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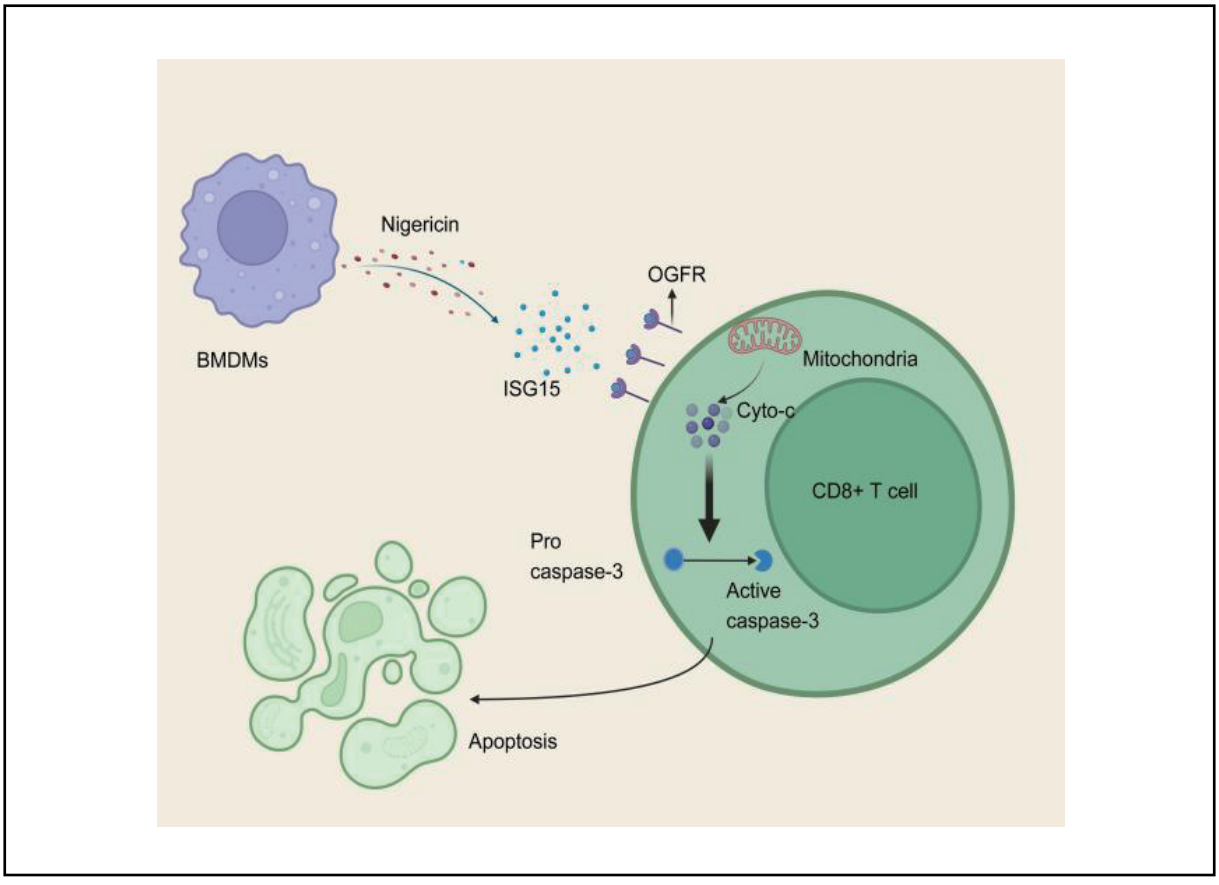
Macrophage-released ISG15 has a stimulatory effect on CD8$^+$ T cell apoptosis

Mao Li[1], Shuhang Li[2] ✉, and Li Bai[1,2] ✉

[1]*Center for Advanced Interdisciplinary Science and Biomedicine of IHM, Division of Life Science and Medicine, University of Science and Technology of China, Hefei 230026, China;*
[2]*Institute of Health and Medicine, Hefei Comprehensive National Science Center, Hefei, China*

✉Correspondence: Li Bai, E-mail: baili@ustc.edu.cn; Shuhang Li, E-mail: lshhlsy@ustc.edu.cn

Graphical abstract



Nigericin-stimulated BMDMs via the ISG15-OGFR axis induce CD8$^+$ T cell apoptosis through caspase activation.

Public summary

- Nigericin induces robust ISG15 expression in BMDMs.
- The ISG15-OGFR axis is critical for driving CD8$^+$ T cell apoptosis.
- The ISG15-OGFR signaling axis promotes cytochrome c release into the cytosol of CD8$^+$ T cells, leading to caspase cascade activation and subsequent apoptosis.

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[2]*Institute of Health and Medicine, Hefei Comprehensive National Science Center, Hefei, China*

✉Correspondence: Li Bai, E-mail: baili@ustc.edu.cn; Shuhang Li, E-mail: lshhlsy@ustc.edu.cn

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Abstract: ISG15, an interferon-stimulated gene product, has been recognized predominantly for its antiviral capabilities. However, the effect of ISG15 on cell survival remains unclear and requires experimental investigation. Our study revealed that ISG15, which is secreted by bone marrow-derived macrophages (BMDMs), has the capacity to induce apoptosis in CD8$^+$ T cells. Notably, we discovered that the proapoptotic effects of ISG15 are facilitated through its interaction with the opioid growth factor receptor (OGFR). This apoptotic pathway can be significantly attenuated by treatment with the opioid antagonist naltrexone. Our findings illuminate a previously uncharacterized ISG15-mediated pathway that regulates CD8$^+$ T cell apoptosis, thereby highlighting the critical role of ISG15 in the orchestration of the innate immune response.

Keywords: Macrophage; CD8$^+$ T cell; apoptosis; ISG15

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1 Introduction

The induction of CD8$^+$ T cell apoptosis constitutes a critical mechanism of negative feedback regulation within the immune system[1–3]. During the immune response, CD8$^+$ T cells are activated and promote the secretion of an assortment of cytokines and molecular signals, orchestrating the scope and persistence of the immune reaction[4–6]. A deficiency in facilitating CD8$^+$ T cell apoptosis may lead to overactivation of CD8$^+$ T cells, potentially culminating in an overactive immune response and the emergence of autoimmune pathologies[7–9]. The regulation of CD8$^+$ T cell apoptosis upon activation is vital for maintaining immune homeostasis, preventing overstimulated CD8$^+$ T cells from initiating disproportionate inflammatory reactions and autoimmunity[10,11]. Furthermore, this regulatory process is integral to the success of antitumor immunotherapies[12–14]. Elucidating the mechanisms and regulatory networks that govern CD8$^+$ T cell apoptosis not only advances our understanding of immune modulation and balance but also paves the way for novel immunotherapeutic interventions and disease treatments.

Interferon-stimulated ubiquitin-like protein 15 (ISG15) has increasingly been recognized as a key player in the host's antiviral defense[15–17]. As a member of the ubiquitin family, ISG15 can covalently attach to target proteins through a series of enzymatic reactions[18]; however, the destiny of these posttranslationally modified proteins is not yet fully understood. In its unconjugated form, ISG15 acts as a cytokine or engages with several intracellular proteins[18]. Current studies suggest that ISG15, through noncovalent interactions and its intrinsic cytokine activity, can modulate viral replication and the host immune response[19–21]. Our research demonstrated that macrophage-secreted ISG15 can induce apoptosis in CD8$^+$ T cell populations, indicating a role for ISG15 in mediating the crosstalk between macrophages and CD8$^+$ T cells in various pathological contexts.

2 Materials and methods

2.1 Mice

C57BL/6 wild-type mice were purchased from the Model Animal Research Center of Nanjing University. *Isg15* knockout mice were generated via CRISPR-Cas9. All the mice were housed under specific pathogen-free conditions with a 12 h dark/12 h light cycle. All the animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Science and Technology of China, and the experiments were performed in accordance with the approved guidelines. Approval number for the animal studies: USTCACUC28110124045.

2.2 Generation of bone marrow-derived macrophages (BMDMs)

Bone marrow cells were extracted from the hind legs of WT or *Isg15* knockout mice. After mixing, the bone marrow cells were used for BMDM differentiation and cultured in DMEM (Gibco) supplemented with 10% FBS and 50 μg/mL M-CSF (Novus Biologicals) for 5 d. When the bone marrow cells were used for bone marrow-derived dendritic cell (BMDC) differentiation, the cells were cultured in RPMI 1640 medium supplemented with 10% FBS, 20 ng/mL GM-CSF (Pepro

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Tech, 315–03), and 10 ng/mL IL4 (Pepro Tech, 214–14) for 8 d.

2.3 Flow cytometry analysis and antibodies

CD8+ T cells were blocked with a purified anti-mouse CD16/32 antibody for 15 minutes on ice. The cells were subsequently stained with the appropriate monoclonal antibodies at 4°C for 30–40 minutes in the dark. For intracellular staining, the cells were fixed and permeabilized with the Foxp3/Transcription Factor Staining Kit (eBioscience, 00–5523–00) for 30 minutes after surface staining and then stained with mAbs against intracellular molecules in permeabilization buffer. For apoptosis detection, the cells were stained with an Annexin V-FITC/PI Apoptosis Detection Kit (Vazyme, A211-02) for 10 minutes at room temperature in the dark. For assessments of mitochondrial ROS, the cells were cultured in RPMI 1640 medium containing MitoSOX Red (1 μmol/L; Thermo Fisher Scientific, M36008) at 37°C for 30 minutes in the dark.

2.4 Cell enrichment

The spleen was aseptically isolated from C57BL/6 mice (6–8 weeks old) and placed in RPMI 1640 with 1% FBS. Next, the spleen was mechanically dissociated via a syringe plunger. The cells were subsequently centrifuged at 500 × g for 5 minutes at 4°C, after which the supernatant was discarded. Splenocytes were resuspended in RPMI 1640 with 1% FBS and blocked with anti-CD16/32 (1 μg/mL) for 15 minutes on ice. For CD8+ T cell enrichment, the cells were stained with anti-APC CD8a for 35 minutes at 4°C in the dark. The cells were washed with RPMI 1640 with 1% FBS and centrifuged at 500 × g for 5 minutes at 4°C. The supernatant was discarded, and this step was repeated. Anti-APC microbeads (Miltenyi Biotech,130-090-855) were added for 40 minutes at 4°C in the dark. The cells were washed twice as described. The cell suspension was applied to an enrichment column (Miltenyi) on a magnet and washed with RPMI 1640 with 1% FBS, and the CD8+ T cells were eluted with RPMI 1640 with 10% FBS. The enrichment procedures were similar, with different staining procedures: CD4+ T cells were stained with anti-CD4-APC, and B cells were stained with anti-B220-APC. All the cell types followed the same enrichment protocol, with antibody specificity being the only variable. For peritoneal macrophage (PM) enrichment, the mice were euthanized and disinfected with 75% ethanol. A small incision was made along the ventral abdomen of each mouse. The abdominal skin was then cut, and the skin on both sides was gently pulled apart to expose the peritoneum. Using prechilled 1× PBS, 5–6 ml was slowly injected into the peritoneal cavity via the lower right quadrant with a syringe. The abdomen was gently massaged 5–8 times and then allowed to stand for 4 minutes. The peritoneal wash fluid was slowly collected by puncturing the peritoneum with a syringe and transferred into a sterile 15 ml centrifuge tube.

2.5 Inhibitors and reagents

Various cell death inhibitors, including Z-VAD-FMK, necrostatin-1, ferrostatin-1 and N-acetylcysteine, were purchased from MedChemExpress. Mito-tempo, anti-CD11a and A-286982 antibodies were also purchased from MedChemExpress. Naloxone HCl was purchased from Selleck.

2.6 Confocal microscopy

CD8+ T cells were stained with anti-cytochrome c (Cell Signaling Technology, 12963) and anti-Tom20 (Cell Signaling Technology, 66401) for 60 minutes at room temperature and fixed in 4% paraformaldehyde (PFA, IBBI, E672002) for 15 minutes. The samples were mounted with ProLong Gold antifade reagent containing 4 ′,6-diamidino-2-phenylindole (DAPI; Thermo Fisher, P36931). Images were taken with a Zeiss LSM 980 microscope with a 60× oil immersion objective and were analyzed with ImageJ software.

2.7 Immunoblotting

The cells were lysed in NP-40 (Beyotime, Shanghai, China) supplemented with complete protease inhibitor cocktail (Thermo Fisher, Waltham, US). SDS–PAGE was used to separate proteins in the cell lysate. The proteins were subsequently transferred to polyvinylidene difluoride (PVDF) membranes, which were blocked in 5% nonfat milk at room temperature for 1 h and incubated with primary antibodies at 4°C overnight. After that, the proteins were incubated with HRP-conjugated secondary antibodies for 45 minutes at room temperature. The primary antibodies used for immunoblotting included anti-ISG15 (Cell Signaling Technology, 2743S, 1:1000) and anti-β-actin (Cell Signaling Technology, 4967, 1:5000) antibodies. The HRP-conjugated secondary antibodies used included AffiniPure goat-anti-mouse IgG (H+L) (Proteintech, SA00012-1, 1:5000) and AffiniPure goat-anti-rabbit IgG (H+L) (Proteintech, SA00001-7H, 1:5000).

2.8 Overexpression of ISG15

293T cells were transfected with a vector containing sh*Isg15* (5'-CGGAATTCCGATGGCCTGGGACCTAAAGGT-3'; 6 μg), along with PMD2. G (2 μg) and PsPAX2 (4 μg), respectively. The lentivirus-containing media were collected and passed through 0.45-μm filters after 2 days. BMDMs were seeded on 6 cm cell culture dishes with 1×10^6 cells per dish, and the lentivirus-containing medium was then added to the cells for 48 h to overexpress ISG15.

2.9 Real-time quantitative PCR

Total RNA extraction from cells was performed according to the manufacturer's protocol for the ReliaPrep RNA Miniprep System (Promega). Following the isolation of total cellular RNA, the concentration of RNA in each sample was measured, and aliquots containing 0.5 μg of RNA were taken for subsequent procedures. Reverse transcription reactions were carried out via the HiScript III RT Supermix for a qPCR kit (Vazyme Biotech). Subsequent quantitative real-time fluorescence PCR was conducted with TB Green Premix Ex Taq II (Takara Bio) on a qPCR instrument.

2.10 Primers for qRT–PCR

The following primers were used in this study: *Itgal*-Forward: CCA GAC TTT TGC TAC TGG GAC, *Itgal*-Reverse: GCT TGT TCG GCA GTG ATA GAG; *Myo1g*-Forward: GGC CCT GAG TAT GGG AAA CC; *Myo1g*-Reverse: GAT ACG AGC ACC TCA CCA ATG; *Esty1*-Forward: CAT CCT CGT TGT CTA CTT GGA C; *Esty1*- Reverse: CAA CAG GCG

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AGC AAG AGG G; *Mctp2*-Forward: CAC GGC AAT GAC GAT CTG AAT; *Mctp2*-Reverse: GGT GAG GAG GTA CGC GAA G; *Ogfr*-Forward: GAG ACC CTA GTA CAG CAC AAA C; *Ogfr*-Reverse: CCT GCC CTA GTC CCA TCA GT.

2.11 Quantitative proteomics analysis

Proteins were extracted from the samples via SDT lysis buffer (4% SDS, 100 mM Tris/HCl (pH=7.6), 0.1 M DTT). The protein concentration was determined via the BCA assay. Aliquots of proteins from each sample were subjected to filter-aided sample preparation (FASP) for tryptic digestion. The peptides were desalted via a C18 cartridge, lyophilized, and then resuspended in 40 μL of 0.1% formic acid solution. The peptide concentration was measured by UV absorbance at 280 nm (OD_{280}). The samples were separated via a nanoflow HPLC system (Easy nLC). Solvent A was 0.1% formic acid in water, and solvent B was 0.1% formic acid in acetonitrile (containing 84% acetonitrile). Peptides were loaded onto a trapping column (Thermo Scientific Acclaim PepMap100, 100 μm × 2 cm, nano Viper C18) and separated through an analytical column (Thermo Scientific EASY column, 10 cm, ID 75 μm, 3 μm, C18-A2) at a flow rate of 300 nL/min. The separated peptides were analyzed via a timsTOF Pro mass spectrometer in parallel accumulated serial fragmentation (PASEF) mode. The raw mass spectrometry data were collected in .d format and processed via MaxQuant software (version 1.6.14) for database searching and quantification analysis.

2.12 Statistical analysis

All flow cytometry data were analyzed via an unpaired Student's t test or one-way/two-way ANOVA, as appropriate. The data are presented as the means ± SDs from at least three independent experiments and were analyzed via GraphPad Prism 9.0.1. Statistical significance was defined as follows: *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

3 Results

3.1 Induction of CD8$^+$ T cell apoptosis by nigericin-treated macrophage-derived supernatants in a dose-dependent manner

When CD8$^+$ T cells were cocultured with bone marrow-derived macrophages (BMDMs), the presence of nigericin significantly increased CD8$^+$ T cell apoptosis (Fig. 1b). Previous studies have reported that nigericin has cytotoxic effects on cells[22]. Consequently, our findings showed that the exposure of CD8$^+$ T cells and BMDMs to nigericin did not directly trigger apoptosis in these cell populations (Fig. 1c), thereby ruling out a direct cytotoxic effect of nigericin. Using a transwell system, we revealed that the presence of nigericin induced CD8$^+$ T cell apoptosis in the presence of BMDMs but did so in a CD8$^+$ T cell–BMDM contact-independent manner (Fig. 1d). These results suggest that soluble factors released by BMDMs might be capable of inducing T-apoptosis. We proceeded to perform dose–response experiments using supernatants from nigericin-treated BMDMs. Compared with cells cultured with supernatants from untreated BMDMs, CD8$^+$ T cells cultured with 30%, 50% or 100% supernatants from nigericin-treated BMDMs gradually increased apoptosis (Fig. 1e), indicating a dose-dependent effect.

Next, we investigated whether immune cells other than BMDMs could induce CD8$^+$ T cell apoptosis upon nigericin treatment. Conditioned media were collected from various nigericin-treated immune cell populations, including CD8$^+$ T cells, B cells, and bone marrow-derived dendritic cells (BMDCs), and only supernatants derived from BMDMs and peritoneal macrophages were able to induce CD8$^+$ T cell apoptosis (Fig. 1f). Together, these data suggest that the response of macrophage-derived molecules to nigericin treatment enhances CD8$^+$ T cell apoptosis.

3.2 CD8$^+$ T cell apoptosis triggered by supernatants of nigericin-treated macrophages depends on caspases

Next, we investigated the mechanism underlying CD8$^+$ T cell apoptosis induced by supernatants from nigericin-treated BMDMs. Ferrostatin-1 (Fer-1) is an inhibitor of ferroptosis[23], Z-VAD(OH)-FMK (Z-VAD-FMK) is a pancaspase inhibitor[24], and necrostatin (Nec-1) inhibits necroptosis[25]. We observed that the administration of Z-VAD-FMK, rather than other inhibitors, significantly blocked the CD8$^+$ T-cell death induced by the supernatants of nigericin-treated BMDMs (as depicted in Fig. 2a and b) in a dose-dependent manner (refer to Fig. 2c). These results indicate a caspase-dependent mechanism leading to CD8$^+$ T cell apoptosis. Given the important role of mitochondria in intrinsic apoptotic regulation[26], we further assessed the impact of nigericin-treated BMDM supernatants on mitochondrial function in CD8$^+$ T cells. The mitochondrial mass and membrane potential (Δψm) were evaluated via MitoTracker Green^{FM} and tetramethylrhodamine (TMRM), respectively[27]. We revealed a reduced TMRM/MitoTracker ratio, indicating mitochondrial damage (Fig. 2d). The generation of mitochondrial reactive oxygen species (ROS), a frequent event in mitochondrial damage[28], was measured via Mito-SOX assays. We observed increased Mito-SOX (red) fluorescence intensity in CD8$^+$ T cells upon exposure to nigericin-treated BMDM supernatants (Fig. 2d). However, neither NAC[29], a broad-spectrum ROS inhibitor nor Mitotempo[30], a mitochondrial-specific ROS inhibitor, alleviated the CD8$^+$ T cell apoptosis induced by nigericin-treated BMDM supernatants (Fig. 2e and f). These results suggest that the CD8$^+$ T cell apoptosis induced by nigericin-treated BMDM supernatants is ROS independent. On the other hand, mitochondrial damage causes the translocation of the proapoptotic protein cytochrome c (Cyt-c) from the mitochondria to the cytoplasm, which leads to caspase activation and apoptosis[31,32]. We examined the cellular localization of Cyt-c, and fluorescence images revealed that Cyt-c levels in the cytoplasm were significantly increased in nigericin-treated BMDM supernatants (Fig. 2g). These data revealed that nigericin-treated BMDM supernatants activated the caspase apoptotic pathway in CD8$^+$ T cells, leading to the release of Cyt-c in a ROS-independent manner.

3.3 Macrophages induce CD8$^+$ T cell apoptosis in an ISG15-dependent manner

To identify the soluble factor in the medium of nigericin-

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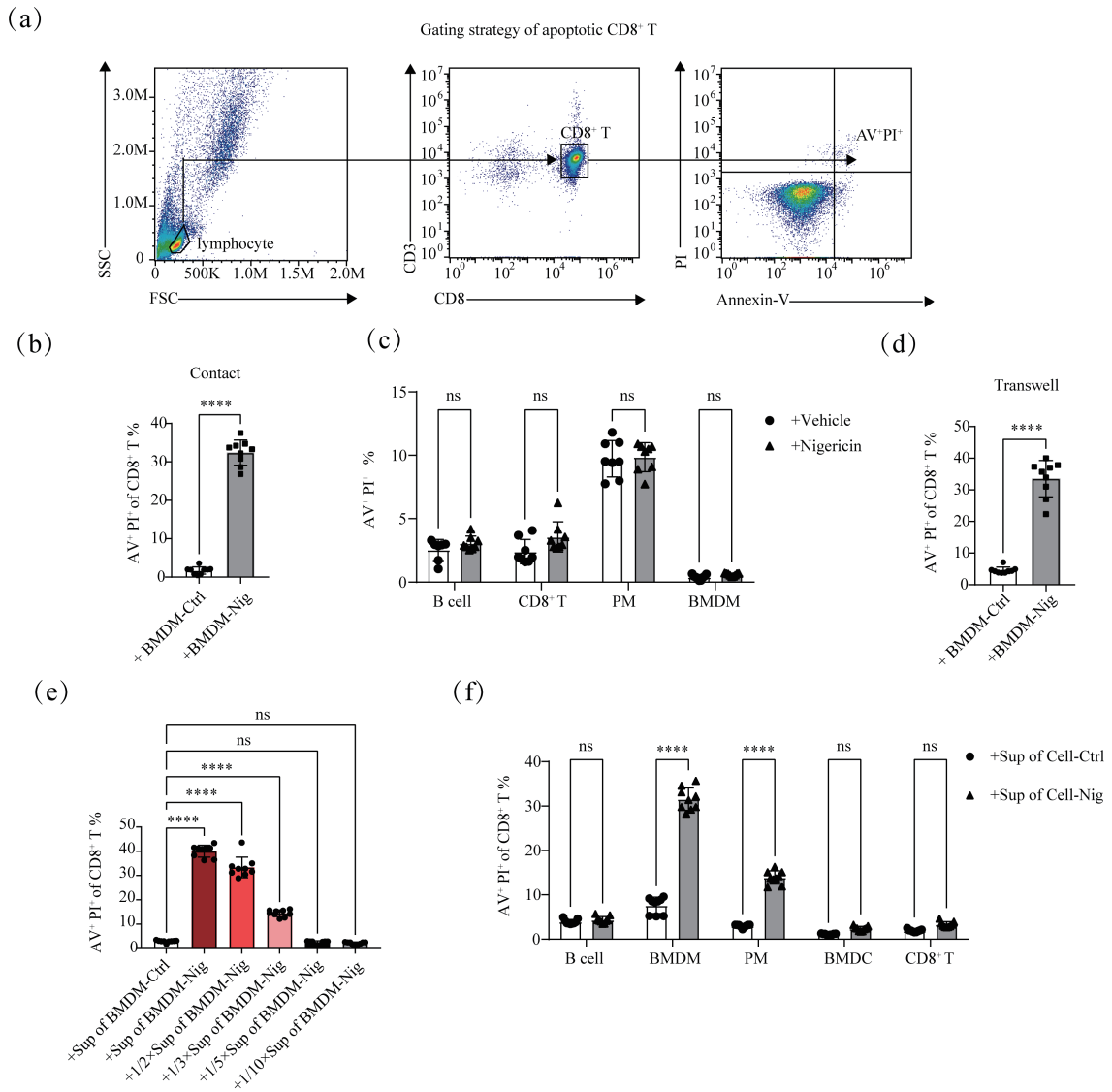

(a) Gating strategy of apoptotic CD8⁺ T cells via flow cytometry analysis. (b) Flow cytometry analysis of CD8⁺ T cell apoptosis following overnight (16 h) coculture with BMDMs treated with nigericin (5 μM). CD8⁺ T cells: CD8α⁺ CD3⁺. The proportion of apoptotic CD8⁺ T cells was determined by measuring the percentage of annexin V (AV)- and propidium iodide (PI)-double-positive (AV⁺PI⁺) cells. (c) Flow cytometry analysis of apoptosis in various immune cells stimulated with nigericin or ethanol (vehicle control). (d) Flow cytometry analysis of CD8⁺ T cell apoptosis after overnight (16 h) coculture with BMDMs in a transwell system. (e) Flow cytometry analysis of CD8⁺ T cell apoptosis following overnight (16 h) coculture with supernatants from nigericin-treated BMDMs. The supernatants were diluted in DMEM containing 10% FBS at various ratios prior to coculture. (f) Flow cytometry analysis of CD8⁺ T cell apoptosis following overnight (16 h) coculture with supernatants from various immune cells treated with nigericin or vehicle. PM: peritoneal macrophage; BMDC: bone marrow-derived dendritic cell. All data above represent the mean ± SEM of three independent experiments. (**** $P < 0.0001$).

Fig. 1. Induction of CD8⁺ T cell apoptosis by nigericin-treated macrophage-derived supernatants occurred in a dose-dependent manner.

treated macrophages responsible for CD8⁺ T cell apoptosis, we harvested supernatants from control BMDMs and nigericin-treated BMDMs and performed proteomic analysis to compare the expression of exosome markers and free proteins across different samples. Together, we identified a total of 38 upregulated proteins and 11 downregulated proteins in nigericin-treated BMDM supernatants, with changes exceeding 2-fold (p value < 0.05) (Fig. 3a). Among the top five most upregulated proteins identified by mass spectrometry (Fig. 3a), ISG15 was the only protein documented in the literature to regulate immune cell proliferation and function. Therefore, we chose ISG15 as the research object because ISG15 has been reported to exist in both conjugated and unconjugated forms, with the unconjugated form being released by macrophages[33,34]. Additionally, ISG15 is proposed to function as a cytokine with various immunomodulatory activities[15]. Western blot analysis demonstrated that the ISG15 protein level was increased in BMDMs following nigericin treatment (Fig. 3b). To investigate the effect of ISG15 on CD8⁺ T cell apoptosis, we constructed *Isg15* knockout (*Isg15*$^{-/-}$ mice (Fig. 3c). The deficiency of ISG15 in nigericin-treated BMDMs significantly alleviated the effect of their supernatants on the induction of CD8⁺ T cell apoptosis (Fig. 3d). On the other hand, overexpression of ISG15 in nigericin-treated BMDMs en-

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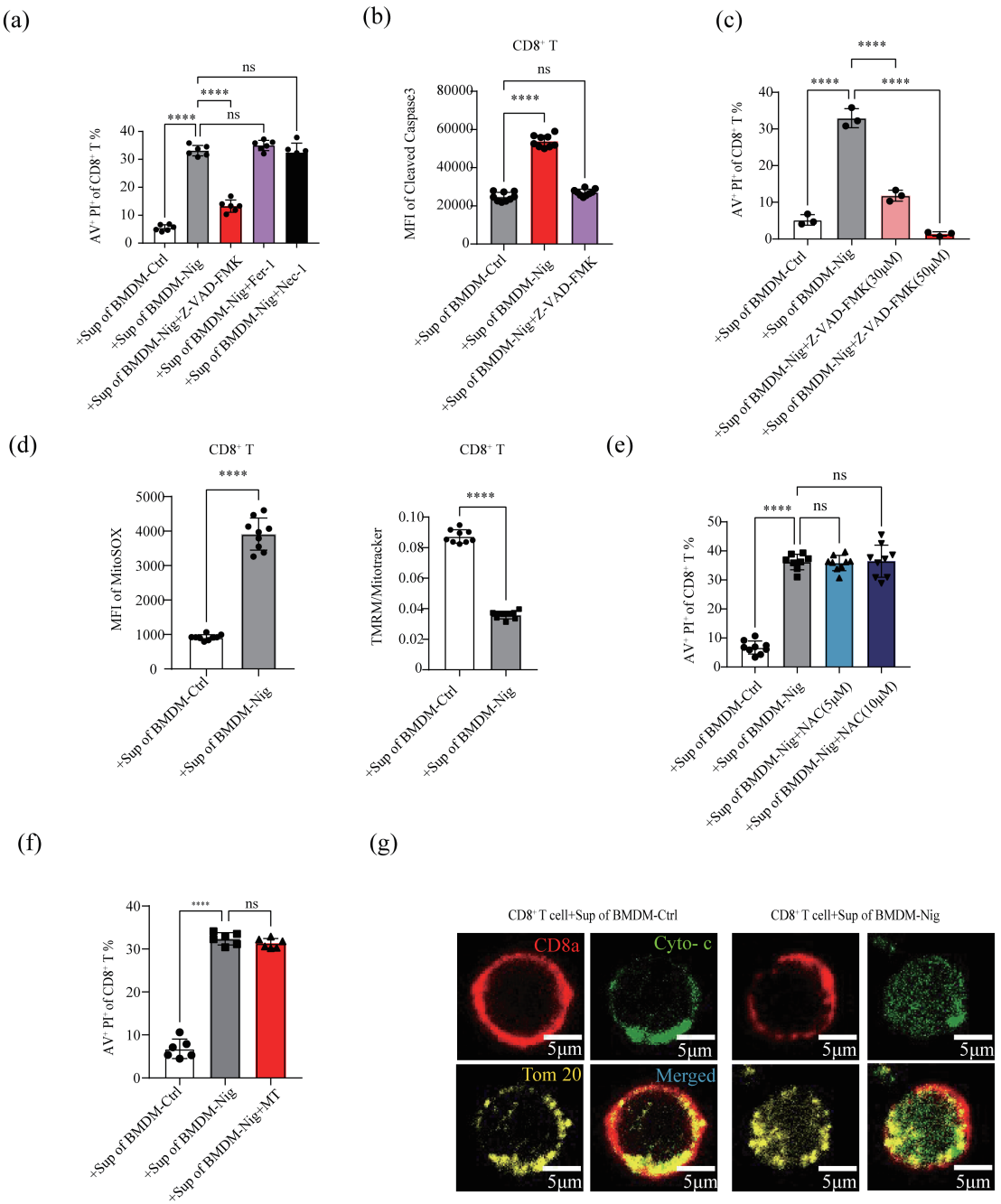

(a) Flow cytometry analysis of CD8$^+$ T cell apoptosis induced by supernatants from nigericin-treated BMDMs in the presence of various cell death inhibitors. Z-VAD-FMK (30 μM) is a pancaspase inhibitor (red). Ferrostatin-1 (Fer-1, 10 μM) is an inhibitor of ferroptotic cell death (purple). Necrostatin-1 (Nec-1, 30 μM) is an inhibitor of necroptotic cell death (black). (b-c): Flow cytometry analysis of the mean fluorescence intensity (MFI) of cleaved caspase-3 (Fig. 2b), and the dose-dependent effect of Z-VAD-FMK on CD8$^+$ T-apoptosis induced by supernatants from nigericin-treated BMDMs was determined (Fig. 2c). (d) Left panel: Flow cytometry analysis of mitochondrial ROS levels in CD8$^+$ T cells treated with *nigericin*-stimulated BMDM supernatants. The cells were stained with MitoSOX™ Red (1 μM final concentration) at 37 °C for 15 min, washed to remove excess dye, and analyzed immediately. Right panel: Ratio of the mitochondrial membrane potential (TMRM) to the mitochondrial mass (MitoTracker™ Green) in CD8$^+$ T cells treated with *nigericin*-stimulated BMDM supernatants. The cells were costained with TMRM (5 μM) for membrane potential detection and MitoTracker™ Green (0.2 μM) for mass quantification following a 30-min incubation at 37 °C in the dark prior to flow cytometry. (e) Flow cytometry analysis of the effect of N-acetylcysteine (NAC, 5/10 μM) on CD8$^+$ T cell apoptosis induced by supernatants from nigericin-treated BMDMs. (f) Flow cytometry analysis of the effect of the mitochondrial ROS inhibitor MT (20 μM) on CD8$^+$ T-apoptosis induced by supernatants from nigericin-treated BMDMs. (g) Representative confocal images reveal changes in the colocalization of cytochrome c and mitochondria within CD8$^+$ T cells. Cellular membranes were stained with an anti-CD8a antibody (red), mitochondria were visualized with a Tom20 antibody (yellow), and cytochrome c was detected with a cytochrome c antibody (green). The figure shows a representative result from three independent experiments. All data above represent the mean ± SEM of three independent experiments. Statistical significance was determined via a two-tailed t test or one-way ANOVA with Tukey's post hoc test (**** $P < 0.0001$).

Fig. 2. CD8$^+$ T cell apoptosis triggered by supernatants of nigericin-treated macrophages depends on caspases.

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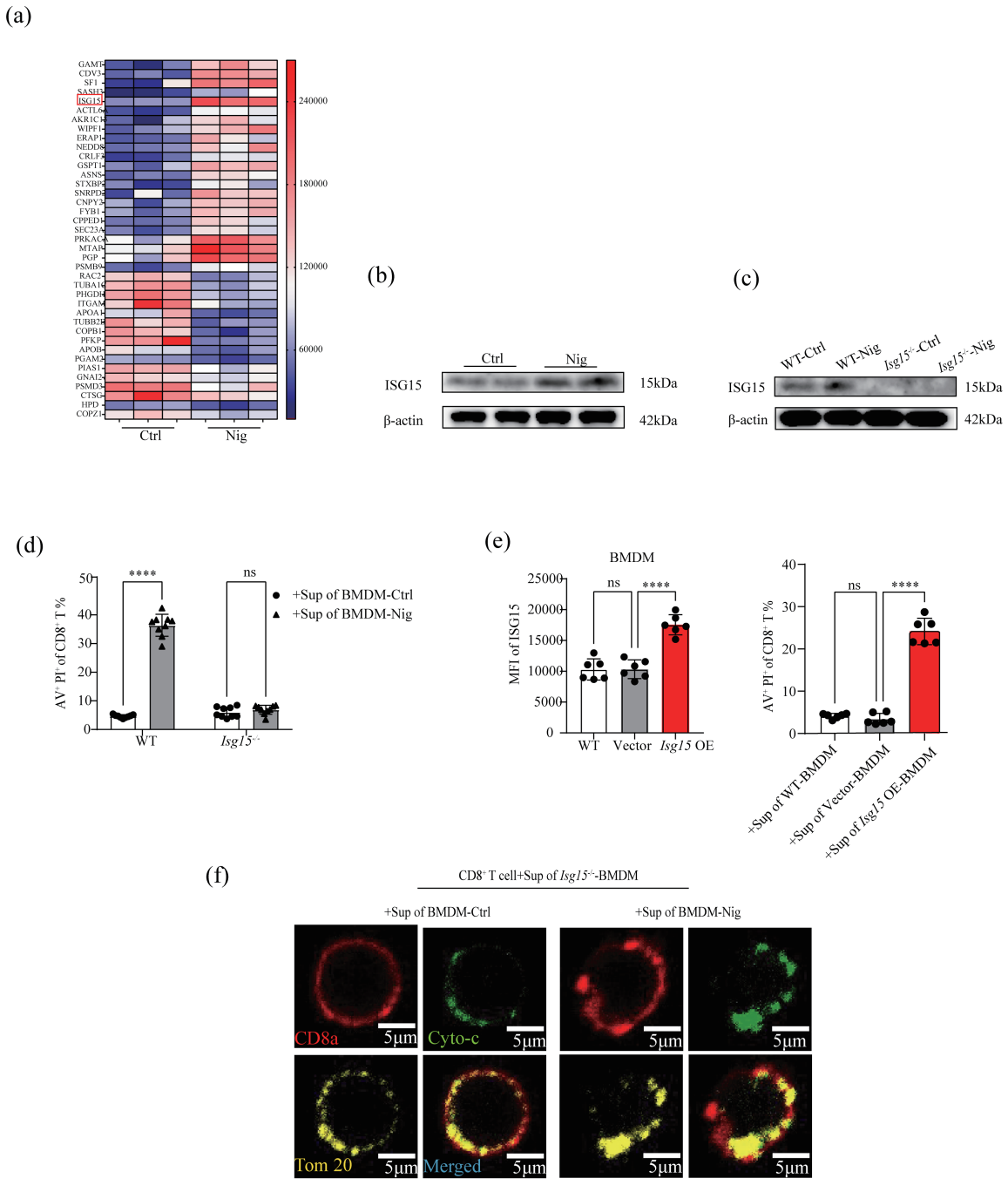

(a) MS analysis of supernatants from nigericin-stimulated BMDMs, with a heatmap displaying proteins whose expression significantly differed between the ctrl and nigericin groups. (b) Western blot analysis of ISG15 protein expression levels in nigericin-stimulated BMDMs compared with control BMDMs. The figure shows a representative result from three independent experiments. (c): The effect of *Isg15* knockout was detected via western blotting. WT or *Isg15* knockout (*Isg15*$^{-/-}$). The figure shows a representative result from three independent experiments. (d) Flow cytometry analysis of CD8^{+} T cell apoptosis following coculture with supernatants from nigericin-stimulated *Isg15*$^{-/-}$ BMDMs and WT BMDMs. (e) Flow cytometry analysis of the effects of *Isg15* overexpression on BMDM and CD8^{+} T cell apoptosis following coculture with supernatants from *Isg15*-overexpressing (*Isg15* OE)-BMDMs. (f) Representative confocal images reveal changes in the colocalization of cytochrome c and mitochondria within CD8^{+} T cells. Cellular membranes were stained with an anti-CD8a antibody (red), mitochondria were visualized with a Tom20 antibody (yellow), and cytochrome c was detected with a cytochrome c antibody (green). The figure shows a representative result from three independent experiments. The results are presented as the means±SEMs from three independent experiments. Statistical significance was determined via one-way/two-way ANOVA with Tukey's post hoc test (***** $P < 0.0001$).

Fig. 3. Macrophages induce CD8^{+} T cell apoptosis in an ISG15-dependent manner

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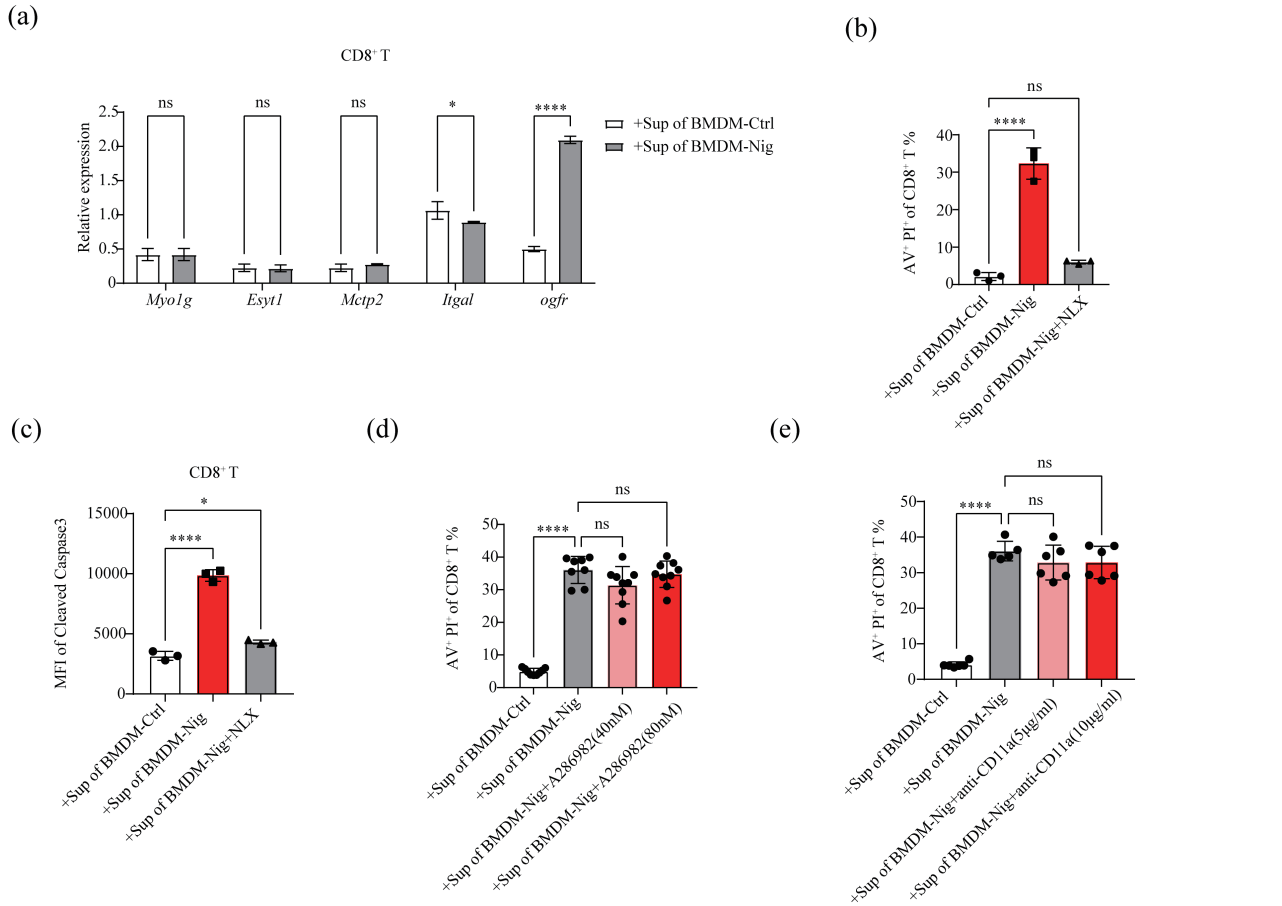
hanced the apoptosis-inducing effect of their supernatants (Fig. 3e). Additionally, when the *Isg15* gene was deleted, nigericin-treated BMDM supernatants failed to induce the translocation of Cyt-c from the mitochondria to the cytoplasm of CD8$^+$ T cells (Fig. 3f). These results indicate that nigericin-treated macrophage supernatants induce CD8$^+$ T cell apoptosis in an ISG15-dependent manner.

3.4 ISG15 Induces CD8$^+$ T cell Apoptosis via the OGFR

To determine whether ISG15 induces CD8$^+$ T cell apoptosis through binding to a cell surface receptor, we examined the mRNA expression levels of five potential ISG15 receptors[35]. As shown in Fig. 4a, *Itgal* (CD11a) and *Ogfr* (OGFR), but not *Mctp2* (MCTP2), *Esyt1* (ESYT1) or *Myo1g* (MYO1G), presented relatively high expression levels in CD8$^+$ T cells, suggesting that CD11a and OGFR may act as receptors for ISG15 in CD8$^+$ T cells (Fig. 4a). Notably, nigericin-treated BMDM supernatants increased the expression of OGFR in CD8$^+$ T cells, indicating that OGFR may be a key receptor for ISG15. Indeed, naltrexone, a specific inhibitor of OGFR, reduced CD8$^+$ T cell apoptosis induced by nigericin-treated BMDM supernatants (Fig. 4b and c). Moreover, CD11a and CD18 form lymphocyte function-associated antigen 1 (LFA-1), which binds to extracellular ISG15[35]. To determine whether LFA-1 is involved in ISG15-mediated CD8$^+$ T cell apoptosis, we used a blocking antibody against CD11a and a small molecule inhibitor (A-286982) of LFA-1 signaling to interfere with ISG15-mediated LFA-1 signaling[36,37]. Neither the anti-CD11a antibody nor A-286982 decreased the CD8$^+$ T cell apoptosis induced by nigericin-treated BMDM supernatants (Fig. 4d and e). These data demonstrate that ISG15 from macrophages induces CD8$^+$ T cell apoptosis via the OGFR.

3.5 Macrophage ISG15 induces CD8$^+$ T cell apoptosis during virus infection

Previous studies have demonstrated that ISG15 plays a cru-



(a) Real-time quantitative PCR (qPCR) was used to analyze the expression levels of five genes (*Myo1g, Esyt, Mctp2, Itgal, and Ogfr*) encoding ISG15 receptors in CD8$^+$ T cells following overnight (16 h) coculture with supernatants from nigericin-stimulated BMDMs. (b, c) Flow cytometry analysis of apoptosis and the mean fluorescence intensity (MFI) of cleaved caspase-3 in CD8$^+$ T cells isolated from the spleen and cocultured with supernatants from nigericin-stimulated BMDMs with or without naltrexone (NLX, 20 μM) treatment to block OGFR. (d) Flow cytometry analysis of CD8$^+$ T cell apoptosis following coculture with supernatants from nigericin-stimulated BMDMs treated with A-286982 (40/80 nM) to block the ISG15 receptor (LFA-1) on CD8$^+$ T cells. (e) Flow cytometry analysis of the induction of apoptosis in CD8$^+$ T cells cocultured with supernatants from nigericin-stimulated BMDMs in the presence of an anti-CD11a antibody. CD8$^+$ T cells were incubated with a CD11a antibody (5/10 μg/ml) to block the interaction between CD11a and ISG15 during the coculture period. The data represent the means±SEMs of three independent experiments. Statistical significance was determined via one-way/two-way ANOVA with Tukey's post hoc test (* P< 0.05, **** P < 0.0001).

Fig. 4. ISG15 induces CD8$^+$ T cell apoptosis via OGFR

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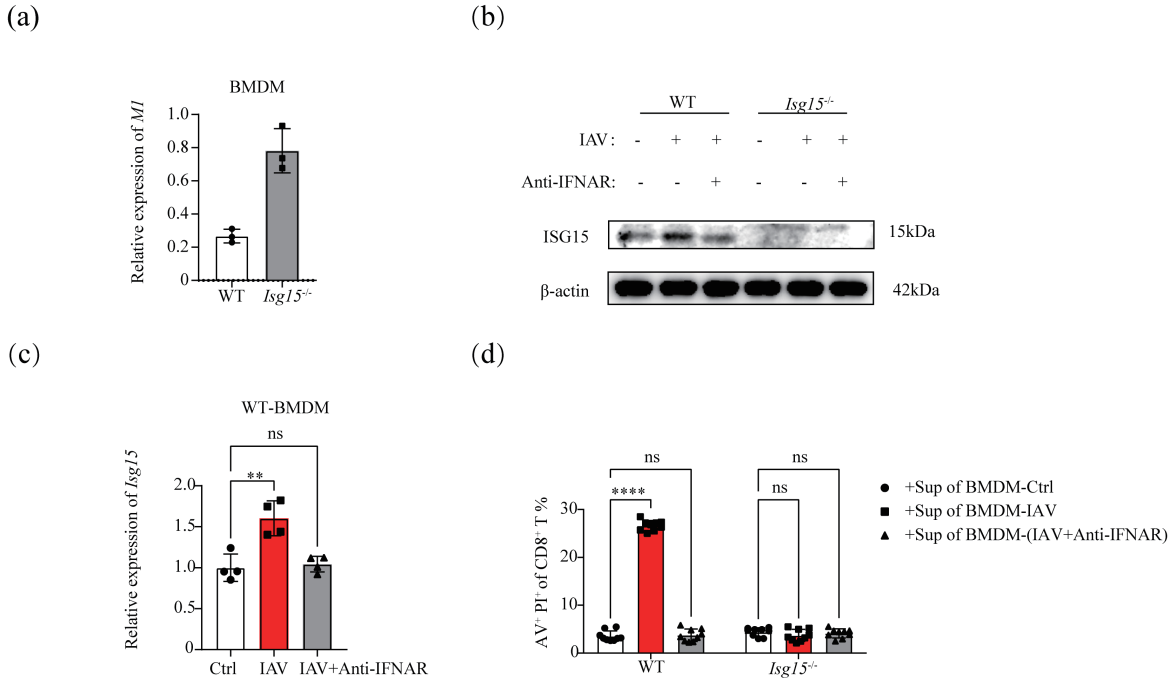
cial role in the innate immune response to viral infections[38]. ISG15 is upregulated following type I interferon induction. It is possible that viral infection might upregulate ISG15 expression and lead to CD8+ T cell apoptosis. To test this hypothesis, we next infected *Isg15* KO BMDMs and control BMDMs with influenza virus A (IAV). Consistent with these findings, *Isg15* KO BMDMs presented greater viral content than control BMDMs did (Fig. 5a). Influenza virus infection increased ISG15 expression in control BMDMs but not in *Isg15* KO BMDMs in an IFNAR-dependent manner, given that anti-IFNAR treatment blocked the upregulation of ISG15 (Fig. 5b and c). Furthermore, the supernatants of BMDMs infected with influenza virus induced CD8+ T cell apoptosis, which was abrogated by the anti-IFNAR antibody and by the deletion of ISG15 in BMDMs (Fig. 5d). These data imply that influenza virus infection induces CD8+ T cell apoptosis through ISG15 from macrophages.

4 Discussion

Within the immune system, the regulation of CD8+ T-cell survival and function by macrophages plays a crucial role in immune responses and disease pathogenesis. The regulatory mechanisms underlying the control of CD8+ T-cell survival by macrophages require further in-depth investigation. This study reveals a novel mechanism whereby macrophage-derived ISG15, upon binding to its receptor OGFR, promotes CD8+ T cell apoptosis by activating the caspase signaling pathway.

We found that soluble factors released by nigericin-stimulated bone marrow-derived macrophages (BMDMs) induced CD8+ T cell apoptosis in a concentration-dependent manner. Mechanistic studies demonstrated that this effect relies on the activation of the caspase signaling pathway and is accompanied by the release of cytochrome c from mitochondria into the cytosol, which is indicative of the activation of the classical mitochondrial apoptotic pathway. Notably, although mitochondrial reactive oxygen species (ROS) levels in CD8+ T cells were significantly elevated following treatment with supernatant from nigericin-stimulated BMDMs, scavenging ROS did not improve CD8+ T-cell survival, indicating that increased ROS levels are not critical factors driving this type of apoptosis.

Through mass spectrometry analysis and functional validation, we identified ISG15 as the key effector molecule in BMDM supernatant that is responsible for inducing CD8+ T-apoptosis. Further investigation revealed that ISG15 exerts its proapoptotic effect by binding to its receptor OGFR. This effect was significantly blocked by the OGFR-specific antagonist naloxone (NLX). In all our experiments, CD8+ T cells were maintained in a naive state. Combined with our experimental results demonstrating that BMDM-released ISG15 induces apoptosis in CD8+ T cells via OGFR engagement, these data indicate that macrophage-secreted ISG15 regulates CD8+ T cell apoptosis without significant antigen specificity. While our study focused on the antigen-nonspecific role of ISG15, it remains possible that ISG15 might synergize with antigen-



(a) Quantitative PCR analysis of influenza virus *M1* gene expression relative to that of the housekeeping gene in WT and *Isg15* knockout (*Isg15*$^{-/-}$) BMDMs following influenza virus A infection (0.5 HA, 48 h). (b) Western blot analysis of ISG15 protein expression in WT and *Isg15*$^{-/-}$ BMDMs postinfluenza virus A infection. β-actin is shown as a loading control. The image is representative of three independent experiments. (c) Quantification of the gray-scale values from the western blot results shown in Fig. 5b. (d) Flow cytometry analysis of CD8+ T cell apoptosis following overnight (16 h) coculture with supernatants from BMDMs harvested under different conditions. The data represent the means ± SEMs of three independent experiments. Statistical significance was assessed via one-way/two-way ANOVA with Tukey's post hoc test (** $P < 0.01$, *** $P < 0.0001$).

Fig. 5. Macrophage ISG15 induces CD8+ T cell apoptosis during virus infection

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specific signals in some contexts and alter TCR sensitivity or costimulatory molecule expression. In general, definitively excluding other potential mechanisms would require extensive experimental investigation. Furthermore, BMDMs infected with influenza virus presented high ISG15 expression, and their conditioned medium similarly promoted $CD8^+$ T cell apoptosis, suggesting that this mechanism may be involved in immune modulation during viral infection.

The significance of this study lies in uncovering the novel ISG15-OGFR axis derived from macrophages as a pathway regulating $CD8^+$ T cell apoptosis, thereby enriching the theoretical framework of macrophage-mediated control of $CD8^+$ T-cell survival. However, the conclusions of this study are currently limited to *in vitro* experiments. Whether ISG15 overexpression *in vivo* suppresses $CD8^+$ T-cell survival and consequently impacts adaptive immune responses requires validation in animal models. The literature suggests that the role of ISG15 in the tumor microenvironment is complex, with its expression levels and functions varying depending on the tumor type and microenvironmental context[39–41]. For example, in breast cancer models, free ISG15 promotes tumor growth by modulating NK cell infiltration and suppressing immune responses[39–41]. Conversely, in a study on ovarian cancer, ISG15 improved the antitumor efficacy of cisplatin by inhibiting the stem-like phenotype of tumor cells and thereby weakening their resistance to cisplatin[42]. Therefore, future research should further investigate the specific mechanisms by which the ISG15-OGFR axis regulates $CD8^+$ T-cell fate under diverse physiological and pathological conditions (e.g., infection and cancer) and its impact on overall immune responses.

5 Conclusions

This study revealed that macrophages stimulated with nigericin induce $CD8^+$ T cell apoptosis via increased ISG15 expression. Genetic ablation of *ISg15* abolished the proapoptotic effect of conditioned medium from nigericin-treated BMDMs on $CD8^+$ T cells. In contrast, conditioned medium from BMDMs overexpressing ISG15 promoted $CD8^+$ T cell apoptosis without nigericin stimulation. Furthermore, upon binding to its receptor OGFR, ISG15 triggers caspase activation and apoptosis by facilitating the release of cytochrome c from mitochondria into the cytosol. These findings provide a theoretical basis for understanding the macrophage-mediated regulation of $CD8^+$ T-cell survival.

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Conflict of interest

The authors declare that they have no conflict of interest.

Biographies

Mao Li is currently a master's student in the Division of Life Sciences and Medicine, University of Science and Technology of China. His research mainly focuses on the regulation of macrophages on the apoptosis of $CD8^+$T cells.

Li Bai is currently a Professor in the Division of Life Sciences and Medicine, University of Science and Technology of China. Her research mainly focuses on the immune and metabolic regulation of NKT cells.

Shuhang Li is an Associate Researcher at the Institute of Advanced Medicine, Hefei Comprehensive National Science Center. She received her Ph.D. degree in Cell Biology from the University of Science and Technology of China. Her research mainly focuses on the development of new immunotherapy drugs for anti-tumor.

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