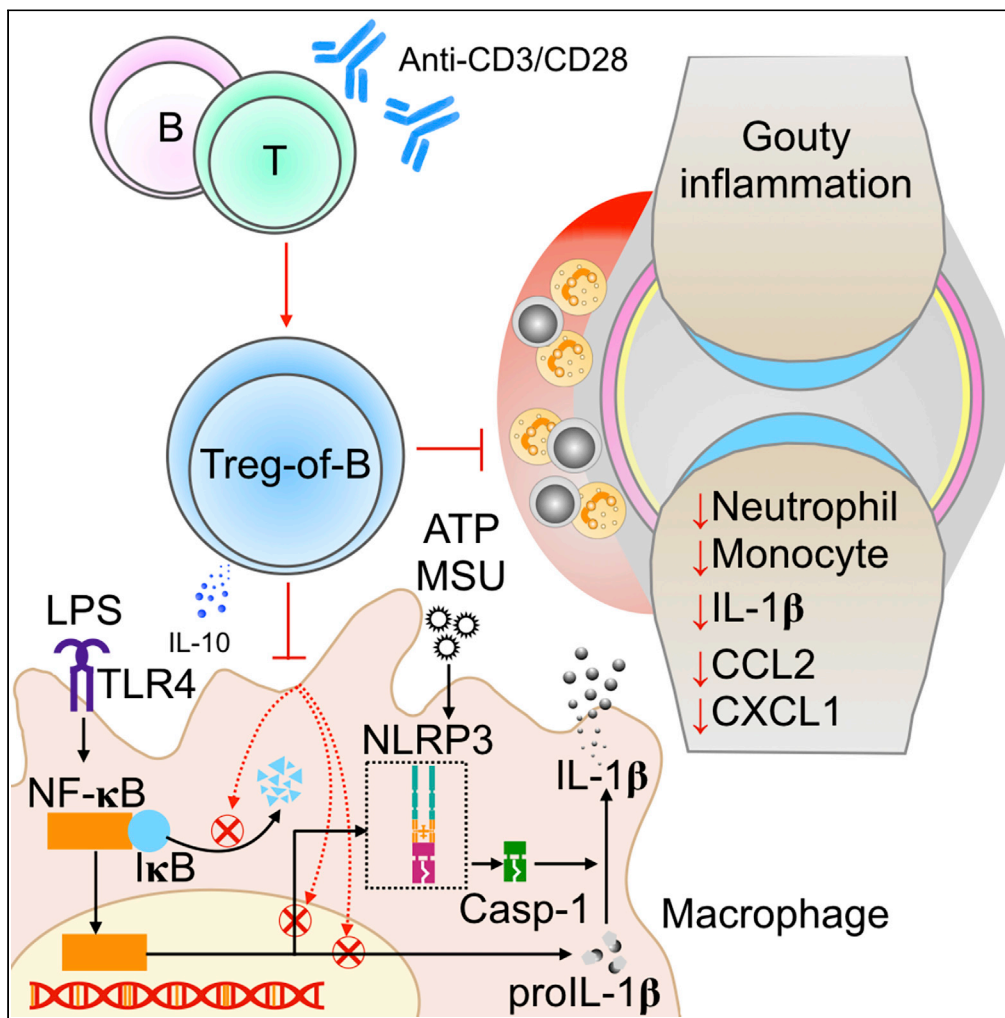


Article

# Regulatory T cells induced by B cells suppress NLRP3 inflammasome activation and alleviate monosodium urate-induced gouty inflammation



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**HIGHLIGHTS**

Foxp3<sup>+</sup> Treg-of-B cells, not tTreg cells, suppress NLRP3 inflammasome activation

Treg-of-B cells inhibit NLRP3 inflammasome activation by repressing NF-κB signaling

Treg-of-B cells ameliorate animal model of gouty inflammation *in vivo*

Treg-of-B cells regulate innate immunity and alleviate NLRP3-associated syndromes

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## Article

## Regulatory T cells induced by B cells suppress NLRP3 inflammasome activation and alleviate monosodium urate-induced gouty inflammation

Jing-Hui Huang<sup>1</sup> and Bor-Luen Chiang<sup>1,2,3,4,\*</sup>

## SUMMARY

**Regulatory T cells induced by B cells (Treg-of-B cells), a distinct Foxp3<sup>+</sup> Treg cell subset, have established the roles in the suppression of inflammatory conditions, including asthma and intestinal inflammation. However, little is known about the regulatory effects of Treg-of-B cells on innate immunity. Herein, we examined whether Treg-of-B cells could regulate macrophage function and prevent NLRP3-associated diseases, particularly inflammatory gouty arthritis. Treg-of-B cells, but not thymus-derived Treg or effector T cells, inhibited inflammasome-mediated IL-1 $\beta$  secretion, caspase-1 activation, and NLRP3 production by LPS/ATP stimulation in a cell contact-dependent manner. In addition, Treg-of-B cells inhibited monosodium urate-induced NLRP3 inflammasome activation *in vitro* via NF- $\kappa$ B signaling. Treg-of-B cells ameliorated gouty inflammation in a mouse air pouch model by reducing neutrophil and leukocyte influx and cytokine and chemokine production. Our results demonstrated that Treg-of-B cells exerted regulatory effects on innate immunity by suppressing NLRP3 inflammasome activation and feasible for future therapeutic applications.**

## INTRODUCTION

Gouty inflammation is an acute and painful type of arthritis caused by the precipitation of monosodium urate (MSU) in the joints. The phagocytosis of MSU by macrophages triggers NLRP3 inflammasome activation, resulting in secretion of IL-1 $\beta$  which plays a key role in the inflammatory cascade (Bach et al., 2019; Kingsbury et al., 2011; Liu-Bryan, 2010; Shin et al., 2019; Steiger and Harper, 2014; Wang et al., 2020). In addition, MSU could directly activate T cells in an antigen-independent manner in gouty arthritis (Webb et al., 2009). These activated T cells promote osteoclast differentiation via RANKL, which causes bone destruction in gouty arthritis (Lee et al., 2011). After 7–10 days, the gout flare is self-limited and inflammation symptoms, including swelling, pain, and synovitis, subside as the disease progresses to the asymptomatic intercritical period (Ragab et al., 2017). However, the mechanism by which immune cells prevent gout flare remains unclear. Current medications for the treatment of gouty inflammation, including colchicine, nonsteroidal anti-inflammatory drugs, steroids, and IL-1 $\beta$  blockers (Ellmann et al., 2020; FitzGerald et al., 2020), have adverse effects, such as gastrointestinal bleeding and renal toxicity. Moreover, these treatments might increase the risk of infection. Therefore, it is important to explore alternative treatments for refractory patients.

The NLRP3 inflammasome is a large multiple protein complex. Its activation requires two signals: (1) toll-like receptor (TLR) activation leading to the synthesis of pro-IL-1 $\beta$  and (2) ATP or MSU resulting in the assembly of the NLRP3 inflammasome, caspase-1 activation, and IL-1 $\beta$  secretion (Aizawa et al., 2020; He et al., 2016; Malik and Kanneganti, 2017; Patel et al., 2017; Schroder and Tschopp, 2010). The constitutive and dysregulated activation of the NLRP3 inflammasome is associated with many inflammatory and autoimmune diseases, such as cryopyrin-associated periodic syndrome, inflammatory bowel disease, coronavirus disease 2019 (COVID-19), and gouty disease (Cartland et al., 2019; Conti et al., 2020; Franchi et al., 2009; Gattorno and Martini, 2013; Guo et al., 2015; Kingsbury et al., 2011; Niyonzima et al., 2020; Zhang et al., 2018). To prevent the constitutive activation of the NLRP3 inflammasome, the immune system also develops a modulatory mechanism to maintain homeostasis (Conti et al., 2020). Recent studies have shown that SARS-CoV2 infection may activate the NLRP3 inflammasome and IL-1 $\beta$  secretion, leading to lung fibrosis (Conti et al., 2020; Perricone et al., 2020). Moreover, memory CD4<sup>+</sup> T cells abolish NLRP3

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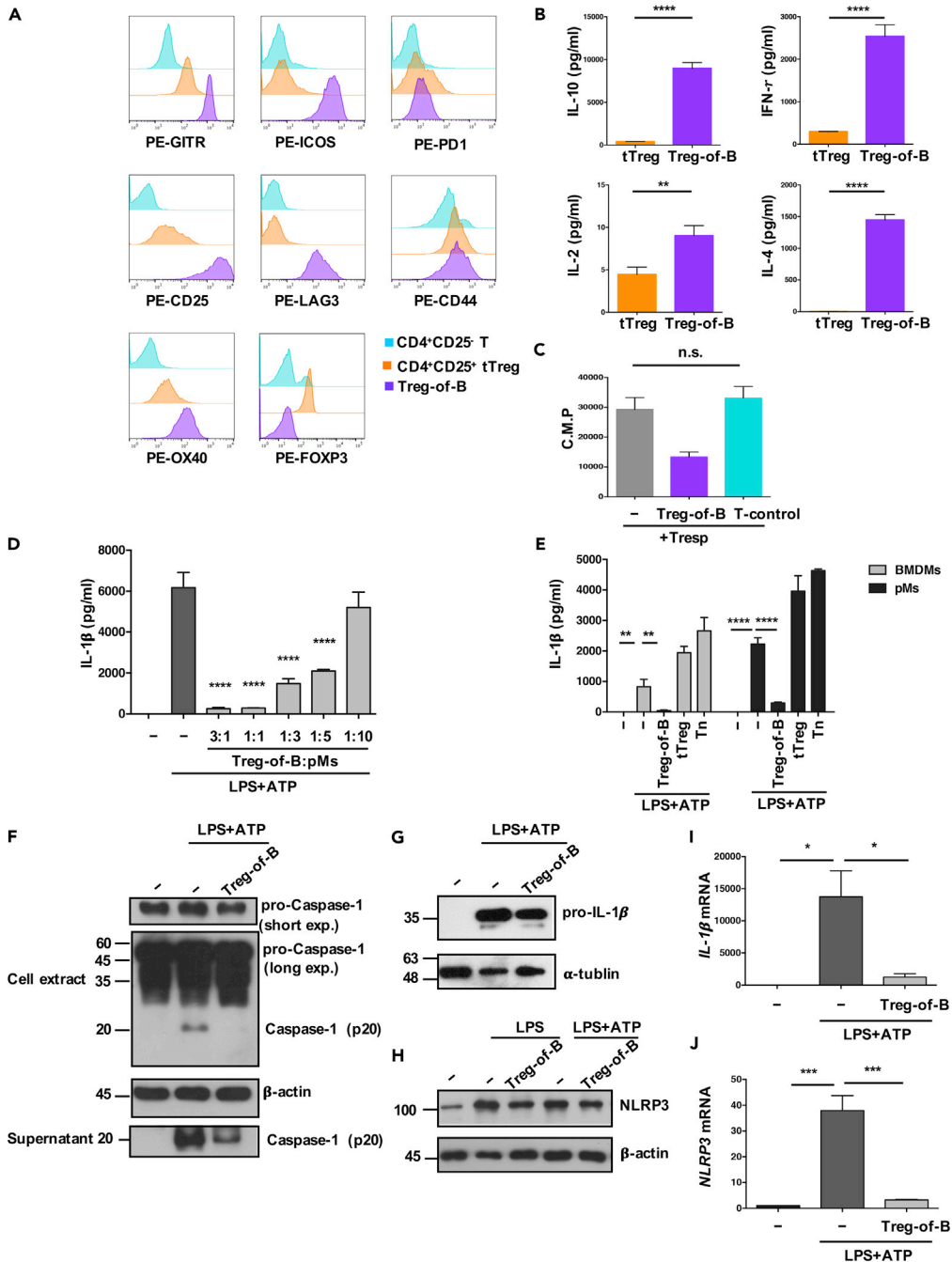
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**Figure 1. Treg-of-B cells suppressed inflammasome activation upon LPS and ATP stimulation**

(A) The expression of surface markers on Treg-of-B cells, CD4<sup>+</sup>CD25<sup>+</sup> tTregs, and CD4<sup>+</sup>CD25<sup>+</sup> T cells was analyzed by flow cytometry.

(B) The cytokine production of Treg-of-B cells and CD4<sup>+</sup>CD25<sup>+</sup> tTreg cells was analyzed by ELISA.

(C) The suppressive ability of Treg-of-B cells was analyzed using CD4<sup>+</sup>CD25<sup>+</sup> T cells as responder T cells.

(D) Treg-of-B cells were cultured together with pMs at the indicated ratio overnight. After that, pMs were primed with LPS for 3.5 hr and then with ATP for 20 min. IL-1 $\beta$  release was measured by enzyme-linked immunosorbent assay (ELISA). The values are expressed as the mean  $\pm$  standard error of the mean (SEM). (\*compared to LPS/ATP-stimulated pMs, \*\*\*\* $p$  < 0.0001, by one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test).

(E) ATP-induced IL-1 $\beta$  secretion by BMDMs cultured together with the indicated T-cell population.

**Figure 1. Continued**

(F) ATP-induced active caspase-1 secretion by BMDMs in the cell lysate and in concentrated supernatants was measured by western blotting analysis.

(G and H) (G) Western blotting analysis of pro-IL1 $\beta$  production and NLRP3 (H) in cell lysates from BMDMs. The western blots are representative of two or three independent experiments.

(I and J) The expression of *IL-1 $\beta$*  and *NLRP3* mRNA in BMDMs cocultured with Treg-of-B cells was quantified by RT-PCR. The data are representative of three independent experiments. The values are expressed as the mean  $\pm$  SEM. (\* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ , by one-way ANOVA with Bonferroni's multiple comparison test). See also [Figure S3](#) and [Table S3](#).

inflammasome activity, including caspase-1 activation and IL-1 $\beta$  production, via cell-cell contact ([Guarda et al., 2009](#)). Therefore, understanding the mechanism underlying immune cell-mediated NLRP3 inflammasome regulation is important.

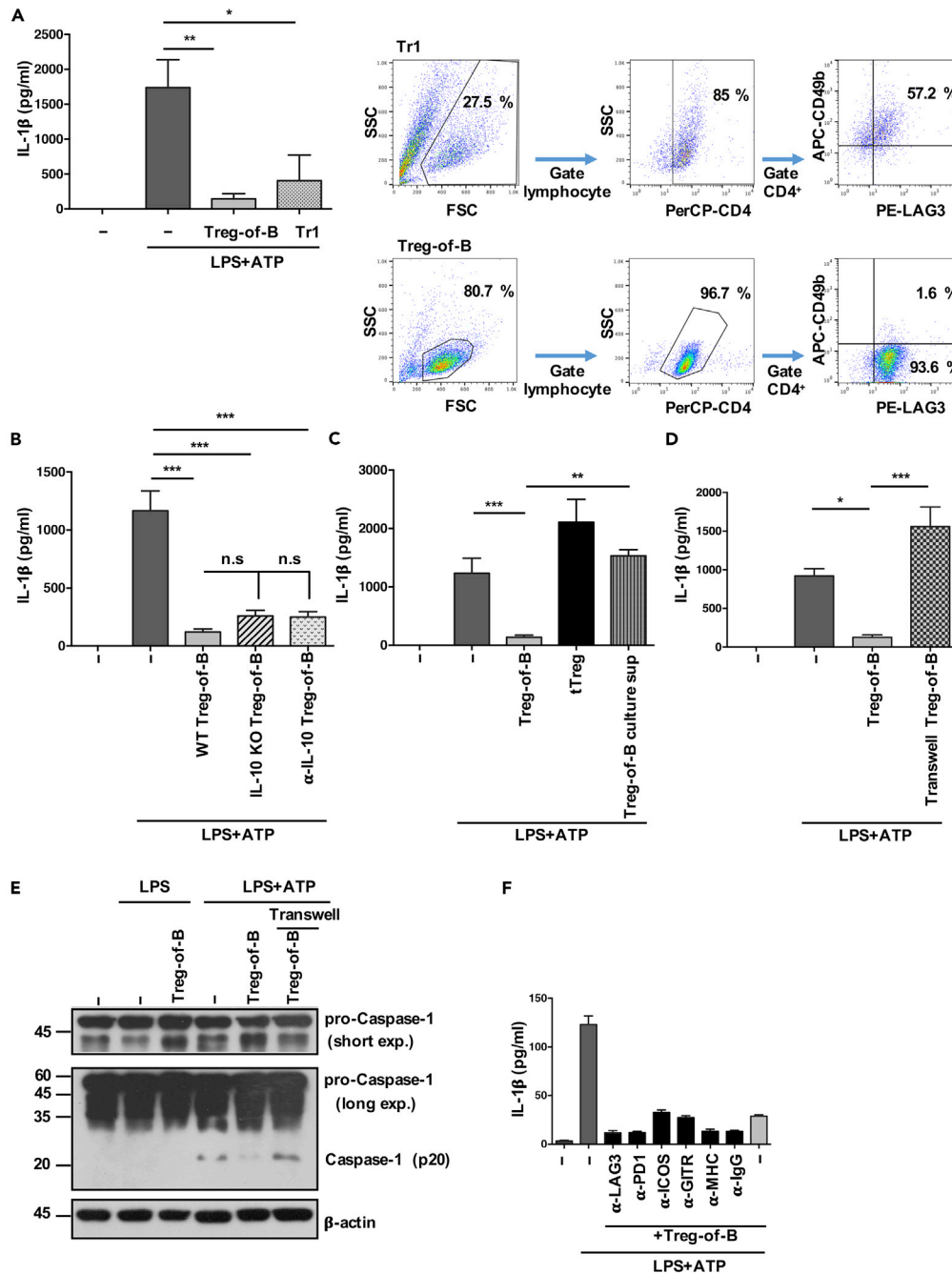
Regulatory T (Treg) cells play a crucial role in maintaining self-tolerance and preventing an excessive hyper-immune response ([Takahashi et al., 2020](#)). Natural Treg cells can be classified into two major groups according to their origin: thymus-derived Treg (tTreg) cells and peripheral Treg (pTreg) cells. FOXP3 expression is crucial for suppressive effects of Treg cells ([Lu et al., 2017](#); [Sakaguchi et al., 2010](#); [Waickman et al., 2020](#)). In contrast, Foxp3<sup>-</sup> type 1 regulatory T cells (Tr1 cells) lack Foxp3 expression and produce a high level of interleukin (IL)-10, which has a suppressive effect ([Pot et al., 2011](#)). A previous study has shown that Tr1 cells suppress NLRP3 inflammasome-mediated caspase-1 cleavage and IL-1 $\beta$  secretion via IL-10 ([Yao et al., 2015](#)).

A previous study indicated that B cells induced the generation of regulatory T cells via an immunological synapse ([Reichardt et al., 2007](#)). We have further confirmed that B cells promote the conversion of naive T cells into Foxp3<sup>+</sup> Treg cells, referred to as Treg-of-B cells ([Chien and Chiang, 2017](#)). Treg-of-B cells have immunosuppressive effects, including the inhibition of T-cell proliferation and Th1 and Th17 cytokine production, and FOXP3 expression is not critical for the development and function of Treg-of-B cells ([Hsu et al., 2014](#); [Shao et al., 2016](#)). Treg-of-B cells have been found to suppress rejection following allogeneic heart transplantation and alleviate asthmatic symptoms, inflammatory bowel disease, and collagen-induced arthritis ([Chen et al., 2016](#); [Chien et al., 2015, 2017](#); [Chu and Chiang, 2012](#); [Shao et al., 2016](#)). Furthermore, our group demonstrated that Treg-of-B cells expressed the Treg-associated markers and produced high levels of IL-10 ([Chien and Chiang, 2017](#)). Although Treg-of-B cells express Treg-associated markers and produce high levels of IL-10 ([Hsu et al., 2014](#); [Shao et al., 2016](#)), IL-10 does not contribute to the suppressive effects of these cells. Therefore, Treg-of-B cells are a unique Foxp3<sup>+</sup> Treg cell subset that differs from Tr1 and natural Treg cells.

Treg-of-B cells clearly suppress adaptive immunity-driven inflammation; however, the ability of Treg-of-B cells to regulate innate immunity remains unclear. We investigated whether Treg-of-B cells could maintain immune homeostasis to prevent inflammasome-induced diseases by the regulation of macrophage function. We established a mouse air pouch model to mimic human gouty synovitis and examined inflammatory biomarker levels in response to MSU. We demonstrated that Treg-of-B cells suppress NLRP3 inflammasome activation both *in vitro* and *in vivo*, potentially alleviating NLRP3-associated gouty arthritis. Our results clarify the role of Treg-of-B cells in the modulation of innate immunity and provide a novel cell-based approach for the treatment of NLRP3-associated diseases.

**RESULTS****Treg-of-B cells suppressed NLRP3 inflammasome activation upon LPS and ATP stimulation**

Flow cytometric analysis showed that Treg-of-B cells expressed high levels of GITR, ICOS, PD1, CD25, LAG3, CD44, and OX40 and rarely expressed Foxp3 ([Figure 1A](#)). Compared to tTreg cells, Treg-of-B cells produced higher levels of IL-10, IFN- $\gamma$ , IL-2, and IL-4 ([Figure 1B](#)). In addition, Treg-of-B cells suppressed T-cell proliferation ([Figure 1C](#)). We investigated whether Treg-of-B cells exert an immunoregulatory effect on myeloid cells and suppress inflammasome activation. Peritoneal macrophages (pMs) were co-cultured with Treg-of-B cells at different ratios overnight and then stimulated with LPS for 3.5 h, followed by ATP for 20 min to activate the NLRP3 inflammasome. Notably, Treg-of-B cells inhibited IL-1 $\beta$  production in a dose-dependent manner ([Figure 1D](#)). In addition, inflammasome activation in bone-marrow-derived macrophages (BMDMs) and pMs was suppressed by Treg-of-B cells but not by tTreg cells or CD4<sup>+</sup>CD25<sup>-</sup>



**Figure 2. Treg-of-B cells suppressed inflammasome activation via a cell contact-dependent mechanism**

(A) Tr1 cells or Treg-of-B cells generated *in vitro* were cultured together with BMDMs at a 3:1 ratio. Subsequently, BMDMs were primed with LPS and stimulated with ATP. IL-1 $\beta$  production was measured by enzyme-linked immunosorbent assay (ELISA). The expression of surface markers of *in vitro*-induced Treg-of-B cells and *in vitro*-induced Tr1 were analyzed by flow cytometry.

(B) BMDMs were cultured together with WT Treg-of-B cells and IL-10 KO Treg-of-B cells at a 1:3 ratio under LPS and ATP stimulation conditions. IL-1 $\beta$  secretion was measured by ELISA.

(C) The BMDMs were cultured with supernatant from Treg-of-B cell cultures and then stimulated with LPS and ATP. IL-1 $\beta$  secretion from BMDMs was measured by ELISA.

(D and E) Treg-of-B cells were cultured with BMDMs at a ratio of 3:1 in the presence or absence of Transwell overnight. The IL-1 $\beta$  production in the supernatant was measured by ELISA (D). Activated caspase-1 in cell lysates was detected by Western blot (E).

**Figure 2. Continued**

(F) Blocking antibodies were added to the Treg-of-B cell-BMDM coculture system under LPS and ATP stimulation conditions, and IL-1 $\beta$  production in the supernatant was measured.

The data are representative of at least three independent experiments. The values are expressed as the mean  $\pm$  standard error of the mean (SEM). (\* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001, by one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test; n.s. = not significant). The western blots are representative of two independent experiments. See also [Figure S4](#) and [Table S3](#).

effector T cells ([Figure 1E](#)). Additionally, western blotting showed that caspase-1 activity in BMDMs was repressed by Treg-of-B cells ([Figure 1F](#)). Furthermore, pro-IL-1 $\beta$  and NLRP3 levels decreased in BMDMs co-cultured with Treg-of-B cells ([Figures 1G](#) and [1H](#)). *IL-1 $\beta$*  and *NLRP3* mRNA expression levels were significantly lower in BMDMs cultured with Treg-of-B cells than in BMDMs alone under LPS and ATP stimulation ([Figures 1I](#) and [1J](#)). These results suggested that the activation of the NLRP3 inflammasome was abolished by Treg-of-B cells by the downregulation of the priming step.

**Treg-of-B cells suppressed inflammasome activation predominantly via a contact-dependent mechanism**

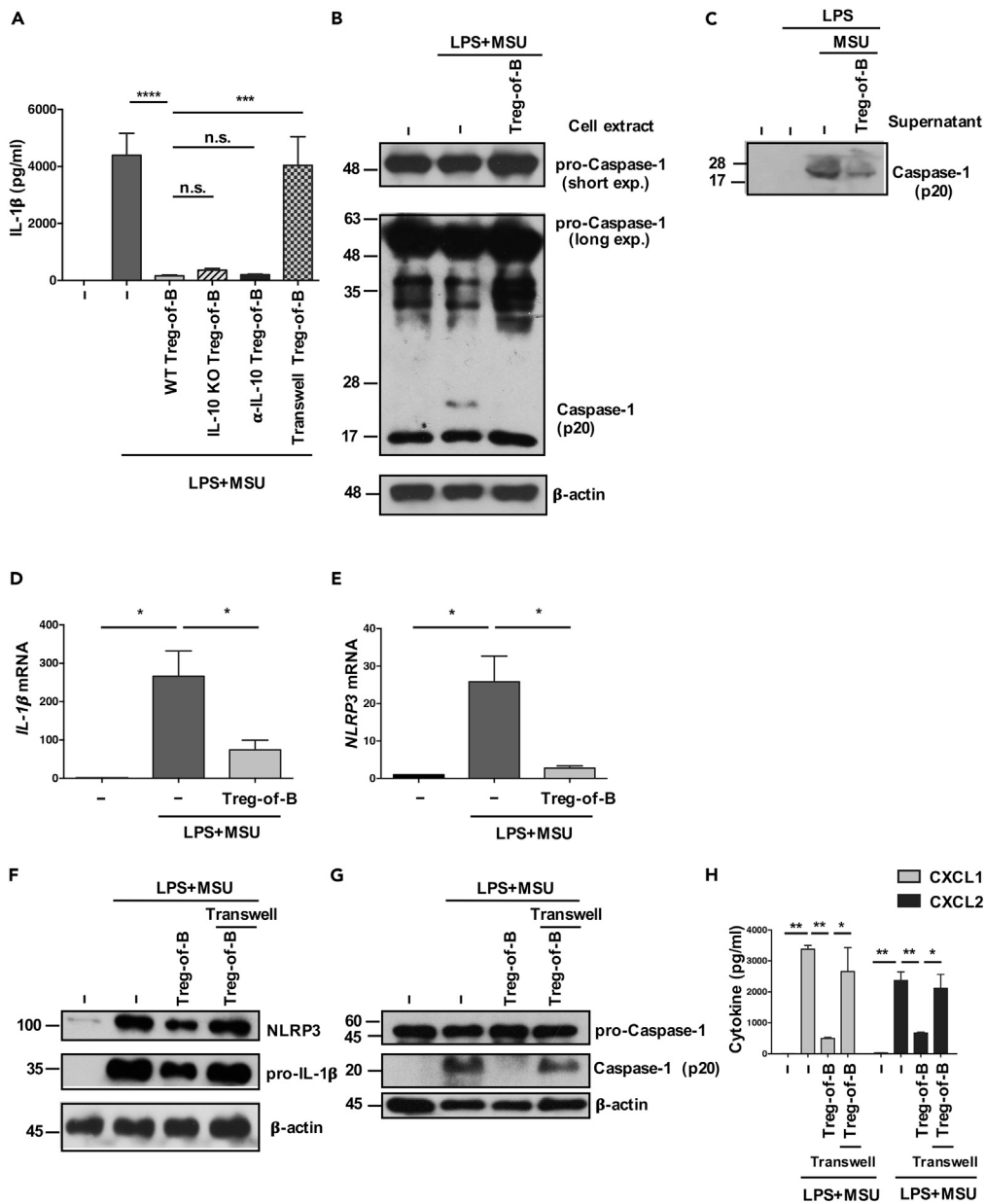
We evaluated the cellular mechanisms underlying the suppression of the NLRP3 inflammasome and found that Treg-of-B and Tr1 cells have similar suppressive effects on inflammasome activation ([Figure 2A](#)). Tr1 cells expressed high levels of CD49b and LAG3; however, the CD49b-expressing Treg-of-B cell population was smaller than the CD49b-expressing Tr1 cell population ([Figure 2A](#)). Therefore, Treg-of-B cells were different from Tr1 cells. To determine whether Treg-of-B cells suppressed inflammasome activation via IL-10, BMDMs were co-cultured with wild-type (WT) Treg-of-B cells and IL-10-deficient Treg-of-B cells (IL-10 KO Treg-of-B cells) under LPS and ATP stimulation. Both the IL-10 KO Treg-of-B and IL-10 neutralization groups showed partially but not significantly reversed IL-1 $\beta$  production compared to that of the WT Treg-of-B cell group ([Figure 2B](#)). These results showed that IL-10 does not play a critical role in the suppression of inflammasome activation by Treg-of-B cells. Moreover, soluble factors from Treg-of-B cultures could not suppress inflammasome activation under LPS and ATP stimulation ([Figure 2C](#)). The co-culture of BMDMs with Treg-of-B cells in the Transwell separation system resulted in a reversal in the production of IL-1 $\beta$  and active caspase-1, suggesting a critical role of cell-cell contact ([Figures 2D](#) and [2E](#)).

To identify the surface molecules necessary for the suppression of inflammasome activation by Treg-B cells, we assessed the expression of membrane-bound markers on Treg-B cells by using a blocking antibody. However, antibodies against LAG3, PD1, ICOS, GITR, and MHCII did not abolish Treg-of-B cell-induced immunosuppression ([Figure 2F](#)).

**Treg-of-B cells inhibited MSU-induced NLRP3 inflammasome activation in a cell contact-dependent manner**

Based on the finding that Treg-of-B cells suppressed NLRP3 inflammasome activation upon LPS and ATP stimulation, we next investigated whether Treg-of-B cells could protect against NLRP3-associated syndromes, such as gouty arthritis. We used an *in vitro* model of MSU-induced NLRP3 inflammasome activation. We found that Treg-of-B cells significantly suppressed IL-1 $\beta$  production under LPS and MSU stimulation ([Figure 3A](#)). Active caspase-1 in the total cell lysate and supernatant was abolished in co-cultures with Treg-of-B cells ([Figures 3B](#) and [3C](#)). An MTT assay revealed that the survival of BMDMs was not affected by Treg-of-B cells ([Figure S1](#)). Furthermore, the mRNA expression of *IL-1 $\beta$*  and *NLRP3* was inhibited by Treg-of-B cells ([Figures 3D](#) and [3E](#)). Treg-of-B cell-mediated inhibition of NLRP3 and pro-IL-1 $\beta$  protein was confirmed by western blot ([Figure 3F](#)). These findings indicated that MSU-induced NLRP3 inflammasome activation was inhibited by Treg-of-B cells *in vitro*.

We next sought to clarify the mechanisms by which Treg-of-B cells suppressed MSU-induced NLRP3 inflammasome activation. We found that IL-1 $\beta$  production in BMDMs was not reversed by the suppressive effect of the IL-10-deficient Treg-of-B cells ([Figure 3A](#)). Moreover, the secretion of IL-1 $\beta$  and the inhibitory function of Treg-of-B cells were completely reversed in the Transwell system ([Figure 3A](#)). Furthermore, western blotting showed that the secretion of the active forms of caspase-1, pro-IL-1 $\beta$ , and NLRP3 in the cell lysate could not be suppressed by Treg-of-B cells in the Transwell system ([Figures 3F](#) and [3G](#)), suggesting an important role of cell-cell contact, rather than IL-10, in Treg-of-B cell-mediated inhibition of MSU-induced NLRP3 activation. In addition, the production of CXCL1 and CXCL2 by BMDMs was inhibited by Treg-of-B



**Figure 3. Treg-of-B cells inhibited MSU-induced NLRP3 inflammasome activation *in vitro***

(A) BMDMs were cocultured with WT Treg-of-B cells or IL-10 KO Treg-of-B cells at a 1:3 ratio overnight under anti-CD3/CD28 stimulation conditions with or without Transwell inserts. Subsequently, BMDMs were primed with LPS for 4 hr, followed by MSU for 2.5 hr. The release of IL-1 $\beta$  was measured by enzyme-linked immunosorbent assay (ELISA).

(B) The activated form of caspase-1 from BMDMs in the cell lysate was detected by western blot.

(C) The activated form of caspase-1 in BMDMs from culture supernatant was detected by western blot.

(D and E) The expression of IL-1 $\beta$  and NLRP3 mRNA in BMDMs was quantified by RT-PCR.

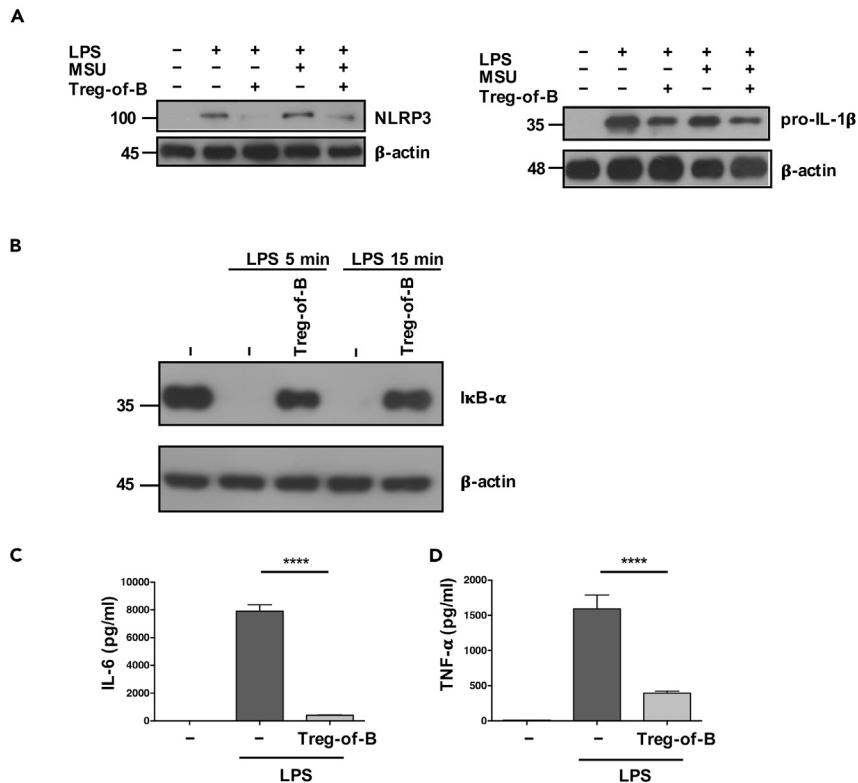
(F–H) In the Treg-of-B cell-BMDM Transwell culture system, NLRP3 and pro-IL-1 $\beta$  (F) production and caspase-1 (G)

cleavage were assessed by western blot. The production of CXCL1 and CXCL2 (H) by BMDMs was measured by ELISA.

The values are expressed as the mean  $\pm$  standard error of the mean (SEM). (\* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001,

\*\*\*\* $p$  < 0.0001 by one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test; n.s. = not significant).

The western blots are representative of three or two independent experiments. See also [Figures S5](#) and [S6](#) and [Table S1](#).



**Figure 4. Treg-of-B cells inhibited the NF-κB signaling pathway**

(A) The protein expression of NLRP3 and pro-IL-1β in cell lysates from BMDMs under LPS stimulation for 4 hr was detected by western blot.

(B) Treg-of-B cells were cocultured with BMDMs at a 3:1 ratio overnight and then stimulated with LPS for 5 min or 15 min. IκBα expression (B) was detected by western blotting.

(C and D) The production of IL-6 and TNF-α by BMDMs co-cultured with Treg-of-B cells was measured under LPS stimulation for 24 hr. The values are expressed as the mean ± standard error of the mean (SEM). (\*\*\*\*p < 0.0001 by one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test). The western blots are representative of three or two independent experiments.

See also [Figure S7](#).

cells under MSU activation ([Figure 3H](#)), implying that neutrophil infiltration was suppressed by Treg-of-B cells under MSU-induced NLRP3 inflammasome activation.

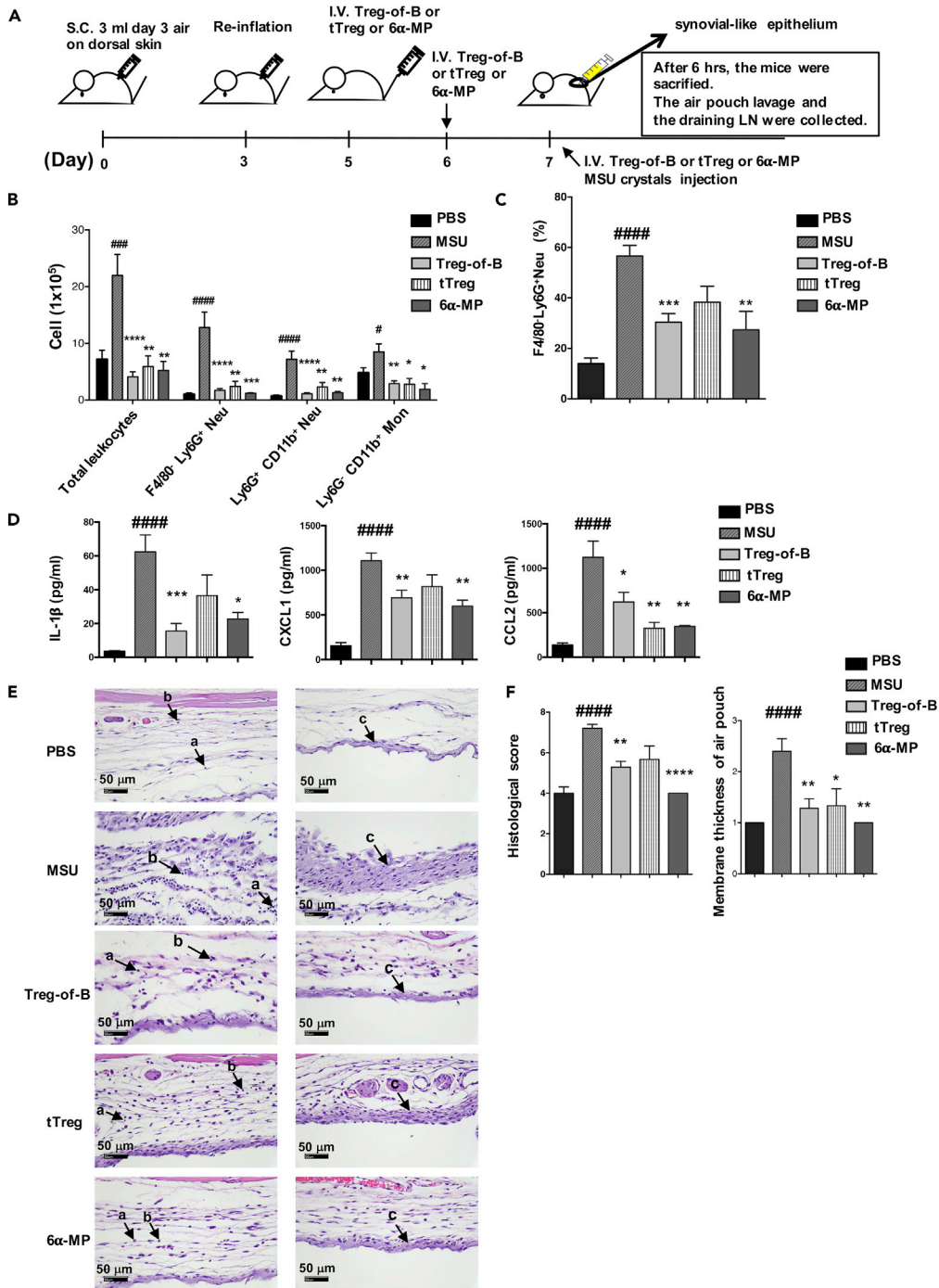
### Treg-of-B cells inhibited MSU-induced NLRP3 inflammasome activation by repressing NF-κB signaling

NLRP3 inflammasome activation is tightly controlled by two-stage signaling. It is worth mentioning that levels of NLRP3 and pro-IL-1β production were decreased in BMDMs co-cultured with Treg-of-B cells under LPS stimulation ([Figure 4A](#)), implying that the suppressive effect of Treg-of-B cells primarily targeted the priming step of inflammasome activation. We further examined whether Treg-of-B cells inhibit NLRP3 inflammasome activation by targeting NF-κB signaling. We observed the degradation of IκBα in BMDMs stimulated with LPS for 5 min and 15 min. In contrast, IκBα degradation was abolished when BMDMs were co-cultured with Treg-of-B cells ([Figure 4B](#)). Moreover, we found that Treg-of-B cells inhibited NF-κB-mediated IL-6 and TNF-α production by BMDMs stimulated with LPS ([Figures 4C and 4D](#)). These results suggested that Treg-of-B cells suppressed the NLRP3 inflammasome by the abrogation of NF-κB signaling.

### Treg-of-B cells suppressed gouty inflammation *in vivo*

Our *in vitro* analyses showed that Treg-of-B cells suppress MSU-induced inflammasome activation. Thus, we evaluated whether Treg-of-B cells alleviate gouty arthritis *in vivo*. To address this question, we established an air pouch model of MSU-induced gouty inflammation. Two control groups were used, mice





**Figure 5. Treg-of-B cells inhibited gouty inflammation in a synovium-like air pouch model**

(A–C) On day 0 and day 3, sterile air was injected into the backs of the mice. For the adoptive transfer experiments, Treg-of-B cells were injected into the mice via intravenous injection on days 5, 6, and 7. Simultaneously, two control groups were used, mice treated with 6α-methylprednisolone (6α-MP, 1.6 mg/kg) and mice receiving adoptive transfer of tTreg via intravenous injection. On day 7, MSU (2 mg) were injected into the air pouches on the back of mice. After 6 hr, the mice were sacrificed, and the air pouch lavage fluid was harvested to count the number of leukocytes and analyzed the populations of neutrophils (F4/80<sup>+</sup>Ly6G<sup>+</sup> and Ly6G<sup>+</sup>CD11b<sup>+</sup>) and monocytes (Ly6G<sup>+</sup>CD11b<sup>+</sup>) by flow cytometry (B and C). The values are expressed as the mean ± SEM (n = 9/PBS group, n = 6/MSU group, n = 7/Treg-of-B group, n = 3/tTreg group, n = 3/6α-MP group).

**Figure 5. Continued**

(D) The production of inflammatory cytokines (IL-1 $\beta$ ) and chemokines (CXCL1 and CCL2) in the air pouch was measured by enzyme-linked immunosorbent assay (ELISA).

(E) The air pouch sections were stained with H&E to examine polymorphonuclear cell (arrow a) and mononuclear cell (arrow b) infiltration in the subcutaneous air pouch. The thickness of the air pouch membrane (arrow c) was detected. Scale bars, 50  $\mu$ m.

(F) The histological score of the air pouch was evaluated, and the membrane thickness of the air pouch was quantified. The values are expressed as the mean  $\pm$  standard error of the mean (SEM). (Bonferroni's multiple comparisons test).

The data are representative of two or three independent experiments. (#compared to the PBS treatment group, \*compared to the MSU treatment group; # $p < 0.05$ , ## $p < 0.01$ , ### $p < 0.001$ , #### $p < 0.0001$ , \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$  by one-way analysis of variance (ANOVA) with Bonferroni's multiple comparison test). See also [Figure S2](#) and [Table S2](#).

treated with 6 $\alpha$ -methylprednisolone (6 $\alpha$ -MP), which is used to treat acute gout (Prasad et al., 2008), and mice receiving adoptive transfer of tTreg cells. In response to air pouch injection with MSU, the total numbers of leukocytes, neutrophils (F4/80<sup>+</sup>Ly6G<sup>+</sup>), and monocytes (Ly6G<sup>+</sup>CD11b<sup>+</sup>) significantly increased (Figure 5B). Further dissection of the neutrophil population confirmed that the cells were Ly6G<sup>+</sup>CD11b<sup>+</sup> neutrophils. We found that mice treated with Treg-of-B cells, 6 $\alpha$ -MP, and tTreg cells showed lower leukocyte, neutrophil, and monocyte counts compared to those in the MSU-treated group. In addition, the proportion of neutrophils was significantly reduced upon Treg-of-B cell and 6 $\alpha$ -MP treatments (Figure 5C). These results indicated that treatment with Treg-of-B cells was as effective as steroid and tTreg cell treatments for the alleviation of subcutaneous air pouch inflammation in response to MSU.

The air pouch fluid was collected, and inflammatory cytokine levels were measured. Compared to the MSU-treated group, the groups receiving the adoptive transfer of Treg-of-B cells and 6 $\alpha$ -MP had significantly lower levels of IL-1 $\beta$ , CXCL1, and CCL2 in the lavage fluid (Figure 5D). There was a significant decrease in the level of IL-6 after the adoptive transfer of Treg-of-B cells (Figure S2A). However, IL-1 $\beta$  level was more significantly lower in Treg-of-B cells and 6 $\alpha$ -MP groups compared to that of tTreg cells group. Thus, tTreg cells may prevent gouty arthritis by the downregulation of the activation of other effector T cells, instead of suppression of inflammasome activation (Figure 5D). Additionally, H&E staining of the subcutaneous tissue in the air pouch showed histological changes. The MSU-treated group presented severe polymorphonuclear and mononuclear cell infiltration in the subcutaneous air pouch, whereas the Treg-of-B cell-treated group showed less immune cell infiltration in subcutaneous tissues than the MSU-treated group (Figures 5E and S2B). The MSU-treated group presented moderate thickening of the air pouch membrane, a biomarker of inflammation (Figure 5E). In contrast, the Treg-of-B cell-, tTreg-, and 6 $\alpha$ -MP-treated groups showed slight air pouch membrane thickening.

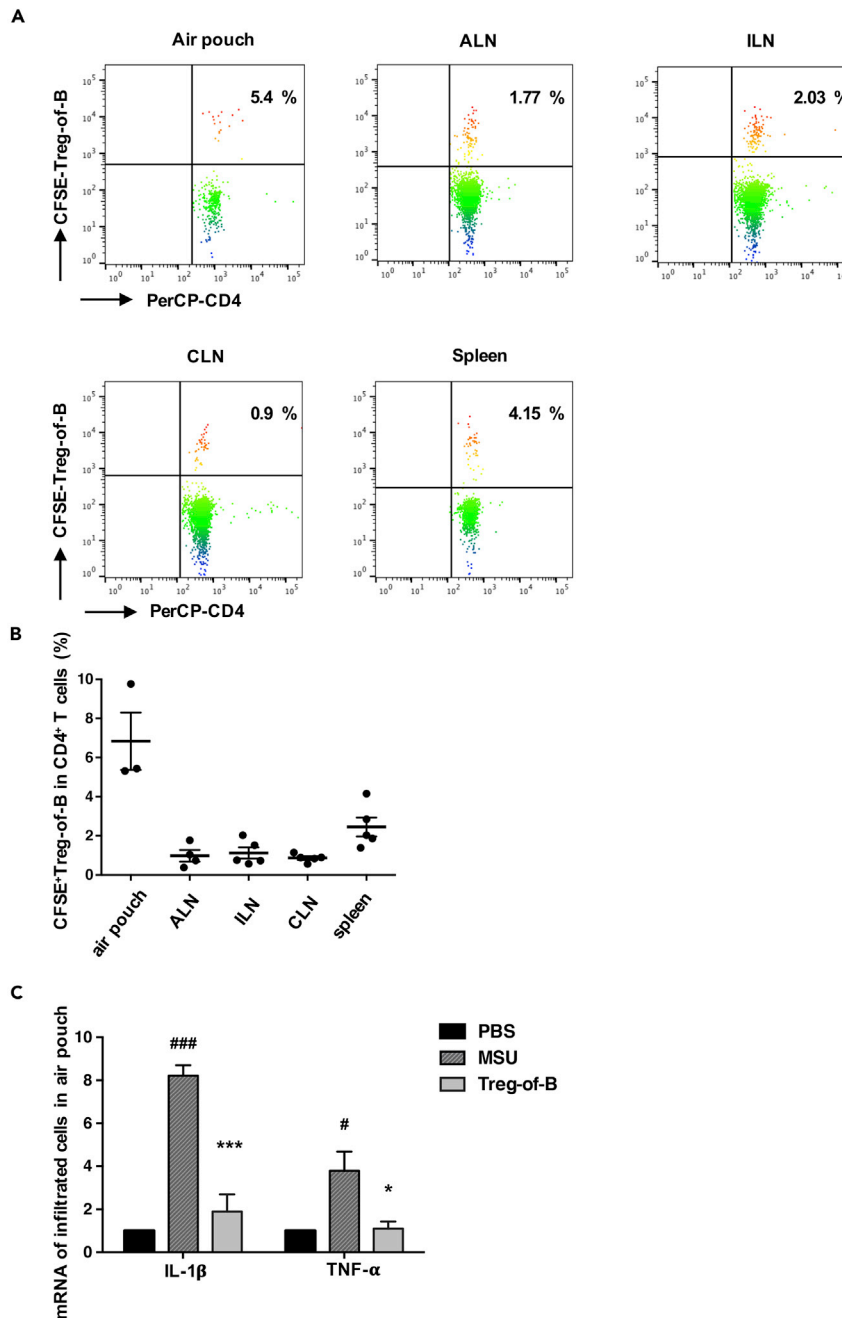
Furthermore, the histological score and membrane thickness in the air pouch were significantly lower in the Treg-of-B cell-treated group than in the MSU-treated group (Figure 5F). These results suggested that Treg-of-B cells had a protective effect against MSU-induced gouty inflammation *in vivo* by decreasing polymorphonuclear and mononuclear cell infiltration and pathological changes. In summary, the downregulation of inflammasome activation might be a mechanism by which Treg-of-B cells ameliorate gouty inflammation; however, Treg-of-B cells may also suppress other immune cells, such as T cells or neutrophils, to manage the gouty inflammation.

**Treg-of-B cells migrated to the air pouch and draining lymph nodes and regulated local inflammation in gout**

We designed an *in vivo* tracking experiment with carboxyfluorescein succinimidyl ester (CFSE)-labeled cells. CFSE-labeled Treg-of-B cells were present in the air pouch, spleen, axillary lymph nodes, inguinal lymph nodes, and cervical lymph nodes in mice with MSU-induced gouty inflammation (Figures 6A and 6B). Moreover, an analysis of inflammatory cells in the air pouch showed that the expression of IL-1 $\beta$  and TNF- $\alpha$  was significantly lower in the Treg-of-B cell-treated group than in the MSU-treated group (Figure 6C). These data suggested that Treg-of-B cells migrated to the air pouch and draining lymph nodes, where they modulated local inflammation in mice with MSU-induced gouty arthritis.

**DISCUSSION**

B cells play a role in the induction of both immune tolerance and activation (Andersson et al., 2016; Ashour and Niederkorn, 2006; Sun et al., 2008). A previous study has indicated that naive B cells induce regulatory



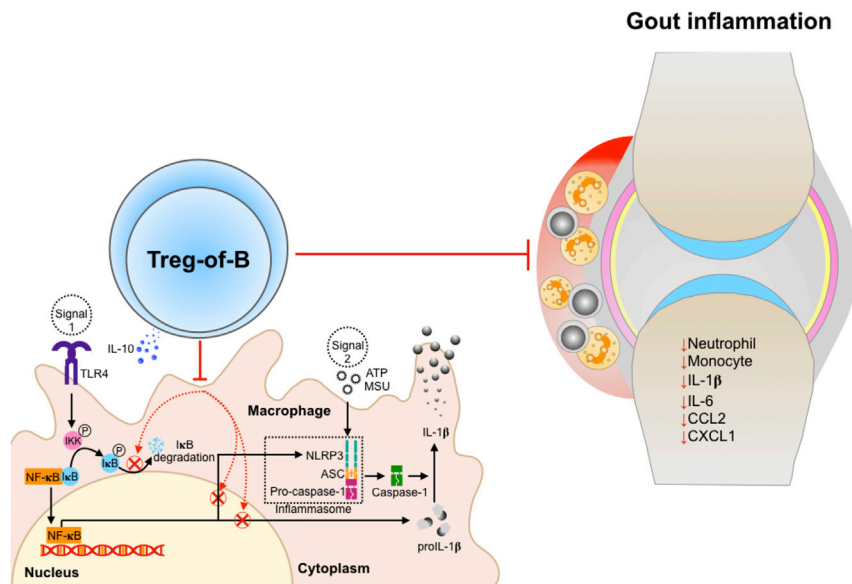
**Figure 6. The Treg-of-B cells administered to mice with gouty inflammation were found in the air pouch and draining lymph node**

(A) Flow cytometry analysis demonstrated the frequencies of CFSE-Treg-of-B cells in air pouch, ALN, ILN, CLN, and spleen in MSU-treated mice.

(B) The frequencies were quantified from flow cytometry analysis.

(C) The infiltrated cells in the air pouch were collected, and the mRNA expression level was analyzed. The data are representative of three independent experiments. The values are expressed as mean  $\pm$  standard error of the mean (SEM). (Bonferroni's multiple comparisons test).

T-cell production by forming a mature immunologic synapse at the contact zone (Reichardt et al., 2007). Primary B cells expand the Treg cell population, exerting increased inhibitory effects in the presence of TGF- $\beta$  or in an allogeneic manner (Chen et al., 2009; Chen and Jensen, 2007; Shah and Qiao, 2008). We



**Figure 7. The proposed model by which Treg-of-B cells alleviated gouty inflammation by inhibiting NLRP3 inflammasome activation**

The activation of the inflammasome requires two signals. The first signal is involved in TLR-mediated NF- $\kappa$ B activation leading to the production of pro-IL-1 $\beta$ . The second signal is ATP or MSU crystals, which results in the assembly of the NLRP3 inflammasome, caspase-1 activation, and IL-1 $\beta$  secretion. Treg-of-B cells inhibit ATP-dependent and MSU-dependent NLRP3 inflammasome activation in a cell-cell contact-dependent manner, and the primary target of the suppressive effect is the NF- $\kappa$ B pathways. Additionally, the administration of Treg-of-B cells reduces the severity of gouty arthritis.

demonstrated that splenic B lymphocytes, peritoneal B lymphocytes, and mucosal B lymphocytes from Peyer's patches induce regulatory T cells named Treg-of-B cells without the addition of any cytokines (Chen et al., 2016; Hsu et al., 2014). In addition, B cells promote Treg expansion to maintain gut homeostasis and suppress colitis (Wang et al., 2015).

The adoptive transfer of Treg-of-B cells protects mice from allergic airway hypersensitivity and collagen-induced arthritis (Chen et al., 2016; Chien et al., 2015). Additionally, Treg-of-B cells inhibit Th1/Th17 cytokine production from mesenteric lymph nodes in a colitis mouse model (Shao et al., 2016) and can suppress the proliferation of effector T cells (Chien and Chiang, 2017; Chien et al., 2015; Chu and Chiang, 2012, 2015; Hsu et al., 2014; Reichardt et al., 2007; Waisman et al., 2013; Wang et al., 2015). These findings indicate that Treg-of-B cells can modulate T-cell-mediated inflammatory diseases. However, it is not clear whether Treg-of-B cells regulate innate immunity. Foxp3<sup>+</sup> Treg cells have an immunomodulatory effect in innate immunity (Yao et al., 2015). Our results demonstrated that Treg-of-B cells suppressed NLRP3 and IL-1 $\beta$  production and caspase-1 activation upon LPS and ATP stimulation. Interestingly, mature IL-1 $\beta$  production was not suppressed by tTreg cells under NLRP3 inflammasome stimulation, consistent with previous findings (Guarda et al., 2009; Yao et al., 2015).

Previous studies have shown that Tr1 cells suppress NLRP3 inflammasome activation by an IL-10-dependent mechanism (Yao et al., 2015). In our system, although IL-10 production was higher in Treg-of-B cells than in tTreg cells, IL-10 was not sufficient to suppress the NLRP3 inflammasome, as evidenced by analyses of IL-10 KO Treg-of-B cells (Figures 2B and 3A). IL-10 production was lesser in Treg-of-B cells than that in Tr1 cells (10 ng/ml versus 200 ng/ml, as reported previously (Yao et al., 2015)); this might explain why IL-10 was not a key mediator of NLRP3 inflammasome inhibition by Treg-B cells. In addition, we previously found that long-term cultured Treg-of-B cells expressed ICOS, LAG3, c-Maf, and CD49b but not CD226 (Chien and Chiang, 2017; Chien et al., 2017). However, the co-expression of CD226, LAG3, and CD49b is a signature of Tr1 cells (Gagliani et al., 2013). Moreover, IL-10 is not required for the development and inhibitory function of Treg-of-B cells; nevertheless, the inhibitory function of Tr1 cells is IL-10 dependent (Hsu et al., 2014;

Shao et al., 2016). Accordingly, Treg-of-B cells differ from Tr1 cells and dampen innate immunity by different underlying mechanisms.

Several diseases are associated with the abnormal activation of the NLRP3 inflammasome, including type II diabetes, COVID-19, Alzheimer disease, inflammatory bowel diseases, multiple sclerosis, and experimental autoimmune encephalomyelitis (Cleophas et al., 2016; Legrand-Poels et al., 2014; Perricone et al., 2020; Yang and Chiang, 2015; Zaki et al., 2011). To prevent the constitutive activation of the NLRP3 inflammasome, the immune system exerts a modulatory mechanism to maintain homeostasis. Memory CD4<sup>+</sup> T cells inhibit IL-1 $\beta$  release and caspase-1 activation triggered by NLRP3 activators, such as LPS and ATP, MSU, alum, or nigericin (Guarda et al., 2009). iNKT cells alleviate NLRP3-associated gouty arthritis by promoting M2 macrophage polarization (Wang et al., 2017). COVID-19-induced IL-37, an anti-inflammatory cytokine, suppresses IL-1 $\beta$  triggered by severe acute respiratory syndrome coronavirus-2 (SARS-CoV2) infection by NLRP3 inflammasome activation (Conti et al., 2020; Perricone et al., 2020). Our both *in vivo* and *in vitro* results demonstrate that Treg-of-B cells ameliorate gouty arthritis by inhibiting NLRP3-mediated inflammation. Therefore, Treg-of-B cells might maintain immune homeostasis to prevent inflammasome-induced diseases.

Recent recommendations for the management of gout by the European League Against Rheumatism include glucocorticosteroids, nonsteroidal anti-inflammatory drugs, colchicine, and IL-1 $\beta$  inhibitors (Ragab et al., 2017). Colchicine is highly effective for the acute gouty attack phase; it functions by the inhibition of NLRP3 assembly. However, a high concentration of colchicine is required, resulting in gastrointestinal side effects. The tolerance and efficacy of IL-1 $\beta$  inhibitors, such as riloncept and canakinumab, are better in patients with acute and chronic gout; however, the incidence of infection is increased. Accordingly, treatments targeting factors upstream of inflammasome activation may be effective for gouty arthritis. Therefore, the administration of Treg-of-B cells, which target inflammasome activation, may effectively cure gouty arthritis, without requiring long-term use, unlike conventional drugs used by patients throughout their lifetime. Moreover, adoptive transfer of Treg-of-B cells showed no organ rejection or hypersensitivity. Compared to Tr1 cells, the generation of Treg-of-B cells was more feasible and efficient. Therefore, our results suggest that Treg-of-B cells can provide a novel approach for the treatment of NLRP3-associated diseases, such as gouty arthritis.

The significance of our study was the identification of a FOXP3<sup>+</sup> regulatory T-cell population induced by B cells that could dampen innate immunity and maintain immune homeostasis. Treg-of-B cells might resolve inflammation by activation of the innate immune response or inflammasome activation. In the future, Treg-of-B cells might be applied for innate immune response mediated inflammation, such as inflammasome-induced inflammatory diseases.

In conclusion, we proposed a pathway for gouty arthritis alleviation via NLRP3 inflammasome inhibition by Treg-of-B cells (Figure 7). Treg-of-B cells suppressed the NLRP3 inflammasome by targeting priming signals and decreasing the degradation of I $\kappa$ B $\alpha$ . Upon MSU stimulation, Treg-of-B cells immediately migrated to the site of inflammation and abolished NLRP3 inflammasome activation, resulting in the amelioration of gouty arthritis. These results provide a basis for the development of a novel approach for the amelioration of inflammasome-related diseases and autoimmune disorders by treatment with Treg-of-B cells.

### Limitations of the study

Our *in vivo* and *in vitro* analyses demonstrated that Treg-of-B cells alleviate gouty arthritis by inhibiting NLRP3-mediated inflammation. However, we cannot exclude the possibility that the suppressive effects of Treg-of-B cells are mediated by other immune cells *in vivo*. Research aimed at characterizing additional mechanisms underlying the immunoregulatory effects of Treg-of-B cells in gouty arthritis will continue in the future.

### Resource availability

#### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Bor-Luen Chiang ([gicmbor@ntu.edu.tw](mailto:gicmbor@ntu.edu.tw))

### Material availability

This study did not generate new unique reagents.

### Data and code availability

This study did not generate large data sets. The relevant data in this study are available from the corresponding author upon reasonable request.

## METHODS

All methods can be found in the accompanying [Transparent methods supplemental file](#).

## SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.isci.2021.102103>.

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## AUTHOR CONTRIBUTIONS

J.H.H. performed the experiments and analyzed the data. B.L.C. contributed to study conception and takes responsibility for the integrity of the data. B.L.C. and J.H.H. supervised the project. All authors were involved in drafting the article and had full access to all of the data in the study. All authors approved the final version to be published.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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iScience, Volume 24

## **Supplemental Information**

**Regulatory T cells induced by B cells suppress  
NLRP3 inflammasome activation and alleviate  
monosodium urate-induced gouty inflammation**

**Jing-Hui Huang and Bor-Luen Chiang**



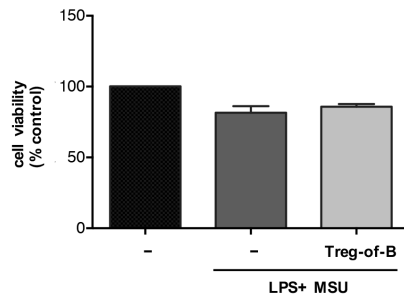
**Supplemental Information**

**Regulatory T cells Induced by B cells Suppress NLRP3 Inflammasome Activation and Alleviate Monosodium Urate-Induced Gouty Inflammation**

Jing-Hui Huang and Bor-Luen Chiang MD, PhD

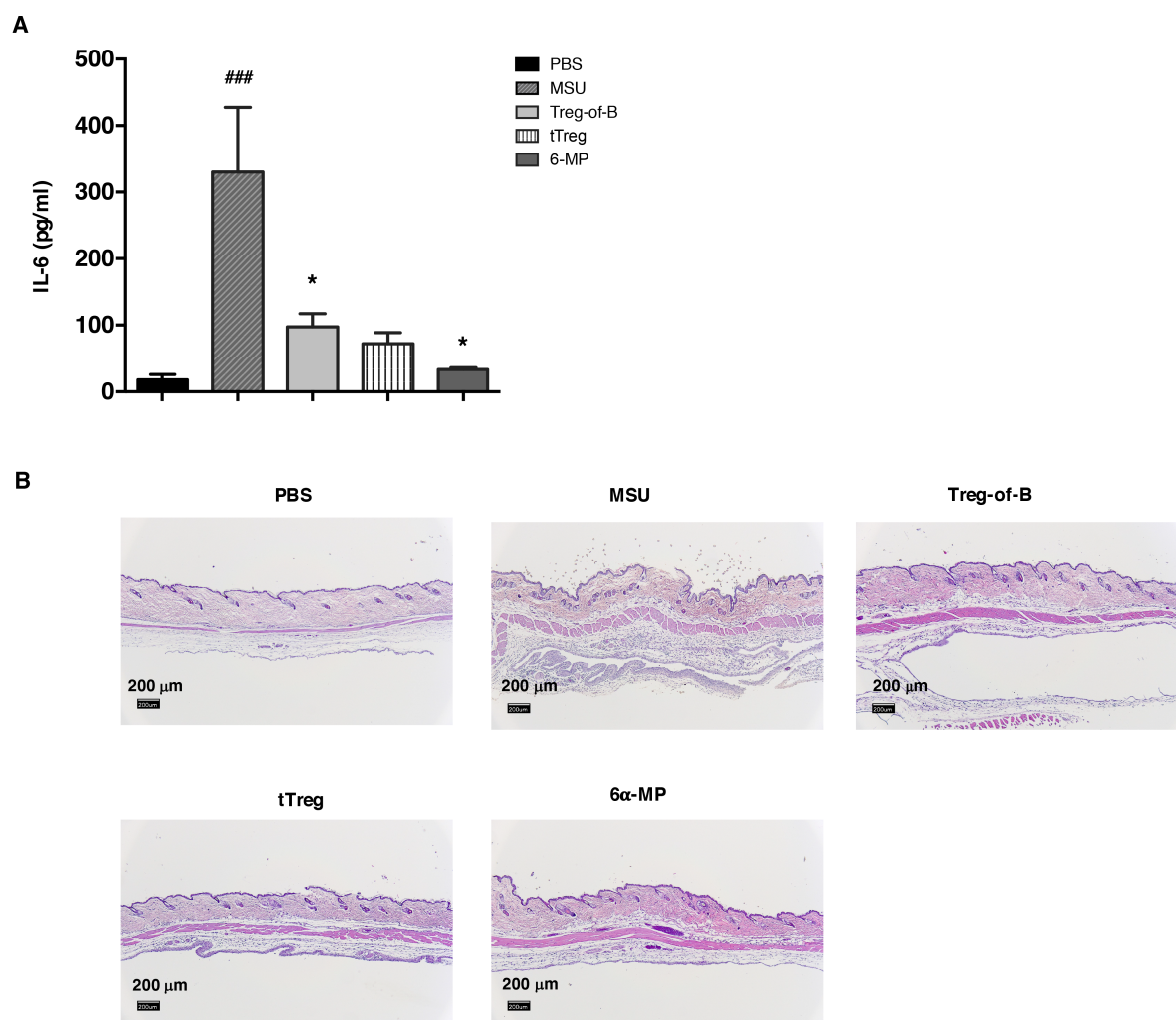
## Supplemental Information

### Supplemental Figures



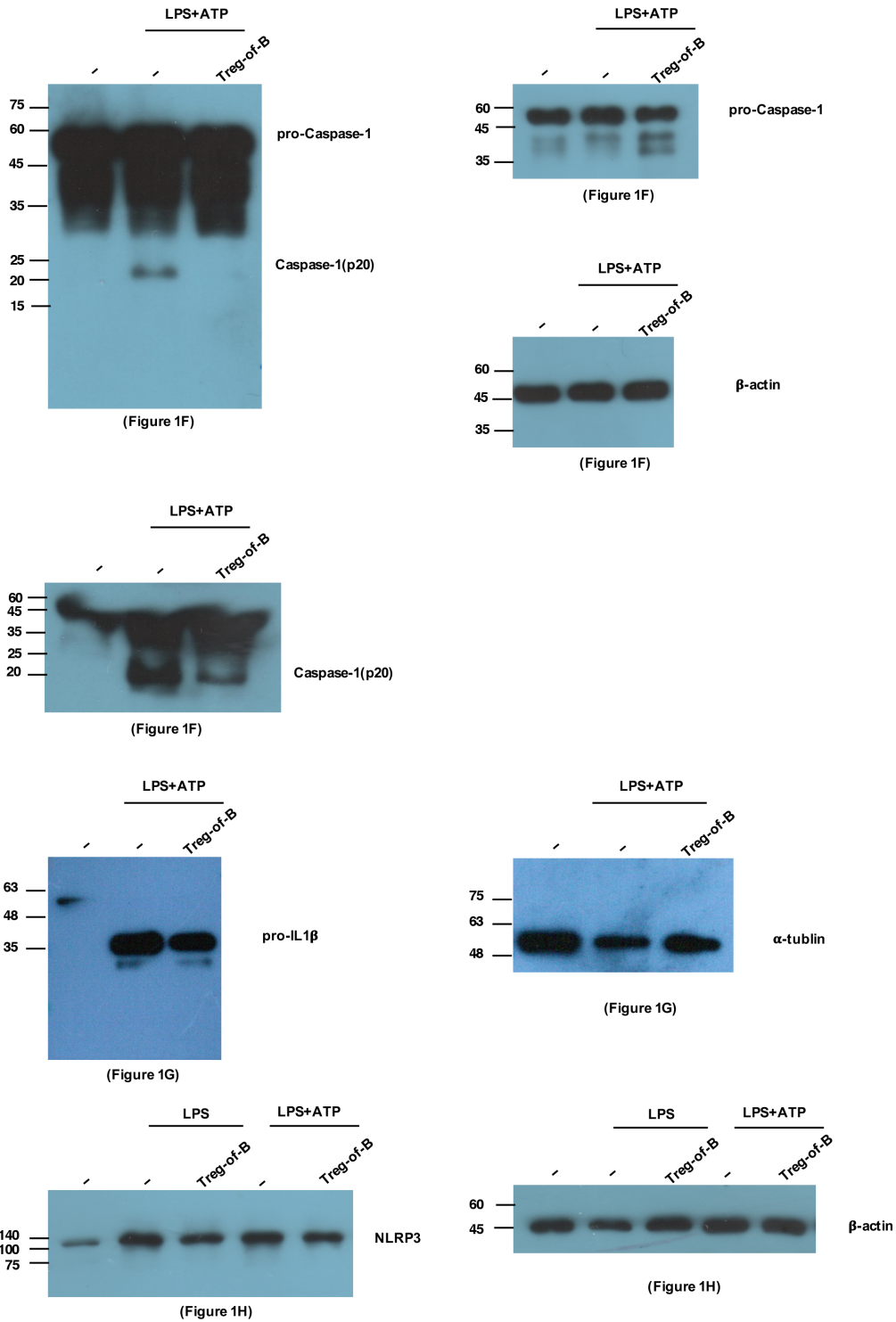
**Figure S1. The cellular viability of BMDMs was not affected by Treg-of-B cells. (Related to Figure 3)**

The BMDMs were cocultured with Treg-of-B cells overnight. After that, the cells were primed with LPS for 4 hours, followed by MSU for 2.5 hours. The survival rate of BMDMs cocultured with Treg-of-B cells was measured by MTT assay.



**Figure S2. Treg-of-B cells suppressed gouty inflammation *in vivo*. (Related to Figure 5)**

(A) The production of IL-6 in the air pouch was measured by ELISA in a mouse model of gouty arthritis. (B) The subcutaneous air pouch showed the infiltration of inflammatory cells by H and E staining (scale bars, 200 μm).



**Figure S3. Uncropped image of western blot used to generate Figure 1F, Figure 1G and Figure 1H. (Related to Figure 1F, Figure 1G, and Figure 1H)**

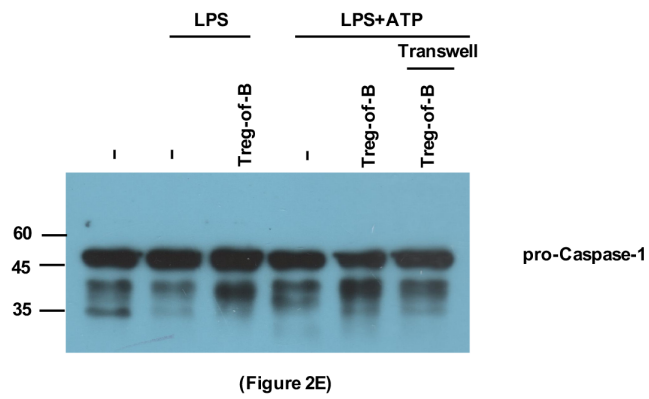
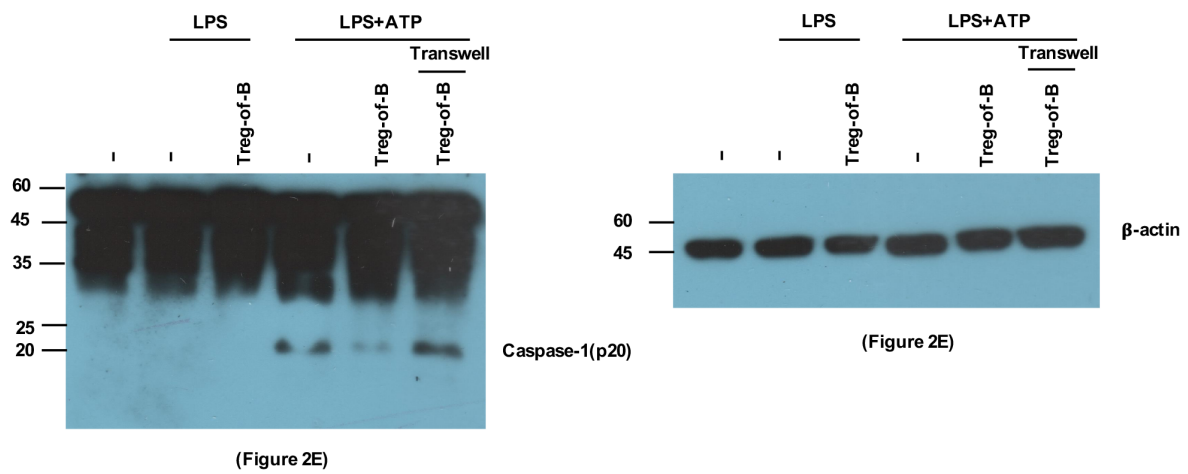


Figure S4. Uncropped image of western blot used to generate Figure 2E. (Related to Figure 2E)

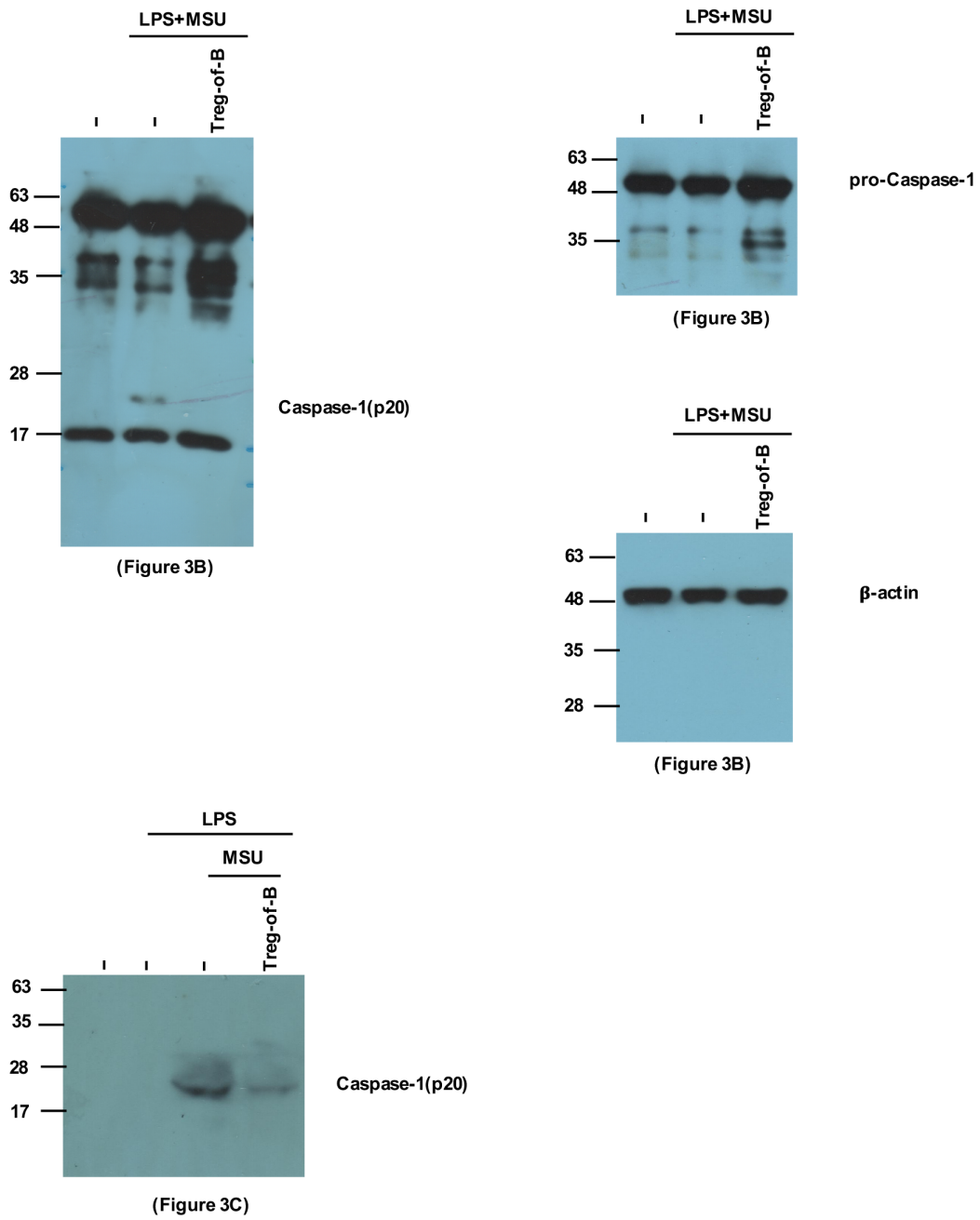
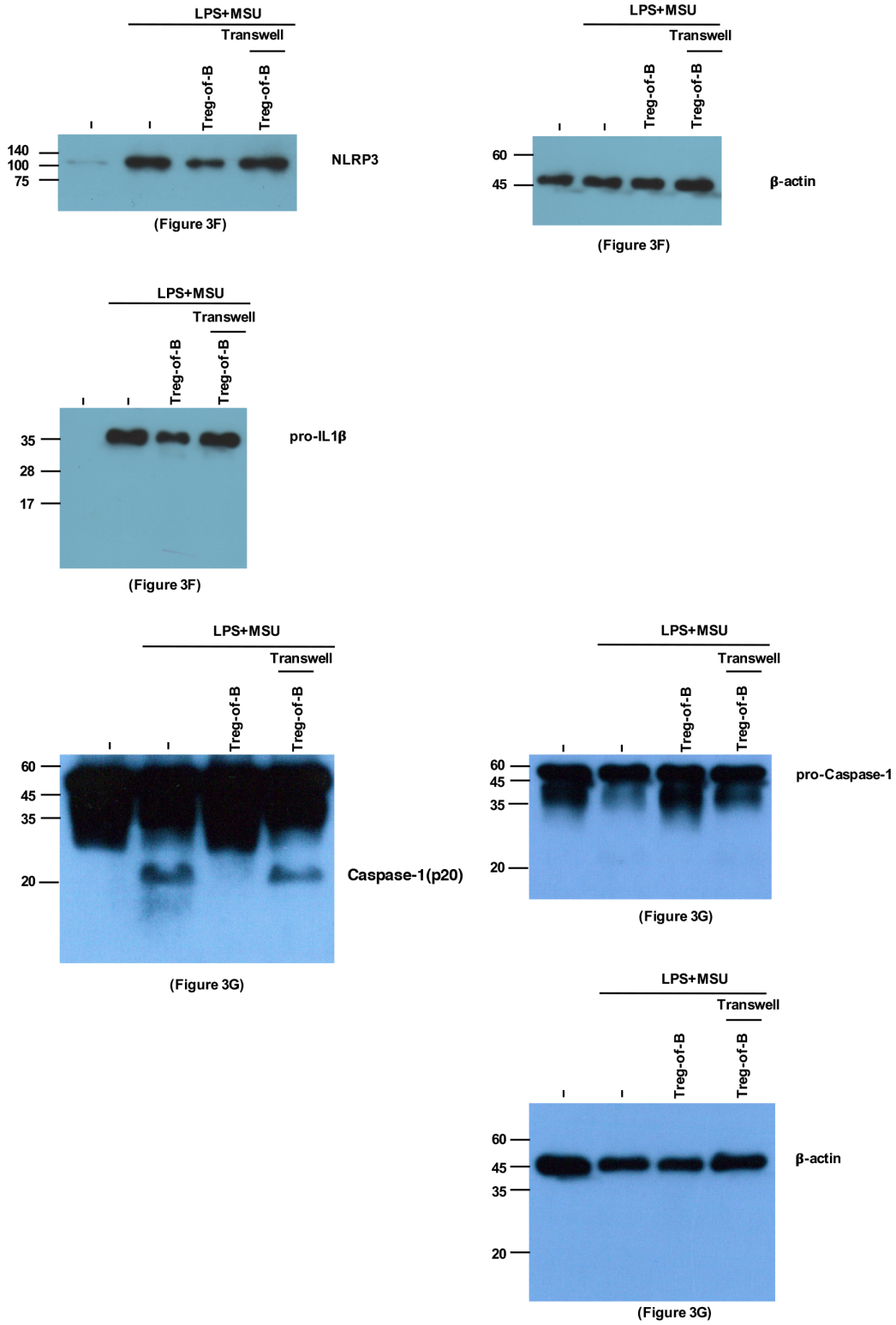


Figure S5. Uncropped image of western blot used to generate Figure 3B and Figure 3C. (Related to Figure 3B and Figure 3C)



**Figure S6. Uncropped image of western blot used to generate Figure 3F and Figure 3G. (Related to Figure 3F and Figure 3G)**

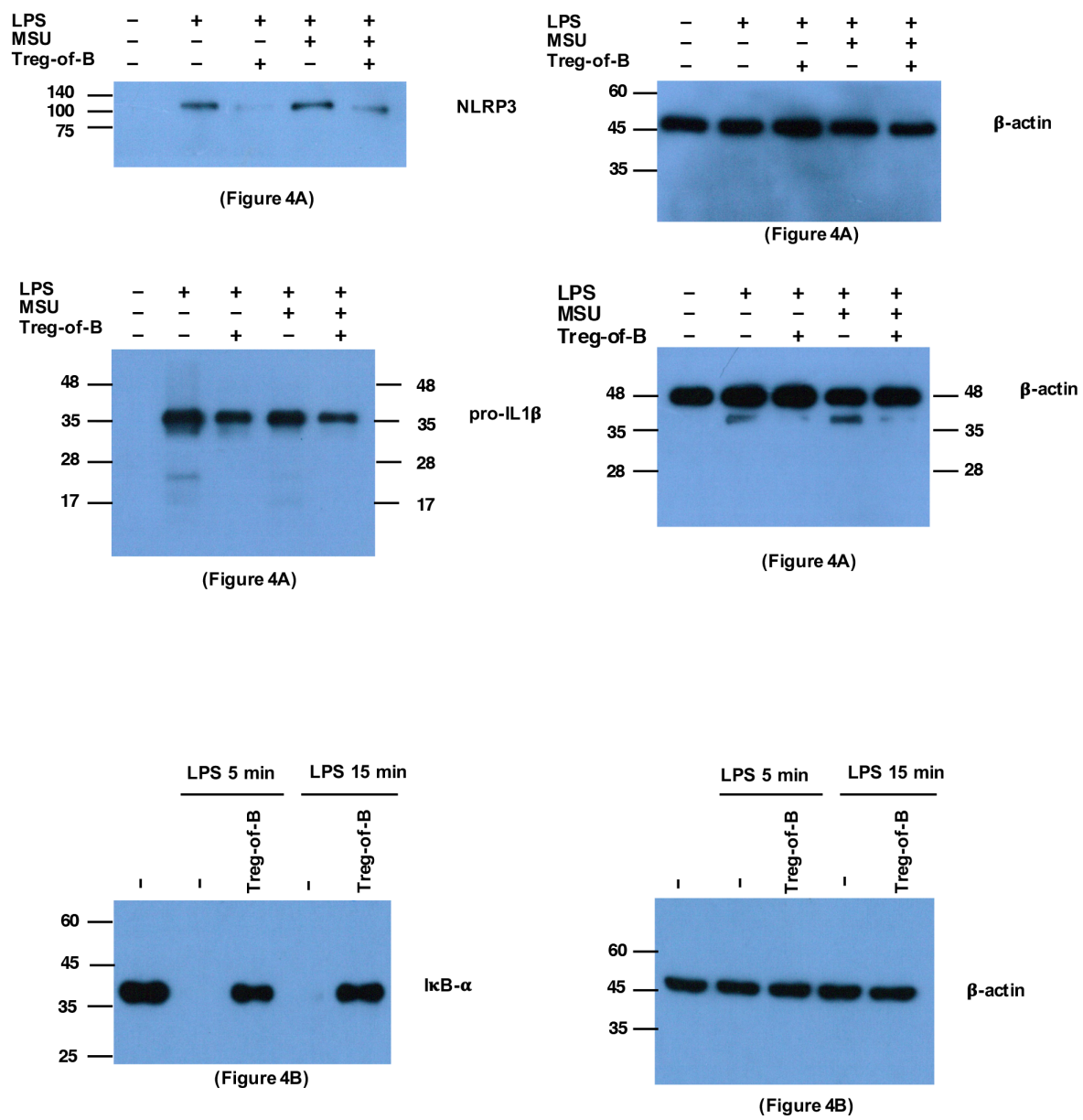


Figure S7. Uncropped image of western blot used to generate Figure 4A and 4B. (Related to Figure 4A and Figure 4B)



## Supplemental Tables

**Table S1. The sequences of primers used for Q-PCR. (Related to Figure 1 and Figure 3)**

Gene name	Forward 5' → 3'	Reverse 5' → 3'
Interleukin-1 $\beta$	agttgacggaccccaaaag	agctggatgctctcatcagg
NOD-, LRR- and pyrin domain-containing 3	cccctcagcaaaccaccaagt	cttgggcagattgacctcagc
Tumour necrosis factor- $\alpha$	tcagccgatttgctatctcat	agtacttgggcagattgacct
Gapdh	gatgggtgtgaaccacg	agatccacgacggacac

**Table S2. Histological scoring system of the murine air pouch model. (Related to Figure 5)**

Histological changes		Score				
		0	1	2	3	4
Inflammation	Polymorphonuclear cells (neutrophils and eosinophils)	None	Minimal, 1-5/per HPF	Slight, 6-15/per HPF	Moderate, 16-25/per HPF	Severe, >25/per HPF
	Mononuclear cells (lymphocytes and macrophages)	None	Minimal, 1-5/per HPF	Slight, 6-15/per HPF	Moderate, 16-25/per HPF	Severe, >25/per HPF
Membrane thickness of murine air pouch		None	<50 $\mu\text{m}$	50-75 $\mu\text{m}$	76-100 $\mu\text{m}$	>100 $\mu\text{m}$

**Table S3. Fluorescence-conjugated antibodies were used. (Related to Figure 1, Figure 2, Figure 5 and Figure 6)**

<b>Ab name</b>	<b>Clone</b>	<b>Supplier</b>
PE-PD1	clone J43	BD
PE-OX40	clone OX-86	Biolegend
PE-GITR	clone DTA-1	Biolegend
PE-ICOS	clone 15F9	Biolegend
PE-LAG3	clone C9B7W	BD
PE-CD25	clone 7D4	Biolegend
FITC-CD25	clone PC61	Biolegend
APC-CD25	clone PC61	BD
PE-CD44	clone IM7	BD
PerCP-CD4	clone RM4-5	BD
PE-Foxp3	clone MF-14	Biolegend
Alexa 488-Ly6G	clone 1A8	Biolegend
Percp-CD11b	clone M1/70	BD
PE-F4/80	clone T45-2342	BD
APC-CD49b	clone HM $\alpha$ 2	BD

## Transparent Methods

### Animals

Six- to eight-week-old male BALB/c mice were obtained from the National Laboratory Animal Center. BALB/c *Il-10*<sup>-/-</sup> mice were purchased from the Jackson Laboratory. These mice were maintained in the Laboratory Animal Center of the College of Medicine, National Taiwan University. The study was performed in accordance with the recommendations in the “Guide for the Care and Use of Laboratory Animals” of the National Institutes of Health. The mice were housed 3-5 mice per cage and maintained on 12-h light/dark cycle under a constant temperature (22 ± 2 °C). All animal experiments were approved by the Institutional Animal Care and Use Committee of National Taiwan University, College of Medicine (#20180065).

### The induction of Treg-of-B cells

Splenic B220<sup>+</sup> B cells were isolated from Balb/c mice, and splenic CD4<sup>+</sup>CD25<sup>-</sup> cells and splenic CD4<sup>+</sup>CD25<sup>+</sup> cells (tTregs) were isolated from BALB/c or BALB/c *Il-10*<sup>-/-</sup> mice. The purification of B220<sup>+</sup> cells was performed by immunomagnetic positivity using a BD IMag™ Cell Separation Magnet. CD4<sup>+</sup>CD25<sup>-</sup> cells were isolated by negative immunomagnetic selection via an EasySep™ Mouse CD4<sup>+</sup> T Cell Isolation Kit. CD4<sup>+</sup>CD25<sup>+</sup> (tTregs) cells were obtained by staining with a PE-anti-CD25 antibody followed by incubation with anti-PE beads. To induction of Treg-of-B cells and IL-10 KO Treg-of-B cells, the isolated B220<sup>+</sup> B cells were cultured together with isolated CD4<sup>+</sup>CD25<sup>-</sup> T cells or IL-10 KO CD4<sup>+</sup>CD25<sup>-</sup> T cells at 1:1 ratio upon anti-CD3/CD28 (0.5 µg/ml) antibodies stimulation. After 3 days, Treg-of-B cells were harvested after depletion of B220<sup>+</sup> B cells.

### The generation of Tr1 cells

The purified CD4<sup>+</sup>CD25<sup>-</sup> T cells were stimulated with plate-coated CD3 (1µg/ml), soluble anti-CD28 (1µg/ml), recombinant IL-10 (100 U/ml, BD Biosciences), and recombinant IL-2 (50 U/ml). After 3 days, one-third of the medium was replaced with fresh medium containing IL-2 (150 U/ml), IL-10 (300 U/ml), and anti-CD28 (3 µg/ml). Three days later, the dead CD4<sup>+</sup>CD25<sup>-</sup> T cells were removed by Ficoll and restimulated with anti-CD3 (1 µg/ml) and anti-CD28 (1µg/ml) supplemented with IL-10 (100 U/ml, BD Biosciences) again. For 3 cultures, Tr1 cells were collected, and CD49b and LAG3 were examined by flow cytometry.

### *In vitro* coculture experiments

For the coculture system, the *in vitro*-induced Treg-of-B cells, *in vitro*-induced Tr1 cells or bead-isolated CD4<sup>+</sup>CD25<sup>-</sup> T and CD4<sup>+</sup>CD25<sup>+</sup> T cells were cocultured with differentiated bone marrow-derived macrophages (BMDMs) or peritoneal macrophages (pMs) overnight at 3:1 ratio unless otherwise

indicated in the presence of anti-CD3 and anti-CD28 antibodies (Abs; 1 µg/ml). To examine suppression in the Transwell assay, BMDMs were cultured in the lower chamber, and anti-CD3- and anti-CD28-stimulated Treg-of-B cells were cultured in the Transwell insert (0.4 µM pore size) overnight. Thereafter, the cultured cells were stimulated with lipopolysaccharide (LPS; 1 µg/ml, Sigma) for 3.5 hours and then treated with ATP (5 mM, InvivoGen) for another 20 min. To activate the NLRP3 inflammasome with crystallized urea, BMDMs were primed with LPS (1 µg/ml) for 4 hours, followed by MSU (150 µg/ml, InvivoGen) for 2.5 hours. The supernatant was collected, and IL-1β production was measured by ELISA. To assess the suppression of NF-κB, BMDMs were stimulated with LPS alone for 5 min, 15 min and 24 hours. In addition, BMDMs were lysed in TRIzol- and Triton X-100-based lysis buffer; the RNA expression was analysed via qPCR, and the protein expression was analysed via western blotting. In addition, the culture supernatant from the BMDM-Treg-of-B coculture system was concentrated with Amicon Ultra centrifugation filters, and the production of protein was measured by western blot. To identify the molecule required for the suppressive function of Treg-of-B cells, anti-IL10 (10 µg/ml), anti-PD1 (10 µg/ml), anti-LAG3 (10 µg/ml), anti-ICOS (10 µg/ml), anti-GITR (10 µg/ml), and anti-MHCII (10 µg/ml) Abs were added to the Treg-of-B cell-BMDM coculture system.

### **The proliferative response of T cells**

For the suppression assay, purified CD4<sup>+</sup>CD25<sup>-</sup> (as responder T cells) cells were cultured with CD4<sup>+</sup>CD25<sup>-</sup> or Treg-of-B cells in the presence of mitomycin C-treated splenocytes, which were stimulated with 2 µg/ml concanavalin A (ConA) for 3 days. [<sup>3</sup>H]-Thymidine (1 µCi in each well, PerkinElmer, Boston, MA) was added to the culture system, and then the cells were harvested after 16-18 hours. [<sup>3</sup>H]-Thymidine incorporation was analysed by a counter for the determination of T cell proliferation.

### **ELISA**

To determine cytokine production, Treg-of-B cells and tTreg cells were restimulated with anti-CD3/anti-CD28 (1 µg/ml) Abs for 3 days. The levels of IL-10, IL-4, IFN-γ and IL-2 cytokines in the supernatant were measured.

To determine the cellular mechanism of inflammasome suppression, the supernatant from the Treg-of-B cell-BMDM coculture system was collected, and IL-1β, CXCL1, CXCL2, IL-6, and TNF-α production were measured. To confirm the suppression of NLRP3 by Treg-of-B cells *in vivo*, the air-pouch fluid was harvested, and IL-1β, CCL2, CXCL1 and IL-6 production were measured. The quantification of cytokine and chemokine levels was performed according to the manufacturer's protocol (R&D).

### **RT-PCR**

Total RNA from BMDM and air pouch cells was extracted by TRIzol reagent. The isolated RNA was

transcribed into cDNA by MMLV reverse transcriptase (Applied Biosystems). The quantification of *IL-1 $\beta$* , *NLRP3*, *TNF- $\alpha$*  and reference genes was performed by using SYBR Green and ABI 7500 Fast Real-time PCR system. The expression of each gene was normalized to GAPDH. The primer sequences are listed in Table S1.

### **Bone marrow-derived macrophage and peritoneal macrophage isolation**

Bone marrow cells were obtained from femoral bone in 4-6-weeks-old mice. These cells were cultured in 10% FBS RPMI containing with 10-15 ng/ml M-CSF (BioLegend, CA, USA) for differentiation. The adherent macrophages were harvested on day 7. For peritoneal macrophage isolation, mice were injected with 4% thioglycollate via intraperitoneal administration. After 4 days, peritoneal macrophages (pMs) were collected by washing the peritoneal cavity with HBSS buffer.

### **Western blotting for IL-1 $\beta$ , caspase-1, NLRP3, I $\kappa$ B $\alpha$ , and p-I $\kappa$ B $\alpha$**

The western blotting assay was carried out as described previously (Lordén et al., 2017) . The BMDMs were lysed in RIPA buffer (Biotech) containing with protease and phosphatase inhibitors (Cell Signaling). The protein was subjected to 10-15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein was transferred to PVDF membranes and probed with Abs against pro-IL-1 $\beta$  (R&D, AB\_416684), procaspase-1 (Adipogen, AB\_2490248), active caspase-1 (p20; Adipogen, AB\_2490248), NLRP3 (Adipogen, AB\_2490202), I $\kappa$ B $\alpha$  (Cell Signaling Technology, AB\_331623), p-I $\kappa$ B $\alpha$  (Cell Signaling Technology, AB\_2566820)  $\beta$ -actin (Merck Millipore, AB\_2223041), and  $\alpha$ -tubulin (Santa CRUZ) .

### **Haematoxylin and eosin staining analysis and scoring system**

The membrane tissue was obtained 6 hours after MSU crystal injection. The sections of skin were stained with haematoxylin and eosin (H&E) to examine the infiltration of leukocytes into subcutaneous tissues. The H&E-stained sections were scored blindly by a pathologist at the NTUCM Graduate Institute of Clinical Medicine. The severity of inflammation was graded on the basis of the sum of polymorphonuclear cell and mononuclear cell infiltration from individual scores. The membrane thickness of the air pouch was graded. The scoring system is shown in Table S2.

### **Air pouch model and adoptive transfer of Treg-of-B cells**

On day 0, sterile air was subcutaneously injected into the backs of the mice after the mice were anaesthetized with tribromoethanol (Sigma). Three days later, an additional 3 ml of sterile air was injected into the pouch. On day 7, MSU crystals (2 mg, InvivoGen) in 1 ml of endotoxin-free PBS were injected subcutaneously into the air pouch, as previously described with modifications (Torres et al., 2009). The mice were randomly assigned to the PBS group, MSU-treated group, Treg-of-B cell-treated

group, tTreg-treated group, and 6 $\alpha$ -MP-treated group. For the adoptive transfer experiments, 5 $\times$ 10<sup>6</sup> Treg-of-B cells were injected into the mice via intravenous injection on days 5, 6, and 7. Simultaneously, two control groups were used, mice treated with 6 $\alpha$ -methylprednisolone (6 $\alpha$ -MP, 1.6 mg/kg) and mice receiving adoptive transfer of tTreg (5 $\times$ 10<sup>6</sup> cells) via intravenous injection. Subsequently, MSU crystals were injected into the mice on day 7. Six hours after the injection of MSU crystals, the mice were sacrificed via CO<sub>2</sub> inhalation, and lavage fluid was harvested for the analysis of cell populations by flow cytometry, gene expression of these cells by Q-PCR, and measurement of the levels of cytokines and chemokines by ELISA.

### **The distribution of Treg-of-B cells**

To track Treg-of-B cells *in vivo*, Treg-of-B cells were stained with CFSE (2.5 mM) and adoptively transferred into mice on days 5, 6 and 7 in air pouch model. After the last administration of Treg-of-B cells, the MSU crystals (2 mg in 1 ml PBS) were injected into air pouch. Six hours later, the mice were sacrificed, and lymphocytes were harvested from the air pouch, and draining lymph nodes (DLNs), such as axillary lymph nodes (ALN), inguinal lymph nodes (ILN), cervical lymph nodes (CLN), and spleen. The CFSE-labelled Treg-of-B cells were analysed by flow cytometry.

### **Flow cytometric analysis**

To analyse the surface marker and intracellular marker, the fluorescence-conjugated Antibodies were used to stain isolated cells. The Abs are listed in Table S3. The data acquisition was performed by BD FACSCalibur (BD Biosciences) and BD FACSLytic (BD Biosciences), and data analysis was performed with FlowJo software (BD Biosciences).

### **MTT assay**

To determine the cellular viability of BMDMs after coculturing with Treg-of-B cells, the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) was added to BMDMs for 6 hours. Next, dimethyl sulfoxide (DMSO) was added to wells to dissolve the purple formazan, and the coloured solution was quantified by measuring the absorbance at 570 nm.

### **Statistical analysis**

The statistical analyses were performed with Graph Prism 6.0. The differences between each group were calculated with one-way ANOVA, followed by Bonferroni's multiple comparison test. A value of p less than 0.05 was considered significant.

## Supplemental References

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