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Soluble OX40L favors tumor rejection in CT26 colon carcinoma model

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ABSTRACT

OX40 receptor-expressing regulatory T cells (Tregs) populate tumors and suppress a variety of immune cells, posing a major obstacle for cancer immunotherapy. Different ways to functionally inactivate Tregs by triggering OX40 receptor have been suggested, including anti-OX40 antibodies and Fc:OX40L fusion proteins. To investigate whether the soluble extracellular domain of OX40L (OX40Lexo) is sufficient to enhance antitumor immune response, we generated an OX40Lexo-expressing CT26 colon carcinoma cell line and studied its tumorigenicity in immunocompetent BALB/c and T cell deficient nu/nu mice.

We found that soluble OX40L expressed in CT26 colon carcinoma favors the induction of an antitumor response which is not limited just to cells co-expressing EGFP as an antigenic determinant, but also eliminates CT26 cells expressing another fluorescent protein, KillerRed. Tumor rejection required the presence of T lymphocytes, as indicated by the unhampered tumor growth in nu/nu mice. Subsequent re-challenge of tumor-free BALB/c mice with CT26 EGFP cells resulted in no tumor growth, which is indicative of the formation of immunological memory. Adoptive transfer of splenocytes from mice that successfully rejected CT26 OX40Lexo EGFP tumors to naïve mice conferred 100% resistance to subsequent challenge with the CT26 EGFP tumor.

1. Introduction

OX40 is a member of the TNFR family of receptors. It has a co-stimulatory function in T cell activation [8,33]. OX40 is preferentially expressed on activated CD4 rather than CD8 T cells, suggesting a more prominent role for OX40 on CD4 T cells [31,35]. The exception is the situation of strong antigenic stimulation, when OX40 may also be expressed on CD8 T cells [4]. Importantly, OX40 is constitutively expressed on murine FoxP3 CD4 naturally occurring T-regulatory cells (nTreg), and is inducible on human Treg cells [29]. The OX40 ligand (OX40L) in turn is expressed on various cell types, including B cells [22], T cell [18] and activated DCs [8,25]. Signaling through the OX40 receptor activates the expression of pro-survival genes and is essential for the long-term survival of CD4 + T cells, however, the OX40L signal alone, without B7:CD28 interaction, is insufficient for full T cell activation [26].

Tregs are considered a major obstacle for the development of an effective antitumor immune response [38]. They are capable of direct contact inhibition of antigen-presenting and effector cells [7]. They also release anti-inflammatory cytokines such as IL-10 or transforming growth factor-β (TGF-β) [32]. Treg cells specific for a particular T cell receptor (TCR) have the ability to suppress several effector cells with distinct TCR specificities when co-localized on the same antigen-presenting cell [9,17,30]. Thymic development of nTreg cells does not require OX40, as the spleens of OX40-/- mice have nTreg cells, which are present at a reduced frequency suggesting that OX40 has a role in nTreg cell homeostasis [29]. In addition to nTreg cells, naïve T cells can become induced Treg cells (iTreg) when activated in the presence of transforming growth factor-beta (TGF-β) [37].

Abbreviations: OX40Lexo, extracellular domain of the OX40 ligand, 50–198 aa; OX40Lfull, the complete OX40 ligand.

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In the development of antitumor immune response, OX40L appears to be a double-edged sword rather than a magic bullet. The OX40 signal antagonizes the TGF-β- and antigen-mediated conversion of naïve $CD25^{-}Foxp3^{-}$ CD4 T cells into $CD25^{+}Foxp3^{+}$ CD4 T cells [28]. The authors showed that *in vitro* stimulation with the anti-OX40 agonistic antibody OX86 can suppress Foxp3 expression in naïve CD4 T cells and antagonize the CD28 and IL2R signals that drive the expression of Foxp3. However, other evidence suggests that OX40 stimulation drives all T cell lineages and may result in Treg accumulation in the context of cytokine blockade [27].

There are numerous examples of OX40 engagement resulting in the inhibition of tumor growth. Injection of Fc:OX40L fusion protein or anti-OX40R *in vivo* during the initial stages of tumor growth results in a significant increase in the percentage of tumor-free survivors in different murine tumor models [19,34]. It has been shown by Bansal-Pakala et al. that a single injection of anti-OX40 antibody can break peptide-induced peripheral tolerance [3]. A subcutaneous CT26 tumor expressing both GM-CSF for APC stimulation and OX40L regressed in 85% of the mice tested [13,14]. Intraperitoneal injection of the mOX40L-mIgGFc fusion protein 3 days after inoculation with the CT26 cells almost completely inhibited tumor growth [1]. In T cell proliferation assay, this fusion protein was significantly superior to OX86 antibody. Human recombinant Fc:OX40L fusion protein linked via a coiled-coil trimerization domain had superior capacity to stimulate human T-cell proliferation compared to the agonist antibody [20]. An agonistic aptamer against OX40 was able to enhance an antigen-pulsed DC-mediated antitumor immune response in murine B16-F10.9 melanoma [10]. Treatment of tumor-bearing mice with an intratumoral injection of full-size murine OX40L-expressing adenovirus resulted in a significant suppression of tumor growth along with survival advantages [2]. APCs with OX40L promoted partial activation of naïve T cells with some IL-2 secretion, but were unable to enhance proliferation, unlike that with B7-1 [12].

The long-lived concept underlying these results is that both anti-OX40 antibody and OX40L:Fc fusions act as OX40 receptor agonists. However recent work suggests that at least part of the *in vivo* effects of OX40 agonists is mediated by an Fc-dependent cellular cytotoxicity mechanism that selectively depletes intratumoral Treg cells [5]. In this connection it would be beneficial to explore the *in vivo* effects of soluble OX40L without the Fc fragment.

We propose a model system where a CT26 cell line is transduced to stably express either soluble murine OX40L (extracellular portion, 50–198 aa) in secretory form or full OX40Lexo in membrane-bound form. BALB/c mice have been inoculated with these OX40L-expressing cells and the tumor incidence and growth parameters individually monitored. This allowed us to explore the influence of OX40L in tumor development.

2. Materials and methods

2.1. Genetic manipulations

A custom multiple cloning site and a sequence for the preprotrypsin leader peptide with a 6xHis tag and FLAG epitope were inserted into XhoI-EcoRI of a pIRES2-EGFP vector (Clontech, USA). The fragment containing the leader sequence, 6x-His tag, multiple cloning site, IRES and EGFP, was amplified by PCR and inserted into the BamHI-SalI sites of the pRRL.SIN. WPRE.hPGK.EGFP vector for lentiviral expression (kind gift by Prof. Terskikh, Stanford-Burham Medical Research Institute). This intermediate product was then used as a control EGFP-only vector. The sequence for OX40L extracellular domain (OX40Lexo, aa 50–198), synthesized by Evrogen, Russia, was inserted into the NheI-EcoRI sites of the polylinker inframe with the leader sequence, producing a vector for the lentiviral expression of OX40Lexo under the control of the hPGK promoter and IRES-driven expression of EGFP. Similarly, for OX40L full construct, the sequence for the multiple cloning site, IRES and EGFP, was amplified with primers containing sites for BglII and XhoI, and inserted into the BamHI and SalI sites of pRRL.SIN. WPRE.hPGK.EGFP in place of EGFP. The OX40Lfull ORF was amplified with primers containing SalI and BamHI recognition sites and cloned into the pRRL.SIN.WPRE.hPGK.IRES.EGFP vector.

2.2. Production of lentiviral vector particles

Lentiviral particles for mammalian cell infection were obtained according to standard procedure. In short, 24–48 h prior to transfection, HEK293T cells at a logarithmic growth stage were plated onto d = 6 cm cell culture dishes (SPL Life sciences, Korea) in DMEM supplemented with 10% fetal calf serum (FCS), 1% glutamine, 10 units/ml penicillin and 10 μg/ml streptomycin. The transfection with 4 μg pR8.91, 1.2 μg pMD.G and 5 μg of the OX40L-carrying plasmid was carried out using a calcium-phosphate transfection kit (Molecular Probes, USA) according to the manufacturer's protocol with a total of 10 μg DNA, when the HEK293T cells had reached about 90% confluency. The culture medium was changed 16 h after transfection and the lentiviral particles were harvested 24 h thereafter. Medium containing the viral particles was concentrated 20 fold using a Pierce concentrator with a 150 KDa cut-off (Thermo Scientific, USA). For lentiviral infection, the CT26 cells were plated onto d = 35 mm cell culture dishes (SPL Life sciences, Korea), at a density of 2.5×10^4 cells/dish, in DMEM with 10% FCS, 1% glutamine, 10 units/ml penicillin and 10 μg/ml streptomycin. After culturing for 24 h the medium was changed for the concentrated medium with viral particles. The fluorescence of the infected cells was analyzed 5–7 days post infection.

2.3. Western blot

Cell culture medium after 72 h cultivation of HEK293 cells transiently expressing OX40Lexo was incubated with Ni sepharose excel (GE Healthcare) (1 mL of medium with 5 μL of Ni-sepharose slurry) for 1 h for selective binding of 6xHis-tagged protein. Then Ni-sepharose beads were washed with TBS (20 mM TrisHCl pH8 150 mM NaCl) and OX40Lexo was eluted with 500 mM imidazole in TBS. Cell culture medium after cultivation of non-OX40L-expressing HEK293 cells was treated in parallel and used as a control. Eluted protein was denatured in SDS loading buffer and subjected to 10–25% gradient SDS-PAGE. Proteins were transferred to a nitrocellulose membrane and probed with monoclonal anti-6xHis-Tag mouse antibodies (Abcam, ab18184). Secondary anti-mouse antibodies conjugated with horseradish peroxidase (Sigma) were detected using a standard luminol-perborate chemiluminescent reaction.

2.4. Anti-OX40L staining and microscopy

Anti-OX40L staining of the CT26 OX40Lfull cell line was performed with rat mAB to CD252 (RM134L) and ab 95656 (Abcam), at a 1:250 dilution. The cells were washed 2 times with ice-cold PBS, and stained in PBS with 1% BSA for 1 h on ice. Then the cells were washed 3 times with PBS and used for the fluorescence microscopy analysis.

Live cell imaging was performed in HEPES-buffered DMEM (PanEco, Russia) supplemented with 10% (v/v) FCS at 37 °C in an atmosphere of 5% CO_2. For the fluorescence microscopy, a Leica AF6000 LX imaging system, based on a DMI 6000 B inverted microscope equipped with a Photo metrics CoolSNAP HQ CCD camera,