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T cell immunoglobulin and mucin domain molecule 4 is dispensable in monosodium urate-induced gouty inflammation in mice

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Objective: Gouty arthritis is a sharp inflammatory arthritis induced by monosodium urate (MSU) crystals. Previous studies indicate that the T cell immunoglobulin and mucin domain molecule 4 (TIM-4) is an adhesive molecule which binds to phosphatidylserine (PS) to mediate phagocytosis of apoptotic cells by macrophages, which also regulate T cell activation and increase proinflammatory cytokine expression. This study aims to investigate whether TIM-4 is involved in MSU-induced gouty inflammation.

Methods: MSU suspensions were injected into ankle joints or foot pads of TIM-4 knock-out (KO) and wild type (WT) mice to induce acute gout. Ankle swelling or paw edema were measured at different time points with digital caliper. MSU suspensions were injected into peritoneal cavity or air pouch. Peritoneal cavity or air pouch exudate cells were then harvested after 3 or 6 hours and the infiltrated macrophages and neutrophils were counted by Fluorescence-Activated Cell Sorting (FACS).

Results: In the ankle joints model of MSU-induced gouty arthritis, ankle joint swelling index in TIM-4 KO mice was not significantly different compared with WT mice. Consistent with the ankle joints model, the paw swelling index also had no difference in TIM-4 KO mice compared with WT controls. Additionally, there were no dramatic changes in total number of peritoneal cavity or air pouch exudate cells, neutrophils influxes and infiltrated macrophages between KO and WT mice.

Conclusion: TIM-4 may not play a crucial role in the development of MSU-induced gouty inflammation.

Key words: TIM-4; Gout; Inflammation

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Introduction

Gout is caused by sterile joint inflammation triggered by the damaging effects of monosodium urate (MSU) crystal accumulation in the synovial space, which often manifests an episode of acute and intense painful arthritis and involves a multicellular inflammatory process, including infiltration of macrophages, neutrophils and lymphocytes. Neutrophils play a crucial role in mediating sterile gouty arthritis. Along with neutrophils, macrophages arise in inflamed joints and contribute to pathogenesis of gout. MSU crystals trigger the nucleotide-binding oligomerization domain-like receptor pyrin domain-containing protein 3 (NLRP3) inflammasome activation in macrophages and mediate the release of interleukin (IL)-1β, which is a crucial cytokine in acute attack of gout and neutrophil recruitment [1,2].

The T cell immunoglobulin and mucin domain

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molecule 4 (TIM-4) is a member of the T cell immunoglobulin and mucin domain molecule family of proteins which have been identified in mice [3-5]. It is a new type of immune regulatory molecule, which is selectively expressed on antigen-presenting cells, particularly on macrophages and mature dendritic cells. TIM-4 was first recognized as a co-stimulatory molecule regulating T-cell activation through its interactions with TIM-1 [6]. Recently, evidence support TIM-4 as a receptor that recognizes apoptotic cells through its binding to phosphatidylserine (PS) on the surface, mediating the phagocytosis of apoptotic cells by macrophages [7,8]. Intriguingly, other TIM family proteins, TIM-1 and TIM-3, have been identified to interact with PS and mediate the phagocytosis of apoptotic bodies by various cell types as well [7-10]. However, unlike the other TIM family member, the cytoplasmic tail of TIM-4 lacks putative signaling motifs and therefore cannot directly mediate inward signaling [11]. TIM-4 mediated phagocytosis of apoptotic cells by macrophages is dependent on ATP and calcium ions [12], and in the process of phagocytosis of apoptosis cells, TIM-4 interacts directly with adenosine monophosphate-activated protein kinase (AMPK)-α1 to induce autophagy [13], which can selectively degrade components of inflammasome such as NLRP3, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) [14] and pro-IL-1β [15]. In diabetes mellitus model, some evidences indicate that TIM-4 could inhibit the activation of NLRP3 inflammasome [16]. Overexpression of TIM-4 could upregulate expression of receptor molecule on surface of macrophage cells and tumor necrosis factor α (TNF-α), while blockade of TIM-4 can inhibit macrophage activation and reduce release of cytokines [17]. However, whether TIM-4 is involved in the inflammatory process and the concise molecular mechanisms remain unclear.

Previous studies have also demonstrated that MSU triggered NLRP3 inflammasome and toll-like receptor 4 (TLR4) signaling are involved in gouty inflammatory response [18-21]. Based on the critical function of macrophages in gout, we hypothesize that TIM-4 could participate in regulation of gouty inflammation. To verify this hypothesis, here we performed four different murine models of gout using TIM-4 knock-out mice.

Methods

Animals

TIM-4$^{-/-}$ C57BL/6 knock-out (TIM-4 KO) mice, kindly provided by Dr. Vijay K. Kuchroo (Brigham and Women's Hospital, Harvard Medical School), have been described previously [22]. C57BL/6 as wild type (WT) mice were purchased from the Jackson Laboratory. TIM-4 KO and WT mice were housed at 24 ± 2 °C under 12-h light/12-h dark cycles in a pathogen-free facility. 8-10 week-old males were used to perform the experiments. Handling of mice and experimental procedures were in accordance with requirements of the Institutional Animal Care and Use Committee.

Reagents

The anti-FcγRII/III (clone 2.4G2) was purchased from BD Biosciences. Ly-6G–PE and F4/80-APC mAb were purchased from eBioscience. Uric acid was purchased from Sigma-Aldrich.

Preparation of MSU crystals

MSU crystals were prepared as described previously [23]. Briefly, 1.0g of uric acid was dissolved in 200ml of boiling distilled water containing 6.0ml of 1M sodium hydroxide (NaOH). After adjusting the pH of the solution to 7.2 with hydrogen chloride (HCl), crystals that formed were sterilized by heating at 180°C for 2h. The solution was gradually cooled by stirring at room temperature and stored overnight at 4°C. The precipitate was filtered from the solution, dried under low heat and suspended in phosphate buffer solution (PBS) at a concentration of 50mg/ml. The crystals obtained by this method were of comparable size (5 to 25μm long) and needle-shaped, negatively birefringent crystals observed by compensated polarized light microscopy. All reagents were prepared under pyrogen-free conditions.

MSU-induced gout model

Mice were placed under anesthesia [150:10mg/kg ketamine: xylazine injected intraperitoneally (IP)] and were injected with MSU crystals into the ankle joint (0.5mg in 20ml PBS) or foot pad (1mg in 40ml PBS). The same volume sterile saline was injected into the other ankle joint or foot pad at the same time as the control. After injection of the MSU crystals, inflammation parameters were evaluated at different time points (3, 6 and 24 hours after injection). The sizes of ankle joints or paw swelling were measured with an electronic caliper at the indicated time points.

MSU crystals (3mg in 0.5ml PBS) were injected into

the peritoneal cavity or air pouch as part of intraperitoneal or air pouch gouty model. The total number of peritoneal cavity or air pouch exudate cells were harvested after 3 or 6 hours and counted by a hematocytometer. The harvested cells were resuspended in PBS and subjected to staining and flow cytometric analysis.

Flow cytometric analysis

The harvested cells (1×10^6) from the peritoneal cavity or air pouch were incubated with mAb 2.4G2 for 15 minutes and stained with mAb Ly-6G–PE (eBioscience) and F4/80-APC (eBioscience) for 30 minutes at 4°C. Following staining, cells were washed twice with PBS, fixed in PBS containing 2% paraformaldehyde, and analyzed on a FACSAria II (BD Biosciences). Data were acquired by CellQuest software (BD Biosciences) and analyzed by FlowJo software (Tree Star Inc.).

Statistical analysis

Data were analysed using Graph pad Prism 5. Differences between experimental groups were tested using the unpaired-t test. Data were expressed as mean ± standard error of measurement (SEM). $P<0.05$ was considered to denote statistical significance.

Results and Discussion

Deposition of MSU induces an sterile inflammation which manifests as acute gouty attack [24]. It has been shown that MSU triggers TLR4 signaling and NLRP3 inflammasome involved in gouty inflammatory response [19,21,25], which causes increased production of pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α and macrophage inflammatory protein (MIP-2), along with increased infiltrated neutrophils and macrophages *in vivo*. Particularly, macrophages play a critical role in mediating IL-1β secretion and regulating immune responses.

In this study, we performed the experiments with different murine gouty models according to our previous studies which investigated the roles of transforming growth factor-β inducible early gene 1 (TIEG1) in gout [26]. TIEG1 not only regulates PU.1 expression to control the development and function of macrophages [27], but also plays a critical role during inflammatory gene expression including TLR4 and cyclooxygenase-2 (COX-2) [28]. Unfortunately, TIEG1 did not affect the phenotype of gouty inflammation [26]. Some evidences have reported that TIM-4 not only mediates phagocytosis by macrophages [7,8] but also inhibits the activation of NLRP3 inflammasome [16]. Therefore, similar to TIEG1, TIM-4 may participate in gouty inflammatory response through regulating functions of macrophages.

In order to address the biological function of TIM-4 in the molecular mechanism of acute gouty inflammatory response, the ankle joint model of MSU-induced gouty arthritis in TIM-4 KO and WT mice were used to mimic acute gouty arthritis in humans. Unexpectedly, the ankle joint swelling index had no obvious difference in TIM-4

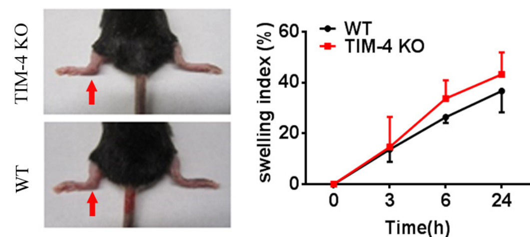

Figure 1. Comparison of swelling index of monosodium urate (MSU)-induced inflammation in ankle joint model between the T cell immunoglobulin and mucin domain molecule 4 (TIM-4) knock out (KO) (n=6) and wild type (WT) (n=6) mice. A suspension of 0.5mg MSU crystals in 20μl of phosphate buffered saline (PBS) was injected into the left ankle joint. The same volume of PBS was injected into the right ankle joint as the control. Inflammation parameters were evaluated at different time points (3, 6 and 24 hours after injection of MSU crystals), ankle joint swelling was measured with an electronic caliper at the indicated time points. The left panel showed at the 24 hours, the peak time point of swollen joints and the right panel showed the ankle joint swelling index.

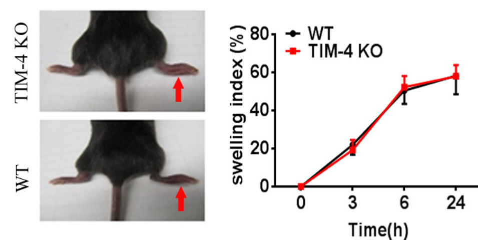

Figure 2. Comparison of swelling index of monosodium urate (MSU)-induced inflammation in foot pad model between the T cell immunoglobulin and mucin domain molecule 4 (TIM-4) knock out (KO) (n=6) and wild type (WT) (n=6) mice. A suspension of 1mg MSU crystals in 40μl of phosphate buffered saline (PBS) was injected into the right foot pad. The same volume of PBS was injected into the left foot pad as the control. Inflammation parameters were evaluated at different time points (3, 6 and 24 hours after injection of MSU crystals). Paw swelling was measured with an electronic caliper at the indicated time points. The left panel showed the paw swelling at the 6 hours, and the right panel showed the paw swelling index.

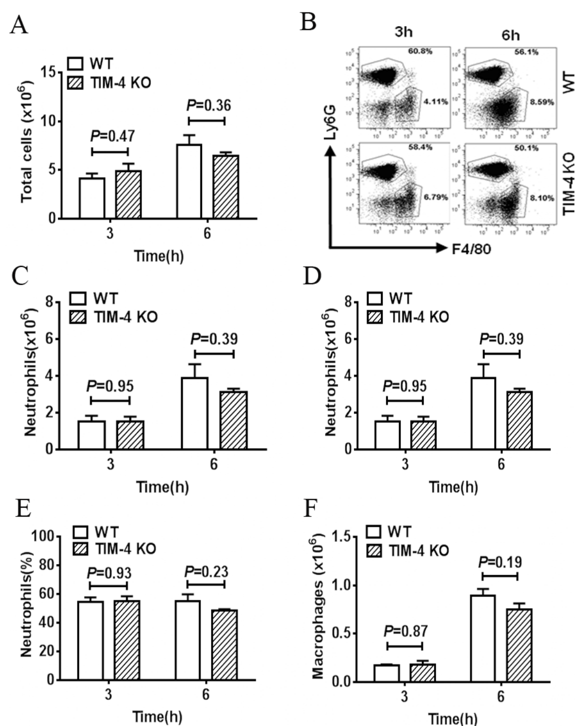

Figure 3. Monosodium urate (MSU)-induced inflammation in the peritoneal cavity of the T cell immunoglobulin and mucin domain molecule 4 (TIM-4) knock out (KO) (n=6) and wild type (WT) (n=6) mice. A suspension of 3mg MSU crystals in 0.5ml phosphate buffered saline (PBS) was injected into peritoneal cavity in TIM-4 KO and WT mice to form peritonitis gouty model. (A) The total number of peritoneal exudate cells harvested at 3 or 6 hours after MSU injection and non-injected MSU mice as control (0h) were counted by a hematocytometer. (B) Infiltrated macrophages and neutrophils analyzed by Fluorescence-Activated Cell Sorting (FACS). Infiltrated macrophages were represented by $F4/80^{+}Ly\text{-}6G^{-}$, while neutrophils were represented by $Ly\text{-}6G^{+}F4/80^{-}$. (C, D) The absolute number and the ratio of neutrophils were analyzed at 3 or 6 hours after MSU injection respectively. (E, F) The absolute number and the ratio of macrophages were also analyzed at 3 or 6 hours after MSU injection respectively.

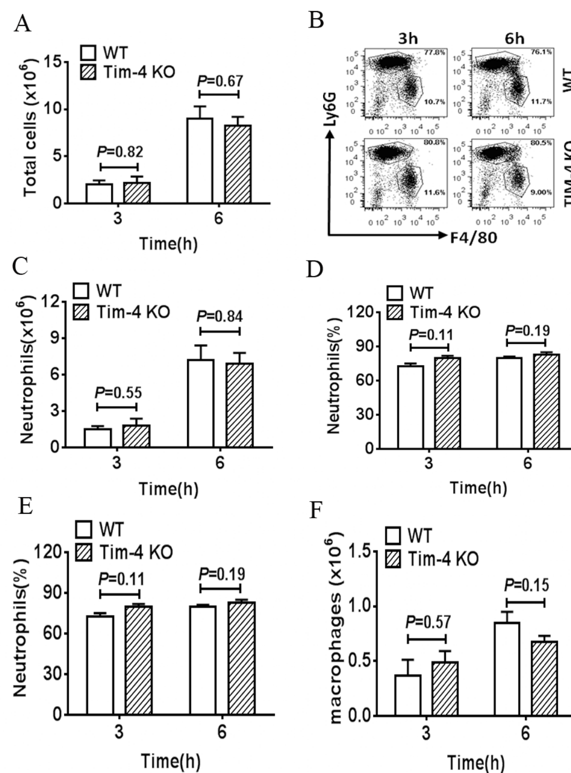

Figure 4. Monosodium urate (MSU)-induced inflammation in the peritoneal cavity of the T cell immunoglobulin and mucin domain molecule 4 (TIM-4) knock out (KO) (n=6) and wild type (WT) (n=6) mice. A suspension of 3mg MSU crystals in 0.5ml phosphate buffered saline (PBS) was injected into air pouch in TIM-4 KO and WT mice to form air pouch model. (A) The total number of air pouch cells was harvested at 3 or 6 hours after MSU injection were counted by a hematocytometer. (B) Infiltrated macrophages were analyzed by Fluorescence-Activated Cell Sorting (FACS). Neutrophils were represented by $F4/80^{-}Ly\text{-}6G^{+}$, while Infiltrated macrophages were represented by $F4/80^{+}Ly\text{-}6G^{-}$. (C, D) the absolute number and the ratio of neutrophils were analyzed at 3 or 6 hours after MSU injection respectively. (E, F) the absolute number and the ratio of macrophages were analyzed at 3 or 6 hours after MSU injection respectively.

KO mice compared with WT controls (Figure 1). To further verify the TIM-4 function, a foot pad model of MSU-induced gouty arthritis was performed. In accordance with the results of the ankle joint model, the paw swelling index from TIM-4 KO mice was almost the same as that from WT mice (Figure 2). These data show that TIM-4 deficiency does not significantly alter the phenotype of MSU-induced gouty inflammation.

As is known, neutrophil influx into the synovium and joint fluid is the pathologic hallmark of acute attack of gout, and are considered to contribute to the pathogenesis of gout [29,30]. Several studies have already suggested that neutrophils in different models of inflammation may be the main effectors of inflammatory pain [31]. An acute inflammatory profile of peritoneal inflammation occurs when reacting to MSU crystals: infiltration of both monocytes/macrophages and neutrophils into the peritoneum were observed after 4 hours, and pro-inflammatory cytokines, such as TNF-a, IL-6, IL-1b, were elevated within 2 hours and peak at 4 hours [32].

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To further demonstrate the function of TIM-4 in gout, a murine peritoneal cavity model of MSU crystal-induced inflammation was performed. 3 or 6 hours after MSU injection, there were no remarkable changes in total number of peritoneal exudate cells (Figure 3A), infiltrated neutrophils (Figure 3C, 3D) and macrophages (Figure 3E, 3F) between TIM-4 KO and WT mice, although the ratio of neutrophils influx decreased slightly in the TIM-4 KO mice compared with WT mice. Additionally, some published reports have demonstrated that the artificial cavity formed by air pouch could be more similar to the synovial cavity of joint in humans [33,34]. We therefore performed the murine air pouch model in MSU crystal-induced inflammation. Consistent with our peritoneal cavity model, we found that there was also no dramatic difference in total number of cells (Figure 4A), infiltrated neutrophils (Figure 4C, 4D) and macrophages (Figure 4E, 4F) between air pouch model of TIM-4 KO and WT mice 3 or 6 hours after MSU injection. Therefore, these data are consistent in the different models of MSU-induced gouty inflammation.

Taken together, the results from these four diverse murine models of MSU-induced gouty inflammation suggest that TIM-4 is dispensable in the development of gouty inflammation.

Conflicts of interest

The authors state no conflict of interest.

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