platelet-derived CXCL4 exacerbates apoptosis of $CD8^+$ T cells in association with upregulation of PD-1 expression, which is dependent upon CXCR3. Finally, we confirmed the apoptotic signaling pathway induced by CXCL4 (Fig. 5C). A time-dependent increase in cleaved-caspase 3, phosphorylated p38 MAPK, p53, Bax, and downregulation of Bcl-2 were detected in CTLL-2 cells treated with CXCL4 protein. Taken together, our data suggest that non-platelet-derived CXCL4 induces apoptosis of $CD8^+$ T cells, possibly using the classical apoptosis pathway through p38-MAPK.

3.6. Non-platelet-derived CXCL4 suppresses tumor-infiltrating $CD8^+$ T cells through CXCR3

After establishing the role of the CXCL4/CXCR3 axis in the regulation of CTLs and Tregs in the spleen [25], we proposed that CXCL4 also suppresses the local tumor immunity.

At 2 weeks, we observed that CXCL4 reduced $Ki67^+CD8^+$ T cells in C57BL/6-T mice, but not $CXCR3^{-/-}$-T mice, compared with the empty-vector controls (Fig. 6A). Furthermore, the percentage of $Ki67^+CD8^+$ T cells dropped in both types of mice at 4 weeks (Fig. 6A). Importantly, the extent of the drop in $CXCR3^{-/-}$—N mice was significantly less than that in C57BL/6-N mice (Fig. 6A). Taken together, these data suggest that non-platelet-derived CXCL4 suppresses the proliferation of tumor-infiltrating $CD8^+$ T cells through CXCR3.

At 4 weeks, CXCL4 exacerbated apoptosis in C57BL/6-T mice but not $CXCR3^{-/-}$-T mice compared to empty-vector controls at 2 weeks (Fig. 6B). Importantly, the apoptosis at 4 weeks was much lower in the $CXCR3^{-/-}$—N mice than in the C57BL/6-N mice (Fig. 6B). Furthermore, CXCL4 increased PD-1 expression on tumor-infiltrating $CD8^+$ T cells in C57BL/6-T mice, but not $CXCR3^{-/-}$-T mice, compared with the empty-vector controls (Fig. 6C). Moreover, the PD-1 expression in C57BL/6-N mice was drastically higher than that in $CXCR3^{-/-}$—N mice (Fig. 6C). Taken together, our findings suggest that non-platelet-derived CXCL4 promotes apoptosis and PD-1 expression in tumor-infiltrating $CD8^+$ T cells via CXCR3.

3.7. Non-platelet-derived CXCL4 enhances the proliferation of tumor-infiltrating Tregs through CXCR3

After observing the role of CXCL4 in tumor-infiltrating $CD8^+$ T cells, we proposed that CXCL4 might also affect tumor-infiltrating Tregs.

At 4 weeks, we observed that CXCL4 increased $Ki67^+$Tregs in C57BL/6-T mice, but not $CXCR3^{-/-}$-T mice, compared with the empty-vector controls (Fig. 7A). Additionally, $Ki67^+$Tregs in the $CXCR3^{-/-}$—N mice were significantly lower than those in C57BL/6-N mice at 2 and 4 weeks (Fig. 7A). Taken together, our findings suggest that non-platelet-derived CXCL4 promotes the expansion of tumor-infiltrating Tregs via CXCR3.

Overall, neither the overexpression of CXCL4 nor the knockout of CXCR3 affected the apoptosis of Tregs (Fig. 7B). Additionally, CXCL4 strongly decreased the expression of PD-1 on Tregs in C57BL/6-T mice, but not $CXCR3^{-/-}$-T mice, compared with the empty-vector controls at both 2 and 4 weeks (Fig. 7C). These findings suggest that CXCL4 inhibits PD-1 expression on tumor-infiltrating Tregs via CXCR3. In the absence of CXCL4 overexpression, PD-1 expression at 4 weeks was lower than that at 2 weeks in both types of mice, indicating that additional signals other than the CXCL4/CXCR3 axis regulate its expression. We speculate that the tumor-infiltrating Tregs may gain a survival advantage through loss of PD-1 expression.

4. Discussion

By increasing the production of CXCL4 in $CXCL4^{-/-}$ and $CXCR3^{-/-}$ mice, we demonstrated that CXCL4 in a platelet-unrelated native form accelerated colon cancer growth through its canonical CXCR3 receptor. Mechanistically, overexpressing CXCL4 suppressed the immune response to colon cancer. Specifically, CXCL4 via CXCR3 exerted several immune regulatory functions, including (1) inhibition of $CD8^+$ T cell activation, (2) promotion of Tregs activation, and (3) increased apoptosis of $CD8^+$ T cells but not Tregs, which was associated with the changes in expression of PD-1.

CXCL4 was first recognized as an angiostatic chemokine that inhibited tumor growth at high concentrations [20,29]. However, clinical trials with recombinant CXCL4 failed to reduce tumor growth [30,31]. Consistently, high plasma CXCL4 levels correlated with poor overall survival in patients with lung cancer [8,32]. Importantly, CRC patients with increased expression of CXCR3 at the tumor site also had poor overall survival [33]. In addition, the receptor CXCR3 was shown to contribute to skin tumorigenesis [34]. In this study, we used non-viral expression cassettes to produce non-platelet-derived CXCL4 at its physiological concentration, which is consistent with previous studies [8,35]. Our repeated electroporation approach in tumor-bearing mice with different genetic backgrounds ($CXCL4^{-/-}$, $CXCR3^{-/-}$, C57BL/6) enabled us to establish a positive association between overproduction of non-platelet-derived CXCL4 and faster tumor growth and identify CXCL4 as a causal factor driving this phenotype.

We primarily evaluated colon cancer in a syngeneic mouse model because CRC is refractory to immunotherapy. The modest success with checkpoint inhibitors in CRC patients highlighted the resistant nature of CRC to immune-based therapies [36]. Low expression of PD-L1 in CRC correlated with a poor response to PD-1 or PD-L1 inhibition [37]. Here, we first reported that the immune response to colon cancer fades away due to (1) apoptosis of CTLs associated with PD-1 upregulation, (2) continued proliferation of Tregs, and (3) enhanced survival of Tregs with PD-1 downregulation. Our studies suggest that PD-1 inhibition alone cannot reverse immunosuppression by Tregs in CRC. Targeting CXCL4 may compensate for the deficiency of PD-1 inhibition in CRC immunotherapy.

In vitro studies of human peripheral T cells showed that CXCL4 at μM concentration in culture suppressed the proliferation and cytokine production of T cells [11,12]. In the present study, we report that in the context of colon cancer in vivo, non-platelet-derived CXCL4 exhibits a

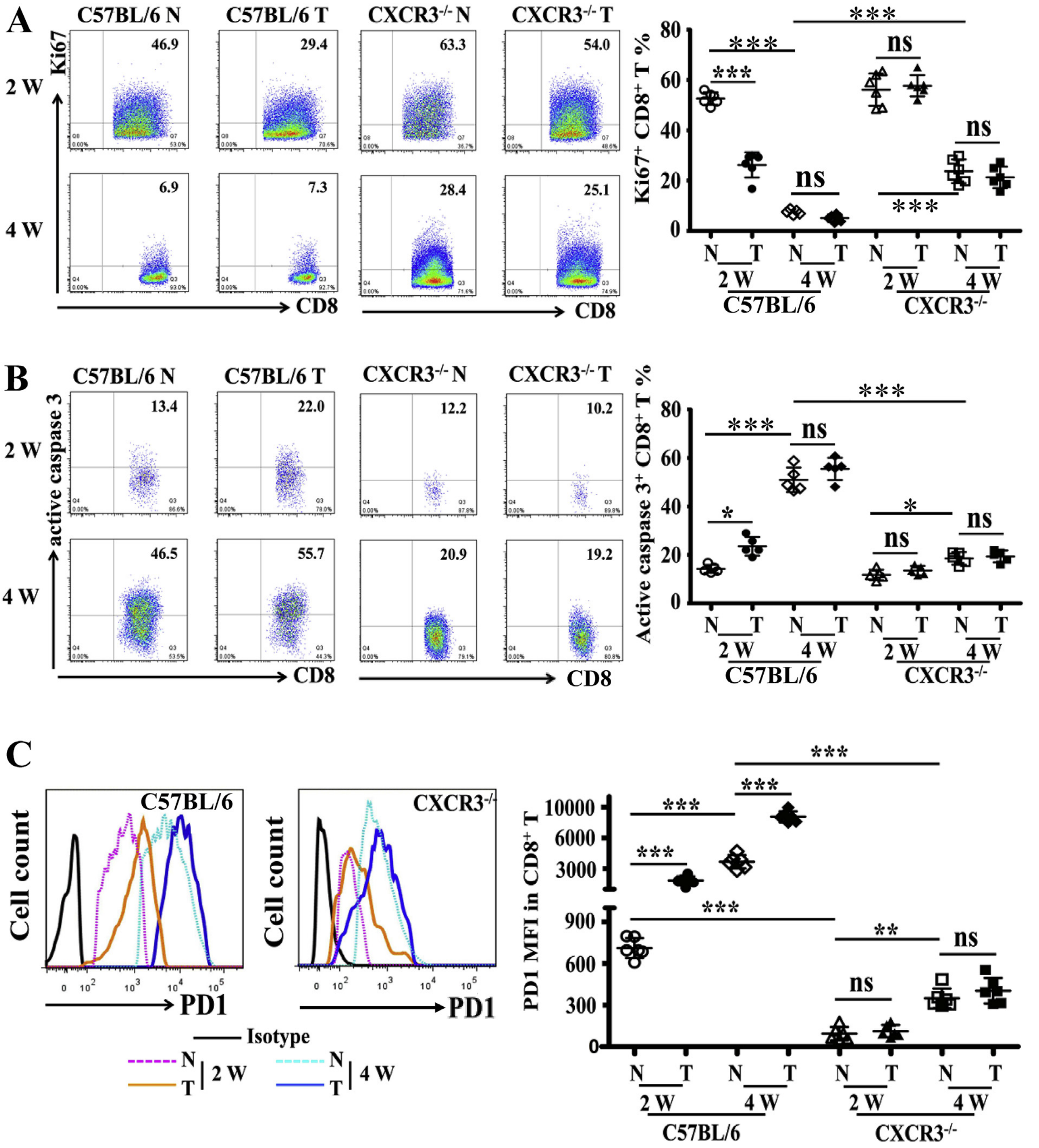

Fig. 6. The CXCL4/CXCR3 axis reduces proliferation and increases apoptosis of tumor-infiltrating CD8$^+$ T cells. C57BL/6 (**C57**) and CXCR3$^{-/-}$ mice were transfected by electroporation with mCXCL4-pcDNA3.1 (**T**) or empty vector (**N**) once a week for four weeks. The mice were inoculated with syngeneic MC38 colon cancer cells three days after the first electroporation. Tumor-infiltrating lymphocytes at 2 and 4 weeks (**w**) after the tumor cell injection were analyzed by flow cytometry. (**A, B**) Representative dot plots (left) and the scatter plot (right) show the percentages of Ki67$^+$CD8$^+$ T in total CD8$^+$ T cells and active caspase-3$^+$CD8$^+$ T in total CD8$^+$ T cells. (**C**) Representative histograms (left) and scatter plot (right) show mean fluorescence intensity (MFI) of PD-1 in CD8$^+$ T cells. Data are shown as the mean ± SD ($n = 6$ mice per group). The following symbols represent the corresponding *P* values determined by two-sided unpaired *t* tests between N and T (*) or N and N ($^\#$): $^{*/\#}$, $P \leq 0.05$; $^{**/\#\#}$, $P \leq 0.01$; $^{***/\#\#\#}$, $P \leq 0.001$.

suppressive role in anticancer immunity by affecting both CTLs and Tregs. Thus, our findings in vivo further expand and strengthen the previous in vitro studies supporting the differential roles of non-platelet-derived CXCL4 in the regulation of CTLs and Tregs in cancer immunology.

CXCR3 has been previously described as a CXCL4 receptor [6,38,39]. In humans, CXCR3-B serves as a receptor for CXCL4, mediating its angiostatic effect in endothelial cells [6], and CXCR3-A shows poor binding to CXCL4 [40]. However, no murine CXCR3 variants have been identified with distinct functions; murine CXCR3 has both the angiostatic and chemotactic activities of CXCL4 [6,41]. CXCR3 is expressed on activated T cells [42,43]. We observed that its expression was elevated only on CD8$^+$ T cells in tumor-bearing mice (Fig. 4A). In contrast, its expression on Tregs was downregulated (Fig. 4B). CXCL4 had no effect on its expression on either cell type (Fig. 4A and B). Although the roles of CXCL4 in the regulation of both CTLs and Tregs required CXCR3, we could not eliminate the possibility that additional receptors, such as the recently reported CCR1, account for the opposite

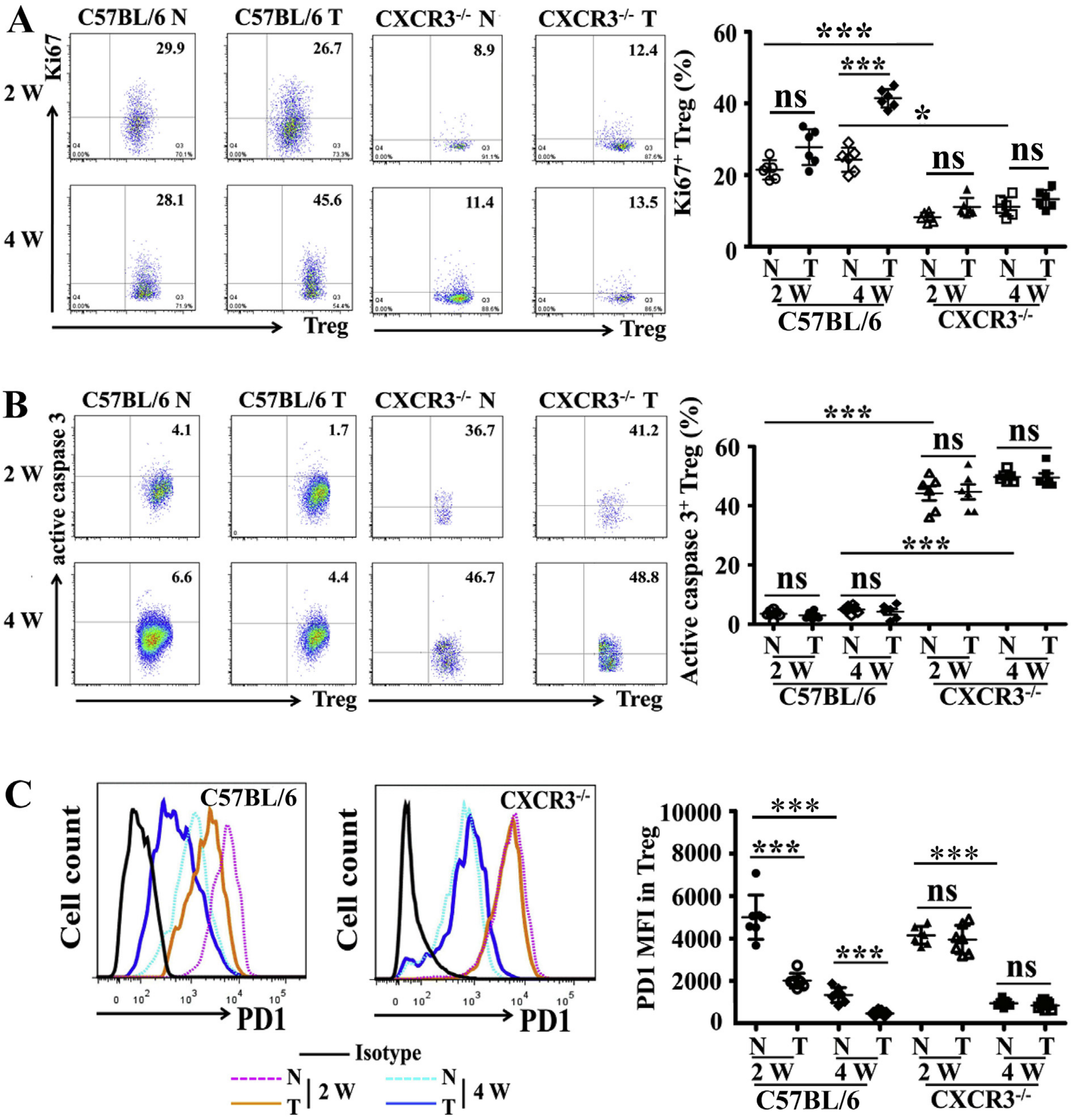

Fig. 7. CXCL4 enhances proliferation of tumor-infiltrating Tregs via CXCR3. C57BL/6 (**C57**) and CXCR3$^{-/-}$ mice were transfected by electroporation with mCXCL4-pcDNA3.1 (**T**) or empty vector (**N**) once a week for four weeks. The mice were inoculated with syngeneic MC38 colon cancer cells three days after the first electroporation. Tumor-infiltrating lymphocytes at 2 and 4 weeks (**w**) after the tumor cell injection were analyzed by flow cytometry. (**A**) Representative dot plots (left) and the scatter plot (right) show the percentages of Ki67$^+$Tregs. (**B, C**) Representative histograms (left) and scatter plot (right) show mean fluorescence intensity (MFI) of active caspase-3$^+$ (**B**) and PD-1 (**C**) in Tregs. Data are shown as the mean ± SD ($n = 6$ mice per group). The following symbols represent the corresponding P values determined by two-sided unpaired t tests between N and T (*) or N and N (#): */#, $P \leq 0.05$; **/##, $P \leq 0.01$; ***/###, $P \leq 0.001$.

roles of CXCL4 in CTLs and Tregs [44].

CXCR3 increases recruitment of CD4$^+$ and CD8$^+$ effector T cells to drive inflammation and Th1-specific Tregs to dampen excess responses [45]. Therefore, CXCR3 through chemotaxis may act as either an immunological facilitator or inhibitor in response to CXCL-9 and -10. The manner in which these differential functions are decided remains unknown. In this study, the immunosuppressive role of CXCR3 activated by non-platelet-derived CXCL4 may provide a rationale. We speculate that the local concentration of CXCL4 secreted by the tumor cells and/or host cells in the microenvironment affects the function of recruited T cells. Future studies are needed to address this hypothesis.

Our findings may shed light on the development of CXCR3 antagonists for Th-1 inflammatory diseases [46]. AMG 487, a CXCR3 inhibitor, failed to show efficacy in a clinical trial treating psoriasis [47]. The rationale for the clinical study was based on CXCR3 expression on T cells infiltrating psoriatic skin lesions. CXCR3 ligands are abundantly expressed in lesions [48,49]. Aside from the chemotaxis function of CXCR3, its immunosuppressive roles reported here need to be investigated in the context of psoriasis.

In conclusion, we identified the immunosuppressive function of the CXCL4/CXCR3 axis on T lymphocytes in both the tumor microenvironment and secondary lymphoid organ (spleen). These findings add complexity to the roles of CXCR3, specifically chemotaxis, the initially identified function of this receptor. Future studies need to reconcile the two functions in the context of Th-1-type adaptive immunity. This study focused on colon cancer in a murine engraftment model. Given the complex interaction between cancer and its host, research using genetic tumor models and other tumor types is warranted. Nevertheless, our findings suggest that pharmacological targeting of the CXCL4/CXCR3 axis using antibodies or small molecules may provide novel treatment for colon cancer.

Conflicts of interest

The authors declare no financial or other conflict of interest with regard to this research.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2018.11.017.

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