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IL-6 restores dendritic cell maturation inhibited by tumor-derived TGF-β through interfering Smad 2/3 nuclear translocation

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ABSTRACT

We previously found, in a canine transferable tumor model, that high concentration of IL-6 produced by tumor-infiltrating lymphocytes effectively restores the MHC expression of the tumor cells and T-cell activation inhibited by tumor-derived TGF-β. This tumor also significantly suppresses monocyte-derived dendritic cells (DCs) differentiation and the functions of differentiated DCs with unknown mechanisms. In this study, we have demonstrated that a strong reaction of IL-6 was present to neutralize TGF-β-down-regulated surface marker expression on DCs (MHC II, CD1a, CD40, CD80, CD83, CD86), TGF-β-hampered DC functions and DC-associated T-cell activation. Western blotting and confocal microscopy results indicated that the presence of IL-6 markedly decreased the nuclear concentration of a TGF-β signaling transducer, Smad 2/3. In addition, while Smad 7 is a potent molecule inhibiting Smad 2/3 nuclear translocation, no significant increase in Smad 7 gene expression upon addition of IL-6 in TGF-β-pretreated DCs was detected, which suggested that the blockage of Smad 2/3 nuclear translocation by IL-6 did not occur through a Smad 7-inhibitory mechanism. In conclusion, IL-6 inhibited TGF-β signaling and concomitantly antagonized the suppression activities of TGF-β on DC maturation and activity. This study enables further understandings of host/cancer interactions an also provide hints facilitating improvements of DC-based cancer immunotherapy.

1. Introduction

Dendritic cells (DCs) are the most potent antigen-presenting cells (APCs). They are crucial to the initiation of Ag-specific T-cell proliferation and other host defensive immune responses [1,2]. A variety of tumors have developed different strategies for the subversion of DCs toward tolerance rather than immune activation by either decreasing the number of effective DCs or by generating abnormal maturation [3,4]. Studies of breast cancer and melanoma have revealed that the numbers of DCs are lower in sentinel lymph nodes than in other lymph nodes [5]. Myeloma patients, though, have normal numbers of DCs, but low CD80 expression, a sign of a maturation defect [6]. TGF-β is one of the tumor-derived factors known to inhibit DC maturation. It was found to attenuate the antigen-presentation activities of DCs by suppressing the expression of antigen-presentation-associated surface marker CD1d [7]. TGF-β is also a potent suppressive modulator of immunologic functions, acting by regulating the development and differentiation of immunocompetent cells including B cells, T cells, and monocytes/macrophages [5,8]. Tumor-derived TGF-β not only inhibits DC maturation, but also induces DC apoptosis [4,5]; it is therefore one of the most potent immunosuppressive cytokines secreted by tumors, and is also important for malignant tumor progression [5,8,9].

Canine transmissible venereal tumor (CTVT), a round-cell tumor, can cross the MHC barrier and is transferable to allogeneic canine individuals [10]. A unique feature of CTVT is that, under normal host immunity, CTVT enters the regression phase spontaneously (SR phase) later in the course of progressive (P) tumor growth. The growth pattern of CTVT is highly related to host immunity; thus, by comparing immune responses toward P and SR phases, how tumor evades immune surveillance to develop and grow up can be deciphered [4,11,12]. CTVT has been found to significantly decrease the number of peripheral monocyte-derived DCs (PMDCs) in the draining lymph nodes as compared with normal dogs during tumor progression. Surface marker expression

Abbreviations: CTVT, canine transmissible venereal tumor; DCs, dendritic cells; iDCs, immature dendritic cells; mDCs, mature dendritic cells; PMDCs, peripheral monocyte-derived dendritic cells; APCs, antigen presenting cells; LAK cells, lymphokine-activated killer cells; TILs, tumor infiltrating lymphocytes; TGF-β, transforming growth factor-β; IL-6, interleukin 6; MHC, major histocompatibility complex; CD, cluster of differentiation.

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and the lymphocyte stimulation ability are also hampered [4]. It is interesting that during spontaneous regression of CTVT, DC activities including surface marker expression and functions are mostly restored [4].

CTVT-derived TGF-β suppresses MHC expression, DC differentiation and activities, and LAK cytotoxicity, while in the SR phase, tumor-infiltrating lymphocytes (TIL) secrete IL-6 [13], a multifunctional cytokine that efficiently restores the TGF-β-associated inhibitory activities including MHC expression and LAK cytotoxicity [14]. In other cancers such as hepatomas, IL-6 protects liver cells against TGF-β-induced apoptosis through elevated Bcl-2 via STAT3 induction, providing another example of the antagonizing effect of IL-6 on TGF-β [15,16]. However, during helper T-cell development, under TGF-β environment, addition of IL-6 is known as an essential factor in coordinating with TGF-β for promoting human naïve $CD4^+$ T cells to differentiate into Th17 cells [17]. Thus, the interactions between TGF-β and IL-6 in diverse cell types remain elusive, and both antagonist and synergistic effects are present. It seems that the interplay between IL-6 and TGF-β varies in distinct cell types; therefore, their effects on DCs deserve further investigation.

In this study, we aimed to explore how IL-6 interacted with TGF-β on DC functions. The results demonstrated that IL-6 reversed the suppressed DC functions through inhibition of Smad 2/3 nuclear transfer by a pathway different to that of activation of inhibitory Smad 7. Recovering DC activity by interrupting TGF-β/Smad 2/3 signaling axis with IL-6 raises a promising rationale for canine cancer therapy.

2. Materials and methods

2.1. Animals and generation of peripheral monocyte-derived DCs

Ten healthy mature beagles, 1–2-year-old, were used as the source for generating monocyte-derived DCs. These studies were approved by the Institutional Animal Care and Use Committee at the National Taiwan University. The DCs were generated following the procedure as previously described [3,18].

2.2. Flow cytometry analysis of DC

Flow cytometric analysis of DCs was performed using fluorescein isothiocyanate (FITC)-labeled anti-human CD1a, CD40 and anti-canine DLA class II monoclonal antibodies (Serotec, Oxford, England). FITC-conjugated mouse IgG2a and IgG1 (Serotec) were used as the isotype control for anti-CD1a and -CD40 abs. Goat anti-mouse IgG labeled with FITC (Serotec) was used as the secondary antibody. The phenotypes of the DCs were analyzed using the procedures described previously [3].

2.3. Quantitative RT-PCR

TRIzol® reagent (Invitrogen) and SuperScript II RT kit (Invitrogen) were utilized for the isolation of total RNA and the following reverse-transcription into cDNA according to the manufacturer's recommendations. Real-time RT-PCR was performed in a Bio-Rad real-time PCR machine using SYBR Green PCR Master Mix according to the manufacturer's instructions (Bio-Rad, CA, USA). The primer sequences used for CD80, CD83 and CD86 were based on our previous publication [3] and are listed in Table 1. A housekeeping gene, β-actin, was utilized as an internal control to perform relative quantification. The results were analyzed using the IQ5 analysis software provided by Bio-Rad.

Table 1
Oligonucleotide primers used for qRT-PCR.

Primers		Sequences
CD 80	Forward	5′-ATGGATTACACAGCGAAGTGGAGAA-3′
	Reverse	5′-AGGCGCAGAGCCATAATCACGAT-3′
CD 83	Forward	5′-CAGTCATATAAAAGCTATGGTGAGATGC-3′
	Reverse	5′-AGATGAAAAGGCCCTGCTGGGG-3′
CD 86	Forward	5′-ATGTATCTCAGATGCACTATGGAAC-3′
	Reverse	5′-TTCTCTTTGCCTCTGTATAGCTCGT-3′
Smad7	Forward	5′-AGCTGCGGGGAGGGGGCG-3′
	Reverse	5′-CGCGGGGCGGGGATGGTGGT-3′
β-actin	Forward	5′-GACCCTGAAGTACCCCATTGAG-3′
	Reverse	5′-TTGTAGAAGGTGTGGTGCCAGAT-3′

2.4. Allogeneic mixed lymphocyte reaction (MLR)

MLR analysis was conducted using a CellTrace™ CFSE Cell Proliferation Kit according to the manufacturer's instructions (Invitrogen). Briefly, allogeneic PBMCs were suspended in pre-warmed PBS/0.1% bovine serum albumin at a final concentration of 1×10^6 cells/mL, and 1 μL of 5 mM CellTrace carboxyfluorescein diacetate succinimidyl ester (CFSE) stock solution per mL was added and incubated at 37 °C for 15 min. The solution was then quenched by the addition of five volumes of ice-cold culture media for 30 min at room temperature. The washed PBMCs, which served as target cells, were co-cultured with DCs or 20 μg/mL concanavalin A (ConA) as a positive control. The DC/PBMC ratios (E/T ratio) at 1:1, 3:1, and 9:1 were used. Cells were harvested on days 4, 5, and 6 after co-culturing, and the T-cell-proliferation decay of CFSE fluorescence was measured using a flow cytometer at an excitation of 488 nm. The MLR was calculated according to the following formula: (experimental data – negative control) × (target cell number/effector cell number)/negative control.

2.5. FITC-dextran uptake assay

DCs were incubated for 2 h with FITC-dextran (100 μg/mL; Sigma) at 4 °C or 37 °C and washed extensively with PBS, and were detected using a flow cytometer at an excitation of 488 nm. Non-specific binding of FITC-dextran to the cell surface was deducted from the data obtained from cells incubated at 4 °C.

2.6. Production of CTVT cell-culture supernatants

1×10^6 CTVT cells were incubated for 3 days in 1 mL of RPMI-1640 medium containing 10% FCS, supplemented with 100 U/mL penicillin, 100 mg/mL streptomycin, and 2 mM L-glutamine. Cell-free supernatants were collected and preserved in a refrigerator at −80 °C for further experiments. Tumor-cell supernatants were forced through a 0.2-μm filter, and 2×10^5 DCs were then co-cultured with l mL tumor supernatant with or without the addition of TGF-β neutralizing antibody (Serotec) or exogenous IL-6 (Peprotech, NJ, USA). The cultures were maintained at 37 °C under 5% CO_2. The tumor cell supernatants were measured their TGF-β amounts with TGF-β_1 Emax Immuno Assay system (Promega, WI, USA) according to the manufacturer's instructions.

2.7. Immunofluorescent staining for confocal microscopy

Immunofluorescent staining was performed on cell spots. A quantity (2.5×10^4) of DCs was centrifuged onto glass slides (Menzel-Glaser, Mainz, Germany) for 5 min at 150*g* using a Cytospin centrifuge to create cell spots [19,20]. The cell spots on coverslips were then fixed and permeabilized for 10 min in acetone at 4 °C and air-dried, followed by incubation with rabbit-anti-Smad 2/3 monoclonal antibody (Cell Signaling, MA, USA) (diluted 1:50)