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TNF receptor associated factor 5 controls oncostatin M-mediated lung inflammation

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ABSTRACT

Oncostatin M (OSM) is involved in pathogenesis of several human inflammatory diseases including lung inflammation and fibrosis. Although accumulating evidence indicates that OSM mediates lung inflammation, the precise mechanism for OSM on lung inflammation still remains unclear. In this study, we found that OSM receptor was abundantly expressed on endothelial and stromal/fibroblast cells in the lung of mice. *In vitro* stimulation with OSM upregulated vascular cell adhesion molecule-1 (VCAM-1), which promotes eosinophil infiltration in the lung tissues, on freshly-isolated lung stromal/fibroblast cells from wild-type mice. However, these cells from TNF receptor associated factor 5 (TRAF5)-deficient mice failed to show the increase in VCAM-1 expression after OSM stimulation. Furthermore, *Traf5*$^{-/-}$ mice showed markedly attenuated lung inflammation in terms of eosinophil infiltration upon intranasal administration with OSM as compared to wild-type mice. These results indicate that TRAF5 is crucially involved in OSM-mediated lung inflammation probably by inducing lung stromal/fibroblast cell activation.

1. Introduction

Oncostatin M (OSM) is a member of the IL-6 cytokine family and has been reported to play pleiotropic roles in pathogenesis for several inflammatory diseases, such as lung fibrosis and inflammatory bowel diseases (IBD). Mozaffarian et al. reported elevated concentration of OSM in bronchoalveolar lavage fluids from patients with idiopathic lung fibrosis [1]. OSM was also highly detected in the synovial fluid from joints of patients with rheumatoid arthritis [2]. A recent report demonstrated that expression levels of both OSM and OSM receptor were closely correlated with the severity of IBD, and that OSM neutralization ameliorated anti-TNF-therapy-resistant colitis in a mouse model [3]. These previous studies suggest that OSM is a possible therapeutic target for the diseases.

OSM is known as a growth factor of Kaposi's sarcoma cells, which are derived from endothelial cells [4]. Indeed, OSM activates endothelial cells and stromal/fibroblast cells *in vitro*, in terms of inducing surface expression of adhesion molecules [5]. Adhesion molecules, such as vascular cell adhesion molecule-1 (VCAM-1), cadherin, and selectin, are crucial for recruiting immune cells into the inflammatory site. In the case of allergic lung inflammation, VCAM-1 is one of the most important effector molecule, which facilitates extravasation and accumulation of eosinophils [6] because eosinophils highly express a VCAM-1 ligand, VLA-4. Indeed, *in vivo* blockade of VCAM-1/VLA-4 ameliorated allergen-induced eosinophil infiltration into the lung [7,8]. In these lines, OSM-mediated tissue inflammation may in part depend on the adhesion molecules, such as VCAM-1. Nevertheless, precise mechanisms of how OSM mediates the lung inflammation remain unclear.

TNF receptor associated factor 5 (TRAF5) belongs to the TRAF family, which plays important roles in transducing intracellular

Abbreviations: OSM, oncostatin M; TRAF, TNF receptor associated factor; IBD, inflammatory bowel disease; MLF, mouse lung stromal/fibroblast cell; DMEM, Dulbecco's Modified Eagle medium; OSMRβ, oncostatin M receptor β chain; VCAM-1, vascular cell adhesion molecule-1.

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signals via TNF receptor superfamily molecules. TRAFs also participate in the signal transduction by the other inflammatory receptors, such as Toll-like receptors and IL-1 receptor family molecules [9,10]. TRAFs thus promote inflammatory responses by transducing signals via the above receptors. On the other hand, it is recently reported that TRAF2 and TRAF5 negatively regulates IL-6 receptor signaling by suppressing STAT3 phosphorylation in naïve $CD4^+$ T cells, resulting in reduced Th17 differentiation [11,12]. The other groups also demonstrated that TRAF3 inhibits IL-2 receptor signaling in $CD4^+$ T cells by recruiting T cell protein tyrosine phosphatase to IL-2 receptor [10]. Therefore, in some situations TRAFs negatively control inflammatory responses in a certain cell type.

In this study, we examined the mechanism of OSM-mediated lung inflammation, and found that *Traf5*$^{-/-}$ lung stromal/fibroblast cells failed to induce surface expression of VCAM-1, an adhesion molecule, upon *in vitro* stimulation with OSM. Although TRAF5 is known to control TNF-α and IL-6/gp130 signaling, the TRAF5-dependent VCAM-1 induction does not appear to depend on TNF-α and IL-6. In an *in vivo* experiment, TRAF5 deficiency in mice remarkably ameliorated lung inflammation that was induced by OSM administration. The ameliorated inflammation in *Traf5*$^{-/-}$ mice was accompanied by impaired upregulation of VCAM-1 on lung stromal/fibroblast cells and endothelial cells. The present results reveal a critical role of TRAF5 in OSM-mediated lung inflammation.

2. Material and methods

2.1. Mice

Wild-type C57BL/6 (B6) mice were purchased from Japan SLC (Shizuoka, Japan). *Traf5*$^{-/-}$ mice on a B6 background have been generated as described previously [13]. All mice were housed in an SPF facility, and 10-12 week-old mice of the same sex were used in the experiments of OSM administration. All experiments were approved by the Institute for Animal Experimentation, Tohoku University Graduate School of Medicine.

2.2. Isolation and culture of mouse lung stromal/fibroblast (MLF) cells

Isolation of mouse lung stromal/fibroblast cells (MLF) for culture was previously described [14]. Briefly, lungs dissected from 7 to 14 days old pups were cut into small pieces and then incubated in 2.5 ml Dulbecco's Modified Eagle medium (DMEM) containing 0.1 mg/ml DNase I (Sigma-Aldrich Japan, Tokyo, Japan) and 0.1 mg/ml collagenase (Sigma-Aldrich) for 45 min at 37 °C. After the incubation, the cells were washed with PBS and resuspended in 15 ml of DMEM supplemented with 10% FCS, 1.0% penicillin-streptomycin. The cell suspension was subsequently transferred to a 75 ml flask and placed in a CO_2 incubator at 37 °C. MLF thus obtained as attached cells onto the flask was maintained and used within 3 passages. MLFs from wild-type or *Traf5*$^{-/-}$ mice were stimulated *in vitro* with mouse OSM (10 ng/ml; R&D Systems, Minneapolis, MN, USA), IL-6 (10 ng/ml; PeproTech, Rocky Hill, NJ, USA), or TNF-α (10 ng/ml; PeproTech).

2.3. Intranasal administration of OSM

Mice were daily treated with 2 μg OSM in 40 μl PBS via intranasal injection for up to 7 days.

2.4. Cell preparation from the lung

Based on previously-described protocols [15,16], two distinct methods were utilized depending on the hematopoietic and non-hematopoietic cell populations. To isolate and analyze hematopoietic cells, the following procedure was used: after perfusing with 10 ml PBS, the dissected lungs were cut into several small pieces and were incubated with 2% FCS, 50 μg/ml Liberase TM (Sigma Aldrich), and 10 μg/ml DNase I for 1 h at 37 °C. Then, all cell suspensions and pieces of lungs were smashed and passed through a 70 μm cell strainer, and washed with PBS to recover cells. The total single cell suspension was enriched by two-layer Percoll gradient centrifugation, and the isolated cells were used as lung hematopoietic cells. To collect non-hematopoietic cells, another method was used as follows: 1 ml (20 U/ml) of dispase (Thermo Fisher Scientific Japan, Tokyo, Japan) were instilled into the lungs, and the tracheas were ligated to prevent leakage of dispase. The lungs were placed in a sterile tube containing 2.5 ml DMEM/lung with 0.1 mg/ml DNase I and 0.1 mg/ml collagenase, and incubated for 45 min at 37 °C. After incubation, lung tissues were separated from large bronchi and heart by mechanical means, and minced well with scissors. The cells were subsequently filtered through a 70 μm cell strainer, washed with PBS to recover cells. Endothelial ($CD45^-CD31^+EpCAM^-$), stromal/fibroblast ($CD45^-CD31^-EpCAM^-$), and epithelial ($CD45^-CD31^-EpCAM^+$) cell populations were separated by using FACSAriaII cell sorter (BD Biosciences Japan, Tokyo, Japan).

2.5. Flow cytometric analysis

Cells were incubated with anti-CD16/CD32 (2.4G2; produced in-house) before being stained with the appropriate antibodies to cell-surface antigens. Living cells were stained with combinations of the following monoclonal antibodies according to manufacturer protocols: anti-OSMRβ-biotin (118125), anti-CD45-PE (30-F11), anti-CD31-FITC (390), anti- CD326-APC (G.8.8), anti-CD106 (VCAM-1)-PE (429), anti-Siglec F-PE (E50-2440), anti-CD3ε-FITC (145-2C11), anti-CD4-FITC (GK1.5), anti-TCRγ/δ-FITC (GL3), anti-CD19-FITC (6D5), anti-B220-FITC (RA3-6B2), anti-CD11c-FITC (N418), anti-CD11b-FITC (M1/70), anti-Gr1-FITC (RB6-8C5), anti-CD49b-FITC(DX5), anti-NK1.1-FITC (PK136), anti-TER119-FITC (TER-119), streptavidin-BV421. Dead cells were excluded by propidium iodide or DAPI. Data were acquired on a FACSCanto II (BD Biosciences Japan) and were analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

2.6. Real-time RT-PCR

THUNDERBIRD SYBR qPCR Mix (TOYOBO, Osaka, Japan) and a 7500 real-time PCR system (ThermoFisher Scientific) were used for quantitative RT-PCR. Total RNA was extracted by using RNAiso Plus (Takara Bio, Kusatsu, Japan), and then reverse transcribed with High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific). All samples were normalized to *Gapdh*. The following primers were used in this study: *Osmr*, 5′-CATCCCGAAGCGAAGTCTTGG-3′ and 5′-GGCTGGGACAGTCCATTCTAAA-3', *Gp130*, 5′-TACATGGTCCGAATGGCCGC-3′ and 5′-GGCTAAGCACACAGGCACGA-3′, *Traf5*, 5′-CCGACACCGAGTACCAGTTTG-3′ and 5′-CGGCACCGAGTTCAATTCTC-3′, *Gapdh*, 5′-CCAGGTTGTCTCCTGCGACTT -3′ and 5′-CCTGTTGCTGTAGCCGTATTCA -3'.

2.7. Statistical analysis

Two-sided Student's t-test was used to determine significance between two groups. *P*-values < 0.05 were considered significant.

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