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Title: TLR2 dependent amelioration of allergic airway inflammation by parasitic nematode type II MIF in mice

Running title: Nematode MIF inhibits allergic airway inflammation via TLR2

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Key words. rAs-MIF, Toll like receptor 2, CD4⁺CD25⁺Foxp3⁺T cell, allergic airway inflammation

Abbreviation used in this study: TLR, Toll like receptor; MIF, macrophage migration inhibitory factor; rAs-MIF, recombinant *Anisakis simplex* macrophage migration inhibitory factor homologues; α -mTLR2 Ab, anti-mouse TLR2 antibody; OVA, ovalbumin; LPS, lipopolysaccharide; hMIF, human MIF; BALF, bronchoalveolar lavage fluid; PBMC, peripheral blood mononuclear cell; MLE12 cells, mouse lung epithelial cells; LDLN, lung draining lymph node;

The online version of this article contains supplemental materials.

Figure S1. Inhibition of production of several allergen-specific cytokines by rAs-MIF in the spleen is associated with TLR2.

Figure S2. rAs-MIF inhibits Th2 cell recruitment and induces T_{reg} cell recruitment in the spleen in a TLR2-dependent manner.

SUMMARY

In our previous studies, the recombinant type II macrophage migration inhibitory factor homologue (rAs-MIF) secreted from *Anisakis simplex* suppressed experimental inflammation mouse model through IL-10 production and CD4⁺CD25⁺Foxp3⁺ T cell recruitment. Also, TLR2 gene expression was significantly increased following rAs-MIF treatment. In order to know the relation between TLR2 and amelioration mechanisms of rAs-MIF, we induced allergic airway inflammation by ovalbumin and alum with or without rAs-MIF under TLR2 blocking systems [anti TLR2 specific antibody (α -mTLR2 Ab) treatment and using TLR2

knockout mice]. As the results, the amelioration effects of rAs-MIF in allergic airway inflammation model (diminished inflammation and Th2 response in the lung, increased IL-10 secretion, CD4⁺CD25⁺Foxp3⁺ T cell recruitment) were diminished under two of TLR2 blocking model. The expression of TLR2 on surface of lung epithelial cell was significantly elevated by rAs-MIF treatment or Pam3CSK (TLR2-specific agonist) treatment, but they might have some competition effect on elevation of TLR2 expression. In addition, the elevation of IL-10 gene expression by rAs-MIF treatment was significantly inhibited by α -mTLR2 Ab or Pam3CSK pre-treatment. In conclusion, anti-inflammatory effects of the rAs-MIF on OVA-induced allergic airway inflammation might be closely related to TLR2.

INTRODUCTION

Parasite-derived molecules have been suggested for use in new therapeutic strategies in the treatment of immune disorders. Several immunosuppressants from parasites have already been isolated and identified as mammalian cytokine homologs, protease inhibitors (including cystatin and serpin), macrophage migration inhibitory factor (MIF)-like protein, abundant larval transcript antigens, and venom allergen-like protein (1-7).

MIF was discovered as a highly conserved mammalian cytokine and was regarded as a pivotal regulator that exerts pro-inflammatory effects on the immune system (8, 9). MIF was first characterized as being derived from activated T cells, inhibiting macrophage migration and acting as a strong pro-inflammatory cytokine (10). CD74 (invariant chain) is a receptor for MIF. CD74 can activate intracellular MAPK pathways via CD44, inflammatory responses against endotoxins or gram-negative bacteria by up regulation of toll-like receptor (TLR) 4, suppression of p53-mediated growth arrest in macrophages challenged by lipopolysaccharide (LPS) (11) MIF is one of three families in the tautomerase superfamily

which are structurally homologous proteins and are unusual in that they employ an amino-terminal proline in a keto-enol tautomerization as a mechanistic feature (12-15). In recent years, expression of MIF homologs has been reported in a range of parasitic organisms infective to humans, including *Plasmodium falciparum*, *Brugia malayi*, *Anisakis simplex*, *Ancylostoma ceylanicum*, and *Trichinella spiralis* (2, 16-18). The role of parasitic MIFs have been mainly studied with regard to host immune evasion. Most parasite MIFs have similar molecular structures compared to human MIF (hMIF), but they have low amino acid sequence similarities to hMIF. However, it has been demonstrated that some parasite MIFs can release pro-inflammatory cytokines from monocytes, similar to hMIF (17, 19). These facts show that parasite MIFs can bind directly to the hMIF receptor CD74 (20, 21). However, parasite MIFs have another function: modulation of the host immune response. Bm-MIF-1 can elicit activated macrophages after injection into the peritoneal cavity (9 times over 3 weeks) (15). These macrophages are not directly suppressive, express Ym-1, and induce a 3-fold rise in eosinophils, consistent with the description of Ym-1 as an eosinophil chemotactic factor (5, 15, 22). In addition, we reported that recombinant As-MIF (rAs-MIF), isolated from *A. simplex* third-stage larva, can down-regulate the OVA-induced allergic airway immune response in mice and suppress Th2 cytokine production in peripheral blood mononuclear cell (PBMC) cultures isolated from patients with asthma (2, 23). rAs-MIF can elicit CD4⁺CD25⁺Foxp3⁺ T cells (regulatory T cell, T_{reg}) recruitment to the spleen and lungs of the rAs-MIF-treated mice, but the recruitment is inhibited by anti-rAs-MIF antibodies. rAs-MIF could elevate the IL-10 secretion of the PBMCs of an asthma patient in an artificial culture medium (23). In addition, rAs-MIF inhibited artificially induced intestinal inflammation in mice and rAs-MIF could elevate TLR2 expression (24).

In this study, we attempted to investigate the mechanisms of amelioration of allergic airway inflammation by rAs-MIF. After blocking the TLR2 with specific antibodies or by

knocking out the TLR2-encoding gene, we evaluated the degree of T_{reg} cell recruitment, IL-10 production, TGF- β production, and allergic airway inflammation response.

MATERIALS AND METHODS

Preparation of rAs-MIF protein isolated from *A. simplex*

In our previous study, rAs-MIF was cloned into a pET-28a expression vector (Novagen, Darmstadt, Germany) and purified according to a previously reported procedure (2).

Induction of allergic airway inflammation in mice

Female C57BL/6 mice, 6 weeks of age, were acquired from Samtako Co. (Gyeonggi-do, Korea), and TLR2 knockout (KO) C57BL/6 female mice were supplied by Prof. Park YM (Pusan National University, Gyeongsangnam-do, Korea). The mice were housed in a laminar flow cabinet throughout the experiments and were maintained in individual ventilation cages (Threes Shine, Korea). All animal studies were approved by the Pusan National University School of Medicine Animal Care and Use Committee. The experiments were conducted in 2 stages. In the first stage, we observed inhibitory effects of rAs-MIF against the induced allergic airway response with or without α -mTLR2 Ab (T2.5, monoclonal antibody, mouse IgG1; Cell Sciences, Canton, MA, USA). In the second stage, we compared the difference of the inhibition effect of rAs-MIF between wild-type (WT) and TLR2 KO mice. All techniques used in the 2 stages were identical. The first stage experiment included 3 groups: an allergic airway inflammation-induced group (OVA+Alum), a rAs-MIF treated allergic airway inflammation-induced group (OVA+Alum+rAs-MIF), and a rAs-MIF plus α -mTLR2 Ab treated allergic airway inflammation induced group (OVA+Alum+ α -mTLR2 +rAs-MIF). The second stage experiment included 4 groups: an allergic airway inflammation-induced WT (WT-

OVA+Alum) and a TLR2 KO (TLR2KO-OVA+Alum) mice group, a rAs-MIF treated and allergic airway inflammation induced WT (WT-OVA+Alum+rAs-MIF), and a TLR2 KO (TLR2KO-OVA+Alum+rAs-MIF) mice group. Allergic airway inflammation was induced as described previously (2, 25). Briefly, the mice were sensitized on days 1, 2, 8, and 9 via intraperitoneal injections of 75 µg of ovalbumin (OVA; Sigma-Aldrich Chemical Co., St. Louis, MO) and emulsified in 2 mg of aluminum hydroxide (alum; Sigma-Aldrich) in a total volume of 100 µl. The negative control group was sensitized with phosphate buffered saline (PBS) on the same day. On days 15, 16, 22, and 23, the mice were intra-nasally treated with 50 µg of OVA in 50 µl of PBS. rAs-MIF (100 ng in 100 µl) was administered via Intra peritoneal injection with or without 10 ug α -mTLR2 Ab 30 min prior to the every OVA challenge.

Determination of airway hyperresponsiveness (AHR)

Measurement of AHR was performed as described previously (26). Briefly, AHR was measured at 1 day after the last OVA challenge. Methacholine (Sigma-Aldrich Chemical Co) was nebulized to mice in increasing concentrations (from 0 mg/ml to 50 mg/ml). AHR was assessed as the percent increase of enhanced pause (Penh) in response to methacholine.

Lung histopathology

Histological analysis was conducted according to standard procedures. In brief, the left lobe of lung tissues was isolated, fixed with formaldehyde, and embedded in paraffin. Thin sections of the embedding tissues were then stained with hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) stain. After staining, we performed microscopic examination of the stained sections.

Analysis of bronchoalveolar lavage fluid (BALF)

After sacrificing the animals, the chest cavity was exposed. The trachea was then carefully intubated with the catheter, which was secured with ligatures. Pre-warmed PBS was slowly inflated into the lungs and withdrawn. The BALF samples were then centrifuged, and the supernatants were maintained at -70 °C until use in the enzyme-linked immunosorbent assay (ELISA). The cell pellets were resuspended with 100 µl of cold PBS and 5×10^4 cells were bound to the slide glass using a cytopsin apparatus (Bio-Rad Laboratories, Hercules, CA). The cells were then stained with Diff-Quick solution (Dade Diagnostics, Japan) for determination of cell differentials in accordance with conventional morphological criteria.

Collection of culture medium from OVA-stimulated lymphocytes

Lymphocytes were individually isolated from the spleens and lung draining lymph nodes (LDLN) of each mice to determine the incidence of specific cytokine-secreting lymphocytes. The spleen and LDLN were disrupted, treated with ACK hypotonic lysis solution (Sigma-Aldrich Chemical Co.) for RBC lysis, and the remaining cells were filtered through a 100-µm mesh (Small Parts, Seattle, WA, USA). Cells (5×10^6 cells/ml) of each mouse were then incubated in two wells of 1 µg/ml OVA pre-coated 48-well plates with RPMI 1640 medium containing 10 % fetal bovine serum (FBS) and penicillin–streptomycin (Hyclone, Logan, UT, USA) for 72 hrs at 37 °C in an atmosphere containing 5 % CO₂. After the incubation, the culture medium was harvested and stored at -70 °C until use.

RNA isolation, cDNA synthesis, PCR, and reverse transcription (RT)-PCR

RNA was extracted from cells and the right lobe of the lung using QIAzol (Qiagen, Valencia, CA, USA), and cDNA was synthesized using moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturer's protocol.

RT-PCR was conducted for all TLR genes. TLR2, IL-10, TGF- β , and Foxp3 mRNA expression levels were measured via real-time PCR using an iCyclerTM (Bio-Rad Laboratories). The glyceraldehyde phosphate dehydrogenase (GAPDH)-encoding gene was used as a reference gene. The primer sequences were listed in Table S1. PCR amplification was conducted with SYBR Premix Ex TaqTM (TAKARA Bio, Otsu, Shiga, Japan). Expression levels were estimated using an iCycler iQTM (Bio-Rad Laboratories), and the relative expression of each gene were calculated using the gene-x program (Bio-Rad Laboratories).

ELISA analysis

Levels of IL-4, IL-5, IL-6, IL-10, IL-13, and TGF- β in the OVA-stimulated lymphocyte culture supernatants and BALF were evaluated using ELISA kits (eBioscience, San Diego, CA, USA) according to the manufacturer's protocol. In addition, OVA-specific immunoglobulin (IgG1, IgG2a, and IgE) levels in serum (dilution factor of 0.5 for IgE and 10^4 to 10^7 for IgG1 and IgG2a) were determined using same protocol.

Cell culture and stimulation

Mouse lung epithelial cell line, MLE12 cells were maintained following a published report (27). MLE12 cells were incubated with or without rAs-MIF (final conc. 1 μ g/ml) for indicated time. For neutralization of TLR2, 10 μ g/ml α -mTLR2 Ab (Cell Sciences) was treated for 2 hrs before rAs-MIF treatment. In addition, 100 ng/ml or 10 μ g/ml of Pam3CSK4 and 50 μ g/ml of polymyxin B was used as a control (InvivoGen, San Diego, CA, USA). In addition, to rule out any bacterial contaminant known so far to signal via CD14/TLR2 to be present in our rAs-MIF preparation, rAs-MIF was boiled for 1 h before use to serve as negative control.

Western blot analysis

Total cell extracts of 5×10^6 cells were prepared as described previously. NP-40 cell lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1.5 mM EDTA, 3 % glycerol, 0.5 % NP-40] was used to prepare total cell lysate. The amounts of proteins were measured by Bradford (Bio-Rad Laboratories). Equal amounts of proteins were fractionated by SDS-PAGE and then transferred to PVDF membrane (Bio-Rad Laboratories). Blots were blocked for at least 1 hr with 5 % non-fat dry milk prior to incubation with TLR2 (R&D systems, Minneapolis, MN) and β -actin (Sigma-Aldrich Chemical Co.) polyclonal antibodies at 4 °C for overnight. After incubation with secondary antibodies conjugated with HRP for 1 hr at room temperature, specific bands of interest were revealed by chemiluminescence (SuperSignal[®] West Femto, Thermo Scientific, IL).

Fluorescence activated cell-sorting (FACS) analysis

Cells were strained with fluorescently conjugated antibodies. Briefly, the cells were stimulated with PMA (Sigma-Aldrich Chemical Co.) and ionomycin (Sigma-Aldrich Chemical Co.) for 4 hrs. To block cytokine release, brefeldin (BD) was co-treated during cell stimulation. Blocked cells were stained with TLR2 (FITC) or CD4 (FITC) and CD25 (Alexa Fluor 700) antibody (eBioscience), in accordance with the manufacturer's recommended protocols. To stabilize surface antibody, cells were incubated with 1% paraformaldehyde (PFA) before permeable buffer (ebioscience) treatment. After that, permeabilization buffer treatment, the cells were stained with anti-mouse Foxp3 (pacific blue), anti-IL-10 (PE), and anti-IL-4 (PE-Cy7) antibody and then fixed by incubation with 1% PFA. Flow cytometry was conducted on a FACS CantoII[™] (BD, Franklin Lakes, NJ, USA) and analyzed using FACS Diva[™] 6.0 software (BD).

Statistical analysis

All data were analyzed using Prism 6 (Graphpad Prism, La Jolla, CA). Means $\pm$ SD were calculated and significant differences were determined using the Student's t test or one way ANOVA with a Dunnett's test multiple comparisons post test comparing all groups with control group.

RESULTS

Amelioration of OVA-induced allergic airway inflammation by rAs-MIF was blocked by TLR2 specific antibody

The percentage of Penh was increased by methacholine administration, in a dose-dependent manner, in allergic airway asthma induced mice. However, this increased sensitivity was reduced in rAs-MIF treated mice. The rAs-MIF and α -mTLR2 Ab treated allergic asthma induced mice showed a higher Penh value than in the rAs-MIF treated group. However, this value was lower when compared with the allergic airway asthma induced mice without any treatment (Fig. 1A). In addition, OVA plus aluminum hydroxide sensitization and OVA challenge induced recruitment and infiltration of immune cells into the lungs, but rAs-MIF treatment prevented recruitment and infiltration, particularly of eosinophils (Fig. 1B). This inhibitory effect was also observed in rAs-MIF plus α -mTLR2 Ab treated allergic asthma induced mice. However, the prevention efficacy to eosinophilia of rAs-MIF was markedly suppressed by TLR2 neutralization (Fig. 1A, B). Thickened epithelia and marked influxes of inflammatory cells into the airway and around blood vessels, as well as remarkably enhanced mucus production with goblet cell hyperplasia in lung bronchial epithelia, were detected in the allergic asthma induced mice (Fig. 1C, data not shown). The rAs-MIF treated mice showed evidence of reduction in inflammatory cell infiltration within

the peribronchiolar and perivascular regions. However, α -mTLR2 Ab pretreated mice did not show ameliorated lung pathogenesis by rAs-MIF treatment (Fig. 1C). Cytokine production in the BALF of allergic airway inflammation induced mice was significantly changed by rAs-MIF or α -mTLR2 Ab treatment. Production of Th2 inflammatory cytokines IL-4, IL-5, and IL-13 was reduced and T_{reg} cell-related cytokines IL-10 and TGF- β increased in rAs-MIF treated allergic asthma induced mice (Fig. 1D). However, in α -mTLR2 Ab pretreated mice, IL-5 production was not reduced by rAs-MIF treatment and the efficacy of IL-13 inhibition was still observed but it was weakened by α -mTLR2 Ab pretreatment; furthermore, the IL-10 and TGF- β levels did not increase in these mice (Fig. 1D). The levels of OVA-specific IgE and IgG1 in serum increased by allergic asthma induction with OVA and aluminum hydroxide, and these increases were down regulated by rAs-MIF treatment. However, in rAs-MIF plus α -mTLR2 Ab treated allergic asthma induced mice, no significant differences in OVA-specific IgE and IgG1 were observed when compared to the allergic asthma induced mice (Fig. 1E). In addition, these alterations in the cytokine levels were detected in the supernatant of cultures of cells isolated from the LDLN and spleen. In the culture supernatant of LDLN cells, the concentrations of IL-4, IL-5, IL-6 and IL-13 were decreased, and the concentration of TGF- β increased in rAs-MIF treated allergic asthma induced mice (Fig. 2). However, some effects (especially IL-4, IL-13 and TGF- β) observed for rAs-MIF treated mice were weaker in α -mTLR2 Ab co-treated mice (Fig. 2). As anticipated, α -mTLR2 Ab treatment prevented rAs-MIF-mediated reductions in Th2 cytokine production (Fig. S1). In addition, we treated normal mouse IgG as control in this experiment, but it is as no difference to untreated mice (data not shown).

Inhibition of OVA-induced airway inflammation by rAs-MIF was not observed in TLR2 KO mice

To confirm the role of TLR2 in rAs-MIF anti-inflammation, we introduced rAs-MIF into TLR2 KO mice while allergic airway inflammation, and the immune responses of these mice were compared with WT mice. Except for rAs-MIF treated WT mice, the mice had breathing difficulties under high doses of methacholine (Fig. 3A). In contrast to TLR2 neutralized mice, TLR2 deficient mice showed decreased cell recruitment than WT mice after ova plus alum exposure, particularly total cell and eosinophil. In addition, there was no significant difference including the number of total cells, macrophages, and lymphocytes in the BALF of the allergic airway inflammation induced TLR2 KO mice with or without rAs-MIF treatment. However, in case of eosinophil, it is not clear whether rAs-MIF has still inhibitory effects on eosinophilia when TLR2 was deficient because of slightly increased eosinophil numbers in allergic airway inflammation induced TLR2 KO mice even though there was significance between rAs-MIF treated or no treated TLR2 KO mice groups. (Fig. 3B). In the histological results, we observed no considerable change in the degree of peribronchiolar and perivascular inflammatory cell recruitment and goblet cell metaplasia after rAs-MIF treatment in TLR2 KO mice (Fig. 3C).

rAs-MIF mediated regulation of cytokine was dependent on TLR2

The rAs-MIF treatment influenced the OVA-specific Ig serum level in allergic airway inflammation induced mice. OVA-specific IgG1 and IgE serum levels increased with allergic airway inflammation induction in both WT and TLR2 KO mice. The elevation of OVA-specific IgE and IgG1 was suppressed by rAs-MIF treatment in WT mice, and these effects were persisted when TLR2 was absent (Fig. 4A). In addition, Th2 related cytokine levels in the BALF decreased by rAs-MIF treatment in allergic airway inflammation induced WT mice,

but these effects, except IL-4 and IL-10, were not detected in TLR2 KO mice (Fig. 4B). Moreover, rAs-MIF enhanced the production of anti-inflammatory cytokines, IL-10 and TGF- β , production in WT mice, but when TLR2 was absent, rAs-MIF lost its ability to induce TGF- β production (Fig. 4B). In addition, we found that the Th2 cytokine inhibition and anti-inflammatory cytokine elevation effects of rAs-MIF in the LDLN and spleen cell culture supernatant in WT mice but not KO mice (data not shown). Furthermore, the relative mRNA expression levels of IL-10, TGF- β , and Foxp3 increased in the lung tissue of rAs-MIF treated WT mice but not in TLR2 KO mice (Fig. 4C).

Regulation of Th2 cells and T_{reg} cell recruitment by rAs-MIF treatment was not observed in TLR2 KO mice

The rAs-MIF treatment significantly decreased IL-4 secreting CD4⁺ T cells in both LDLN and spleen (Fig. 5 and Fig. S2). However, this suppressive effects of rAs-MIF in LDLN and spleen of TLR2 KO mice was similar to those of WT mice. Moreover, CD4⁺CD25⁺ Foxp3⁺ T cell recruitment was stimulated by rAs-MIF treatment in the LDLN of allergy-induced WT mice (Fig. 5).

rAs-MIF induced in TLR2 expression on cell surface of lung epithelial cell

To assess whether the effect of rAs-MIF on allergic airway inflammation was related to lung epithelial cell and TLR2, we evaluated TLR2 gene expression levels, TLR2 protein concentration in the cell, and TLR2 expression on the cell surface of the MLE12 cells after rAs-MIF treatment. Results showed that the mRNA expression of TLR2 was significantly elevated by rAs-MIF treatment, but this increase in TLR2 mRNA expression by rAs-MIF was not observed after denaturation of rAs-MIF by heat (Fig. 6A). In addition, TLR2 production of lung epithelial cell was enriched by rAs-MIF treatment, but it was not inhibited by

polymixin B or Pam3CSK4, a known agonist of TLR2, co-treatments (Fig. 6B). The TLR2 expression of lung epithelial cell surface also significantly increased by rAs-MIF, it was not related with LPS contamination (Fig. 6C). Interestingly, Pam3CSK4 could more highly elevate the expression level of TLR2 than those of rAs-MIF treatment (Fig. 6D), but the expression level was slightly decreased after co-treatment with Pam3CSK4 and rAs-MIF (Fig. 6D).

Induction of IL-10 expression by rAs-MIF by TLR2 activation, and TLR6 and TLR9 gene expression was increased by rAs-MIF

To rule out bacterial contaminants, we compared IL-10 levels between natural rAs-MIF and boiled rAs-MIF treated cells. The induction of IL-10 mRNA expression was observed in only intact rAs-MIF treated cells (Fig. 7A). To identify a relation between IL-10 gene expression and TLR2, we added rAs-MIF after α -mTLR2 Ab pre-treatment. The elevation of IL-10 gene expression by rAs-MIF treatment was significantly inhibited by α -mTLR2 Ab pre-treatment, in a dose dependent manner in MLE12 cells (Fig. 7B). In addition, the enhanced IL-10 gene expression level by rAs-MIF was inhibited with Pam3CSK4 pre-treatment (Fig. 7B). A slight competition effect was detected between rAs-MIF and Pam3CSK4 against TLR2 and IL-10 expression (Fig. 6D & 7B). In order to know, which TLR gene expression was affected by rAs-MIF treatment, we evaluated TLR1 ~ TLR9 gene expression in lung epithelial cell after rAs-MIF treatment. As a results, TLR6 and TLR 9 gene expression was significantly increased after rAs-MIF treatment (Fig. 7C).

DISCUSSION

In previous studies, it was demonstrated that rAs-MIF had powerful anti-inflammatory effects in an allergic airway inflammation mouse model (2). In addition, rAs-MIF inhibited artificially induced intestinal inflammation and T_{reg} cells, and the IL-10 levels were greatly increased in the rAs-MIF-treated mice compared with mice that had not been treated with rAs-MIF (24). The elevated IL-10 production of intestinal epithelial cells induced by rAs-MIF was generated via TLR2. Therefore, we evaluated the correlation between the anti-inflammatory effects of rAs-MIF and TLR2 by blocking the TLR2 pathway.

The cytokine MIF plays a critical role in many inflammatory disorders (8, 12). MIF is an integral component of the host antimicrobial alarm system and stress response, promoting the pro-inflammatory functions of immune cells (12). Interestingly, many parasites also express MIF-like proteins. Why does a parasite express MIF-like proteins that may induce inflammation and would therefore be self-toxic? Although parasite MIF can elicit pro-inflammatory cytokine production similar to the host MIF, some parasite MIFs play roles in the anti-inflammatory response (2, 15, 17, 28). These two different functions of parasite MIFs indicate that parasite MIFs might bind to another molecule/receptor of the host immune system. Recently, Cho et al. noted that the structure of nematode parasite MIF (rAceMIF, type II MIF) was similar to hMIF and type I MIF, and that they both bind to a common receptor, CD74, although the binding activity of rAceMIF is 4.5-fold lower than that of hMIF (20, 29). However, parasite type II MIF was not inhibited by the mammalian MIF inhibitor ISO-1. Thus, the parasite MIF was hypothesized to exert its biological effect via direct interaction with the MIF receptor CD74 and that this complex may interact with another receptor. It has recently been reported that cytokine hMIF can be bound to several chemokine receptors, for example CXCR2 and CXCR4 in the cytoplasm, which are expressed on many

CD74⁺ cell types (30). In addition, hMIF also binds to c-Jun activation domain-binding protein-1, preventing the latter from activating the proinflammatory transcription factor AP-1 (31). Therefore, parasite MIF might have other receptors in their host cell to elicit the anti-inflammatory immune response.

In this study, *in vivo* experiments showed that rAs-MIF could not activate the anti-inflammatory immune response in two of TLR2 blocking models. First, we treated mice with rAs-MIF and/or α -mTLR2 Ab during OVA challenges. Second, we treated allergic airway inflammation induced WT and TLR2 KO mice with rAs-MIF. rAs-MIF prevented the increase in PenH, inflammatory cell recruitment (particularly eosinophils into the lung tissue), and lung injury induced by OVA. Inflammatory cytokine (IL-4, IL-5, and IL-6) production was also reduced, and T_{reg} relative cytokines, including IL-10 and TGF- β , were increased in the lung, LDLN, and spleen by rAs-MIF treatment. However, these effects were ameliorated by α -mTLR2 Ab treatment (Fig. 1, 2 and Fig. S1) or in TLR2 KO mice (Fig. 3 and 4). Moreover, TLR2 was involved in recruitment of immune cells into the spleen and LDLN. The fraction of CD25⁺Foxp3⁺ cells in the CD4⁺ cell subset increased, and IL-4⁺ cells in the CD4⁺ cell population decreased by rAs-MIF treatment in both the LDLN and spleen. However, no considerable differences in these cell populations were observed between allergic asthma induced TLR2 KO mice and rAs-MIF treated and allergic asthma induced TLR2 KO mice (Fig. 5 and Fig. S2). These results showed that IL-10 and T_{reg} cell recruitment are the most important factors in amelioration effects of airway inflammation by rAs-MIF, and rAs-MIF could not enhance the events and ameliorate the inflammation under TLR2 blocking.

IL-10, which is referred to as the cytokine synthesis inhibitory factor, is an anti-inflammatory cytokine capable of inhibiting pro-inflammatory cytokine synthesis. IL-10 is generated primarily by T_{reg} cells (32). T_{reg} cells were the key for amelioration of lung

inflammation by rAs-MIF treatment in this study. T_{reg} cells regulate peripheral tolerance and play a critical role in the control of the immune response in infection, tumor defense, organ transplantation, and allergy (33). Molecules secreted during parasite infection interact with TLR, which may drive the differentiation of naive T cells into distinct CD4⁺ T cell subtypes, including T_{reg} phenotypes (34). The *Schistosoma mansoni*-specific phosphatidyl serine is recognized by TLR2 and may promote the induction of T_{reg} cells (35). Oberg et al. suggested that TLR2 modulates the suppressive activity of naturally occurring CD4⁺CD25^{high} T_{reg} cells (36). Oberg et al. demonstrated that direct modulation of human T_{reg} cell activity by TLR2 ligands, and the suppressive capacity, was completely abolished when T_{reg} cells but not responder T cells were pre-incubated with TLR2 agonist Pam2CSK4, Pam3CSK4, or FSL-1.

In addition *in vitro* experiments, we observed that rAs-MIF could elicit TLR2 gene expression, TLR2 production and TLR2 expression on the lung epithelia cell surface by rAs-MIF treatment (Fig. 6) and these effects blocked by α -mTLR2 Ab and TLR2 agonist Pam3CSK4 co-treatment (Fig. 7). In addition, Pam3CSK4 could not elicit IL-10 gene expression but inhibited IL-10 expression after rAs-MIF treatment (Fig. 7). These results showed that rAs-MIF might need the TLR2 pathway to elicit IL-10 secretion and that rAs-MIF might need another receptor or several receptors to elicit IL-10 secretion. TLR2 forms functional dimers with either TLR1 or TLR6, may have different signaling capacities (37). We did not investigate which receptor is involved in IL-10 elevation by rAs-MIF treatment with TLR2. TLR6 is a candidate specific receptor for IL-10 expression by rAs-MIF treatment because TLR6 gene expression was activated by rAs-MIF (Fig. 7C). TLR2 senses pathogen-associated molecular patterns in bacteria and fungi and is unique among TLR receptors because TLR2 functions as a heterodimer with either TLR1 or TLR6 (38). TLR2 is closely related to asthma prevalence, and genetic variation in TLR2 is a major determinant of the susceptibility to asthma in children (39, 40). Clearly, TLR2 signaling may coordinate the

development of a Th1 phenotype in some individuals, and where this function is reduced there could be an increased risk of asthma (41). Also, in several pathogen infections in airways, TLR2 is important in the early innate immune response (42). Reconstitution of airway epithelial TLR2 signaling in TLR2^{-/-} mice significantly restores the lung defense against bacteria (43). Therefore, TLR2 is a good therapeutic target for the treatment of lung diseases (44).

In conclusion, we showed that rAs-MIF could activate TLR2 and inhibit OVA induced allergic airway inflammation through a TLR2-dependent mechanism. In a previous study, we also confirmed that rAs-MIF could directly suppress the production of Th2 cytokines in PBMC cultures isolated from patients with atopic asthma, patients with non-atopic asthma, and healthy subjects (23). In summary, rAs-MIF is beneficial for use in the treatment of allergic asthma. Although we confirmed that rAs-MIF inhibits allergic airway inflammation through TLR2, the detailed mechanism of the interaction between rAs-MIF and TLR2/TLR6 will be pursued in a future study.

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FIGURE LEGENDS

Figure 1. The inhibition effect of rAs-MIF on allergic airway inflammation is diminished by the anti-TLR2 antibody. (A) The enhanced pause (PenH) was evaluated by nebulization to mice with increasing concentrations (from 0 mg/ml to 50 mg/ml) of methacholine) (B) The cells were isolated from the BALF and were differentiated by morphological features. (C) Thin section of the lung stained with H&E and then subjected to microscopic examination. (D) Cytokine levels in the BALF and (E) OVA-specific Ig levels in serum were measured by ELISA. Lung inflammation as assessed by PenH, cell recruitment, inflammatory cytokine levels, and OVA-specific Ig levels were moderated by rAs-MIF, but the inhibition was suppressed after anti-TLR2 antibody treatment. The data are expressed as the mean $\pm$ SD from one of three independent experiments (n= 5 mice per group). Asterisks show statistical differences (one way ANOVA). (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$)

Figure 2. Allergen specific cytokine production of lung draining lymph nodes was inhibited in rAs-MIF treated mice but not rAs-MIF and anti-TLR2 antibody treated mice. Lymphocytes were isolated from the LDLN of each group, and the cells were stimulated by OVA (final conc. 1 μ g/ml) or not stimulated by OVA for 3 days. The concentration of cytokines was measured using an ELISA kit (OVA-; OVA non-treated cell, OVA+; OVA treated cell. The data are expressed as the mean $\pm$ SD from one of three independent experiments (n= 5 mice per group). Asterisks show statistical differences (one way ANOVA). (*; $p < 0.05$, ***, $p < 0.001$)

Figure 3. TLR2 contributes to the anti-lung inflammation ability of rAs-MIF. WT and TLR2 KO mice were treated with rAs-MIF after allergic airway inflammation induction. (A) PenH was evaluated at baseline and after treatment with increasing doses of aerosolized

methacholine (0, 12.5, 25.0, 37.5, and 50 mg/ml). (B) The immune cells were isolated from the BALF and were differentiated by morphological features. (C) Thin sections of the lung were stained with H&E and PAS. Down regulated PenH, immune cell recruitment into the lung, and mucus production were blocked in TLR2 KO mice. Data is expressed from one of three independent experiments (n = 5 mice per group). (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$)

Figure 4. rAs-MIF requires TLR2 activation to suppress inflammatory immune responses and enhances regulatory molecules *in vivo*. (A) The serum was diluted, and the OVA-specific IgE and IgG1 levels were then measured by ELISA. (B) Inflammatory cytokine and regulatory cytokine concentrations in the BALF evaluated using an ELISA kit. Inflammation regulatory ability of rAs-MIF weakened in TLR2 KO mice. (C) After the right lobe of the lung had been collected from each mouse, total RNA was isolated. The alteration of T_{reg} cell related gene (IL-10, TGF- β , and Foxp3) expression in the lung was measured by real-time PCR. The GAPDH gene was used as a control gene. The regulatory genes were increased by rAs-MIF in WT mice but not in TLR2 KO mice. The data are expressed as the mean $\pm$ SD from one of three independent experiments (n= 5 mice per group). Asterisks show statistical differences (one way ANOVA). (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$)

Figure 5. rAs-MIF induces T_{reg} cell recruitment in the LDLN, mainly via TLR2. The absolute cell number (A) and percentage (B) of the $CD4^+IL-4^+$ and $CD4^+CD25^+Foxp3^+$ cell populations by rAs-MIF treatment in WT and TLR2 KO mice was evaluated by FACS. To analyze T cell subpopulation, $CD4^+$ or $CD4^+CD25^+$ cells were gated first and then $IL-4^+$ cells or $Foxp3^+$ cells were gated respectively. The data are expressed as the mean $\pm$ SD from one of three independent experiments (n= 5 mice per group). Asterisks show statistical differences

(one way ANOVA). (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$)

Figure 6. rAs-MIF enhances TLR2 expression levels in MLE12 cells. The rAs-MIF (MIF) and boiled rAs-MIF (Boiled MIF) were incubated with MLE12 cells (final conc. 1 $\mu\text{g/ml}$) for 2 hrs, and the expression of TLR2 was determined by real-time PCR (A). TLR2 protein expression on lung epithelial cell surface was determined after rAs-MIF, and/or polymyxin B, Pam3CSK4 using western blotting, the ratio of band intensity with control was analysed using Image J program (B) and FACS (C). MLE12 cells were incubated under 1 $\mu\text{g/ml}$ (final conc.) of rAs-MIF with/without Pam3CSK4 (final conc.; 10 $\mu\text{g/ml}$) for 24 hrs, or after pre-treatment of polymyxin B (final conc.; 50 $\mu\text{g/ml}$) for 1 hr. Relative TLR2 mRNA expression level in MLE12 cells evaluated after treatment of rAs-MIF and/or Pam3CSK4 by real-time PCR (D). (*, $p < 0.05$; **, $p < 0.01$; the value were compare to each group (A), or negative control (D), all experiments were performed in 3 independent experiments)

Figure 7. rAs-MIF up regulated IL-10 gene expression by TLR2 dependent manner and other TLRs also activated by rAs-MIF treatment. (A) The rAs-MIF (MIF) and boiled rAs-MIF (Boiled MIF) were incubated with MLE12 cells (final conc. 1 $\mu\text{g/ml}$) for 2 hrs and the IL-10 gene expression level was measured. (B) rAs-MIF was incubated with MLE12 cells after 1 hr anti-TLR2 antibody (0 ~ 20 $\mu\text{g/ml}$) pre-treatment, or Pam3CSK4 (final conc.; 10 $\mu\text{g/ml}$) treatments. After 2 hr, relative IL-10 mRNA gene expression was evaluated by real-time PCR (B). TLR1 ~ TLR9 gene expression in MLE12 cell were evaluated rAs-MIF treatment for 2 hr, the ratio of band intensity with PBS was analysed using Image J program (C). GAPDH gene was used as standard expression control (*, $p < 0.05$; **, $p < 0.01$; all experiments were performed in 3 independent experiments).

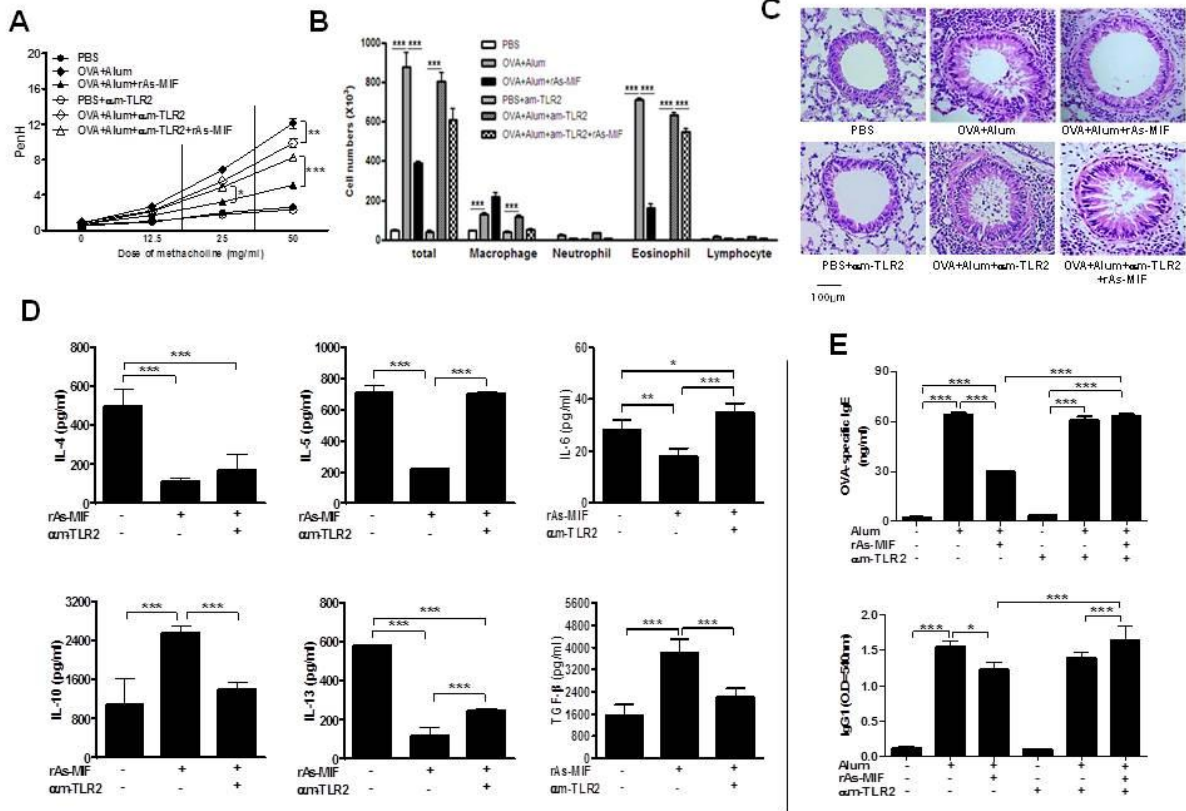


Figure 1

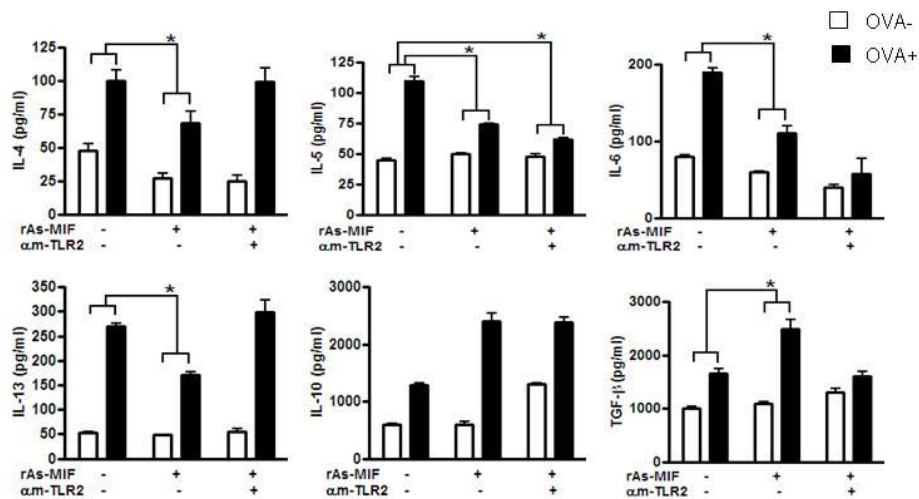


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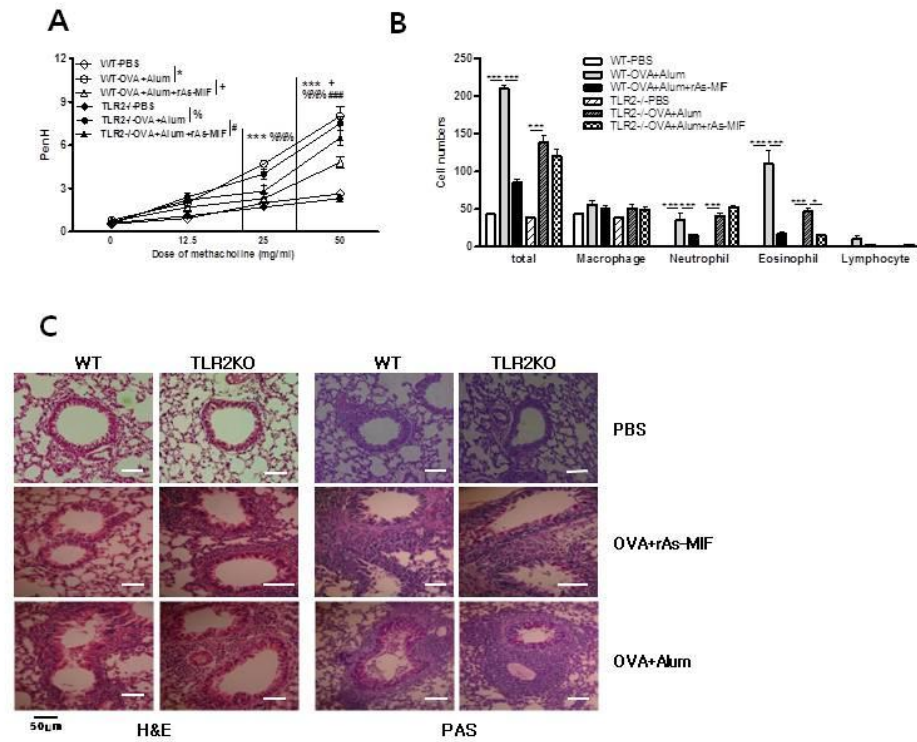


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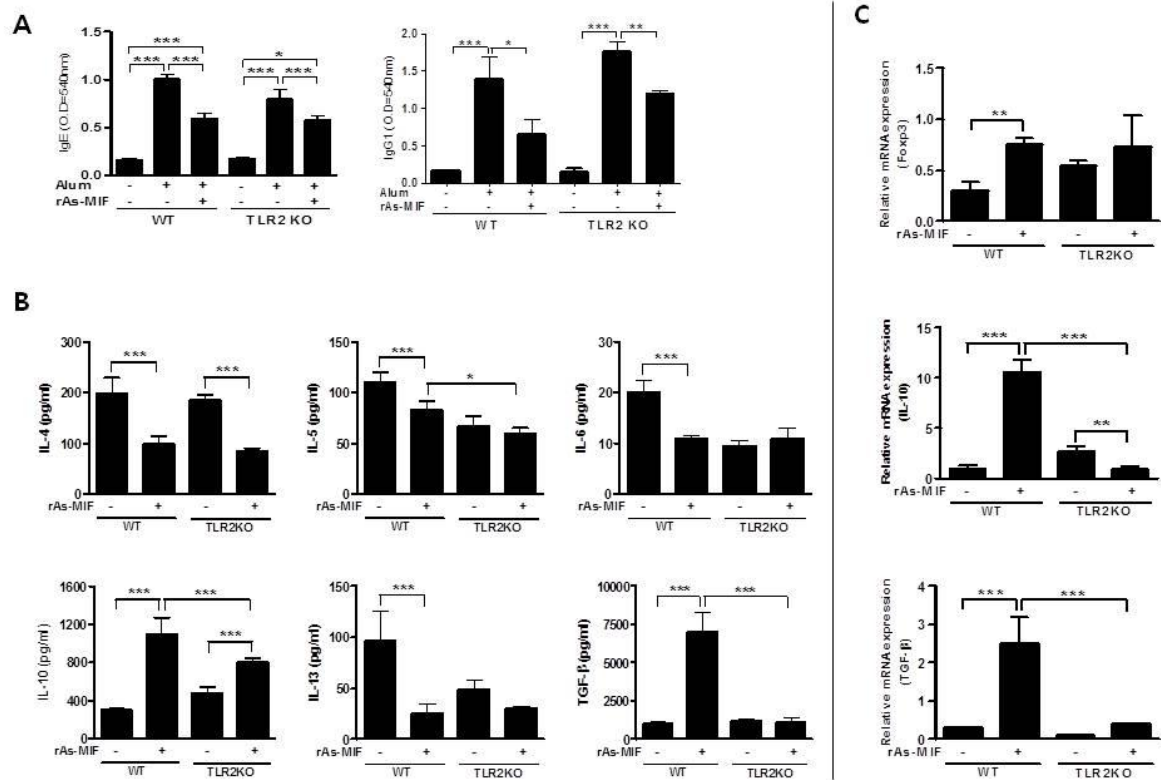


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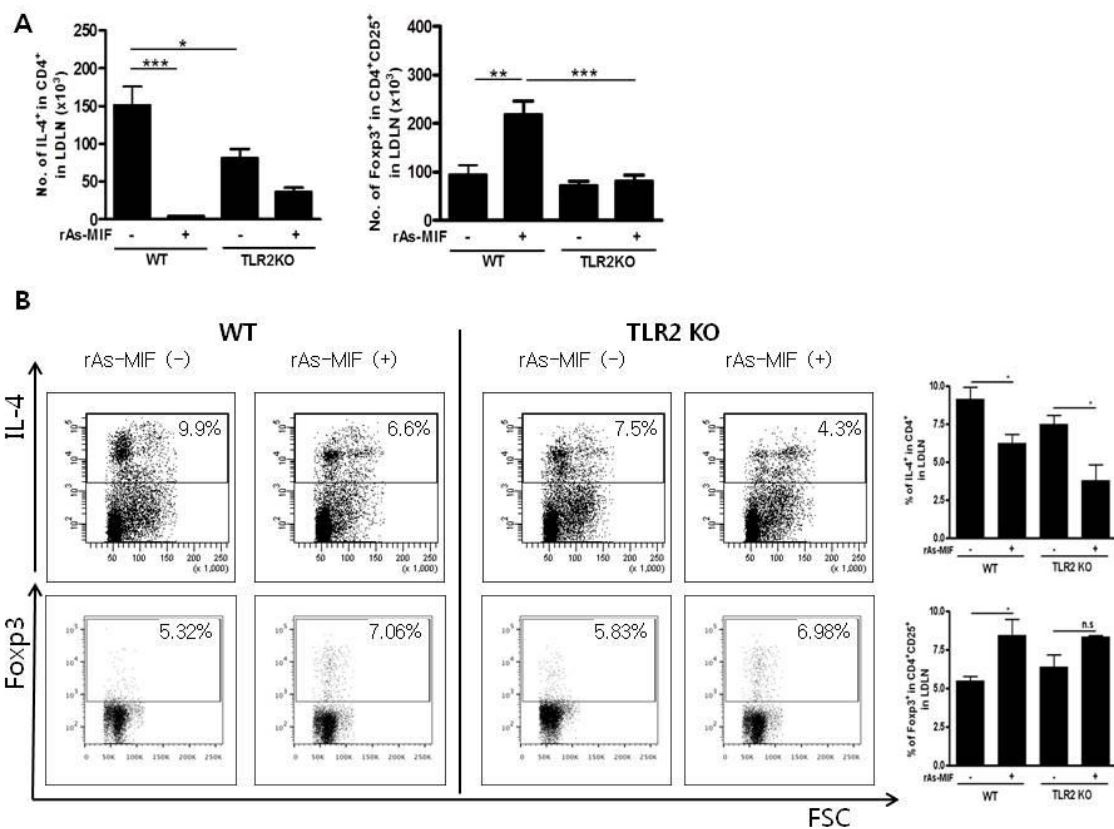


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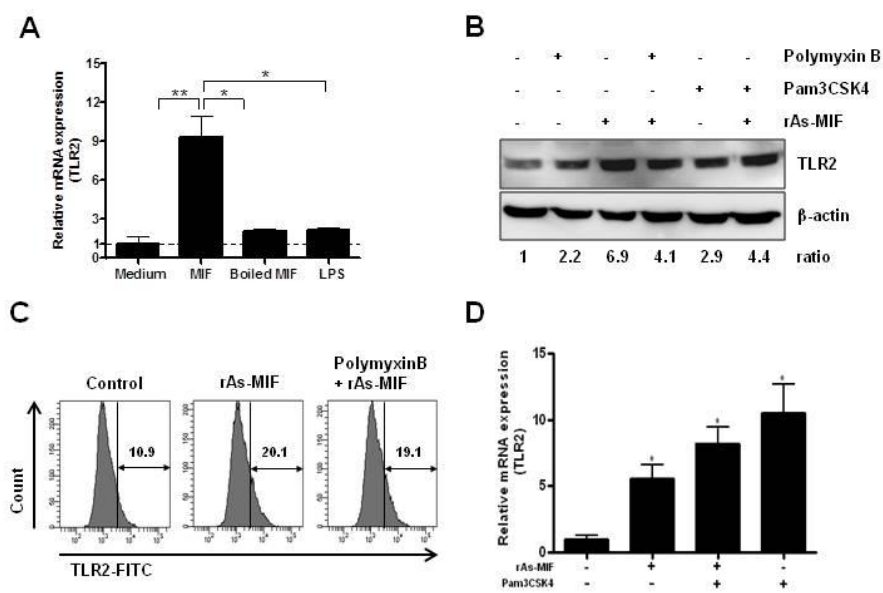


Figure 6

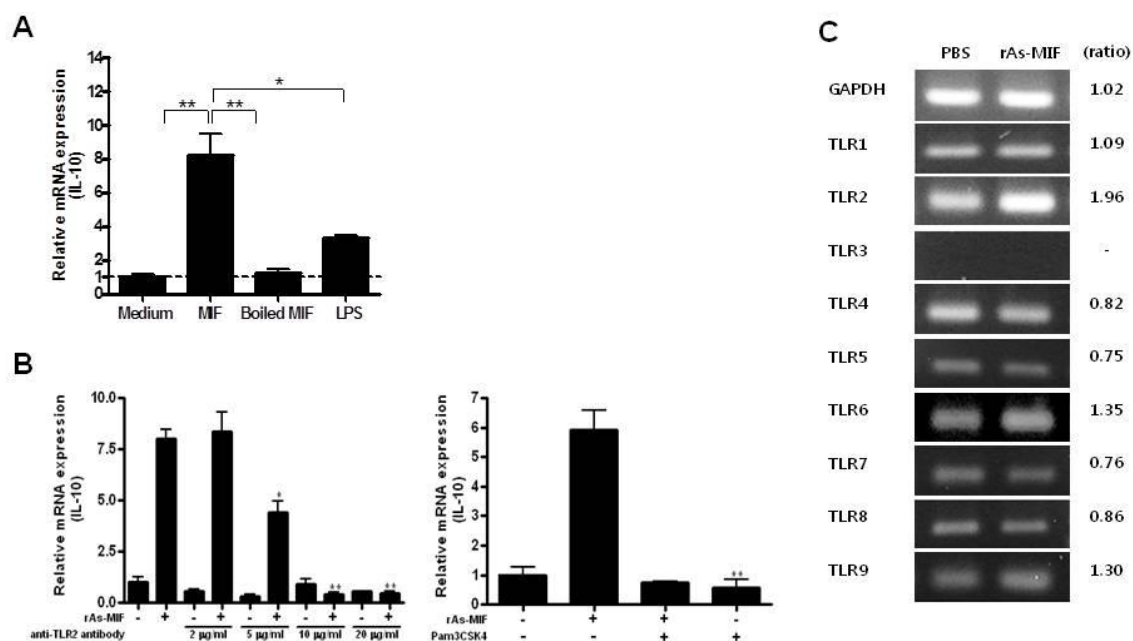


Figure 7