



Th2-inducing cytokines IL-4 and IL-33 synergistically elicit the expression of transmembrane TNF- α on macrophages through the autocrine action of IL-6

Hiromi Ogawa^{a,1}, Kaori Mukai^{a,1,2}, Yohei Kawano^a, Yoshiyuki Minegishi^{a,b}, Hajime Karasuyama^{a,b,*}

^a Department of Immune Regulation, Tokyo Medical and Dental University Graduate School, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan

^b JST, CREST, Tokyo Medical and Dental University Graduate School, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan

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ABSTRACT

Tumor necrosis factor- α (TNF- α) is a potent proinflammatory cytokine produced predominantly by activated macrophages, and plays a central role in the protective immunity against intracellular pathogens and the pathogenesis of autoimmune and inflammatory diseases. While both the soluble and transmembrane forms of TNF- α (sTNF- α and tmTNF- α) are biologically functional, the latter but not the former acts as a receptor besides as a ligand, and transmit a retrograde signal in a cell-to-cell contact manner. The production of TNF- α by macrophages under Th2-type (allergic) inflammatory conditions has been ill defined, compared to that under Th1-type inflammatory conditions. Here we examined the effect of representative Th2-inducing cytokines IL-4 and IL-33 on the TNF- α expression in macrophages. IL-4 induced the production of neither sTNF- α nor tmTNF- α while IL-33 promoted the production of sTNF- α with no detectable tmTNF- α . Notably, the combination of IL-4 and IL-33 elicited the tmTNF- α expression on macrophages, in addition to the enhanced production of sTNF- α and IL-6. The IL-4/IL-33-elicited tmTNF- α expression was not observed in IL-6-deficient macrophages, suggesting the involvement of macrophage-derived IL-6 in the tmTNF- α expression. Indeed, the stimulation of macrophages with the combination of IL-4 and IL-6 induced the tmTNF- α expression with no detectable production of sTNF- α . Thus, IL-4 and IL-33 synergistically elicit the tmTNF- α expression on macrophages through the autocrine action of IL-6.

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1. Introduction

TNF- α , one of the most abundant early mediators in inflamed tissues, is produced by many different types of cells, particularly macrophages [1,2]. TNF- α shows pleiotropic effects on various cell lineages and play crucial roles in the host defense against intracellular pathogens [3,4] and the pathogenesis of autoimmune and inflammatory disorders [5,6]. Indeed, several anti-TNF- α agents have been successfully introduced for treating rheumatoid arthritis and inflammatory bowel diseases such as Crohn's disease [5,6]. TNF- α is first generated as a type II transmembrane polypeptide (tmTNF- α) that is in turn processed by TNF- α -converting enzyme (TACE, also known as ADAM17) on the cell surface to release

sTNF- α [7,8]. sTNF- α exerts its biological function through binding to two types of receptors (TNFR1 and TNFR2) expressed on target cells [9]. Of note, it has been shown that tmTNF- α , in addition to sTNF- α , is involved in cytotoxicity and inflammatory responses, in a cell-to-cell contact manner [10–13]. tmTNF- α acts as a ligand by binding to TNF receptors on target cells as does sTNF- α . Moreover, tmTNF- α , in contrast to sTNF- α , functions as a receptor that transmits outside-to-inside (reverse) signals back into the tmTNF- α -expressing cells [14]. Although both sTNF- α and tmTNF- α can bind to TNFR1 and TNFR2, TNFR2, that is typically expressed by immune cells, can be fully activated only by tmTNF- α and not by sTNF- α [15]. Thus, tmTNF- α shows activities that are distinct from as well as overlapping with those displayed by sTNF- α [16].

Macrophages are distributed throughout many different tissues and organs, and play a central role in homeostasis, host defense, inflammatory reactions, and tissue remodeling [17]. They are heterogeneous in their phenotype and function, and can be polarized to various distinct subsets, depending on the microenvironment [17,18]. Polarized macrophages have been broadly classified as M1 or M2 macrophages. M1 macrophages are induced by microbial products such as lipopolysaccharide (LPS) and IFN- γ during Th1-type responses, and function as effector cells against microorganisms and tumor cells. M2 macrophages, in contrast,

Abbreviations: TNF- α , tumor necrosis factor- α ; tmTNF- α , transmembrane TNF- α ; sTNF- α , soluble TNF- α ; TACE, TNF- α -converting enzyme; LPS, lipopolysaccharide; WT, wild-type.

* Corresponding author at: Department of Immune Regulation, Tokyo Medical and Dental University Graduate School, 1-5-45 Yushima, Bunkyo-ku, Tokyo 113-8519, Japan. Fax: +81 3 3814 7172.

E-mail address: karasuyama.mbch@tmd.ac.jp (H. Karasuyama).

¹ These authors contributed equally to this work.

² Current address: Department of Pathology, Stanford University School of Medicine, Stanford, CA, United States.

are typically induced by Th2 cytokines such as IL-4 during Th2-type responses, including allergic reactions and parasitic infections [19,20]. IL-33, a recently-identified member of the IL-1 family of cytokines, has been shown to amplify the M2 polarization of macrophages, and promote the production of Th2 cytokines such as IL-4, IL-5 and IL-13 by innate-type lymphoid cells, basophils, mast cells, and Th2 cells [21–24].

The production of TNF- α by macrophages under Th2-type inflammatory conditions has been ill defined, compared to that under Th1-type inflammatory conditions. Therefore, in the present study, we examined the expression of tmTNF- α and sTNF- α by macrophages that were stimulated with Th2-inducing cytokines, IL-4 and IL-33, either alone or in combination. Whereas IL-4-stimulated macrophages, a prototype of M2 macrophages, produced no detectable TNF- α , regardless of a transmembrane or soluble form, the combinatorial stimulation of macrophages with IL-4 and IL-33 induced the tmTNF- α expression on the cell surface, even though IL-33 alone failed to do so. The IL-4/IL-33-elicited tmTNF- α expression turned out to be dependent on a proinflammatory cytokine IL-6 produced by macrophages, in contrast to the LPS-elicited tmTNF- α expression. Thus, Th2-inducing cytokines IL-4 and IL-33 synergistically elicit the tmTNF- α expression on macrophages through the autocrine action of IL-6.

2. Materials and methods

2.1. Mice

BALB/c and C57BL/6 mice (6–10 week old) were purchased from CLEA Japan (Tokyo, Japan). *Il6*^{-/-} mice [25] were maintained in our animal facilities under specific pathogen-free conditions. All animal experiments were performed according to the guidelines of the Institutional Animal Care and Use Committees of the Tokyo Medical and Dental University. The protocols for animal experiments were approved by the Institutional Animal Care and Use Committees of the Tokyo Medical and Dental University.

2.2. Antibodies

FITC-conjugated anti-mouse F4/80 (BM8), anti-CD11b (M1/70) and control rat IgG1 and IgG2a, biotinylated control rat IgG1 and IgG2a were purchased from eBioscience (San Diego, CA). PE-conjugated anti-mouse F4/80 (BM8), Pacific Blue™ anti-mouse CD11b (M1/70), biotinylated anti-mouse TNF- α (MP6-XT22), APC-conjugated streptavidin were from BioLegend (San Diego, CA). Anti-CD16/32 (2.4G2) was prepared from hybridoma culture supernatants in our laboratory.

2.3. Preparation and stimulation of macrophages

Cells were harvested from the peritoneal cavity 4 days after mice were injected i.p. with 1.5 ml of 3% thioglycolate broth (BD Diagnostic Systems), and cultured at 37 °C for 2 h in RPMI 1640 containing 10% FCS, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μ M nonessential amino acids, 0.05 M 2-ME, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Adherent cells (as peritoneal macrophages, 1×10^6 cells/1 ml/well) were stimulated with IL-4 (50 ng/ml, R&D), IL-6 (50 ng/ml, Peprotech), IL-33 (50 ng/ml, R&D), or LPS *Escherichia coli* 055:B5 (100 ng/ml, Sigma-Aldrich) individually or in combination for the indicated time periods.

2.4. Flow cytometric analysis

Cells were preincubated with anti-CD16/32 mAb and normal rat serum on ice for 15 min prior to incubation with the indicated

combination of Abs in order to prevent the non-specific binding of irrelevant Abs. Stained cells were analyzed on a FACSCanto II machine (BD) using FACSDiva software (BD). Macrophages were identified as F4/80⁺CD11b⁺ cells.

2.5. Evaluation of cytokine production

The concentration of TNF- α and IL-6 in culture supernatants was determined by ELISA using Ready-SET-Go! (eBioscience) and Mouse IL-6 ELISA MAX™ Standard (BioLegend), respectively, according to the manufacturer's instructions.

2.6. Statistical analysis

Data were analyzed by unpaired Student *t* test to determine the difference between the samples. *P* < 0.05 was considered as significant.

3. Results

3.1. IL-4 and IL-33 synergistically induce the tmTNF- α expression on macrophages

Peritoneal macrophages isolated from BALB/c mice were stimulated with representative Th2-inducing cytokines, IL-4 and IL-33, either individually or in combination (Fig. 1). Neither IL-4 nor IL-33 alone induced any significant expression of tmTNF- α on macrophages during the 48 h culture. In contrast, the surface expression of tmTNF- α became detected 24 h and 48 h after the stimulation of macrophages with the combination of IL-4 and IL-33. LPS stimulation is well known to induce the TNF- α production. However, the LPS-elicited tmTNF- α expression on the cell surface was confined to the early phase (2–6 h) of the culture, and became undetectable at the late phase (24 h and 48 h) (Fig. 1). Thus, the tmTNF- α expression on macrophages can be induced even under Th2 conditions if both IL-4 and IL-33 coexist, although the timing of the tmTNF- α expression is much delayed compared to that observed in LPS-stimulated macrophages. The similar pattern of tmTNF- α expression was observed in macrophages isolated from C57BL/6 mice (as shown later in Fig. 3), even though they had a tendency to show lower levels of tmTNF- α expression compared to BALB/c macrophages.

IL-4 alone induced little or no production of sTNF- α in macrophages while IL-33 alone was sufficient for inducing the sTNF- α production (Fig. 2A). Notably, the combination of IL-4 and IL-33 stimulation significantly augmented the sTNF- α production compared to the stimulation with IL-33 alone (Fig. 2A). This was also true for the IL-6 production by macrophages, and the combination of IL-4 and IL-33 stimulation promoted the IL-6 production to the extent comparable to that induced by the LPS stimulation (Fig. 2B).

3.2. The IL-4/IL-33-elicited production of tmTNF- α but not sTNF- α is abolished in IL-6-deficient macrophages

To examine the possible influence of macrophage-derived IL-6 on the TNF- α production by macrophages, we compared the production of sTNF- α and tmTNF- α between macrophages derived from wild-type mice and those from IL-6-deficient mice, when stimulated with either LPS or IL-4 plus IL-33 (Fig. 3). Because the IL-6-deficient mice we obtained have the C57BL/6-type genetic background, C57BL/6 instead of BALB/c mice were used as wild-type control mice in this experiment. No significant difference in the sTNF- α production was observed between wild-type and IL-6-deficient macrophages when stimulated with the combination of IL4 and IL-33 (Fig. 3A). Intriguingly, however, the IL-4/IL-33-elic-

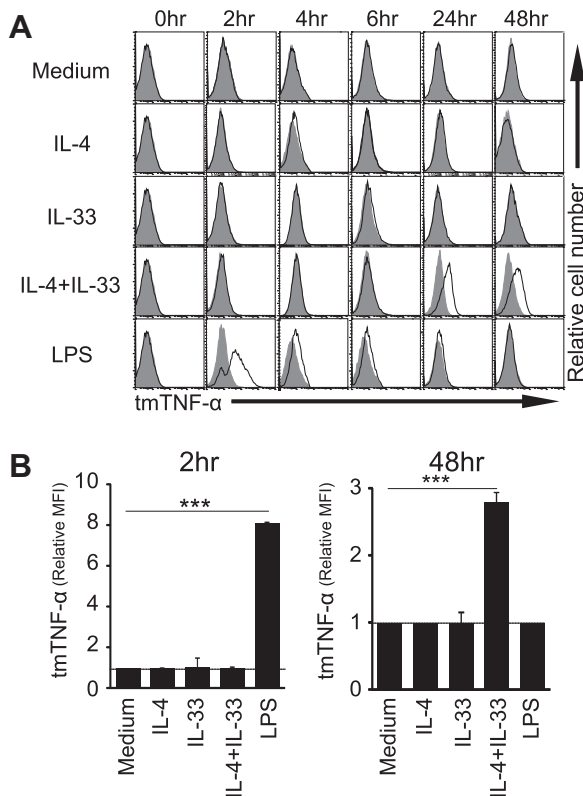


Fig. 1. IL-4 and IL-33 synergistically induce the tmTNF- α expression on macrophages. (A) Peritoneal macrophages isolated from BALB/c mice were cultured with either control medium, IL-4, IL-33 or LPS alone or in combination for the indicated time periods. Data shown are representative of the surface expression of tmTNF- α (open histograms) compared with control staining with an isotype-matched control antibody (filled histograms) at each time point. (B) The level of the tmTNF- α expression on macrophages that were stimulated as in A for 2 h (left panel) or 48 h (right panel) is shown as the relative mean fluorescence intensity (MFI), that is, the MFI of staining with the anti-TNF- α antibody/the MFI of control staining with the isotype-matched control antibody, in which 1 stands for no tmTNF- α expression. The mean $\pm$ SEM, $n = 5$ (2 h) and $n = 3$ (48 h), *** $P < 0.001$. Data shown in A and B are representative of five independent experiments.

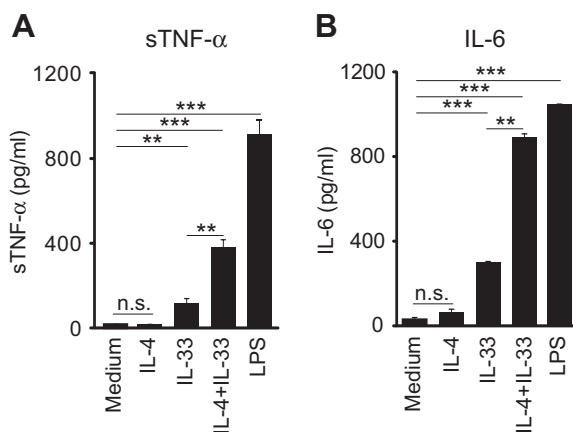


Fig. 2. IL-4 augments the production of sTNF- α and IL-6 by IL-33-stimulated macrophages. Peritoneal macrophages from BALB/c mice were cultured as in Fig. 1 for 48 h, and their culture supernatants were assayed for the production of sTNF- α (A) and IL-6 (B). Data shown are the mean $\pm$ SEM ($n = 4$ each) and representative of five independent experiments. ** $P < 0.01$; *** $P < 0.001$; n.s., not significant.

intact even in the absence of IL-6 (Fig. 3B and C). This observation suggested that IL-6 is critically involved in the tmTNF- α expression induced by the stimulation with the IL-4 and IL-33 combination but not with LPS.

3.3. IL-4 and IL-6 cooperatively induce the tmTNF- α expression with no detectable sTNF- α production

The stimulation of macrophages with IL-6 alone induced little or no production of tmTNF- α (Fig. 4A and B) or sTNF- α (Fig. 4C) as did the IL-4 stimulation. In contrast, the combination of IL-4 and IL-6 stimulation elicited the tmTNF- α expression on macrophages at the late phase (24–48 h) but not early phase (2–6 h) of culture (Fig. 4A and B) as did the IL-4/IL-33 stimulation. Of note, no significant production of sTNF- α was detected in the IL-4/IL-6-stimulated macrophages (Fig. 4C) unlike in the IL-4/IL-33-stimulated ones (Fig. 2A). Thus, IL-4 and IL-6 cooperatively induced the production of tmTNF- α but not sTNF- α . The combination of IL-6 and IL-33 failed to induce the tmTNF- α expression (data not shown). These results suggested that the IL-4/IL-33-elicited tmTNF- α is mediated by the autocrine action of macrophage-derived IL-6 in combination with IL-4.

4. Discussion

M2 macrophages have been shown to produce little or no pro-inflammatory cytokines, including TNF- α , in contrast to M1 macrophages [19,20]. However, this may not reflect what happens *in vivo* under Th2-type inflammatory conditions such as allergic responses, because macrophages could be stimulated *in vivo* with typical Th2 cytokines, such as IL-4, in combination with other cytokines present in the microenvironment. Th2 cells, mast cells, and basophils are important players in Th2-type (allergic responses, and can produce an array of cytokines, including IL-4 and IL-6, upon activation [26–28]. In the present study, we demonstrated for the first time, to our knowledge, that IL-4 and IL-6 in combination but not individually elicit the tmTNF- α expression on macrophages with no detectable production of sTNF- α . This suggests that M2 macrophages differentiated under Th2-type inflammatory conditions can exert their function through tmTNF- α expressed on their surface.

The mechanism underlying the synergistic effect of IL-4 and IL-6 on the expression of tmTNF- α remains to be determined. Peritoneal macrophages used in the present study express IL-4 receptor (IL-4R) on their surface even without addition of exogenous cytokines, and the IL-6 stimulation showed little or no effect on the level of *Il4ra* mRNA or surface IL-4R α expression (data not shown). Therefore, the synergy of IL-4 and IL-6 does not seem to be attributed to the IL-6-mediated induction or up-regulation of IL-4R on macrophages. The IL-4/IL-6-stimulated macrophages express tmTNF- α and produce no detectable sTNF- α . This phenotype is similar to that observed in iRhom2-deficient macrophages when stimulated with LPS [29,30]. iRhom2 is a member of the rhomboid family, and its expression is induced by LPS stimulation. iRhom2 promotes the intracellular trafficking and activation of TACE, leading to the conversion of tmTNF- α to sTNF- α [29,30]. Further studies are needed to clarify the mechanism by which IL-4 and IL-6 regulate the production and processing of TNF- α , including the involvement of iRhom2.

IL-33 has been recognized as one of the key cytokines enhancing Th2-type immune responses [21–24]. It amplifies the M2 polarization of macrophages, and promotes the production of Th2 cytokines such as IL-4, IL-5 and IL-13 by innate-type lymphoid cells, basophils, mast cells, and Th2 cells. Pretreatment of macrophages with IL-33 has been shown to enhance the LPS-induced

ited tmTNF- α expression (after the 48 h stimulation) was almost completely abolished in IL-6-deficient macrophages while the LPS-elicited tmTNF- α expression (after 2 h stimulation) remained

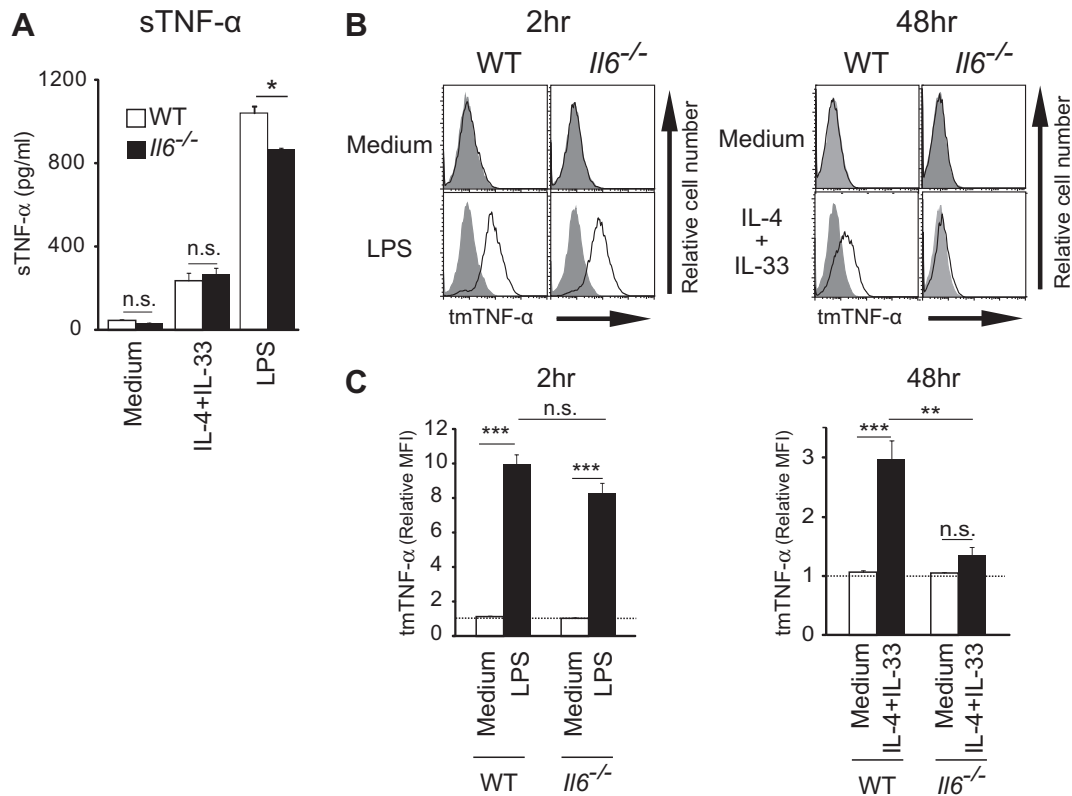


Fig. 3. The IL-4/IL-33-elicited production of tmTNF- α but not sTNF- α is abolished in IL-6-deficient macrophages. Peritoneal macrophages isolated from wild-type (WT) or *Il6*^{-/-} C57BL/6 mice were stimulated with either LPS or IL-4 plus IL-33. (A) The sTNF- α production by WT (open bars) and *Il6*^{-/-} (filled bars) macrophages during the 48 h-culture is shown (the mean $\pm$ SEM, $n = 3$ each). (B and C) Representative data of the tmTNF- α expression on macrophages stimulated with LPS for 2 h and those stimulated with IL-4 plus IL-33 for 48 h are shown in B, and all the data are summarized in C (displayed as in Fig. 1B, the mean $\pm$ SEM, $n = 3$ each). Data shown in A–C are representative of at least three independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; n.s., not significant.

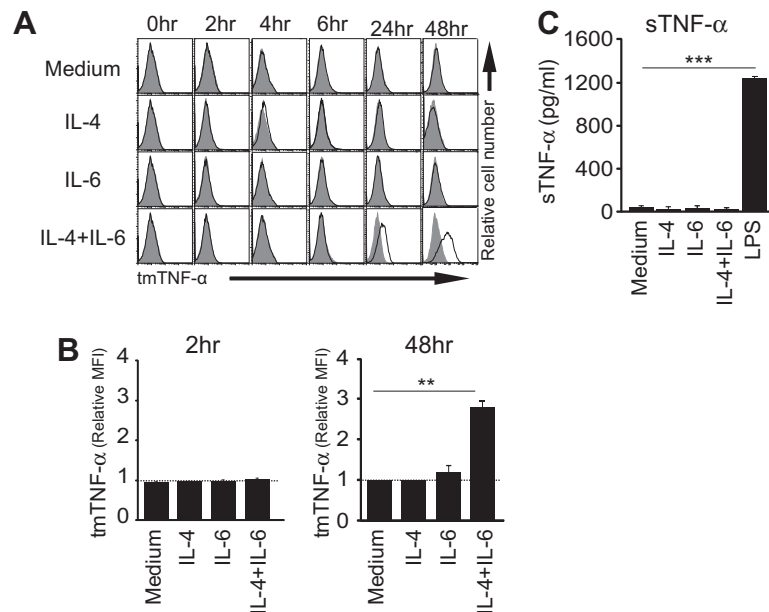


Fig. 4. IL-4 and IL-6 cooperatively induce the tmTNF- α expression with no detectable sTNF- α production. (A) Peritoneal macrophages from BALB/c mice were cultured with either control medium, IL-4, IL-6 or LPS alone or in combination for the indicated time periods. Data shown are representative of the surface expression of tmTNF- α (open histograms) compared with control staining with an isotype-matched control antibody (filled histograms) at each time point. (B) The level of the tmTNF- α expression on macrophages that were stimulated as in A for 2 h (left panel) or 48 h (right panel) is displayed as in Fig. 1B. The mean $\pm$ SEM, $n = 5$ (2 h) and $n = 3$ (48 h). (C) The sTNF- α production by macrophages during the 48 h-culture is shown (the mean $\pm$ SEM, $n = 3$ each). Data shown in A–C are representative of three independent experiments. ** $P < 0.01$; *** $P < 0.001$.

sTNF- α production by macrophages [31]. We found in the present study that while IL-4-elicited M2 macrophages produced little or no sTNF- α or IL-6, the combination of IL-4 and IL-33 stimulation

significantly enhanced the production of sTNF- α and IL-6 above the level of production by IL-33-stimulated macrophages. This might be explained by the fact that Th2 cytokines enhance the

expression of an IL-33 receptor, ST2 [21]. Notably, IL-4 and IL-33 in combination, but not individually, induced the tmTNF- α expression on macrophages in an IL-6-dependent manner. Given that IL-4 and IL-6 synergistically induce the tmTNF- α expression, IL-6 produced by IL-4/IL-33-stimulated macrophages appears to act on macrophages, in combination with IL-4, leading to the tmTNF- α expression. Thus, Th2-inducing cytokines IL-4 and IL-33 can synergistically elicit the tmTNF- α expression on macrophages through the autocrine action of IL-6.

Taken together, the present study underscores the need for considering possible involvement of both tmTNF- α and sTNF- α even in Th2-type inflammatory responses, in addition to Th1-type responses, although it remains to be clarified whether they exert pro-inflammatory or anti-inflammatory functions under Th2 conditions.

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