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# Targeting of the Tec kinase ITK drives resolution of T cell-mediated colitis and emerges as potential therapeutic option in ulcerative colitis

*Short title: Blocking ITK drives resolution of colitis*

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## Abbreviations:

CD: Crohn's disease, CsA: Cyclosporine A, ITK: Interleukin-2 inducible tyrosine kinase, NFAT: Nuclear factor of activated T cells, UC: Ulcerative Colitis

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### **Declaration of Interests**

The authors declare no competing interests.

### **CRedit Authorship contributions:**

Kristina Lechner, M.Sc. (Conceptualization: Lead, Investigation: Lead, Formal analysis: Lead, Project Administration: Lead, Validation: Lead, Visualization: Lead, Writing – original draft: Lead), Stefanie Mott, M.Sc. (Investigation: Supporting, Formal Analysis: Supporting), Ragheed Al-Saifi, TA (Investigation: Supporting), Lisa Knipfer, PhD (Investigation: Supporting), Stefan Wirtz, MD PhD (Resources: Supporting), Raja Atreya, MD Prof. PhD (Resources: Supporting), Michael Vieth, MD Prof. PhD (Validation: Supporting), Timo Rath, MD Prof. PhD (Resources: Supporting), Tina Fraass, PhD (Methodology: Supporting), Zoltan Winter, PhD (Methodology: Supporting), Avery August, MD Prof. PhD (Resources: Supporting), Jeremy Luban, MD Prof. PhD (Resources: Supporting), Valérie S. Zimmermann, MD Prof. PhD (Resources: Supporting), Benno Weigmann, MD PhD (Conceptualization: Equal, Writing – original draft: Supporting, Validation: Equal, Principal Investigator: Equal), Markus Neurath, MD Prof. PhD (Conceptualization: Equal, Writing – original draft: Equal, Validation: Equal, Co-Principal Investigator: Equal).

## Abstract

**Background & Aims:** The molecular checkpoints driving T cell activation and cytokine responses in ulcerative colitis are incompletely understood. Here, we studied the Tec kinase ITK in ulcerative colitis and explored the link between Cyclosporine A (CsA) function and ITK activation.

**Methods:** We analyzed patients with inflammatory bowel disease (n=202) and evaluated ITK expression and activity as well as the functional effects of CsA. Moreover, three independent murine colitis models were used to investigate the functional role of ITK. Finally, the activity of ITK was blocked via pharmacological inhibitors and genetically engineered mice. Readout parameters were mini-endoscopy, histopathology, mucosal T cell apoptosis and cytokine production.

**Results:** We found an expansion of ITK expressing mucosal CD4<sup>+</sup> T cells in ulcerative colitis rather than Crohn's disease that correlated with disease severity. CsA suppressed activation of ITK in cultured CD4<sup>+</sup> T cells and calcineurin-containing microclusters adjacent to the T cell receptor signaling complex. Functionally, the capacity of CsA to suppress activity of experimental colitis was critically dependent on ITK. Moreover, genetic inactivation of *Itk* via gene targeting or induction of allele-sensitive *Itk* mutants suppressed experimental colitis in three colitis models, while pharmacological ITK blockers could be used for treatment of established colitis and triggered resolution of inflammation. Hereby, ITK controlled apoptosis and activation of mucosal Th2 and Th17 lymphocytes via NFATc2 signaling pathways.

**Conclusion:** ITK activation was detected in ulcerative colitis and could be downregulated in cultured T cells by CsA administration. Selective targeting of ITK emerges as an attractive approach for treatment of chronic intestinal inflammation and potentially ulcerative colitis by driving resolution of mucosal inflammation.

**Keywords:** ITK, Cyclosporine A, IBD, Cytokines, Therapeutic Strategies

## Introduction

The Tec kinase ITK belongs to the Tec family of non-receptor tyrosine kinases (TFK) <sup>1,2</sup>. ITK in T cells is activated by T cell receptor signaling and Lck resulting in active phospholigand-bound ITK. PLC-gamma is a key substrate for the kinase activity of ITK thereby generating IP<sub>3</sub> and increased DAG and intracellular calcium levels <sup>3</sup>. Thereby, ITK drives the activation of Act and RAS/MAPK pathways and various transcription factors including NF-kappaB, IRF4, RORgammat and *nuclear factor of activated T cells* (NFAT) <sup>3</sup>.

The loss of *Itk* in genetically engineered mice has a strong effect on the production and accumulation of cytokines characteristic of Th2, Th9 and Th17 cells. Furthermore, ITK plays a pivotal pro-inflammatory role in the development of auto-inflammatory and chronic inflammatory diseases like allergic asthma, atopic dermatitis or neuro-inflammatory diseases like multiple sclerosis. Moreover, studies in the mucosal immune system demonstrated an important role of ITK in controlling function of mucosal CD8<sup>+</sup> T cells and innate lymphoid cells type 2 <sup>4,5</sup>. In spite of these findings, little is known about the role of ITK in the pathogenesis of inflammatory bowel diseases (IBD). However, previous studies looked at the role of ITK and its homologue RLK in experimental models of intestinal inflammation. In these studies, a small molecule inhibitor of both RLK and ITK reduced the inflammatory phenotype in the CD4<sup>+</sup>CD45Rbhi transfer model, which is associated with Th1-mediated mucosal immune responses <sup>6,7</sup>. Moreover, decreased levels of T cells were seen in the colon as well as a reduced percentage of IFN $\gamma$ -expressing CD4<sup>+</sup> T cells in mesenteric lymph nodes, lamina propria and IELs. <sup>6</sup> In contrast, little is known about the role of ITK in other forms of T cell-dependent experimental colitis associated with different T cell cytokine responses.

The immunosuppressive drug cyclosporine A (CsA) is clinically used to prevent immune-mediated organ rejection as well as for therapy of chronic inflammatory disorders <sup>8,9</sup>. CsA blocks the enzymatic activity of calcineurin and calcium-dependent transcription factors like NFAT <sup>8</sup>. In this way, CsA inhibits T cell activation and causes immunosuppression. CsA has also been previously used to treat patients with IBD. However, this drug showed beneficial effects in ulcerative colitis rather than Crohn's disease <sup>10,11</sup>. The reasons for these differential responses are not clearly understood.

In the present study, we observed an expansion of ITK expressing mucosal T cells in ulcerative colitis and define ITK activation as a key molecular target of CsA.

## **Material and Methods**

### **Human samples**

Human samples from patients with UC (n=99) and CD (n=103) as well as controls (n=27) were analysed. Detailed patient information can be found in the supplementary table 1. The collection of samples was approved by the ethical committee of the Friedrich-Alexander-Universität Erlangen-Nürnberg. A pathologist blinded to all clinical and endoscopic data of the respective patient performed histological classification of the intestinal biopsies<sup>12</sup>. Severity of inflammation was determined by histopathological grading that included absent [score 0], mild [score 1], moderate [score 2] and severe [score 3] inflammation.

### **Mouse strains**

Male and female C57BL/6, *Itk*<sup>-/-</sup>, *Itkas*, *Nfatc1* Cre, *Nfatc2*<sup>-/-</sup> and *Rag1*<sup>-/-</sup> mice were housed under specific pathogen-free conditions. Experiments were performed with permission of the government of Middle Franconia in accordance with institutional guidelines. For experiments, 6-15 week old mice were used. All mice were on a C57BL/6 background. We declare that we have adhered to the ARRIVE guidelines.

### **Human PBMC isolation**

Human peripheral blood mononuclear cells (PBMC's) were isolated either from healthy controls or from UC and CD patients by density gradient centrifugation with Pancoll (PanBiotec). CD4<sup>+</sup> T cells were purified with immunomagnetic beads (Miltenyi Biotec), analyzed or plated at a density of 10<sup>6</sup> cells/ml in RPMI media (10% FCS, 1% Pen-Strep, 1% L-Glutamin) with 1µg/ml anti-human CD3/CD28 antibodies (Biolegend) or 20µg/ml CsA for 24h.

### **Cell polarisation**

PBMC's were plated with 1µg/ml anti-human CD3/CD28 antibodies. For Th2 differentiation cells were cultured with IL-2 (20ng/ml), IL-4 (100ng/ml), anti-IFNγ (5µg/ml) and anti-IL-12 antibodies (1µg/ml). For Th9 differentiation cells were cultured with IL-2 (20ng/ml), IL-4 (100ng/ml), TGF-β (100ng/ml) and anti-IFNγ (5µg/ml). For Th17 differentiation cells were cultured with recombinant human IL-1β (20ng/ml), IL-6 (100ng/ml), IL-23 (50ng/ml) and anti-human IFNγ (5µg/ml) plus anti-human IL-4 (5µg/ml). At day 6 cells were incubated with either 20µg/ml CsA, 30µM ATX-021 or

DMSO for 24h. Cells were re-stimulated with *Cell Stimulation Cocktail* plus protein transport inhibitors (Invitrogen) for 3h in order to prevent cytokine secretion. The cells were stained for CD4, AnnexinV, 7AAD, pITK and cytokines (Biolegend) for flow cytometry analysis.

### **Protein isolation and Protein Binding Assay**

Cells were lysed with UTA buffer containing protease inhibitor together with phosphoSTOP (Roche). Protein (50µg) was loaded to anti-CypA (R&D, goat) coated plates and incubated at 4°C overnight. Bound protein was either incubated with a mouse-anti-ITK antibody (Biolegend) or a rabbit-anti-pITK antibody (Invitrogen). For the detection HRP-coupled secondary antibodies were used (CST, anti-mouse-HRP; anti-rabbit-HRP).

### **Experimental oxazolone induced colitis model**

Mice were sensitized epicutaneously with 3% oxazolone (Sigma-Aldrich) diluted in a 4:1 acetone-olive oil solution. 4 days later mice were challenged rectally with a concentration of 0.5-1% oxazolone in 50% ethanol. In preventive settings mice were injected 10mg/kg CsA (Novartis) i.p. at the day of sensitization and received a daily injection of CsA until end of the experiment. In therapeutic settings, CsA was first injected 48h after the first challenge with oxazolone and subsequently on a daily basis. To induce overexpression of IL-6, mice were injected with 0.1µg IL-6 mini-circle vector 4 days before sensitization. This vector was created with full open reading frame of the murine gene IL-6 as previously described<sup>13</sup>. The vector was applied i.v. via hydrodynamic injection<sup>14</sup>.

### **Administration of *Itk* siRNA, the inhibitor 1-NM-PP1 or ATX-021 in oxazolone colitis model**

Two days before sensitization as well as on the day of sensitization 100nM negative siRNA or *Itk* siRNA (siTools), was administrated intrarectally into mice. In a preventive setup 20mg/kg 1-NM-PP1 was daily injected i.p. 24h before sensitization until end of the experiment. In a therapeutic setup 20mg/kg 1-NM-PP1 was daily injected i.p. 48h after the first challenge until end of the experiment. In a preventive setup, the covalent ITK inhibitor ATX-021 (provided by Aclaris Therapeutics) was daily given at a concentration of 50mg/kg via oral gavage 24h before sensitization and in a therapeutic



setup the same concentration of ATX-021 was given daily via oral gavage 48h after the first challenge.

### **Experimental trinitrobenzene sulfonic acid induced colitis model**

Mice were sensitized epicutaneously with 3% TNBS (Sigma Aldrich) in H<sub>2</sub>O. 4 days later mice were challenged intrarectally with TNBS in 50% ethanol. The challenges were repeated for 8 cycles with concentrations of TNBS ranging between 0.5-2.5% <sup>15</sup>. Mice additionally treated with CsA were daily injected i.p. with 10mg/kg CsA 24h before sensitization until 8 weeks later.

### **Experimental transfer colitis in combination with oxazolone induced colitis**

Donor mice were sensitized epicutaneously with 3% oxazolone. 4 days later the mice were euthanized and spleen cells were isolated. CD4<sup>+</sup> T cells were purified with immunomagnetic beads and 5 x 10<sup>6</sup> cells were injected i.p. into Rag1<sup>-/-</sup> mice.

### **Endoscopic analysis**

24h and 48h after every challenge with the haptens, respectively, mini-endoscopy (Coloview, Storz) was performed <sup>16</sup>.

### **Analysis of myeloperoxidase (MPO) expression by an *in vivo* imaging system (IVIS)**

24h after challenge with the haptens, respectively, *in vivo* IVIS staining to detect MPO expression was performed <sup>17</sup>.

### **Lamina propria mononuclear cell (LPMC) isolation**

To isolate LPMCs, colon pieces were incubated in digestion solution (HBSS, 5% FCS, LPMC isolation enzymes (Miltenyi Biotec)) for 25 minutes while rotating. Afterwards, the pieces were shredded in the gentleMACS Octo Dissociator (Miltenyi Biotec) and washed with PBS. For cell culture 2.5 x 10<sup>6</sup> cells/ml were plated onto 48 well plates and incubated for 48 hours.

### **Spleen cell isolation**

To isolate spleen cells, the spleen was mechanically disrupted and erythrocytes were lysed. Cells were then used for CD4 and AnnexinV/7AAD flow cytometry staining.

### **Murine PBMC isolation**

Blood was drawn from mice via heart puncture. The erythrocytes were lysed and the remaining cells were used for CD4 and AnnexinV/7AAD flow cytometry staining.

### **Histopathology**

The slides were stained with hematoxylin and counterstained with eosin. Samples were analyzed by histopathology for grading of colitis activity in a blinded fashion<sup>18</sup>. For histopathologic grading of experimental colitis the distribution of infiltrating cells, the chronicity and the activity were scored from 0 to 2 yielding an additive score between 0 (no colitis; score 0) and 6 (maximal colitis activity; score 3).

### **Immunofluorescence staining**

Sections were incubated with the antibody solution overnight (MPO, pCD247, calcineurin, pITK) and secondary antibodies conjugated with AlexaFluor488 or AlexaFluor594 (Invitrogen) were applied. Additionally, the tissue was stained for CD3 (mouse, AlexaFluor488, Biolegend) and with Dapi. Immunofluorescence staining was detected with a confocal microscope (Leica SP5 II and Leica Stellaris 8) and all HPFs (12-27 per sample) were quantified.

### **TUNEL staining**

Cryosections were treated as described before and the DeadEnd<sup>TM</sup> Fluorometric TUNEL System (Promega) was used. Additionally, cells were stained for CD4 (1. Antibody: mouse, eBioscience; 2. Antibody: Alexa Fluor 594, donkey-anti-mouse, Life Technologies).

### **qPCR**

cDNA was mixed with 2x SYBR Green (Bioline), DNase-free water and primer for *Itk* (Qiagen) or the housekeeping gene 18s RNA (Qiagen), respectively.

### **ELISA**

ELISA's were performed for IL-5, IL-6, IL-13, IL-17A, TNF-alpha (Biolegend) and IL-9 (Cusabio). After the respective incubation time, detection antibody, Avidin-HRP and the substrate solution were administered according to the manufacturer's protocol.

### **Flow Cytometry**

For apoptosis and necrosis detection, isolated cells were stained for CD4, AnnexinV and 7AAD (Biolegend). For the pITK staining cells were treated with 2mM H<sub>2</sub>O<sub>2</sub> for 20 minutes at 37°C<sup>19</sup> and stained for phospho-ITK (Invitrogen) or the respective isotype control at RT for 30 minutes. Flow cytometry analysis was done with the LSRFortessa (BD). For each measurement, compensation with beads (Invitrogen), stained with the respective fluorochrome, was performed.

### **Statistics**

Statistical differences between two groups were determined by using Student's t-tests. Statistical differences between three or more groups were determined by using a one-way ANOVA and a post-hoc Tukey test. P values <0.05 were considered as statistically significant and identified with asterisks (\*<0.05, \*\*<0.01, \*\*\*<0.001, \*\*\*\*<0.0001). Results are expressed as mean values with SEM.

All authors had access to the study data and reviewed and approved the manuscript.

## Results

### **ITK expression is upregulated in mucosal T cells from patients with ulcerative colitis and its activation can be blocked by cyclosporine A**

In initial studies, we observed significantly higher expression of *ITK* mRNA in patients with active UC as compared to patients with CD and healthy controls (Fig. 1a, Suppl. Table 1). However, there was a considerable heterogeneity of mRNA expression levels and highest expression levels were seen in severe mucosal inflammation in UC (Fig. 1b). In contrast, all patients with active UC showed significantly more CD3/pITK double positive T cells in the lamina propria than patients with CD and healthy controls (Fig. 1c). Consistently, flow cytometry showed higher percentages of CD3<sup>+</sup> T cells with activated pITK in lamina propria (LPMCs) and blood cells (PBMCs) in UC as compared to controls (Fig. 1d-e; Suppl. Fig. 1).

Next, we explored the possibility that CsA might regulate ITK function in T cells of UC patients. While ITK was highly phosphorylated in UC blood CD4<sup>+</sup> T cells, treatment with CsA strongly diminished the phosphorylation of ITK. In contrast, CD CD4<sup>+</sup> T cells showed little or no phosphorylation of ITK in the presence or absence of CsA (Fig. 1f). As calcineurin forms microclusters close to the TCR<sup>20</sup>, we determined effects of CsA in UC via triple staining for calcineurin, pITK and pCD247 (pCD3 zeta). While ITK phosphorylation clustered with calcineurin and pCD247 in UC T cells, ITK phosphorylation was greatly reduced in calcineurin microclusters upon CsA treatment (Fig. 1g) suggesting that this drug affects ITK activation in TCR-related calcineurin microclusters.

Next, we analysed if CsA regulates ITK function in Th2, Th9 and Th17 cells, as these cells are induced in UC<sup>21, 22</sup>. Accordingly, we treated Th2/Th9/Th17 cells (Suppl. Fig. 2) with CsA or the covalent ITK inhibitor ATX-021, stained them for CD4/pITK and investigated T cell apoptosis via flow cytometry. We could detect significantly higher phosphorylation of ITK in Th2, Th9 and Th17 cells as compared to naïve Th0 cells (Fig. 1h) and phosphorylation in Th2, Th9 and Th17 cells was significantly suppressed by treatment with CsA and ATX-021, respectively. This finding was associated with a significant induction of apoptosis in Th2 and Th17 cells (Suppl. Fig. 3). Since CypA can interact with ITK<sup>23</sup>, we wanted to evaluate if CsA directly impacts the expression

of ITK via CypA. Therefore, we added protein from CsA-treated CD4<sup>+</sup> PBMCs to anti-CypA coated wells. While CypA-bound total ITK expression was not altered by CsA treatment, phosphorylated ITK was significantly reduced upon CsA administration (Fig. 1i, Suppl. Fig. 4) demonstrating that ITK activation could be a target of this drug.

To investigate the effect of CsA on human colonic cells, LPMCs were isolated from biopsies of IBD patients and treated with CsA for 24h. Treatment with CsA led to reduced phosphorylation of ITK in T cells from UC but not in CD patients or controls (Fig. 1j). Moreover, CsA resulted in a significant induction of apoptosis in mucosal CD4<sup>+</sup> T cells from UC (Suppl. Fig. 5a-b). This observation was associated with impaired production of IL-6 (Suppl. Fig. 5c), a key survival factor of mucosal T cells<sup>24</sup>. Moreover, successful clinical CsA therapy of patients with UC was accompanied by the presence of apoptotic mononuclear cells (Suppl. Fig. 5d) demonstrating that CsA induces apoptosis of mucosal immune cells in the inflamed gut.

### ***Itk* deficiency suppresses mucosal Th2, Th9 and Th17 cytokine production and protects from experimental oxazolone-induced colitis**

Next, we studied the role of ITK in murine oxazolone colitis, a model characterized by abnormal Th2 and Th9 cytokine production with similarities to UC in humans<sup>25</sup>. Hereby, elevated *Itk* mRNA levels were observed (Fig. 2a) and mucosal CD4<sup>+</sup> T cells expressed higher levels of active pITK protein as compared to controls (Fig. 2b). Moreover, while wild-type mice treated with oxazolone showed severe signs of inflammation, *Itk* knockout mice were significantly protected from endoscopic and histologic signs of inflammation (Fig. 2c) and had lower expression of myeloperoxidase (MPO) as compared to controls *in vivo* (Fig. 2d).

Next, we analyzed cytokine content in culture supernatants of LPMCs after T cell stimulation (Fig. 2e). We observed a marked induction of Th2 (IL-5, IL-6, IL-13), Th9 (IL-9), TNF and to a lesser extent Th17 (IL-17A) cytokines in oxazolone colitis as compared to untreated controls. However, *Itk* deficiency led to significant reduction in pro-inflammatory cytokine production.

## **Cyclosporine A treatment suppresses ITK activity in mucosal CD4<sup>+</sup> T cells and protects from oxazolone colitis**

To further explore the effects of CsA on pITK, we performed preventive CsA treatment (Fig. 3a). CsA-induced suppression of inflammation was comparable to that seen in *Itk*<sup>-/-</sup> mice, while additional treatment of CsA in *Itk* knockout mice had little effects. Similar effects of CsA treatment and *Itk* deficiency on colitis activity were noted by histology (Fig. 3a), *in vivo* luminescence analyses (Fig. 3b) and immunofluorescence stainings for MPO (Fig. 3c). As these findings were consistent with the concept that CsA reduces colitis activity via pITK blockade, we next determined mucosal ITK expression. We noted markedly reduced ITK phosphorylation in mucosal CD4<sup>+</sup> T cells of oxazolone-treated wild-type mice upon CsA treatment as compared to controls (Fig. 3d). Moreover, the administration of CsA in wild-type mice caused a significant reduction of TNF and several Th2, Th9 and Th17 cytokines when compared to untreated controls (Fig. 3e) and similar effects were noted in *Itk* knockout mice. CsA treatment was associated with a significant induction of apoptosis in lamina propria CD4<sup>+</sup> T cells compared to untreated controls, as determined by Annexin V/7AAD staining (Suppl. Fig. 6a). Apoptosis levels in CsA treated mice were comparable to those seen in *Itk* deficient mice. In contrast to mucosal cells, CsA treatment or *Itk* deficiency in oxazolone colitis did not cause significant upregulation of AnnexinV<sup>+</sup>/7AAD<sup>-</sup> cells in CD4<sup>+</sup> T spleen and blood cells (Suppl. Fig. 6b-c).

CsA treatment to mice with active mucosal inflammation led to suppression of established colitis (Fig. 3f, Suppl. Fig. 7a). MPO staining via IVIS (Fig. 3g) or immunofluorescence staining (Fig. 3h) showed a significantly higher expression of MPO in untreated wild-type mice compared to all other groups. Moreover, the expression of phosphorylated ITK protein in isolated mucosal CD4<sup>+</sup> T cells was significantly suppressed by CsA treatment (Fig. 3i). This effect was associated with a significant suppression of IL-5, IL-6, IL-13 and IL-17A production by mucosal T cells (Fig. 3j). Such effects on cytokine production were associated with an induction of T cell apoptosis upon CsA treatment (Suppl. Fig 7b).

## **Cyclosporine A activity in experimental colitis is critically dependent on the presence of ITK and NFATc2 (NFAT1)**

To gain further mechanistic insights on CsA function, we looked at the role of IL-6, as this cytokine has been shown to play a key role in mucosal T cell apoptosis<sup>24</sup>. Accordingly, we generated IL-6 minicircles<sup>26,27</sup> and injected wild-type and *Itk* deficient mice before oxazolone challenge with vector DNA (Fig. 4a). Assessment of IL-6 levels in serum confirmed overexpression upon vector injection (Suppl. Fig. 8a). Mini-endoscopic analysis as well as histologic stainings from colon tissue showed that the injection of IL-6 mini-circles leads to more severe inflammation in wild-type mice and a loss of the protective effects of *Itk* deficiency on colitis activity (Fig. 4b). While the treatment of wild-type mice with IL-6 did not affect the protective results of CsA therapy, CsA administration had no effect on colitis activity in *Itk*-deficient mice given IL-6 minicircle DNA demonstrating that CsA function is critically dependent on the presence of ITK (Fig. 4b-d).

We noted a significant reduction of IL-6, IL-9, IL-13 and TNF in the cellular supernatants of wild-type mice with oxazolone colitis treated with CsA after IL-6 overexpression as compared to control wild-type mice given oxazolone only. However, no reduction was detectable in supernatants from lamina propria cells of *Itk* deficient mice treated with CsA after IL-6 injection compared to *Itk*<sup>-/-</sup> control mice in the absence of CsA treatment (Fig. 4e) suggesting that effects of CsA treatment on cytokine production are dependent on ITK. While CsA treatment resulted in a significant induction of T cell apoptosis (Suppl. Fig. 8b), *Itk* deficiency caused increased T cell apoptosis and this phenotype was not affected by CsA treatment.

We next studied whether the absence of NFAT in T cells controls colitis activity. Therefore, splenic CD4<sup>+</sup> T cells from NFATc1 (NFAT2), NFATc2 (NFAT1) deficient and control mice were transferred into Rag1<sup>-/-</sup> mice followed by oxazolone challenge in the presence or absence of CsA treatment. We observed that the loss of NFATc1 in CD4<sup>+</sup> cells had no beneficial effects on colitis severity, while the inactivation of NFATc2 caused a significant suppression of colitis activity (Suppl. Fig. 9). However, the treatment with CsA had a significant effect upon transfer of NFATc1 but not NFATc2 deficient T cells, suggesting that regulation of NFATc2 rather than NFATc1 activity is important for the efficacy of CsA. NFATc2 deficiency caused reduced mucosal inflammation and significant downregulation of TNF plus Th2, Th9 and Th17 cytokines in this colitis model (Suppl. Fig. 10). Flow cytometric analyses of ITK/pITK in mucosal

CD4<sup>+</sup> cells showed reduced ITK activity in NFATc2 deficient mice, while ITK expression remained unchanged (Fig. 4 f-g + Suppl. Fig. 10 c-d).

### **The loss of *Itk* prevents inflammation in a haptenized adoptive cell transfer and chronic TNBS colitis models**

To determine the function of ITK in independent models of colitis, we transferred isolated CD4<sup>+</sup> T cells from wild-type and *Itk* deficient mice, upon sensitization with oxazolone, into syngenic *Rag1*<sup>-/-</sup> hosts followed by oxazolone challenge. These studies indicated a key role of ITK in T cells in mediating colitis activity (Fig. 5a). While CsA treatment effectively suppressed activity of T cell transfer colitis in the wild-type group, no effects were noted in the *Itk*<sup>-/-</sup> group (Figs. 5b-c) consistent with the idea that CsA acts via pITK inhibition to regulate colitis activity. Subsequent analyses of mucosal cytokine production showed significant suppression of Th2, Th17 and TNF production in CsA treated mice as compared to untreated controls (Fig. 5d). Finally, there was a marked induction of apoptotic mucosal T cells in mice given wild-type T cells upon CsA treatment that was indistinguishable from the *Itk*<sup>-/-</sup> groups (Suppl. Fig. 11).

In studies on chronic 2,4,6-Trinitrobenzenesulfonic acid (TNBS) colitis, wild-type and *Itk* deficient mice were treated with TNBS for 8 cycles to induce a Th2 inflammatory response<sup>15</sup>. After the first cycle of TNBS, associated with a Th1 response, both wild-type and *Itk* knockout mice showed comparable levels of mucosal inflammation (Suppl. Fig. 12). However, after the 8<sup>th</sup> cycle, treatment of wild-type mice with CsA led to significant suppression of colitis activity as compared to untreated controls and *Itk* deficiency suppressed colitis activity, while additional CsA treatment had no significant effect (Figs. 5e-g). Except for IL-9, pro-inflammatory cytokine secretion was significantly reduced for all tested cytokines in wild-type mice upon CsA treatment as compared to untreated controls and similar effects were noted in the *Itk*<sup>-/-</sup> groups (Fig. 5h). Furthermore, higher levels of mucosal T cell apoptosis were detected in wild-type mice upon CsA administration as compared to untreated wild-type controls (Suppl. Fig. 13).



## **ITK blockade via 1-NM-PP1 or *Itk* siRNA leads to a protected phenotype in *Itk* allele sensitive and wild-type mice**

In further studies, we explored the effects of ITK blockade using *Itk* allele sensitive (*Itkas*) mice with an allele sensitive ITK kinase function. In these mice, injection of the small molecule inhibitor 1-NM-PP1 suppresses kinase function of ITK<sup>28</sup>. In a preventive setup, reduced oxazolone inflammation was noted in *Itkas*<sup>+/+</sup> mice treated with the inhibitor compared to the vehicle-treated controls (Figs. 6a-c). Analysis of ITK phosphorylation showed reduced percentages of pITK expressing lamina propria CD4<sup>+</sup> T cells in mice given 1-NM-PP1 (Fig. 6d). Moreover, the administration of the inhibitor led to a reduced secretion of IL-9, IL-13 and TNF and a significant induction of mucosal T cell apoptosis in comparison to controls (Suppl. Fig. 14a-b).

Next, we determined the effects of ITK inhibition in mice with established colitis (Fig. 6e-g, Suppl. Fig. 15a). *Itkas*<sup>+/+</sup> mice treated with 1-NM-PP1 and *Itkas*<sup>-/-</sup> mice had reduced pITK levels and colitis activity as compared to *Itkas*<sup>+/+</sup> mice given vehicle (Fig. 6h). Therapeutic administration of 1-NM-PP1 led to a significant reduction of IL-5, IL-6, IL-13 and TNF secretion by mucosal cells in *Itkas*<sup>+/+</sup> mice (Fig. 6i). Finally, mucosal T cell apoptosis was elevated upon 1-NM-PP1 treatment as compared to controls (Suppl. Fig. 15b).

As an alternative approach to inhibit ITK function, we injected *Itk* specific or control siRNA intrarectally into wild-type mice in oxazolone colitis. Mini-endoscopic and histologic analyses revealed significantly reduced inflammation in wild-type mice upon *Itk* siRNA administration compared to the control group. Overall, wild-type mice treated with *Itk* siRNA showed reduced MPO expression, lower ITK and ITK phosphorylation levels, reduced secretion of IL-5, IL-6, IL-9 and IL-13 and higher levels of apoptotic CD4<sup>+</sup> T cells as compared to the control group (Suppl. Fig. 16).

## **A covalent ITK inhibitor effectively suppresses oxazolone-induced colitis**

Finally, we investigated the effects of the covalent ITK blocker ATX-021 on colitis activity. Mini-endoscopic analysis and histological stainings revealed significant suppression of mucosal inflammation in ATX-021 treated mice compared to vehicle-treated controls (Figs. 7a-b). The percentage of T cells with phosphorylated ITK in the

mucosa was significantly diminished in mice treated with ATX-021 to levels comparable to CsA-treated mice (Fig. 7c). Furthermore, average IL-5, IL-6, IL-9, IL-13, IL-17 and TNF secretion was reduced, while mucosal T cell apoptosis was elevated in ATX-021-treated mice (Fig. 7d; Suppl. Fig. 17a).

Treatment of mice with established oxazolone colitis with the ITK blocker ATX-021 led to significant suppression of colitis activity as compared to controls given vehicle. Additionally, CsA treatment caused a similar reduction of mucosal inflammation (Fig. 7e-f). Both treatment approaches resulted in significant reduction of mucosal CD4<sup>+</sup> T cells expressing pITK (Fig. 7g). Moreover, average production of IL-5, IL-6, IL-13, IL-17 and TNF was reduced upon therapy with both agents (Fig. 7h) indicating that they affect pro-inflammatory cytokine production. Lastly, both CsA and ATX-021 caused apoptosis (Suppl. Fig. 17b).

## Discussion

Although ITK has been implicated in the development of T cell-dependent inflammatory diseases, its role in the pathogenesis of IBD remains poorly understood. Here, we found that mucosal T cells from patients with UC rather than CD display elevated activation of ITK. Moreover, ITK activation was identified as a molecular target of CsA, a drug that has been successfully used for clinical therapy of UC <sup>11</sup>. Studies in three independent models of colitis showed a crucial role of ITK in driving pro-inflammatory cytokine production and apoptosis resistance in mucosal T cells. Finally, a covalent ITK blocker was successfully used for treatment of established experimental colitis suggesting that ITK blockade is a promising new therapeutic approach for chronic intestinal inflammation.

Although genetic polymorphisms did not provide a strong differential link to cytokines in IBD subsets <sup>29-34</sup>, previous studies suggested differences in mucosal T cell cytokine production between CD and UC. While CD was associated with Th1 and Th17 cytokine responses, T cells in UC demonstrated elevated production of some Th2, Th9 and Th17 cytokines <sup>22, 35-37</sup>. In spite of marked heterogeneity at *ITK* mRNA levels, we found increased phosphorylation of ITK in mucosal T cells from UC rather than CD patients. As ITK blockade suppressed a panel of proinflammatory cytokines including mucosal Th2 and Th9 responses, our findings suggested that ITK drives the characteristic mucosal cytokine profile in UC.

CsA has been used for treatment of patients with IBD. However, it mainly exerts therapeutic effects in patients with UC rather than CD <sup>38</sup>, although the molecular mechanism for this observation is unclear. As CypA has been shown to interact with ITK via the peptidyl-prolyl isomerase active enzymatic site <sup>23</sup>, we explored the hypothesis that ITK function might be affected by CsA treatment. We found that administration of CsA to human UC LPMCs as well as to human polarized Th2, Th9 and Th17 cells significantly reduced levels of ITK phosphorylation. Protein assays showed that CsA reduces ITK activity via CypA interaction without impacting ITK expression. Although CsA did not directly suppress ITK levels, the CsA/CypA complex can bind to TCR-related calcineurin which regulates the inversion of the inhibitory phosphorylation of Lck on Ser59 in the TCR <sup>20</sup>. Blocking of calcineurin activity by CsA

can upregulate pLck<sup>S59</sup>, which inhibits Zap-70 and SLP76 activity and impairs ITK phosphorylation<sup>39, 40</sup> thus providing a potential mechanism for CsA-induced blockade of ITK activity via regulation of calcineurin-associated ITK phosphorylation. This concept is supported by preliminary studies showing that Zap-70 deficient mice are protected from oxazolone colitis like *Itk* knockout mice and that CsA treatment had no effect on colitis activity in these mice (Weigmann et al., unpublished observations). Therefore, CsA utilizes a specific pathway to impair ITK activity and subsequent cytokine production. Consistently, we found that CsA treatment suppressed IL-6 production and caused marked induction of T cell apoptosis in LPMCs derived from UC patients. Thus, our findings do not only identify ITK as a key effector molecule of CsA in mucosal T cells in this disease but also give an explanation to why CsA preferentially regulates mucosal inflammation in UC. In contrast, in CD, low levels of ITK in T cells and possibly lack of CsA effects on CD-related cytokines may impair clinical efficacy of this drug. As the use of CsA in the clinic in UC is limited by marked potential toxicity<sup>41</sup>, we hypothesized that therapeutic modulation of the CsA target ITK might be helpful for optimized therapy in colitis.

To test this concept under *in vivo* conditions, we investigated the function of ITK in experimental colitis. We noted significant protection from colitis activity upon genetic inactivation of *Itk* (using *Itk* deficient mice or *Itkas* mice) as compared to *Itk* proficient controls in three independent models of Th2-associated colitis. *Itk* deficiency yielded similar effects like CsA treatment. Several aspects might contribute to these beneficial effects. *Itk* deficiency is associated with decreased numbers of mature T cells which display reduced responses to anti-TCR cross-linking<sup>42</sup> suggesting that impaired T cell activation may contribute the reduced colitis activity. Moreover, we found here reduced pro-inflammatory cytokine production in the absence of ITK and similar results were noted upon CsA treatment. Interestingly, some of these cytokines such as IL-6 have been previously shown to play an important role in experimental colitis<sup>24, 27, 43</sup>. Besides reducing Th2-related cytokines, CsA administration also resulted in a decreased concentration of Th9- and Th17-related cytokines. However, suppression of Th2, Th9 and Th17 cytokine production was dependent on the model system, the ITK targeting approach and the time points of ITK inactivation. This could explain the differential effects on cytokine secretion seen in the experimental setups where ITK was blocked via genetic approaches or via inhibitors such as ATX-021, NM-PP1 and siRNA. Additional effects of ITK were noted on TNF and IL-9 production<sup>44</sup>, although reduced

production of this cytokine was mainly seen in preventive rather than therapeutic settings.

Previous studies have shown that ITK may regulate Th1 cytokine production in vitro and in vivo <sup>6, 28</sup>. However, a recent study found that ITK negatively regulates the differentiation of natural IFN-gamma producing Th1 cells, a population of thymic T cells with innate-like phenotype <sup>45</sup>. Moreover, additional studies suggested that ITK is dispensable for peripheral Th1 development but plays a key role in Th2, Th9 and Th17 development <sup>3, 44, 46-48</sup>. Further studies demonstrated that polarized Th2 rather than Th1 cells are sensitive to the inhibition of ITK via Ibrutinib <sup>49</sup>. Consistently, we found here that ITK deficiency or inhibition led to marked suppression of Th2 cytokine production highlighting a major role of this Tec kinase for mucosal Th2 responses. Moreover, *Itk* deficiency was associated with augmented induction of mucosal T cell apoptosis in experimental colitis. This finding is in agreement with previous studies showing that the loss or inhibition of ITK leads to a higher apoptotic rate in murine splenic iNKT cells <sup>50</sup> and human T cell lymphoma cell lines <sup>51</sup>. Hereby, ITK inactivation resulted in reduced expression of anti-apoptotic proteins with concomitant increase of pro-apoptotic proteins. With regard to mucosal T cell apoptosis, however, we detected an important functional role of IL-6 in ITK-mediated regulation of cell death, as IL-6 levels were suppressed in the absence of ITK and injection of IL-6 mini-circles abrogated apoptosis effects in *Itk* deficient T cells. This observation is in line with previous findings showing that IL-6 can prevent T cell apoptosis and may prolong the survival and expansion of CD4<sup>+</sup> cells <sup>52, 53</sup>. However, IL-6 effects are critically dependent on the cell type studied, as this cytokine can also induce apoptosis in neutrophils and STAT3-deficient malignant T cell lymphoma <sup>54, 55</sup>. In mucosal T cells, however, ITK emerges as regulator of cell death in effector T cells. As some studies suggested that ITK inactivation can actually enhance CD4<sup>+</sup> FoxP3<sup>+</sup> Treg differentiation <sup>56, 57</sup>, ITK inactivation might also affect the balance between effector and regulatory T cells in colitis and further studies are required to address this point.

CsA treatment led to suppression of colitis activity in all three models tested and its efficacy was critically dependent on the presence of ITK. Our findings are in contrast to a previous study showing that CsA administration increases ITK activity in Jurkat cells <sup>58</sup> possibly due to the tumor origin of this cell line (acute lymphoblastic leukemia) with various mutations in T-cell receptor signaling pathways (*PTEN*, *INPP5D*, *CTLA4*,

and SYK) that severely affect TCR signaling <sup>59</sup>. Additional studies revealed that the transcription factor NFATc2 (NFAT1), a protein whose activity is regulated by both CsA and ITK-induced cellular Ca<sup>2+</sup> influx <sup>1, 60</sup>, is important in controlling colitis activity, while the inactivation of NFATc1 in T cells had little or no effects. These findings suggested that an ITK-NFATc2 signalling pathway in mucosal T cells regulates chronic intestinal inflammation. This link between ITK and NFATc2 is in agreement with a previous study showing that the inhibition of ITK leads to impaired levels of NFATc2 in CD8<sup>+</sup> cells <sup>61</sup>.

To further explore the therapeutic potential of ITK blockade in experimental colitis, we utilized *Itk* allele sensitive (*Itkas*) mice to investigate targeted ITK inhibition at different time points in experimental colitis via the administration of 1-NM-PP1 <sup>28</sup>. Both preventive and therapeutic suppression of ITK function via 1-NM-PP1 caused reduced mucosal cytokine production, induced T cell apoptosis and ameliorated colitis activity. Moreover, we used siRNA <sup>62</sup> to target *Itk* expression in the colon upon topical delivery. These studies revealed that inactivation of *Itk* via siRNA can be used for treatment of colitis and is associated with the induction of T cell apoptosis and reduced mucosal cytokine production. Finally, the covalent ITK blocker ATX-021 was used to target ITK function in experimental colitis. ATX-021 led to suppression of pITK, prevented colitis activity and was successfully used for therapy of established oxazolone colitis. These observations highlighted the potential use of ITK blockade for therapy of active colitis. In agreement with this concept, a small molecule inhibitor of ITK and RLK has been used for prevention of colitis upon CD4<sup>+</sup>CD45Rbhi T cell transfer <sup>6</sup>.

In conclusion, our findings demonstrate a pivotal role of ITK in experimental colitis and UC in humans. Although murine models of IBD have limitations with regard to the situation in human IBD, our studies revealed a broad relevance of ITK signalling in mediating Th2 cell survival, pro-inflammatory cytokine production and colitis activity in vivo. Targeting of ITK function in established colitis was effective in suppressing colitis severity and mediated resolution of mucosal inflammation. Although we can not rule out the possibility that certain infections may be facilitated upon ITK blockade, we did not observe any spontaneous infections in the absence of ITK in vivo. Our findings highlight the therapeutic potential of ITK inhibition as a possible new approach for therapy of UC in humans.

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## Figure Legends

### Figure 1: *ITK* is highly expressed in ulcerative colitis patients and CsA administration blocks *ITK* phosphorylation

**A-B:** The mRNA of mucosal samples from patients with IBD was isolated and subsequently analysed. **A:** *ITK* expression in ulcerative colitis (UC), Crohn's disease (CD) patients and healthy controls was determined by qPCR. **B:** *ITK* expression in UC was stratified by endoscopic levels of mucosal inflammation (eMayo score). **C:** Immunofluorescence staining of CD3 and pITK on human colonic tissue of IBD patients and healthy controls. Data are representative of three independent. **D:** *ITK* phosphorylation in CD3<sup>+</sup> LPMCs isolated from IBD patients or healthy controls was determined via flow cytometry. **E:** *ITK* phosphorylation in CD3<sup>+</sup> PBMCs isolated from IBD patients or healthy controls was determined via flow cytometry. **F-G:** PBMC CD4<sup>+</sup> cells were treated with DMSO or CsA for 24h before staining. Representative pictures are shown. **F:** CD4<sup>+</sup> cells were stained for calcineurin (red), pITK (white) and Dapi (blue). Expression and clustering of calcineurin and pITK was analysed via confocal microscopy. **G:** Cells were stained for pCD247 (green), calcineurin (red), pITK (white) and Dapi (blue). Expression and clustering of these molecules was assessed via the Leica Stellaris 8 confocal microscopy. **H:** Sorted human CD4<sup>+</sup> T cells were cultured under Th2, Th9 or Th17 polarising conditions for 7 days. Subsequently, cells were treated with DMSO, CsA or ATX-021 for 24h. Cells were re-stimulated and harvested. Naïve Th0 cells served as a negative control. Polarised cells were stained for CD4 plus pITK and analysed via flow cytometry. **I:** Human CD4<sup>+</sup> PBMCs were cultured in the presence or absence of CsA for 24h. The protein was isolated and put in anti-CypA coated wells. The measured oD revealed the impact of CsA on CypA bound *ITK*/pITK. **J:** LPMCs from human colon biopsies were isolated and cultured with or without CsA for 24h. LPMCs were stained for CD4 and pITK and analysed via flow cytometry.

Mean values  $\pm$  SEM are shown (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).

### Figure 2: *Itk* deficient mice are protected from oxazolone-induced colitis

**A:** Colonic *Itk* expression in untreated and oxazolone-treated wild-type mice was determined by qPCR. **B:** Isolated LPMCs were stained for CD4 and pITK and analysed



via flow cytometry. **C:** Endoscopic analysis of wild-type and *Itk* deficient mice with or without oxazolone challenge. The murine endoscopic index of colitis severity (MEICS) was determined to quantify the level of intestinal inflammation. The corresponding histologic stainings of colon tissue with assessment of inflammation are shown. **D:** *In vivo* MPO bioluminescence staining via IVIS was performed in mice upon luminol injection. **E:** Isolated LPMCs were cultured for 48h. The supernatant was harvested and cytokine content was analysed via ELISA.

Mean values  $\pm$  SEM are shown (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).

### **Figure 3: Preventive and therapeutic administration of CsA leads to reduced inflammation in oxazolone colitis**

**A-E:** Mean values  $\pm$  SEM are shown. **A:** Endoscopic analysis of wild-type and *Itk* deficient mice in the presence or absence of CsA treatment. MEICS score as well as histologic stainings and scoring of colon tissue are shown. **B:** *In vivo* MPO bioluminescence staining via IVIS was performed after injection with luminol. **C:** Colon tissue was immunostained with MPO antibodies and analysed via confocal microscopy. **D:** Isolated LPMCs were stained for CD4 and pITK and analysed via flow cytometry. **E:** Isolated LPMCs were cultured for 48h. Supernatant were analysed for cytokine content via specific ELISAs. **F-J:** Mean values  $\pm$  SEM are shown. **F:** Endoscopic analysis of wild-type and *Itk* knockout mice with or without therapeutic CsA treatment. MEICS scores, H&E stainings of colon tissue and histological scorings are shown. **G:** *In vivo* MPO bioluminescence staining via IVIS was detected after Luminol injection. **H:** Colon tissue was stained for MPO and analysed via confocal microscopy. **I:** Isolated LPMCs were stained for CD4 and pITK followed by flow cytometry analysis. **J:** Isolated LPMCs were cultured for 48h. The supernatant was harvested and cytokine content was analysed via ELISA (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).

### **Figure 4: Lack of efficacy of CsA treatment in intestinal inflammation in the absence of ITK and the regulatory role of NFATc2 on ITK activity**

**A:** Schematic timeline for injection of IL-6 mini-circle DNA and the administration of CsA. **B-E:** Mean values  $\pm$  SEM are shown. **B:** Endoscopic analysis of wild-type and *Itk*

knockout mice with or without CsA treatment. Endoscopic MEICS scores, histologic stainings and histological scorings are shown. **C:** *In vivo* MPO bioluminescence staining via IVIS was performed after Luminol injection. **D:** Colon tissue was stained for MPO and analysed via confocal microscopy. **E:** Isolated LPMCs were cultured for 48h. The supernatant was harvested and cytokine content was analysed via ELISA. **F-G:** Isolated LPMCs from oxazolone-treated wild-type and NFATc2 deficient mice were analysed via flow cytometry. LPMCs were stained for CD4 and ITK/pITK. Mean values  $\pm$  SEM are shown (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).

**Figure 5: The loss of ITK prevents inflammation in haptenized adoptive T cell transfer colitis and chronic TNBS-induced colitis**

**A-D:** Mean values  $\pm$  SEM are shown. **A:** Endoscopic analysis of recipient Rag1<sup>-/-</sup> mice in the presence or absence of CsA is depicted. Endoscopic MEICS scores, histologic stainings and histological scores are shown. **B:** *In vivo* MPO bioluminescence staining via IVIS was made after Luminol injection. **C:** Colon tissue was stained for MPO and analysed via confocal microscopy. **D:** Isolated LPMCs were cultured for 48h. The supernatant was harvested and cytokine content was analysed via ELISA. **E-H:** Mean values  $\pm$  SEM are shown. **E:** Endoscopic analysis of wild-type and *Itk* knockout mice with or without CsA treatment in TNBS-induced colitis. Endoscopic MEICS scores, histologic stainings and histological scores are shown. **F:** *In vivo* MPO staining via IVIS. **G:** Colon tissue was stained for MPO and analysed via confocal microscopy. **H:** Isolated LPMCs were cultured for 48h. The supernatant was harvested and cytokine content was analysed via ELISA (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).

**Figure 6: Treatment with 1-NM-PP1 reduces oxazolone colitis in *Itk* allele sensitive mice**

**A-D:** Mean values  $\pm$  SEM are shown. **A:** Endoscopic analysis of *Itk* mice with or without preventive 1-NM-PP1 treatment. Endoscopic MEICS scores, histologic stainings and histological scores are shown. **B:** *In vivo* MPO bioluminescence staining via IVIS. **C:** Colon tissue was stained for MPO and analysed via confocal microscopy. **D:** Isolated LPMCs were stained for CD4 and pITK and analysed via flow cytometry.

**E-I:** Mean values  $\pm$  SEM are shown. **E:** Endoscopic analysis of *Itkas* mice with or without therapeutic 1-NM-PP1 treatment. Endoscopic MEICS scores, histologic stainings and histological scores are shown. **F:** *In vivo* MPO bioluminescence staining via IVIS. **G:** Colon tissue was stained for MPO and analysed via confocal microscopy. **H:** Isolated LPMCs were stained for CD4 and pITK and analysed via flow cytometry. **I:** Isolated LPMCs were cultured for 48h. The supernatant was harvested and cytokine content was determined by ELISA (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).

**Figure 7: The covalent ITK inhibitor ATX-021 prevents inflammation and drives resolution of established inflammation in oxazolone colitis**

**A-D:** Mean values  $\pm$  SEM are shown. **A:** Endoscopic analysis of wild-type mice with or without preventive CsA or ATX-021 treatment during oxazolone colitis. Endoscopic MEICS scores, histologic stainings and histological scores are shown. **B:** Colon tissue was stained for MPO and analysed via confocal microscopy. **C:** Isolated LPMCs were stained for CD4 and pITK and analysed via flow cytometry. **D:** Isolated LPMCs were cultured for 48h. The supernatant was harvested and cytokine content was analysed via ELISA. **E-H:** Mean values  $\pm$  SEM are shown. **E:** Endoscopic analysis of wild-type mice with established colitis in the presence or absence of therapeutic CsA or ATX-021 treatment. Endoscopic MEICS scores, histologic stainings and histological scores are shown. **F:** Colon tissue was stained for MPO and analysed via confocal microscopy. **G:** Isolated LPMCs were stained for CD4 and pITK and analysed via flow cytometry. **H:** Isolated LPMCs were cultured for 48h. The supernatant was harvested and cytokine content was determined via ELISA (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).

## **Supplementary Figures:**

### **Supplementary Figure 1: Compensation and gating scheme for the isotype control staining of human LPMCs**

**A:** Compensation was performed with beads stained with the respective fluorochrome. This compensation scheme for PE is representative for the compensation performed for ITK phosphorylation staining. **B:** Human LPMCs were isolated, stained for CD3 and an isotype control for ITK phosphorylation. This gating scheme is representative for the staining of the isotype control and for the staining of ITK phosphorylation.

### **Supplementary Figure 2: Th2, Th9 and Th17 cytokine production is downregulated after administration of CsA and ATX-021**

**A-E:** Naïve T cells as well as Th2, Th9 and Th17 cells were re-stimulated, harvested and stained for cytokines characteristic for the respective T cell subset to test if the polarisation was successful. CsA and ATX-021 treatment reduced cytokine expression in Th2 and Th9 cells. **A:** IL-4 cytokine levels. **B:** IL-5 cytokine levels. **C:** IFN-gamma cytokine levels. **D:** IL-9 cytokine levels. **E:** IL-17A cytokine levels.

Mean values  $\pm$  SEM are shown (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ ).

### **Supplementary Figure 3: CsA increases apoptosis in Th2 and Th17 cells**

Naïve T cells as well as Th2, Th9 and Th17 cells were stained for CD4, AnnexinV and 7AAD and analysed via flow cytometry. Mean values  $\pm$  SEM are shown (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ ).

### **Supplementary Figure 4: CsA indirectly reduces ITK activity via CypA**

Human CD4<sup>+</sup> PBMCs were cultured for 24h. The protein was isolated and put in anti-CypA coated wells. Additionally, CsA was added to the bound protein. The measured

optical density (oD) revealed the impact of added CsA on CypA bound total ITK. Mean values  $\pm$  SEM are shown.

### **Supplementary Figure 5: CsA induces apoptosis and decreases IL-6 secretion in LPMCs and PBMCs from UC patients**

**A:** LPMCs from human colon biopsies were isolated and cultured with or without CsA for 24h. LPMCs were stained for CD4, AnnexinV and 7AAD and analysed via flow cytometry. **B:** Human PBMCs from patients or healthy controls were treated with CsA for 24h. Cells were stained for CD4 and checked for apoptosis via TUNEL staining. Immunofluorescence was analysed via confocal microscopy. Data are representative of three experiments. **C:** Human PBMCs from patients or healthy controls were cultured in the presence or absence of CsA for 24h. The supernatant was analysed for IL-6 secretion via ELISA. **D:** Colonic cryosections of a representative patient with UC before and after clinically effective CsA therapy were stained for TUNEL and Caspase3.

Mean values  $\pm$  SEM are shown. (\*  $p < 0.05$ ).

### **Supplementary Figure 6: Preventive CsA treatment of oxazolone colitis induces apoptosis in LPMCs but not in splenic or blood lymphocytes**

**A:** Isolated LPMCs were stained for CD4, AnnexinV and 7AAD and analysed via flow cytometry. **B:** Spleen cells were isolated, stained for CD4, AnnexinV and 7AAD and analysed via flow cytometry. **C:** Lymphocytes were isolated from peripheral blood, stained for CD4, AnnexinV and 7AAD and analysed via flow cytometry.

Mean values  $\pm$  SEM are shown. (\*  $p < 0.05$ , \*\*  $p < 0.01$ ).

### **Supplementary Figure 7: Therapeutic administration of CsA in oxazolone colitis increases apoptosis in LPMCs**

**A:** Schematic timeline for the therapeutic administration of CsA. **B:** Isolated LPMCs were stained for CD4, AnnexinV and 7AAD and analysed via flow cytometry. Mean values  $\pm$  SEM are shown. (\*  $p < 0.05$ , \*\*  $p < 0.01$ ).

### **Supplementary Figure 8: Serum concentration of IL-6 is increased after administration of IL-6 mini-circle vectors**

**A:** 24h after administration of IL-6 mini-circle DNA, blood serum was taken from mice and tested for IL-6 cytokine secretion via ELISA. Mice without IL-6 overexpression were used as controls. **B:** Isolated LPMCs were stained for CD4, AnnexinV and 7AAD and analysed via flow cytometry.

Mean values  $\pm$  SEM are shown. (\*  $p < 0.05$ , \*\*  $p < 0.01$ ).

### **Supplementary Figure 9: NFATc2 but not NFATc1 is a decisive factor for the induction of intestinal inflammation**

**A:** CD4<sup>+</sup> T splenic cells from oxazolone treated donor mice were injected into Rag1<sup>-/-</sup> mice. Recipient mice were treated with oxazolone and CsA. Endoscopic analysis of recipient Rag1<sup>-/-</sup> mice is depicted. The MEICS score determined endoscopic levels of inflammation severity. The respective histologic stainings and histological scores of colonic tissue are shown. **B:** Colon tissue was stained for MPO and analysed via confocal microscopy. **C:** Isolated LPMCs were cultured for 48h. The supernatant was harvested and cytokine secretion was analysed via ELISAs.

Mean values  $\pm$  SEM are shown. (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).

### **Supplementary Figure 10: NFATc2 deficiency ameliorates the inflammatory phenotype of oxazolone colitis and leads to less ITK activity**

**A:** Endoscopic analysis of wild-type and NFATc2-deficient mice. The MEICS score determined endoscopic levels of inflammation severity. The respective histologic stainings and histological scores of colon tissue are shown. **B:** Colonic tissue was immunostained with MPO antibody and analysed via confocal microscopy. **C-D:** Isolated LPMCs were stained for CD4 and ITK/pITK and analysed via flow cytometry. **C:** Representative flow cytometry plots for CD4/pITK staining. **D:** Representative flow cytometry plots for CD4/ITK staining. **E:** Isolated LPMCs were cultured for 48h. The supernatant was harvested and cytokine secretion was analysed via ELISAs.

Mean values  $\pm$  SEM are shown. (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ , \*\*\*\*  $p < 0.0001$ ).

**Supplementary Figure 11: CsA administration and *Itk* deficiency induce apoptosis of LPMCs in a haptenized T cell transfer-colitis model**

Isolated LPMCs were stained for CD4, AnnexinV and 7AAD and analysed via flow cytometry. Mean values  $\pm$  SEM are shown. (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ ).

**Supplementary Figure 12: *Itk* deficiency and administration of CsA does not inhibit the development of a Th1-mediated colitis**

**A:** Endoscopic analysis of wild-type and *Itk*-deficient mice in the presence or absence of CsA after the first cycle of TNBS. Endoscopic MEICS scores, histologic stainings and histological scores are shown. **B:** Colon tissue was stained for MPO and analysed via confocal microscopy. **C:** Isolated LPMCs were cultured for 48h. The supernatant was harvested and cytokine content was analysed via ELISA. **D:** Isolated LPMCs were stained for CD4, AnnexinV and 7AAD and analysed via flow cytometry.

Mean values  $\pm$  SEM are shown. (\*  $p < 0.05$ ).

**Supplementary Figure 13: CsA treatment increases the percentage of apoptotic LPMCs in chronic TNBS-induced colitis**

Isolated LPMCs were stained for CD4, AnnexinV and 7AAD and analysed via flow cytometry. Mean values  $\pm$  SEM are shown. (\*  $p < 0.05$ ).

**Supplementary Figure 14: Preventive administration of 1-NM-PP1 in *Itk* allele sensitive mice impacts cytokine secretion and apoptosis**

**A:** Isolated LPMCs were cultured for 48h. The supernatant was harvested and the cytokine secretion was measured using ELISAs. **B:** Isolated LPMCs were stained for CD4, AnnexinV and 7AAD and analysed via flow cytometry.

Mean values  $\pm$  SEM are shown (\*  $p < 0.05$ , \*\*  $p < 0.01$ ).

### **Supplementary Figure 15: Therapeutic administration of 1-NM-PP1 in *Itk* allele sensitive mice impacts apoptosis**

**A:** Schematic timeline for the therapeutic administration of 1-NM-PP1. **B:** Isolated LPMCs were stained for CD4, AnnexinV and 7AAD and analysed via flow cytometry. Mean values  $\pm$  SEM are shown (\*  $p < 0.05$ ).

### **Supplementary Figure 16: Administration of *Itk* siRNA suppresses mucosal inflammation in oxazolone colitis**

**A:** Endoscopic analysis of wild-type mice given oxazolone after treatment with negative siRNA or *Itk* siRNA, respectively. Endoscopic MEICS scores, histologic stainings and histological scores are shown. **B:** Colonic *Itk* expression in wild-type mice treated with negative siRNA or *Itk* siRNA was determined by qPCR. **C:** *In vivo* MPO bioluminescence staining via IVIS. **D:** Colon tissue was stained for MPO and analysed via confocal microscopy. **E:** Isolated LPMCs were stained for CD4 and pITK and analysed via flow cytometry. **F:** Isolated LPMCs were cultured for 48h. The supernatant was harvested and cytokine content was analysed via ELISA. **G:** Isolated LPMCs were stained for CD4, AnnexinV and 7AAD and analysed via flow cytometry.

Mean values  $\pm$  SEM are shown. (\*  $p < 0.05$ , \*\*  $p < 0.01$ ).

### **Supplementary Figure 17: Preventive or therapeutic administration of ATX-021 increases the percentage of apoptotic LPMCs in an oxazolone-induced colitis**

**A:** Isolated LPMCs were stained for CD4, AnnexinV and 7AAD and analysed via flow cytometry. Mean values  $\pm$  SEM are shown. **B:** Analysis of cell apoptosis via TUNEL stainings of colon cryosections. Immunofluorescence was studied via confocal microscopy. (\*  $p < 0.05$ ).



**Supplementary Table:**

**Supplementary Table 1: Information about healthy controls, UC and CD patients having taken part in this study**

Data from patients and healthy controls, from which biopsies, colonic cryo sections or blood were used, are displayed in this table. Information about the age, sex, inflammation score, location or inflammation and current medication are given. N.d. = not defined.

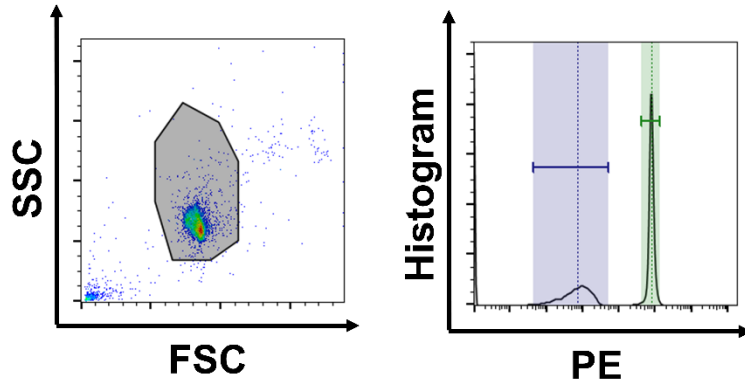
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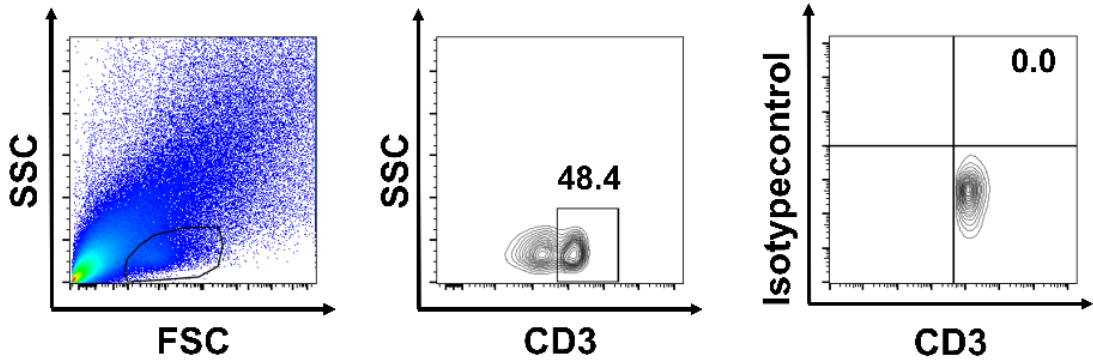
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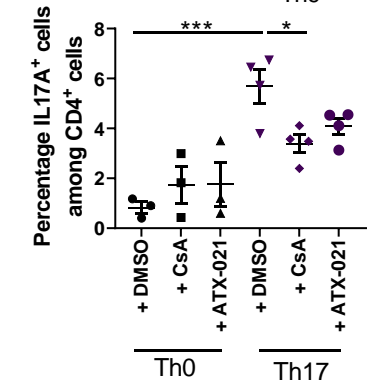
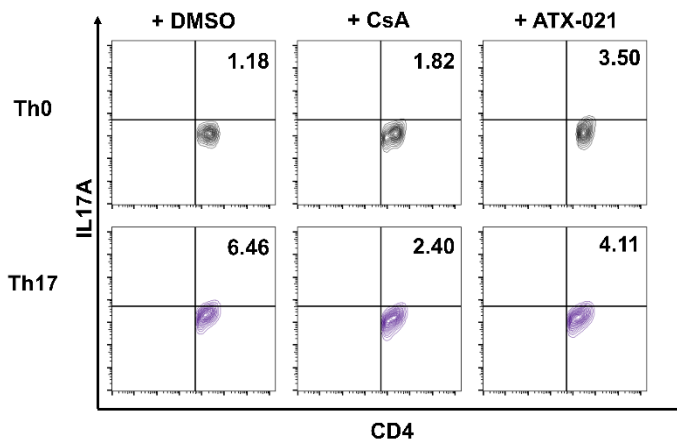
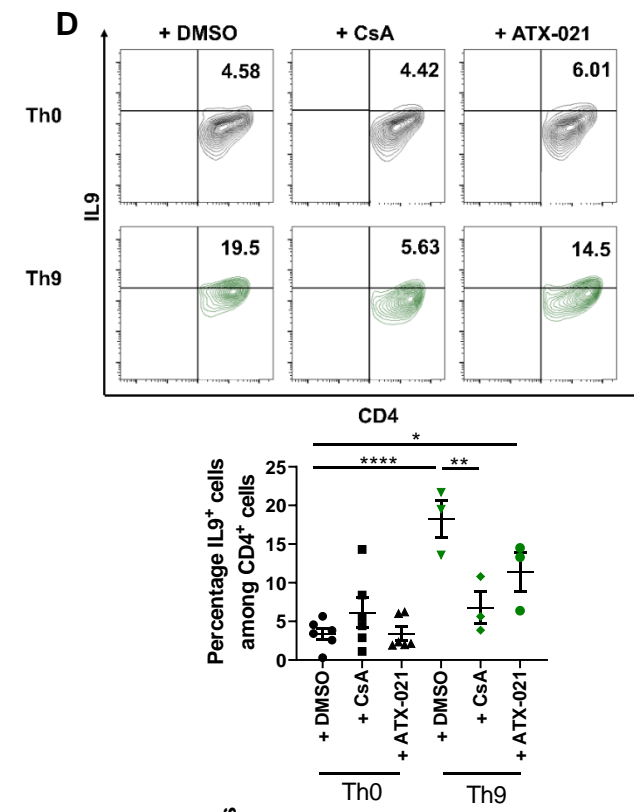
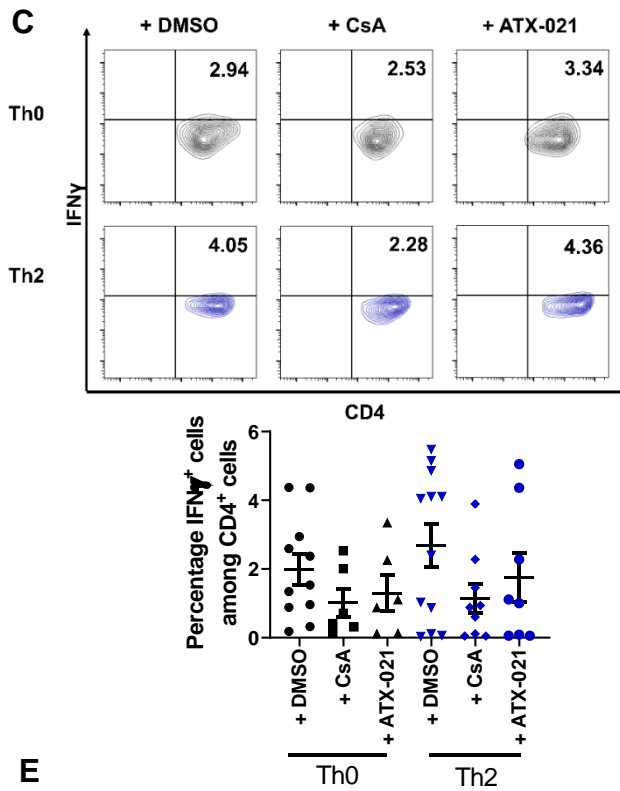
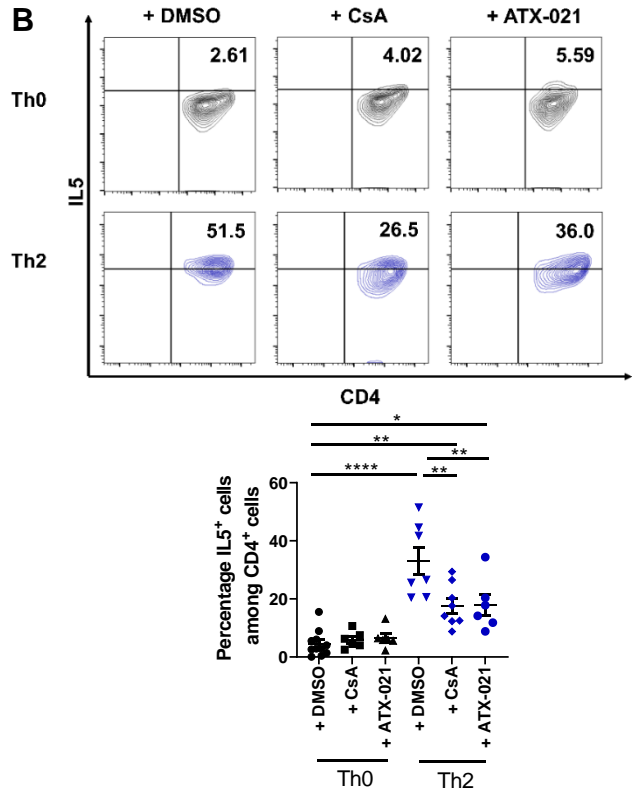
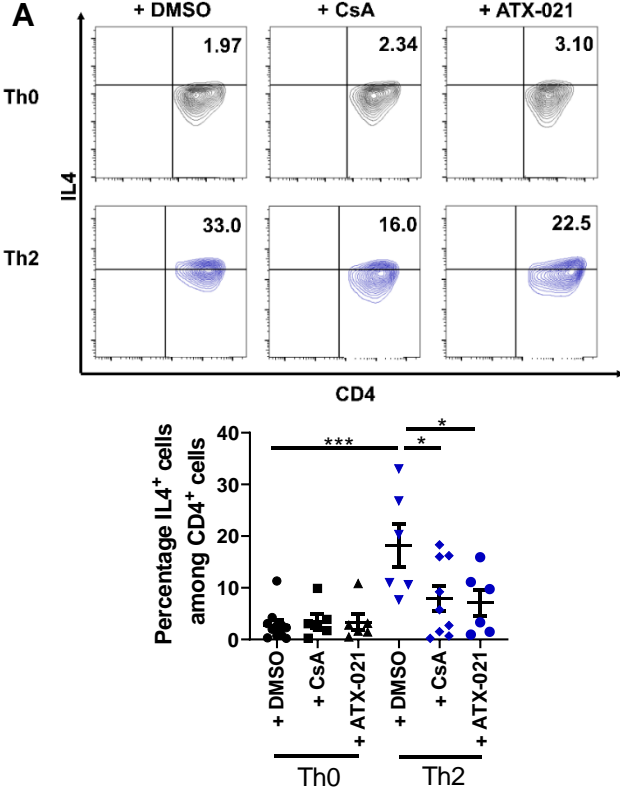
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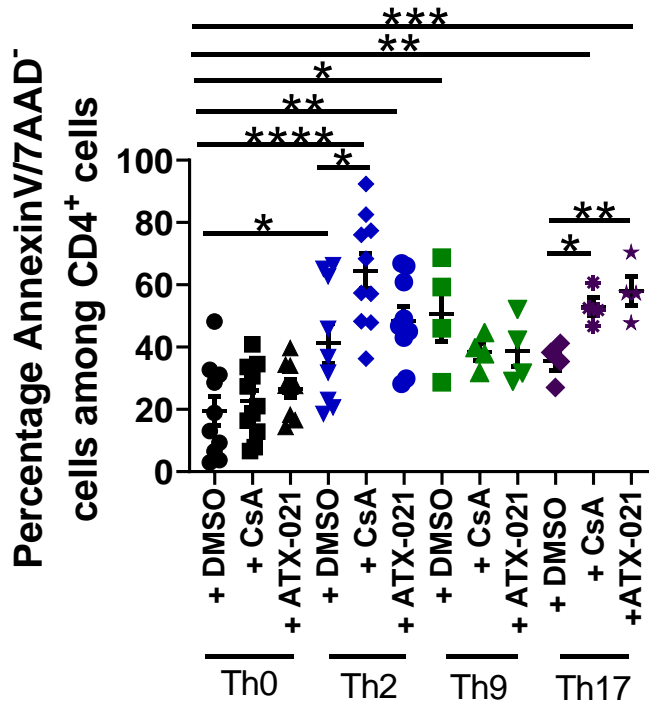
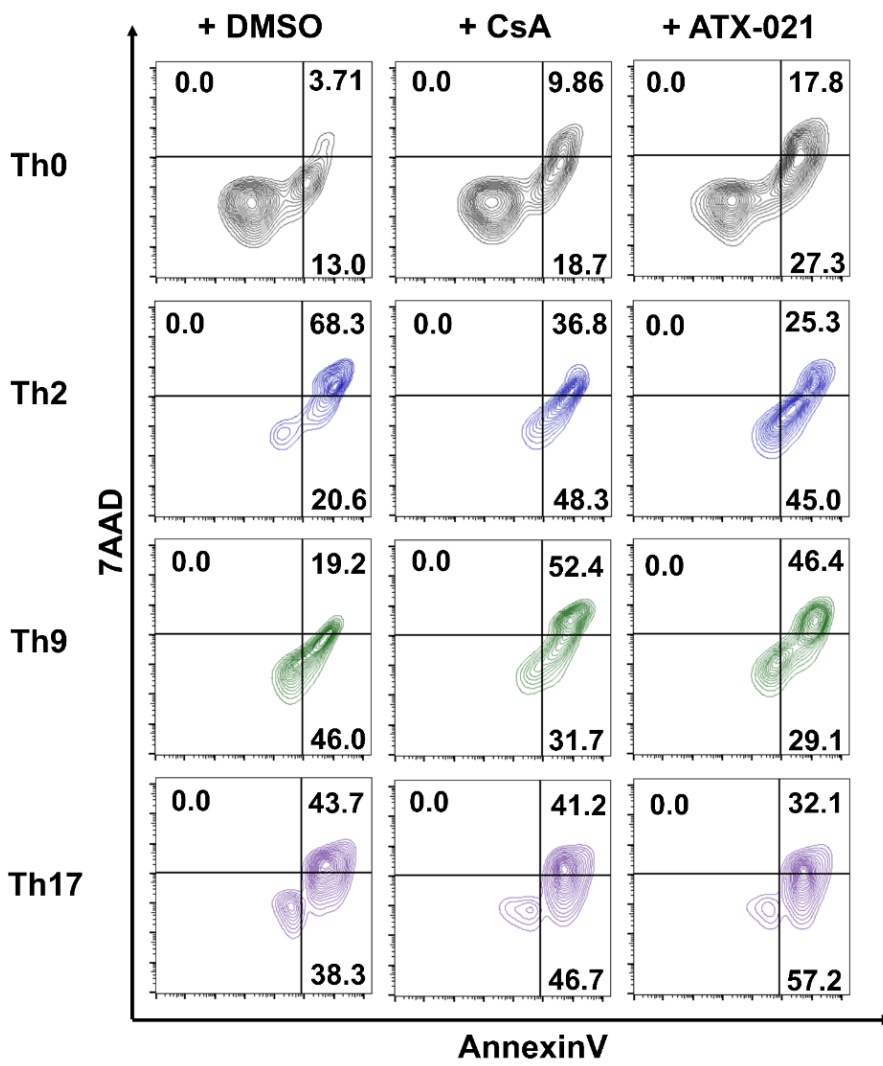
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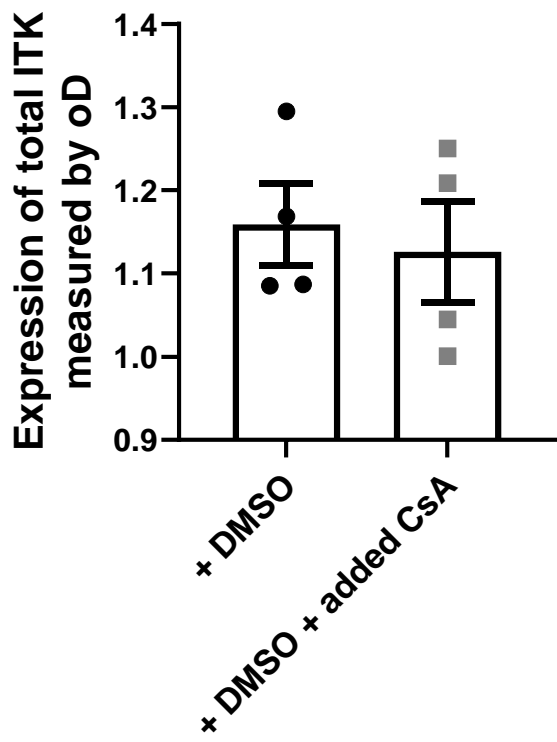




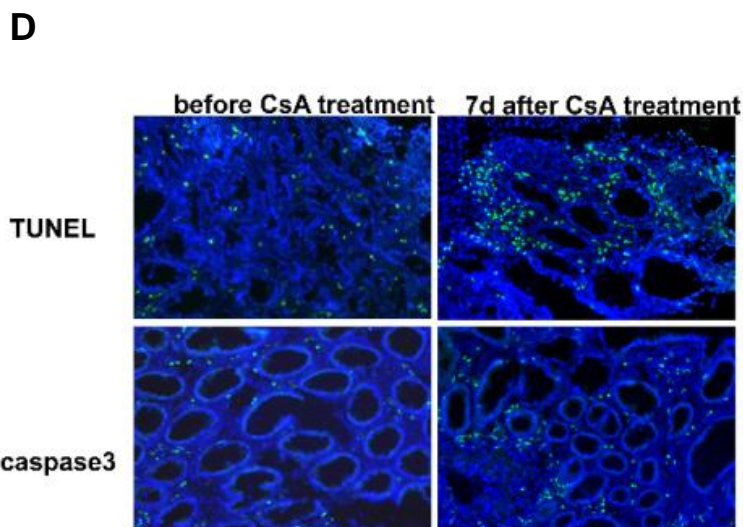
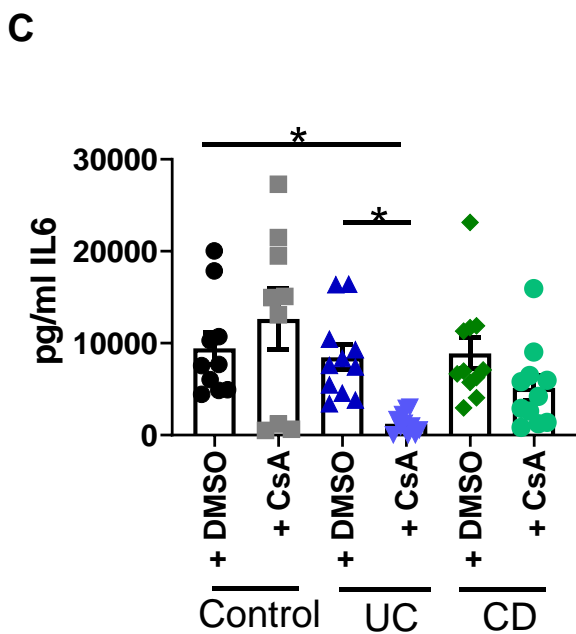
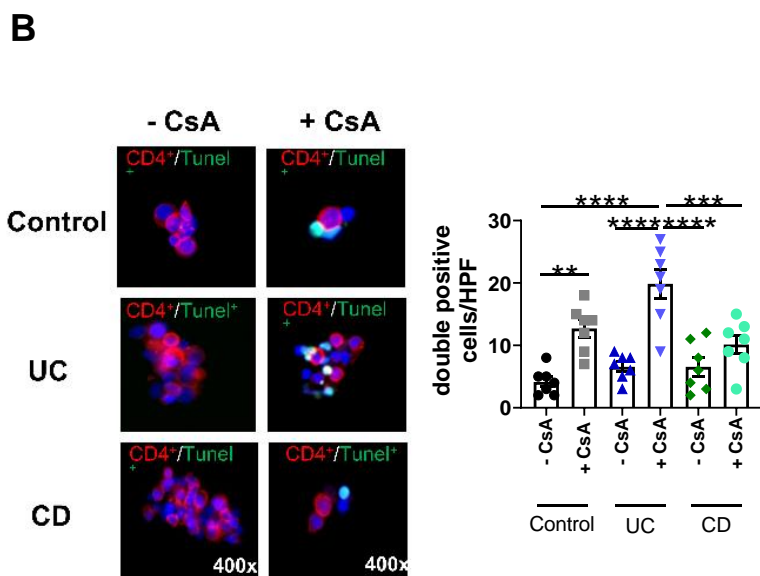
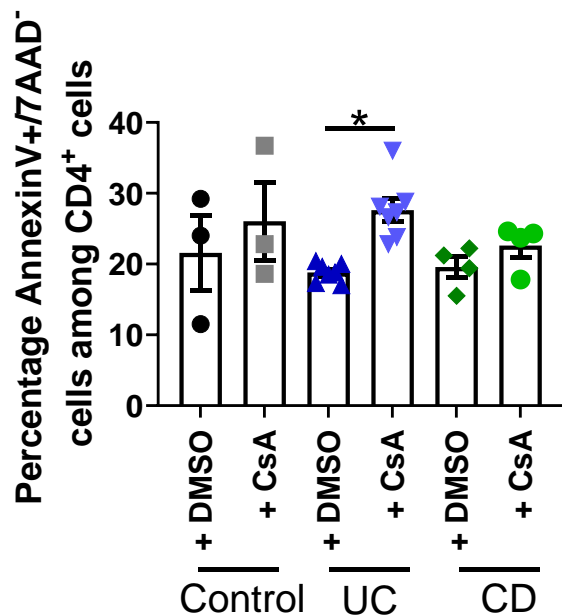
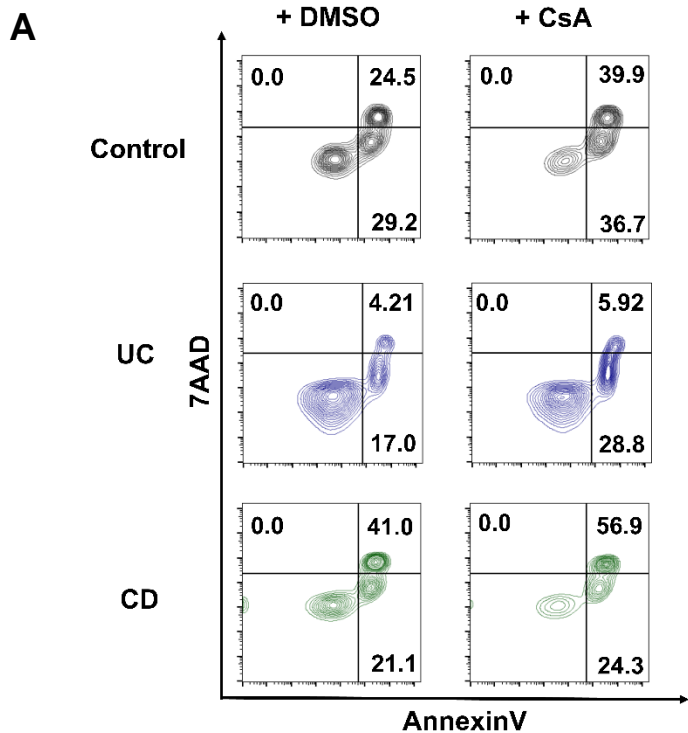
Supplementary Figure 2

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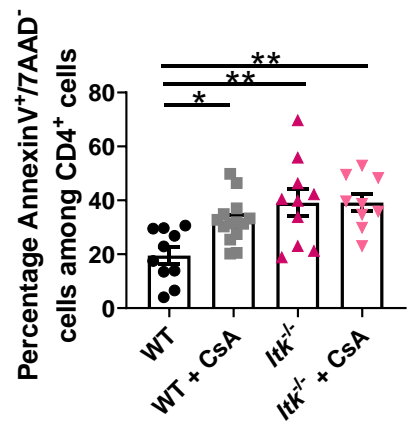
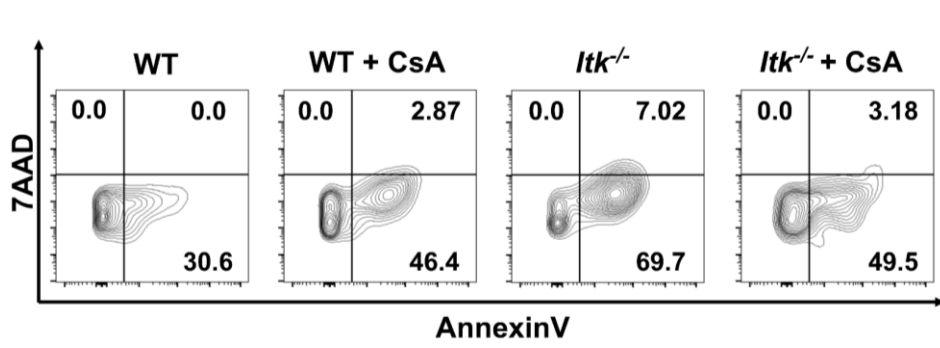




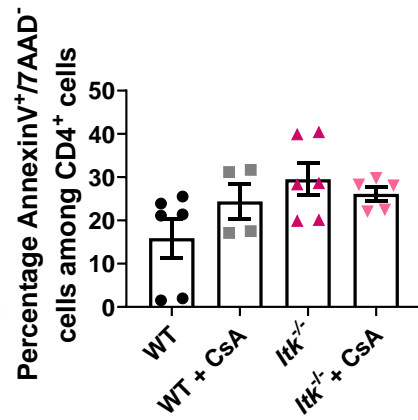
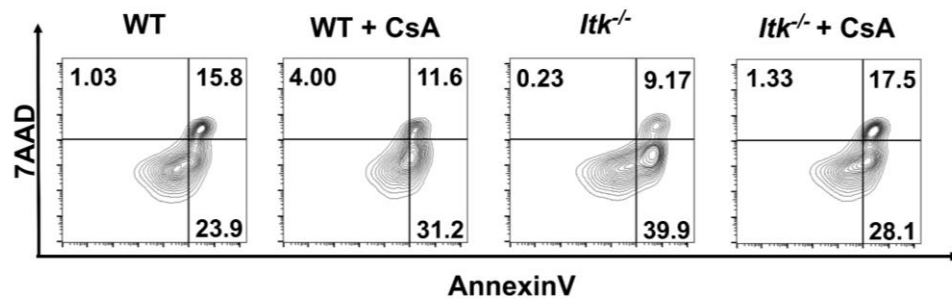




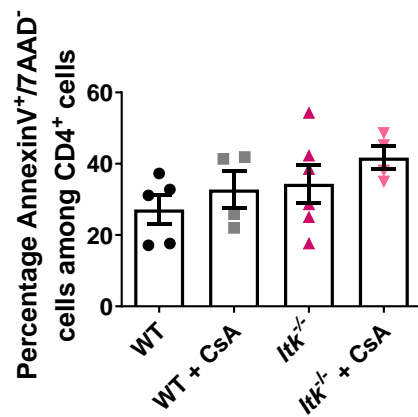
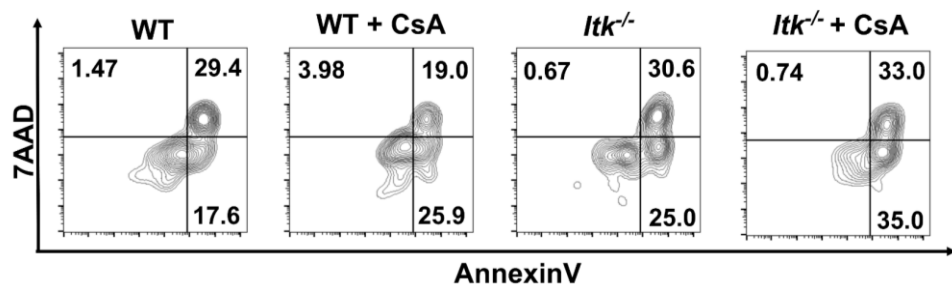
Supplementary Figure 5

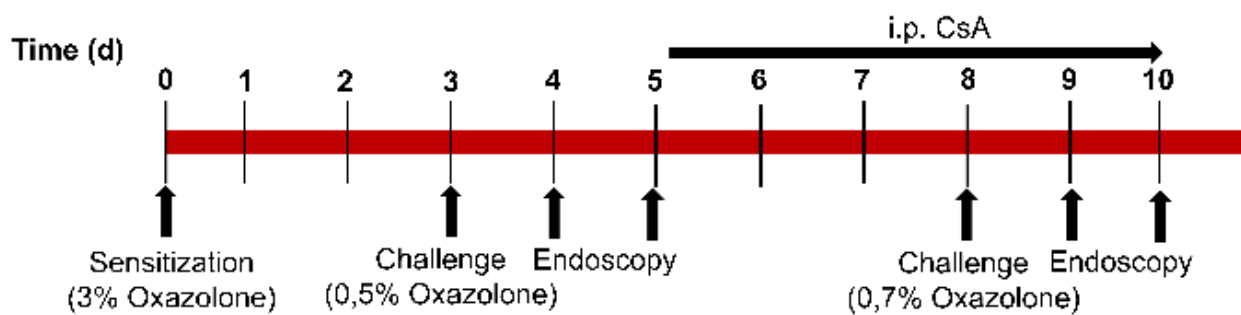
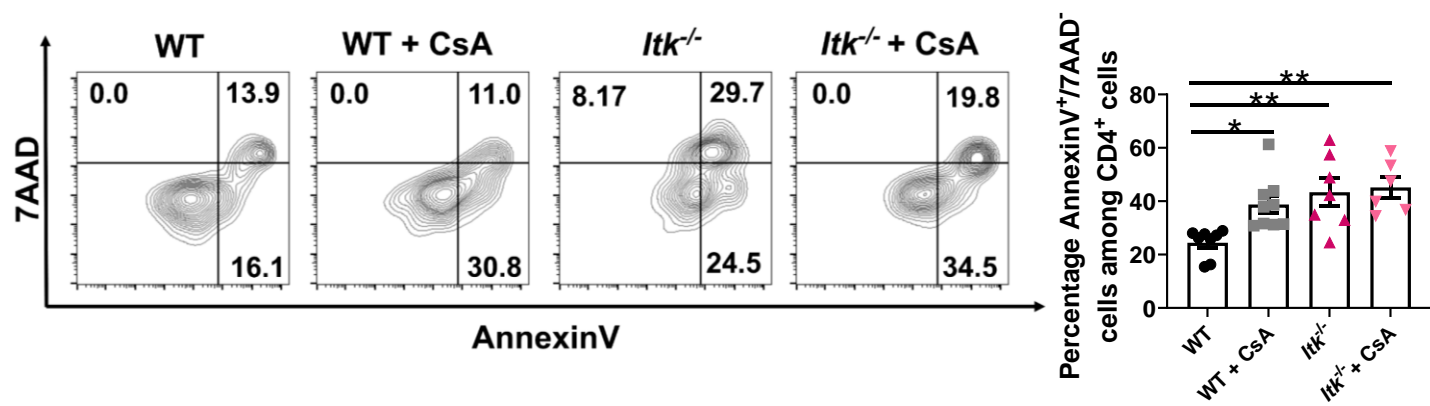
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Spleen

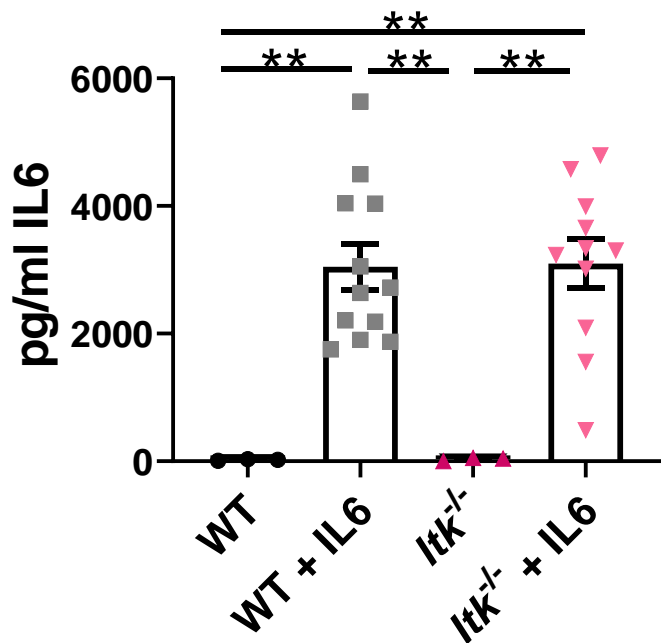
**C**

Blood

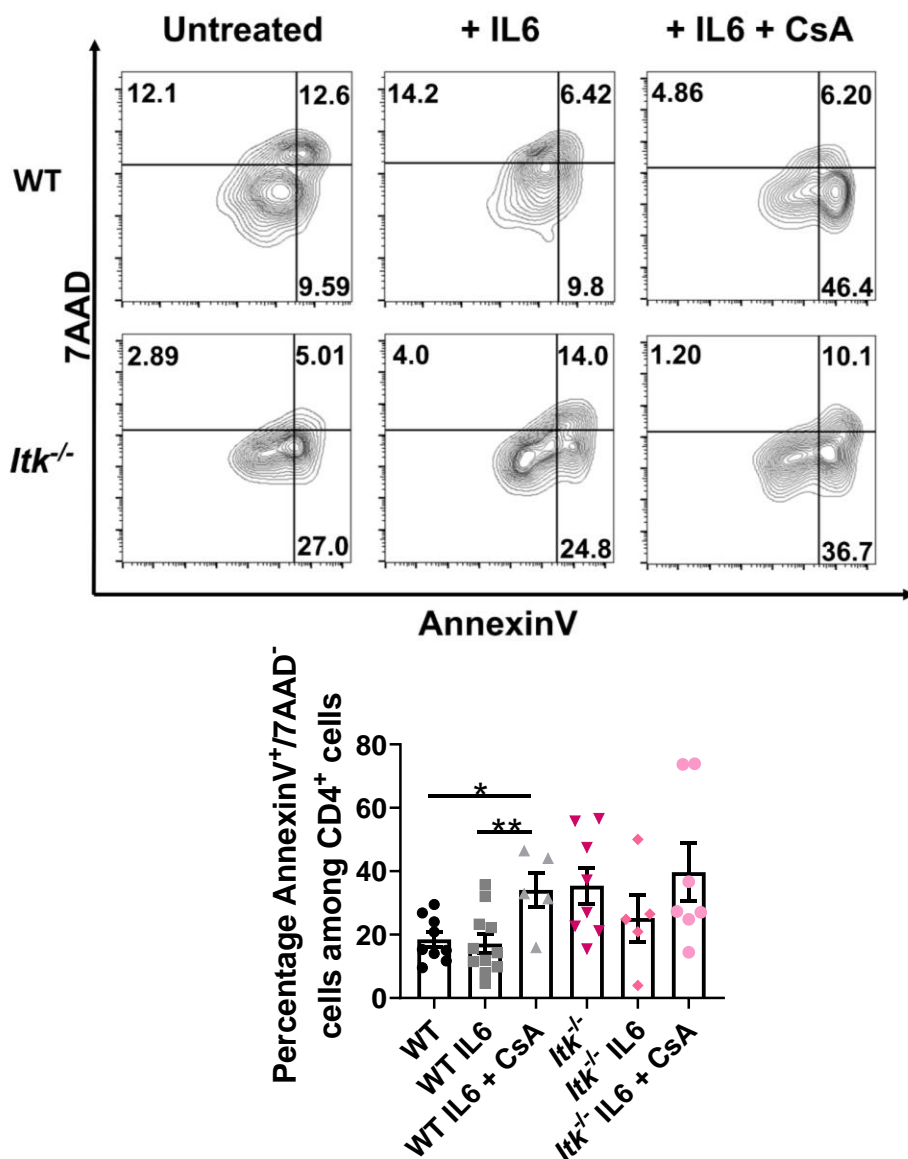


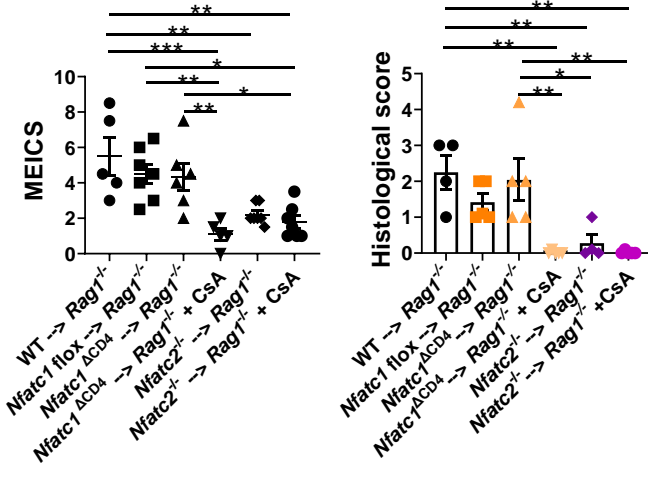
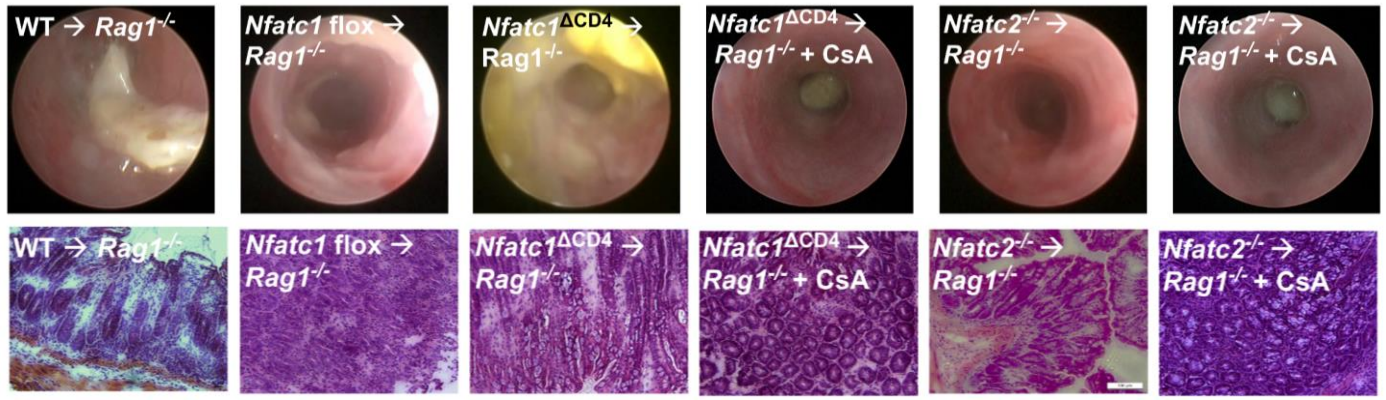
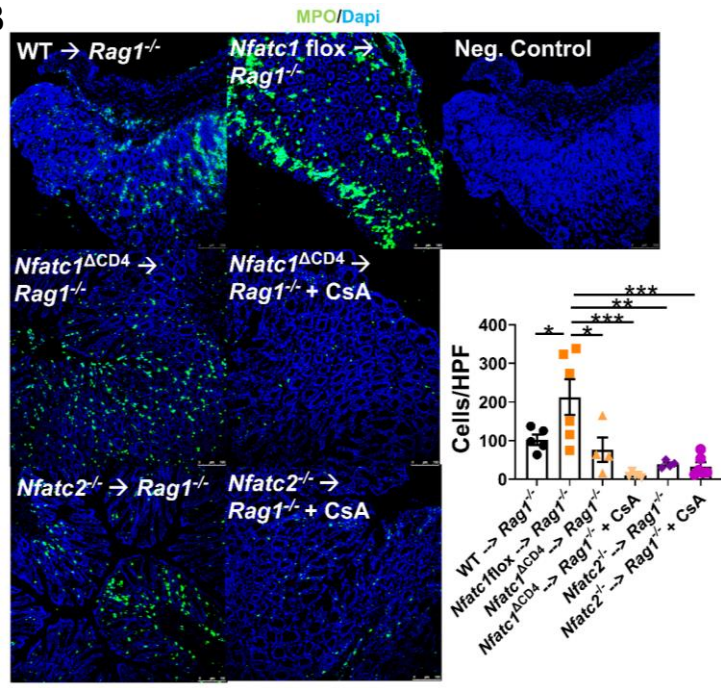
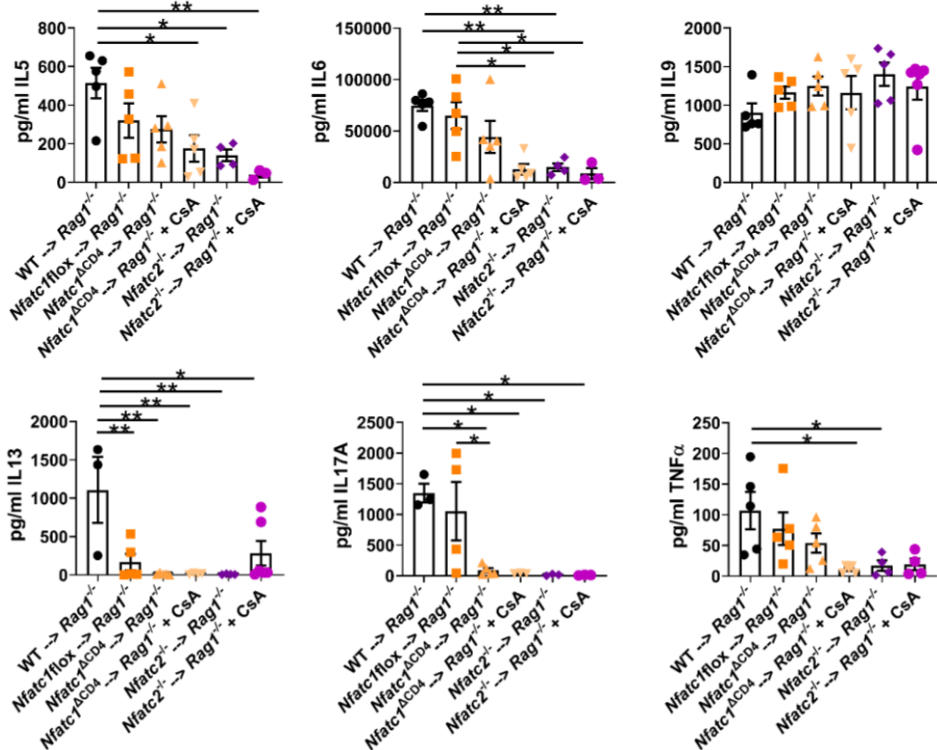
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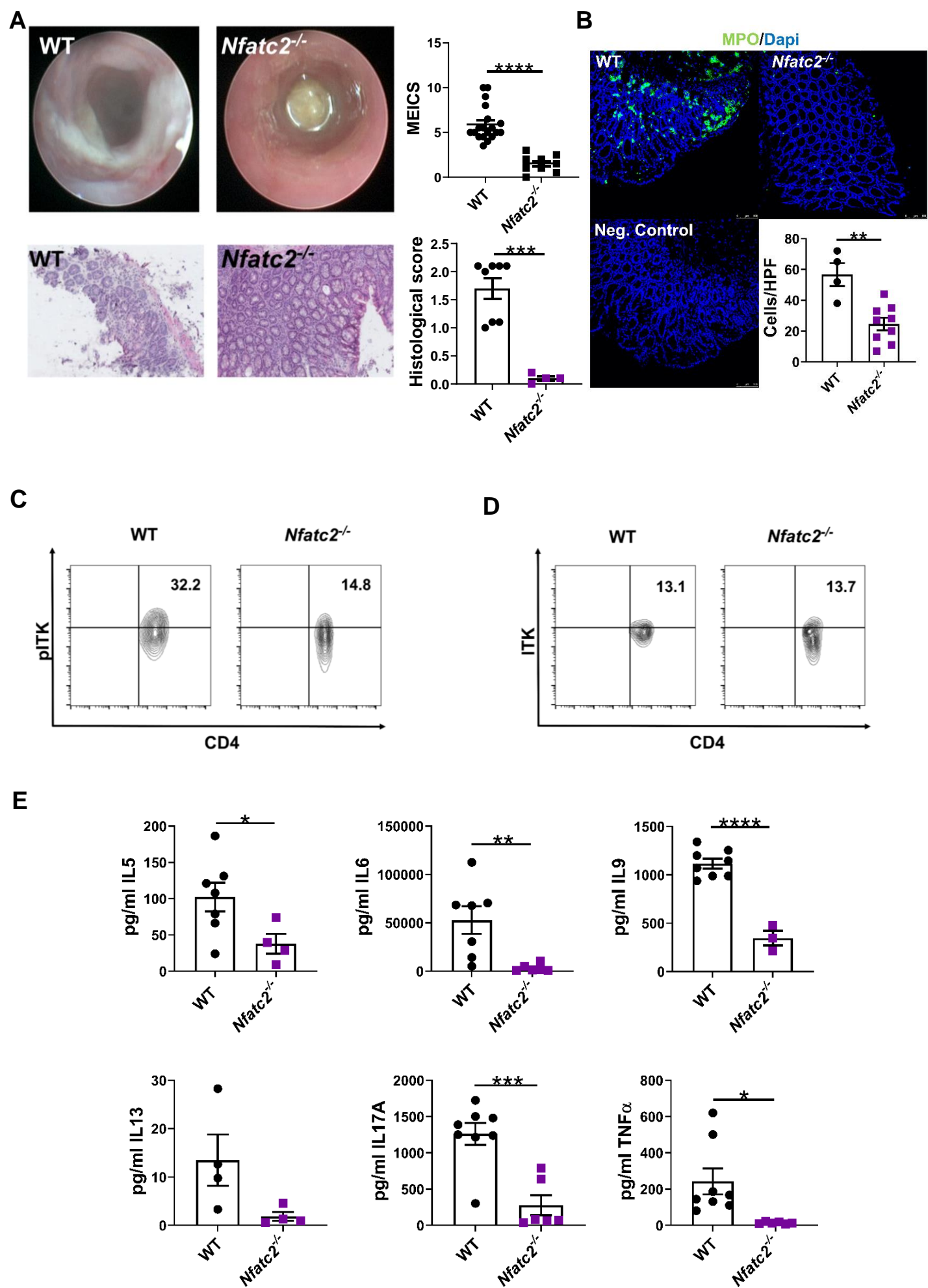


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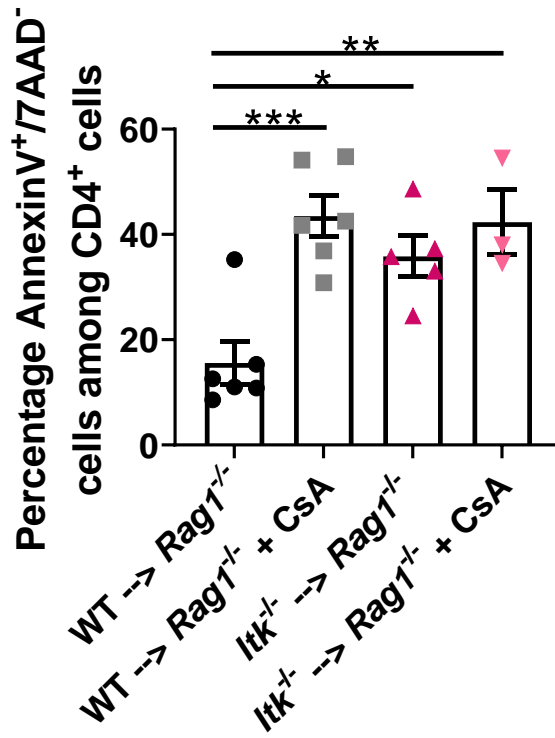
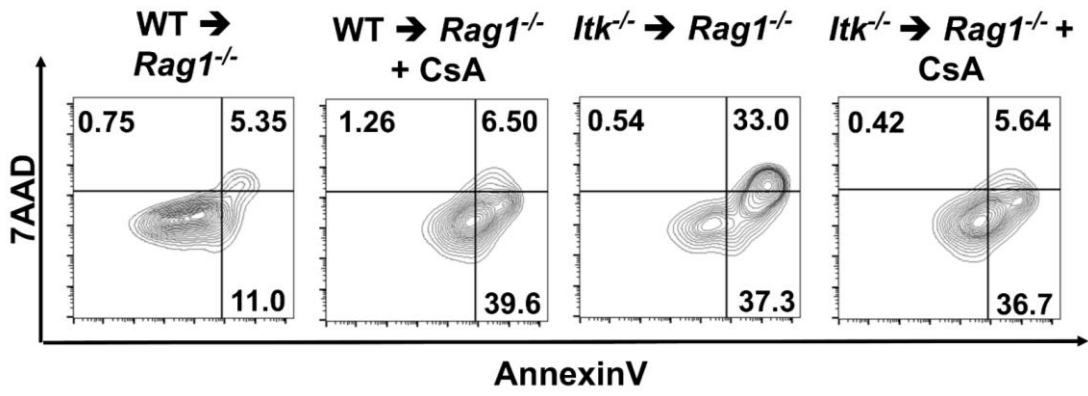


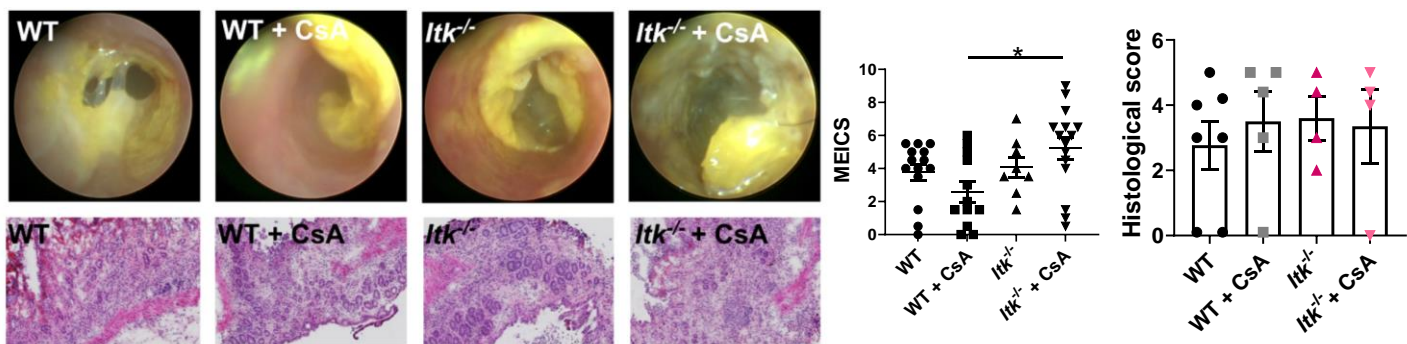
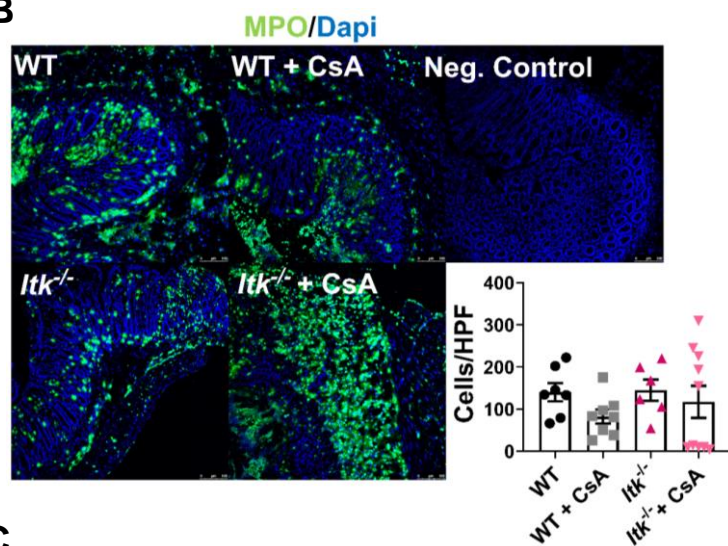
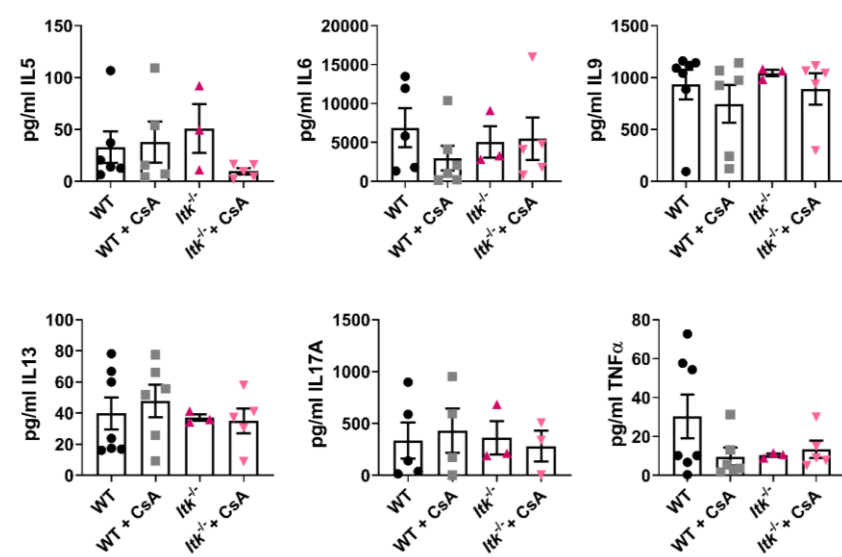
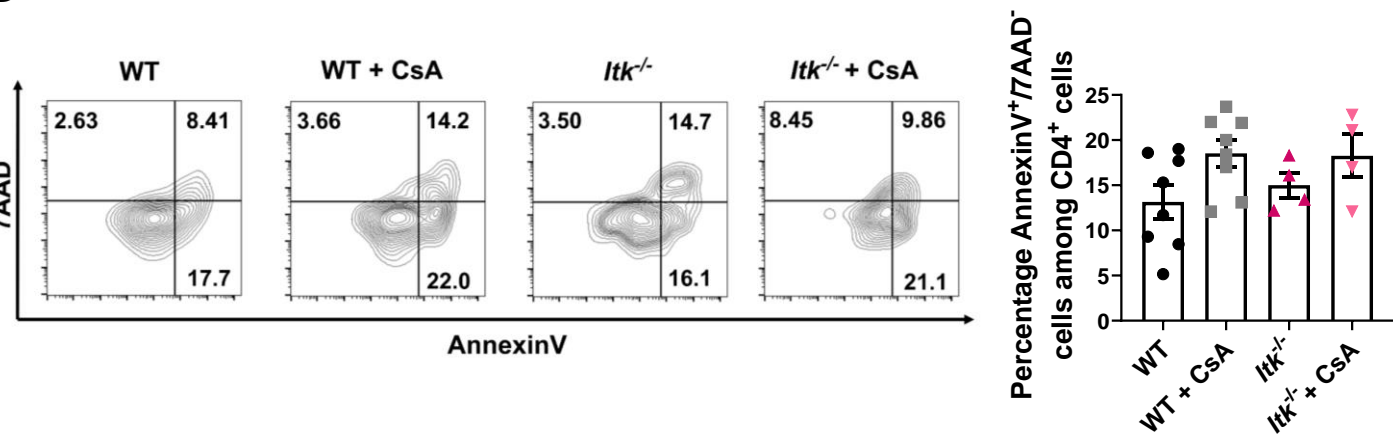
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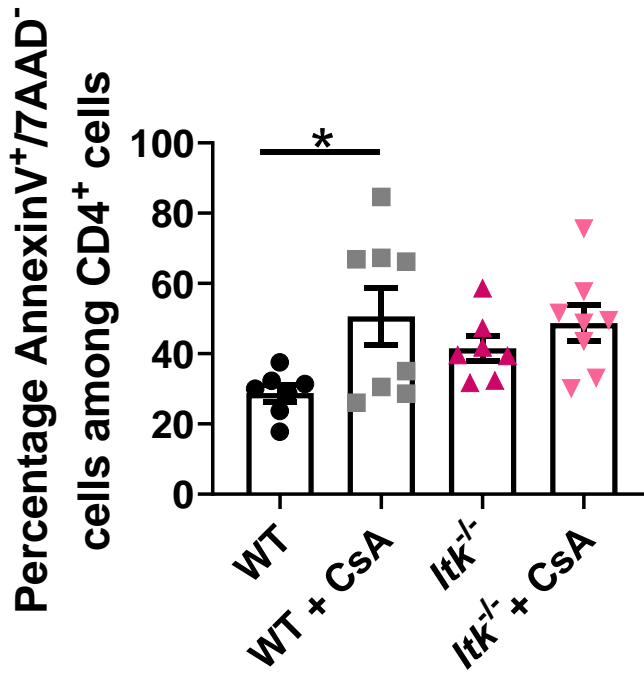
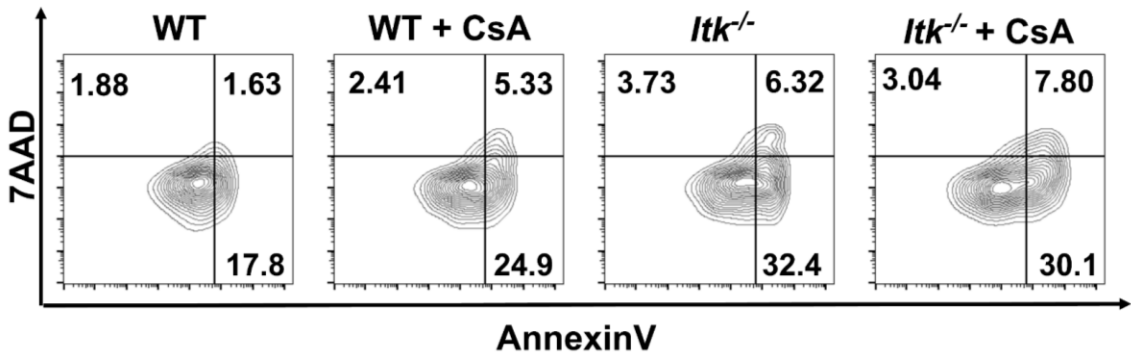


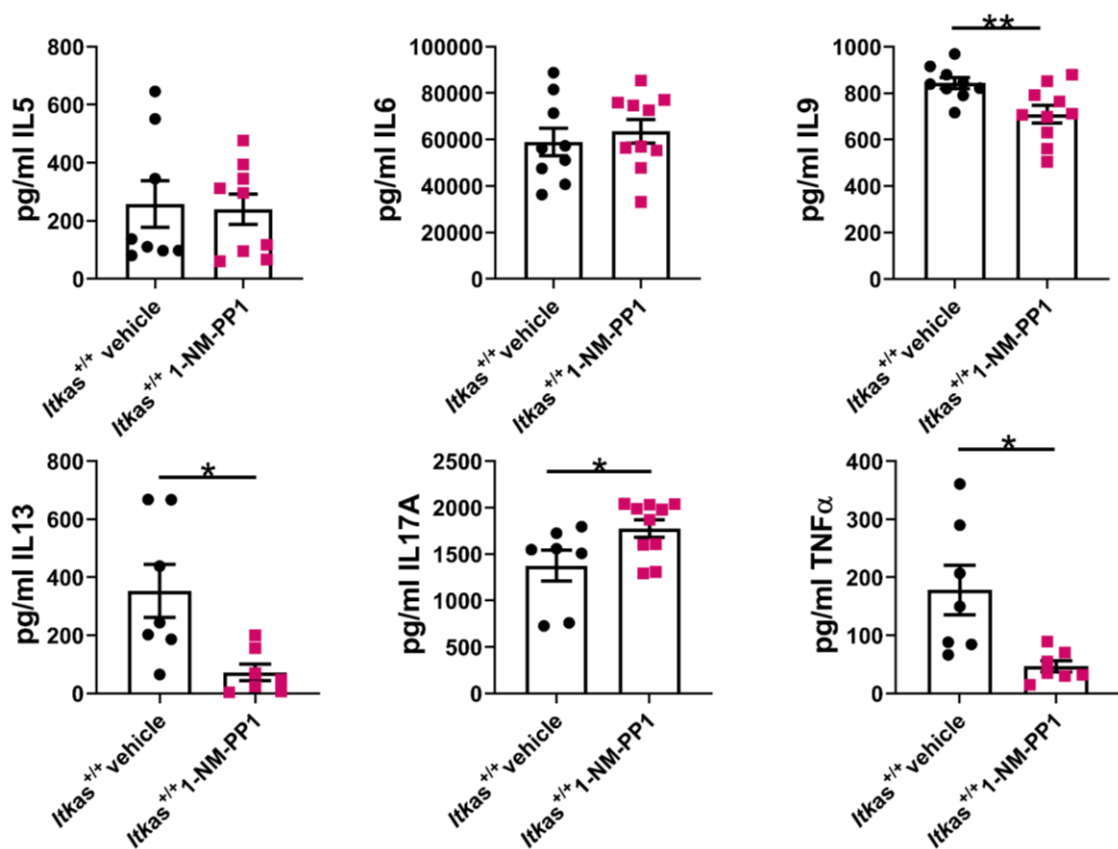
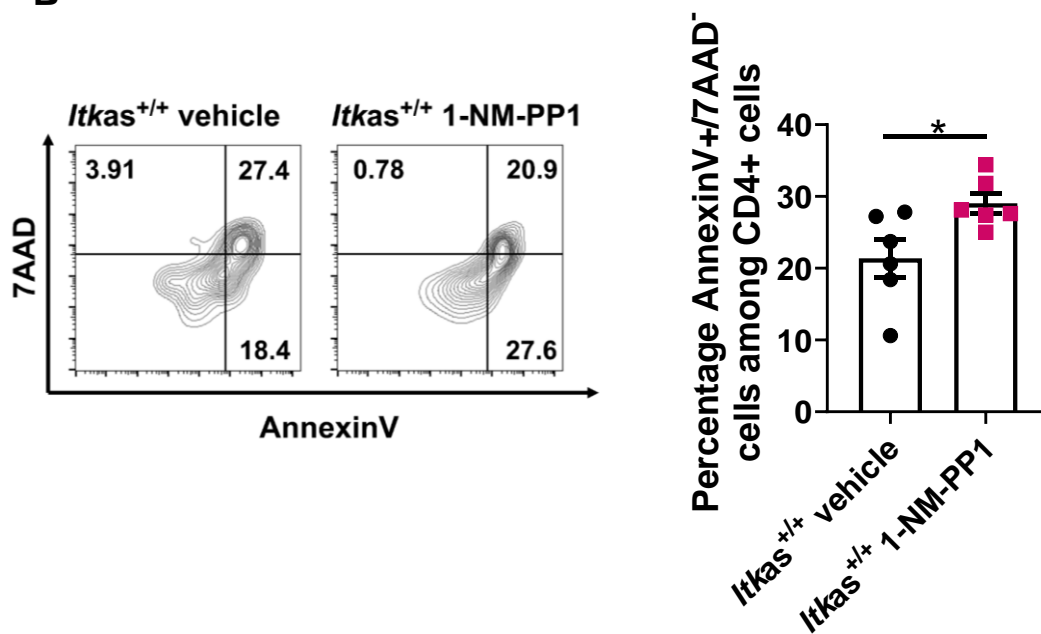
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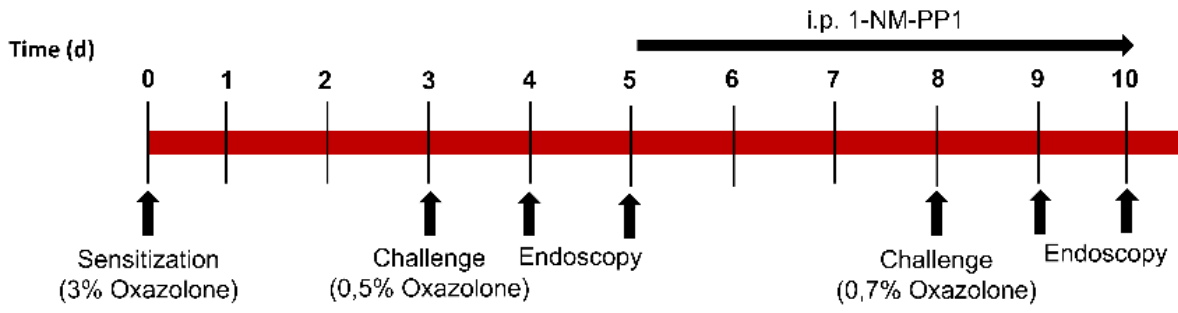
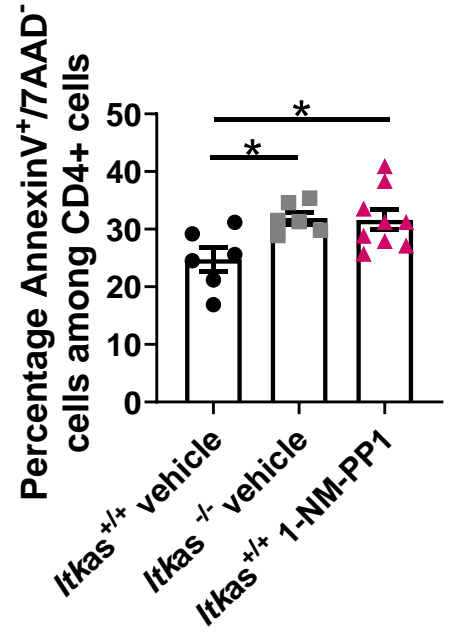
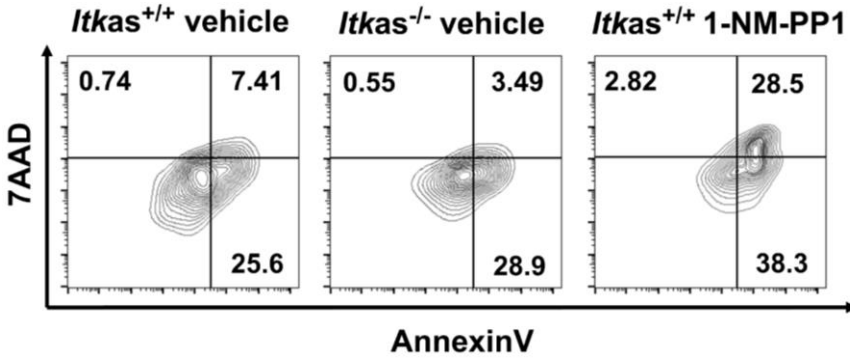


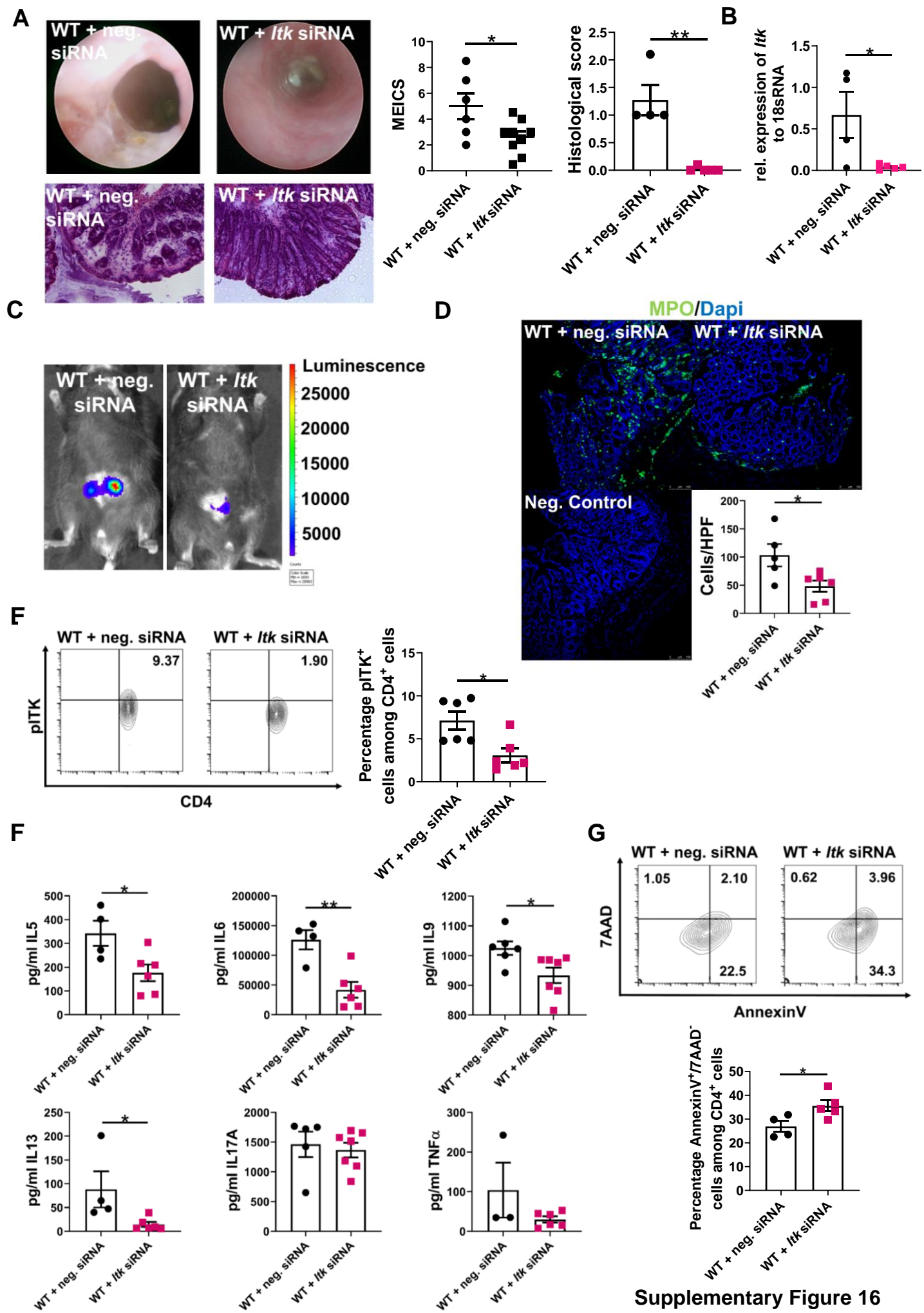
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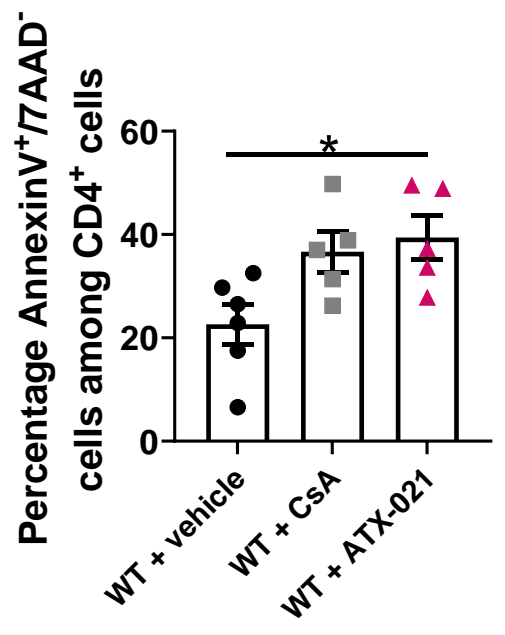
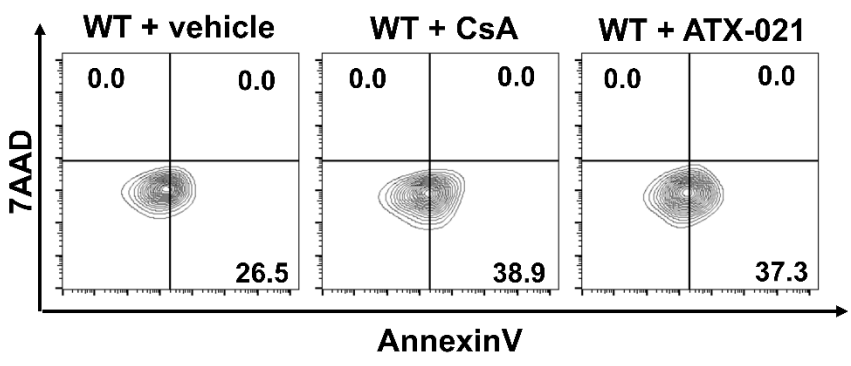
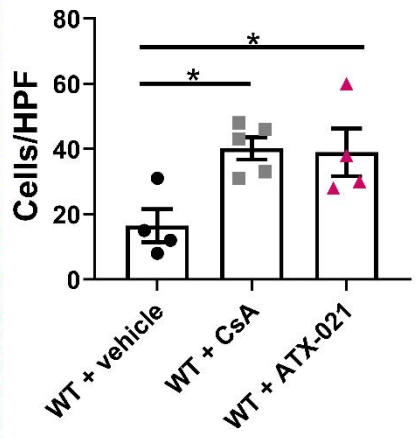
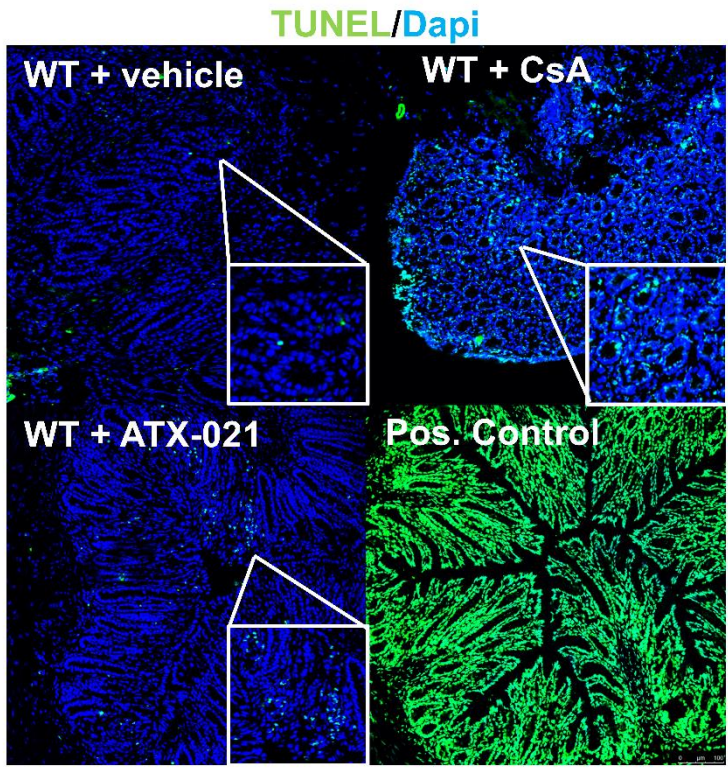


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**A****B**



Supplementary Figure 16

**A****B**



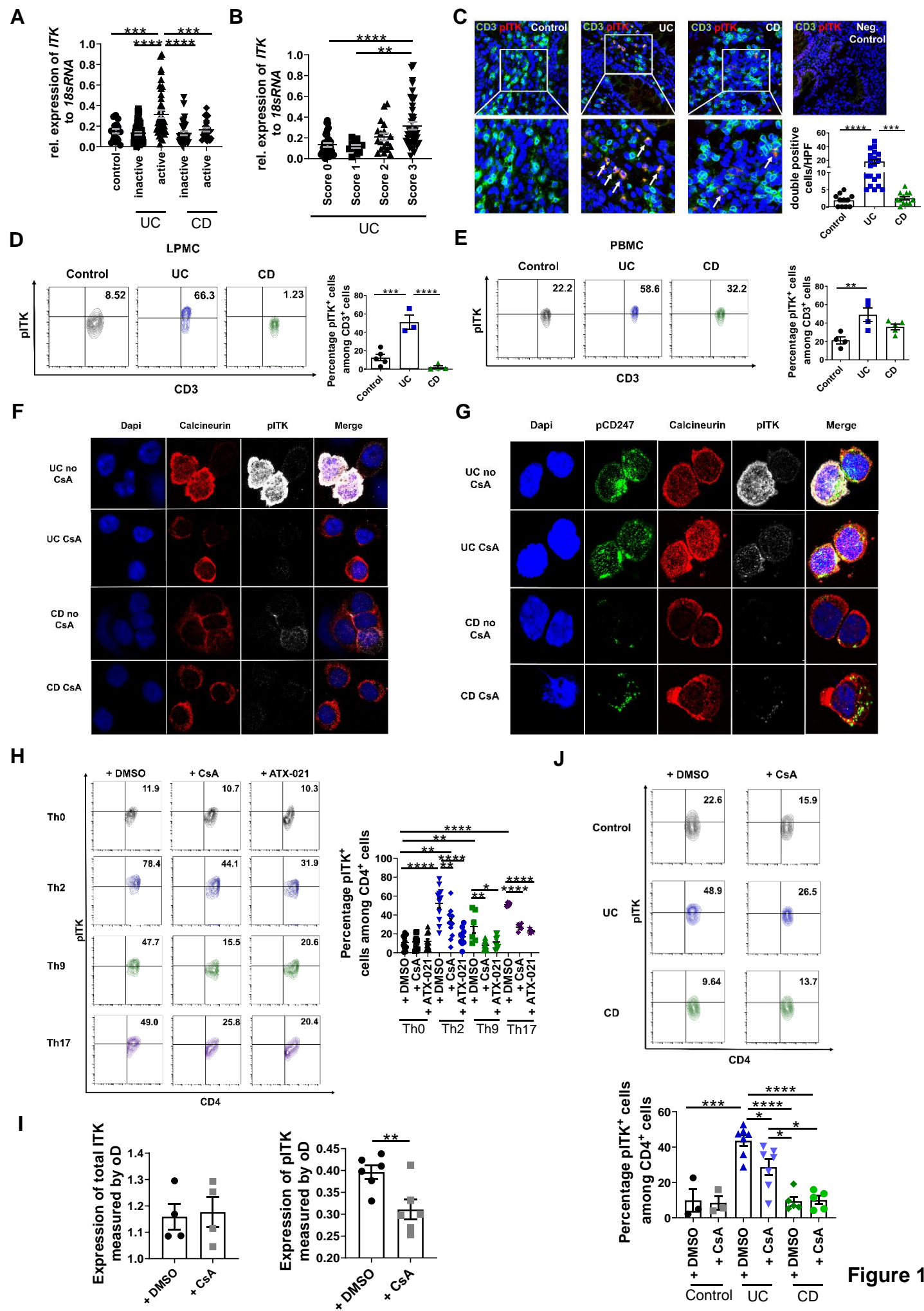


Figure 1

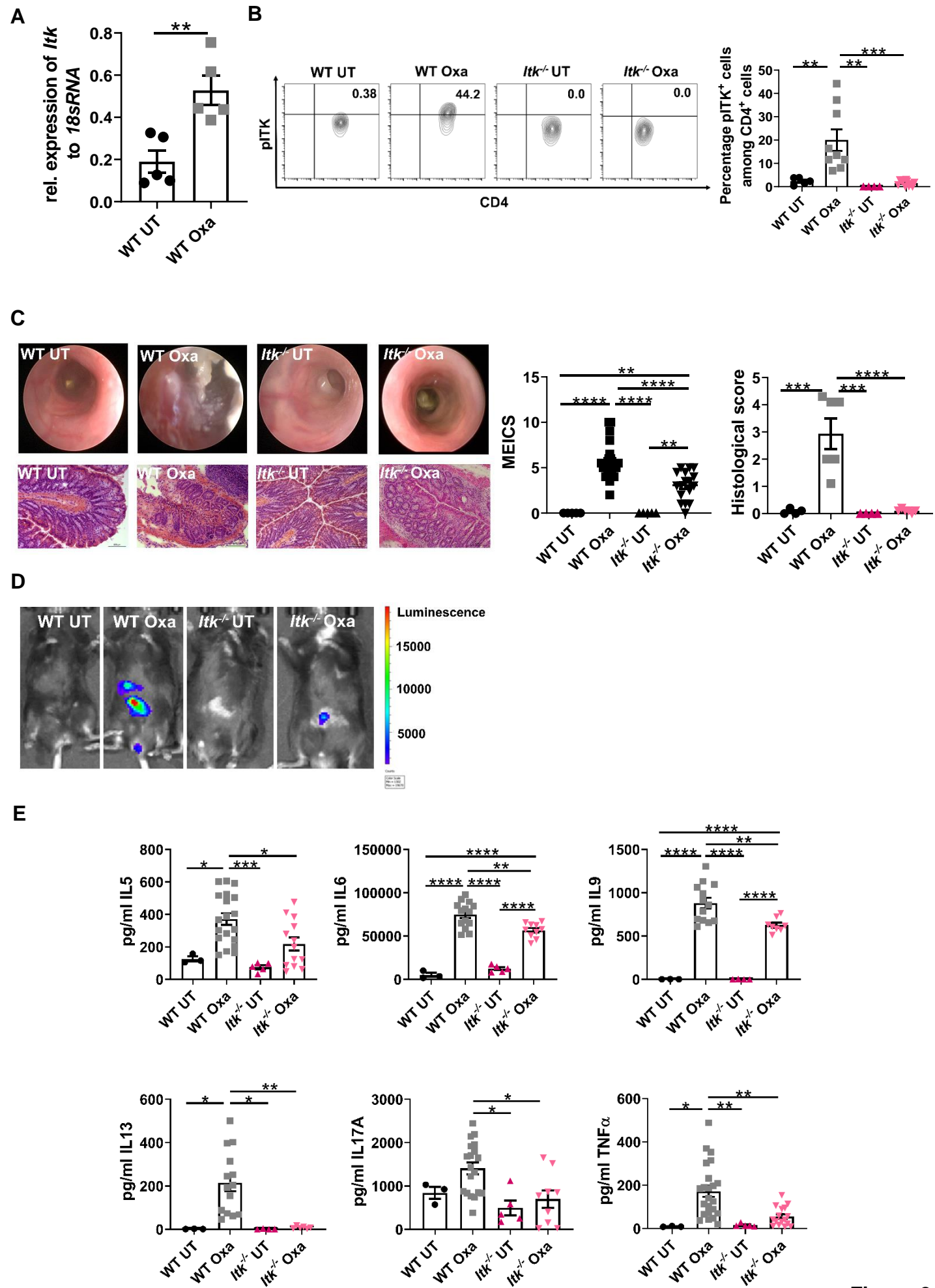


Figure 2

