Original Paper

TNF- α Regulates Mast Cell Functions by Inhibiting Cell Degranulation

Yuwei Gao^{a,b} Baixue Xu^b Peng Zhang^b Yanlong He^c Xin Liang^{a,b} Jianwen Liu^b
Jiyu Li^{a,c}

^aDepartment of Clinical Laboratory, People's Hospital of Shanghai Putuo District, Shanghai, ^bState Key Laboratory of Bioreactor Engineering & Shanghai Key Laboratory of New Drug Design, School of pharmacy, East China University of Science and Technology, Shanghai, ^cDepartment of General Surgery, Shanghai Tenth People's Hospital, Tongji University School of Medicine, Shanghai, PR China

Key Words

Tnf- α • Mast cells • ICOSL • Degranulation

Abstract

Background/Aims: The aim of this study was to investigate the involvement of inducible co-stimulatory ligand (ICOSL) expression in stimulation of mast cells (MCs) by TNF- α and the ability of TNF- α stimulation of MCs to influence CD4⁺ T cell differentiation and function. The mechanisms underlying TNF- α stimulation of MCs were also explored. **Methods:** Mast cells and CD4⁺ T cells were prepared from C57BL/6 mice (aged 6–8 weeks). ICOSL expression by MCs was measured by real-time PCR and flow cytometry, and levels of IL-4, IL-10 and IFN- γ were measured by ELISA. **Results:** ICOSL expression by MCs was increased by TNF- α stimulation, and resulted in interaction with CD4⁺ T cells. The IL-4 and IL-10 levels in the co-culture system increased, while IFN- γ levels decreased. Furthermore, CD4⁺CD25⁺Foxp3⁺ T cell proliferation was induced by co-culture with TNF- α -stimulated MCs. The mechanism by which TNF- α stimulated MCs was dependent on the activation of the MAPK signaling pathway. **Conclusion:** TNF- α upregulated the expression of ICOSL on mast cells via a mechanism that is dependent on MAPK phosphorylation. TNF- α -treated MCs promoted the differentiation of regulatory T cells and induced a shift in cytokine expression from a Th1 to a Th2 profile by up-regulation ICOSL expression and inhibition of MC degranulation. Our study reveals a novel mechanism by which mast cells regulate T cell function.

© 2017 The Author(s)
Published by S. Karger AG, Basel

Introduction

Mast cells (MCs) are widely distributed in the connective and vascularized tissues or cavities throughout the body and play an important role in the interaction between the immune system and environmental antigens and allergens [1, 2]. Following exposure to a variety of pathological conditions, the phenotypic profile of MCs is influenced by various

Xin Liang, Jianwen Liu
and Jiyu Li

Dept. of Clinical Laboratory, People's Hospital of Shanghai Putuo District, State Key Laboratory of Bioreactor Engineering & Shanghai Key Laboratory of New Drug Design, Shanghai, (China); E-Mail leejiyu@sina.com, liujian@ecust.edu.cn, xin.liang@ecust.edu.cn

micro-environmental stimuli [3] leading to different functional effects [4-6]. This versatility is reflected in the IgE-dependent and -independent activation pathways that modulate MC responses [7]. Thus, through interactions with immune cells, MCs mediate different regulatory impacts on the immune system through the release of a wide variety of pre-existing and de novo-synthesized mediators [2, 6].

In recent years, co-stimulatory signals in the immune system have shown promise for the targeted treatment of diseases [8-10]. Actually, the MC surface also expresses a broad array of molecules involved in cell-cell interactions that mediate the delivery of essential signals in response to multiple types of exogenous stimulation [11, 12]. However, the relationships between co-stimulatory molecules and MCs are still unclear. The influences of changes in the expression of co-stimulatory molecules by MCs and secreted products on various T cells functions and the underlying mechanisms remain to be clarified. According to previous studies, toll-like receptors (TLRs) activate MCs to recognize pathogen-associated molecular patterns, resulting in the secretion of cytokines that promote adaptive immune responses [13-15]. These findings provided convincing evidence for a pro-inflammatory role of MCs by focusing mainly on the acute phase response or the recurrence of autoimmune and inflammatory diseases [7, 16]. TNF- α is a common factor involved in different pathological conditions [11, 17]; thus, elucidation of its impact on MCs in regulating immune responses is an important and challenging task.

In this study, we investigated MC expression of ICOSL, which is the third member of B7 superfamily [18-20] to clarify its relationship with TNF- α . Furthermore, we explored the impacts of changes in MC expression of ICOSL on T cells. To address this question, initially, we used TNF- α to stimulate MCs and analyzed the level of ICOSL expression. We hypothesized that changes in MC expression of ICOSL could influence MC membrane stabilization, which suggests a function in immune cell regulation.

Materials and Methods

Antibodies and reagents

Anti-DNP monoclonal IgE, DNP-HSA and p-nitrophenyl-N-acetyl-b-D-glucosaminide were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mouse monoclonal anti-MC tryptase antibody was purchased from Abcam (Cambridge, UK). Antibodies against β -actin, p38, phospho-p38, ERK1/2, phospho-ERK1/2, and JNK, phospho-JNK were purchased from Cell Signaling Technology (Beverly, MA, USA). Dulbecco's modified Eagle's medium and fetal bovine serum (FBS) were purchased from GIBCO (Grandland, NY, USA). Recombinant murine IL-3, recombinant murine stem cell factor (SCF) and recombinant murine TNF- α were purchased from Pretech (Rocky Hill, NJ, USA).

Mice

Male C57BL/6 mice (aged 6–8 weeks) were purchased from Schleck Experimental Animals Co. (Shanghai, China). ICOSL^{-/-} mice (C57BL/6) were purchased from Jackson Laboratory. All mice were housed in a specific pathogen-free facility and used in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals of the Chinese Academy of Sciences.

Cell preparation

Murine bone marrow-derived mast cells (BMMCs). Murine BMMCs were obtained as described previously [21]. Briefly, bone marrow cells were isolated from femurs of C57BL/6 mice, cultured in complete RPMI (Gibco, Grand Island, NY, USA), and supplemented with 10% FBS (Gibco, Grand Island, NY, USA), 100 U/mL penicillin/streptomycin (Gibco, USA), 10 ng/mL recombinant murine IL-3 (Pretech, Rocky Hill, NJ), and 10 ng/mL recombinant murine SCF (Pretech, Rocky Hill, NJ) at 37°C under 5% CO₂. Seven days later, non-adherent cells were carefully removed and replaced with fresh culture medium to enhance the purity of the MCs; this step was repeated every 7 days until adherent cells disappeared (after 5–6 passages). The purity of BMMCs was assessed by flow cytometric analysis of the expression of c-kit (CD117) and Fc ϵ RI α . BMMCs were used at a purity exceeding 90%.

CD4⁺ T cells. CD4⁺ T cells were prepared from spleens using the CD4+CD25⁺ T Cell Isolation Kit (Miltenyi Biotech, Shanghai, China) according to the manufacturer's instructions. According to flow cytometric analysis, the final purity of CD4⁺ T cells exceeded 90%.

MTT assays

Cytotoxicity of different doses of TNF- α for 24 h on MCs were assessed by colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay as described in the previous report [22].

Western blot analysis

Western blotting was conducted as described previously [23]. In brief, cell lysates were centrifuged at 12,000 rpm for 30 min at 4°C. Protein concentrations of cell lysates were determined with the BCA assay (Pierce) and 40 μ g of proteins was loaded onto SDS polyacrylamide gels (7.5%–15%). Following separation by SDS-PAGE, the proteins were transferred to nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The membranes were blocked in blocking buffer (5% non-fat dried milk/1% Tween 20 in PBS) for 2 h at room temperature and then incubated with appropriate primary antibodies (1:1000) in blocking buffer overnight at 4°C. The blot was incubated with appropriate alkaline phosphatase (AP)-conjugated secondary antibodies. Immunoreactivity was detected by the addition of 5 mL AP buffer containing 16.5 mL BCIP and 33 mL of NBT at room temperature for 10 to 20 min. Blots were then photographed. β -Actin was used as a loading control.

Real-time PCR

RNA samples were reverse transcribed into cDNA using a DNA Synthesis kit (Trans Gen, Beijing, China). To determine the quantity of mRNA, the cDNA was amplified by real-time PCR with Top Green qPCR SuperMix (Trans Gen, Beijing, China) and GAPDH was used as the internal control. The SYBR Green assays were performed in duplicate or triplicate on an iCycler® thermal cycler (Bio-Rad, Hercules, USA). The relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method.

The following primers were used for amplification of ICOSL and the endogenous control (GAPDH): ICOSL forward 5'-GTGACTTACTACCTGCCTTAC-3'; reverse 5'-CAGCCTGACCACCTCTT-3'; GAPDH forward 5'-GGTTGTCTCTGCGACTTCA-3'; reverse 5'-GGTGGTCCAGGGTTTCTTACT-3'.

Flow cytometry

BMMCs were washed twice with PBS and stained with FITC-labeled Fc ϵ RI α and PE-labeled CD117 for 30 min at room temperature. After washing, the samples were subjected to flow cytometric analysis (Cytomics FC500, Beckman Coulter). For analysis of T cell differentiation, cells were collected, washed with PBS twice, and stained with FITC-labeled anti-mouse CD4 monoclonal antibody (mAb) and PE-labeled anti-mouse CD25 mAb for 30 min at room temperature. Cells were subjected to fixation and permeabilization followed by staining with APC-labeled anti-mouse Foxp3 mAb for identification of regulatory T cells.

BMMC degranulation assay

After treating MCs with PBS and TNF- α at different concentrations for 24 h, BMMCs were pre-incubated with anti-DNP-IgE (100 ng/mL) for 24 h then challenged with DNP-HSA (200 ng/mL). After 1 h, conditioned media from these IgE/Ag-degranulated BMMCs were mixed into the culture system. After solubilization with 0.5% Triton X-100 in Tyrode's buffer, the enzymatic activities of β -hexosaminidase in supernatants and cell pellets were measured with p-nitrophenyl N-acetyl-b-D-glucosaminide in 0.1 M sodium citrate (pH 4.5) for 60 min at 37°C. The reaction was stopped by addition of 0.2 M glycine (pH 10.7). The release of the 4-p-nitrophenol product was detected by absorbance at 405 nm. The extent of degranulation was represented as the 4-p-nitrophenol absorbance in the supernatants as a percentage of the sum of absorbance in the supernatants and in cell pellets solubilized in detergent. To evaluate the impact of changes in ICOSL expression by MCs on CD4⁺T cells, MCs were treated with PBS and TNF- α for 24 h, washed twice, then added to T cells with an anti-DNP-IgE mAb associated with DNP-HSA.

T cell–MC co-culture

BMMCs were stimulated with TNF- α (10 ng/mL, 20 ng/mL, 40 ng/mL, 80 ng/mL) for 24 h in complete RPMI 1640 medium. T cells (1×10^5 cells/well) were plated into 6-well flat-bottomed plates (Falcon; BD Biosciences) coated with 1 mg/mL anti-mouse CD3 mAb and then co-cultured with TNF- α stimulated or unstimulated BMMCs (1×10^5 cells/well) for 24 h.

Cytokine detection assays

Concentrations of IL-4, IL-10 and IFN- γ were measured in various cell co-culture supernatants by ELISA using cytokine specific kits according to the manufacturers' instructions. The optical density was measured at 450 nm using a microplate reader (BioTek, UK).

Statistical analysis

All experiments were repeated no less than three times and the results were presented as the mean of those independent experiments. All data were described as (mean $\pm$ SD). SPSS 22.0 software was used for the statistical analysis. Comparisons between two independent groups were performed by Student's t-test. The Spearman test was used for correlation analyses. Statistical analysis was carried out using Prism 5.0 (GraphPad Software, San Diego, CA, USA) to evaluate the significance of differences between groups; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Results

Characteristics of bone marrow mast cells and purification of CD4+ T cell

To explore the effects of pre-treated MCs on CD4+ T cells, mast cells from bone marrow were generated in the presence of IL-3 and SCF following a 5-week culture. Fig. 1A–D illustrate the morphology of BMMCs, which contained an abundance of purple granules after toluidine blue staining. Identification of mast cells and by flow cytometric analysis of Fc ϵ RI α and CD117 suggested that the purity of mast cells exceeded 90% (Fig. 1E). Similarly, flow cytometric analysis of the isolated CD4+ T cells revealed the purity of these sorted CD4+ T cell populations was more than 90%.

TNF- α upregulates ICOSL expression on MCs

To establish a model of high ICOSL expression by MCs, we first examined expression levels of ICOSL mRNA on MCs treated with PBS, TNF- α (50 g/mL), ox-LDL (50ng/mL), LPS (50 ng/mL) and ConA (50 ng/mL) for 24 h. Fig. 2A shows that only TNF- α upregulated ICOSL expression on MCs. We then stimulated MCs with different TNF- α concentrations (1 ng/ml, 5 ng/ml, 10 ng/mL 20 ng/mL, 40 ng/mL, and 80 ng/mL). RT-PCR (Fig. 2B) and flow cytometry analysis (Fig. 2C, D) showed that TNF- α affected ICOSL level through a dose-independent manner. The higher doses of TNF- α may induce the change of other factors. These results showed that the expression levels of ICOSL increased in MCs due to the presence of lower doses of TNF- α . Therefore, we chose 10ng/ml as TNF- α concentration in the next function experiments.

TNF- α inhibits MC degranulation through upregulation of ICOSL expression

To examine the effect of ICOSL on the degranulation of MCs, we measured MC degranulation after TNF- α treatment (Fig. 3). After stimulation with anti-DNP-IgE mAb and DNP-HSA, the β -hexosaminidase release represented the extent of degranulation. Pretreatment of MCs with TNF- α for 24 h led to a decrease in β -hexosaminidase release (Fig. 3 A, B) and the reduction of the degranulation response does not depend on treatment toxicity (Fig. 3C). Moreover, in ICOSL $^{-/-}$ MCs, pretreat with TNF- α did not affect MC degranulation (Fig. 3D). These results demonstrated that TNF- α inhibited MC degranulation and stabilized MC membranes through upregulation of ICOSL expression.

Fig. 1. Characteristics of bone marrow mast cells and purification of CD4⁺ T cells. (A–C) Characteristics of mast cells after five weeks in culture viewed under a light microscope (A: 40 $\times$, B: 200 $\times$, C: 400 $\times$). (D) Bone marrow mast cells showing abundant purple granules following toluidine blue staining (400 $\times$). (E) Mast cell expression of Fc ϵ R1 α and CD117 analyzed by flow cytometry. (F) The final purity of CD4⁺ T cells was shown to exceed 90% by flow cytometry analysis.

Fig. 2. ICOSL expression is up-regulated by lower doses of TNF- α on MCs. MCs were pre-treated with PBS (control),

TNF- α (50 ng/mL), ox-LDL (50 ng/mL), LPS (50 ng/mL) and ConA (50 ng/mL) and different TNF- α concentrations for 24 h. (A–B) Expression of ICOSL mRNA after MC pre-treatment. (C) Numbers in the flow cytometry histogram represent the percentage of cells expressing ICOSL. (D) Flow cytometry histograms showing ICOSL expression on MCs after PBS (control), 10, 20, 40 and 80 ng/ml TNF- α treatment. Each histogram is a representative of three independent experiments. Data were combined from three different experiments. Significant differences: * P <0.05; ** P <0.01; *** P <0.001.

Fig. 3. TNF- α inhibits MC degranulation through up-regulation of ICOSL expression. (A) MCs were pre-treated with PBS (control), TNF- α (50 ng/mL), ox-LDL (50 ng/mL), LPS (50 ng/mL) and ConA (50 ng/mL) and (B) different TNF- α concentrations for 24 h, and the level of degranulation was examined. (C) Cytotoxicity of different doses of TNF- α for 24 h on MCs was determined by MTT assay. (D) MCs from wild-type or ICOSL^{-/-} mice were pre-treated with PBS or TNF- α (10 ng/mL), and the level of degranulation was examined. Up: ICOSL expression in ICOSL^{-/-} MC was examined by western blot. Data are representative of three independent experiments. Significant differences: *P<0.05, **P<0.01.

TNF- α upregulates ICOSL expression on MCs by activation of MAPK signaling pathway

To determine the signaling mechanisms involved in the effects of ICOSL on MCs, the expression of the phosphorylated and unphosphorylated forms of SAPK/JNK, ERK1/2, and p38 were investigated. As shown in Fig. 4A, there were notable differences in MAPK phosphorylation in the MCs after TNF- α treatment. The degree of phosphorylation of ERK1/2 was not as dramatic as that of p38 and SAPK/JNK. However, recovery after degranulation of TNF- α -treated MCs did not affect the MAPK signal or its phosphorylation level. As shown in Fig. 4B, a p38 MAPK inhibitor SB203580 [24] and a JNK MAPK inhibitor SP600125 [25] suppressed phosphorylation of p38 and JNK, respectively. These MAPK inhibitors also suppressed up-regulation of ICOSL by the TNF- α treatment (Fig. 4C, D). This result suggested that TNF- α up-regulated ICOSL expression on MCs by activation of MAPK phosphorylation via a degranulation-independent mechanism.

TNF- α -treated MCs promote the differentiation of regulatory T cells

We investigated the effects of ICOSL on the differentiation of T cells in six experimental groups: (1) MCs co-cultured with T cells (MC+T); (2) MC degranulation (stimulation with anti-DNP-IgE mAb and DNP-HSA) and then co-cultured with T cells (deMC+T); (3) TNF- α -treated MCs and then co-cultured with T cells (TNF- α -MC+T); (4) degranulation-recovered TNF- α -treated MCs (treated with TNF- α and then stimulated with anti-DNP-IgE mAb and DNP-HSA) co-cultured with T cells (deTNF- α -MC+T); (5) TNF- α -treated ICOSL^{-/-} MCs and then co-cultured with T cells (TNF- α -ICOSL^{-/-}-MC+T) and (6) degranulation-recovered TNF- α -treated ICOSL^{-/-} MCs co-cultured with T cells (deTNF- α -ICOSL^{-/-}-MC+T). CD4⁺ T cells were co-cultured with MCs with or without treatment at the ratio of 1:1 for 24 h in a 6-well plate pre-coated with anti-CD3 mAb (Fig. 5A). Subsequently, the percentages of CD4⁺CD25⁺Foxp3⁺ T cells were determined by flow cytometry analysis. The MC+T group served as the control. Compared with the control, T cells in the stimulated group (TNF- α -MC+T group) showed a higher differentiation rate (Fig. 5B). Meanwhile, the percentage of regulatory T cells decreased in the deTNF- α -MC+T group. In ICOSL^{-/-} MCs co-cultured with T cells, TNF- α -treatment or degranulation-recovery did affect the differentiation of regulatory T cells (Fig. 5C). According to these findings, TNF- α -treated MCs promote CD4⁺ T cell differentiation to CD4⁺CD25⁺Foxp3⁺ T cells through up-regulation of ICOSL expression, while degranulation inhibited the ability of TNF- α to induce the differentiation of regulatory T cells.

Fig. 4. TNF- α up-regulates ICOSL expression on MCs by activation of MAPK signaling pathway. (A) MCs were treated with only 10ng/ml TNF- α (TNF- α -MC), or treated with TNF- α and then stimulated with anti-DNP-IgE mAb and DNP-HSA (deTNF- α -MC), and the expression levels of p38, p-p38, ERK1/2, p-ERK1/2, JNK and p-JNK proteins were detected by western blotting. β -Actin was used as the control. (B) Western blot analysis for the levels of p-p38, p38, p-JNK and JNK in the absence (-) or presence (+) of 10 μ M SB203580 or 15 μ M SP600125 30 min after TNF- α stimulation. (C, D) ICOSL expression in the absence (-) or presence (+) of 10 μ M SB203580 or 15 μ M SP600125 30 min after TNF- α stimulation was determined by RT-PCR and flow cytometry. Data are representative of three independent experiments. Significant differences: *P<0.05.

TNF- α treated MCs induce a shift in cytokine expression from a Th1 to a Th2 profile

Based on the finding that MCs influence T cell differentiation, we also analyzed the concentrations of Th1-type (IFN- γ) and Th2-type (IL-4 and IL-10) cytokines in the co-culture. Compared with the MC+T group, IFN- γ secretion and mRNA expression levels of IFN- γ and VEGF were decreased in the TNF- α -MC+T group, while the secretion of IL-4 and IL-10 and mRNA expression level of IL-10 were increased (Fig. 6A,B). Interestingly, when compared with TNF- α -MC+T group, IL-4 and IL-10 were significantly decreased in the deTNF- α -MC+T group, while there was no significant difference in IFN- γ secretion. IL-4, IL-10 and IFN- γ were significantly decreased via TNF- α concentration-dependence in the deTNF- α -MC+T group (Fig. 6C). In ICOSL^{-/-} MCs co-cultured with T cells, TNF- α -treatment or degranulation-recovery did affect the shift in cytokine expression from a Th1 to a Th2 profile (Fig. 6D). These results indicate that TNF- α -treated MCs influence the Th1/Th2 distribution by up-regulation of ICOSL expression and inhibition of MC degranulation.

Discussion

Recently, BMMCs have become a focus of research due to their ubiquitous distribution in the connective and vascularized tissues or cavities throughout the body and their critical roles in the regulation in variety of immune processes and many inflammatory diseases [2, 26, 27]. Currently, mast cells are considered to function in a manner that is similar to antigen presenting cells in host defense and homeostasis via the release of cytokines and other mediators that recruit T cells to sites of infection [27, 28]. According to previous studies, the interaction of ICOS with its sole ligand, ICOSL (also known as B7-related protein-1) triggers key T cell activities, including cytokine production and differentiation into the T follicular helper (T_{fh}) cell lineage over effector lineages [29-32]. Therefore, mast cells may play other important roles in homeostasis and allergic inflammation. It is well-known that TNF- α plays a

Fig. 5. TFN- α -treated MCs promote the differentiation of regulatory T cells. (A) 10^5 naive CD4 $^+$ T cells were co-cultured with 10^5 pre-treated BMMCs. (B) Representative and cumulative data on FoxP3 expression by CD4 $^+$ CD25 $^+$ T cells after stimulation with MCs co-cultured with T cells (MC+T), MC degranulation (stimulation with anti-DNP-IgE mAb and DNP-HSA) and then co-cultured with T cells (deMC+T), TNF- α -treated MCs and then co-cultured with T cells (TNF- α -MC+T) or degranulation-recovered TNF- α -treated MCs (treated with TNF- α and then stimulated with anti-DNP-IgE mAb and DNP-HSA) co-cultured with T cells (deTNF- α -MC+T). (C) Cumulative data on FoxP3 expression by CD4 $^+$ CD25 $^+$ T cells after stimulation with MCs co-cultured with T cells (MC+T), TNF- α -treated MCs and then co-cultured with T cells (TNF- α -MC+T), TNF- α -treated ICOSL $^{-/-}$ MCs and then co-cultured with T cells (TNF- α -ICOSL $^{-/-}$ -MC+T) or degranulation-recovered TNF- α -treated ICOSL $^{-/-}$ MCs co-cultured with T cells (deTNF- α -ICOSL $^{-/-}$ -MC+T). Data are representative of three independent experiments. Significant differences: ** P <0.01; *** P <0.001.

key role in these conditions; however, the ability of TNF- α to influence MC functions in T cells differentiation remains to be elucidated [33]. In this study, we showed that TNF- α stimulation upregulated ICOSL expression on MCs and inhibited degranulation, while several other exogenous stimulators such as ox-LDL, LPS and ConA did not (Fig. 2–3). Co-culture of MCs and CD4 $^+$ T cells revealed that TNF- α stimulation enhances the immunoregulatory functions of MCs, with corresponding changes in the percentage of regulatory T cells. Specifically, TNF- α treated MCs promoted CD4 $^+$ T cell differentiation to CD4 $^+$ CD25 $^+$ Foxp3 $^+$ T cells, while this effect was inhibited by MC degranulation (Fig. 5). Furthermore, we identified changes in the secretion of Th1/Th2 type cytokines in the supernatants collected from the co-culture system. Fig. 6 shows that the levels of the Th1 marker cytokine, IFN- γ , decreased significantly, while expression of the Th2 marker cytokines, IL-4 and IL-10, increased. However, in the

Fig. 6. TNF- α -treated MCs induce a shift in cytokine expression from a Th1 to a Th2 profile. (A) IL-4, IL-10 and IFN- γ levels in the supernatants of the co-culture system: MC+T, deMC+T, TNF- α -MC+T or deTNF- α -MC+T. (B) After MCs co-cultured with T cells, RT-PCR analysis detected mRNA expression level of IFN- γ , IL-10 and VEGF in isolated MCs, (C) Cytokine changes in supernatants of degranulation-recovered TNF- α -treated MCs co-cultured with T cells. (D) IL-4, IL-10 and IFN- γ levels in the supernatants of the co-culture system: MC+T, TNF- α -MC+T, TNF- α -ICOSL $^{-/-}$ -MC+T or deTNF- α -ICOSL $^{-/-}$ -MC+T. Each group are presented as the mean $\pm$ SD of at least three independent experiments. Significant differences: *P<0.05; **P<0.01; ***P<0.001.

presence of degranulated MCs, the levels of IL-4 and IL-10 decreased compared with that in the presence of undegranulated DCs, while there was no significant difference in the levels of IFN- γ . We also found that, in the MC-T co-culture system with TNF- α -untreated MCs, the levels of IFN- γ , IL-4 and IL-10 increased in the presence of degranulated MCs, compared with the levels detected in the presence of undegranulated MCs. Based on these findings, we conclude that MCs promote the differentiation and functions of regulatory T cells by upregulating the expression of ICOSL. MCs regulate T cell cytokines secretion through degranulation commonly; however, in the present study, we revealed that stimulation of MC-expressed molecules such as ICOSL may participate in the regulation of these processes via cell-cell interactions. This novel role for MC-derived ICOSL in the regulation of the immune microenvironment has important implications for the design of therapeutic strategies.

The mechanism underlying the inducible expression of ICOSL is not clear. A recent study demonstrated increased ICOSL expression during the maturation of plasmacytoid DCs [34],

although the exact mechanism was unknown. Here, we found that inducible expression of ICOSL on MCs was TNF- α -dependent for a short period. We suspected that cumulative effects leading to the functional modification of MCs occurred during the stimulation. Fig. 4A shows that the ICOSL expression is related to MAPK and p-MAPK signals; thus, we conclude that TNF- α upregulates ICOSL by activation of the MAPK signaling pathway. Furthermore, degranulation of MCs has no influence on MAPK expression and its phosphorylation status, which may correlate with other signaling pathways. Unfortunately, a direct connection between MAPK signaling activation and ICOSL expression could not be investigated due to the restrictions of time and conditions. Further studies are required to confirm TNF receptor expression on MCs for clarification of the mechanism underlying TNF- α -mediated stimulation of MCs as well as the potential expression of other mediators that could connect MAPK/p-MAPK activation and ICOSL induction in the TNF- α culture system and play key roles in this process.

Conclusion

In summary, although the exact mechanism of TNF- α dependent ICOSL expression on MCs was not fully elucidated, we demonstrate novel regulatory roles of MCs in the differentiation of CD4⁺ T cells to regulatory T cells via the ICOSL/ICOS interaction. These findings provide insights into the molecules and signaling pathways involved in the interaction of MCs with other immune cells, which it is hoped will lead to the development of different therapeutic strategies aimed at boosting or suppressing specific MC functions, depending on the pathological context.

Disclosure Statement

The authors have no competing financial or intellectual interests.

Abbreviations

MCs (mast cell), ICOSL (inducible co-stimulatory ligand), T-reg (regulatory T cell), TNF- α (tumor necrosis factor- α), TNF- α -MC (TNF- α -stimulated MCs), deTNF- α -MCs (degranulation of TNF- α stimulated MCs).

Acknowledgements

This work was supported by National Natural Science Foundation of China, No. 81270555, 81470897, 81502540 and 81503434, Program for New Century Excellent Talents in University, No. NECT-13-0422 and Fundamental Research Fund for the Central Universities of China, No. 222201514333.

References

- 1 Krystelwhittemore M, Dileepan KN, Wood JG: Mast Cell: A Multi-Functional Master Cell. *Front Immunol* 2016;6:620.
- 2 Mekori YA, Metcalfe DD: Mast cells in innate immunity. *Immunol Rev* 2000;173:131-140.
- 3 Frossi B, De CMC: The mast cell: an antenna of the microenvironment that directs the immune response. *J Leukocyte Biol* 2004;75:579-585.
- 4 Marshall JS: Mast-cell responses to pathogens. *Nat Rev Immunol* 2004;4:787-799.

- 5 Galli SJ, Grimaldeston M, Tsai M: Immunomodulatory mast cells: negative, as well as positive, regulators of immunity. *Nat Rev Immunol* 2008;8:478-486.
- 6 Dudeck A, Dudeck J, Scholten J, Petzold A, Surianarayanan S, Köhler A, Peschke K, Vöhringer D, Waskow C, Krieg T: Mast cells are key promoters of contact allergy that mediate the adjuvant effects of haptens. *Immunity* 2011;34:973-984.
- 7 Tsai M: Mast cells in the development of adaptive immune responses. *Nat Immunol* 2005;6:135-142.
- 8 Chen D, Nurieva RI, Prasad DVR: Immune regulation by novel costimulatory molecules. *Immunol Res* 2003;28:39-48.
- 9 Zhu Y, Lai X, Zhao S, Zheng J, Wang X, Weng X, Li J, Ye Y, Chen Q, Lin X: Antitumor enhancement by irradiated haploidentical donor lymphocyte infusion of mice with melanoma. *Cell Physiol Biochem* 2015;35:2181-2191.
- 10 Liu Y, Zhao X, Zhong Y, Meng K, Yu K, Shi H, Wu B, Tony H, Zhu J, Zhu R, Peng Y, Mao Y, Cheng P, Mao X, Zeng Q: Heme oxygenase-1 restores impaired GARP CD4(+)CD25(+) regulatory T cells from patients with acute coronary syndrome by upregulating LAP and GARP expression on activated T lymphocytes. *Cell Physiol Biochem* 2015;35:553-570.
- 11 Nakae S, Suto H, Iikura M, Kakurai M, Sedgwick JD, Tsai M, Galli SJ: Mast cells enhance T cell activation: importance of mast cell costimulatory molecules and secreted TNF. *J Immunol* 2006;176:2238-2248.
- 12 Girvin AM, Dal Canto MC, Rhee L, Salomon B, Sharpe A, Bluestone JA, Miller SD: A critical role for B7/CD28 costimulation in experimental autoimmune encephalomyelitis: a comparative study using costimulatory molecule-deficient mice and monoclonal antibody blockade. *J Immunol* 2000;164:136-143.
- 13 Dietrich N, Akira S: Mast cells elicit proinflammatory but not type I interferon responses upon activation of TLRs by bacteria. *Proc Natl Acad Sci USA* 2010;107:8748-8753.
- 14 Takenaka H, Ushio H, Niyonsaba F, Jayawardana STM, Hajime S, Ikeda S, Ogawa H, Okumura K: Synergistic augmentation of inflammatory cytokine productions from murine mast cells by monomeric IgE and toll-like receptor ligands. *Biochem Biophys Res Commun* 2010;391:471-476.
- 15 Xu X, Gao X, Zhao X, Liao Y, Ji W, Li Q, Li J: PU.1-Silenced Dendritic Cells Induce Mixed Chimerism and Alleviate Intestinal Transplant Rejection in Rats via a Th1 to Th2 Shift. *Cell Physiol Biochem* 2016;38:220-228.
- 16 Frossi B, Gri G, Tripodo C, Pucillo C: Exploring a regulatory role for mast cells: 'MCREGS'? *Trends Immunol* 2010;31:97-102.
- 17 Cheng W, Yu X, Yan Y, Wei Y, Zhang S, Xiang Y, Jian Z, Wang W: Tumor necrosis factor- α : a key contributor to intervertebral disc degeneration. *Acta Biochim Biophys Sin* 2017;49:1-13.
- 18 Greenwald RJ, Freeman GJ, Sharpe AH: The B7 Family Revisited. *Immunology* 2005;23:515-548.
- 19 Swallow MM, Wallin JJ, Sha WC: B7h, a novel costimulatory homolog of B7.1 and B7.2, is induced by TNF α . *Immunity* 1999;11:423-432.
- 20 Sharpe AH, Freeman GJ: The B7-CD28 superfamily. *Nat Rev Immunol* 2002;2:116-126.
- 21 Von KM, Goldmann O, Thulin P, Heinemann K, Norrby-Teglund A, Rohde M, Medina E: Phagocytosis-independent antimicrobial activity of mast cells by means of extracellular trap formation. *Blood* 2008;111:3070-3080.
- 22 Liang X, Wu A, Xu Y, Xu K, Liu J, Qian X: B1, a novel naphthalimide-based DNA intercalator, induces cell cycle arrest and apoptosis in HeLa cells via p53 activation. *Invest New Drug* 2011;29:646-658.
- 23 Zhang J, Shen Y, Liu J, Wei D: Antimetastatic effect of prodigiosin through inhibition of tumor invasion. *Biochem Pharmacol* 2005;69:407-414.
- 24 Cuenda A, Rouse J, Doza YN, Meier R, Cohen P, Gallagher TF, Young PR, Lee JC: SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Lett* 1995;364:229-233.
- 25 Bennett BL, Sasaki DT, Murray BW, O'Leary EC, Sakata ST, Xu W, Leisten JC, Motiwala A, Pierce S, Satoh Y, Bhagwat SS, Manning AM, Anderson DW: SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. *Proc Natl Acad Sci USA* 2001;98:13681-13686.
- 26 Qiao SK, Ren HY, Shi YJ, Liu W: Allogeneic Compact Bone-Derived Mesenchymal Stem Cell Transplantation Attenuates the Severity of Idiopathic Pneumonia Syndrome in a Murine Bone Marrow Transplantation Model. *Cell Physiol Biochem* 2016;40:1656-1669.

- 27 Chen Y, Xu Y, Du J, Guo J, Lei X, Cui J, Liu C, Cheng Y, Li B, Gao F, Ju J, Cai J, Yang Y: Radioprotective Effects of Heat-Killed Mycobacterium Tuberculosis in Cultured Cells and Radiosensitive Tissues. *Cell Physiol Biochem* 2016;40:716-726.
- 28 Malaviya R, Ikeda T, Ross E, Abraham SN: Mast cell modulation of neutrophil influx and bacterial clearance at sites of infection through TNF-alpha. *Nature* 1996;381:77-80.
- 29 Mazzoni A, Siraganian RP, Leifer CA, Segal DM: Dendritic cell modulation by mast cells controls the Th1/Th2 balance in responding T cells. *J Immunol* 2006;177:3577-3581.
- 30 Chen L: Co-inhibitory molecules of the B7-CD28 family in the control of T-cell immunity. *Nat Rev Immunol* 2004;4:336-347.
- 31 B S, Lenschow DJ, Rhee L, Ashourian N, Singh B, Sharpe A, Bluestone JA: B7/CD28 Costimulation Is Essential for the Homeostasis of the CD4+CD25+ Immunoregulatory T Cells that Control Autoimmune Diabetes. *Immunity* 2000;12:431-440.
- 32 Wang L, Wu G, Qin X, Ma Q, Zhou Y, Liu S, Tan Y: Expression of Nodal on Bronchial Epithelial Cells Influenced by Lung Microbes Through DNA Methylation Modulates the Differentiation of T-Helper Cells. *Cell Physiol Biochem* 2015;37:2012-2022.
- 33 Sayed BA, Brown MA: Mast cells as modulators of T-cell responses. *Immunol Rev* 2007;217:53-64.
- 34 Ito T, Yang M, Wang YH, Lande R, Gregorio J, Perng OA, Qin XF, Liu YJ, Gilliet M: Plasmacytoid dendritic cells prime IL-10-producing T regulatory cells by inducible costimulator ligand. *J Exp Med* 2007;204:105-115.