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## 4-1BBL regulates the polarization of macrophages and inhibition of 4-1BBL signaling alleviates imiquimod-induced psoriasis

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### Abstract

4-1BBL, a member of the tumor necrosis factor superfamily, regulates the sustained production of inflammatory cytokines in macrophages triggered by TLR signaling. In this study, we have investigated the role of 4-1BBL in macrophage metabolism and polarization, and in skin inflammation using a model of imiquimod (IMQ)-induced psoriasis in mice. Genetic ablation or blocking of 4-1BBL signaling by Ab or 4-1BB-Fc alleviated the pathology of psoriasis by regulating the expression of inflammatory cytokines associated with macrophage activation, and regulated the polarization of macrophages *in vitro*. We further linked this result with macrophage by finding that 4-1BBL expression during the immediate TLR response was dependent on glycolysis, mitochondrial oxidative phosphorylation and fatty acid metabolism, while the late phase 4-1BBL-mediated sustained inflammatory response was dependent on glycolysis and fatty acid synthesis. Correlating with this, administration of a fatty acid synthase inhibitor cerulenin also alleviated the pathology of psoriasis. We further found that 4-1BBL-mediated psoriasis development is independent of its receptor 4-1BB, as a deficiency of 4-1BB augmented the severity of psoriasis, linked to a reduced Treg population and increased IL-17A expression in  $\gamma\delta$  T cells. Additionally, co-blocking of 4-1BBL signaling and IL-17A activity additively ameliorated psoriasis. Taken together, 4-1BBL signaling regulates macrophage polarization and contributes to IMQ-induced psoriasis by sustaining inflammation, providing a possible avenue for psoriasis treatment in patients.

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#### CONFLICT OF INTEREST:

The authors have no financial conflicts of interest.

## Keywords

Rodent; Monocytes/Macrophages; Cell Surface Molecules; Inflammation; Skin

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## INTRODUCTION

We have previously identified a novel signaling mechanism involving two distinct and sequential signaling cascades that regulate the initiation and persistence of inflammation by TLR-activated macrophages (1). 4-1BBL, a member of the TNF superfamily, allows the sustained production of inflammatory cytokines driven by TLR signaling. For example, LPS-induced production of cytokines such as TNF, IL-6, IL-12, and IL-23 occurred for 24 h in wild-type macrophages, whereas 4-1BBL-deficient macrophages produced cytokines similar to WT cells for the first few hours after LPS treatment but then ceased production. Cytokine production was also significantly reduced when 4-1BBL KO macrophages were treated with other TLR ligands, but not by other cytokines such as IL-1 $\beta$ , indicating the specific role of 4-1BBL in TLR responses. Expression of 4-1BBL is induced immediately upon TLR signaling and it subsequently interacts with TLRs to allow secondary late-phase signaling. Notably, genetic ablation of 4-1BBL or inhibition of the TLR-4-1BBL interaction by antagonists such as 4-1BBL Ab or 4-1BB-Fc protein significantly reduced cytokine production by macrophages and improved survival in mouse models of sepsis (1–3). These data suggest a possible role for 4-1BBL in the development of inflammatory diseases linked to macrophage activity and that inhibition of 4-1BBL might be used for the treatment of such inflammatory diseases.

Cell metabolism plays a crucial role in determining the phenotypes of macrophages, and subsequent development of inflammatory diseases (4–8). Despite the heterogeneity of macrophages associated with diverse functions, two well-established phenotypes are often referred to as classically activated pro-inflammatory M1 and alternatively activated anti-inflammatory M2 macrophages that induce T<sub>H</sub>1 and T<sub>H</sub>2 responses, respectively. In addition to distinct functions and gene expression profiles, M1 and M2 macrophages exhibit contrasting metabolic activities. M1 macrophages rely on glycolysis for ATP generation partly due to the disrupted TCA cycle, and fatty acid synthesis is activated during inflammation (9–11). In contrast, M2 macrophages have an intact TCA cycle and use oxidative phosphorylation (OXPHOS) for energy generation (12–14), indicating that different aspects of cell metabolism can regulate macrophage polarization. Although many studies have shown the role of cell metabolism in the regulation of polarization and activation of macrophages in inflammatory disease development, how cell metabolism sustains macrophage activation and if this contributes to inflammatory disease has not been fully elucidated. Moreover, how 4-1BBL activity that drives sustained macrophage responses is linked to macrophage metabolism and polarization remain unknown.

In this study, we investigated the role of 4-1BBL in the polarization and metabolism of macrophages, and in skin inflammation using an IMQ-induced psoriasis mouse model. We find that 4-1BBL induces pro-inflammatory macrophage polarization and the development of psoriasis by contributing to the expression of inflammatory cytokines. We also find that

sustained inflammation mediated by 4-1BBL is regulated by glycolysis and fatty acid synthesis, indicating the distinct role of cell metabolism in the late phase of macrophage activation for sustaining inflammatory responses. Consequently, inhibition of fatty acid synthase by a pharmacological inhibitor alleviates psoriasis and reduces the expression of 4-1BBL. Additionally, co-blockade of 4-1BBL signaling and IL-17A activity additively ameliorates psoriasis, implying that combination treatment for blocking inflammatory cytokine activity could be an option for the treatment of diseases such as psoriasis.

## METHODS AND MATERIALS

### Reagents

2-Deoxyglucose, cerulenin, etomoxir, and rotenone were purchased from Calbiochem, and antimycin A was from Sigma. Recombinant murine IFN- $\gamma$ , murine IL-4, and murine M-CSF were obtained from Peprotech, and LPS (*E. coli* 0111:B4) was from List Biological Laboratories. Recombinant mouse 4-1BB conjugated with human hIgG<sub>2</sub>-Fc2 (4-1BB-Fc) was prepared as previously described (3), and agonist rat anti-mouse 4-1BB (clone 3H3, rat IgG2a) antibodies were prepared in-house. Endotoxin levels were tested (< 0.01 EU/ $\mu$ g). Blocking anti-mouse IL-17A (clone 17F3, IgG1,  $\kappa$ ) and anti-mouse 4-1BBL (clone TKS-1, Rat IgG2a,  $\kappa$ ) were purchased from BioXcell. Corresponding isotype antibodies were obtained from the same source.

### Animals

Sex-matched 8- to 12-week old male or female C57BL/6 mice were purchased from Jackson Laboratory and used as wildtype. 4-1BB-deficient mice ((15), C57BL/6 background) were bred at the La Jolla Institute for Immunology. Mice were housed in individually ventilated, pathogen-free cages with 12:12-h light-dark cycles, and fed *ad libitum*. Animal experiments were in compliance with the regulations of the Scripps Research Institute and the La Jolla Institute for Immunology Animal Care Committee in accordance with guidelines of the Association for the Assessment and Accreditation of Laboratory Animal Care.

### IMQ-induced psoriasis-like skin inflammation.

To induce skin inflammation, 62.5 mg of 5% IMQ cream (Aldara; 3M Pharmaceuticals) or control cream (Vaseline) were applied to the shaved dorsal skin of mice once daily for 7 days. Mice were anesthetized in an isoflurane chamber connected to an anesthetic machine, and injected i.p. with isotype Ab, anti-4-1BBL Ab, anti-IL-17A Ab (100  $\mu$ g/25g body weight), or 4-1BB-Fc (10  $\mu$ g/25g body weight) daily before applying IMQ. Dorsal skin areas were scored for signs of psoriasis-like inflammation such as scaling, erythema, and thickness on a scale from 0 (none) to 4 (very severe), and a cumulative severity score was calculated (scale 0–12), as previously described (16). Sera, lymph nodes, and dorsal skin specimens were collected at the end of the experiment (day 8). Experiments were performed in a double-blinded manner.

### Histology

Specimens from the dorsal skin were fixed in 10% neutral-buffered formalin, embedded in paraffin, and sectioned at 4  $\mu$ m followed by H & E staining.

## Cell culture

RAW 264.7 mouse macrophage cell line and immortalized WT or 4-1BBL-deficient macrophage cell lines (2) were cultured in DMEM supplemented with 10% FBS and antibiotics (Gibco). Peritoneal macrophages were obtained from peritoneum cavity of mice after the intraperitoneal injection of 4% thioglycollate. Bone marrow cells were obtained from femur and tibia of mice, and cultured in DMEM supplemented with 10% FBS, antibiotics, and 10 ng/ml M-CSF to differentiate into macrophages for 7 days. Mouse 4-1BBL-expressing adenovirus (Adv-4-1BBL) was kindly provided by Tania Watts (University of Toronto) and used to induce the overexpression of 4-1BBL in macrophages as in our previous publications (1, 2).

RAW 264.7 cells ( $5 \times 10^5$ /ml), WT or 4-1BBL-deficient macrophages ( $5 \times 10^5$ /ml), peritoneal macrophages ( $1 \times 10^6$ /ml), or BMDMs ( $1 \times 10^6$ /ml) were treated with LPS (100 ng/ml), IMQ (2  $\mu$ g/ml), LPS + IFN- $\gamma$  (100 g/ml), or IL-4 (10 ng/ml) for the indicated times for the preparation of RNAs, cell lysates, or culture supernatants. For the inhibition of cell metabolism, peritoneal macrophages were incubated with vehicle, 2-deoxyglucose (1 mM), rotenone (10  $\mu$ M), cerulenin (20  $\mu$ M), antimycin A (1  $\mu$ M), etoximir (100  $\mu$ M) or metformin (2 mM) for 1 h, and followed by LPS or IMQ stimulation, or Adv-4-1BBL infection ( $3 \times 10^4$  pfu/ $10^6$  cells).

## Reverse transcription and quantitative PCR

Total RNA was isolated from axillary draining lymph nodes, dorsal skins, macrophages, or cultured BMDMs using TRIzol reagent (Invitrogen). cDNA was prepared using a SuperScript IV reverse transcriptase kit (Thermo Fisher). Quantitative PCR was performed using PowerUp SYBR Green Master Mix (Thermo Fisher). Gene expression was calculated by normalizing to GAPDH mRNA levels. Primer sequences are provided in Supplemental Table 1.

## Flow cytometry

For intracellular staining of cytokines, cells from axillary lymph nodes were stimulated with 20 ng/ml of PMA and 1  $\mu$ g/ml of ionomycin in the presence of Monensin (Biolegend) for 6 h. Cells were incubated with anti-mouse CD16/CD32 Ab (clone 2.4G2, Tonbo Biosciences) to prevent non-specific binding, and further stained with CD45-AF700 (clone 30-F11, Biolegend), CD3-PerCP-Cy5.5 (clone 145-2C11, Biolegend), CD4-PB (clone GK1.5, Biolegend), and CD25-PE (clone PC61.5, Thermo Fisher) Abs followed by FoxP3-FITC Ab (clone FJK-16s, Thermo Fisher) using FoxP3/Transcription Factor staining kit (Thermo Fisher) to examine the intracellular FoxP3 expression, or CD45-AF700, CD3-PerCP-Cy5.5, CD4-PB, and  $\gamma\delta$  TCR-FITC (clone GL3, Biolegend) Abs followed by the intracellular staining using IL-17A-APC (clone TC11-18H10.1, Biolegend) and IFN- $\gamma$ -PE (clone XMG1.2, Biolegend) Abs. Isotype antibodies were purchased from the same source.

For the analysis of macrophage polarization, WT or 4-1BBL-deficient macrophages were incubated with anti-mouse CD16/CD32 Ab and followed by cell surface staining with CD11b-FITC (clone M1/70, Thermo Fisher), F4/80-PerCPCy5.5 (clone BM8, Thermo Fisher), CD11c-AF700 (clone N418, Thermo Fisher) and CD206-APC (clone C068C2,

BioLegend). After fixation and permeabilization, cells were stained intracellularly with NOS2 (iNOS)-PE eF610 (clone CXNFT, Thermo Fisher) and Egr2-PE (clone erongr2, Thermo Fisher).

Flow cytometry analysis was performed on an LSR-II Flow Cytometer and data were analyzed using FlowJo Software (version 10, FlowJo, LLC).

### Extracellular flux analysis

Metabolic changes were monitored by an extracellular flux analyzer XFe24 (Seahorse Bioscience). For optimization, 3 doses of cell densities and concentrations of FCCP (carbonyl cyanide-p-trifluoromethoxyphenylhydrazone) were tested in both stress tests for the best metabolic performance of macrophages, and 125,000 cells were determined to be the best concentration along with 1  $\mu$ M FCCP. Cells were resuspended in XF Base Media (DMEM, Seahorse Bioscience) supplemented with 2 mM glutamine, and seeded in a Seahorse Bioscience 24-well plate. After 3 h, cells were treated with medium, LPS + IFN- $\gamma$ , IMQ, or IL-4, and incubated for 24 h at 37°C in an atmosphere of 5% CO<sub>2</sub>. During extracellular flux analysis, cells were sequentially treated with 10 mM glucose, 2  $\mu$ M oligomycin A (OA), 1  $\mu$ M FCCP + 1 mM pyruvate, and 1  $\mu$ M rotenone + antimycin A (R/A) to measure the extracellular acidification rate (ECAR, glycolysis indication) and the oxygen consumption rate (OCR, mitochondrial respiration indication) following a previously published protocol (17).

### Short hairpin RNAs, lentivirus, and generation of *Glut1* or *Fasn* knockdown macrophages

Lentiviral vectors expressing the short hairpin RNAs (shRNAs) that target *Glut1* (clone ID TRCN0000079328) or *Fasn* (clone ID TRCN0000075703) genes were obtained from Sigma (MISSION<sup>®</sup> TRC shRNAs). Lentiviruses were prepared in 293T cells by co-transfection of shRNA-encoding plasmids and helper plasmids such as pRSV-REV, pMDLg and pVSV-G (Addgene).

To generate knockdown (KD) macrophage cells, RAW 264.7 cells were infected with scramble (control, cat # SHC 201), *Glut1*, or *Fasn* shRNAs lentiviruses. After infection, cells were further incubated with puromycin (5  $\mu$ g/ml, Invivogen), and KD of genes was confirmed by immunoblotting using rabbit anti-GLUT1 (Abcam) or mouse anti-Fasn (Santa Cruz Biotechnology) antibodies. GAPDH levels were detected as a loading control using anti-GAPDH antibody (Santa Cruz Biotechnology).

### Cytokine measurements

Concentrations of TNF, IL-23, IFN- $\gamma$  and IL-17A were measured by ELISA kits (Thermo Fisher).

### Intracellular succinate quantification

Succinate levels in macrophages were quantified using a colorimetric Succinate Assay Kit (Abcam) according to the manufacturer's protocol. WT or 4-1BBL-deficient macrophages were stimulated with PBS, LPS + IFN- $\gamma$ , or IMQ for 6 or 12 h, and cell lysates were prepared to determine the intracellular succinate levels.

## Statistical analysis

Statistical significance was analyzed by unpaired *t*-test for comparison of means between two independent groups or by one-way ANOVA followed by Tukey's post hoc multiple comparison test for differences of means between multiple groups using Prism 8 software (version 8, GraphPad, San Diego, CA). A *p* value <0.05 was considered statistically significant and can be found in the figure legends. For *in vitro* experiments, *n* = number of samples. For *in vivo* studies, *n* = number of animals in each group. Each experiment was repeated as indicated in the figure legends, and the representative results are shown.

## RESULTS

### Inhibition of 4-1BBL-mediated signaling alleviates IMQ-induced psoriasis in mice.

In our previous studies, we showed that inhibition of 4-1BBL signaling, by administration of blocking 4-1BBL Ab or antagonist 4-1BB-Fc, reduces the sustained inflammatory response of macrophages *in vitro* and alleviates the pathology of endotoxin-induced sepsis in mice, similar to the response of 4-1BBL-deficient macrophages or 4-1BBL-deficient mice (1–3). To extend this data, we assessed the role of 4-1BBL in skin inflammation by inhibiting 4-1BBL in a mouse model of psoriasis. Topical application of the TLR7/8 ligand IMQ induces psoriasis-like skin inflammation. In patients with psoriasis and in mice with IMQ-induced skin inflammation, inflammatory cells and mediators such as lymphocytes, macrophages, plasmacytoid DCs, IL-17A, IL-22, IL-23, and TNF all contribute to the pathology of disease (18). Histological analysis and severity scoring showed that skin inflammation was severe in IMQ-treated mice compared with control cream-treated mice, but IMQ-driven inflammation was significantly reduced by administration of 4-1BBL Ab or 4-1BB-Fc (Fig. 1 A&B). Expression of pro-inflammatory cytokines *Tnf*, *Nos2*, *Il17a*, and *Il23* was increased in draining lymph node (LN) cells and skin of IMQ-treated control Fc-injected mice, and was reduced by 4-1BBL Ab or 4-1BB-Fc administration. mRNA expression of *Il10*, *Arg1*, and *Tgfb* did not change in LN cells, while expression of these molecules was reduced in the skin tissues of 4-1BBL Ab or 4-1BB-Fc-injected mice (Fig. 1 C&D). IMQ treatment also induced the expression of *4-1BBL* in LN and skin tissues, and this was reduced by administration of 4-1BBL Ab or 4-1BB-Fc in LN, but not in skin. We further found that production of TNF, IL-23, and IL-17A in sera was significantly reduced by 4-1BBL Ab or 4-1BB-Fc administration, while IFN- $\gamma$  production was comparable between isotype Ab-, and 4-1BBL Ab or 4-1BB-Fc-injected mice (Fig. 1E). Collectively, our results suggest that blocking of 4-1BBL signaling ameliorates the pathology of psoriasis by regulating the expression of inflammatory genes associated with skin pathology.

### 4-1BBL regulates the polarization of macrophages.

Because we found that blocking 4-1BBL signaling reduced the expression of *Tnf*, *Nos2*, and *Il23* in IMQ-treated mice, molecules that are hallmark genes of M1 macrophages, we further tested whether 4-1BBL regulates the polarization of macrophages *in vitro*. WT or 4-1BBL KO macrophages (2) were treated with LPS plus IFN- $\gamma$  or IL-4 for 24 h. We found that a 4-1BBL-deficiency resulted in the reduced expression of *Tnf*, *Nos2*, *Il23*, *Il6*, and *Cxcl10* in LPS plus IFN- $\gamma$ -treated macrophages (M1). In contrast, the levels of expression of *Il10*,

*Arg1*, *Fizz1*, *Ym1*, *Egr2*, and *Mrc1* (*Cd206*) were increased in IL-4-treated 4-1BBL KO cells (M2) (Fig. 2A).

We further tested whether blocking 4-1BBL signaling by 4-1BB-Fc regulates the polarization of macrophages. M-CSF-cultured BMDMs from WT mice were treated with LPS plus IFN- $\gamma$  or IL-4. Similar to 4-1BBL KO macrophages, blocking of 4-1BBL resulted in the reduced expression of *Tnf*, *Nos2*, *Il23*, *Il6*, and *Cxcl10* in LPS plus IFN- $\gamma$ -treated BMDMs while IL-4-induced expression of *Il10*, *Arg1*, *Fizz1*, *Ym1*, *Egr2*, and *Mrc1* was increased compared with control Fc-treated macrophages (Fig. 2B).

We also found that IMQ, the TLR7/8 ligand that induces psoriasis-like skin inflammation, regulates the polarization of macrophages in vitro. Similar to LPS plus IFN- $\gamma$ -treated macrophages, IMQ treatment induced the expression of *Tnf*, *Nos2*, *Il23*, *Il6*, and *Cxcl10* in WT macrophages. However, a deficiency of 4-1BBL or blocking of 4-1BBL resulted in the reduced the expression of these inflammatory genes (Fig. 2 A&B). Additionally, the number of F4/80+CD11b+CD11c+ cells and intracellular NOS2 levels were significantly reduced in LPS plus IFN- $\gamma$ - or IMQ-treated 4-1BBL-deficient macrophages compared with WT cells, whereas those of F4/80+CD11b+CD206+ and expression of *Egr2* were rather increased in IL-4-treated 4-1BBL-deficient cells, further supporting the role of 4-1BBL in the regulation of macrophage polarization (Supplemental Fig. 1A).

Since the intracellular accumulation of the TCA cycle intermediate succinate is one of the most profound metabolic changes in M1-like polarized macrophages (19), we examined the changes in succinate levels in macrophages. Treatment with LPS plus IFN- $\gamma$  or IMQ increased the intracellular succinate levels in WT and 4-1BBL-deficient macrophages similarly for the first 6 h, while succinate levels were lower in 4-1BBL-deficient macrophages compared with WT cells after 12 h of stimulation (Supplemental Fig. 1B), indicating that 4-1BBL induced by TLR signaling regulates cell metabolism at the late phase of macrophage activation and polarization.

To further examine the role of 4-1BBL in macrophage polarization, we performed glycolysis stress tests using the Seahorse analyzer. Following glucose injection, a large increase in glycolysis was detected in LPS plus IFN- $\gamma$ - or IMQ-treated cells, with the level of increase being higher with LPS plus IFN- $\gamma$ . In contrast, 4-1BBL-deficient macrophages displayed significantly lower ECAR after glucose injection (Fig. 2C,  $p < 0.001$ , unstimulated vs. LPS plus IFN- $\gamma$ -stimulated;  $p < 0.005$ , unstimulated vs. IMQ-stimulated;  $p < 0.01$ , LPS plus IFN- $\gamma$ - vs. IMQ-stimulated;  $p < 0.005$ , LPS plus IFN- $\gamma$ -stimulated WT vs. 4-1BBL KO cells;  $p < 0.01$ , IMQ-stimulated WT vs. 4-1BBL KO cells). IL-4-polarized WT or 4-1BBL-deficient macrophages displayed lower rates of glycolysis compared with LPS plus IFN- $\gamma$ - or IMQ-treated M1-like cells (Fig. 2C,  $p < 0.001$ ).

Next, we determined potential changes in oxidative metabolism driven by 4-1BBL. LPS plus IFN- $\gamma$ - or IMQ-treated WT macrophages exhibited lower OCR compared with unstimulated or IL-4-stimulated cells and were less responsive to FCCP injection, while IL-4-stimulated macrophages displayed higher responses (Fig. 2D,  $p < 0.01$ , unstimulated WT vs. LPS plus IFN- $\gamma$ - or IMQ-stimulated WT or 4-1BBL KO;  $p < 0.005$ , unstimulated 4-1BBL KO vs. LPS

plus IFN- $\gamma$ - or IMQ-stimulated WT or 4-1BBL KO;  $p < 0.01$ , IL-4-stimulated WT vs. unstimulated WT;  $p < 0.001$ , IL-4-stimulated 4-1BBL KO vs. unstimulated 4-1BBL KO macrophages). In this case, a deficiency of 4-1BBL did not significantly change the OCR of LPS plus IFN- $\gamma$ - or IMQ-treated WT macrophages while the OCR of 4-1BBL-deficient macrophages significantly increased in response to FCCP in IL-4-stimulated cells compared with WT macrophages (Fig. 2D,  $p < 0.001$ , IL-4-stimulated 4-1BBL KO vs. IL-4-stimulated WT macrophages). Collectively, our data support a role of 4-1BBL in cell metabolism associated with macrophage polarization.

### **Glycolysis and fatty acid synthesis regulate the 4-1BBL-mediated sustained inflammatory responses in macrophages.**

Since 4-1BBL expression is initially induced in macrophages by TLR signaling (1, 2), we tested whether 4-1BBL expression driven by TLR signaling is regulated by cell metabolism. In macrophages, expression of *Tnf* and *4-1BBL* induced by LPS was significantly reduced by inhibiting glycolysis by 2-deoxyglucose (2-DG), fatty acid synthesis by cerulenin, fatty acid oxidation by etomoxir, electron transport chain (ETC) complexes by antimycin A or rotenone, or ETC/gluconeogenesis by metformin (Fig. 3A). The optimal concentration of each inhibitor was determined in our pilot experiment (data not shown) and similar to that used in other studies. We previously showed that induction of 4-1BBL is driven by the same signaling pathway that promotes *Tnf* expression (1) and these inhibitors similarly reduced *Tnf*. Additionally, IMQ-induced expression of *4-1BBL* mRNA and production of TNF were also reduced by these inhibitors in macrophages (Supplemental Fig. 2 A&B). These data indicate that cell metabolism regulates the expression of 4-1BBL during the early phase of TLR-mediated macrophage activation.

We further confirmed the role of glycolysis and fatty acid synthesis in the regulation of inflammatory responses by knocking down *Glut1* (glucose transporter 1) or *Fasn* (fatty acid synthase) (Supplemental Fig. 2 D & E). LPS plus IFN- $\gamma$ - or IMQ-induced expression of *Tnf*, *Il23*, *Il6*, and *4-1BBL* was reduced in *Glut1* or *Fasn* KD macrophages, while *Nos2* expression was reduced in *Glut1* KD macrophages but not in *Fasn* KD cells. In addition, IMQ stimulation did not induce the expression of *Cxcl10*, whereas LPS plus IFN- $\gamma$ -induced *Cxcl10* expression, which was reduced by *Glut1* or *Fasn* KD.

When 4-1BBL is induced in macrophages it then associates directly with TLRs to allow sustained cytokine production. Overexpression of 4-1BBL by infecting cells with 4-1BBL-encoding adenovirus can also result in this late phase signaling to maintain the inflammatory response (1, 2). Thus, we tested whether cell metabolism regulates TNF production following overexpression of 4-1BBL. We found that this was significantly reduced by 2-DG or cerulenin treatment, but not by the other inhibitors (Fig. 3B), suggesting that late phase 4-1BBL-mediated response is regulated by glycolysis and fatty acid synthesis. The latter was further investigated by timed treatment with these inhibitors. While LPS- or IMQ-induced TNF production was reduced in macrophages preincubated with all of the inhibitors, addition of only 2-DG or cerulenin after 4 h of stimulation blocked TNF production (Fig. 3 C&D and Supplemental Fig. 2C).



Next, we compared the expression levels of essential genes in the cell metabolism pathways to examine whether blocking of 4-1BBL signaling affected the expression in psoriasis. *Glut1* facilitates the transport of glucose across the plasma membranes of mammalian cells for the conversion of glucose to pyruvate for energy generation via citric acid cycle in mitochondria, to lactate in the presence of oxygen (Warburg Effect), or back to carbohydrate or fatty acids. Pyruvate dehydrogenase kinase 1 (*Pdk1*) and pyruvate dehydrogenase-alpha 1 (*Pdha1*) regulate the further conversion of pyruvate into acetyl-CoA. ATP citrate lyase (*Acly*) links glycolysis and fatty acid synthesis by converting citrate from glycolysis to acetyl-CoA for fatty acid synthesis. *Fasn* is a critical enzyme in fatty acid synthesis that catalyzes the synthesis of palmitate from acetyl-CoA and malonyl-CoA in the presence of NADPH (5, 6, 20). Expression of *Fasn* was significantly reduced in LN or skin of 4-1BBL Ab- or 4-1BB-Fc-injected IMQ-treated mice, while *Glut1* levels were comparable (Supplemental Fig. 3). Expression of *Pdk1*, *Pdha1*, and *Acly* was not induced in IMQ-treated LN or skin, and inhibition of 4-1BBL did not affect the expression (Supplemental Fig. 3). Collectively, these results suggest a role for specific macrophage metabolic pathways in sustaining inflammatory cytokine production upstream and downstream of 4-1BBL.

### Inhibition of *Fasn* activity ameliorates psoriasis

A recent study showed the contribution of *Glut1* to psoriasis development (21) as *Glut1* inactivation by an inhibitor WZB117 reduced the severity of psoriasis in mice. Based on our results above, we then tested whether inhibition of *Fasn* activity using cerulenin can also reduce the severity of IMQ-induced psoriasis. Interestingly, IMQ-induced skin inflammation was less severe in cerulenin-administered mice compared with vehicle-injected animals, indicating the contribution of fatty acid synthesis to the pathology of psoriasis (Fig. 4 A&B). IMQ-induced expression of *Tnf*, *Nos2*, and *Il17a* was also significantly reduced in LN and skin by cerulenin administration, whereas expression of *Il23*, *Il6*, *Il10*, and *Tgfb* was reduced in LN, but not in the skin (Fig. 4 C&D). It should be noted that cerulenin reduced the expression of 4-1BBL in LN, but not in the skin of IMQ-treated mice, indicating the tissue-specific role of fatty acid synthesis for the expression of 4-1BBL in psoriasis. Furthermore, IMQ-induced expression of *Fasn*, not *Glut1*, was significantly reduced by cerulenin administration (Fig. 4 E&F). Cerulenin injection from day 4 onwards also reduced skin inflammation, although the effect was lower and the reduction of inflammatory cytokine expression was less than cerulenin administration from day 1 (Fig. 4 A–F). Collectively, our data suggest that fatty acid synthesis contributes to the development of psoriasis both during the initiation of disease as well as once disease is established.

### 4-1BB deficiency augments the severity of IMQ-induced psoriasis

Previous studies have demonstrated that 4-1BBL acts as a costimulatory molecule by binding to its receptor 4-1BB on activated T cells, but only a moderate amount of data suggests that this 4-1BB-dependent T cell activation by 4-1BBL plays a role in inflammatory disease (22, 23). In contrast, emerging evidence suggests a role for 4-1BBL in some inflammatory diseases independent of its interaction with 4-1BB (1–3). To expand on this concept, WT or 4-1BB-deficient mice were treated with IMQ, and we found that skin inflammation was actually more severe in 4-1BB KO mice compared with WT mice (Fig. 5

A&B). Expression of *Nos2*, *Il17a*, *Il23*, and *Il6* was increased while *Tnf* expression was not affected in LN cells of IMQ-treated 4-1BB KO mice; however, that of *Il10*, *Arg1*, and *Tgfb* was reduced in LN cells of 4-1BB KO mice (Fig. 5C). In the skin tissues of 4-1BB KO mice, expression of *Tnf*, *Nos2*, *Il17a*, *Il23*, *Il6*, *Il10*, and *Arg1* was significantly increased while *Tgfb* expression was reduced compared with WT skin (Fig. 5D).

Additionally, the serum level of IL-17A was increased in 4-1BB KO mice compared with WT mice at day 8 (Fig. 5E). Expression of IFN- $\gamma$  and IL-17A in  $\gamma\delta$  T cells in LN that play a pivotal role in skin inflammation (24) were higher in IMQ-treated 4-1BB KO mice than WT mice at day 4 (Fig. 5F). Treg cells are critical for the regulation of inflammatory disease development, including in psoriasis (25, 26), and we found that the population of CD4+CD25+Foxp3+ Treg cells was significantly reduced in LN of IMQ-treated 4-1BB-deficient mice compared with WT mice (Fig. 5G). This supports the role of 4-1BB in promoting Treg that can suppress inflammatory diseases as previously reported (27, 28), and suggests that the activity of 4-1BBL in contributing to psoriasis is independent of its interaction with 4-1BB.

### **Administration of agonist 4-1BB Ab alleviates the severity of IMQ-induced psoriasis by increasing Treg cells.**

Previous studies have shown that agonist 4-1BB Ab can improve the pathology of several inflammatory and autoimmune diseases with the prevailing view being that it expands Treg cells (28–31). For example, in EAE models, administration of agonist 4-1BB Ab prevented the development of disease by regulating the balance between T<sub>H</sub>17 and Treg cells (32, 33).

In agreement with a previous study (34), we also found that administration of agonist 4-1BB Ab significantly alleviated the severity of IMQ-induced skin inflammation (Fig. 6 A&B). Expression of *Il17a* in LN and skin, and production of IL-17A in sera were reduced while *Tnf* and *Il23* expression was comparable between isotype- and 4-1BB Ab-injected mice (Fig. 6 C–E). However, expression of IL-17A in CD4+ or  $\gamma\delta$  T cells in LN was comparable between isotype- or 4-1BB Ab-administered mice at day 4 of IMQ-treatment (Fig. 6F). The number of CD4+CD25+Foxp3+ Treg cells was significantly increased in LN of 4-1BB Ab-injected mice compared with isotype Ab-administered mice (Fig. 6G), suggesting a crucial role of 4-1BB signaling in Treg activation for the regulation of IL-17A expression in  $\gamma\delta$  T cells later stage in the development of psoriasis. Taken together, our data suggest that 4-1BBL and 4-1BB play opposite roles in the development of psoriasis, as 4-1BBL promotes while 4-1BB inhibits IMQ-induced skin inflammation.

### **Co-administration of 4-1BB-Fc and IL-17A Ab further ameliorates the severity of skin inflammation.**

Inflammatory cytokines such as TNF, IL-23, and IL-17A play a crucial role in the pathogenesis of inflammatory diseases, and therapeutic strategies targeting cytokines have been found to be effective for the treatment of psoriasis (35–39). As we found that inhibition of 4-1BBL signaling by 4-1BB-Fc significantly alleviated the pathology of psoriasis, we further tested whether co-blockade of 4-1BBL and IL-17A could exert a greater effect on the treatment of IMQ-induced psoriasis. Histological analysis and severity scoring showed that

skin inflammation was significantly reduced in 4-1BB-Fc- or IL-17A Abs-treated mice, while co-administration of 4-1BB-Fc and IL17A Ab reduced skin inflammation more effectively compared with administration of either alone (Fig. 7 A&B).

Administration of 4-1BB-Fc, IL-17 Ab, or the combination reduced the expression of inflammatory cytokines in skin tissues of IMQ-treated mice (Fig. 7C). 4-1BB-Fc or 4-1BB-Fc with anti-IL-17A administration was more effective than the anti-cytokine Ab in regulating the expression of *Tnf*, *Il23*, and *Il17a*, while IL-17A Ab administration did not affect the expression of *Il23*. Furthermore, expression of cytokines was reduced more by 4-1BB-Fc plus anti-IL-17A treatment than that by the treatment of either alone.

These results indicate the possibility that co-inhibition of 4-1BBL signaling and cytokine activity can be a highly effective anti-inflammation strategy for the treatment of psoriasis and possibly other inflammatory diseases.

## DISCUSSION

Inflammation is a beneficial process to protect hosts from microbial infection and tissue damage, thereby maintaining homeostasis (40, 41). However, uncontrolled, excessive and sustained inflammation causes the development and progression of a range of inflammatory diseases (35, 42). Sustained inflammation is not the repetition of acute inflammation; it is distinct in a variety of pathological conditions, and its biological complexity limits the development of anti-inflammatory therapies (43). We have previously identified a novel signaling mechanism involving two distinct and sequential signaling cascades that regulate the initiation and persistence of inflammation in macrophages (1). TLR signaling drives the early response through MyD88/Trif-mediated NF- $\kappa$ B and MAPK signaling pathways and then 4-1BBL regulates the sustained production of inflammatory cytokines via binding to TLRs and promoting the MAPKs and protein kinase signaling pathways independently of NF- $\kappa$ B activation (1, 2). In line with this, here and in previous studies, we found that a deficiency or blocking 4-1BBL signaling alleviates several inflammatory conditions by dampening the sustained production of inflammatory cytokines (1–3). Thus, our results support the 4-1BBL signaling pathway as a target of anti-inflammation treatments in situations where TLR are active.

The anti-inflammatory activity of a 4-1BB-Fc fusion protein is achieved by inhibiting the oligomerization of TLRs and 4-1BBL (1–3), thereby alleviating sepsis and psoriasis in mice. Compared to a blocking anti-4-1BBL Ab, 4-1BB-Fc exhibited higher anti-inflammatory activity and binding affinity (~ 1000 fold) to 4-1BBL, while they share the same binding epitope of 4-1BBL. Thus, 4-1BB-Fc reduced the severity of skin inflammation more effectively. Most importantly, blocking of 4-1BBL significantly reduced the production of inflammatory cytokines such as TNF and IL-23 by macrophages. This is similar to the response of 4-1BBL-deficient macrophages. Thus we suggest that inhibition or blocking of 4-1BBL in innate immune cells reduces the production of pro-inflammatory cytokines that subsequently leads to dampened induction of other inflammatory cytokines, like IL-17 in psoriasis, that are key features of disease.

Cell metabolism plays a crucial role in determining the phenotypes of macrophages, and the development of inflammatory diseases (8–14). Previous studies have shown the role of cell metabolism in the polarization of macrophages in inflammatory diseases. However, how metabolic changes contribute to sustained inflammatory responses in macrophages and subsequent development of inflammatory disease are not fully elucidated. Our study demonstrates that genetic ablation of 4-1BBL or blocking of 4-1BBL signaling resulted in the reduced expression of pro-inflammatory genes associated with polarization of macrophages to the M1 phenotype. The metabolic impact of 4-1BBL in macrophages was demonstrated in that a 4-1BBL deficiency dramatically blunted glycolysis, as measured by ECAR, that was increased by TLR stimulation. OCR measurements indicated that respiration was substantially increased by the lack of 4-1BBL in the uncoupled state after FCCP injection in IL-4-stimulated 4-1BBL-deficient macrophages compared with WT cells. Thus, our data suggest that 4-1BBL contributes to the regulation of macrophage metabolism and polarization. Additionally, stimulation of macrophages with LPS or IMQ increased the intracellular levels of succinate, an indicative metabolite of M1-like polarized macrophages. 4-1BBL deficiency resulted in reduction of intracellular levels after 12 h while the accumulation of succinate was comparable between WT and 4-1BBL-deficient macrophages at early times after stimulation, further linking late 4-1BBL activity to metabolic changes and the polarization of macrophages.

Metabolic activity was also involved in TLR-mediated expression of 4-1BBL, as this was substantially reduced by a variety of inhibitors or knocking down of metabolism genes. However, the late phase of macrophage activation driven by 4-1BBL was also found to be dependent on glycolysis and fatty acid synthesis, showing the distinct role of cell metabolism in sustaining as well as initiating the inflammatory response of macrophages.

Although inhibition of glycolysis or genetic modification of *Glut1* regulates the pro-inflammatory responses in macrophages, a previous study showed that treatment with the glycolysis inhibitor 2-DG reduced IL-1 $\beta$ , while *Tnf* expression was not affected in macrophages (19), which is inconsistent with our finding. Additionally, a recent study showed that IL-1 $\beta$  expression was rather increased while expression of TNF was not affected in LPS plus IFN- $\gamma$ -stimulated *Gut1*-deficient macrophages (44). However, inhibition or knockdown of *Glut1* reduced the expression of *Tnf* in LPS-treated macrophages in our system. In contrast, expression of *Nos2* was significantly reduced by knockdown or genetic ablation of *Glut1* in this and another study (44). Most likely this inconsistency is due to the experimental conditions of each study, such as the use of primary macrophages or cell lines, and the use of chemicals, or shRNA or genetic deletion of genes. Further studies will help us to better understand the inconsistency.

IMQ-induced expression of *Fasn* was downregulated by blocking of 4-1BBL signaling, implicating the fatty acid synthesis pathway in psoriasis. *Fasn* is expressed strongly in normal and inflamed human epidermis including psoriasis, and it may regulate epidermal differentiation (45). Additionally, inhibition of acetyl-CoA carboxylase that is an enzyme of the fatty acid synthesis pathway decreased IL-17-expressing T cell differentiation and IL-17 production by murine T cells (46), further suggesting a role of fatty acid synthesis in Th17 responses and inflammatory disease development (47). Inhibition of *Fasn* activity with

cerulenin ameliorated psoriasis significantly by regulating the expression of inflammatory cytokines, including *Tnf*, *Nos2*, *Il23*, *Il6*, and *Il17a* in LN. Interestingly, cerulenin has been shown to suppress colorectal cancer development and liver metastasis (48–50), and reverse hepatic steatosis in obese mice (51). In innate immunity, cerulenin increases survival and decreases bacterial load in *S. aureus*-induced sepsis (52) and alleviates LPS-induced sepsis by suppressing the pro-inflammatory activation of macrophages (53, 54). However, cerulenin has side effects in cancer patients, such as anorexia and body weight loss due to a neuronal effect that reduces appetite (55). In mice, cerulenin did not show any detectable toxicity (54), but the next generation of Fasn inhibitors is currently being developed to avoid the adverse effect (56, 57).

A recent study has shown that Glut1 contributes to psoriasis development (21). Both genetic and pharmacological Glut1 inactivation by an inhibitor WZB117 reduced skin inflammation, suggesting that Glut1 is required for inflammation-associated keratinocyte proliferation. Although the expression of *Glut1* was not affected by blocking 4-1BBL signaling in our study, inhibition of Glut1 might also inhibit glucose uptake in other cell types during skin inflammation, including immune cells, and thereby limit inflammation as previously reported roles for Glut1 and glucose metabolism in macrophages and T cells (44, 58, 59).

As 4-1BBL binding to its receptor 4-1BB on activated T cells has been shown to enhance T cell activity in a number of systems, this suggests that 4-1BB interaction with 4-1BBL could be involved in the development of some autoimmune or inflammatory diseases (22, 23). However, we found different phenotypes in 4-1BBL and 4-1BB knockout mice suggesting that 4-1BB and 4-1BBL can regulate the psoriasis inflammatory response independently. Specifically, genetic ablation of 4-1BB resulted in more severe skin inflammation and correspondingly stimulation of 4-1BB by agonist Ab alleviated pathology. 4-1BB deficiency increased the expression of inflammatory genes and reduced the Treg population in LN and skin of IMQ-treated mice, while the agonist Ab showed the opposite effect consistent with a previous report (34).

Inflammatory cytokines such as TNF, IL-23, and IL-17A play a crucial role in the pathogenesis of psoriasis and other diseases. However, despite the benefit of the current therapies targeting these cytokines in treating patients, a significant proportion fail to respond to the treatment and may suffer side effects (35–37, 60–65). Therefore, development of alternate therapeutic strategies is still needed. While the current therapeutics directly inhibit the activities of these individual cytokines, our strategy of targeting 4-1BBL may inhibit the production of multiple cytokines like TNF and IL-23 made by innate immune cells. Moreover, the combination of 4-1BB-Fc and IL-17A Ab was more effective in blocking psoriasis presumably by shutting down both the production of inflammatory cytokines and the activity of existing cytokines. Thus, combination therapy could be an advanced option for the treatment of psoriasis patients.

In summary our data show that 4-1BBL contributes to the pathology of psoriasis, and promotes macrophage M1 polarization, dependent on glycolysis and fatty acid synthesis. Our data suggest that fatty acid synthesis can be a potential target for the treatment of this

disease, and that combination therapy with 4-1BB-Fc and anti-cytokine Abs may exhibit greater effectiveness.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Abbreviations used in this article,

<b>IMQ</b>	imiquimod
<b>TCA cycle</b>	tricarboxylic acid cycle
<b>OXPHOS</b>	oxidative phosphorylation
<b>WT</b>	wild-type
<b>KO</b>	knockout
<b>BMDM</b>	Bone marrow-derived macrophages
<b>M-CSF</b>	Macrophage Colony Stimulating Factor
<b>2-DG</b>	2-deoxyglucose
<b>Ceru</b>	Cerulenin
<b>ETO</b>	etomoxir
<b>ETC</b>	electron transport chain
<b>AA</b>	antimycin A
<b>Rot</b>	rotenone
<b>Met</b>	metformin

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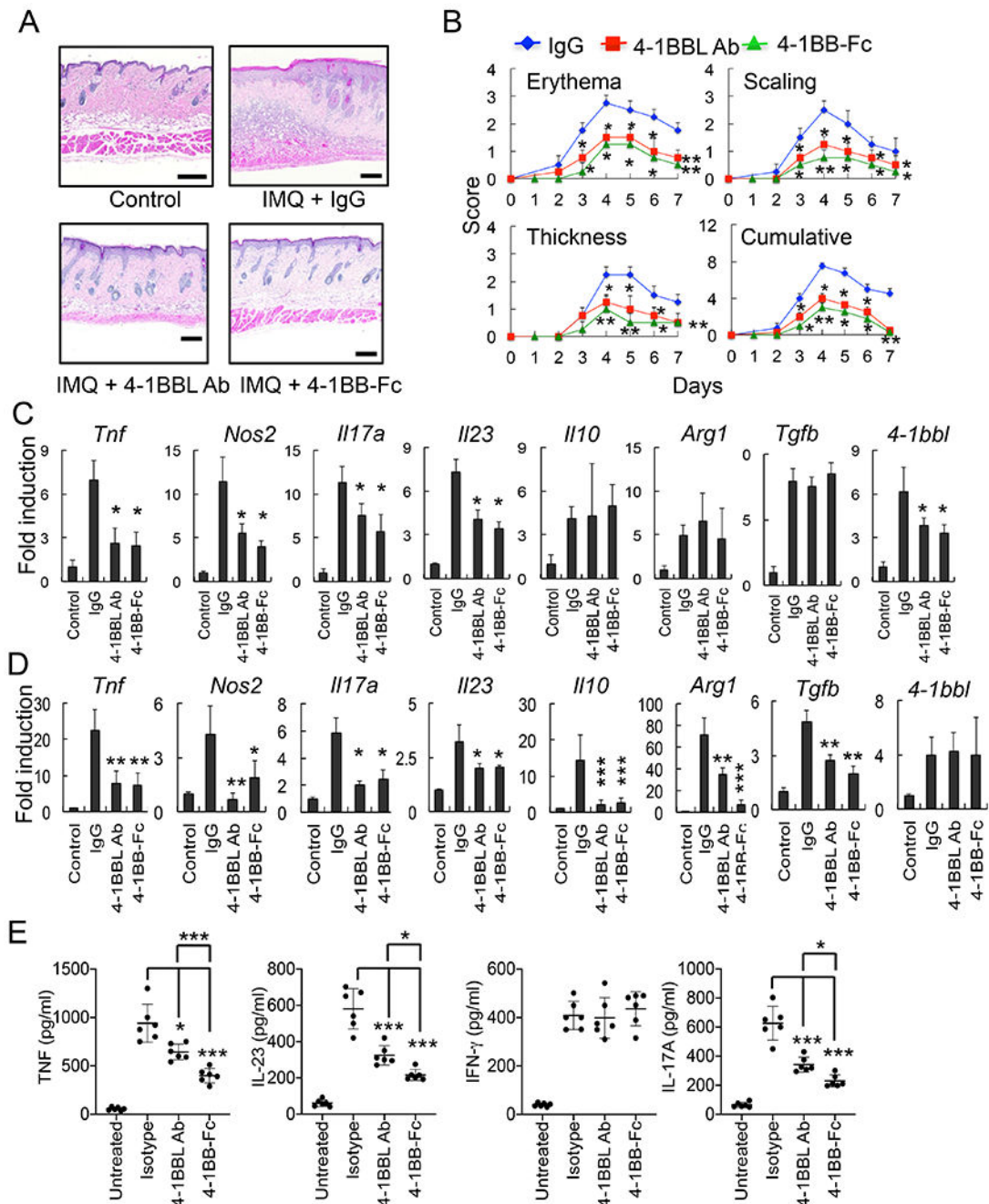


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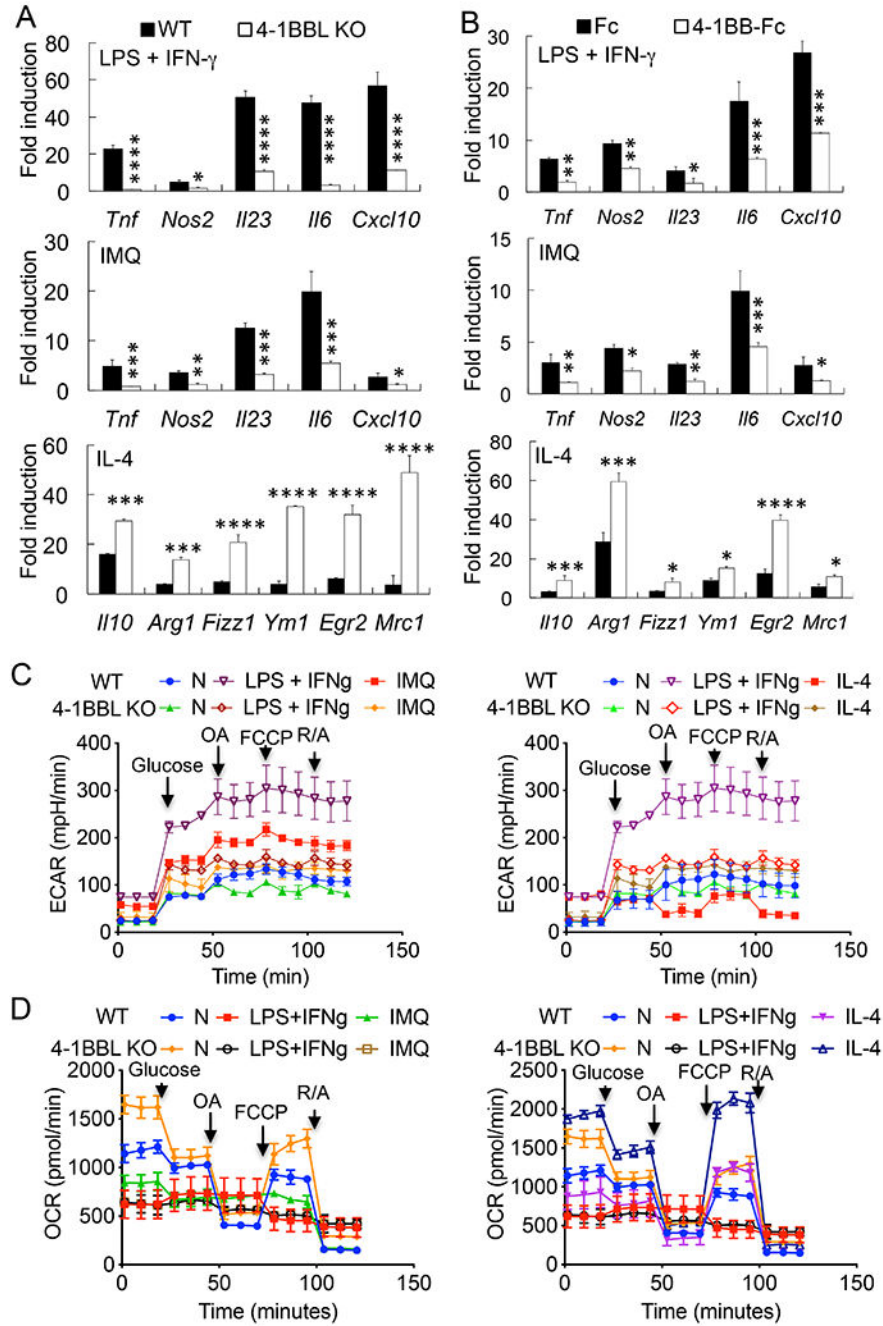
**KEY POINTS**

1. 4-1BBL signaling regulates metabolism and polarization in macrophages.
2. Blocking 4-1BBL signaling alleviates the severity of psoriasis in mice.
3. Targeting 4-1BBL signaling can be an option for treating psoriasis in patients.

**Figure 1.**

Inhibition of the 4-1BBL-mediated signaling alleviates the IMQ-induced psoriasis in mice. C57BL/6 mice (6 mice per group) were injected i.p. with isotype IgG (100  $\mu$ g), 4-1BBL Ab (100  $\mu$ g) or 4-1BB-Fc (10  $\mu$ g) daily, and treated with IMQ cream or control cream on the shaved back skin for 7 days. *A*, H&E staining of the mouse back skin. *B*, Erythema, scaling, and thickness of the back skin was scored daily and the cumulative score is depicted. *C* & *D*, RNA samples were prepared from LNs or skin tissues on day 8, and expression of cytokine genes in LNs (*C*) and skin (*D*) was analyzed by qPCR (6 mice per group). Control cream-

treated mice were used as a control. *E*, Sera were collected from mice after 5 days, and cytokine levels were measured by ELISA (6 mice per group). Data are shown as mean  $\pm$  SD. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\* $p < 0.005$ . Scale bar, 200  $\mu\text{m}$ . Result shown is representative of repeated experiments.



**Figure 2.** 4-1BBL regulates the macrophage metabolism and polarization. *A*, WT or 4-1BBL-deficient macrophages were treated with LPS (100 ng/ml) + IFN- $\gamma$  (100 ng/ml), IMQ (2  $\mu$ g/ml), or IL-4 (10 ng/ml) for 24 h, or *B*, WT BMDMs were cultured in M-CSF, and treated with LPS + IFN- $\gamma$ , IMQ, or IL-4 with Ig Fc or 4-1BB-Fc (5  $\mu$ g/ml) for 24 h. Gene expression was analyzed by qPCR (n = 4). Fold induction was compared to each unstimulated control. *C* & *D*, Cell metabolism analysis. Glycolysis (*C*) and mitochondrial (*D*) stress tests were used to measure the contribution of 4-1BBL to macrophage metabolism. Unstimulated (N), LPS +

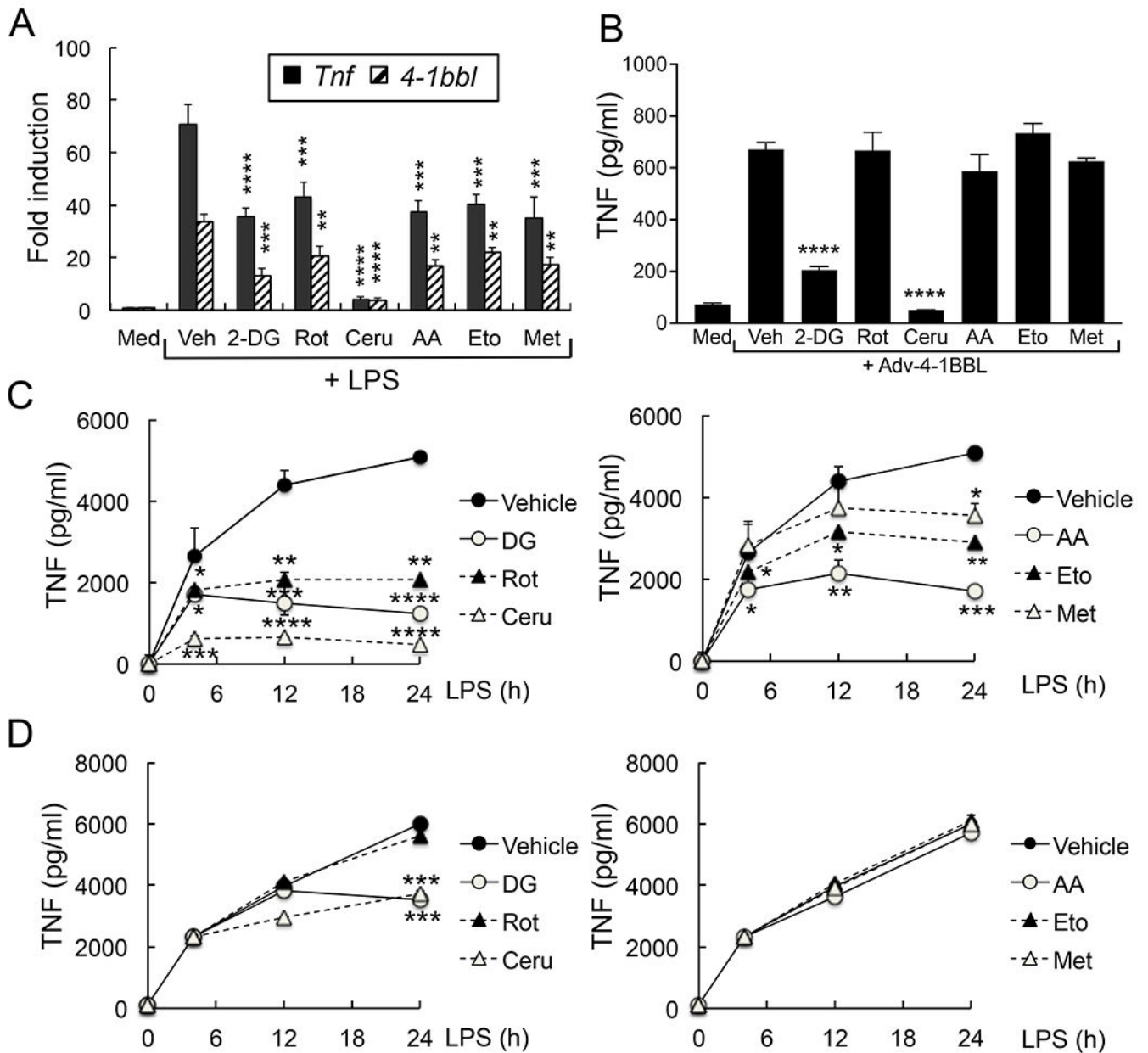
IFN- $\gamma$ -, IMQ-, or IL-4-stimulated WT or 4-1BBL-deficient (KO) macrophages were analyzed on a Seahorse XFe24 Extracellular Flux Analyzer (n=3). Data are shown as mean  $\pm$  SD. \*, p<0.05; \*\*p<0.01; \*\*\*p<0.005; \*\*\*\*p<0.001. Statistical analysis of C & D is described in the text. Result shown is representative of 3 (A & B) or 2 (C) independent experiments.

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**Figure 3.** Macrophage metabolism regulates the 4-1BBL-mediated sustained inflammatory responses. *A&B*, Peritoneal macrophages ( $10^6$  cells/ml) were incubated with vehicle, 2-deoxyglucose (2-DG, 1 mM), rotenone (Rot, 10  $\mu$ M), cerulenin (Ceru, 20  $\mu$ M), antimycin A (AA, 1  $\mu$ M), etomoxir (Eto, 100  $\mu$ M) or metformin (Met, 2 mM) for 1 h. Cells were further stimulated with LPS (100 ng/ml) and 4-1BBL expression was analyzed by qPCR after 4 h (A,  $n = 4$ ), or infected with Adv-4-1BBL ( $3 \times 10^4$  pfu/ $10^6$  cells). TNF levels in culture supernatant were measured by ELISA after 24 h (B,  $n = 4$ ). *C & D*, Peritoneal macrophages were incubated with inhibitors 1 h before (C) or 4 h after (D) LPS stimulation. TNF levels in culture supernatants were measured at the indicated times by ELISA. Fold induction was compared



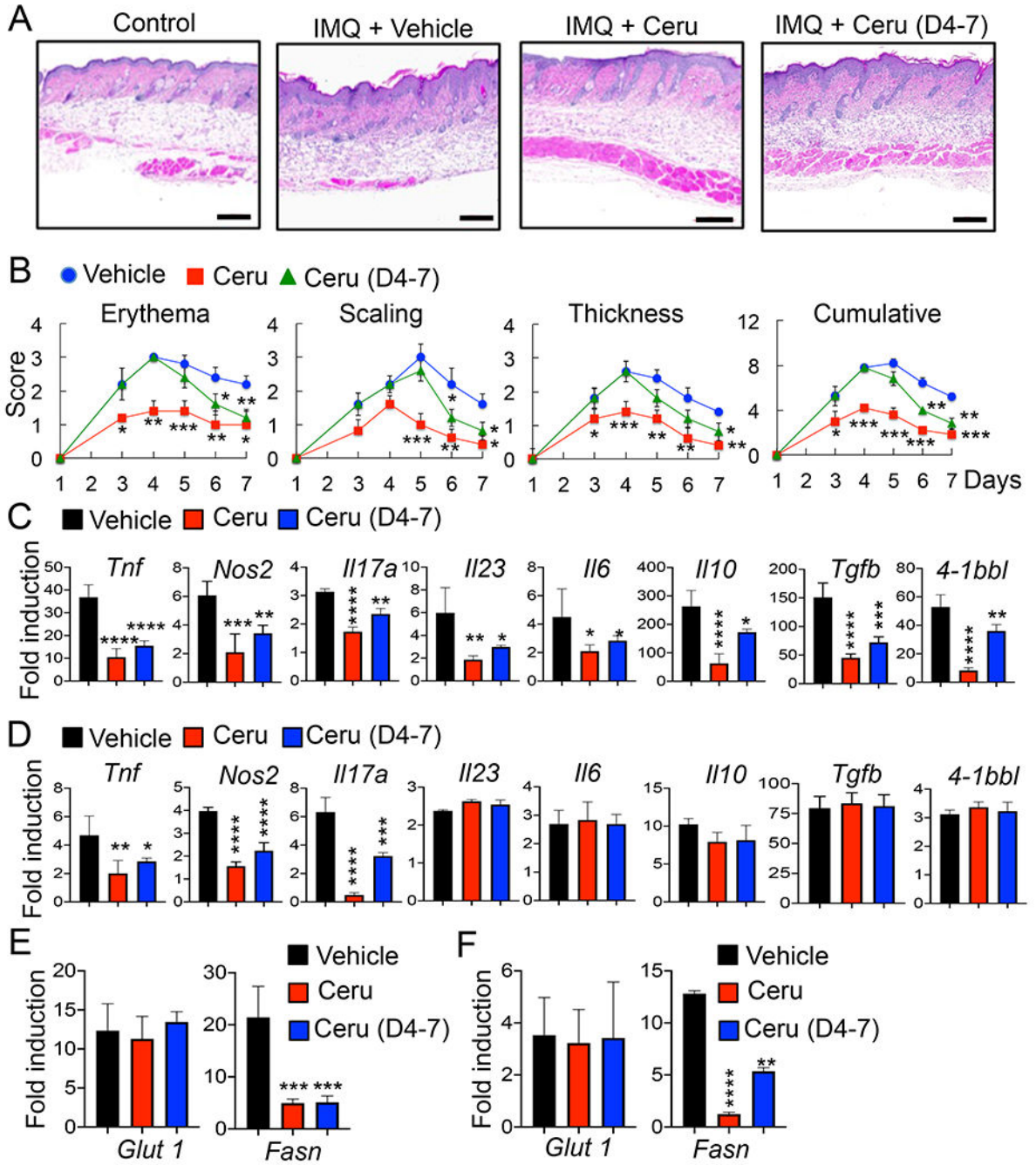
to unstimulated control. Data are shown as mean  $\pm$  SD. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\* $p < 0.005$ ; \*\*\*\* $p < 0.001$ . Representative result of 3 independent experiments is shown.

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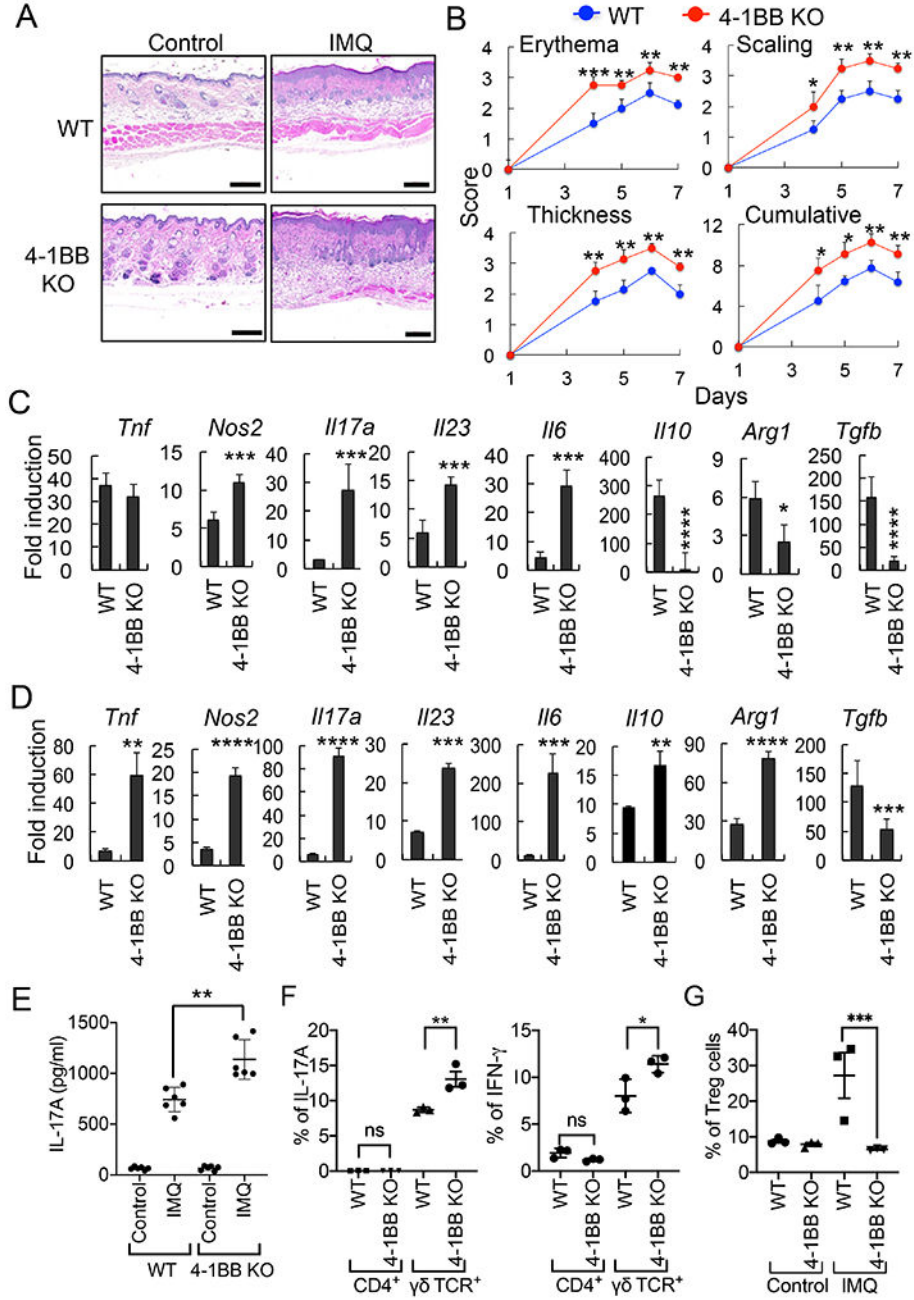
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**Figure 4.** Inhibition of the fatty acid synthesis pathway alleviates the IMQ-induced psoriasis in mice. C57BL/6 mice (6 mice per group) were injected i.p. with vehicle (10% EtOH) or Ceru (30 mg/kg body weight in 10% EtOH) daily followed by control or IMQ cream application on the shaved back skin daily for 7 days. *A*, H&E staining of the back skin. *B*, Scoring of erythema, scaling, and thickness of the back skin. The cumulative score is depicted. *C - F*, RNA samples were prepared from LNs or skin tissues on day 8. Quantitative PCR analysis was performed to determine the expression of cytokine genes (*C & D*) in LNs (*C*) and skin

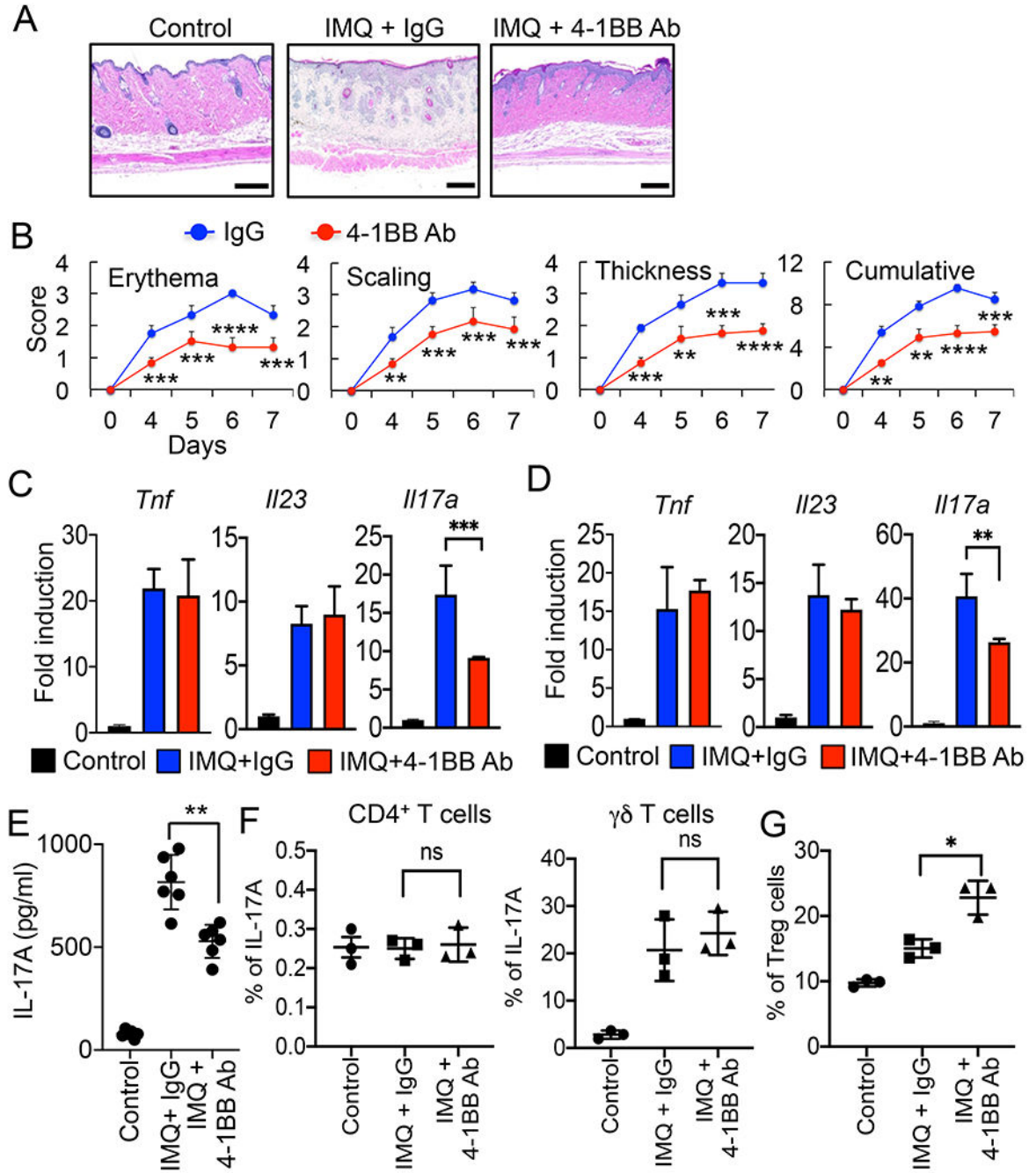
(D), or *Glut1* and *Fasn* (E & F) in LNs (E) or skin (F) (n = 4). Control cream-treated mice were used as control. Fold induction was compared to control. Data are shown as mean  $\pm$  SD. \*, p<0.05; \*\*, p<0.01; \*\*\*p<0.005; \*\*\*\*p<0.001. Scale bar, 200  $\mu$ m. Representative result of repeated experiments is shown.



**Figure 5.**

4-1BB regulates the induction of psoriasis. WT and 4-1BB-deficient mice (6 mice per group) were treated epicutaneously on the shaved back with IMQ or control cream for 7 days to induce psoriasis-like skin inflammation. *A*, H&E staining of the mouse dorsal skin. *B*, Erythema, scaling, and thickness of the back skin was scored daily from day 4 and the cumulative score is depicted. *C & D*, RNA samples were prepared from LN (*C*) or back skin (*D*) of mice on day 8, and the mRNA induction of inflammatory genes was analyzed by qPCR. Control cream-treated mouse samples were used as controls. *E*, Sera were collected

from mice at day 8, and IL-17A levels were determined by ELISA (6 mice per group). *F*, Expression of IL-17A is regulated by 4-1BB in  $\gamma\delta$  T cells in psoriasis. Lymphocytes isolated from LN were analyzed by flow cytometry for intracellular IL-17A or IFN- $\gamma$  expression. Cells were gated for CD45+CD3+CD4+ or CD45+CD3+ $\gamma\delta$  TCR+, and expression of IL-17A or IFN- $\gamma$  in each population was analyzed (n = 3). *G*, Treg induction in LN cells was analyzed by flow cytometry. Cells were gated CD45+CD3+CD4+CD25+ cells, and the intracellular levels of FoxP3 were analyzed (n=3). Data are shown as mean score  $\pm$  SD. \*, p<0.05; \*\*, p<0.01; \*\*\*p<0.005. Scale bar, 200  $\mu$ m. Result shown is representative of repeated experiments.



**Figure 6.** Administration of agonist 4-1BB Ab alleviates the pathology of skin inflammation. C57BL/6 mice (6 mice per group) were injected i.p. with isotype IgG (100  $\mu$ g), 4-1BB Ab (100  $\mu$ g) daily, and treated with IMQ cream or control cream on the shaved back skin for 7 days. *A*, Histology of the mouse back skin by H&E staining. *B*, Erythema, scaling, and thickness of the back skin was scored daily from day 4 and the cumulative score is depicted. *C & D*, Expression of inflammatory cytokines in LN (*C*) or back skin (*D*) of mice was analyzed by qPCR (4 mice per group). Control cream-treated mouse samples were used as

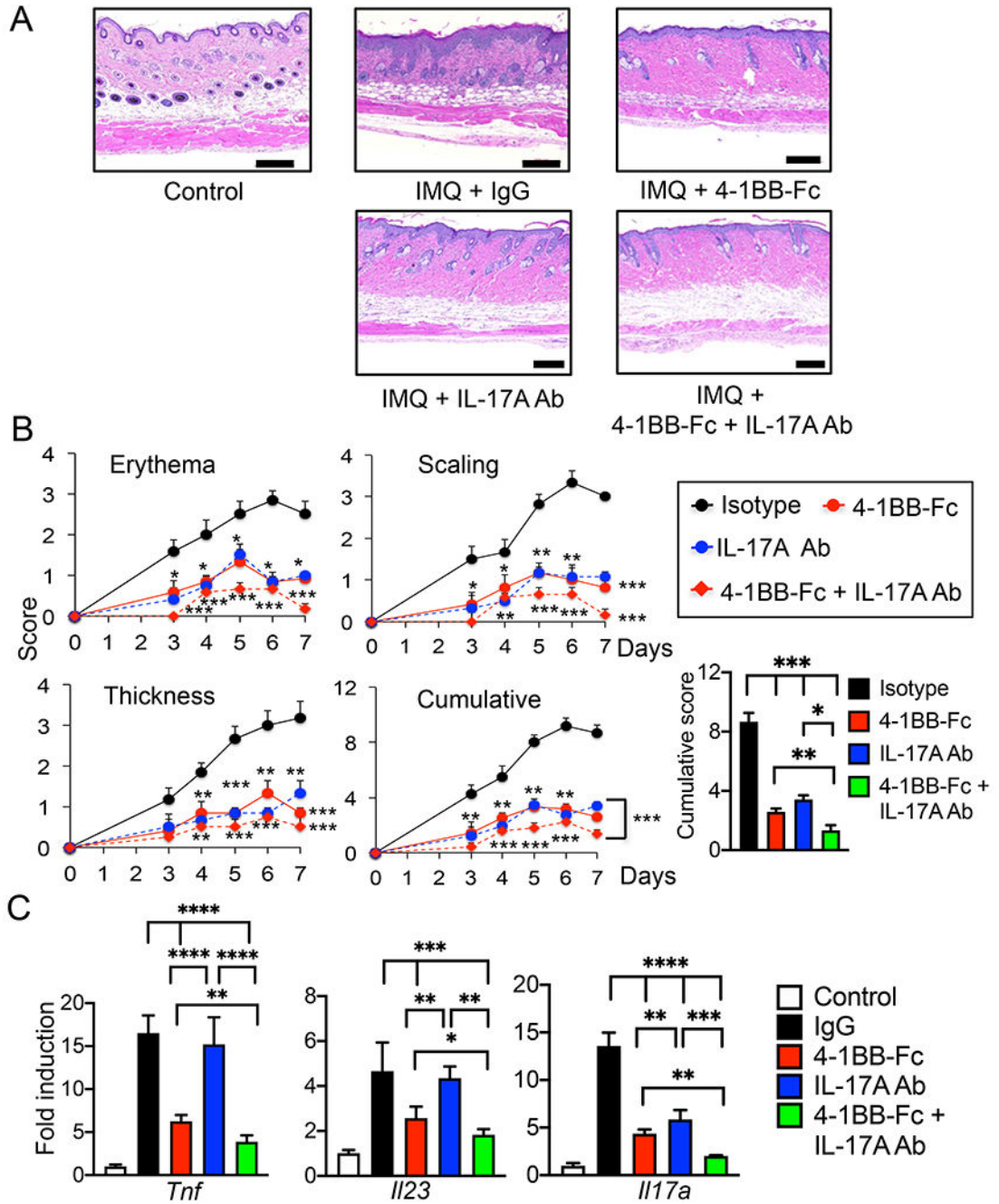
controls. *E*, Serum IL-17A levels were determined by ELISA (n = 6). *F*, FACS analysis for IL-17A expression in 4-1BB Ab-treated mice. LN cells were gated for CD45+CD3+CD4+ or CD45+CD3+ $\gamma$  $\delta$  TCR+, and intracellular level of IL-17A in each population was analyzed (n = 3). *G*, FACS analysis of Treg induction. LN cells were gated CD45+CD3+CD4+CD25+, and intracellular levels of FoxP3 was analyzed (n = 3). Data are shown as mean  $\pm$  SD. \*, p<0.05; \*\*, p<0.01; \*\*\*p<0.005, and ns, not significant. Scale bar, 200  $\mu$ m. Result shown is representative of repeated experiments.

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**Figure 7.** Co-administration of 4-1BB-Fc and IL-17A Ab shows greater efficacy in reduction of the psoriasis-like skin inflammation in mice. C57BL/6 mice (4 mice per group) were injected i.p. with isotype IgG (50 µg), 4-1BB-Fc (10 µg), IL-17A Ab (50 µg) or 4-1BB-Fc plus IL-17A Ab daily, and treated with IMQ cream or control cream on the shaved back skin and right ear daily for 7 days. *A*, H&E staining of the mouse dorsal skin. *B*, Erythema, scaling, and thickness of the dorsal skin was scored daily and the cumulative score is depicted, and the cumulative score at day 7 is shown. *C*, RNA samples were prepared from skin tissues on



day 8, and expression of inflammatory cytokines was analyzed by qPCR (4 mice per group). Data are shown as mean  $\pm$  SD. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.005$ ; \*\*\*\*,  $p < 0.001$ , and ns, not significant. Scale bar, 200  $\mu\text{m}$ . Result shown is representative of repeated experiments.

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