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Original Articles

Interleukin-10 attenuates tumour growth by inhibiting interleukin-6/signal transducer and activator of transcription 3 signalling in myeloid-derived suppressor cells

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ABSTRACT

Interleukin-10 (IL-10) is a well-characterized anti-inflammatory cytokine, but its role in anti-cancer immunity is controversial. After injection with TC-1 cancer cells, we observed more rapid tumour growth and significantly higher interleukin-6 (IL-6) production in IL-10 knockout (IL-$10^{-/-}$) mice than wild-type (WT) mice. Blocking IL-6 with an anti-IL-6 receptor (IL-6R) monoclonal antibody (mAb) inhibited tumour growth and myeloid-derived suppressor cell (MDSC) generation, which were significantly increased in IL-10-deficient mice. MDSCs and tumour cells from IL-$10^{-/-}$ mice had increased phosphorylated signal transducer and activator of transcription 3 (p-STAT3) levels. Treatment with a STAT3 inhibitor, S3I, reduced tumour growth, inhibited MDSC expansion, reduced IL-6 in tumours, and relieved T cell suppression. The combination of anti-IL-6R mAb and S3I further inhibited tumour growth compared to S3I treatment alone. These results suggested that the inhibition of the IL-6/STAT3 signalling axis is a candidate anti-cancer strategy, especially under systemic inflammatory conditions with high IL-6.

Introduction

Myeloid-derived suppressor cells (MDSCs) are an immunosuppressive population consisting of precursors of macrophages, dendritic cells, granulocytes, and so on [1]. MDSCs expand and accumulate in pathological conditions, including infections, inflammation, and cancer [1,2]. In the case of cancer, MDSCs, which are induced by both tumour-derived and host-derived factors, inhibit anti-tumour immune responses and contribute to the establishment of an immunosuppressive tumour environment [3,4]. MDSCs suppress T cell proliferation and inhibit T cell function. They also induce regulatory T cell (Treg cell) activation and expansion [5]. Immune suppression of MDSCs is not restricted to T cell responses, and MDSCs downregulate natural killer (NK) cell activity [6] and dendritic cell function [7], which play critical roles in anti-tumour immunity. Therefore, the regulation of MDSCs is considered one of the most important steps in anti-tumour immunotherapy.

IL-10 is clearly indispensable in the regulation of inflammation and may be related to the immunosuppressive functions of MDSCs [8]. However, since it was discovered in the early 1990s, there is conflicting evidence regarding the precise role of IL-10 in cancer development. It has been reported that IL-10 mediates tumour-inhibitory effects in various cancer types. Although increased levels of IL-10 have been detected in biopsies and premalignant lesions of cervical cancer patients [9], decreased IL-10 levels are also associated with the risk of cervical cancer [10]. In other cancer types, a hypomorphic variant that produces low levels of IL-10 is a high risk factor for gastric adenocarcinoma [11], and low levels of IL-10 are associated with prostate cancer development [9,12]. In several cases, IL-10 is involved in tumour promotion. For example, IL-10 expression is highly associated with the development of HPV-positive cervical cancer [13]. In a mouse model of tumour transplantation, tumour-infiltrating macrophages produce IL-10, which might promote the induction of Treg cells and MDSCs as immune evasion mechanisms to facilitate tumour growth [14].

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The tumour-inhibitory effect of IL-10 might be related to its ability to modulate other cytokines or pro-tumoural mediators. IL-10 inhibits tumour growth via the inhibition of several inflammatory and angiogenic factors, including vascular endothelial growth factor, interleukin-1β (IL-1β), TNF-α, and IL-6 [11]. Blocking IL-1β ameliorates tumour growth enhanced by IL-10 deficiency [15]. Unexpectedly, IL-10 inhibits the production of inflammatory cytokines and dampens the generation of MDSCs as well as Treg cells in the tumour environment, and the depletion of MDSCs restores tumour growth in IL-10-deficient conditions [15]. Lipopolysaccharide-stimulation increases the production of IL-1α and IL-1β by MDSCs, and IL-1 blockade by IL-1 receptor α (IL-1Rα) efficiently inhibits tumour growth in IL-10-deficient conditions.

Similar to IL-10 deficiencies, IL-1R-deficient mice exhibit delayed accumulation of MDSCs after tumour transplantation and show reduced tumour growth with decreased potential for inflammation [16]. Interestingly, the decreased accumulation of MDSCs and tumour progression in IL-1R-deficient mice are restored via the additional expression of IL-6 by tumour cells, suggesting that IL-6 is a downstream mediator of IL-1R signalling [16]. In addition, the decreased IL-6 production and reduced expression of p-STAT3 in C/EBP-homologous protein (CHOP)-deficient MDSCs may be associated with the decreased suppressive function of MDSCs, conferring antitumour immunity to T cells [17]. IL-6 overexpression on cancer cells restores the immune-suppressive activity of CHOP-deficient MDSCs. Based on these previous reports, we inferred that IL-6R is a plausible target to restrict the MDSC expansion observed in IL-10-deficient mice.

In the current study, we examined the mechanisms of increased tumour growth in IL-10-deficient mice. We adopted a TC-1 cervical cancer model with subcutaneously (s.c.) transplanted cancer cells. Based on our initial finding that IL-10-deficient mice with TC-1 tumour transplantation produced significantly higher levels of IL-6, we administered anti-IL-6R monoclonal antibodies (mAbs) to tumour-bearing mice and found that IL-6R blockade efficiently slowed TC-1 tumour growth in wild-type (WT) and IL-10-deficient mice. The levels of p-STAT3 in MDSCs from IL-10-deficient mice were significantly higher than those from control mice, and were decreased by the IL-6R blockade. Treatment of mice with a STAT3 inhibitor (S3I) dampened the production of IL-6 in tumour tissues, and co-treatment with the IL-6R blockade and STAT3 inhibitor had greater anti-tumour effects than S3I treatment alone in IL-10-deficient mice. Collectively, these data suggest that IL-10 attenuate tumour growth by inhibiting IL-6/STAT3 axis on MDSCs.

Materials and methods

Mice

All experiments were approved by the Institutional Animal Care and Use Committee of Kangwon National University. C57BL/6 mice were purchased from Charles River Laboratories (Orient Bio Inc., Sungnam, Korea). IL-10$^{-/-}$ mice (C57BL/6 background) were purchased from Jackson Laboratories (Bar Harbor, ME, USA). Mice were bred and housed in specific pathogen-free conditions, and IL-10$^{-/-}$ mice and their littermate controls were used for tumour inoculation. All mice used in the experiments were 6–8 weeks of age at the beginning of the experiments. The mice were kept in the Animal Center for Pharmaceutical Research at Kangwon National University. To establish tumours, mice were s.c. injected with 2×10^5 tumour cells on the left flank.

Cell line

Human papillomavirus (HPV) E6/E7-expressing TC-1 cells were kindly provided by Prof. Tae Woo Kim (Korea Univ., South Korea) [18], and which was originally established by Tzyy-Choou Wu [18,19]. TC-1 cells were maintained in RPMI 1640 medium with 10% foetal bovine serum, 0.01% antibiotic-antimycotic solution and grown at 37 °C with 5% CO_2. Antibiotic-antimycotic solution, foetal bovine serum and RPMI 1640 were supplied by Gibco BRL (Grand Island, NY, USA). To establish tumours, 5×10^5 TC-1 cancer cells were s.c. injected in the left flank of C57BL/6 mice and IL-10$^{-/-}$ mice. Tumour growth was measured using callipers 3 times a week.

Reagents and antibodies for flow cytometry

Mice were treated with 5 mg/kg STAT3 inhibitor (S3I; Selleck Chemicals, Houston, TX, USA) every other day. For *in vivo* use, mice were injected intraperitoneally (i.p.) with 2.5 mg/kg anti-IL-6R mAb (Chugai Pharmaceutical Co., Ltd., Tokyo, Japan) or vehicle once a week, starting when the tumour reached approximately 50 mm^3. Abs used for the flow cytometry analysis were purchased from BD Biosciences (San Jose, CA, USA).

Isolation of tissue-infiltrating MDSCs

To obtain tissue-infiltrating MDSCs, tumour tissue samples were homogenized with the gentleMACS Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany) and treated with 0.5 mg/ml collagenase type IV (Sigma, St. Louis, MO, USA) for 30 min. Cells were harvested by gentle pipetting and subjected to a 40–70% Percoll gradient to enrich mononuclear cells. To isolate MDSCs, cells were stained with anti-CD11b$^+$ microbeads (Miltenyi Biotec) and isolated by Magnetic-activated cell sorting cell separation.

Western blot

Total protein lysates from tissue samples or MDSCs were prepared by sonication in lysis buffer (iNtRON, Seongnam, Korea). Equal amounts of lysates were boiled at 100 °C and resolved by 8–12% sodium dodecyl sulphate polyacrylamide gel electrophoresis. Proteins were transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA) and blocked with 5% bovine serum albumin in Tris-buffered saline and Tween 20. Membranes were incubated with the primary antibody and detected with horseradish peroxidase-conjugated antibody (Cell Signaling Technology, Danvers, MA, USA). Membranes were developed using the enhanced chemiluminescence method using femtoLUCENT™ PLUS-HRP (G-Biosciences, St. Louis, MO, USA).

Cytokine measurement by ELISA

Cytokines in tissue homogenates were assayed by enzyme-linked immune sorbent assays (ELISA). The assays were conducted using the mouse IL-6, TNF-α, IL-1β, and IFN-γ set (eBiosciences, San Diego, CA, USA), according to the manufacturer's instructions.

Intracellular cytokine staining

To analyse T cell responses, splenocytes were incubated for 4 h in GolgiPlug (BD Pharmingen, San Diego, CA, USA) with phorbol 12-myristate 13-acetate (50 ng/ml) and ionomycin (750 ng/ml) (all from Sigma-Aldrich). After surface staining with PE/Cy7-conjugated anti-CD8 Abs and FITC-conjugated anti-CD4 Abs, splenocytes were fixed and permeabilized using Cytofix/Cytoperm reagents (BD Pharmingen). Permeable cells were stained with APC-conjugated anti-IFN-γ and PE-conjugated anti-TNF-α Abs. Cells were detected by FACSVerse (BD Biosciences) and analysed using the BD FACSuite.

Statistical analysis

GraphPad prism software (GraphPad, La Jolla, CA) was used for statistical analysis. Student's *t*-tests were used to compare mean values between 2 groups, while a one-way analysis of variance with Tukey's HSD post-hoc test was used for comparisons among more than 2 groups. Values of $P < 0.05$ were considered significant.

Results

IL-10-deficiency accelerated tumour growth via the upregulation of IL-6 in mice

Several previous studies have suggested that tumour growth is significantly more rapid in IL-10$^{-/-}$ mice than WT mice [15,20]. To confirm this pattern for TC-1 cervical cancer cells expressing HPV E6 and E7, we s.c. injected 5×10^5 TC-1 cells/mouse into the left flank of WT and IL-10$^{-/-}$ mice and measured tumour volume over a 5-week period. TC-1 tumour growth was significantly faster in IL-10$^{-/-}$ mice than in WT mice (Fig. 1A). Based on a previous report showing that IL-6 levels are highly increased in IL-10-deficient conditions and that increased IL-6 is involved in accelerated tumour growth [21], we assessed the levels of serum IL-6 in both WT and IL-10$^{-/-}$ mice at 4 weeks after TC-1 cell inoculation. We found that the levels of serum IL-6 in TC-1 tumour-bearing IL-10$^{-/-}$ mice were significantly higher than those in TC-1 tumour-bearing WT mice (Fig. 1B). In the absence of tumour burden, no detectable levels of serum IL-6 were found in WT and IL-10$^{-/-}$

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