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Damage-induced NAD release activates intestinal CD4+ and CD8+ T cell via P2X7R signaling

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ABSTRACT

Background: Postoperative ileus (POI) is characterized by the activation of inflammation triggered by tissue damage. Damage-associated molecular patterns (DAMPs) reportedly induce local inflammation after injury. However, the impact of DAMPs on intestinal resident lymphocytes during POI remains poorly elucidated.
Methods: POI in mice was induced via intestinal manipulation (IM). The concentration of nicotinamide adenine dinucleotide (NAD) was detected after IM. The gastrointestinal motility of the mice was assessed after IM or NAD injection. Cytokine production and calcium influx in T cells were investigated after NAD stimulation using flow cytometry.
Results: The concentration of extracellular NAD significantly increased after IM administration, and NAD directly impaired gastrointestinal motility. Intraperitoneal injection of NAD promoted the expression of TNF-α in intestinal CD8+ and CD4+ T cells, but only IFN-γ production by CD8+ T cells was significantly promoted by NAD injection. Granzyme B production in CD8+ and CD4+ T cells decreased after administration. Concordantly, the same results were observed in NAD stimulation of intestinal CD3+ T cells *in vitro*. Blocking the P2X7R-related membrane enzyme ART2.2 significantly diminished the pro-inflammatory effect of NAD.
Conclusion: IM includes the release of NAD derived from damaged tissues, consequently promoting pro-inflammatory cytokine production in intestinal CD4+ and CD8+ T lymphocytes. NAD-induced intestinal T cells activation may be associated with POI progression in the mouse.

1. Introduction

Postoperative ileus (POI) is a well-known complication of abdominal surgery. Patients with POI usually experience nausea, bloating, and an inability to tolerate transoral feeding [1]. POI progression is divided into early neurogenic and late inflammation phases. Surgical trauma-induced adrenergic nerve activation dominates the neurogenic phase of inhibition of motility. Stimulation of nociceptors and mechanoreceptors ceases at abdominal closure. Inflammation dominates the progression of POI for 2–4 days postoperatively.

Numerous studies have elucidated the role of myeloid cells in POI [2–4]. A recent study observed focal *T*-cell aggregation in the duodenum of patients with functional dyspepsia [5]. Moreover, memory Th1 cells are vital for the progression of POI [6]. However, the mechanism underlying lymphocyte activation after mechanical stimulation in POI remains enigmatic.

Surgery may lead to unavoidable tissue damage and release of damage-associated molecular patterns (DAMPs), such as dsDNA, nicotinamide adenine dinucleotide (NAD), and adenosine triphosphate (ATP) [7]. DAMPs are recognized by pattern recognition receptors (PRRs), which trigger inflammatory cascades. Myeloid cells activated by DAMPs facilitate the upregulation of inflammation-related genes,

Abbreviations: NAD, Nicotinamide adenine dinucleotide; POI, Postoperative ileus.

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including interleukin-6 (IL-6), cyclooxygenase-2 (COX-2), and inducible nitric oxide synthase (iNOS) [8]. Previous studies have reported the effects of DAMPs on lymphocytes. Extracellular ATP can facilitate calcium influx and enhance IL-2 production in activated T cells [9]. High mobility group box 1 (HMGB1) is crucial in controlling the CXCL11-induced accumulation of CD8+ T cells in the tumor microenvironment [10].

NAD is mainly located in the mitochondria and cytoplasm and performs its biological activity under physiological conditions [11]. During hypoxia, stress, and acute inflammation, NAD is released into the extracellular matrix [12] and recognized as one of the components of DAMPs. A high concentration of extracellular NAD agitates the purinergic receptor P2X7R. As a calcium channel, P2X7R activated by NAD triggers calcium influx and activation of related in-cell calcium signals. P2X7R signaling has also been reported to mediate the activation of the NLRP3 inflammasome, resulting in cytokine release (including IL-1β, TNFα, and IL-6) [13]. A P2X7R inhibitor was reported to improve gastrointestinal (GI) motility and ameliorate local inflammation in mice with POI; however, the detailed mechanism remains unelucidated [14].

In present study, we confirmed the release of DAMPs after surgery by measuring the concentration of NAD in abdomen and tissue. Furthermore, NAD caused impaired gastrointestinal (GI) transit independently of surgery. P2X7R was highly expressed in intestinal T lymphocytes compared with lymph nodes. Exogenous NAD promoted the expression of IFN-γ and TNF-α in intestinal T lymphocytes via the P2X7R signal. Our study unveiled the proinflammatory mechanism of NAD to intestinal residential T cells via the P2X7R pathway, which may provide a clue for the treatment of POI.

2. Materials and methods

2.1. Animals

C57BL/6J (6–8-week-old) male mice were used for the experiments. All mice were housed under specific pathogen-free conditions with constant humidity and temperature at a 12 h/12 h light/dark cycle and allowed *ad libitum* access to feed and water. All animal experiments were performed in accordance with the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Ethics Committee of Beijing Friendship Hospital, Capital Medical University.

2.2. Animal models

POI was induced by standardized intestinal manipulation (IM) as described previously [3]. The fasted mice were anesthetized with tribromoethanol (Sigma-Aldrich, St. Louis, MO, USA, CAT#T48402). For POI, the small intestine was rubbed twice after median laparotomy from the duodenum to the ileocecal region for 5 min using a moist cotton swab. For the sham group, the abdomen was opened for 5 min without manipulation. The abdomen was closed using 6–0 sutures. All mice were placed on a 37 °C thermostat plate from anesthesia to awakening.

Mice were injected intraperitoneally with 300 mg/kg NAD (Sigma-Aldrich, CAT#481915) or ATP (Sigma-Aldrich, CAT#A1852). The mice in the control group were injected with the same volume of saline.

2.3. Measurement of NAD+ NADH and ATP levels

Mice were sacrificed 6 h after surgery. The peritoneal cavities were washed with 500 μL sterile PBS, and the lavage fluid was harvested. The small intestine was removed and homogenized on ice using ultrasound. The concentration of NAD+ NADH in the lavage and homogenate samples were measured using NAD/NADH assay kit (Beyotime, Nanjing, China, CAT#S0175) and advanced ATP assay kit (Beyotime, Nanjing, China, CAT#S0026), according to the manufacturer's instructions. The results were normalized to the total protein concentration in the small intestine measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA. CAT#23225).

2.4. GI transit assay

FITC-dextran (70 kWM; Sigma-Aldrich, CAT#FD70-100MG) solution (200 μL of 5 mg/mL) was administered by oral gavage to the fasted mice. Ninety minutes later, the mice were sacrificed, and the whole GI tract (from the stomach to the rectum) was dissected. The GI tract was divided into 15 segments (stomach to the colon) and assigned a number from 1 to 15 according to its position. Each fragment was cut into 2–3 mm pieces and placed into a 1.5 mL EP tube containing 1 mL DPBS. After centrifugation at 500 × g for 1 min, the supernatant was collected to quantify fluorescence using Varioskan LUX (Thermo Fisher Scientific, Waltham, MA, USA). GI transit was described by the geometric center and calculated using the following formula:

$$\sum \%FD70 \textit{ in each segment} * \textit{segment number}$$

2.5. Isolation of lymphocytes

2.5.1. Intestine

Mice were sacrificed 6 h after surgery or 3 h after NAD/ATP injection. Small intestines, fecal contents removed, were opened longitudinally and cut into 1 cm pieces in ice-cold PBS. The intestines in the predigestion solution (Ca^{2+}/Mg^{2+}-free Hank's balanced salt solution supplemented with 1 mM dithiothreitol and 10 mM ethylenediaminetetraacetic acid) were placed on an orbital shaker for 20 min at 37 °C. Segments were incubated in a digestion solution (500 μg/mL collagenase IV (Sigma-Aldrich, CAT#C5138), 200 μg/mL DNAase I (Sigma-Aldrich, CAT#DN25-100MG), 100 μM HEPES buffer, and 4 % FBS in RPMI 1640) for 30 min under slow rotation (For grind group, the tissue was put into digestion solution without Collagenase IV and DNAase I, and then was ground immediately on a 70 μM cell strainer), and then filtered through 70 μm nylon mesh cell strainers. The supernatant was collected and centrifuged for 10 min at 500 × g and 20 °C. The precipitate was resuspended in 40 % Percoll (Sigma-Aldrich, CAT#P4937) solution, centrifuged without brake for 30 min at 800 × g and 20 °C, and the supernatant discarded.

2.5.2. Mesenteric lymph nodes

Lymph nodes were placed on a 70-μm filter and ground using the end of a syringe plunger. The cell pellet was washed and resuspended in cold fluorescence-activated cell sorting (FACS) buffer (PBS with 2 μM EDTA and 0.5 % bovine serum albumin).

2.5.3. CD3+/CD4+/CD8+ lymphocyte isolation

The cells were incubated with an anti-mouse CD3/CD4/CD8a antibody (PE-conjugated 17A2/GK1.5/S18018E BioLegend, San Diego, CA, USA). After washing twice with FACS buffer, cells were incubated with anti-PE microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany, 130–048-801). Cell isolation was performed using EasySep ™ Magnet (STEMCELL Technologies, Vancouver, Canada, CAT#18000).

2.6. In vitro activation

The cells were resuspended in a complete medium containing NAD (0, 0.1, 1, and 10 μM) and incubated at 37 °C for 30 min.

For the inhibition experiment, cells were pretreated with 20 μM 1,4-naphthoquinone (MedChemExpress, Wilkinson Way, Princeton, NJ, USA, CAT#HY-W015490) for 10 min and then stimulated with 10 μM NAD for 30 min.

For intracellular staining, all cells were incubated in a complete medium with the cell activation cocktail [PMA (phorbol 12-myristate-13-acetate), ionomycin, and protein transport inhibitor (Brefeldin A)]. (BioLegend, CAT#423304) at 37℃ for 4 h.

For ELISA experiment, the cells were incubated in a complete medium with PMA(Sigma-Aldrich, CAT#P1269) at 37℃ for 4 h. The supernatant was collected after stimulation.

2.7. Elisa

The detection was under the guidance of instruction manual. Granzyme B (MULTI SCIENCES Hangzhou, Zhejiang Province, China, CAT# EK2173 – 48), IFN-γ (MULTI SCIENCES Hangzhou, Zhejiang Province, China, CAT# EK280/3–48) and TNF-α (Thermo Fisher Scientific, Waltham, MA, USA.CAT#BMS607-3) kit were used to determine the cytokine production. The data was detected by Varioskan LUX (Thermo Fisher Scientific, Waltham, MA, USA). The concentration of each cytokine was calculated by the standard curve.

2.8. Flow cytometry

The Zombie Aqua™ Fixable Viability kit (BioLegend, CAT# 423102) was used to exclude dead cells. Antibody staining was performed at 4℃ with fluorochrome-conjugated clone mAb according to standard methods: anti-CD3 (PE-conjugated, clone 17A2), anti-CD45 (FITC conjugated, clone S18009F), anti-CD45 (BV711 conjugated, clone 30-F11), anti-TCRγδ (APC conjugated, clone GL3), anti-CD4 (Pacific Blue conjugated, clone GK1.5), anti-CD8a (BUV396 conjugated, clone 53–6.7), anti-CD3 (BUV737 conjugated, clone 500A2), anti-Ly6C (FITC conjugated, clone HK1.4), anti-MHC-II (PerCP/Cyanine5.5 conjugated, clone M5/114.15.2), anti-CD64 (APC conjugated, clone S18017D), anti-Ly6G (APC/Cyanine7 conjugated, clone 1A8), anti-CD11c (BV421 conjugated, clone N418), anti-CD11b (BV605 conjugated, clone M1/70), and anti-P2X7 (extracellular) receptor (PE-conjugated, clone Q9Z1M0).

For intracellular staining, cells were incubated in a complete medium with the Cell Activation Cocktail (BioLegend, CAT#423304) at 37 °C for 4 h. Cells were permeabilized with the Cyto-Fast™ Fix/Perm Buffer Set (BioLegend, CAT# 426803), following the manufacturer's protocol. Fixed cells were incubated with an antibody cocktail overnight at 4 °C for intracellular staining: anti-granzyme B (FITC conjugated, clone QA16A02), anti-IFN-γ (APC/Cyanine7 conjugated, clone XMG1.2), anti-Granzyme B (PE-conjugated, clone QA16A02), anti-TNF-α (PE/Cyanine7 conjugated, clone MP6-XT22), anti-IFN-γ (BV605 conjugated, clone XMG1.2), anti-TNF-α (PE-conjugated, clone MP6-XT22), and anti-CD206 (PE/Cyanine7 conjugated, clone C068C2). Data were acquired using a FACS Symphony A5 system (BD Biosciences, Franklin Lakes, NJ, USA) and analyzed using FlowJo X.

Anti-P2X7 receptor antibody was purchased from Alomone (Jerusalem, Israel), and the rest of the antibodies were purchased from BioLegend. The working dilution of all antibody was 1:200.

2.9. Calcium flux assay

Sorted CD3+ T cells were loaded with 2 μM Fluo-4 (Beyotime, CAT#S1060) in Hank's Balanced Salt Solution (without Ca^{2+} and Mg^{2+}) at 37 °C for 1 h. Flow cytometry was used to detect calcium mobilization in response to stimulation. Data were collected from unstimulated cells up to 300 s after 10 μM NAD stimulation.

2.10. Statistical analysis

Statistical analysis was performed using the two-tailed Student's *t*-test or one-way ANOVA with Dunn's multiple comparisons and analyzed using GraphPad Prism V9.0 (San Jose, CA, USA). Data are presented as mean ± standard error of the mean (SEM). Statistical significance was set at $p < 0.05$.

3. Results

3.1. Extracellular NAD is associated with POI

Consistent with previous studies, IM led to impaired mouse GI motility (Fig. 1**A**). To determine whether NAD played a role in POI, we evaluated the expression of NAD+ NADH in intestinal and peritoneal lavage fluid (PLF) harvested 6 h after surgery. The concentration of NAD+ NADH was significantly decreased in the intestine; however, the NAD+ NADH concentration in PLF increased (Fig. 1B). The different concentrations of NAD+ NADH in the intestinal tissue and PLF suggested the increase of extracellular NAD after IM. The ATP, known as the ligand of P2X7R, was found to increase in PLF 6 h after surgery (Fig.S1 A).

Next, we investigated whether extracellular NAD affects GI function. Mice were injected with NAD, and GI transit was measured 3 h post-injection. It was found that compared to the vehicle, NAD caused impairment in GI transit independent of surgical manipulation (Fig. 1**C**). However, intraperitoneal injection of ATP caused a slightly impairment of GI transit (**Fig. S1 B**). These results suggest that extracellular NAD contributes to the impairment of GI motility.

3.2. NAD increases the expression of TNF-α and IFN-γ in intestinal CD4+ and CD8+ T cells in vivo

To investigate whether the increase in extracellular NAD affects intestinal immune cells, we injected NAD intraperitoneally into C57 mice. NAD injection significantly increased the expression of TNF-α and IFN-γ in intestinal CD8+ T cells. However, NAD decreased granzyme B production (Fig. 2**A–C**). Regarding intestinal CD4+ T cells, granzyme B and TNF-α showed the same changes as CD8+ T cells, but IFN-γ levels did not significantly differ (Fig. 2**D–F**). Extracellular NAD has been suggested to promote local inflammation through intestinal CD4+ and CD8+ T cells. Considering the large proportion of intestinal macrophages which may be stimulated by NAD, we analyzed the polarization of macrophages. It was also found that NAD injection did not alter the expression of TNF-α and CD206 in macrophages (Fig. 2**G**). Therefore, NAD does not modulate the polarization of intestinal macrophages. Thus, the intraperitoneal injection of NAD promoted pro-inflammatory cytokine production of intestinal CD4+ and CD8+ T cells.

Production of granzyme B (A), IFN-γ (B), and TNF-α (C) in intestinal

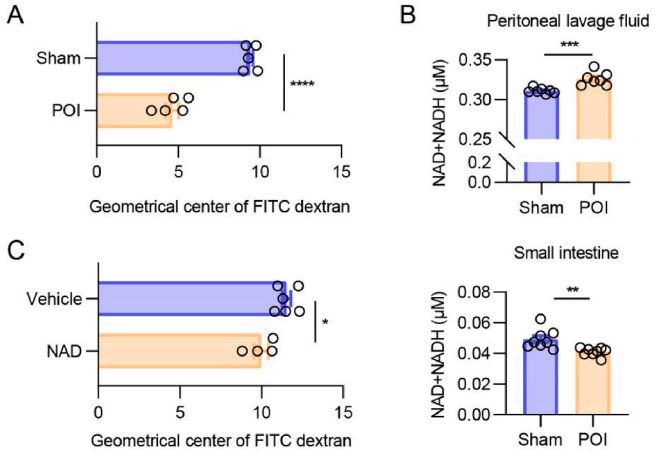

Fig. 1. Extracellular NAD is associated with the postoperative ileus. (A) The GI transit was assessed by the geometric center of FITC-dextran in the POI and sham group. (B) Normalized concentration of NAD+ NADH in PIF and small intestine at 6 h after surgery. (C) The GI transit at 3 h after intraperitoneal injection of NAD. Each point represents one replicate, and each experiment was repeated 2 times. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ or, ***$p < 0.0001$. Each column shows the mean ± SEM.

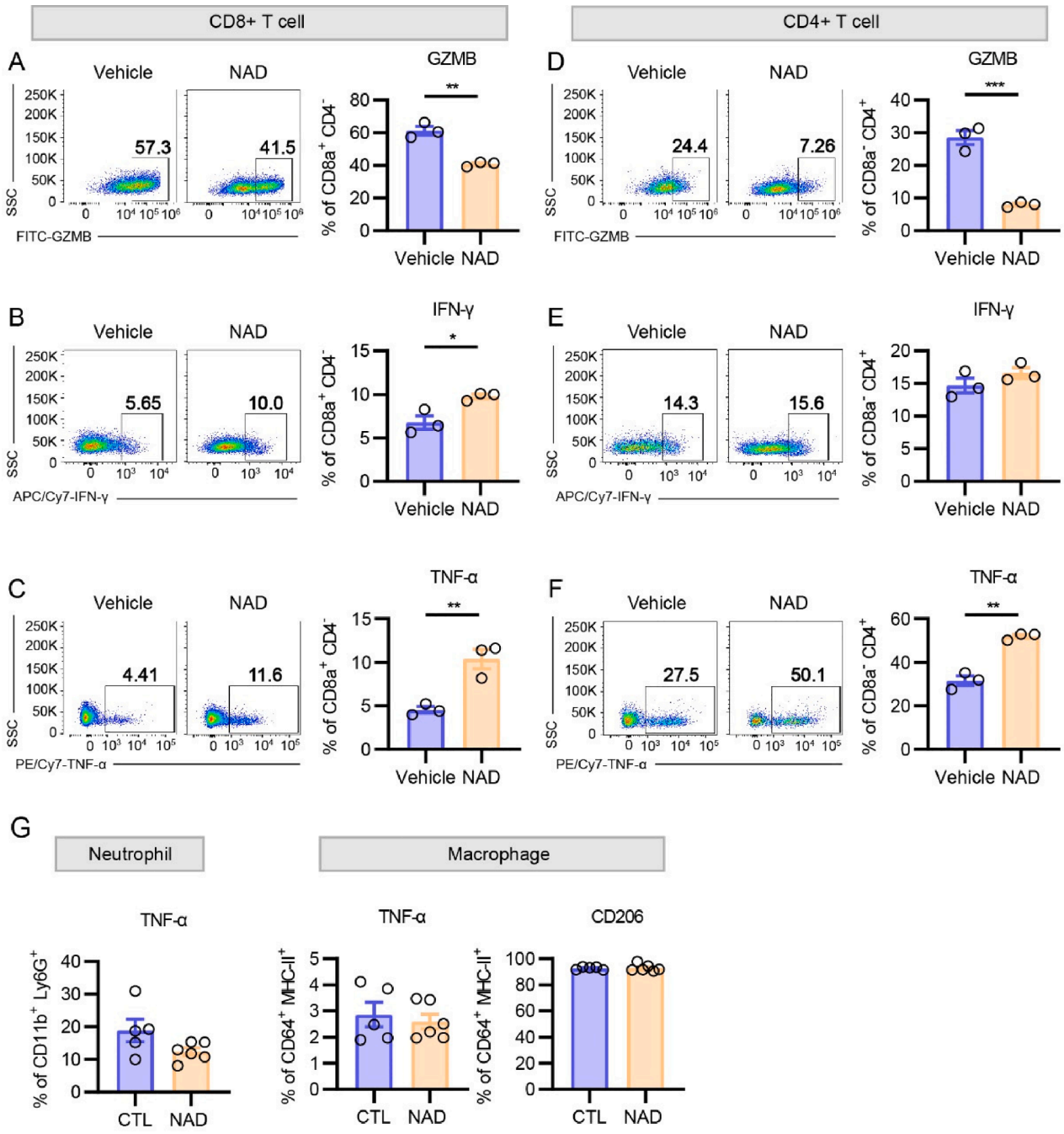

Fig. 2. Alteration of cytokine production of intestinal T cells and myeloid cells after intraperitoneal injection of NAD.

CD8+ lymphocytes after NAD+ injection. Production of granzyme B (D), IFN-γ (E), and TNF-α (F) in intestinal CD4+ lymphocytes after NAD injection. (G) TNF-α and CD206 levels in neutrophils and macrophages after NAD+ injection. Live Aqua- cells were gated for analysis. Each point represents one replicate, and each experiment was repeated 2 times. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, or $***p < 0.0001$. Each column depicts the mean ± SEM.

3.3. NAD directly increased the expression of TNF-α and IFN-γ in intestinal T cells in vitro

To further determine the effects of NAD on lymphocytes, we performed an *in vitro* stimulation assay. CD3+ intestinal T cells were isolated and stimulated *in vitro* with NAD. We observed a dose-dependent reduction in the viability of intestinal CD8+ T cells (Fig. 3**A and B).** Intracellular staining showed that TNF-α and IFN-γ were significantly increased in live CD8+ T cells with a concomitant decrease in granzyme B upon exposure to NAD (Fig. 3**C–H).** Similar alterations in cytokine levels were also observed in intestinal CD4+ T cells (Fig. 4**A–H).** However, sorted mesenteric lymph node (mLN) CD3+ T cells did not exhibit appreciable changes upon NAD stimulation (Fig. 5**A and B).** The supernatant detection of cytokines showed the increased IFN-γ produced by intestinal CD8+ T cells (**Fig. S2A and B).** These data suggest that NAD directly promoted pro-inflammatory cytokine production in intestinal T cells.

3.4. Intestinal T cells express the purinergic receptor P2X7R

To further explain the varying effects of NAD on intestinal and mLN T cells, we investigated the expression of the purinergic receptor P2X7R as

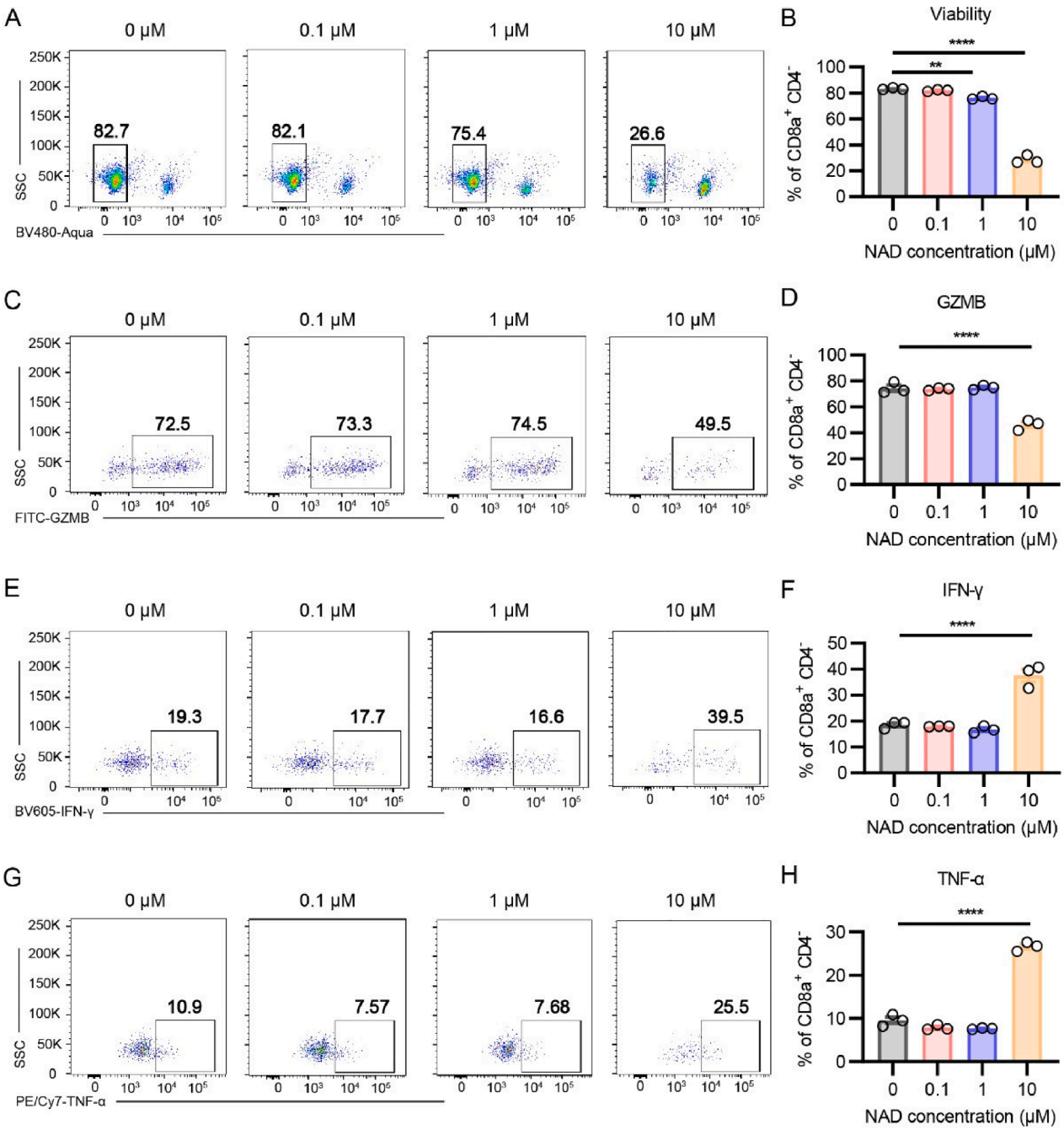

Fig. 3. Alteration of intestinal CD8+ T cells after NAD stimulation *in vitro*. (A, B) Viability of intestinal CD8+ lymphocytes after NAD stimulation *in vitro*. Production of granzyme B (C, D), IFN-γ (E, F), and TNF-α (G, H) in intestinal CD8+ lymphocytes after NAD stimulation *in vitro*. Live (Aqua-) cells were gated for protein production analysis. Each point represents one replicate, and each experiment was repeated 2 times. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, or $^{***}p < 0.0001$. Each column shows the mean ± SEM.

a receptor for NAD using flow cytometry. Compared with mLN T cells, intestinal CD8+ and CD4+ T cells expressed higher levels of P2X7R on the membrane surface **(**Fig. 6**A and B)**. The digest program did not impact the difference in expression of P2X7R on T cells between intestinal and mLN. **(**Fig. S3 **A-C)**. Previous studies have indicated that P2X7R induces calcium influx into T cells [15]. To further elucidate the intracellular signal induced by NAD, the calcium fluorescent probe Fluo-4 was used to detect calcium flux in intestinal T lymphocytes. The results indicated that NAD functioned as an agonist to the ion channel receptor P2X7R in intestinal T cells **(**Fig. 6**C)**. The rapid and noteworthy response to extracellular NAD stimulation may be due to the high expression of P2X7R in intestinal T cells. Furthermore, we investigated the magnitude of cytokine production in mLN and intestinal T cells. The results demonstrated that NAD stimulation potently induced IFN-γ and TNF-α production in CD8+ T cells in the intestine than in mLN *in vitro*. **(**Fig. 6**D)**.

3.5. NAD promotes the activation of intestinal CD4+ and CD8+ T cells via NAD+ -ART2.2-P2X7R signaling

Extracellular NAD induces ATP-independent activation of the ionotropic P2X7 purinergic receptor in murine T lymphocytes. This activation is catalyzed by ART2.2, a glycosylphosphatidylinositol-anchored ADP-ribosyltransferase (ART) that is constitutively expressed in murine

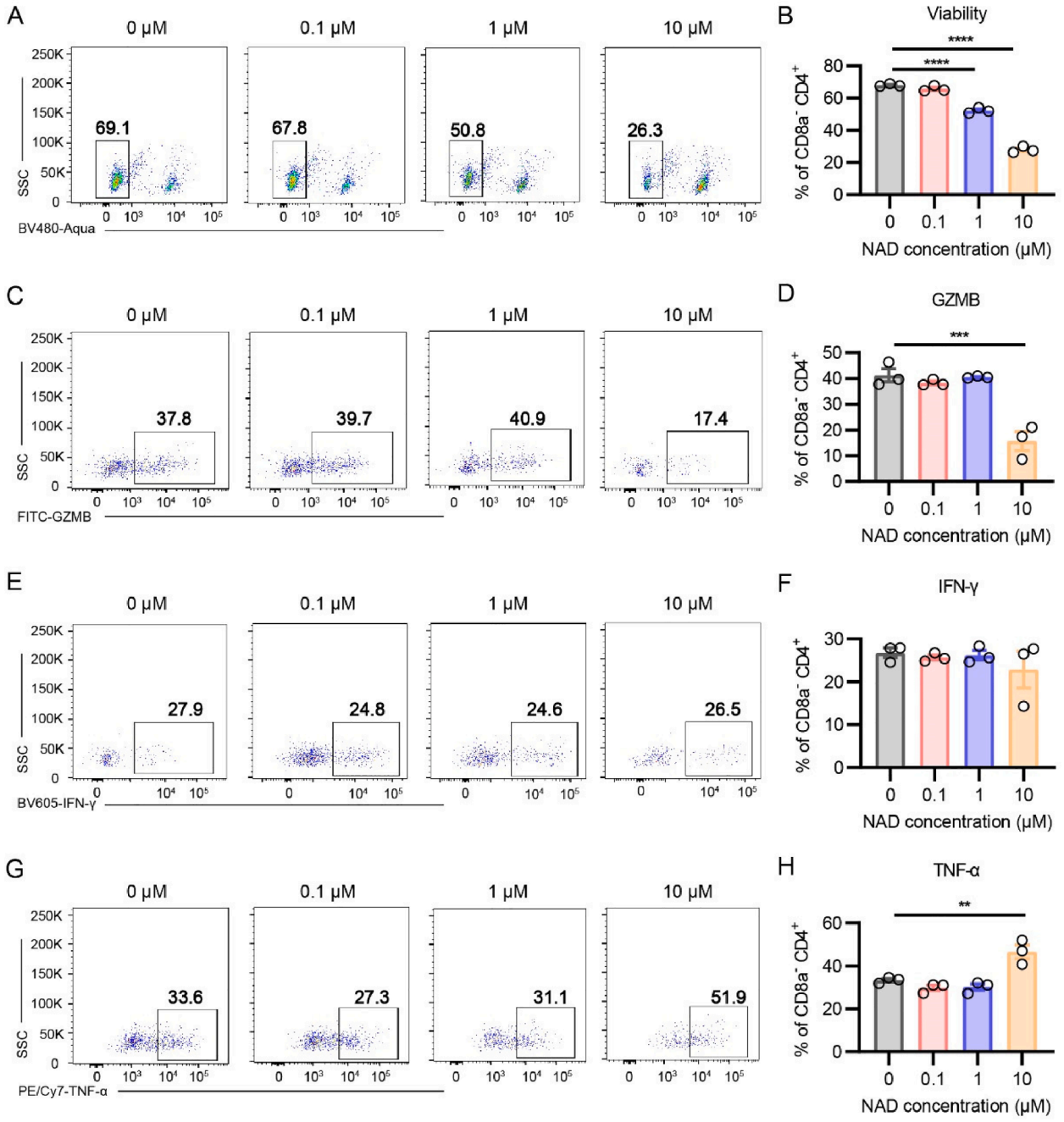

Fig. 4. Alteration of intestinal CD4+ T cells after NAD stimulation *in vitro*. (A, B) Viability of CD4+ lymphocytes after NAD stimulation *in vitro*. Production of granzyme B (C, D), IFN-γ (E, F), and TNF-α (G, H) in intestinal CD4+ lymphocytes after NAD stimulation *in vitro*. Each point represents one replicate, and each experiment was repeated 2 times. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, or $***p < 0.0001$. Each column depicts the mean ± SEM.

T cells [16]. To explore whether NAD activated intestinal CD4+ and CD8+ T cells via ART2.2-P2X7R, we blocked the ART2.2-P2X7R interaction with the ART2.2 inhibitor 1,4-naphthoquinone. Sorted CD3+ intestinal T cells were pretreated with 1,4-naphthoquinone before stimulation with NAD. Intracellular staining showed that the ART2.2 inhibitor reversed NAD-induced cytokine alterations in intestinal CD8+ T cells (Fig. 6**E**). Regarding CD4+ T cells, NAD-induced alterations in granzyme B and TNF-α were also reversed (Fig. 6**F**). The cytokines were also found to correlate positively with the expression of P2X7R in intestinal CD4+ and CD8+ T cells (**Fig. S4B and S4C**). Collectively, these results indicate that NAD promoted the production of pro-inflammatory cytokines by intestinal CD4+ and CD8+ T cells through NAD-ART2.2-P2X7R signaling.

4. Discussion

In the present study, we revealed the novel role of extracellular NAD in activating intestinal CD4+ and CD8+ T cells. We demonstrated that surgical damage led to NAD release into the extracellular space, and extracellular NAD consequently impaired GI motility. Furthermore, NAD promoted the production of the pro-inflammatory cytokines TNF-α and IFN-γ in intestinal T cells via NAD-ART2.2-P2X7R signaling. Our data suggest that extracellular NAD release may promote pro-inflammatory cytokine production in intestinal T cells and exacerbate the progress of POI.

We constructed a canonical model of POI by IM, which induces GI damage. Various DAMPs, such as HMGB1, mitochondrial DNA, heat

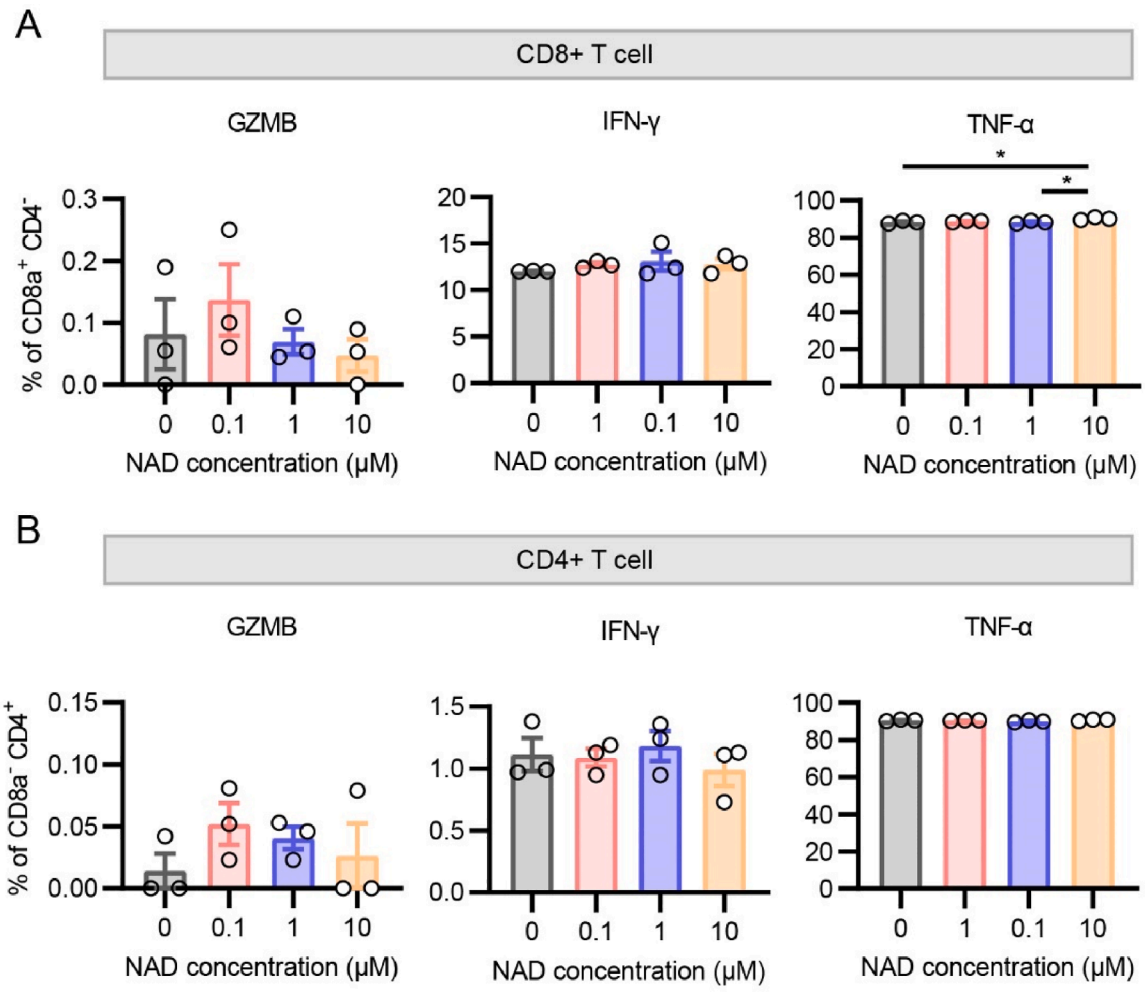

Fig. 5. Alteration of T cells in mesenteric lymph nodes after NAD stimulation *in vitro*. (A) Production of granzyme B, IFN-γ, and TNF-α in CD8+ lymphocytes of mesenteric lymph nodes (mLN) after NAD stimulation *in vitro*. (B) Production of granzyme B, IFN-γ, and TNF-α in CD4+ lymphocytes of mLN after NAD stimulation *in vitro*. Each point represents one replicate, and each experiment was repeated 2 times. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ or, $^{***}p < 0.0001$. Each column depicts the mean ± SEM.

shock proteins, and ATP, are reportedly released because of surgical damage [17–19]. However, we lack reports on DAMPs released during POI. Our data validated the increased extracellular NAD as a component of DAMPs after IM. In the current study, the significant inverse correlation of NAD+ NADH concentrations in intestinal tissues and the peritoneal cavity suggested the release of NAD from the intracellular to the extracellular space. Our data indicate that injection of exogenous NAD impairs GI motility, suggesting that a single component of DAMPs is sufficient to induce local inflammation with POI-like symptoms. Moreover, extracellular NAD promoted pro-inflammatory cytokine production in intestinal T cells. This finding may support the hypothesis that NAD could be the causative factor of acute inflammation, as reported in a previous study [12]. Furthermore, NAD biosynthesis exacerbates acute and chronic inflammation [20–22]. NAD supplementation has also been reported to exert a therapeutic effect on chronic inflammation [23–27]. These studies demonstrated the amelioration of chronic inflammation several weeks after NAD administration. A possible explanation for the difference between our results and their findings is that the long-term administration of NAD differs from acute release. As a vital hydrogen carrier in the cytoplasm, NAD also plays a pivotal role in the metabolism and function of the immune cell [22,23]. Our research focused on the immediate effect, not the long-term efficacy, of damage-induced extracellular NAD in intestinal CD4+ and CD8+ T cells. Further exploration of the underlying mechanism of extracellular or intracellular NAD in the short- or long-term should be conducted.

Our data revealed that NAD injection increased the production of TNF-α and IFN-γ in intestinal CD8+ and CD4+ T cells. The expression of granzyme B in intestinal CD8+ and CD4+ T cells was reduced after NAD injection. Similar results were observed in *in vivo* and *in vitro* experiments, suggesting that the alteration of cytokines may be due to the direct action of NAD on intestinal CD4+ and CD8+ T cells. Counterintuitively, the alteration of granzyme B and TNF-α levels exhibited the opposite trend. Pro-inflammatory T cells tend to upregulate the expression of granzyme B, TNF-α, and IFN-γ after activation with the complete TCR signal [28]. The P2X7R signal promotes IFN-γ production in tissues with acute inflammation [29]. NAD+ treatment is believed to reduce CD8+ T cell-mediated cytotoxicity in mice [30]. Therefore, NAD may inhibit granzyme B in CD8+ and CD4+ T cells. Related research has indicated that calcineurin inhibitors inhibit IFN-γ production in T lymphocytes [31]. In contrast, the withdrawal of calcineurin inhibitors leads to a decrease in granzyme B in T cells [32], which provides a potential explanation for the mechanism of our finding. Combined with the calcium influx verified after NAD stimulation, these data suggest that NAD-induced calcium signaling leads to an increase in IFN-γ and a decrease in granzyme B. In other words, NAD promotes the expression of pro-inflammatory cytokines rather than cytotoxicity in intestinal CD4+ and CD8+ T cells. In addition, mLN T cells were more insensitive to extracellular NAD, probably because of reduced P2X7R expression than

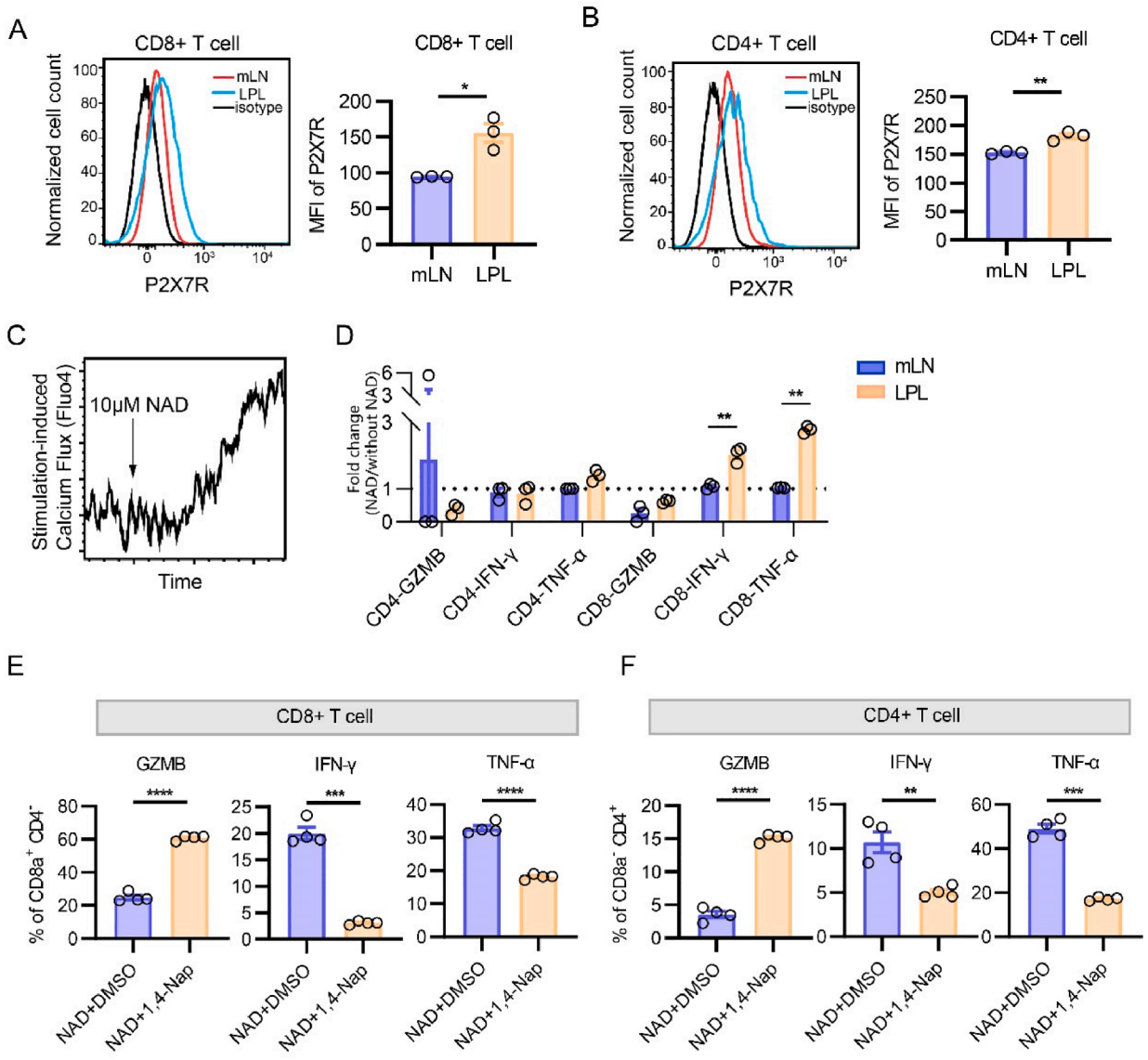

Fig. 6. Intestinal CD4+ and CD8+ T cells express the purinergic receptor P2X7R and calcineurin inhibition experiment. (A) The P2X7R expression of CD8+ T cells in the intestine and mesenteric lymph node. B. The P2X7R expression of CD4+ T cells in the intestine and mesenteric lymph node (mLN). (C) Calcium flux induced by extracellular NAD in CD3+ intestinal T cells. (D) Flow cytometry analysis of alteration of cytokine production in mLN T cells and lamina propria lymphocytes (LPL) after NAD stimulation *in vitro*. Relative fold changes were evaluated by the cytokine-positive percentage of NAD-stimulated cells divided by that in unstimulated cells. Flow cytometry analysis of cytokine alteration in (E) CD8+ and (F) CD4+ lymphocytes pretreated with 1,4-naphthoquinone before NAD. Live (Aqua-) cells were gated for analysis. Each point represents one replicate, and each experiment was repeated 2 times. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ or, $***p < 0.0001$. Each column depicts the mean ± SEM.

in LPL.

Myeloid cells play a crucial role in POI. Mast cells in the muscular layer activate macrophages in the intestinal via degranulation [33]. Endotoxin-stimulated macrophage activation is also induced by surgical manipulation [34]. Activated dendritic cells and M1-polarized macrophages are thought to be responsible for intestinal dysfunction due to increased prostaglandins and nitric oxide [35]. In contrast, dendritic cells promote ileus expansion by activating mTH1 via IL-12 [6]. Moreover, intestinal resident CD4+ and CD8+ memory lymphocytes respond rapidly to pathogens [36,37]. Furthermore, our data indicate that NAD increased the production of pro-inflammatory cytokines in intestinal T cells. However, the limitation of this research is that the study was conducted only in mouse intestinal T cells. The mechanism of extracellular NAD affects human intestinal T cells is still unknown due to the lack of ADP-ribosyltransferase activity in human immune cells [38,39]. Intestinal resident lymphocytes may play a role similar to innate myeloid cells in acute intestinal inflammation. NAD is only one of the numerous DAMPs, and the effects of other DAMPs on intestinal T cells warrant further investigation.

5. Funding statement

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6. Ethics review

Animal care and experimental procedures were conducted in accordance with the "Guiding Principles in the Care and Use of Animals" (China) and were approved by the Ethics Review Committee of Beijing Friendship Hospital (NO. 20-2056).

CRediT authorship contribution statement

Fandi Bu: Conceptualization, Methodology, Writing – original draft. **Shiyang Huang:** Investigation, Validation. **Xiaobao Yang:** Investigation. **Luyang Wei:** Investigation. **Dong Zhang:** Conceptualization, Supervision. **Zhongtao Zhang:** Writing – review & editing, Funding acquisition. **Dan Tian:** Conceptualization, Methodology, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Author contributions.

Fandi Bu and Shiyang Huang conducted the research and wrote the manuscript. Xiaobao Yang and Luyang Wei helped with the animal experiments. Dong Zhang provided scientific advice throughout this study. Zhongtao Zhang and Dan Tian supervised and guided this study.

Data availability statement.

The data supporting the findings of this study are available in the Supplementary Material. Further details can be obtained by contacting the corresponding author.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellimm.2023.104677.

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