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ORIGINAL RESEARCH

Synergistic Effects of Azithromycin and STING Agonist Promote IFN-I Production by Enhancing the Activation of STING-TBK1 Signaling

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Background: Azithromycin (AZM) is a macrolide antibiotic that exhibits anti-inflammatory and anti-viral infection properties by enhancing type-I interferon (IFN-I) responses. The stimulator of interferon genes (STING) can directly induce IFN-I production. However, elevated IFN-I induces auto-immune phenotypes such as systemic lupus erythematosus (SLE). The effects of AZM and STING on the production of IFN-I are unclear.

Objective: Therefore, this study aims to evaluate the role of AZM and STING on IFN-I responses in macrophages.

Methods: RAW 264.7 macrophages were treated with AZM with and without a STING-agonist (DMXAA), and the maturation of macrophages was determined using flow cytometry. Gene expression and pro-inflammatory cytokines were analyzed using qPCR and ELISA, respectively. Moreover, protein expression was investigated using Western blot assays and immunofluorescence.

Results: Our results show that AZM significantly induced M1 phenotypes, promoting surface molecule expansion of CD80 and MHC-II and production of IL-6 and TNF-α cytokines on DMXAA-stimulated macrophages. Furthermore, we found that AZM-increased mRNA levels of interferon-stimulated genes (ISGs) could be due to the high expression of STNG-TBK1 signaling in the presence of DMXAA.

Conclusion: Our data suggest that AZM enhancement of IFN-I responses was STING dependent in DMXAA-stimulated macrophages. These data underline a novel approach to AZM action-mediated STING-TBK1 signaling for regulating IFN-I responses and may further augment the scientific basis and potential use of AZM in clinical applications.

Keywords: azithromycin, stimulator of interferon genes, TANK binding kinase 1, type-I interferon

Introduction

Type I interferon (IFN-I) is a type of inflammatory cytokine predominantly produced by innate immune cells, such as plasmacytoid dendritic cells (pDC) and macrophages,[1,2] to combat viral infection.[3] Furthermore, high circulation of IFN-I levels is associated with pathogenesis of SLE.[4,5] The molecular mechanism of IFN-I induction is stimulated through several intracellular DNA, which is recognized by cytosolic DNA binding proteins such as cyclic GMP–AMP synthase (cGAS), DNA-dependent activator of IFN-regulatory factors (DAI), Interferon gamma inducible protein 16 (IFI16), Melanoma differentiation-associated gene 5 (MDA5), Retinoic acid-inducible gene I (RIG-I), and Toll-like receptor 9 (TLR9).[6–8] Upon activation, DNA sensors bind to their ligand leading to IFN-I expression. The secreted IFN-I signals through an IFN-I receptor (IFNAR) to stimulate the phosphorylation of JAK-STAT signaling and regulate IFN-γ induction and expression of many interferon-stimulated genes (ISGs),[9,10] such as MX Dynamin Like GTPase 1 (Mx1), C-X-C Motif Chemokine Ligand 10 (CXCL10) and Interferon-stimulated gene 15 (ISG15), which can fight against infection.[11] Moreover, IFN-I plays a vital role in innate antiviral responses against early COVID-19 infection.[12,13]

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Azithromycin (AZM) belongs to a group of macrolide antibiotics most commonly used for bacterial infection by targeting the 50S ribosomal RNA subunit, which can inhibit bacterial protein synthesis.[14,15] It exhibits well-documented anti-inflammatory activity, which can reduce the activation of NF-kB and AP-1 activity in several immune cells, alleviating cytokine-mediated inflammation.[16,17] Additionally, azithromycin may facilitate the M2 phenotype of macrophages under lupus stimulation by attenuating the PI3K/Akt signaling pathway in vitro.[18,19] AZM also enhances the expression of type I and II interferons and ISGs, which can mitigate rhinoviral[20] and Zika virus infection[21] by upregulating expression of RIG-I and MDA5 and boosting the activation of downstream TBK1 and IRF3 proteins. Currently, several studies have shown the immunomodulatory effect of AZM on antiviral effects in patients with COVID-19[22–24] by up-regulation of IFN-I production through IRF3 signaling.[22] Likewise, AZM was demonstrated to suppress the binding between angiotensin-converting enzyme 2 (ACE2) and spike protein of SARS-CoV-2, which can reduce viral infection.[25] Moreover, AZM can reduce the SARS-CoV-2 viral replication[26] lysosomal pH change, leading to uncoating envelopes of viruses.[24]

The Stimulator of Interferon Genes (STING) is a cytosolic DNA sensor that is recognized by intracellular DNA-mediated IFN-I responses and inflammatory cytokines,[27] and which signals through the phosphorylation of TANK-binding kinase 1 (TBK1) and interferon regulatory factor 3 (IRF3) to fight against microbial infection.[28–30] STING activation-mediated IFN-I and ISG expression can also respond to DNA and RNA viral infection.[31,32] It has been shown that STING is a major driver of IFN-I and ISG production, which can cause SLE through activation of dendritic cells.[33] Moreover, activation of STING can induce the maturation of macrophages and produce cytokines, which can stimulate T cell activation.[34] Several studies revealed that STING agonists, such as 5.6-dimethylxanthenone-4-acetic acid (DMXAA) and derivatives, interacted directly with STING and enhanced the IFN-I production to anti-cancer properties[35,36] and against viral infection.[37] Furthermore, activation of STING by DMXAA can promote severe SLE,[33] Rheumatoid arthritis[38,39], and Sjögren's syndrome[40] in vivo study by increasing IFN-I production. Therefore, the induction of IFN-I with the combination of AZM and STING agonists-DMXAA could be elucidated to understand the mechanism in autoimmune diseases. Interestingly, current the literature has demonstrated the relevance of the STING signaling pathway associated with type I IFN responses in COVID-19.[41] Moreover, the STING agonist has shown potent inhibition of SARS-CoV-2 infection.[42]

The association of AZM with the STING-TBK1 signaling pathway regarding the regulation of IFN-I and ISGs responses is still lacking data. Therefore, we have focused on the role of AZM action via the STING signaling pathway by DMXAA activation to increase the scientific basis of information regarding AZM regulation of IFN-I exhibition. These data may be a profitable restoration target for STING-mediated IFN-I production, which could be used in clinical applications.

Materials and Methods

Chemicals

Azithromycin was purchased from Sigma (Sigma-Aldrich, Darmstadt, Germany). STING agonist (DMXAA), namely 5.6-Dimethylxanthenone-4-acetic acid, was purchased from Invitrogen (InvivoGen, San Diego, CA, USA). The chemical structure of these two reagents is presented in Figures 1A and B.

Cell Culture

RAW 264.7 macrophages were cultured in DMEM high glucose medium supplemented with 10% fetal bovine serum (HyCclone, Logan, UT, USA), and a final concentration of 1X anti-antibiotics (Gibco–Thermo Fisher Scientific, MA, USA). Cells were cultured in an incubator at 37°C with 5% CO_2. For stimulation, 80 ug/mL of azithromycin and 10 ug/mL of DMXAA were added to the cells for 24 hours. DMSO-treated cells (Sigma-Aldrich, Darmstadt, Germany) were used as the control in the following experiments.

Determination of Cell Viability by the MTS Assay

200 µL ($2x10^4$ cells) of RAW 264.7 macrophages were cultured in 96 well-culture plates and maintained in an incubator at 37°C and 5% CO_2 for 24 hr. The drug cytotoxicity assessment of AZM was performed using the MTS assay (Promega, cat. G3580, Madison, USA). In brief, RAW 264.7 macrophages were added at various doses of AZM between 5–100 ug/

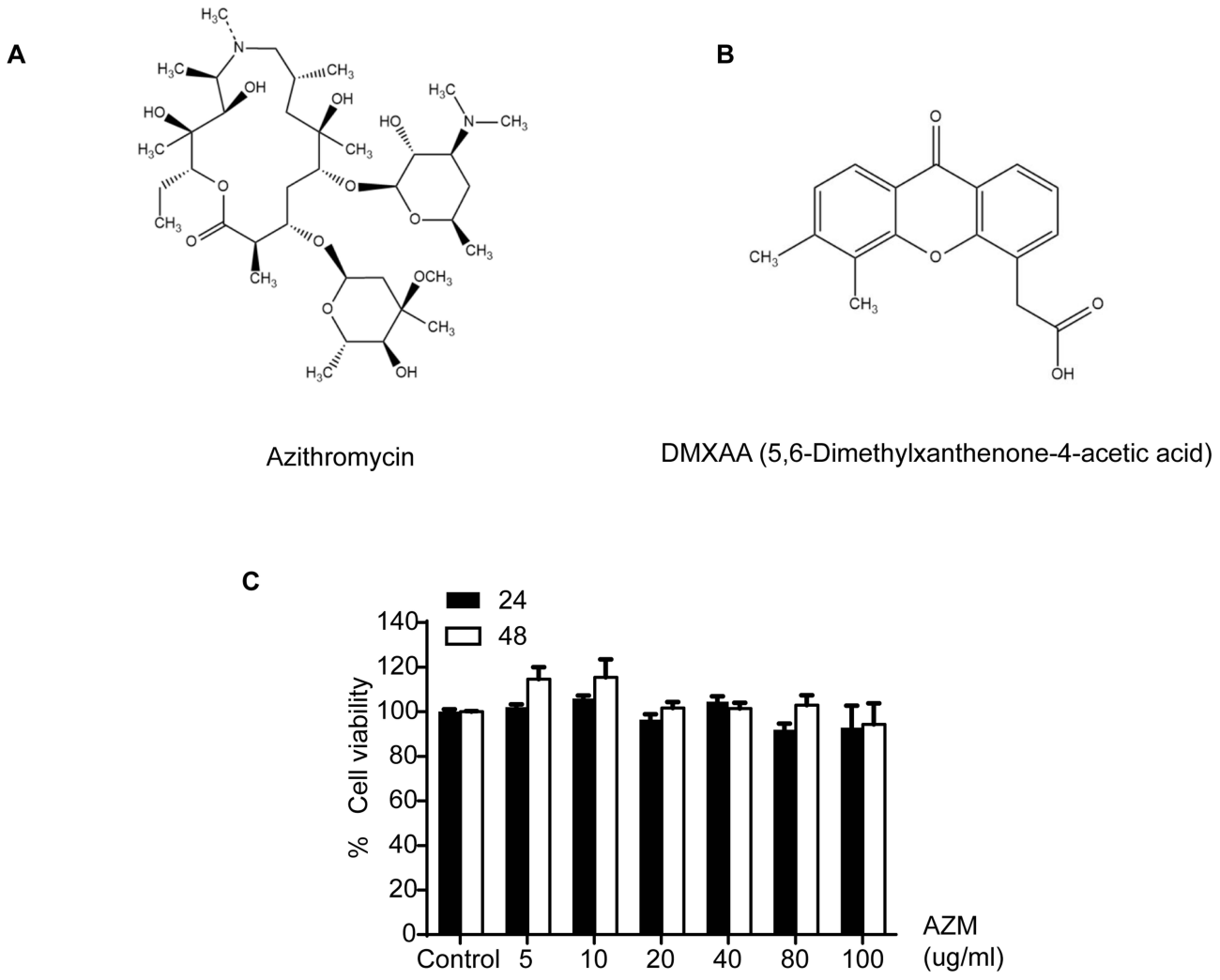

Figure 1 The effects of AZM on cell cytotoxicity. The chemical structure of (**A**) azithromycin and (**B**) DMXAA. (**C**) Cell viabilities were examined using MTS assay for 24 and 48 hr (N=3). Data are presented as mean ± SEM.

mL for 24 and 48 hours. Then, cells were washed with a complete medium. After washing, each sample was added with 100 µL of complete medium. Next, 20 µL of MTS reagents were added and incubated for 3 hours in an incubator. Then, the reaction signals were observed using a microplate reader at 490 nm (Thermo Fisher Scientific, MA USA). The percentage of cell viability was evaluated using DMSO (vehicle)-treated cells as the control.

Determination of Luciferase Activity

STING$^{+/+}$ and STING$^{-/-}$ RAW-Lucia ISG cells (InvivoGen, San Diego, CA, USA) were cultured in a DMEM high glucose solution containing 10% fetal bovine serum, 2 Mm L-glutamine (HyCclone, Logan, UT, USA), 100 µg/mL Zeocin and 50 µg/mL Normocin (InvivoGen, San Diego, CA, USA) in an incubator, as described above. Next, STING wild-type and knockout cells were stimulated with 40 µg/mL and 80 µg/mL of AZM with and without 10 µg/mL of DMXAA for 3, 24, and 48 h. The luciferase activity in the culture medium was observed with the QUANTI-Luc luciferase detection kit according to the manufacturer's protocol (InvivoGen, San Diego, CA, USA). In brief, 20 µL of culture medium from all experiments was collected in the black plate. Then, 50 µL of luciferase detector was added to each sample. Finally, luminescence activity was immediately measured by microplate reader (Thermo Fisher Scientific, MA USA).

Determination of Mature Macrophages

To determine mature macrophages, 1×10^6 cells of macrophages were cultured in a 6-well plate and stimulated with 80 µg/mL of AZM with and without 10 µg/mL of DMXAA for 24 h. Moreover, to confirm the effect of DMXAA on mature phenotypes of macrophages via STING signaling, 10 µg/mL of DMXAA and 1 µg/mL of LPS (Sigma-Aldrich, Darmstadt, Germany) were treated in STING-/- RAW-Lucia ISG cells for 24 h. Next, activated cells were collected and stained with anti-mouse F4/80 (clone: BM8), anti-mouse I-Ab (clone: AF6-120.1; cat. 116406), and anti-mouse CD80 (clone: 16–10A1; cat. 104733) (Bio Legend, San Diego, CA, USA). Fixable Viability Dye eFluor 780 (Thermo Fisher Scientific, MA USA) was used to stain excluded dead cells. Then, the maturation of stained macrophages was investigated using a BD LSR-II flow cytometer (BD Biosciences, North Brunswick, NJ, USA). The percentage of activated macrophages was evaluated with FlowJo software (version 10, USA).

Determination of Cytokines Production

To determine cytokine production, 1×10^6 $STING^{+/+}$ and $STING^{-/-}$ RAW-Lucia ISG cells were cultured in the 6-well plate described above. Cells were treated with 80 µg/mL of AZM with or without 10 µg/mL of DMXAA in an incubator at 37°C at 5% CO_2 for 24 h. IL-6 and TNF-α were then investigated in the supernatant using ELISA MAX Deluxe Set Mouse IL-6 (cat. 431304) and ELISA MAX Deluxe Set Mouse TNF-α (cat. 430904) (Bio Legend, San Diego, CA, USA) following the manufacturer's protocol. Finally, the signals were measured using a microplate reader at 450 nm (Thermo Fisher Scientific, MA USA). The concentration of cytokines was calculated and presented as pg/mL.

Determination of Genes Expression

mRNA expression of interferon-inducible genes was investigated in 80 ug/mL of AZM-treated cells with and without 10 ug/mL of DMXAA. In brief, total RNA from all experiments was extracted using Trizol reagent (InvivoGen, San Diego, CA, USA) followed by RNA purification using the RNeasy mini kit (QIAGEN, Germantown, MD, USA) according to the manufacturer's instructions. Next, cDNA synthesis from 1 µg of total RNA was performed using iScript RT Supermix (Bio-Rad, Hercules, CA, USA). The mRNA expression of genes of interest was conducted with quantitative PCR using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) and Applied Biosystems 7500 (Applied Biosystems). β-actin was used to normalize relative mRNA expression. The fold induction was calculated using the $2^{(-ddCt)}$ formula. All primers are shown in Table 1.

Determination of Protein Expression

1×10^6 cells of RAW 264.7 macrophages were cultured in a 6-well plate, and then 80 ug/mL AZM was added with and without DMXAA for 180 min. Activated cells were lysed for 30 min on ice with a lysis buffer (10 mM Tris-HCL, 1% Triton-X100, 150 mM NaCl, 5 mM, and EDTA pH 8). Then, protein from the cell lysates was collected by centrifuging at 13,000 × g for 15 min at 4°C. The protein was collected from the supernatant. The amount of protein was measured using BCA assay (Thermo Fisher Scientific, Waltham, MA, USA). Next, total protein (25 µg) was separated on a 10% polyacrylamide gel. Then, proteins from the gel were transferred into nitrocellulose membranes and the antibodies' unspecific binding was blocked with a blocking buffer for 1 h and then probed with Total TBK1 antibody (clone: D1B4 cat: 3504S, 1:1000) and Phospho-TBK1/NAK (Ser172) (clone: D52C2 cat: 5483S, 1:1000), Total STAT1 antibody (clone: 42H3 cat: 9175S, 1:1000), Phospho-Stat1 (Tyr701) (clone: D4A7 cat: 7649S, 1:1000) and β-ACTIN (clone: 13E5 cat: 4970S, 1:2500) (Cell Signaling, Danvers, MA, USA) overnight at 4°C. After washing with 1X TBS-T, membranes fluorescent secondary antibody IRDye 680RD donkey anti-rabbit IgG (1:10000) (LI-COR, Lincoln, NE, USA) was added for 1 h in the dark at room temperature. Finally, the targeted protein from the membrane was detected with ODYSSEY CLx (LI-COR, Lincoln, NE, USA). The quantitative intensity of the proteins was calculated with the ODYSSEY CLx program.

Table 1 The Primer Sequences for Quantitative Real Time-PCR

Primer Name	Forward Sequence	Reverse Sequence
IFN-α	5'-TCTGATGCAGCAGGTGGG-3'	5'-AGGGCTCTCCAGACTTCTGCTCTG-3'
IFN-β	5′-ATGAGTGGTGGTTGCAGGC-3′	5′-TGACCTTTCAAATGCAGTAGATTCA-3′
Sting	5′-TGCCGGACACTTGAGGAAAT-3′	5′- GTTTCCGTCTGTGGGTTCTTG-3′
TBK1	5′-GACATGCCTCTCTCCTGTAGTC-3′	R: 5′-GGTGAAGCACATCACTGGTCTC-3′
Cxcl10	5′-CAGTGAGAATGAGGGCCATAGG-3′	5′-CGGATTCAGACATCTCTGCTCA-3′
Mx1	5′-GATCCGACTTCACTTCCAGATGG-3′	5′-CATCTCAGTGGTAGTCCAACCC-3′
ISG15	5′-GAGCTAGAGCCTGCAGCAAT-3′ R	5′-TAAGACCGTCCTGGAGCACT-3′
Irf3	5′-GCTTGTGATGGTCAAGGTTGT-3′	5′- AGATGTGCAAGTCCACGGTT-3′
Irf7	5′-CCCAGACTGCCTGTGTAGACG-3′,	5′-CCAGTCTCCAAACAGCACTCG-3′
NF-kB p65	5′-TCCTGTTCGAGTCTCCATGCAG-3′	5′-GGTCTCATAGGTCCTTTTGCGC-3′
IL-6	5′-TACCACTTCACAAGTCGGAGGC-3′ R	5′-CTGCAAGTGCATCATCGTTGTTC-3′
TNF- α	5′-CCTCACACTCAGATCATCTTCTC-3′	5′-AGATCCATGCCGTTGGCCAG-3′
Actin	5′-GGCTGTATT CCCCTCCATCG-3′	R: 5′-CCAGTTGGTAACAATGCCATGT-3′

Immunofluorescence Staining

1×10^5 cells of macrophages were seeded in a cell culture slide. Cells were treated with AZM with and without DMXAA for 3 h. Then, 200 µL of 10% formalin (Sigma-Aldrich, Darmstadt, Germany) was added for 10 min at room temperature. Next, 200 µL of 0.2% Triton X-100 in PBS was added to the fixed cells for 10 min, followed by addition of 5% BSA in PBS for 1 h to block unspecific binding. Fixed macrophages were probed with STING antibodies (clone: D2P2F cat: 13647, 1:200) and Phospho-TBK1/NAK (Ser172)(clone: D52C2 cat: 5483S, 1:200) (Cell Signaling, Danvers, MA, USA) at 4°C for overnight. Then, secondary antibodies of Alexa Fluor 488 rabbit IgG (Thermo Fisher Scientific, Waltham, MA, USA) were added to stained cells in the dark for 1 h. Finally, DNA was stained with 1 µM DAPI in the dark (Thermo Fisher Scientific, Waltham, MA, USA) for 5 min, and fluorescence signals were observed by confocal microscope (Carl Zeiss LMS800, Oberkochen, Germany).

Statistical Analysis

All data are presented as mean ± standard error (SEM). Statistical analyses between multiple groups were performed with the one-way ANOVA analysis, and the comparison between groups was performed with a two-tailed Mann–Whitney using GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA). The statistically significant data with $P < 0.05$ were considered.

Results

Cell Cytotoxicity of Azithromycin

We first examined drug cytotoxicity effects in macrophages for 24 and 48 hr using MTS assay. The results indicate that cell viabilities were not significantly affected by azithromycin at different concentration between 5–100 µg/mL relative to the untreated cells, as shown in Figure 1C.

Azithromycin Increases Interferon Secretion in DMXAA-Induced Macrophages

Next, we determined the effect of azithromycin on IFN-I production in DMXAA-stimulated macrophages, which was produced through activation of the STING/TBK1/IRF3 pathway. Our data indicate that AZM significantly increased

the levels of Lucia luciferase in DMXAA-stimulated RAW-Lucia ISG cells relative to the DMXAA-treated and untreated cells (Figure 2A). However, AZM did not induce luciferase secretion in the culture medium of STING-/- RAW-Lucia ISG cells after 3 and 24 hr of stimulation. Interestingly, AZM alone was able to induce IFN-I production after 48 hr of activation (Figure 2B). Moreover, AZM-treated cells with DMXAA stimulation did not affect cell viability (Figure 2C). These results suggest that AZM-mediated IFN-I production by synergistic STING/TBK1/IRF3 signaling.

Azithromycin Enhances the Maturation of DMXAA-Stimulated Macrophages

In order to determine whether AZM affects the maturation of macrophages after co-treatment with and without DMXAA, the surface molecules of macrophages were stained with CD80 and an I-Ab, indicating the mature macrophages. The results revealed that DMXAA-stimulated cells exhibited a significant expansion of I-Ab and CD80 relative to untreated cells. Interestingly, co-treatment with DMXAA and AZM significantly increased these activated markers compared to treatment with DMXAA alone, as shown in Figure 3A–E. Moreover, we confirmed the AZM-mediated maturation of macrophages using flow cytometry in STING-/- RAW-Lucia ISG cells. The results showed that AZM did not affect the activation of DMXAA-induced STING-/- cells, while LPS was able to induce this activation in STING-/- cells (Figure 3F and G). These data suggest that the synergy of AZM and DMXAA induced the maturation of macrophages.

AZM Increases Cytokines Production in DMXAA-Stimulated Macrophages

Levels of IL-6 and TNF-α were significantly higher in DMXAA-stimulated cells after 24 hours than in AZM- and untreated cells. Furthermore, co-treatment of AZM and DMXAA showed a significantly increase in these

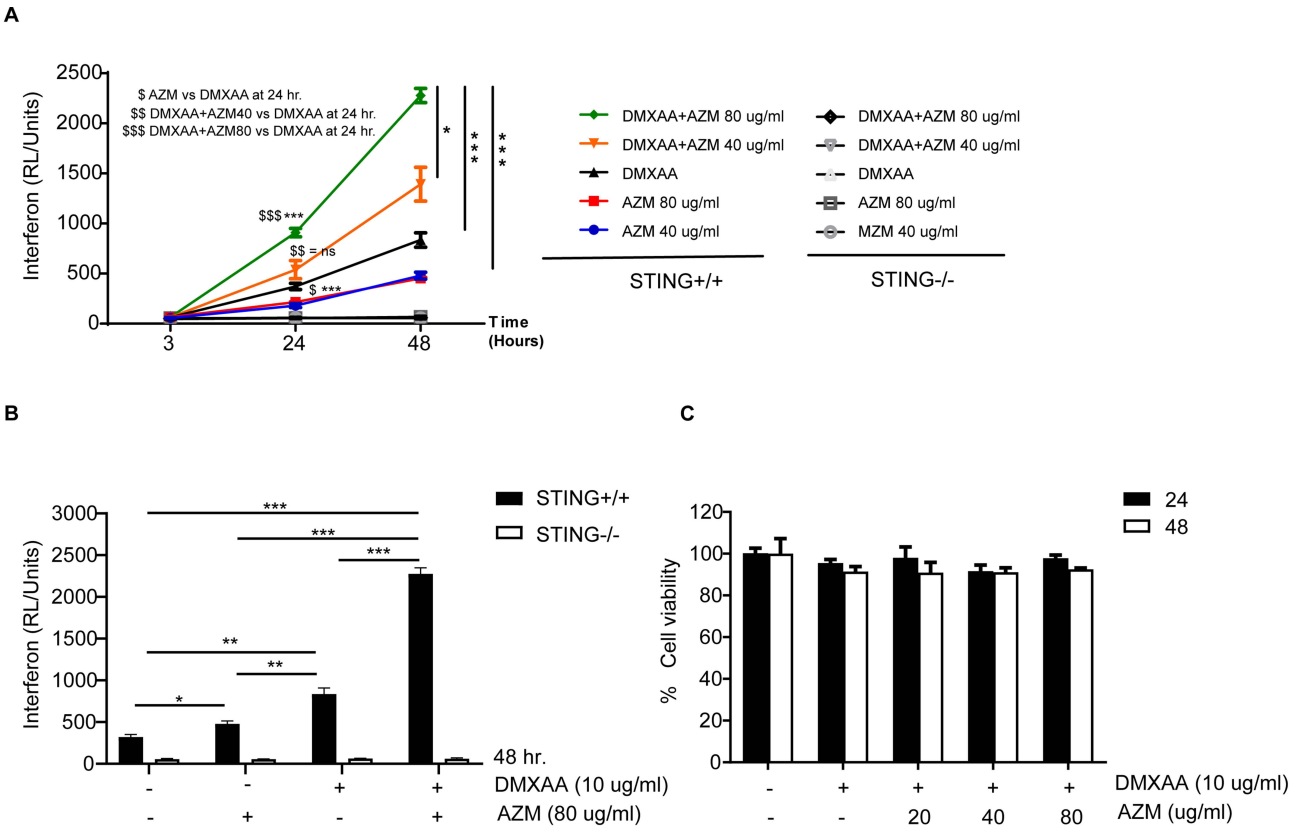

Figure 2 The effects of AZM on IFN-I secretion. (**A**) Secreted luciferase of STING+/+ and STING-/- RAW-Lucia ISG cells in the culture medium after activation by AZM with or without DMXAA were investigated with luciferase detection reagent (N=4). (**B**) IFN-I production at 48 hr (N=3). (**C**) Cell viability of AZM-treated cells with or without DMXAA stimulation for 24 and 48 hr (N=3). Data are shown as mean ±SEM; *p < 0.05, *p < 0.01, and ***p < 0.001. The dollar sign ($) represents the comparison between groups.

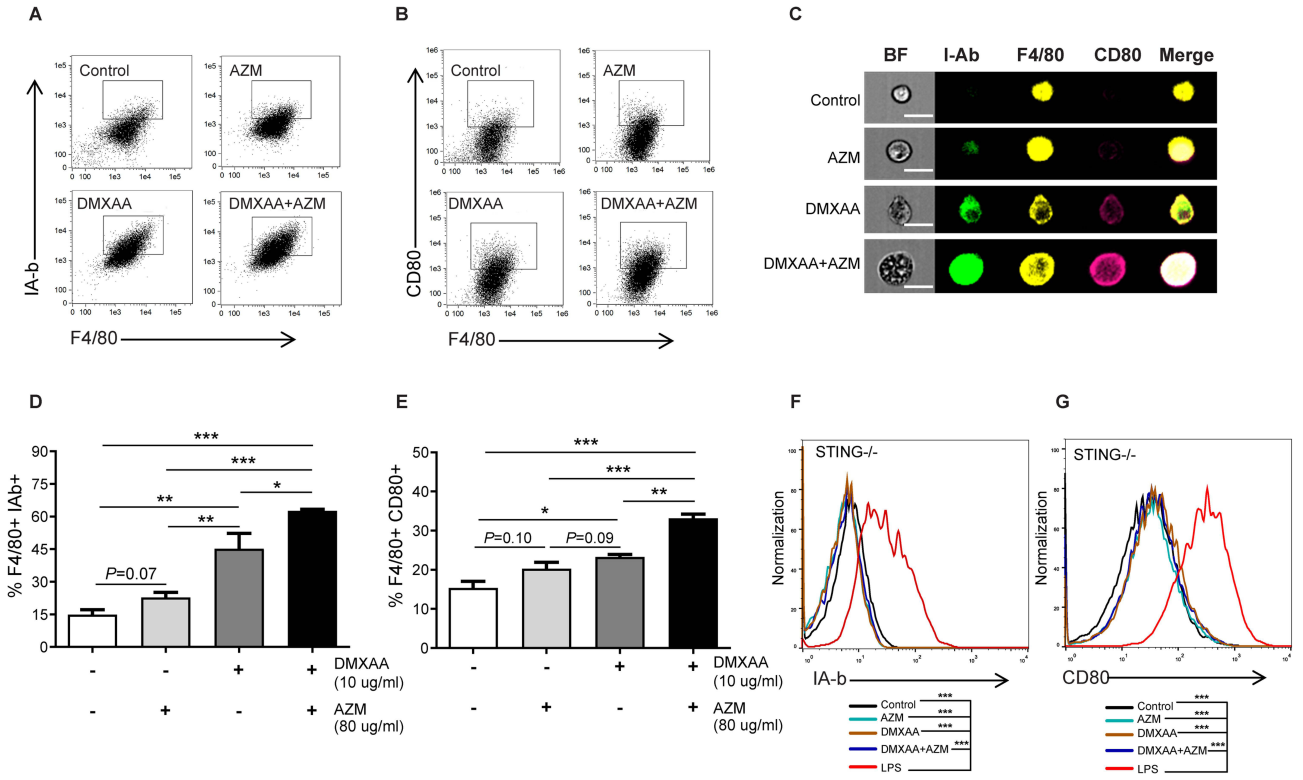

Figure 3 The effect of AZM on the maturation of DMXAA-induced macrophages. Flow cytometry was used to examine the expansion of (**A**) I-Ab and (**B**) CD80 (N=3-4). (**C**) The representative of image flow was investigated; scale bar = 20 μm (N=3). Data are indicated in the percentage of (**D**) F4/80$^+$ I-Ab$^+$ cells and (**E**) F4/80$^+$ CD80$^+$ cells (N=3-4). (**F** and **G**) Representation of flow cytometry analysis of activated macrophages from STING-/- RAW-Lucia ISG cells (N=3). Data are shown as ± SEM; *p < 0.05, **p < 0.01, and ***p < 0.001.

proinflammatory cytokines to DMXAA alone. However, inflammatory cytokine levels of STING-/- cells did not differ significantly from those of AZM with or without DMXAA- and untreated controls, as presented in Figures 4A and B.

Effects of AZM on Interferon Signature Genes (ISG) Expression

Our data show that co-treatment with AZM and DMXAA led to significant induction of ISG mRNA levels after 24 hours of activation in macrophages relative to DMXAA-treated and untreated controls, including *Ifn-α, Ifn-β, Cxcl10, Mx-1*, and *Isg15*. Nevertheless, AZM-treated cells did not show significant induction of ISG expression compared to untreated cells (Figure 5A–E). Interestingly, DMXAA-stimulated cells exhibited a significantly higher mRNA expression of *NF-kβ*

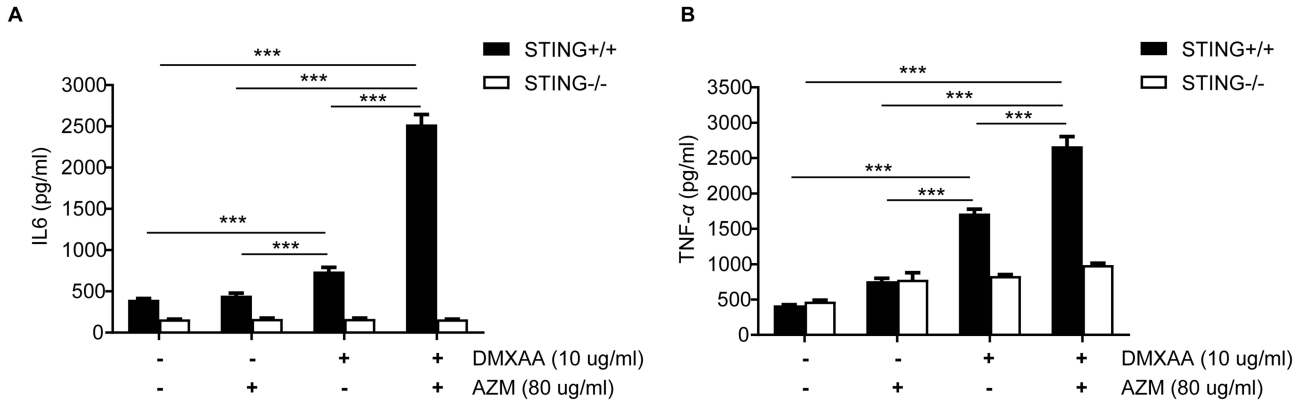

Figure 4 Effects of AZM on cytokine production after DMXAA-stimulated macrophages. Supernatants from STING+/+ and STING-/- RAW-Lucia ISG cells after activation by AZM with or without DMXAA for 24 hr were collected and analyzed: (**A**) IL-6 and (**B**) TNF-α secretion by ELISA (N=3-5). Data are shown as mean ± SEM; ***p < 0.001.

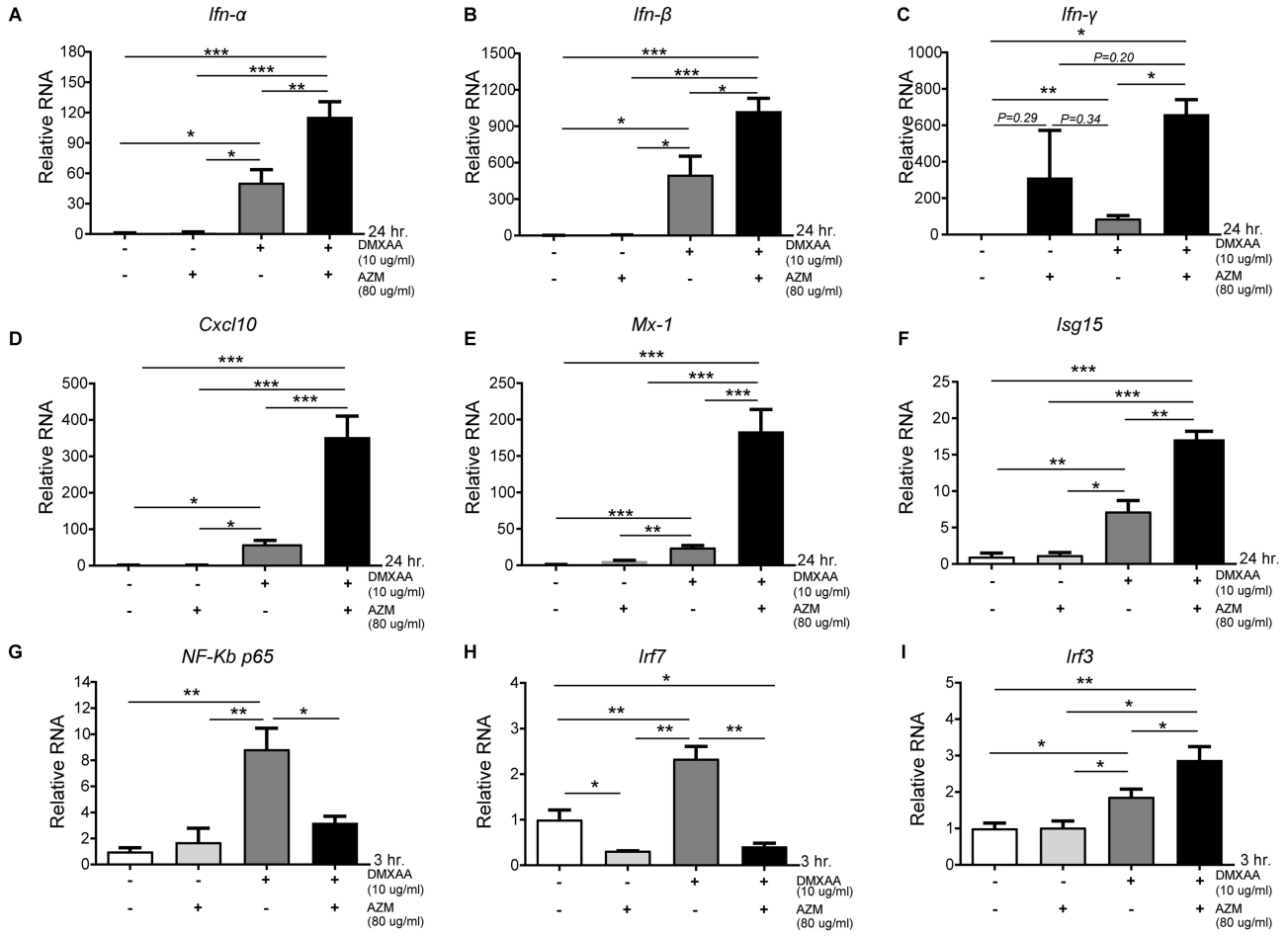

Figure 5 Co-treatment of AZM and DMXAA induced ISG expression in activated macrophages. (**A**–**E**) Relative mRNA transcription of (**A**) *Ifn-α*, (**B**) *Ifn-β*, (**C**) *Ifn- γ*, (**D**) *Cxcl10*, (**E**) *Mx1*, (**F**) *Isg15*, (**G**) *NF-kβ p65*, (**H**) *Irf7*, and (**I**) *Irf3* from AZM-stimulated cells with and without DMXAA for 3 and 24 hr (N = 3–6 per group). Data are shown as mean ±SEM; *p < 0.05, **p < 0.01, and ***p < 0.001.

p65 and *Irf7* than DMXAA-treated cells with AZM (Figure 5G and H). In contrast, we found a significant increase in *Irf3* expression in these groups, as presented in Figure 5I.

Effect of AZM on STING Activation

It is well established that STING is essential to initiation of IFN-I production. Here, an increase in IFN-I as a result of synergistic AZM and STING-TBK1 was observed. The data show that the mRNA level of *Sting* after co-treatment with AZM and DMXAA was significantly higher than in DMXAA-treated and untreated cells (Figure 6A). In addition, the activation of STING was confirmed by immunofluorescence staining using confocal microscopy. The results show a significantly higher expression of STING following co-treatment with AZM and DMXAA relative to DMXAA-treated and untreated cells, as shown in Figure 6B and C.

Effect of AZM on TBK-1 Activation

Next, the activation of TBK-1, which is downstream of STING signaling, was investigated. The data show a significantly higher transcription of mRNA levels of *Tbk1* in co-treatment of AZM and DMXAA compared with DMXAA-treated and untreated cells (Figure 7A). Moreover, the phosphorylation of TBK1 (Ser172) after 3 hr of activation by AZM with or without DMXAA was observed. Cells treated with a combination of AZM and DMXAA showed a significantly higher expression of pTBK-1 relative to cells treated with DMXAA alone (Figure 7B and C). Additionally, we confirmed this activation by immunofluorescence using confocal microscopy. The data demonstrated a significantly higher expression of pTBK-1 in the

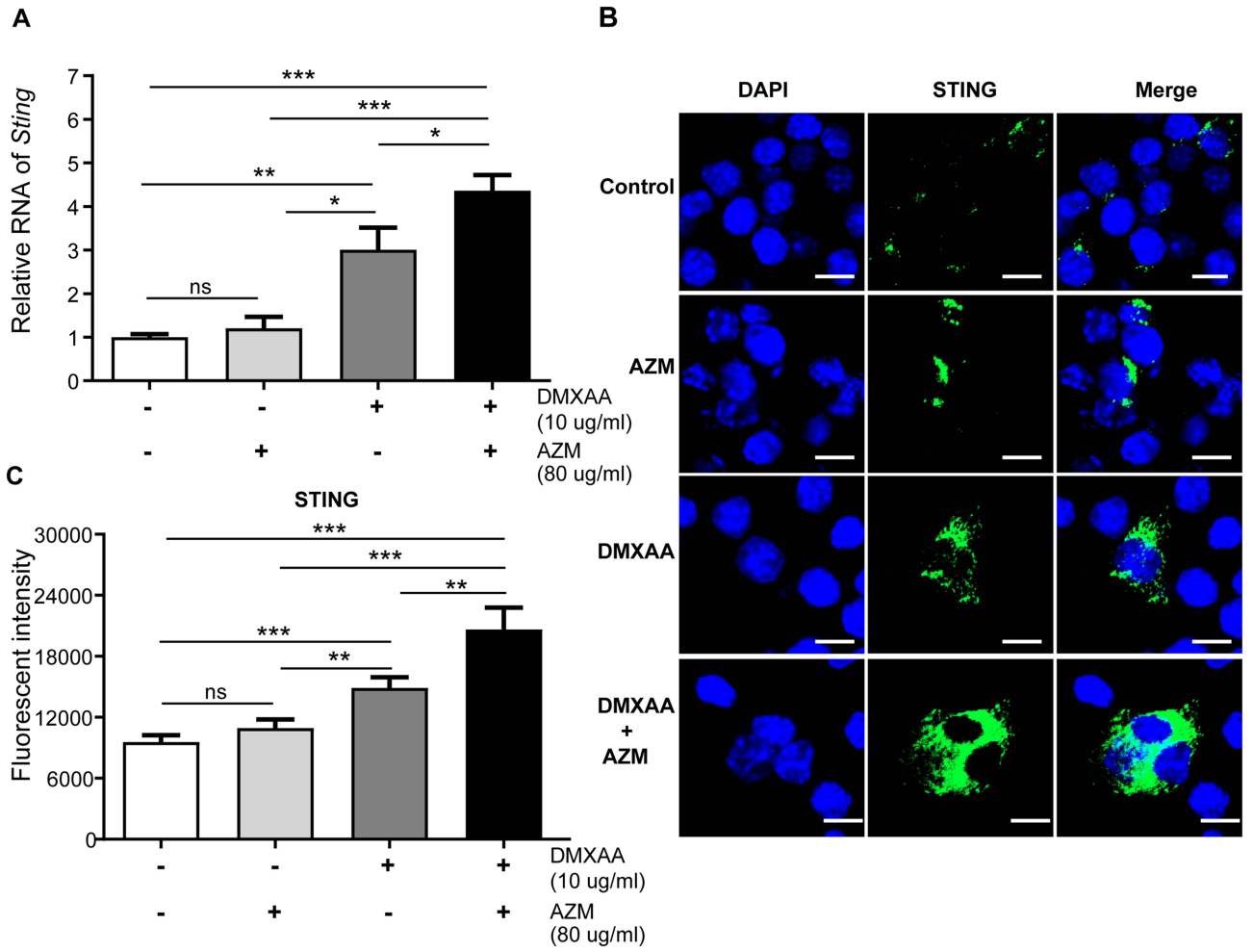

Figure 6 Co-treatment of AZM and DMXAA increased STING expression in activated macrophages after 3 hr of activation. (**A**) Cells were collected and mRNA levels of *Sting* were investigated by quantitative PCR (N=3-5). (**B**) Immunofluorescence staining of STING was examined using confocal microscopy (N=3); confocal microscope images show DAPI (blue), and STING (green). (**C**) Quantification of immunofluorescence signals (N = 3). Data are shown as mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001, and ns, not significant.

co-treatment of AZM and DMXAA than in DMXAA-treated cells with Western blot assay (Figures 7D and E). These data suggested that AZM induced IFN-I production by activating the STING/TBK1 pathway.

Effect of AZM on STAT1 Signaling

To examine the mechanism of ISG expression, mainly caused by the activation of IFN- I. The phosphorylation of STAT1 (Tyr701) after 3 hr of activation by AZM with or without DMXAA was investigated. Macrophages treated with a combination of AZM and DMXAA exhibited a significantly higher expression of pSTAT-1 than in cells treated with DMXAA alone (Figure 7F and G). These results demonstrated that a combination of AZM and DMXAA induced IFN-I production through STING/TBK1/ signaling, leading to the expression of ISGs via the activation of STAT1 signaling.

Discussion

Azithromycin is an antibiotic agent[14] exhibiting anti-inflammatory properties in macrophages.[19,43] Moreover, AZM can induce IFN-I secretion against viral infection.[21,44] It is well documented that the activation of cGAS-STING by intracellular DNA regulates IFN-I production.[27] Our previous data have shown that STING may increase levels of IFN-I, leading to autoimmune phenotypes, such as SLE[33] and rheumatoid arthritis.[39] Thus, we further investigated whether STING, which is mainly expressed in macrophages, can be affected by treatment with AZM.

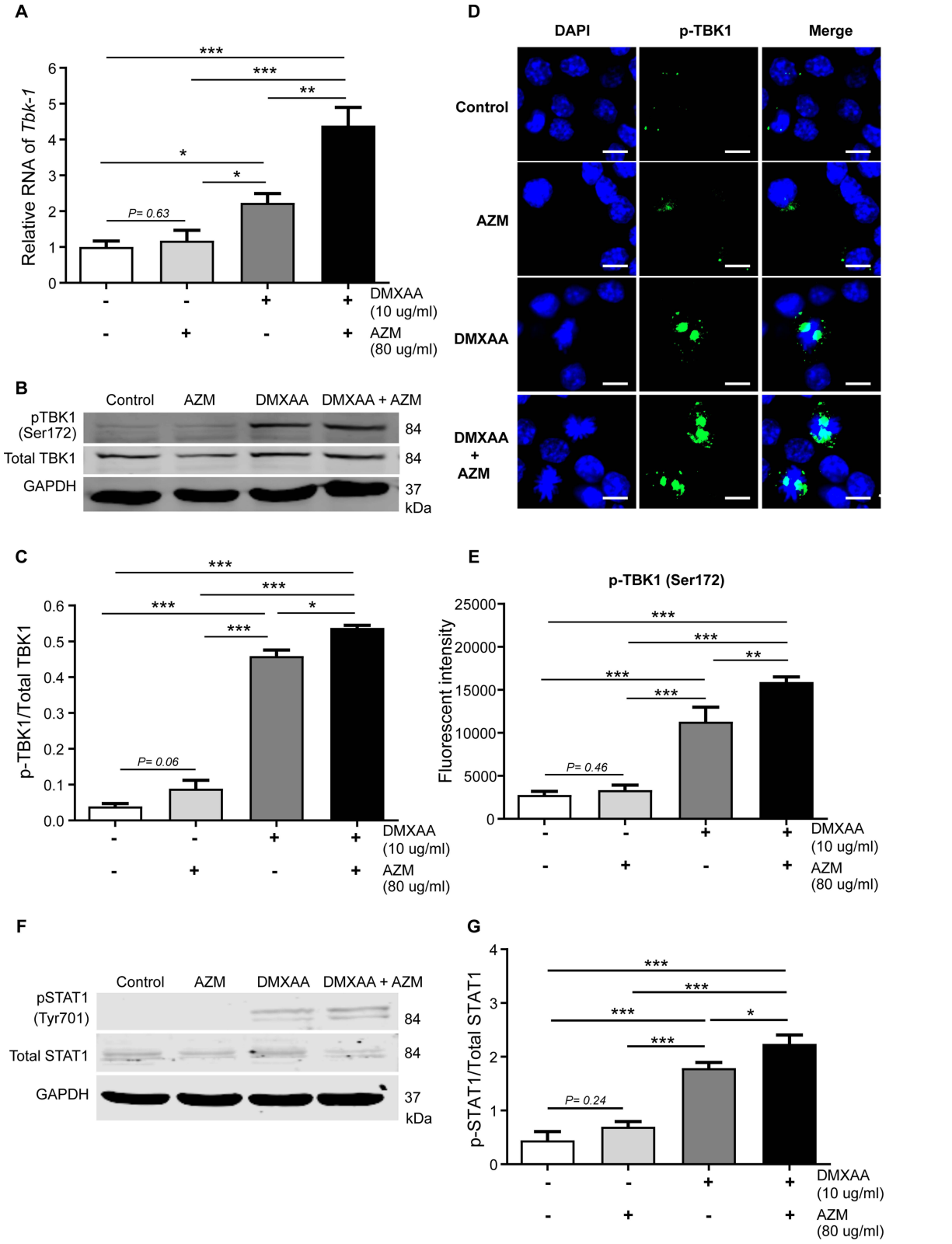

Figure 7 Co-treatment of AZM and DMXAA increased pTBK1 expression in activated macrophages after 3 hr of activation. (**A**) Cells were collected and mRNA levels of TBK1 were investigated by quantitative PCR (N=3-5). (**B** and **C**) Western blot analysis of AZM-activated macrophages with or without DMXAA showed phosphorylation of TBK1 (Ser172). Data are representative of independent over three experiments. (**D**) Confocal microscope images show DAPI (blue) and pTBK1 (Ser172) (green). (**E**) The quantification of immunofluorescence signals (N = 3). (**F** and **G**) Western blot analysis of AZM-activated macrophages with or without DMXAA showed phosphorylation of STAT1 (Tyr701). Data are presented as mean ± SEM; *p < 0.05, **p < 0.01, and ***p < 0.001.

Our results showed that co-treatment with AZM and DMXAA increased IFN-I release in the culture medium. Moreover, we detected the expansion of CD80 and I-Ab (MHC-II) molecules, suggesting the maturation of macrophages as the antigen-presenting cells (APCs) in AZM with DMXAA-treated cells. These data suggest that AZM and DMXAA-treated cell-induced IFN-I responses and secrets into the culture medium, which binds to IFN-I receptors (IFNAR) and subsequently increases the expansion of co-stimulatory molecules CD80[45] via regulation of interferon regulatory factor-1 (IRF1).[46] Moreover, increasing IFN-I in a culture environment can induce MHC II expression[10,33,47] by inducing the phosphorylation of STAT1, which is generally stimulated IFN-γ production,[10,48] leading to enhancement of surface molecules of MHC-II on macrophages.[49] Additionally, our results show that AZM enhanced mRNA levels of IFN-γ in DMXAA-treated cells. This may be due to IFN-γ activation via receptors (IFNGR) being dependent on IFNAR signaling.[9,50] In contrast, AZM can reduce the expression of co-stimulatory molecules CD80 and MHC class II molecules in LPS-stimulated DCs by inhibiting the translocation of NF-κB.[51] These data suggest that azithromycin alternatively promotes the maturation of macrophage phenotypes via STING-dependent IFN-I signaling.

The previous data have shown that AZM is an anti-inflammatory drug and attenuates pro-inflammatory cytokine production by reducing NF-κB and AP-1 activation in LPS-induced cells.[16,17] Consistency with previous results indicated that AZM can reduce the expression of *NF-κB*[17] and *Irf7*[52] in DMXAA-activated cells, which stimulates the induction of pro-inflammatory cytokines. However, we found that the pro-inflammatory cytokines IL-6 and TNF-α were significantly enhanced in the AZM and DMXAA-cotreated cells. These data might be due to elevated IFN-I and IFN-γ, which stimulate the polarization of M1 macrophages and enhance cytokine production through mediated IFN-γ /IRF1/IRF5 signaling.[53–55] Additionally, the releasing of IFN-γ by AZM with DMXAA can activate the STAT1 signaling through the IFN-γ receptor (IFNGR), leading to the phosphorylation of STAT1 at Tyr701, resulting in translocation into the nucleus and initiating the induction of TNF-α and IL-6 production.[56] Thus, our data suggest combining DMXAA and AZM enhances cytokine production through STING-dependent IFN- γ/STAT1 signaling. Moreover, AZM administration on *P. aeruginosa*-infected mice increased levels of IFN-γ, resulting in decreased bacterial infection.[57] However, we also observed that AZM alone did not promote the levels of IL-6 and TNF-α in the culture medium. These results imply that a combination of AZM and DMXAA induces IFN-I and IFN-II responses that lead to establishment M1 phenotypes of macrophages that are STING-dependent.

Furthermore, we found high expression of *Ifn-α, Ifn-β*, Ifn- γ and interferon-inducible genes (ISGs), including *Cxcl10, Mx1*, and *Isg15* in the AZM and DMXAA-cotreated cells, while cells treated with AZM alone did not affect the mRNA levels of these genes. Similarly, AZM can enhance IFN-I and ISGs gene expression in rhinovirus (RV) 1B-infected cells.[20,58,59] These results might be due to the STING-dependent IFN-I and IFN-γ production, which induce ISGs expression through STAT1 signaling.[10,60] AZM has also been shown to increase the expression of pathogen recognition receptors (PRRs), including RIG-I and MDA5, which induce ISGs expression in HT-29 cells after ZIKV infection.[21] These results indicate that the effect of AZM-associated IFN-I and ISGs signaling could temporarily augment inflammatory response in the presence of DMXAA.

Nevertheless, the effects of AZM on STING signaling-mediated IFN-I responses in macrophages has not yet been clarified. Thus, we further investigated these effects. Our results showed that AZM could upregulate mRNA levels of *Sting* in DMXAA-stimulated cells consistently with high expression of STING by immunofluorescence. AZM has previously been reported to increase the levels of phosphorylated TBK1 and IRF3, downstream of RIG-I and MDA5 after stimulation by viral infection.[20,21,26] Here, we found that AZM increased the phosphorylation of TBK1, which was activated by STING,[29,61] leading to induction of IFN-I responses in the presence of DMXAA-stimulated macrophages. It is well-recognized that AZM is a weakly basic drug that accumulates in the lysosome, leading to lysosome function change, which can induce apoptosis[62,63] and mitochondrial DNA leakage.[64] These effects of AZM might stimulate the cGAS-STING pathway[65] and increase the activation by combination with STING agonist (DMXAA). Our results showed that AZM-treated cell alone at 24 hr did not affect this mechanism. However, AZM-treated cells at 48 hr can enhance IFN-I luciferase activity via STING signaling compared with untreated cells (Figure 2B).

These results suggest that the combination of AZM and DMXAA may alternatively affect activation of IFN-I via STING/TBK1/IRF3 signaling, resulting in enhance the activation of interferon-stimulated genes and pro-inflammatory cytokines through STING-dependent IFN-γ/STAT1 pathway, as presented in Figure 8. In this study, we provide evidence for a probable new mechanism of azithromycin. Our study also showed that the efficacy of AZM on IFN-I response and ISG signaling on the regulation of macrophages was STING dependent.

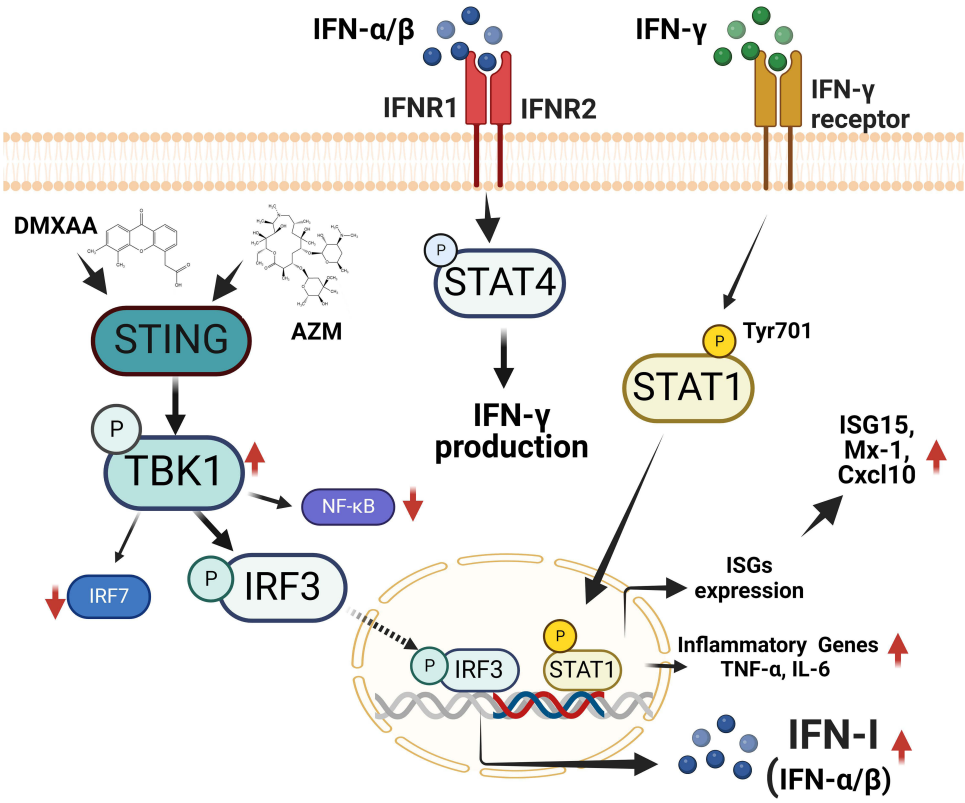

Figure 8 The proposed effect of Azithromycin and STING agonist (DMXAA) on macrophages. Co-treatment of AZM and DMXAA increased the expression of STING-TBK1-IRF3 signaling, leading to the up-regulation of IFN-I production. IFN-I can induce the activation of IFN- γ, resulting in the induction of STAT1 signaling and increasing ISG and pro-inflammatory cytokines expression. This figure was constructed by biorender.com.

Conclusion

In summary, Azithromycin is an antibiotic drug that exerts anti-inflammatory activity. We hypothesize that AZM could inhibit IFN-I via STING/TBK1/IRF3 signaling, which might be a targeted treatment for lupus. In contrast, the combination of AZM and DMXAA reveals the synergistic effects on the IFN-I responses by enhancing the activation of STING expression. This activation increases the phosphorylation of downstream TBK-1 and might stimulate IRF3 to translocate to the nucleus. Therefore, these effects increased interferon-stimulated gene expression, including *Cxcl10, Isg15*, and *Mx1*, through IFN-γ/STAT1 signaling. These data emphasize a novel approach to AZM use and may further augment the clinical application of AZM in terms of activating via the cGAS-STING pathway.

Ethical Approval

In this study, no ethics approval was required. RAW-Lucia ISG cells mouse macrophages (STING+/+; cat. Rawl-isg) and RAW-Lucia ISG-KO-STING cells mouse macrophages (STING-/-; cat. Rawl-kostg) were purchased from InvivoGen (InvivoGen, San Diego, CA, USA).

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Disclosure

The authors declare no conflict of interest.

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