HOKKAIDO UNIVERSITY

Title	The mechanism of Tyk2 deficiency-induced immunosuppression in mice involves robust IL-10 production in macrophages
Author(s)	Hirashima, Koki; Muromoto, Ryuta; Minoguchi, Hiroya et al.
Citation	Cytokine, 130, 155077 https://doi.org/10.1016/j.cyto.2020.155077
Issue Date	2020-06
Doc URL	https://hdl.handle.net/2115/82117
Rights	©2020. This manuscript version is made available under the CC-BY-NC-ND 4.0 license https://creativecommons.org/licenses/by-nc-nd/4.0/
Rights(URL)	https://creativecommons.org/licenses/by-nc-nd/4.0/
Type	journal article
File Information	WoS_94259_Muromoto.pdf

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

Title: The mechanism of Tyk2 deficiency-induced immunosuppression in mice involves robust IL-10 production in macrophages

Authors: Koki Hirashima[a], Ryuta Muromoto[a,*], Hiroya Minoguchi[a], Tomohiro Matsumoto[a], Yuichi Kitai[a], Jun-ichi Kashiwakura[a], Kazuya Shimoda[b], Kenji Oritani[c] and Tadashi Matsuda[a,*]

Affiliation:
[a] Department of Immunology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Sapporo, 060-0812, Japan
[b] Department of Internal Medicine II, Faculty of Medicine, University of Miyazaki, Kiyotake, Miyazaki 889-1692, Japan
[c] Department of Hematology, International University of Health and Welfare, 4-3 Kouzunomori, Narita, Chiba 286-8686, Japan

[*] Corresponding authors:
Dr. Ryuta Muromoto, Ph.D.
Department of Immunology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-Ku Kita 12 Nishi 6, Sapporo 060-0812, Japan
TEL: 81-11-706-3245; FAX: 81-11-706-4990; E-mail: muro@pharm.hokudai.ac.jp
Prof. Tadashi Matsuda, Ph.D.
Department of Immunology, Graduate School of Pharmaceutical Sciences, Hokkaido University, Kita-Ku Kita 12 Nishi 6, Sapporo 060-0812, Japan
TEL: 81-11-706-3243; FAX: 81-11-706-4990; E-mail: tmatsuda@pharm.hokudai.ac.jp

Abbreviations: BMDM, bone marrow-derived macrophage; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; IFN, interferon; IL, interleukin; KO, knock out; LPS, lipopolysaccharide; mAb, monoclonal antibody; *P. acnes*, *Propionibacterium acnes*; PBS, phosphate-buffered saline; PGE_2, prostaglandin E_2; PKA, protein kinase A; RT-PCR, reverse transcription–polymerase chain reaction; SEM, standard error of the means; Tyk2, tyrosine kinase-2; WT, wild-type.

Abstract

Macrophages are highly plastic in their pro-inflammatory/anti-inflammatory roles. Type I and II interferons (IFNs) are known to modulate macrophage activation. Tyrosine kinase 2 (Tyk2) has an intimate relationship with type I and II IFN signaling. Animal studies have shown that Tyk2 knock-out (KO) in mice is associated with reduced inflammatory responses in various mouse models of diseases. To investigate the role of Tyk2 in inflammation in more detail, we intraperitoneally injected heat-killed *Propionibacterium acnes* (*P. acnes*) to Tyk2 KO mice. *P. acnes*–induced acute peritoneal inflammation, assessed by neutrophil infiltration, was reduced in Tyk2 KO mice. The reduction was accompanied with diminished productions of inflammatory cytokines and an enhanced production of anti-inflammatory IL-10. Unexpectedly, pre-treatment of wild-type mice with the neutralizing antibodies for IFNs did not affect *P. acnes*-induced neutrophil infiltration. A neutralizing antibody for the IL-10 receptor in Tyk2 KO mice restored *P. acnes*-induced peritoneal inflammation. Enhanced production of IL-10 from Tyk2 KO peritoneal cells was suppressed by either the cyclooxygenase inhibitor diclofenac or protein kinase A inhibitor H-89. The level of prostaglandin E_2 (PGE_2) in the steady-state peritoneal cavity in Tyk2 KO mice was higher than that in wild-type mice. Tyk2 KO macrophages showed an enhanced CREB phosphorylation induced by *P. acnes* plus PGE_2. Taken together, these results showed that Tyk2 deficiency potentiates the PGE_2-protein kinase A-IL-10 pathway in macrophages, and thereby contributes to potentiation of the immunosuppressive phenotype.

Keywords: Tyk2; macrophage plasticity; inflammation; interferon; IL-10

Highlights

- Heat-killed *P. acnes*-induced peritonitis was reduced in Tyk2 KO mice independently of IFNs.
- Tyk2 KO macrophages produced a greater amount of IL-10 than WT macrophages.
- The enhanced IL-10 production was correlated to an enhanced activation of PGE_2-PKA signaling.
- These data provide an additional explanation for the immunosuppression seen in Tyk2 KO mice.

1. Introduction

Inflammation is part of a defense mechanism that protects higher organisms from harmful stimuli such as infection and injury. In the process, the immune system plays key roles in various steps to recognize and remove harmful stimuli. Macrophages are an immune cell population that plays fundamental roles in the regulation of inflammation, and are highly plastic in their pro-inflammatory/anti-inflammatory roles [1-3]. During the course of inflammation, the microenvironment surrounding macrophages varies over time, affects the balance of the pro-/anti-inflammatory roles of macrophages, and tunes macrophages to exert appropriate functioning [1-3].

The type II interferon (IFN), IFN-γ, is a well-known, potent macrophage-activating factor that promotes antigen presentation, microbial killing, and cytokine production [4]. The type I IFNs (e.g., IFN-α and IFN-β) are constitutively produced by various kinds of cells, including macrophages. The constitutively produced type I IFNs, although produced only in very small amounts, regulate the cell function in an autocrine/paracrine manner [5,6]. The genetic deletion of the type I IFN receptor subunit (Ifnar1) gene has been reported to cut off the constitutive type I IFN signal and attenuate the pro-inflammatory function of macrophages by downregulating the expression of IFN-inducible inflammatory genes such as *Cxcl10* [7]. This spontaneous autocrine type I IFN signaling is crucial not only for driving its own pathway but also for enhancing IFN-γ responsiveness [5,6]. Thus, both type I and II IFNs have pro-inflammatory roles.

Autocrine type I IFN signaling in macrophages has been suggested to play not only a pro-inflammatory role, but paradoxically, an anti-inflammatory role as well. The notion that such signaling plays an alternative role is supported by the previous observation that the expression of IL-10, which is a strong anti-inflammatory cytokine that plays a role in limiting tissue injury during inflammation by regulating the duration and intensity of immune and inflammatory reactions [8,9], is upregulated by autocrine type I IFN signaling [7,10], but not by IFN-γ signaling [11]. Collectively, these facts indicate that the autocrine type I IFN signaling can be considered to potentiate both pro- and anti-inflammatory responses, while IFN-γ signaling exclusively stimulates the pro-inflammatory function of macrophages.

Tyk2 is a member of the JAK family of tyrosine kinases that has an intimate relationship with type I and II IFNs. Tyk2 associates with cytokine receptors and is activated in response to various cytokines such as type I IFNs, interleukin (IL)-12, and IL-23 [12-15]. Meta-analysis of genetic associations in the *TYK2* region across human autoimmune diseases has revealed that a genetic variant in the *TYK2* gene (rs34536443)

has a protective effect against 10 different autoimmune conditions, indicating that the modulation of Tyk2 function could be a strategy for the treatment of certain common autoimmune disorders [16]. We and others have reported that Tyk2 knock-out (KO) in mice is associated with reduced inflammatory responses in various mouse models of human diseases [17-24].

One important role for Tyk2-mediated signaling is the production of IFN-γ, which induces activation of macrophages. In T and natural killer cells, Tyk2 appears to be central for the appropriate functioning of the IL-12/IFN-γ axis and T and NK cell-mediated host defense against viral and/or mycobacterial infections in humans and mice [12,13,25-27]. In addition to the role of Tyk2 in IFN-γ production, Tyk2 has been demonstrated to control the production of type I IFNs in macrophages, and the innate immune response against bacterial components. In Tyk2 KO macrophages, basal and lipopolysaccharide (LPS)-induced expressions of type I IFNs were impaired [22]. Tyk2 KO mice as well as Ifnb (encoding IFN-β) KO mice were resistant to the lethal septic shock induced by high doses of LPS [21,22], indicating that Tyk2-mediated type I IFN production/signaling is an essential effector module in LPS-induced vigorous inflammation and lethal shock. In addition, in a microarray analysis comparing global gene expression changes between wild-type peritoneal macrophages and Tyk2 KO peritoneal macrophages under steady-state and LPS-stimulated conditions, the expression of type I IFN-stimulated genes, particularly under the steady-state condition, was decreased in Tyk2 KO macrophages [28]. These observations indicate that Tyk2 in innate immune cells plays a fundamental role in the inflammatory responses through the production of IFNs.

Based on these previous findings, it seemed reasonable to speculate that Tyk2 has some influence on macrophage activation through its contribution to autocrine type I IFN production and/or exogenous IFN-γ production from other neighboring immune cells such as T and NK cells. In the present study, we sought to confirm this point and further delineate the role of Tyk2 in inflammatory responses in vivo. In the process, we unexpectedly found that Tyk2 KO macrophages produced a greater amount of IL-10 than WT macrophages. The mechanism underlying the augmented IL-10 production was clearly related to the enhancement of anti-inflammatory protein kinase A (PKA) signaling in Tyk2 KO macrophages, but seemed not to be linked directly with a change in IFN signaling by Tyk2 deficiency. Therefore, this mechanism could represent an extended Tyk2 function in macrophages that works independently and simultaneously with IFN-mediated effects and could provide an additional explanation for the Tyk2 deficiency-induced anti-inflammatory and immunosuppressive phenotypes.

2. Materials and Methods

2.1 Mice

Gene-targeted Tyk2 KO mice were back-crossed for at least eight generations onto BALB/c mice [12]. The mice were kept under specific pathogen-free conditions and provided with food and water *ad libitum*. All experiments were performed according to the guidelines of the Institutional Animal Care and Use Committee of Hokkaido University.

2.2 In vivo challenge with heat-killed *P. acnes*

Heat-killed *Propionibacterium acnes* (*P. acnes*) [29] were kindly provided by Dr. Hiroko Tsutsui (Department of Microbiology, Hyogo College of Medicine). Mice were injected intraperitoneally with heat-killed *P. acnes* (500 μg per mouse) or phosphate-buffered saline (PBS). At 12 h post-injection, the peritoneal lavages were collected, and the numbers of neutrophils and levels of inflammatory cytokines (TNF-α and IL-6) were determined. The IL-6 and TNF-α protein contents in the peritoneal lavage were measured by enzyme-linked immunosorbent assay (ELISA) kits (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. The level of IL-10 in the peritoneal lavages at 6 h after heat-killed *P. acnes* injection was measured using a mouse IL-10 ELISA Ready-SET-Go kit (eBioscience, San Diego, CA) according to the manufacturer's instructions. To analyze the production of IFN-γ, mice were stimulated intraperitoneally with heat-killed *P. acnes* for 0, 1, 3, 5 and 7 days, and the amounts of IFN-γ in serum were determined by a BD CBA Mouse Th1/Th2/Th17 Cytokine Kit (BD Biosciences, San Jose, CA). Alternatively, total RNA was extracted from the livers of heat-killed *P. acnes*-injected mice and subjected to real-time reverse transcription–PCR (RT-PCR) analysis for the expression of the genes encoding IFN-γ and Cxcl10.

Neutralizing monoclonal antibodies (mAbs)—i.e., anti-IFNAR1 (clone MAR1-5A3; 200 μg per mouse), anti-IFN-γ (clone R4-6A2; 200 μg per mouse) and anti-IL-10R (clone 1B1.3A; 300 μg per mouse) mAbs—were intraperitoneally injected 3 h before heat-killed *P. acnes* injection. These mAbs were purchased from Bio X Cell (West Lebanon, NH). Control mice received the same doses of mouse IgG1 or rat IgG1, respectively. On day 7 after heat-killed *P. acnes* injection, the spleens were removed and weighed. The livers were also removed, and fixed in 4% buffered formaldehyde.

Organ slices were prepared and stained using hematoxylin and eosin. Microscopic images were taken using a BZ-8000 microscope (Keyence, Osaka, Japan). The levels of prostaglandin E_2 in the peritoneal lavages of mice were determined using a prostaglandin E_2 ELISA kit (Cayman Chemical, Ann Arbor, MI).

2.3 Flow cytometry

Peritoneal exudate cells (PECs) were resuspended in PBS containing 2% fetal bovine serum (FBS). Prior to staining, samples were Fc-blocked with anti-CD16/CD32 (clone 2.4G2; BD Pharmingen) for 20 min on ice. Cells were washed with PBS plus 2% FBS and incubated with 0.5 μg/mL of the following fluorochrome-tagged primary antibodies for 30 min on ice: anti-CD11b (clone M1/70; Biolegend, San Diego, CA), anti-F4/80 (clone BM8; Biolegend), anti-Gr-1 (clone RB6-8C5; BD Biosciences), and anti-B220 (clone RA3-6B2; Miltenyi Biotec, Berhischh Gladbach, Germany). Cells were washed with PBS plus 2% FBS, resuspended in PBS, and kept on ice until they were analyzed by flow cytometry (FACSCalibur; Becton Dickinson, Franklin Lakes, NJ). Data were analyzed using Cell Quest software (Becton Dickinson).

For intracellular IL-10 staining, brefeldin A (100 μg per mouse) was injected intraperitoneally 2 h after injection of heat-killed *P. acnes.* PECs were collected 5 h after *P. acnes* injection. Cells were stained with surface markers as described above. They were then washed, treated with BD Cytofix/Cytoperm solution (BD Biosciences), and kept on ice for 30 min. Finally, the cells were washed with PBS plus 0.04% saponin and incubated with PE-conjugated anti-mouse IL-10 mAb (clone JES5-16E3; Biolegend) overnight at 4°C.

2.4 Cell culture, preparation of macrophages, and IL-10 detection in the culture supernatant

To prepare bone marrow-derived macrophages (BMDMs), bone marrow cells from WT and Tyk2 KO mice were cultured in RPMI supplemented with 10% FBS and recombinant murine macrophage colony stimulating factor (100 ng/ml; PeproTech, Rocky Hill, NJ) for 6 days. BMDMs and peritoneal resident cells collected from the peritoneal lavage were plated in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 0.1 mg/ml streptomycin. IL-10 levels in the culture supernatants of BMDMs or peritoneal resident cells were determined using an IL-10 ELISA kit (eBioscience). The following agents were tested for their effects on IL-10 production: diclofenac sodium (10 μM; Wako Pure Chemical Industries, Osaka, Japan), and H-89

(10 μM; Cell Signaling Technologies, Beverly, MA).

2.5 Real-time reverse transcription–polymerase chain reaction analysis

Total RNA was prepared with a GenElute Mammalian Total RNA Miniprep Kit (Sigma, St. Louis, MO), and complementary DNA (cDNA) was generated with ReverTra Ace reverse transcriptase (Toyobo, Osaka, Japan). Quantitative real-time PCR analyses of the respective gene transcripts were performed using KAPA SYBR FAST qPCR master mix (KAPA Biosystems, Woburn, MA) and an Mx3005P real-time PCR system (Stratagene, Santa Clara, CA). The following primer pairs were used: for *Actb*, 5′-TGTTACCAACTGGGACGACA (sense) and 5′-GGGGTGTTGAAGGTCTCAAA (antisense); for *Cxcl10*, 5′-GTGAGAATGAGGGCCATAGG (sense) and 5′-TTTTGGCTAAACGCTTTCATT (antisense); for *Ifng*, 5′-ACTGGCAAAAGGATGGTGAC (sense) and 5′-TGAGCTCATTGAATGCTTGG (antisense); for *Irf7*, 5′-CCAGTTGATCCGCATAAGGT (sense) and 5′-GAGCCCAGCATTTTCTCTTG (antisense); for *Gbp2b*, 5′-GGAAAAGCAGATTGAAATGGAACG (sense) and 5′-TCTGGAATCCTTGCTTGAGAAGT (antisense). Data were normalized to the amount of beta-actin (*Actb*) mRNA.

2.6 Cytokine detection by antibody array

Cytokine expression profiling of peritoneal lavages from *P. acnes*-injected WT and Tyk2 KO mice was performed by membrane-based antibody array kit (Proteome Profiler Mouse XL Cytokine Array, ARY028, R&D Systems, Minneapolis, MN) according to manufacturer's instructions.

2.7 Western blotting

BMDMs were pretreated without or with H-89 (10 μM; Cell Signaling Technologies) for 1 h and stimulated with either *P. acnes* (20 μg/mL) or prostaglandin E_2 (PGE_2) (10 μM; AdipoGen, Switzerland) alone, or the combination of *P. acnes* and PGE_2 for 0.5 h. Total cell lysate samples were prepared as previously described [30]. The samples were resolved by SDS-PAGE and transferred to PVDF membranes (PerkinElmer, Boston, MA). The membranes were then incubated with primary antibodies. Horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG secondary antibodies were purchased from GE Healthcare (Little Chalfont, UK) and used as received. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Millipore; Bedford, MA) and an ImageQuant LAS 4000 imaging

system (GE Healthcare Japan, Tokyo). The primary antibodies used were anti-phospho-CREB (clone 87G3) and anti-CREB (clone 86B10) antibodies (both from Cell Signaling Technologies) and anti-actin (clone AC15) mAb (Sigma-Aldrich, St. Louis, MO).

2.8 Statistical evaluation

Data are expressed as means ± standard errors of the means (SEMs). Student's two-tailed *t*-tests were performed for statistical analyses of the data. A value of $p < 0.05$ was used as an indication of statistical significance.

3. Results

3.1 Involvement of Tyk2 in heat-killed *P. acnes*-induced inflammatory response.

Intraperitoneal injection of heat-killed *P. acnes* into mice has been shown to induce intrahepatic granuloma and splenomegaly via a mechanism involving robust IL-12 and IFN-γ production [31-33]. Tyk2 mediates IL-12 signaling that is required for IFN-γ production [12,13,25]. These facts led us to believe that Tyk2-mediated IFN-γ production is required for *P. acnes*-induced inflammation.

To investigate this hypothesis and obtain clues to the specific ways in which Tyk2 participates in the modulation of *P. acnes*-induced inflammation, we injected heat-killed *P. acnes* into WT or Tyk2 KO mice to induce inflammatory responses in vivo. We then assessed the leukocyte infiltrate as an indicator of the early phase of inflammation. The intraperitoneal injection of heat-killed *P. acnes* increased the number of total peritoneal cells and of $CD11b^+$ $Gr\text{-}1^+$ cells (neutrophils) at 12 h post-injection (Fig. 1A). This neutrophil infiltration was significantly suppressed in Tyk2 KO mice compared with that in WT mice (Figure 1A).

At a later phase of *P. acnes*-induced inflammation, i.e., day 7 after injection of heat-killed *P. acnes*, we observed massive splenomegaly (Fig. 1B) and liver granuloma formation (Fig. 1C) of WT mice. By contrast, both the splenomegaly and the number of granulomas were significantly reduced in Tyk2 KO mice. In the livers of *P. acnes*-injected WT mice, the mRNA expressions of *Ifng* (encoding IFN-γ) and an IFN-inducible gene *Cxcl10* increased over time (Fig. 1D). These responses were repressed in Tyk2 KO mice (Fig. 1D). In addition, the levels of serum IFN-γ after *P. acnes* injection were lower in Tyk2 KO mice compared to WT mice (Fig. 1E). These results suggested that Tyk2 has a role in mediating *P. acnes*-induced IFN-γ production and immune responses as predicted.

3.2 Neutrophil infiltration into the peritoneal cavity induced by heat-killed *P. acnes* injection was not suppressed by IFN neutralization.

Given the known contribution of Tyk2 to type I and II IFN productions, we reasoned that the impairment of *P. acnes*-induced responses in Tyk2 KO mice might have been attributable to the poor signaling of type I and II IFNs in Tyk2 KO mice. The formation of hepatic granulomas by *P. acnes* injection has been demonstrated to be impaired in IFN-γ KO mice [34], and such impairment of granuloma formation was recapitulated with anti-IFN-γ monoclonal antibody (mAb) treatment (supplementary Fig. S1), suggesting that *P. acnes*-induced formation of hepatic granulomas is more or less IFN-γ-dependent. Meanwhile, it is unclear whether IFNs are responsible for the early

phase response to *P. acnes*. To evaluate the role of IFN-mediated signaling in *P. acnes*-induced responses, we intraperitoneally injected the neutralizing antibodies for IFNs or control Ig into WT mice before heat-killed *P. acnes* injections. Pre-treatment of mice with either anti-IFNAR1 or anti-IFN-γ mAb did not suppress the *P. acnes*-induced neutrophil infiltration into the peritoneal cavity (Fig. 2A and B), even though each mAb effectively reduced the expression of type I IFN-inducible *Irf7* and IFN-γ-inducible *Gbp2*, respectively, in the spleen of *P. acnes*-injected WT mice (Fig. 2C). Also, pre-treatment of mice with the combination of anti-IFNAR1 and anti-IFN-γ mAbs did not affect *P. acnes*-induced neutrophil infiltration (Fig. 2D), indicating that this early-phase response to *P. acnes* is not significantly mediated by IFNs. These results suggested that *P. acnes*-induced immune responses are mediated not only by IFNs but also by other mechanism(s) in which IFNs play a smaller role, and that Tyk2 might contribute to both types of mediation.

3.3 Increased IL-10 is responsible for the inflammation-suppressive phenotype in heat-killed *P. acnes*-treated Tyk2 KO mice.

To delineate the character of the attenuated inflammation caused by Tyk2-deficiency, the production of pro- and anti-inflammatory cytokines induced by heat-killed *P. acnes* injection was investigated. The *P. acnes*-induced productions of IL-6 and TNF-α were significantly lower in Tyk2 KO mice than in WT mice (Fig. 3A), whereas the production of anti-inflammatory cytokine IL-10 in peritoneal lavage was enhanced in *P. acnes*-injected Tyk2 KO mice compared to WT mice (Fig. 3B). On the other hand, pre-treatment of WT mice with either anti-IFNAR1 or anti-IFN-γ mAb did not potentiate *P. acnes*-induced IL-10 production (Fig. 3B). These results suggested that *P. acnes*-induced IL-10 production is negatively regulated by Tyk2 but not by IFNs.

In our attempt to obtain information about the difference(s) in *P. acnes*-induced responses between WT and Tyk2 KO mice, we conducted an antibody array experiment to evaluate the levels of 111 soluble factors/cytokines simultaneously. Intraperitoneal injection of heat-killed *P. acnes* into WT mice increased the amounts of more than forty soluble factors (Supplementary Figure S2A, B). The amounts of several of these factors—namely, neutrophil function-related factors such as myeloperoxidase, CXCL1 and matrix metalloproteinase 9—were partly decreased in the peritoneal lavage of Tyk2 KO mice compared to that of WT mice (Supplementary Figure S2). These findings were in accordance with the aforementioned attenuated neutrophil infiltration in Tyk2 KO mice. Tyk2 KO mice showed a higher expression of Serpin F1 (also known as pigment epithelium-derived factor: PEDF) and a lower expression of FGF-acidic compared to

WT mice, both in the presence and absence of *P. acnes*-stimulation (Supplementary Figure S2C). As for cytokines, although the spot intensities of almost all interleukin cytokines were faint or undetectable, a slight increase of IL-10 and a decrease of CXCL10, which is an IFN-inducible protein, were detected in Tyk2 KO compared to WT mice (Fig. 3C and Supplementary Figure S2B, D). These results support the notion that *P. acnes*-induced IL-10 production in the peritoneal cavity was enhanced more in Tyk2 KO mice than WT mice.

The increased level of peritoneal IL-10 in Tyk2 KO mice compared with that in WT mice might account for the reduction in the early-phase response to *P. acnes* seen in Tyk2 KO mice. To directly test this, we injected anti-IL-10 receptor neutralizing antibody into Tyk2 KO mice and monitored its effect on *P. acnes*-induced neutrophil infiltration. The treatment of Tyk2 KO mice with anti-IL-10R antibody significantly restored the neutrophil infiltration to a level comparable to that in WT mice in the peritoneal cavity of Tyk2 KO mice (Fig. 3D). These results suggested that the increased IL-10 in heat-killed *P. acnes*-treated Tyk2 KO mice may be responsible for the inflammation-suppressive phenotype of Tyk2 KO mice.

An intracellular IL-10 staining analysis of the peritoneal cells collected from *P. acnes*-treated Tyk2 KO mice was performed to identify specific cell populations showing differential IL-10 production between WT and Tyk2 KO cells (Fig. 3E). The percentage and absolute number of IL-10-positive cells among the peritoneal F4/80-positive cell population was significantly increased in Tyk2 KO compared with WT mice. IL-10-positive cells among the F4/80-negative population or B220-positive population were not significantly increased in the Tyk2 KO peritoneum. These results suggested that at least macrophages were a cell population exhibiting enhanced IL-10 production in Tyk2 KO mice.

3.4 Enhanced IL-10 production in Tyk2 KO peritoneal resident cells was significantly suppressed by cyclooxygenase inhibition or PKA inhibition.

An ELISA experiment of in vitro *P. acnes* stimulation of resident peritoneal cells showed an enhanced IL-10 production in Tyk2 KO compared to WT peritoneal cells (Fig. 4A). IL-10 production by macrophages has been reported to be enhanced by prostaglandin E_2 (PGE_2) signaling through protein kinase A (PKA) activity [35]. In agreement with this previous report, we found that the IL-10 productions in both WT and Tyk2 KO peritoneal cells were significantly suppressed by the addition of diclofenac, an inhibitor of cyclooxygenases, which are required for prostaglandin production. In addition, H-89, a specific PKA inhibitor [36], abrogated *P. acnes*-induced

IL-10 production in both WT and Tyk2 KO peritoneal cells. These results suggested that PGE_2/PKA signaling is the endogenous key mediator of *P. acnes*-induced IL-10 production in both WT and Tyk2 KO peritoneal cells and that Tyk2 KO cells might utilize an enhanced PKA activity to potentiate IL-10 secretion.

PGE_2 is an endogenous immunosuppressant that reprograms macrophages to increase their IL-10 production [37]. Interestingly, we observed that the peritoneal lavage from steady state Tyk2 KO mice contained an approximately four-fold greater amount of PGE_2 compared to that in WT mice (Fig. 4B), although peritoneal PGE_2 levels in both WT and Tyk2 KO mice were elevated after heat-killed *P. acnes* injection and became indistinguishable (Fig. 4B). These results suggested that the enhanced IL-10 production seen in Tyk2 KO mice might have been attributable to the immunosuppressive microenvironment created by the spontaneously high level of PGE_2 production in the peritoneal cavity of Tyk2 KO mice. This seemed to be in accordance with a previous report showing that the anti-inflammatory effect of PGE_2 on mononuclear phagocytes was mediated by an autocrine feedback mechanism involving IL-10 [38].

3.5 Tyk2 KO macrophages exhibited enhanced PKA activation in response to combined stimulation with *P. acnes* and PGE_2.

We further investigated how Tyk2 deficiency affects *P. acnes*-induced cellular responses using bone marrow-derived macrophages (BMDMs). We observed that CREB phosphorylation, which is a hallmark of PKA activation, was induced by *P. acnes* alone and further enhanced in the presence of exogenously added PGE_2 in WT BMDMs (Fig. 4C). Treatment with the PKA inhibitor H-89 abrogated the CREB phosphorylation induced by *P. acnes* plus PGE_2, confirming that this CREB phosphorylation is mediated by PKA. Interestingly, Tyk2 KO BMDMs showed an enhancement of CREB phosphorylation in response to *P. acnes* alone, PGE_2 alone, or combined stimulation with *P. acnes* plus PGE_2 (Fig. 4C). These results suggested that PKA activity is more greatly enhanced in Tyk2 KO BMDMs than in WT.

4. Discussion

The observation that Tyk2 KO mice exhibited reduced inflammatory responses against intraperitoneally injected heat-killed *P. acnes* compared with WT mice indicates that Tyk2 has a role in acute inflammation. This phenomenon might be attributed to the enhanced production of IL-10 in the peritoneal cells of Tyk2 KO mice. We found that enhancements of PGE_2-signaling and PKA activity might be important for IL-10 enhancement in Tyk2 KO macrophages.

In the heat-killed *P. acnes* challenges, while the impairment of IFN signaling by Tyk2 deficiency (Fig. 1) was observed as expected, the enhanced IL-10 production in the peritoneal cavity of Tyk2 KO mice (Fig. 3B) was unexpected, since IL-10 production from macrophages has been reported to require autocrine type I IFN signaling [7], and such autocrine IFN signaling is known to be attenuated in Tyk2-deficient macrophages [28]. Also, we showed that combined pre-treatment of anti-IFNAR1 and anti-IFN-γ neutralizing antibodies did not attenuate neutrophil infiltration in the peritoneal cavity of heat-killed *P. acnes*-injected WT mice (Fig. 2D), suggesting that the initial phase of *P. acnes*-induced inflammation is not essentially mediated by type I and II IFNs. Collectively, these results implied that, although it would be true that Tyk2 plays roles in IFN production and signaling, Tyk2 may also have a role for IL-10 regulation through an IFN-independent mechanism. Thus Tyk2 might not function merely as an IFN signaling mediator.

We demonstrated that the peritoneal lavage from steady-state Tyk2 KO mice contained an increased amount of PGE_2 compared to that of WT mice (Fig. 4B). In addition, compared with WT BMDMs, Tyk2 KO BMDMs showed an enhanced CREB phosphorylation, which was mediated by PKA activity, in the cells stimulated with the combination of *P. acnes* and PGE_2 (Fig. 4C). The *P. acnes*-induced IL-10 production in both WT and Tyk2 KO cells was significantly inhibited by either a cyclooxygenase inhibitor or a PKA inhibitor (Fig. 4A). These results indicated that Tyk2 deficiency may potentiate PGE_2-induced PKA activation in macrophages, and thereby enhance IL-10 production (Fig. 4D).

Tyk2 KO mice are known to be resistant to LPS-induced septic shock [21,22]. This resistance was thought to be principally mediated by a suppression of IFN-β-dependent pathway [22]. However, we conducted only a neutralizing antibody experiment to evaluate the impact of IFNs on acute inflammation by *P. acnes* in the present study. In addition, previous studies have reported that Tyk2 is involved in the production of IL-1β and IL-27 during *Listeria monocytogenes* infection and LPS-induced experimental sepsis, respectively [39,40], suggesting that these cytokines might also play a role in *P.*

acnes-induced acute peritonitis. In our cytokine detection by a proteome profiler antibody array (supplementary Fig. S2), we observed neither clear inductions of IL-1β and IL-27 p40 proteins upon heat-killed *P. acnes* injection nor differences between WT and Tyk2 KO mice. Nevertheless, for a more reliable evaluation of the roles of IL-1β, IL-27, and IFNs in *P. acnes*-induced acute peritonitis, future studies using gene-disrupted mice for these cytokines would be useful.

Based on the results of the present study, we propose an aspect of the Tyk2-mediated regulation of inflammation that is centered on IL-10 production. Our model has similarities and consistencies with the previous studies showing that a mouse model of septic shock was negatively regulated by PGE_2 [37] and IL-10 [41]. Although the present study did not sufficiently elucidate the molecular mechanism linking Tyk2-deficiency with the potentiation of IL-10 production, it can be speculated that some of the soluble factors that are differentially expressed between WT and Tyk2 KO mice might play a role in the enhanced IL-10 production in Tyk2 KO mice. For example, Serpin F1 (also known as PEDF), which was increased in the Tyk2 KO peritoneal cavity (Supplementary Figure S2C), has been reported to promote IL-10 production from macrophages [42,43]. Therefore, the information obtained from the antibody array in the present study might contribute to a more detailed elucidation of the mechanism underlying the relationship between Tyk2-deficiency and immunosuppression in future studies.

5. Conclusions

The present study demonstrated that Tyk2-deficiency skewed macrophage potential towards an anti-inflammatory state, resulting in the potentiation of IL-10 production. Thus, Tyk2 inactivation might be regarded as an effective new approach, which has a dual immunosuppressive mechanism of suppressing the IFN pathway and enhancing IL-10, for treating a broad spectrum of inflammatory diseases.

Acknowledgments

This work was supported in part by Japan Society for the Promotion of Science (JSPS) KAKENHI grants (numbers 2546005603, 17K08263 and 19H03364).

Disclosures

The authors have no financial conflicts of interest.

References

[1] F. Ginhoux, J.L. Schultze, P.J. Murray, J. Ochando, S.K. Biswas, New insights into the multidimensional concept of macrophage ontogeny, activation and function, Nat Immunol 17 (2016) 34-40. 10.1038/ni.3324.

[2] P. Italiani, D. Boraschi, From Monocytes to M1/M2 Macrophages: Phenotypical vs. Functional Differentiation, Front Immunol 5 (2014) 514. 10.3389/fimmu.2014.00514.

[3] A. Shapouri-Moghaddam, S. Mohammadian, H. Vazini, M. Taghadosi, S.A. Esmaeili, F. Mardani, B. Seifi, A. Mohammadi, J.T. Afshari, A. Sahebkar, Macrophage plasticity, polarization, and function in health and disease, J Cell Physiol 233 (2018) 6425-6440. 10.1002/jcp.26429.

[4] K. Schroder, P.J. Hertzog, T. Ravasi, D.A. Hume, Interferon-gamma: an overview of signals, mechanisms and functions, J Leukoc Biol 75 (2004) 163-189. 10.1189/jlb.0603252.

[5] D.J. Gough, N.L. Messina, C.J. Clarke, R.W. Johnstone, D.E. Levy, Constitutive type I interferon modulates homeostatic balance through tonic signaling, Immunity 36 (2012) 166-174. 10.1016/j.immuni.2012.01.011.

[6] T. Taniguchi, A. Takaoka, A weak signal for strong responses: interferon-alpha/beta revisited, Nat Rev Mol Cell Biol 2 (2001) 378-386. 10.1038/35073080.

[7] A.J. Fleetwood, H. Dinh, A.D. Cook, P.J. Hertzog, J.A. Hamilton, GM-CSF- and M-CSF-dependent macrophage phenotypes display differential dependence on type I interferon signaling, J Leukoc Biol 86 (2009) 411-421. 10.1189/jlb.1108702.

[8] S.S. Iyer, G. Cheng, Role of interleukin 10 transcriptional regulation in inflammation and autoimmune disease, Crit Rev Immunol 32 (2012) 23-63.

[9] R. Kühn, J. Löhler, D. Rennick, K. Rajewsky, W. Müller, Interleukin-10-deficient mice develop chronic enterocolitis, Cell 75 (1993) 263-274. 10.1016/0092-8674(93)80068-p.

[10] E.Y. Chang, B. Guo, S.E. Doyle, G. Cheng, Cutting edge: involvement of the type I IFN production and signaling pathway in lipopolysaccharide-induced IL-10 production, J Immunol 178 (2007) 6705-6709. 10.4049/jimmunol.178.11.6705.

[11] X. Hu, P.K. Paik, J. Chen, A. Yarilina, L. Kockeritz, T.T. Lu, J.R. Woodgett, L.B. Ivashkiv, IFN-gamma suppresses IL-10 production and synergizes with TLR2 by regulating GSK3 and CREB/AP-1 proteins, Immunity 24 (2006) 563-574. 10.1016/j.immuni.2006.02.014.

[12] K. Shimoda, K. Kato, K. Aoki, T. Matsuda, A. Miyamoto, M. Shibamori, M. Yamashita, A. Numata, K. Takase, S. Kobayashi, S. Shibata, Y. Asano, H. Gondo, K. Sekiguchi, K. Nakayama, T. Nakayama, T. Okamura, S. Okamura, Y. Niho, Tyk2 plays

a restricted role in IFN alpha signaling, although it is required for IL-12-mediated T cell function, Immunity 13 (2000) 561-571.
[13] M. Karaghiosoff, H. Neubauer, C. Lassnig, P. Kovarik, H. Schindler, H. Pircher, B. McCoy, C. Bogdan, T. Decker, G. Brem, K. Pfeffer, M. Müller, Partial impairment of cytokine responses in Tyk2-deficient mice, Immunity 13 (2000) 549-560.
[14] C. Parham, M. Chirica, J. Timans, E. Vaisberg, M. Travis, J. Cheung, S. Pflanz, R. Zhang, K.P. Singh, F. Vega, W. To, J. Wagner, A.M. O'Farrell, T. McClanahan, S. Zurawski, C. Hannum, D. Gorman, D.M. Rennick, R.A. Kastelein, R. de Waal Malefyt, K.W. Moore, A receptor for the heterodimeric cytokine IL-23 is composed of IL-12Rbeta1 and a novel cytokine receptor subunit, IL-23R, J Immunol 168 (2002) 5699-5708.
[15] C.M. Bacon, D.W. McVicar, J.R. Ortaldo, R.C. Rees, J.J. O'Shea, J.A. Johnston, Interleukin 12 (IL-12) induces tyrosine phosphorylation of JAK2 and TYK2: differential use of Janus family tyrosine kinases by IL-2 and IL-12, J Exp Med 181 (1995) 399-404.
[16] C.A. Dendrou, A. Cortes, L. Shipman, H.G. Evans, K.E. Attfield, L. Jostins, T. Barber, G. Kaur, S.B. Kuttikkatte, O.A. Leach, C. Desel, S.L. Faergeman, J. Cheeseman, M.J. Neville, S. Sawcer, A. Compston, A.R. Johnson, C. Everett, J.I. Bell, F. Karpe, M. Ultsch, C. Eigenbrot, G. McVean, L. Fugger, Resolving TYK2 locus genotype-to-phenotype differences in autoimmunity, Sci Transl Med 8 (2016) 363ra149. 10.1126/scitranslmed.aag1974.
[17] M. Ishizaki, T. Akimoto, R. Muromoto, M. Yokoyama, Y. Ohshiro, Y. Sekine, H. Maeda, K. Shimoda, K. Oritani, T. Matsuda, Involvement of Tyrosine Kinase-2 in Both the IL-12/Th1 and IL-23/Th17 Axes In Vivo, Journal of Immunology 187 (2011) 181-189. 10.4049/jimmunol.1003244.
[18] M. Ishizaki, R. Muromoto, T. Akimoto, Y. Ohshiro, M. Takahashi, Y. Sekine, H. Maeda, K. Shimoda, K. Oritani, T. Matsuda, Tyk2 deficiency protects joints against destruction in anti-type II collagen antibody-induced arthritis in mice, International Immunology 23 (2011) 575-582. 10.1093/intimm/dxr057.
[19] M. Ishizaki, R. Muromoto, T. Akimoto, Y. Sekine, S. Kon, M. Diwan, H. Maeda, S. Togi, K. Shimoda, K. Oritani, T. Matsuda, Tyk2 is a therapeutic target for psoriasis-like skin inflammation, International Immunology 26 (2014) 257-267. 10.1093/intimm/dxt062.
[20] R. Muromoto, K. Tawa, Y. Ohgakiuchi, A. Sato, Y. Saino, K. Hirashima, H. Minoguchi, Y. Kitai, J.I. Kashiwakura, K. Shimoda, K. Oritani, T. Matsuda, IκB-ζ Expression Requires Both TYK2/STAT3 Activity and IL-17-Regulated mRNA

534 Stabilization, Immunohorizons 3 (2019) 172-185. 10.4049/immunohorizons.1900023.
535 [21] K. Kamezaki, K. Shimoda, A. Numata, T. Matsuda, K. Nakayama, M. Harada, The
536 role of Tyk2, Stat1 and Stat4 in LPS-induced endotoxin signals, Int Immunol 16 (2004)
537 1173-1179. 10.1093/intimm/dxh118.
538 [22] M. Karaghiosoff, R. Steinborn, P. Kovarik, G. Kriegshäuser, M. Baccarini, B.
539 Donabauer, U. Reichart, T. Kolbe, C. Bogdan, T. Leanderson, D. Levy, T. Decker, M.
540 Müller, Central role for type I interferons and Tyk2 in lipopolysaccharide-induced
541 endotoxin shock, Nat Immunol 4 (2003) 471-477. 10.1038/ni910.
542 [23] T. Hashiguchi, A. Oyamada, K. Sakuraba, K. Shimoda, K.I. Nakayama, Y. Iwamoto,
543 Y. Yoshikai, H. Yamada, Tyk2-dependent bystander activation of conventional and
544 nonconventional Th1 cell subsets contributes to innate host defense against Listeria
545 monocytogenes infection, J Immunol 192 (2014) 4739-4747.
546 10.4049/jimmunol.1303067.
547 [24] A. Oyamada, H. Ikebe, M. Itsumi, H. Saiwai, S. Okada, K. Shimoda, Y. Iwakura,
548 K.I. Nakayama, Y. Iwamoto, Y. Yoshikai, H. Yamada, Tyrosine kinase 2 plays critical
549 roles in the pathogenic CD4 T cell responses for the development of experimental
550 autoimmune encephalomyelitis, J Immunol 183 (2009) 7539-7546.
551 10.4049/jimmunol.0902740.
552 [25] K. Shimoda, H. Tsutsui, K. Aoki, K. Kato, T. Matsuda, A. Numata, K. Takase, T.
553 Yamamoto, H. Nukina, T. Hoshino, Y. Asano, H. Gondo, T. Okamura, S. Okamura, K.
554 Nakayama, K. Nakanishi, Y. Niho, M. Harada, Partial impairment of interleukin-12
555 (IL-12) and IL-18 signaling in Tyk2-deficient mice, Blood 99 (2002) 2094-2099.
556 [26] A.Y. Kreins, M.J. Ciancanelli, S. Okada, X.F. Kong, N. Ramírez-Alejo, S.S. Kilic, J.
557 El Baghdadi, S. Nonoyama, S.A. Mahdaviani, F. Ailal, A. Bousfiha, D. Mansouri, E.
558 Nievas, C.S. Ma, G. Rao, A. Bernasconi, H. Sun Kuehn, J. Niemela, J. Stoddard, P.
559 Deveau, A. Cobat, S. El Azbaoui, A. Sabri, C.K. Lim, M. Sundin, D.T. Avery, R.
560 Halwani, A.V. Grant, B. Boisson, D. Bogunovic, Y. Itan, M. Moncada-Velez, R.
561 Martinez-Barricarte, M. Migaud, C. Deswarte, L. Alsina, D. Kotlarz, C. Klein, I.
562 Muller-Fleckenstein, B. Fleckenstein, V. Cormier-Daire, S. Rose-John, C. Picard, L.
563 Hammarstrom, A. Puel, S. Al-Muhsen, L. Abel, D. Chaussabel, S.D. Rosenzweig, Y.
564 Minegishi, S.G. Tangye, J. Bustamante, J.L. Casanova, S. Boisson-Dupuis, Human
565 TYK2 deficiency: Mycobacterial and viral infections without hyper-IgE syndrome, J
566 Exp Med 212 (2015) 1641-1662. 10.1084/jem.20140280.
567 [27] Y. Minegishi, M. Saito, T. Morio, K. Watanabe, K. Agematsu, S. Tsuchiya, H.
568 Takada, T. Hara, N. Kawamura, T. Ariga, H. Kaneko, N. Kondo, I. Tsuge, A. Yachie, Y.
569 Sakiyama, T. Iwata, F. Bessho, T. Ohishi, K. Joh, K. Imai, K. Kogawa, M. Shinohara, M.

570 Fujieda, H. Wakiguchi, S. Pasic, M. Abinun, H.D. Ochs, E.D. Renner, A. Jansson, B.H.
571 Belohradsky, A. Metin, N. Shimizu, S. Mizutani, T. Miyawaki, S. Nonoyama, H.
572 Karasuyama, Human tyrosine kinase 2 deficiency reveals its requisite roles in multiple
573 cytokine signals involved in innate and acquired immunity, Immunity 25 (2006)
574 745-755.
575 [28] C. Vogl, T. Flatt, B. Fuhrmann, E. Hofmann, B. Wallner, R. Stiefvater, P. Kovarik,
576 B. Strobl, M. Müller, Transcriptome analysis reveals a major impact of JAK protein
577 tyrosine kinase 2 (Tyk2) on the expression of interferon-responsive and metabolic genes,
578 BMC Genomics 11 (2010) 199. 10.1186/1471-2164-11-199.
579 [29] M. Imamura, H. Tsutsui, K. Yasuda, R. Uchiyama, S. Yumikura-Futatsugi, K.
580 Mitani, S. Hayashi, S. Akira, S. Taniguchi, N. Van Rooijen, J. Tschopp, T. Yamamoto, J.
581 Fujimoto, K. Nakanishi, Contribution of TIR domain-containing adapter inducing
582 IFN-beta-mediated IL-18 release to LPS-induced liver injury in mice, J Hepatol 51
583 (2009) 333-341. 10.1016/j.jhep.2009.03.027.
584 [30] R. Muromoto, K. Sugiyama, A. Takachi, S. Imoto, N. Sato, T. Yamamoto, K.
585 Oritani, K. Shimoda, T. Matsuda, Physical and functional interactions between Daxx
586 and DNA methyltransferase 1-associated protein, DMAP1, Journal of Immunology 172
587 (2004) 2985-2993.
588 [31] C. Kalis, M. Gumenscheimer, N. Freudenberg, S. Tchaptchet, G. Fejer, A. Heit, S.
589 Akira, C. Galanos, M.A. Freudenberg, Requirement for TLR9 in the
590 immunomodulatory activity of Propionibacterium acnes, J Immunol 174 (2005)
591 4295-4300.
592 [32] J. Kim, M.T. Ochoa, S.R. Krutzik, O. Takeuchi, S. Uematsu, A.J. Legaspi, H.D.
593 Brightbill, D. Holland, W.J. Cunliffe, S. Akira, P.A. Sieling, P.J. Godowski, R.L. Modlin,
594 Activation of toll-like receptor 2 in acne triggers inflammatory cytokine responses, J
595 Immunol 169 (2002) 1535-1541.
596 [33] K. Matsui, T. Yoshimoto, H. Tsutsui, Y. Hyodo, N. Hayashi, K. Hiroishi, N.
597 Kawada, H. Okamura, K. Nakanishi, K. Higashino, Propionibacterium acnes treatment
598 diminishes CD4+ NK1.1+ T cells but induces type I T cells in the liver by induction of
599 IL-12 and IL-18 production from Kupffer cells, J Immunol 159 (1997) 97-106.
600 [34] H. Tsuji, N. Mukaida, A. Harada, S. Kaneko, E. Matsushita, Y. Nakanuma, H.
601 Tsutsui, H. Okamura, K. Nakanishi, Y. Tagawa, Y. Iwakura, K. Kobayashi, K.
602 Matsushima, Alleviation of lipopolysaccharide-induced acute liver injury in
603 Propionibacterium acnes-primed IFN-gamma-deficient mice by a concomitant reduction
604 of TNF-alpha, IL-12, and IL-18 production, J Immunol 162 (1999) 1049-1055.
605 [35] H. Cheon, Y.H. Rho, S.J. Choi, Y.H. Lee, G.G. Song, J. Sohn, N.H. Won, J.D. Ji,

606 Prostaglandin E2 augments IL-10 signaling and function, J Immunol 177 (2006)
607 1092-1100. 10.4049/jimmunol.177.2.1092.
608 [36] T. Chijiwa, A. Mishima, M. Hagiwara, M. Sano, K. Hayashi, T. Inoue, K. Naito, T.
609 Toshioka, H. Hidaka, Inhibition of forskolin-induced neurite outgrowth and protein
610 phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent
611 protein kinase, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89),
612 of PC12D pheochromocytoma cells, J Biol Chem 265 (1990) 5267-5272.
613 [37] K. Németh, A. Leelahavanichkul, P.S. Yuen, B. Mayer, A. Parmelee, K. Doi, P.G.
614 Robey, K. Leelahavanichkul, B.H. Koller, J.M. Brown, X. Hu, I. Jelinek, R.A. Star, E.
615 Mezey, Bone marrow stromal cells attenuate sepsis via prostaglandin E(2)-dependent
616 reprogramming of host macrophages to increase their interleukin-10 production, Nat
617 Med 15 (2009) 42-49. 10.1038/nm.1905.
618 [38] G. Strassmann, V. Patil-Koota, F. Finkelman, M. Fong, T. Kambayashi, Evidence
619 for the involvement of interleukin 10 in the differential deactivation of murine
620 peritoneal macrophages by prostaglandin E2, J Exp Med 180 (1994) 2365-2370.
621 10.1084/jem.180.6.2365.
622 [39] M. Bosmann, B. Strobl, N. Kichler, D. Rigler, J.J. Grailer, F. Pache, P.J. Murray, M.
623 Müller, P.A. Ward, Tyrosine kinase 2 promotes sepsis-associated lethality by facilitating
624 production of interleukin-27, J Leukoc Biol 96 (2014) 123-131.
625 10.1189/jlb.3A1013-541R.
626 [40] M. Radwan, R. Stiefvater, T. Grunert, O. Sharif, I. Miller, M.
627 Marchetti-Deschmann, G. Allmaier, M. Gemeiner, S. Knapp, P. Kovarik, M. Müller, B.
628 Strobl, Tyrosine kinase 2 controls IL-1ß production at the translational level, J Immunol
629 185 (2010) 3544-3553. 10.4049/jimmunol.0904000.
630 [41] S.Q. Latifi, M.A. O'Riordan, A.D. Levine, Interleukin-10 controls the onset of
631 irreversible septic shock, Infect Immun 70 (2002) 4441-4446.
632 10.1128/iai.70.8.4441-4446.2002.
633 [42] S.L. Yang, S.L. Chen, J.Y. Wu, T.C. Ho, Y.P. Tsao, Pigment epithelium-derived
634 factor induces interleukin-10 expression in human macrophages by induction of PPAR
635 gamma, Life Sci 87 (2010) 26-35. 10.1016/j.lfs.2010.05.007.
636 [43] P. Zamiri, S. Masli, J.W. Streilein, A.W. Taylor, Pigment epithelial growth factor
637 suppresses inflammation by modulating macrophage activation, Invest Ophthalmol Vis
638 Sci 47 (2006) 3912-3918. 10.1167/iovs.05-1267.
639

Figure Legends

Fig. 1. Inflammatory cell infiltration, splenomegaly and hepatic granuloma formation in heat-killed *P. acnes*-injected mice. (A) Peritoneal lavages were collected from WT and Tyk2 KO mice at 12 h after intraperitoneal injection with 500 μg of heat-killed *P. acnes* or PBS, and peritoneal cells were collected and counted by flow cytometry. The results for total peritoneal cells and neutrophils ($CD11b^+$ $Gr\text{-}1^+$) are shown (mean ± SEM, n = 9, pooled from four independent experiments). *$p < 0.05$. (B) Spleens of WT and Tyk2 KO mice were removed 7 days after intraperitoneal injection with 500 μg of heat-killed *P. acnes* or PBS. Spleen weights were measured. Data were pooled from three independent experiments and are shown as the mean values ± SEM (n = 5). **$p < 0.01$. (C) Representative hematoxylin and eosin-stained liver sections on day 7 after intraperitoneal injection of heat-killed *P. acnes* (500 μg). Arrowheads indicate granuloma formation. 200 × magnifications. Scale bars, 100 μm. Data are representative of three independent experiments. (D) Real-time RT-PCR analysis of the abundances of mRNAs of *Ifng* (IFN-γ) and *Cxcl10* in the livers of mice that were treated with heat-killed *P. acnes* (500 μg) for the indicated periods. Shown are the mean values ± SEM (n = 3, pooled from two independent experiments). **$p < 0.01$. (E) Analysis of IFN-γ levels in the serum of mice that were treated with heat-killed *P. acnes* (500 μg) for the indicated periods. Shown are the mean values ± SEM (n = 3-4, pooled from two independent experiments). *$p < 0.05$. ND: not detected.

Fig. 2. Neutralization of type I and II IFNs did not suppress *P. acnes*-induced acute neutrophil infiltration in WT mice. (A and B) WT mice were intraperitoneally injected with either 200 μg of anti–IFNAR1 mAb (clone MAR1-5A3), 200 μg of anti–IFN-γ mAb (clone R4-6A2), or the respective control IgG1. Three hours later, the mice were intraperitoneally injected with 500 μg of heat-killed *P. acnes*. Twelve hours after the injection, peritoneal exudate cells were collected. The numbers of total peritoneal cells (A) and neutrophils (B) were counted by flow cytometry. Shown are the mean values ± SEM (n = 3, pooled from three independent experiments). NS: not significant. (C) Real-time RT-PCR analysis for the assessment of neutralizing capacity of anti-IFN mAbs treatment. The mRNA levels of a type I IFN-induced gene (*Irf7*) and IFN-γ-induced gene (*Gbp2b*) were suppressed by treatment with anti-IFNAR1 and anti-IFN-γ mAb in the spleen of WT mice that were injected with heat-killed *P. acnes* (500 μg) for 12 h. Data were pooled from two independent experiments and are shown as the mean values ± SEM (n = 3). **$p < 0.01$. (D) WT mice were intraperitoneally injected with the combination of 200 μg of anti–IFNAR1 mAb and 200 μg of

anti–IFN-γ mAb, or control IgG1. Three hours later, the mice were intraperitoneally injected with 500 μg of heat-killed *P. acnes*. Twelve hours after the injection, peritoneal exudate cells were collected and the numbers of neutrophils were counted by flow cytometry. Shown are the mean values ± SEM (n = 3, pooled from two independent experiments). NS: not significant.

Fig. 3. Tyk2-deficiency caused an enhanced IL-10 production in peritoneal cavity. (A) ELISA analysis of IL-6 and TNF-α production in the peritoneal lavages of mice that were treated with PBS or heat-killed *P. acnes* (500 μg) for 12 h. Shown are the mean values ± SEM (n = 4-11). Data were pooled from four independent experiments. ***$p < 0.001$. ND: not detected. (B) ELISA analysis of IL-10 production in the peritoneal lavages of mice that were treated with PBS or heat-killed *P. acnes* (500 μg) for 6 h. Anti–IFNAR1 mAb, anti–IFN-γ mAb or the respective control IgG1 was administered 3 h before injection of heat-killed *P. acnes*. Shown are the mean values ± SEM (n = 4-7). Data were pooled from three independent experiments. NS: not significant. (C) A mouse cytokine array panel showing differences in the level of IL-10 and CXCL10 in the peritoneal lavage of WT and Tyk2 KO mice 12 h after heat-killed *P. acnes* injection, as determined by a Proteome profiler antibody array. Peritoneal lavage samples were collected from three mice in each group and pooled for the assay. (D) The effect of anti-IL-10R antibody on neutrophil number in *P. acnes*-treated Tyk2 KO mice. Tyk2 KO mice were intraperitoneally injected with 300 μg of anti–IL-10R mAb (clone 1B1.3A) or control IgG1. Three hours later, the mice were intraperitoneally injected with heat-killed *P. acnes* (500 μg). Twelve hours after the injection, peritoneal cells were collected and the numbers of neutrophils were counted by flow cytometry. As a control, the cell numbers in peritoneal lavage of WT mice given the heat-killed *P. acnes* injection for 12 h are shown. Shown are the mean values ± SEM (n = 3-4). Data were pooled from two independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. (E) WT or Tyk2 KO mice were intraperitoneally injected with 500 μg of heat-killed *P. acnes* for 5 h. For fluorescence staining of intracellular IL-10 protein, 100 μg of brefeldin A was injected into the peritoneal cavity of each mouse 3 h before euthanasia. The percentages and absolute numbers of the IL-10-producing population among peritoneal $F4/80^+$ cells, $F4/80^-$ cells and $B220^+$ cells were determined by flow cytometry. Data were pooled from three independent experiments and are shown as the mean values ± SEM (n = 3). *$p < 0.05$. NS: not significant.

Fig. 4. Enhanced IL-10 production in Tyk2 KO peritoneal resident cells was

suppressed by PKA- and cyclooxygenase-inhibitors. (A) ELISA analysis of IL-10 production in the culture supernatant of in vitro cultured peritoneal resident cells (3.0×10^5 cells) from WT or Tyk2 KO mice that were not treated or treated with heat-killed *P. acnes* (20 μg/mL) for 6 h. The treatments with diclofenac (DF; 10 μM) or H-89 (10 μM) were applied 1 h before the addition of heat-killed *P. acnes* into the culture. Data were pooled from three independent experiments and are shown as the mean values ± SEM (n = 3-6). *$p < 0.05$. ND: not detected. (B) ELISA analysis of the PGE_2 level in steady-state and in *P. acnes*-stimulated peritoneal lavages from WT and Tyk2 KO mice. Data were pooled from two independent experiments and are shown as the mean values ± SEM (n = 3). *** $p < 0.001$. (C) Western blot detection of phosphorylated and total CREB protein amounts in WT and Tyk2 KO BMDMs pretreated without or with H-89 (10 μM) for 1 h and stimulated with either *P. acnes* (20 μg/mL) or PGE_2 (10 μM) alone, or the combination of *P. acnes* and PGE_2 for 0.5 h. Relative intensity values were calculated from densitometry values of phosphorylated CREB and total CREB. Data are representative of three independent experiments. (D) Schematic mechanism of the involvement of Tyk2 in IL-10 regulation in macrophages. The Tyk2 KO peritoneal cavity exhibited a spontaneously high level of PGE_2, resulting in an immunosuppressive microenvironment. PGE2-induced activation of the PKA pathway in macrophages was also enhanced by Tyk2 deficiency.

Figure 1

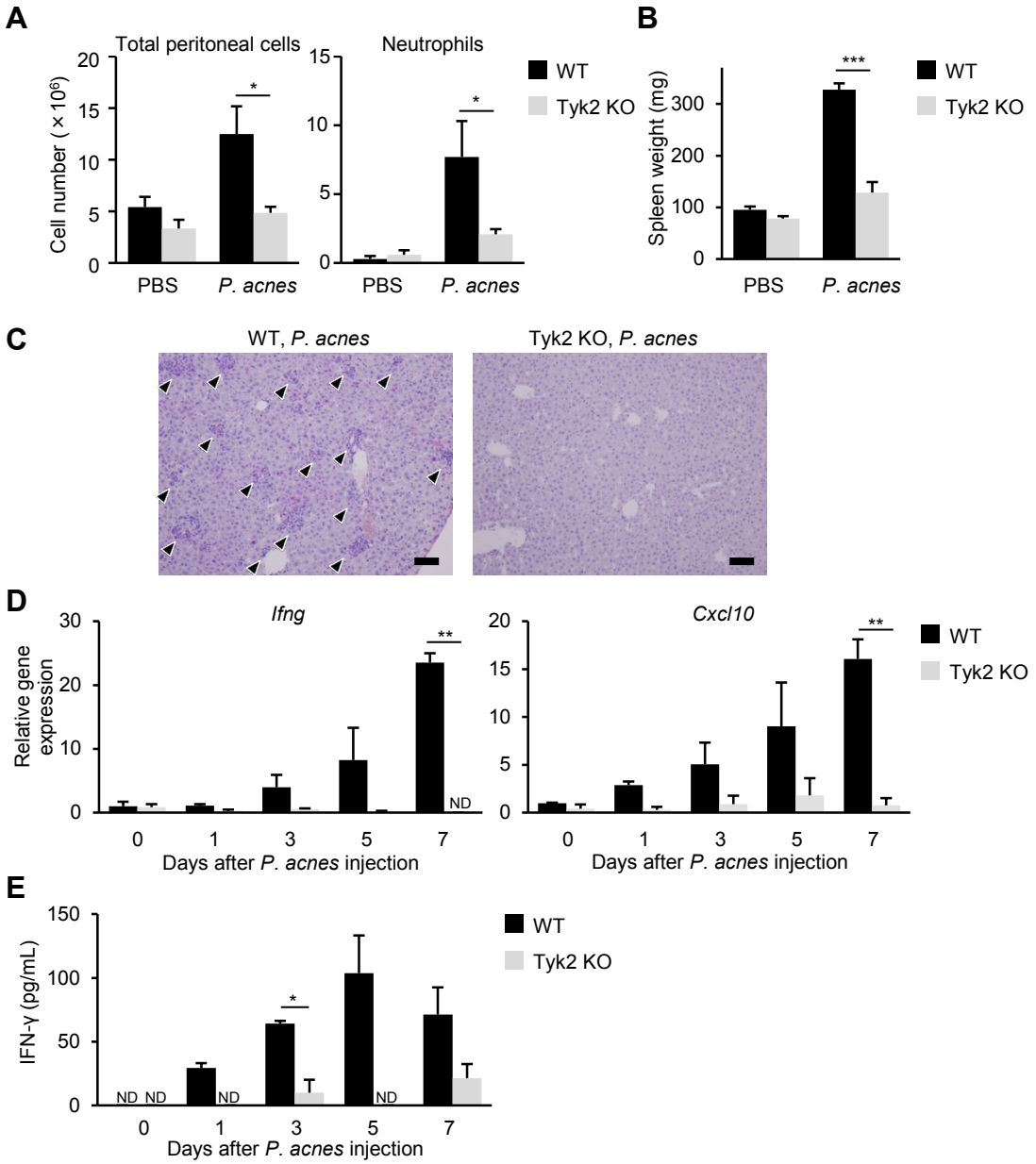

A
Total peritoneal cells
Neutrophils
Cell number (× 10^6)
PBS
P. acnes
WT
Tyk2 KO
B
Spleen weight (mg)
C
WT, P. acnes
Tyk2 KO, P. acnes
D
Ifng
Cxcl10
Relative gene expression
Days after P. acnes injection
ND
E
IFN-γ (pg/mL)

Figure 2

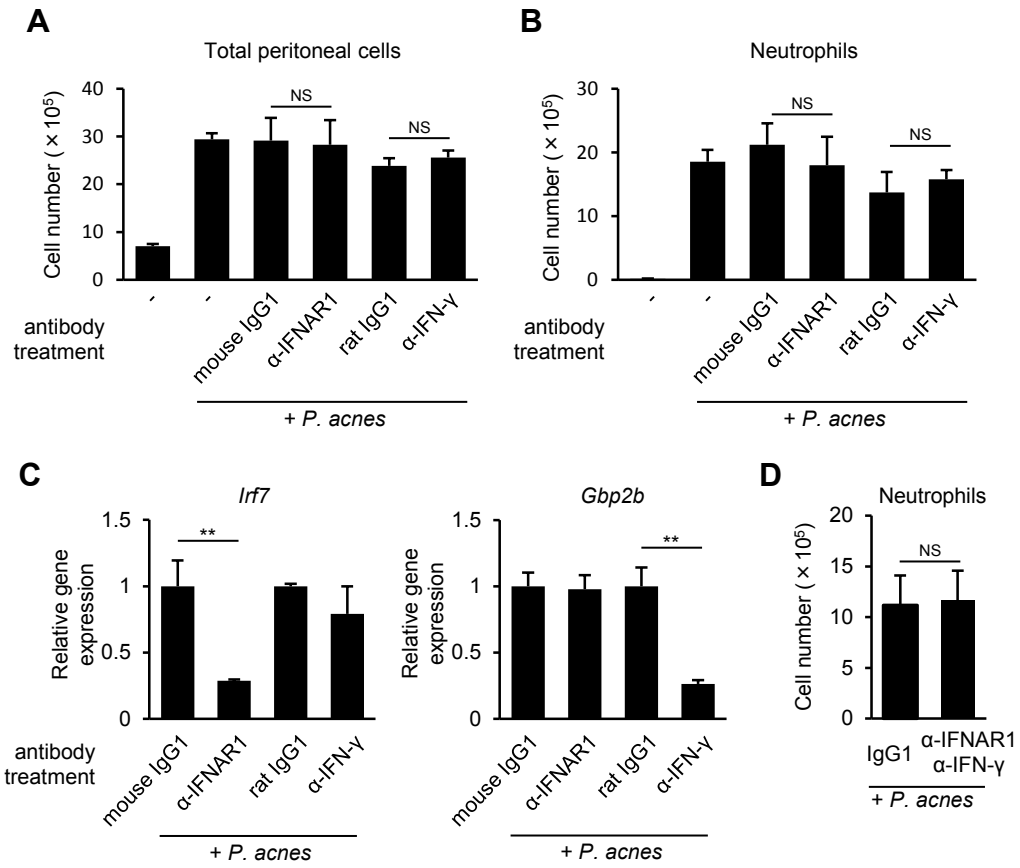

A
Total peritoneal cells
Cell number (× 10^5)
40
30
20
10
0
NS
NS
-
-
mouse IgG1
α-IFNAR1
rat IgG1
α-IFN-γ
antibody treatment
+ P. acnes
B
Neutrophils
Cell number (× 10^5)
30
20
10
0
NS
NS
-
-
mouse IgG1
α-IFNAR1
rat IgG1
α-IFN-γ
antibody treatment
+ P. acnes
C
Irf7
Relative gene expression
1.5
1
0.5
0
**
mouse IgG1
α-IFNAR1
rat IgG1
α-IFN-γ
antibody treatment
+ P. acnes
Gbp2b
Relative gene expression
1.5
1
0.5
0
**
mouse IgG1
α-IFNAR1
rat IgG1
α-IFN-γ
+ P. acnes
D
Neutrophils
Cell number (× 10^5)
20
15
10
5
0
NS
IgG1
α-IFNAR1
α-IFN-γ
+ P. acnes

Figure 3

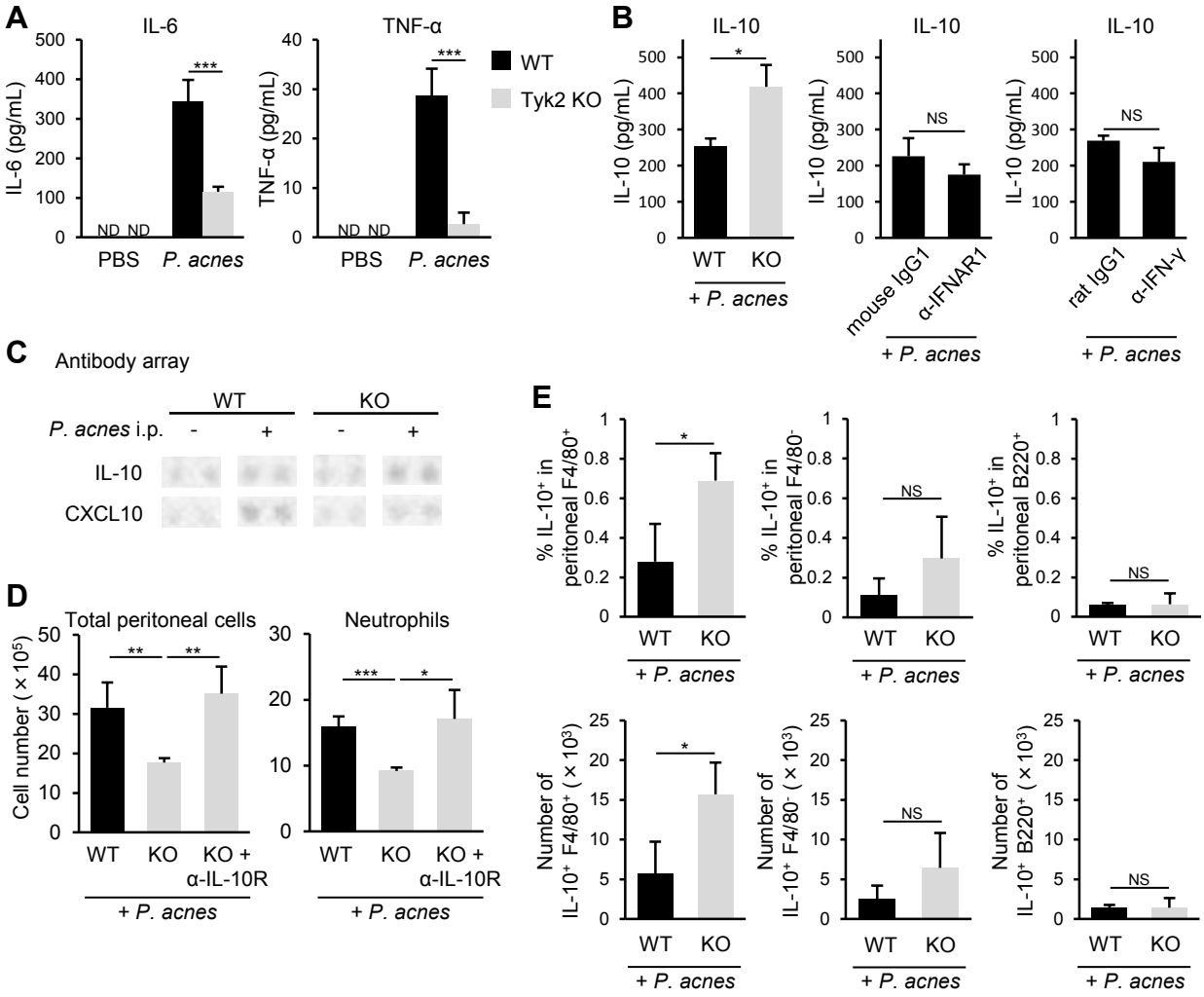

A
IL-6
TNF-α
IL-6 (pg/mL)
TNF-α (pg/mL)
WT
Tyk2 KO
ND ND
PBS
P. acnes
B
IL-10
IL-10 (pg/mL)
WT
KO
+ P. acnes
NS
mouse IgG1
α-IFNAR1
rat IgG1
α-IFN-γ
C
Antibody array
WT
KO
P. acnes i.p.
IL-10
CXCL10
D
Total peritoneal cells
Neutrophils
Cell number (×10⁵)
KO + α-IL-10R
E
% IL-10⁺ in peritoneal F4/80⁺
% IL-10⁺ in peritoneal F4/80⁻
% IL-10⁺ in peritoneal B220⁺
Number of IL-10⁺ F4/80⁺ (×10³)
Number of IL-10⁺ F4/80⁻ (×10³)
Number of IL-10⁺ B220⁺ (×10³)

Figure 4

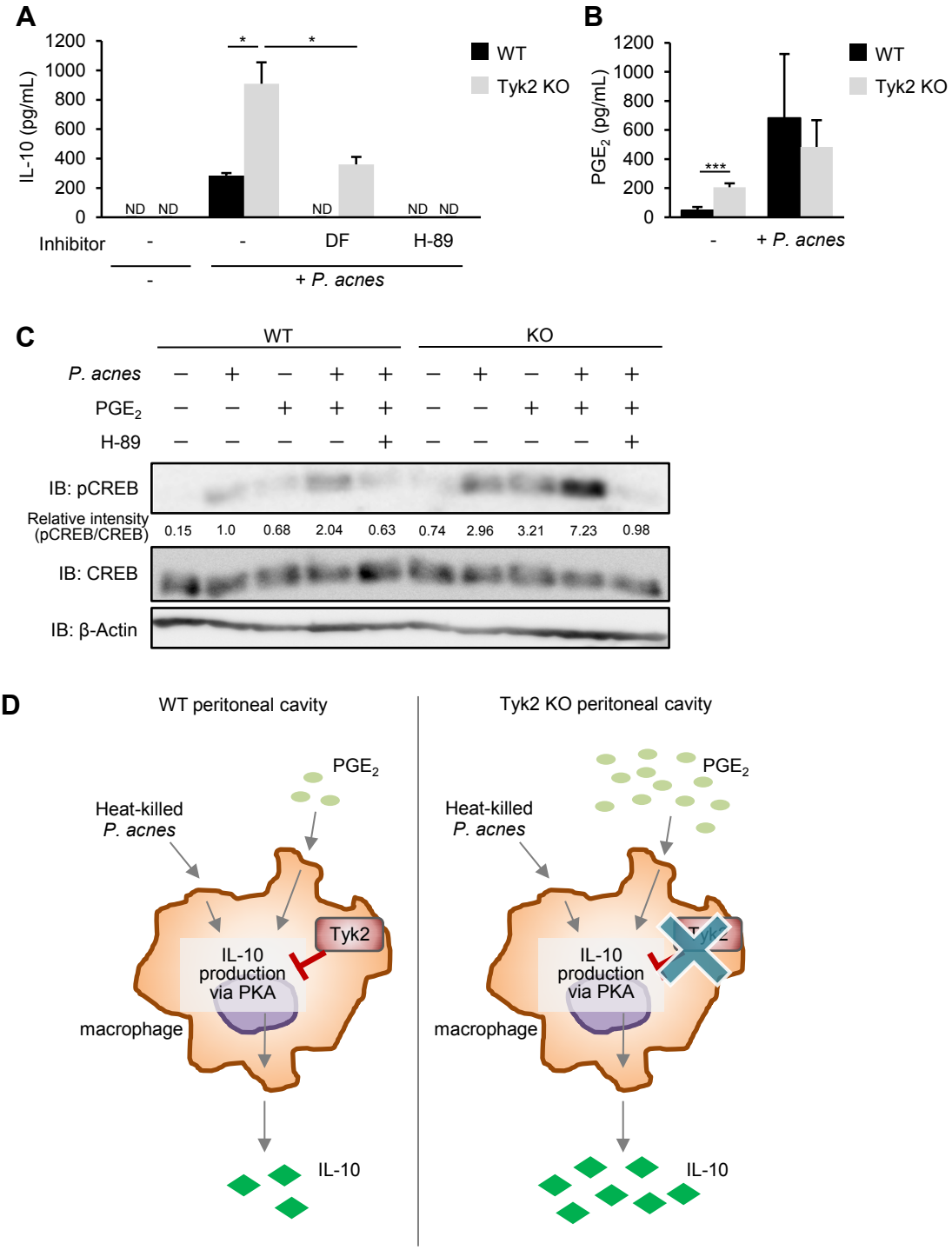

A
IL-10 (pg/mL)
1200
1000
800
600
400
200
0
*
*
ND ND
ND
ND ND
WT
Tyk2 KO
Inhibitor
-
-
DF
H-89
-
+ P. acnes
B
PGE2 (pg/mL)
1200
1000
800
600
400
200
0
***
WT
Tyk2 KO
-
+ P. acnes
C
WT
KO
P. acnes
PGE2
H-89
IB: pCREB
Relative intensity (pCREB/CREB)
0.15 1.0 0.68 2.04 0.63 0.74 2.96 3.21 7.23 0.98
IB: CREB
IB: β-Actin
D
WT peritoneal cavity
Tyk2 KO peritoneal cavity
PGE2
Heat-killed P. acnes
Tyk2
IL-10 production via PKA
macrophage
IL-10

Figure S1

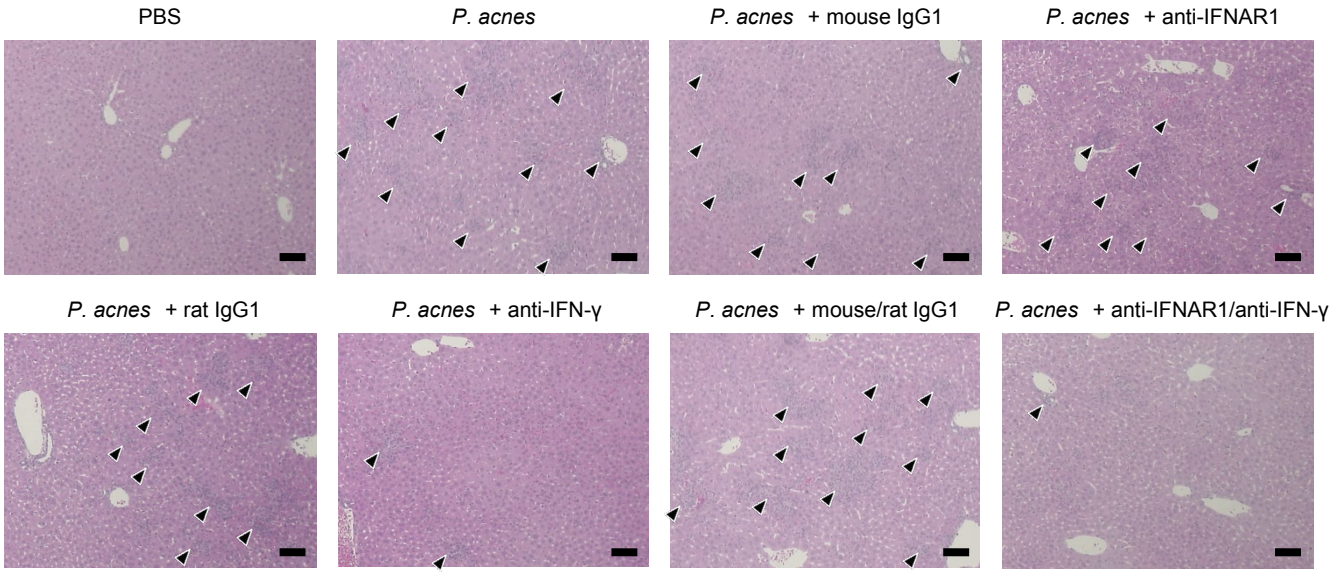


Figure S1: The effect of anti-IFNAR1 or anti-IFN-γ mAb on *P. acnes*-induced formation of hepatic granulomas. Representative hematoxylin and eosin-stained liver sections on day 7 after intraperitoneal injection of heat-killed *P. acnes* (500 μg) are shown. Arrowheads indicate granuloma formation. Scale bars, 100 μm.

Figure S2

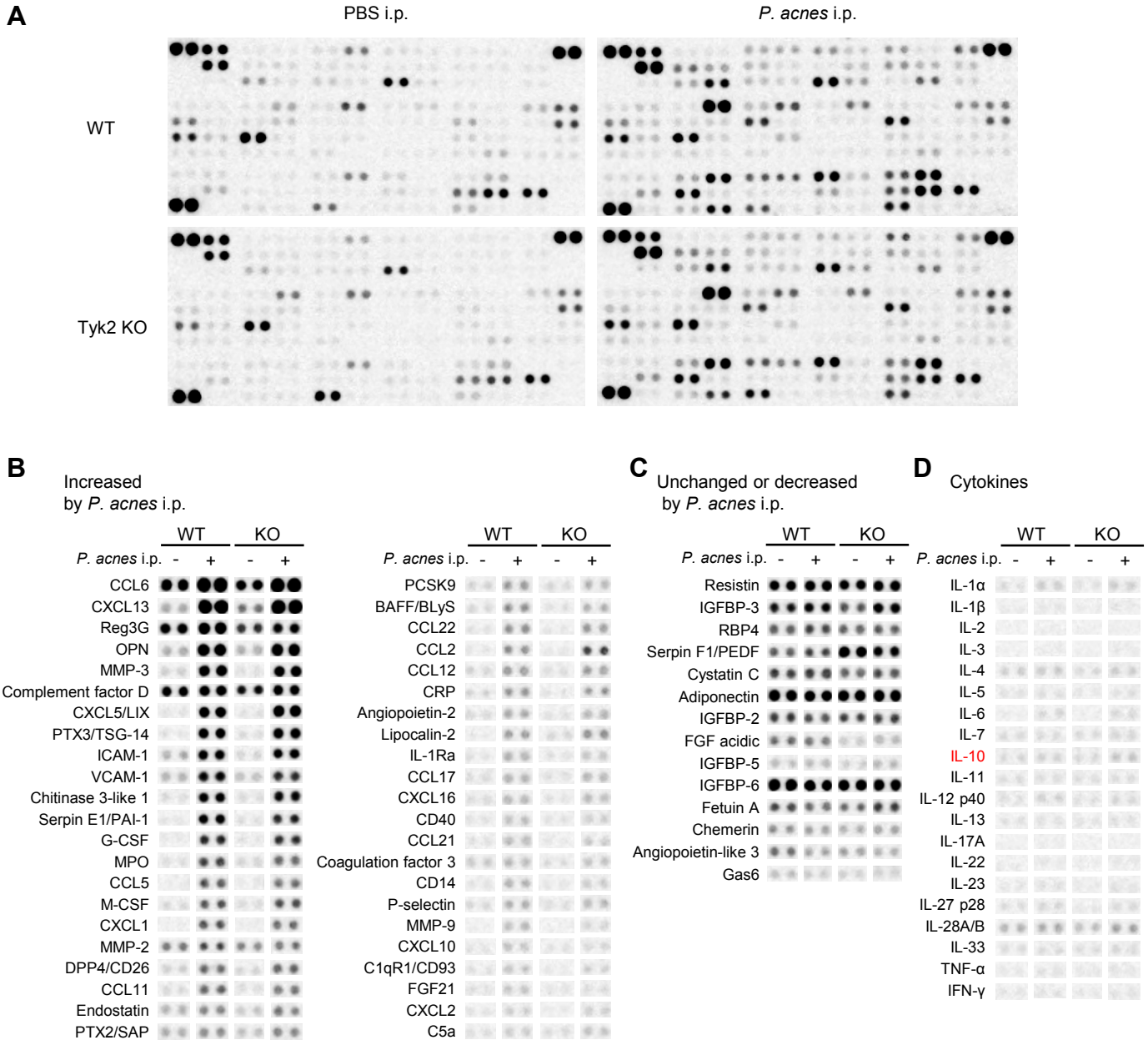


Figure S2: Mouse cytokine array panel showing differences in the level of soluble factors in peritoneal lavage of WT and Tyk2 KO mice 12 h after heat-killed *P. acnes* injection, determined by Proteome profiler antibody array. Peritoneal lavage sample of each group was collected from three mice and pooled for the assay. (A) The uncropped blot image for whole membranes. (B) *P. acnes*-induced soluble proteins. (C) Constitutively expressed proteins that were not upregulated by *P. acnes* injection. (D) Major Cytokines.