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NAIP/NLRC4 inflammasome activation in human small intestinal organoids and murine tuft cells

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Abstract:

The NAIP/NLRC4 inflammasome is crucial for the restriction of pathogenic bacteria. Especially activation in intestinal epithelial cells provides a first line of defense by inducing cell extrusion of infected cells and releasing eicosanoids. As NLRC4 mutations result in severe autoinflammation, inflammasome activation needs to be tightly regulated. This makes it essential to understand the mechanisms of NAIP/NLRC4 activation not only in immune cells, but also in other cell types. Gastroenteritis, often induced by inflammasome formation, shows the highest death rates in regions with increased prevalence of parasite infections. The sensing of parasites and induction of a type 2 immune response involves intestinal tuft cells. Very interestingly, these cells in contrast to other intestinal epithelial cells do express all inflammasome components and the enzymes for the synthesis of the eicosanoid prostaglandin D2. However, the role of inflammasome formation in this particular cell type remains unknown.

Therefore, our main aim was to investigate tuft cell specific NAIP/NLRC4 inflammasome activation and eicosanoid release both in human and murine intestinal epithelial cells.

To be able to use human intestinal organoids as a more physiologically relevant model system, we tried to influence inflammasome expression with different cell culture media and an air-liquid-interface cultivation system, which in general resulted in upregulation of caspase 1, gasdermin D and NAIP, whereas NLRC4 was barely detectable.

To study tuft cell specific roles, we used a co-infection model in WT and NLRC4- deficient mice resulting in higher translocation of Salmonella to lymph nodes, when tuft cell numbers were increased. Moreover, inflammasome activation specifically in tuft cells resulted in reduced ILC3 cytokine mRNA in the small intestine. Additionally we could prove that our HPGDS knockout mouse shows similar small intestinal tuft cell counts, is not able to produce PGD2 and does not show an aberrant shift to the production of other eicosanoids.

These preliminary results give a hint on how inflammasome activation in tuft cells might influence the surrounding tissue, in particular innate lymphoid cells. However, further *in vivo* and *in vitro* experiments are required to investigate the underlying molecular mechanisms.

Introduction:

The immune system is constantly faced with pathogenic invaders and has therefore evolved numerous defense mechanisms. Sensing of highly conserved pathogen- associated molecular patterns (PAMPs) or damage- associated molecular patterns (DAMPs) is mediated by pattern recognition receptors (PRRs) [1]. Amongst others, the best characterized PRRs include the extracellular Toll- like receptors (TLRs) and the intracellular nucleotide- binding domain (NBD) and leucine- rich repeat (LRR) containing receptors (NLRs). Upon recognition of PAMPs such as bacterial or fungal cell-wall components or viral nucleic acids by macrophages, dendritic cells (DCs) or other antigen- presenting cells, the adaptive immune response is shaped specifically to the type of pathogen usually resulting in clearance of the invader [2].

NLRs share the NACHT domain consisting of the NBD, helical domain 1 (HD1), winged helix domain (WHD) and helical domain 2 (HD2) and the C- terminal LRRs. There are several N-terminal domains such as the pyrin domain (PYD), the caspase activation and recruitment domain (CARD) or the baculovirus- inhibitor of apoptosis repeat (BIR) domain, which vary within the different NLRs [3]. Up to date, there are more than 20 NLRs with quite distinct functions known. Data suggests that at least three of them (CIITA, NLRC5, NLRP3) can act as transcription factors [4-6]. Several other NLRs such as NLRP1, NLRP3, NLRP6 or NAIP and NLRC4 form cytosolic multi-protein complexes called inflammasomes [7]. Activation of inflammasomes by PAMPs results in recruitment and activation of Caspase 1 with or without the adaptor protein ASC (apoptosis- associated speck- like protein containing a CARD). Caspase 1 cleaves the precursor forms of gasdermin D (GSDMD) which forms pores into the cell membrane through which the cleaved cytokines IL-1β and IL-18 are released. This lytic and inflammatory cell death is also called pyroptosis [8].

The NAIP/NLRC4 inflammasome is somewhat unusual as two different NLRs are required to form a functional inflammasome [9]. Mice encode four functional NAIPs (neuronal apoptosis inhibitory proteins) whereas humans only express one full-length protein [10]. It was only shown a few years ago that murine NAIP1 detects the needle protein of the bacterial type 2 secretion system (T3SS), NAIP2 recognizes the T3SS rod protein and NAIP5 and NAIP6 are required for the detection of flagellin [11, 12]. In humans, experiments in primary immune cells and transfection of cell lines indicate that one NAIP isoform is sufficient to detect T3SS needle or rod and flagellin, although it still needs to be determined if other isoforms or splice variants might be involved in recognition of these ligands as well [13-15]. Apart from the NBD and the LRR, NAIP contains three N-terminal BIR domains, whose function is still unknown, although this region might be involved in binding of flagellin [16, 17]. Upon ligand recognition, NAIP is relieved from its auto- inhibited state by rotation, exposing hydrophobic and basic amino acid residues, which in turn bind to a complementary region on NLRC4. NLRC4 is relieved from its auto-inhibited conformation as well, thereby revealing hydrophobic residues resulting in a "domino" reaction of recruiting other NLRC4 molecules until a multi-protein complex, the inflammasome, is formed [17-20]. NLRC4 - as the name implies - contains a N-terminal CARD domain, which is oligomerized upon inflammasome formation and allows direct binding to the CARD domain of Caspase 1, although ASC recruitment is possible and results in a more efficient response [21, 22].

As recently reviewed by Bauer and Rauch, the NAIP/NLRC4 inflammasome is known to be activated *in vivo* by various strains of bacteria resulting in restriction of for example *Burkholderia sp.*, *Chromobacterium violaceum*, *Citrobacter rodentium* or *Salmonella enterica* serovar Typhimurium.

Additional *in vitro* data shows recognition of *Listeria monocytogenes* or *Shigella flexneri* as well, and recently, Mitchell et al. identified NAIP/NLRC4- deficient mice as a physiologically relevant infection model as they show similar susceptibility to shigellosis as humans [9, 23]. Very interestingly, in contrast to most other bacteria, *Helicobacter pylori* benefits from NAIP/NLRC4- induced inflammation and IL-18- mediated downregulation of antimicrobial peptides [24].

However, one major limitation of using these bacteria for research on NAIP and NLRC4 functions is the fact that they can also be recognized by extracellular TLRs, which makes it quite difficult to distinguish between TLR- and NLR- induced effects. The Vance lab at the University of California, Berkeley came up with a great solution to specifically target NAIP/NLRC4. They fused flagellin, T3SS needle or rod to the N-terminal domain of the *Bacillus anthracis* lethal factor (LFn). The resulting fusion proteins FlaTox, NeedleTox and RodTox can be administered together with the anthrax protective antigen (PA). PA forms pores through which the different proteins are translocated into the cytosol, where they can be detected by human NAIP or murine Naip 5/6, 1 or 2, respectively [25].

When they first injected wildtype (WT) mice with FlaTox, the animals succumbed within 30 minutes due to systemic inflammasome activation. One of the underlying causes for this severe phenotype is the release of prostaglandins and leukotrienes, also known as eicosanoid storm, which initiate inflammation and vascular fluid loss into the peritoneal cavity and the intestine [25]. Recently, inflammasome activation in macrophages and the subsequent release of tissue factor, which mediates blood clotting, was identified as another reason for host death [26]. A similar phenotype is observed in humans with NLRC4 mutations within the HD1, WHD or LRR domain thereby affecting the autoinhibition and resulting in a constitutively active conformation. This is the cause for a severe and in most cases lethal autoinflammation. In comparison to other inflammasopathies, these mutations show three distinct symptoms: macrophage activation syndrome (MAS), high IL-18 levels and enterocolitis [9, 27].

Initially, activation of inflammasome in myeloid cells such as macrophages was thought to be the major driving factor for the severe phenotypes observed in humans and mice. However, also murine intestinal epithelial cells (IECs) show a high expression of NAIP and NLRC4 and the intestinal phenotypes of both bacterial infections and NLRC4 hyper mutations suggest a role of inflammasome activation in these cells as well [28, 29]. Indeed, both *Salmonella* infection models and FlaTox injection experiments reveal that NLRC4 activation induces cell extrusion of infected IECs, which is a very efficient and quick way of inhibiting dissemination of bacteria [30, 31]. Very interestingly, NLRC4 in IECs but not in myeloid cells is sufficient for the restriction of Salmonella [30, 32]. By injecting FlaTox into mice expressing NLRC4 in either myeloid cells or IECs, Rauch et al observed the typical loss of body temperature in both mouse strains, but diarrhea is mainly driven by inflammasome activation in IECs [30]. Unpublished data from the Rauch lab in collaboration with the Gronert lab at UC Berkeley additionally shows the release of PGD2 upon inflammasome activation by FlaTox. This lipid mediator derives from arachidonic acid and is specifically synthesized by the Hematopoietic Prostaglandin D Synthase (HPGDS). PGD2 is mainly known for its role in type 2 inflammation against parasites and in promoting development and progression of allergies [33].

These findings are quite interesting, as NLRs were initially not connected to type 2 inflammation. Only a few years ago, a group described NLRP3 as an important transcription factor in murine T_H2 cells [4]. Additionally, unpublished data from the Horejs-Hoeck lab displays that DCs of birch-pollen allergic people show significantly reduced messenger RNA (mRNA) levels of NAIP and NLRC4 compared to healthy individuals.

Allergies are highly increasing, but the mechanism of allergy development remains poorly understood. However, the observations that the allergen alone in contrast to the allergen in its natural context often fails to induce an immune response and that bacteria, which could be recognized by NLRs, colonize the allergen source suggests a role of PRRs in type 2 inflammation as well [34, 35]. Moreover, allergens usually first encounter tissue and not immune cells. This is also the reason why scientists start to focus more and more on innate defense mechanisms in epithelial cells.

Depending on the purpose of an organ, epithelia are very specialized. This is particularly obvious when looking at the gastro- intestinal tract. As shown in Figure 1, the majority of small intestinal cells are enterocytes responsible for the uptake of nutrition, but the tissue is also composed of several different cell types with distinct functions. Stem cells in the crypts are lined by paneth cells secreting antimicrobial peptides. Enteroendocrine cells are responsible for hormons and goblet cells are producing mucus. The last very rare cell type found in the intestine are tuft cells, which are known for decades. However, their role remained unknown until 2016, when three groups discovered that these cells are very important in inducing anti-parasite immune responses by acting on ILC2s [36-38].

Tuft cells can be identified with several different biomarkers as reviewed by Ting and von Moltke. The major transcription factors, which are constitutively expressed are POU2F3 (POU class 2, homeobox 3) and GFI1B (Growth factor independent protein 1B). Moreover, DCLK1- (DCAMKL1, Doublecortin- like and CAM kinase- like 1) expressing tuft cells comprise more than 95% of all DCLK1- expressing IECs. These three together with IL-25 and SIGLECF (Sialic acid-binding Ig-like lectin F) allow identification of tuft cells with several molecular biological methods such as flow cytometry or immunohistochemistry [39]. Additionally, tuft cells express a G-protein coupled receptor (SUCNR1), which detects succinate, a metabolite produced by protists such as *Tritrichomonas* and very likely also bacteria and helminths. Upon recognition, they secrete the cytokine IL-25, which is important for the activation and IL-13 production of type 2 innate lymphoid cells (ILC2). IL-13 in turn induces proliferation of mucus-producing cells. This induced goblet cell hyperplasia and smooth- muscle hypercontractility results in the typical “weep and sweep” response to rid the host of parasites [38, 39]. Moreover, succinate treatment of mice results in an almost tenfold increase of tuft cell numbers [40].

Interestingly, data from the von Moltke lab at the University of Washington identifies tuft cells to not only express high levels of enzymes involved in the arachidonic acid metabolism such as HPGDS but also all NAIP/NLRC4 inflammasome components [40]. Furthermore, in a collaboration experiment of the von Moltke, Gronert and Rauch lab with WT and Pou2f3- deficient IECs, they observed that PGD2 release is tuft cell specific.

PGD2 binds to its receptors DP1 or CRTH2 (or PTGDR2, Chemoattractant receptor- homologous molecule expressed on Th2 cells). The latter one is mainly expressed on T_H2 cells, basophils or eosinophils [41]. However, single cell RNA sequencing (RNAseq) data also shows an expression of CRTH2 on ILC3s [42]. Whereas ILC2s - similar to T_H2 cells - secrete IL-4, IL-5 and IL-13, ILC3s are the innate counterpart of T_H17 cells (responsible for pathogen clearance) and produce IFN-γ, IL-22 and IL-17 [43, 44].

Taken together, these results suggest a connecting role of tuft cells, especially as regions with a higher prevalence of parasite infections (hookworm) also show a higher death rate due to gastroenteritis [45, 46]. Inflammasome activation is very important in defense against invading bacteria. However, the role of NAIP/NLRC4 in tuft cells, which are involved in anti-helminth responses, is not known. Moreover,

data on human cells is usually obtained from immortalized cell lines but more *in vivo*- related models are still missing.

Therefore, we wanted to establish a more physiologically relevant human *in vitro* model using intestinal stem cells in spheroid culture (organoids, "mini guts") which allows research on inflammasomes. Furthermore, our main aim was to analyze *in vivo* inflammasome activation and eicosanoid release in IECs with a focus on tuft cells using mouse strains deficient in NLRC4, POU2F3 or HPGDS.

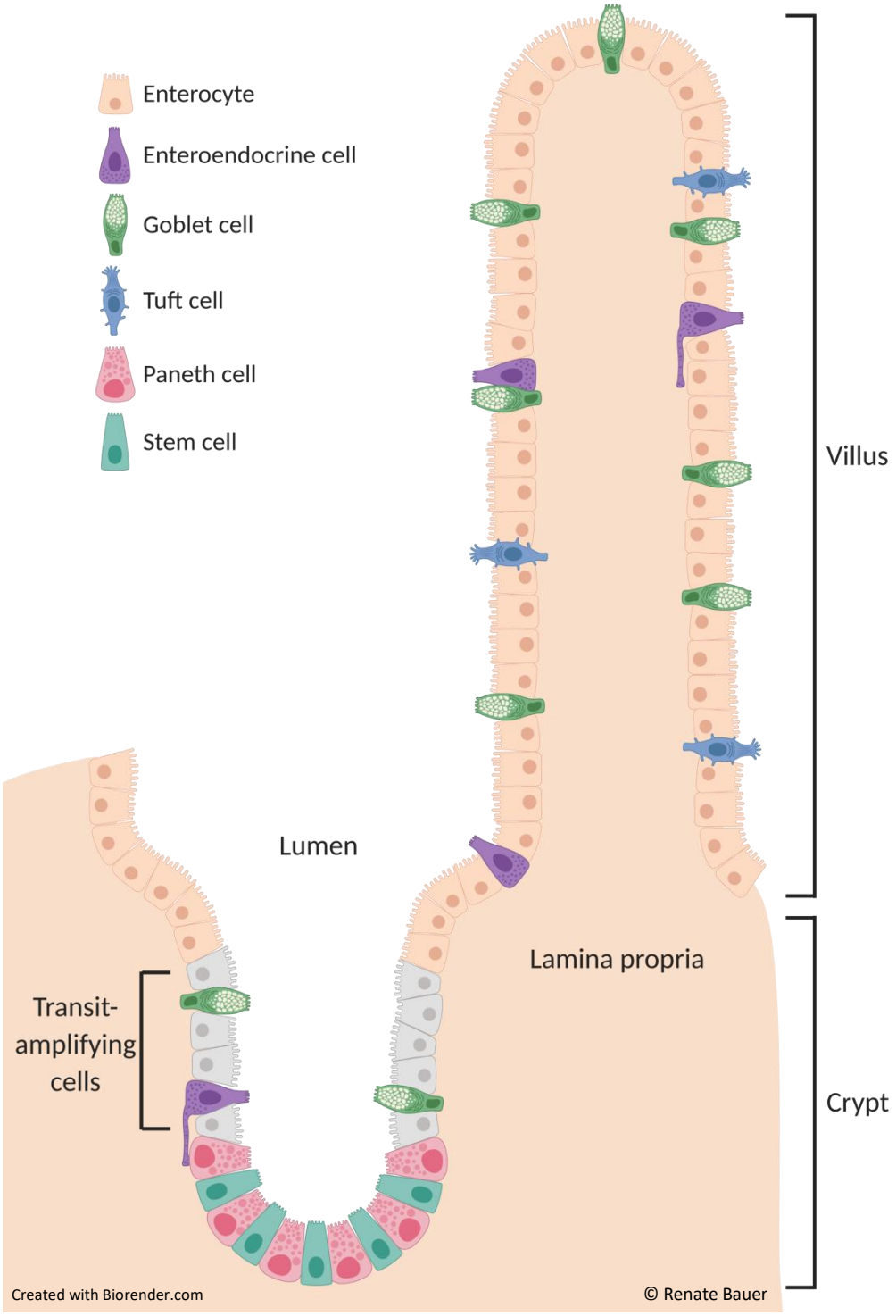

Figure 1: Morphology and cellular composition of the small intestine

Methods:

Isolation of primary human monocytes

Peripheral blood mononuclear cells (PBMCs) from one blood donor were isolated using density gradient centrifugation. Lymphoprep™ (Alere Technologies AS) was overlayed with blood mixed 1:2 with 1x PBS (pH 7.4, Gibco) and centrifuged at 800xg for 20 minutes at room temperature (RT). After lysis of residual erythrocytes with 1x RBC lysis buffer (10x, BioLegend), PBMCs were washed three times with fresh PBS for 10 minutes at 250xg. Subsequently cells were resuspended in monocyte medium (RPMI Medium 1640 (Gibco), 10% heat-inactivated (ia) FBS (Sigma), 1x L-Glutamine (L-Glu), 1x Penicillin/Streptomycin (P/S, Gibco)) and seeded on 6-well plates to allow monocytes to adhere. After incubation for 70 minutes at 37°C, all other cells were washed off and monocytes left at 37°C overnight.

The next day, the detached monocytes were collected, counted and seeded to 24- and 96-well plates at a concentration of $1x10^5$ cells/mL. Cells in 24-well plates were treated with 1 ng/mL LPS or 2 µg/mL Pam3CSK4 for 4h. Subsequently, the cell pellets were resuspended in TRIzol™ Reagent (Thermo Fisher Scientific) for RNA analysis. Cells in a 96-well plate were stimulated with 1 ng/mL LPS or the respective amount of PBS for 4 hours. Subsequently, propidium iodide (PI, 1:500, Sigma) and either PBS, PA (4 µg/mL, insect cell)- LFn-Fla (1 µg/mL, V. para, insect cell), PA (4 µg/mL)- LFn-PrgJ (100 ng/mL, insect cell), PA (4 µg/mL)- LFn-Needle (2 µg/mL, V. para, insect cell) or 1% Triton X-100 (Fisher Scientific) were added for the indicated timepoints to measure cell death via PI uptake.

Handling of human IECs in spheroid culture

Growing cells in spheroid culture allows long-term cultivation of gastro-intestinal stem cells. Spheroids were derived from human duadenum by our collaborator Jared Fisher and cultivated in special conditioned medium as described by Myoshi & Stappenbeck [47]. Briefly, spheroids were grown in a special matrix in 24-well plates for 3-4 days. For splitting, domes were washed with PBS-EDTA (0.5 mM) and subsequently suspended in Trypsin-EDTA (1x PBS, 0.25% trypsin, 0.5 mM EDTA). After incubation at 37°C for 5 minutes to dissociate spheroids, washing medium (DMEM/F12 with HEPES (Sigma), 10% FBS (ia), 1x P/S) was added to stop trypsinization. Cells were washed at least once with washing medium at 200xg for 5 minutes at room temperature (RT). Afterwards the supernatant was completely aspirated and the cell pellet resuspended in Corning® Matrigel® Basement Membrane Matrix (VWR). 20 µL matrigel-cell drops were placed into 24-well plates and polymerized for 5 minutes at 37°C upside down. Afterwards cells were cultured in organoid medium consisting of 50% primary culture medium (Advanced DMEM/F12 (Gibco), 20% FBS, 1x P/S, 1x L-Glu) and 50% conditioned medium (L-WRN supernatant) supplemented with 10 µM of rock-inhibitor Y27632 (Sigma) and 10 µM TGF-β-inhibitor SB431542 (Millipore).

To analyze inflammasome expression under different media conditions, spheroids were additionally cultured in differentiation medium consisting of DMEM/F12 supplemented with 20% murine R-Spondin1 supernatant, 10% murine Noggin supernatant, 50 ng/mL recombinant murine EGF (Fisher Scientific), 1x P/S, 1x L-Glu, 10 mM HEPES (Himedia), 1x N2 (Life Technologies), 1x B27 (Life Technologies), 1 mM N-Acetylcysteine (Fisher Scientific) and 5 µM DAPT.

For stimulation, 100 ng/mL of recombinant human TNF-α (R&D), IL-22 (Tonbo Biosciences) and IFN-λ2 (Peprotech) were used. After 6 hours of incubation at 37°C, matrigel domes were dissolved in PBS-EDTA (5 mM) for 1 hour at 4°C on an orbital shaker. After centrifugation at 300xg for 5 minutes at 4°C, the cell pellet was resuspended in TRIzol™ to analyze mRNA expression.

Air-liquid-interface cultivation system

Several recent publications suggest that growth of stem cells in monolayers under air-liquid conditions supports differentiation of intestinal or colonic cells in mice [48, 49]. To test, whether also human intestinal stem cells are able to differentiate, we used a similar model comparing growth and inflammasome expression on transparent and translucent transwells (ThinCert™ Cell Culture Inserts 24 well, pore size: 0.4 µm, Greiner bio-one) under different media conditions.

Transwells were coated with 10% Matrigel in 1x PBS. Spheroids were dissociated to a single-cell solution with Trypsin-EDTA at 37°C including several vigorous pipetting steps. $3x10^4$ cells were added per transwell and incubated at 37°C with organoid medium on both sides, changing medium on basolateral side as required. After 7 days, medium in upper part was removed to create an air-liquid interface. Medium on basolateral side was replaced with fresh one every 3 days and medium in transwell was aspirated as required. After 5-7 days, the medium of half of the samples was changed to differentiation medium.

After a total of 21 days, cells of each condition were harvested in TRIzol to analyze mRNA expression. Additional wells of each condition were treated with propidiumiodide (1:100) and stimulated with a mix of 4 µg/mL PA (bacterial), 2 µg/mL LFn-Fla (bacterial) and 1 µg/mL LFn-Needle (*V. para*) or the respective amount of PBS for 45 minutes. Cells were fixed with 4% PFA. After a washing step, membranes were cut out using a razor blade, placed on microscopy slides (VWR® Frosted Microscope Slide) and stained with DAPI (1:1000) for 10 minutes at RT. After another washing step, membranes were covered in mounting medium (Vectashield HardSet™ Antifade Mounting Medium, Fisher Scientific) and sealed with a cover glass.

Mouse lines

Male and female NLRC4$^{-/-}$ and WT (NLRC4$^{+/+}$ or NLRC4$^{+/-}$) as well as iNLRC4-Pou2f3Cre (Cre$^-$ or Cre$^+$) and HPGDS PreCre$^{+/-}$ (WT) and PreCre$^{+/+}$ (HPGDS$^{-/-}$) mice (C57BL/6 background) were maintained in specific-pathogen-free animal facilities at Oregon Health & Science University (OHSU) and provided with food and water ad libitum on a 12 hour light/dark cycle. To prevent abnormalities caused by differences in the microbiomes of the animals, littermates were used for the individual experiments. Animal protocols (#IP00002053) were approved by the institutional animal care and use committee (IACUC) according to national guidelines.

Salmonella infection

Seven days before infection, 4 out of 8 WT and 3 out of 6 NLRC4$^{-/-}$ mice (8-9 weeks old) were administered 150 mM succinate (Sodium succinate hexahydrate, 99%, Alfa Aesar™, Fisher Scientific) in their drinking water. Drinking water was changed every other day. Two days before infection, *S.*

enterica serovar Typhimurium (glms:Ptrc-SfGFPST::FRT) were streaked out on McConkey agar (Fisher Scientific) + 50 µg/mL Streptomycin (Streptomycin sulfate powder in H_2O, Fisher Scientific) and incubated at 37°C overnight. The next day, food and water of the mice was withdrawn 4 hours prior to oral gavage of Streptomycin (250 mg/mL) in H_2O in order to prepare a niche for Salmonella. One colony of the bacteria grown on the agar plate was inoculated in Luria-Bertani (LB) broth (Merck) with 50 µg/mL Streptomycin and grown overnight at 37°C, shaking.

At the day of infection, bacteria were sub-cultured for 3 hours and food and water was again withdrawn 4 hours prior to oral gavage of $5x10^7$ colony-forming units (CFUs). CFU number was determined by measuring the OD at a wavelength of 600 nm.

After 18 hours of infection, mice were sacrificed according to animal protocol and mesenteric lymph nodes, the ileal part of the small intestine (SI), cecum content and one half of the cecum were incubated for 30 minutes at RT in PBS + 400 µg/mL Gentamycin to kill all extracellular bacteria. Subsequently, tissues were washed at least five times with PBS to wash off all residual antibiotics, homogenized in 1 mL PBS and plated on McConkey Agar in different concentrations for 24 hours to determine CFU numbers. Additionally, the second half of the cecum was fixed in 4% PFA overnight at 4°C.

In vivo inflammasome activation

iNLRC4Pou2f3Cre mice were administered 150 mM succinate in their drinking water 7 days before treatment. Additionally they were orally gavaged three times in two-day intervals with 100 µL of 20 mg/mL Tamoxifen (Sigma) in corn oil (Sigma). Animals were retro-orbitally injected with 4 µL of high dose PA-LFnFla per g bodyweight for 6 hours. For mRNA analysis, small pieces of the duodenum, jejunum and ileum without Peyer's patches were isolated and homogenized in TRIzol. The rest of the small intestine was cut open and fixed with PLP (4% PFA, $NaIO_4$, 0.2 M L-Lysine (in P-buffer), P-buffer (40.5% of 0.2 M Na_2HPO_4, 9.5% of 0.2 M NaH_2PO_4, 50% deionized H_2O) for histology.

Histology

PLP-fixed tissue was washed twice with PLP-buffer and incubated in 30% Sucrose (in P-buffer) overnight at 4°C. Subsequently, tissue was embedded in Tissue Plus® O.C.T. Compound (Fisher Health Care) in Tissue-Tek® Cryomolds® (Sakura) and frozen at -80°C. Tissue was cut in a cryotome, placed on Fisherbrand™ Superfrost™ Plus Microscope Slides (Fisher Scientific) and stored at -20°C until staining procedure.

After permeabilizing the cells on the slides with PBS-T (PBS, 0.05% Tween-20) for 3 minutes, free binding sites are blocked for 30 minutes at RT with 10% goat serum (Jackson Immuno Research Laboratories) diluted in PBS-T containing 1% BSA (PBS-T-BSA). The tissue was incubated for 1 hour at RT with 1:1000 rabbit anti- mouse DCAMKL1 (Abcam) in PBS-T-BSA. The slides were washed three times in PBS-T and subsequently Alexa Fluor® 647 AffiniPure Goat Anti- Rabbit IgG (Jackson Immuno Research Laboratories) diluted 1:500 in PBS-T-BSA was added for 1 hour at RT. After three washing steps in PBS-T, cell nuclei were stained for 10 minutes at RT with 100 ng/mL DAPI in PBS followed by another washing step with PBS-T. The tissue was covered in mounting medium (Vectashield HardSet™ Antifade Mounting Medium, Fisher Scientific) and sealed with a cover glass. Fluorescent stainings were

analyzed with the Zeiss ApoTome.2 and ZEN Blue microscopy software at the Advanced Light Microscopy Core at OHSU.

PFA-fixed tissue was washed twice with 70% EtOH and embedded in 2% Agarose and stored in histocassettes in 70% EtOH. Paraffin- embedment and H&E staining was performed by the Histopathology Shared Resource at OHSU. Tissue was graded from 1 (minor effects) to 4 (major effects) for epithelial damage and edema formation.

RNA extraction, cDNA synthesis and real-time quantitative polymerase chain reaction (RT-qPCR)

After thawing the TRIzol™ samples, chloroform was added and the tubes centrifuged for 15 minutes at 12,000xg at 4°C. The aequous phase was transferred to a new tube containing linear polyacrylamide (Gene-Elute™ LPA, Sigma). After addition of isopropanol the samples were incubated for 10 minutes to allow RNA precipitation and subsequently centrifuged for 10 minutes at 12,000xg. The supernatant was aspirated and the RNA pellet washed once with 75% ethanol for 5 minutes at 7,500xg at 4°C. The supernatant was aspirated and the dried pellet resuspended in Molecular Biology Grade Water (Corning). RNA content and quality (260/280 and 260/230 ratios) were determined and 1 µg of RNA was reversely transcribed into cDNA. Reaction steps were performed in Biorad T100 Cycler. First, residual DNA was removed using RQ1 RNAse free DNase (Promega) in RQ1 DNase 1x Reaction buffer (Promega) and incubation for 30 minutes at 37°C. After stopping the reaction with RQ1 DNase Stop Solution (Promega) for 10 minutes at 65°C, OligodTs (Sigma) and dNTPs (Sigma) were added for 5 minutes at 65°C. SuperScript™ IV Reverse Transcriptase (Invitrogen), 5 mM DTT and SuperScript™ IV Reaction Buffer (Invitrogen) was added for 10 minutes at 50-55°C. Subsequently the enzyme was inactivated at 80°C for 10 minutes.

To analyze RNA expression, cDNA was mixed with forward and reverse primers and PowerUp™ SYBR™ Green Master Mix (Applied Biosystems). The following primer sequences were used:

Human:

RPLP0:	forward:	5’- GGCACCATTGAAATCCTGAGTGATGTG -3‘
	reverse:	5’- TTGCGGACACCCTCCAGGAAG -3‘
CASP1:	forward:	5’- GAGGCATTTGCACACCGCCC -3‘
	reverse:	5’- GGATCTCTTCACTTCCTGCCCACA -3‘
GSDMD:	forward:	5’- CAGTTTCACTTTTAGCTCTGGGC -3‘
	reverse:	5’- ATTGAGGTGCTGGAGCTGTC -3‘
NAIP:	forward:	5’- AAGCATCCGCCCAGCTCTTGA -3‘
	reverse:	5’- TATTGCCCTCCAGATCCACAGACAGTTC -3‘
NLRC4:	forward:	5’- CATAGTCAAGTCTCTGTCAAGTGAACCCTGT -3‘
	reverse:	5’- GCTGTTCTAGCACGTTCATCCTGTCG -3‘

Mouse:

BACT:	forward:	5’- CGCAGCCACTGTCGAGTC -3‘
	reverse:	5’- CCTTCTGACCCATTCCCACC -3‘

IL-4:	forward:	5'- TCACAGCAACGAAGAACACCA -3'
	reverse:	5'- CAGGCATCGAAAAGCCCGAA -3'
IL-5:	forward:	5'- GCAATGAGACGATGAGGCTTC -3'
	reverse:	5'- GCCCCTGAAAGATTTCTCCAATG -3'
IL-13:	forward:	5'- TGAGCAACATCACACAAGACC -3'
	reverse:	5'- GGCCTTGCGGTTACAGAGG -3'
IFNg:	forward:	5'- GGCAAAAGGATGGTGACATGAA -3'
	reverse:	5'- TTTTCGCCTTGCTGTTGCTG -3'
IL-22:	forward:	5'- TGCGATCTCTGATGGCTGTC -3'
	reverse:	5'- CCTTAGCACTGACTCCTCGG -3'
TNFa:	forward:	5'- GTCCCCAAAGGGATGAGAAGT -3'
	reverse:	5'- TTTGCTACGACGTGGGCTAC -3'
CHGA:	forward:	5'- TGGTGTCGCAGGATAGAGA -3'
	reverse:	5'- CCAAGGTGATGAAGTGCGT -3'
DCLK1:	forward:	5'- TCCAGATTCCTGCCACAATAA -3'
	reverse:	5'- GCAGTCGACCTCTCTATACATTC -3'
DEFA5:	forward:	5'- GCTGCTCCTCAGTATTAGTCTCT -3'
	reverse:	5'- CTCCTGCTCAACAATTCTCCA -3'
LGR5:	forward:	5'- GTCAAAGCATTTCCAGCAAGA -3'
	reverse:	5'- CTCCAACCTCAGCGTCTTC -3'
MUC2:	forward:	5'- TCAAAGTGCTCTCCAAACTCTC -3'
	reverse:	5'- CCTCTCAGAATTCCACACTCTT -3'
VIL1:	forward:	5'- GATAGCCAGGACTACATAGCAG -3'
	reverse:	5'- CTCTCTCAACATCACCACTCC -3'

Amplification was measured in real-time with StepOne Software v2.3. In brief, samples were incubated for 10 minutes at 95°C, followed by 40 cycles of heating to 95°C for 15 seconds and cooling to 60°C for 1 minute. To monitor specificity of the run, the melt curves were determined by keeping the samples at 95°C for 15 seconds, cooling to 60°C for 1 min and then increasing the temperature every 15 seconds by 0.3°C up to 95°C. MRNA expression relative to the house keeping genes (RPLP0, BACT) was calculated using the formula $x = 2^{-\Delta ct}$.

Intestinal epithelial cell isolation for flow cytometry:

The small intestine of WT and HPGDS$^{-/-}$ mice was isolated, fat and Peyer's patches removed and sliced lengthwise. Feces were washed out with cold PBS/ 3% FBS. To drain off the mucus, the tissue was incubated for 15 minutes at 37°C shaking (60 rounds per minute (rpm)) in DTT wash solution (HBSS (w/o Ca/Mg, Gibco), 5% FBS, 10 mM HEPES, 5 mM DTT) and subsequent vortexing for 10 seconds. EDTA wash solution (HBSS (w/o Ca/Mg), 5% FBS, 10 mM HEPES, 5 mM EDTA) was added for 15 minutes at 37°C, shaking (60 rpm) followed by vortexing. The supernatant containing the murine IECs was

transferred to a fresh tube and the EDTA washing step was repeated to isolate more cells, which were pooled. IECs were counted using a Neubauer counting chamber and $1x10^6$ cells were stained for flow cytometry.

After blocking of free binding sites with 2.5% TruStain in flow buffer (DPBS, 3% FBS) for 10 minutes at RT, cells were stained with the following antibodies in flow buffer for 30 minutes at 4°C in the dark:

Ep-CAM	Alexa 647	1:100	Biolegend
CD24	PE	1:100	Biolegend
Siglec F	BV421	1:100	Biolegend
c-kit (CD117)	PerCP	1:100	Biolegend

Subsequently, cells were stained with a viability dye diluted in DPBS for 15 minutes at RT in the dark:

Zombie Aqua fixable viability dye	Zombie Aqua	1:1000	Biolegend

After washing with flow buffer once, cells were fixed with 0.5% PFA for 10 minutes at 4°C in the dark and washed with flow buffer again. To prevent cell clumps, cells were pipetted through a 70 µM cell strainer and immediately surface marker expression was measured with a flow cytometer (BD LSRII Diva) and analyzed using FlowJo v10.7.0. Figure 2 describes the gating strategy applied.

After doublet exclusion, all living cells were gated for Ep-CAM positive intestinal epithelial cells. The tuft cell population can be distinguished by their expression of SiglecF and because they highly express CD24.

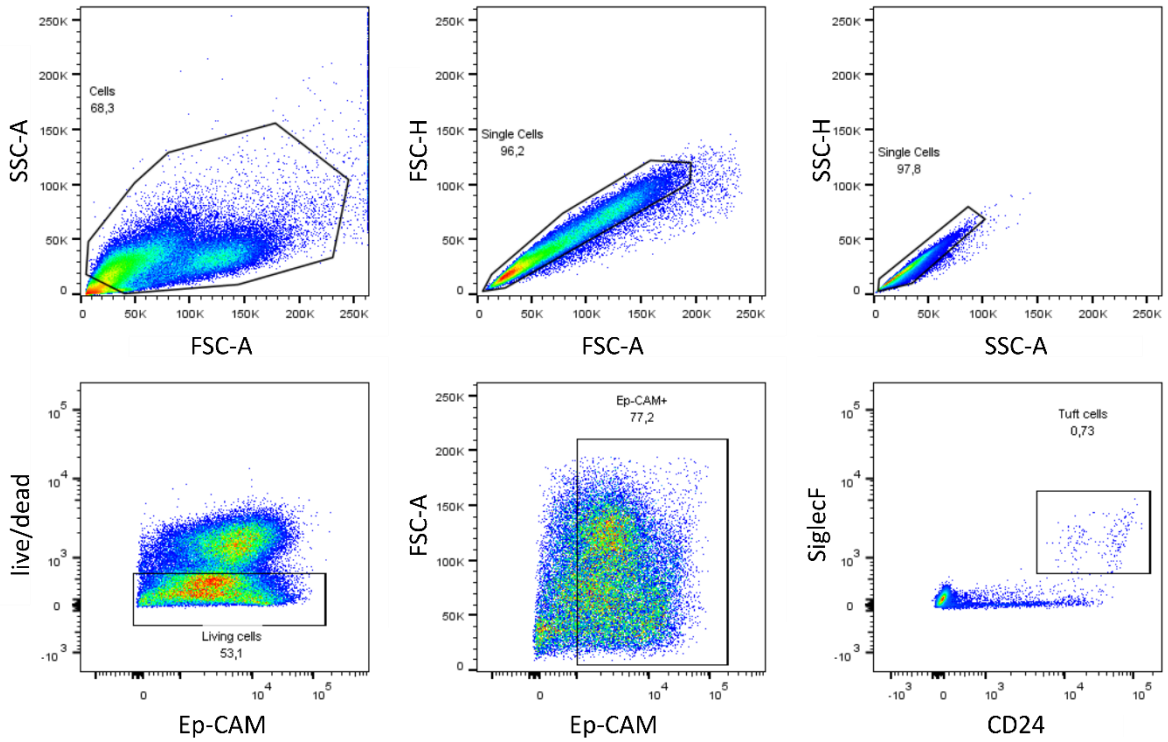

Figure 2: Gating strategy for tuft cells out of intestinal epithelial cells (IECs)
Comparing forward and side scatter area with height allowed distinguishing single cells from doublets. Afterwards only living cells were gated for Ep-CAM positive IECs. Tuft cells highly express the surface marker CD24 and are positive for SiglecF.

Intestinal crypt isolation and stimulation for lipidomics

The small intestines of WT and HPGDS$^{-/-}$ mice were isolated, cut open and flushed with cold PBS. The villi were scraped off using a microscopy slide and washed again three times with cold PBS. Afterwards, tissue was incubated in crypt isolation buffer for 30 minutes at 4°C shaking gently. The supernatant was removed and the tissue shaken in PBS to release remaining villi. Fresh PBS was added, intestine was shaken at medium speed and the supernatant containing the crypts was transferred to a fresh tube through a FBS-coated 70 µM cell strainer. This step was repeated once again. Subsequently, cells were centrifuged, resuspended in organoid medium supplemented with 10 µM Y-27632 and plated in 2% matrigel coated 48-well plates. After incubation overnight, cells were stimulated with HBSS (w/ $Ca2^{+}$/$Mg2^{+}$, Sigma) only (mock treatment) or 4 µg/mL PA, 2 µg/mL LFn-Fla in HBSS for 30 minutes at 37°C. Supernatants were isolated and frozen immediately in liquid nitrogen, stored at -80°C until eicosanoid release was analyzed by our collaborator Karsten Gronert at the University of California, Berkeley.

Data evaluation and statistics

Data was analyzed using GraphPad Prism v8. Statistical significance was determined using One-way ANOVA, Tukey Post-hoc test.

Figures and illustrations

Figure 1 was created with Biorender.com.

All other illustrations were created with Inkscape.

Results:

NAIP/NLRC4 inflammasome activation in human intestinal organoids

Inflammasome activation was predominantly investigated in primary human immune cells. However, also other cell types do express the required components although the exact role in different tissues is still far from being understood. The fact that patients with NLRC4 hypermutations show gastro-intestinal symptoms, similar to what is described after IEC-specific inflammasome activation in mice, suggests a role of the NAIP/NLRC4 inflammasome in the intestine of humans as well. As human gastro-intestinal organoids are much more physiologically relevant than immortalized cell lines, this would be a great model to examine the molecular functions of NAIP/NLRC4 activation. However, for unknown reasons, human intestinal stem cells cultivated as spheroids do not react to FlaTox. If this is a result of a lack of growth or differentiation factors, still needs to be determined. Therefore, we tried various approaches to induce inflammasome expression by using different cultivation media and -systems.

To have an internal control of expression levels and activity of the specific inflammasome activators, we first analyzed mRNA expression of the inflammasome- related genes Caspase 1 (CASP1), Gasdermin D (GSDMD), NAIP and NLRC4 in primary human monocytes.

Priming of monocytes with LPS or Pam3CSK4 induces mRNA expression of Caspase 1 but results in reduced levels of the other inflammasome- related mRNAs of GSDMD, NAIP and NLRC4 (Figure 3A). Nevertheless, inflammasome activation and therefore pyroptosis of LPS-primed monocytes with the different inflammasome activators is still possible as indicated in Figure 3B. While cells react very quickly within the first 2 hours to LFn-PrgJ and LFn-Needle, the effect of LFn-Fla is only visible after several hours.

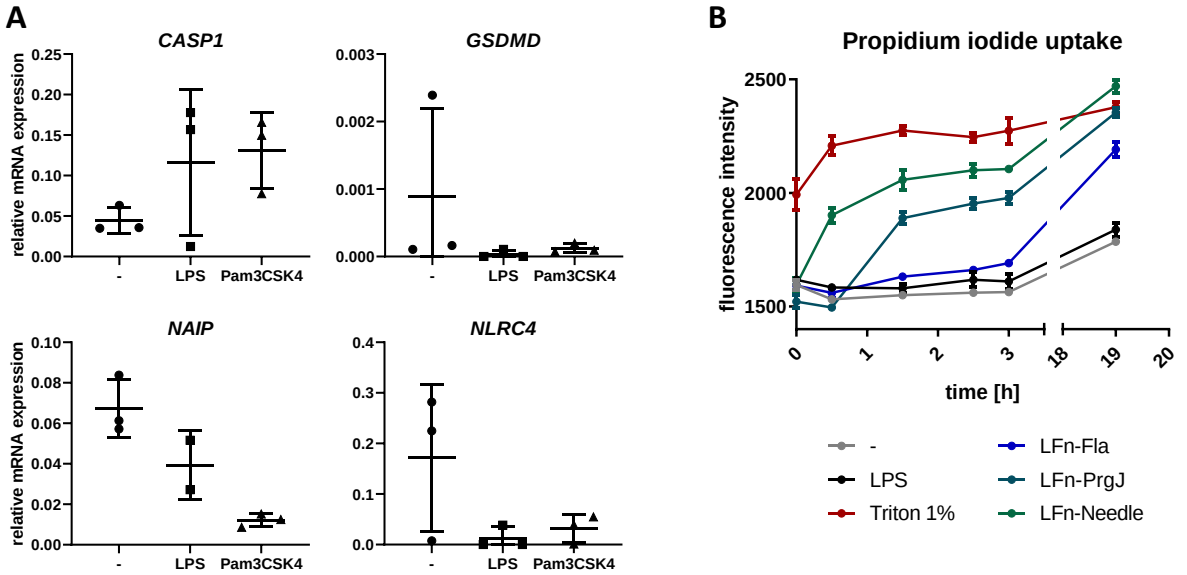

Figure 3: Inflammasome expression and activation of primary human monocytes
Primary human monocytes were isolated from blood of one donation. After resting for 1 day, cells were treated in triplicates with LPS or Pam3CSK4 for 4 hours (A). LPS-pretreated cells were stimulated with the inflammasome activators PA-LFn-Fla, PA-LFn-PrgJ and PA-LFn-Needle. Propidium iodide uptake by dead cells was measured at indicated time points (B). Data is represented as single values including mean value + SD.

As already mentioned before, murine IECs nicely react to specific inflammasome activators, but human intestinal epithelial cells cultivated in spheroid culture fail to react so far. To test whether human cells need special growth and differentiation factors to induce inflammasome components, we stimulated organoids in spheroid culture with differentiation medium for 5 days. Figure 4A shows that expression of Caspase 1 and NAIP is increased whereas NLRC4 is barely detectable especially in comparison to PBMCs. Additionally we wanted to investigate the influence of various pro-inflammatory cytokines on differentiated organoids. A mix of TNF-α and IL-22 as well as IFNλ for 6 hours are sufficient to significantly increase levels of Caspase 1. GSDMD and NAIP expression do not change. However, NLRC4 is still not induced.

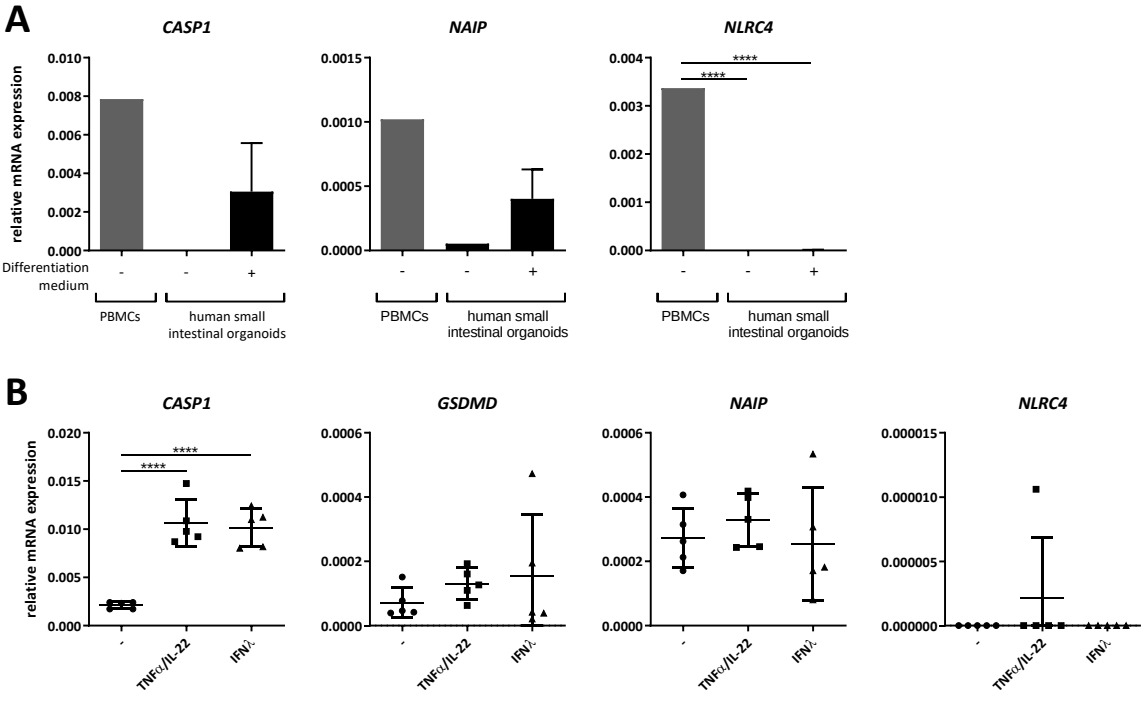

Figure 4: Inflammasome expression of differentially treated human intestinal stem cells in spheroid culture
*Human small intestinal organoids are cultured in matrigel domes with normal organoid medium or special differentiation medium for 5 days. Inflammasome component expression in comparison to human PBMCs from 1 donor was measured via RT-qPCR (A). Organoids treated with differentiation medium for 5 days were stimulated with different cytokine mixes for 6 hours. Expression of mRNA was analyzed (B). Data is represented as mean + SD of 5 independent experiments. Statistical significance was determined using Ordinary one-way ANOVA, Tukey-post hoc test (****=p<0.0001)*

These results made us wonder if human cells are still too stem cell like and if a different cultivation method will induce differentiation and expression of all inflammasome components. Several publications were suggesting an air-liquid interface cultivation system to induce cell differentiation of murine intestinal and colonic stem cells. To check if human intestinal stem cells differentiate as well, we compared cell growth and inflammasome component expression of cells cultivated in transparent and translucent transwells and stimulated with normal organoid medium or special differentiation medium. The cells form a confluent monolayer after approximately 8-10 days (Figure 5). Stimulation with differentiation medium for the last 5-7 days results in a different phenotype as observed by light microscopy. The cell layer is not flat anymore but seems to extend vertically. Additionally there is fluid observable on top of the cells.

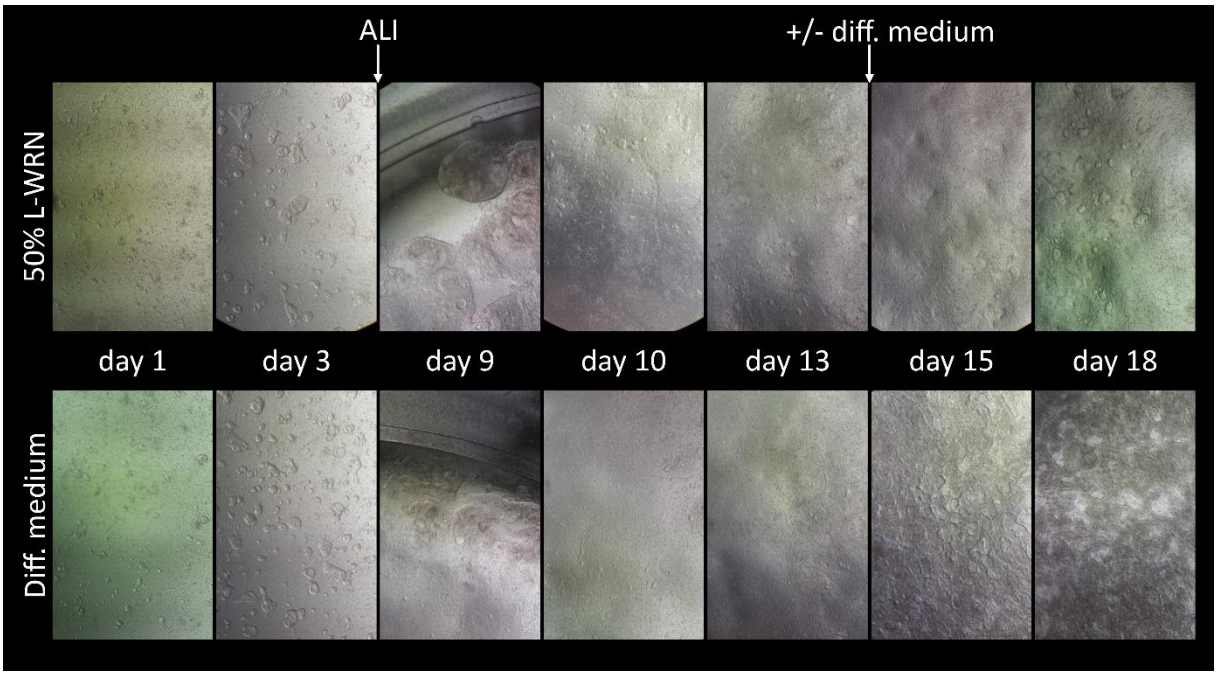

Figure 5: Microscope pictures of human intestinal stem cells grown in monolayers on transparent transwells
Cells were seeded on transwells and cultivated for 7 days with medium on both sides. Afterwards, air-liquid-interface was created by removing medium in the transwell for further 7 days. On day 14, half of the cells were cultivated in differentiation medium. Pictures were taken through the oculars of a common light microscope with a magnification factor of 10x.

To check whether these optical changes also imply an effect on mRNA level, we next investigated expression of inflammasome-related genes upon cultivation in differentiation medium. Caspase 1 and NAIP are increased, but there is no change in GSDMD expression and NLRC4 is again barely detectable. The mRNA yield of cells in translucent transwells was very low (data not shown). Very interestingly, human IECs in contrast to murine cells seem to grow much better on transparent transwells with a lower pore density than translucent ones (data not shown).

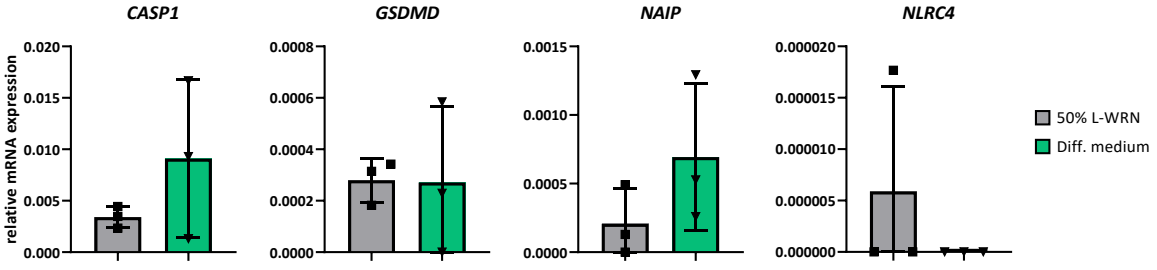

Figure 6: Expression of inflammasome- related genes in human intestinal stem cells grown in monolayers
Cells were seeded on transwells and cultivated for 7 days with medium on both sides. Afterwards, air-liquid-interface was created by removing medium in the transwell for further 7 days. On day 14 or 16, half of the cells were cultivated in differentiation medium. Cells were harvested in TRIzol after 21 days of cultivation and analyzed for mRNA expression of inflammasome-related genes via RT-qPCR. Data shows mean + SD of 3 individual experiments.

Although mRNA of NLRC4 is not detectable, NAIP would be able to detect inflammasome activators. However, inflammasome activation/ pyroptosis determined by propidium iodide staining was not observed in response to PA-LFn-Fla/-Needle (data not shown).

Taken together, these experiments show the limitations of using human intestinal stem cells in spheroid culture as a model system presumably because the cultivation conditions are just too different from the *in vivo* situation. Further improvement measures and refinements need to be achieved to be able to analyze the molecular mechanism of inflammasome activation in human intestinal organoids.

NAIP/NLRC4 activation in murine tuft cells

In mice, it is known that inflammasome activation in IECs is crucial for the restriction of specific bacteria. However, the intestinal epithelium is composed of functionally very different cell types such as paneth cells, goblet cells, enteroendocrine cells or tuft cells. Up to date, the latter ones are mainly known for their function in parasite infections, but they also express inflammasome components. However, it is not known what inflammasome activation in these cells would trigger. Therefore, our main aim was to analyze the effects of inflammasome activation and the subsequent secretion of lipid mediators, in particular PGD2, of tuft cells using three different mouse models.

The fact that regions with the highest prevalence of worm infections also show the highest death rates due to gastroenteritits, hints at a connection of inflammasome activation in an established parasite infection. Recently it was shown that succinate treatment induces tuft cell hyperplasia comparable to parasites. In our “co-infection” model, we wanted to test the influence of succinate pre- treatment on a bacterial Salmonella infection.

We observed more colony forming units (CFU) in the cecum and the mesentheric lymph nodes of NLRC4$^{-/-}$ mice, whereas edema formation and damage of epithelium is marginally higher in wild type mice (Figure 7), similar to what was observed before [30]. Succinate treatment does not change the amount of CFUs in the ileum or the cecum. Very interestingly, succinate pretreatment results in slightly increased translocation to the mesenteric lymph nodes both in WT and in NLRC4$^{-/-}$ mice.

In summary, we did not observe a change in the effect of the NLRC4 inflammasome on bacterial restriction upon expansion of tuft cells.

To investigate inflammasome activation in tuft cells only, we used another mouse line on a NLRC4 deficient background that allows induction of NLRC4 via Cre recombinase only in Pou2f3 positive cells. As it is known that tuft cells on one hand act on ILC2s and on the other hand secrete PGD2 that might act on ILC3s, we injected mice with low dose PA-LFn-Fla for 6 hours, extracted whole tissue mRNA from the intestine and analyzed it for typical ILC2 or ILC3 cytokines and different IEC transcription factors.

The levels of the ILC2- related cytokines IL-4, IL-5 and IL-13 do not change upon inflammasome activation, but expression of ILC3- related IL-22 and IFNγ slightly decreases. Very interestingly, Chga, a main transcription marker for enteroendocrine cells is significantly lower in mice where the inflammasome was induced. Although there is a tendency of less tuft and stem cells as well, there are no significant differences.

In histology we could not observe physiological changes such as varying numbers of tuft cells, whether mice did express the Nlrc4 inflammasome or not.

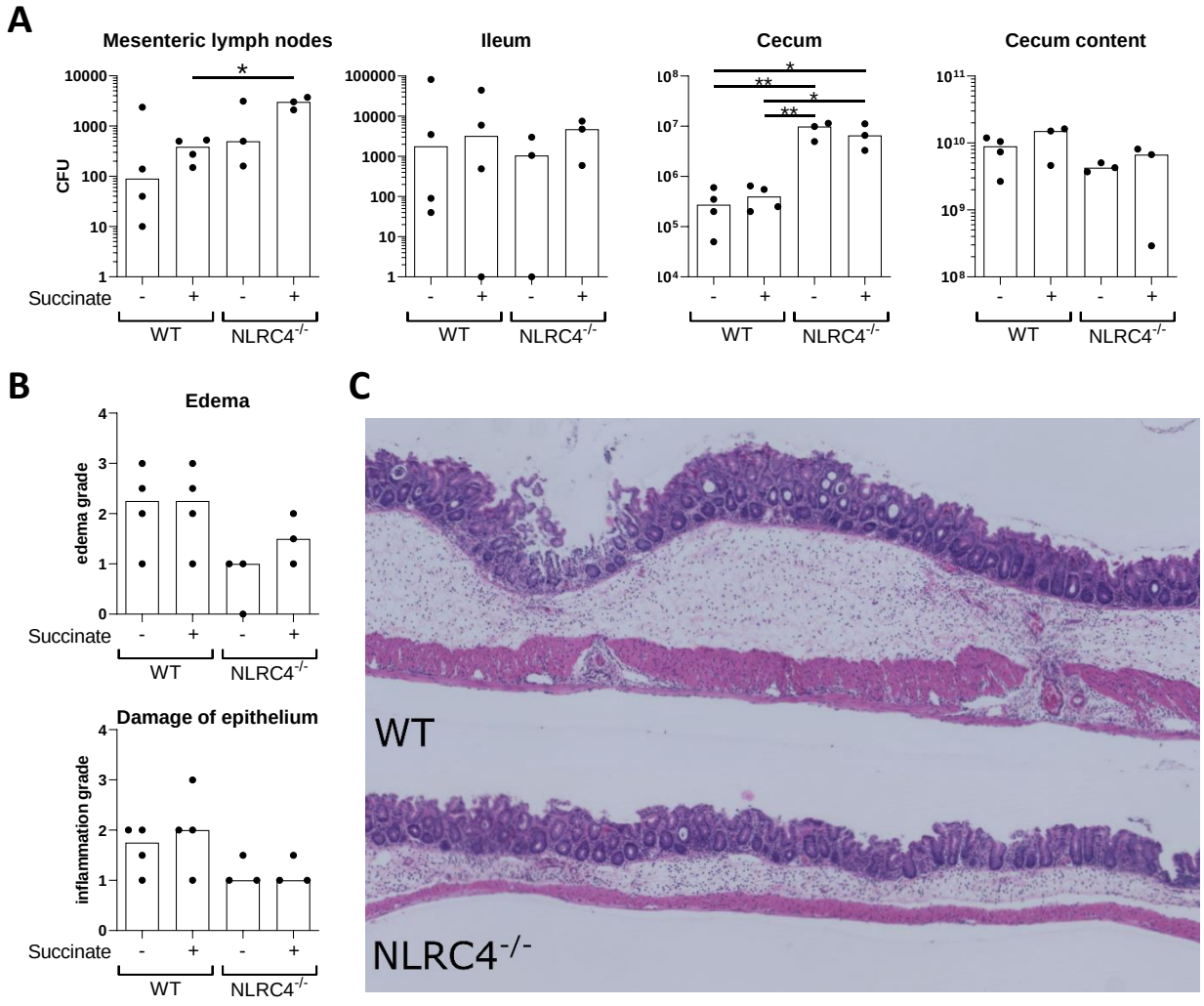

Figure 7: CFU count and histograding of succinate-pretreated, Salmonella-infected WT and NLRC4$^{-/-}$ mice
WT and NLRC4-/- mice were administered succinate in their drinking water, 7 days prior to Salmonella infection. One day before infection mice were orally gavaged Streptomycin to create a niche for the bacteria. 18 hours after oral gavage of 1x10^7 bacteria per mouse, colony forming units (CFUs) in different parts of the gastro-intestinal tract (A) and edema formation and damage of epithelium (B,C) were analyzed. Data represents median of 3-4 mice per group (A-C). H&E staining shows the cecum of non- succinate- pretreated WT and NLRC4$^{-/-}$ mice.

As mentioned before, one mediator secreted by tuft cells is PGD2. The synthesis of this lipid mediator requires the converting enzyme HPGDS. We obtained Hpgds$^{tm1a(KOMP)Wtsi}$ mice, which contain a gene cassette flanked by flippase recognition target (*FRT*) sites that blocks *Hpgds* transcription making this mouse HPGDS- deficient. Upon later cross to a flippase expressing mouse line, this line can be transformed into a conditional mouse strain.

We first wanted to check with flow cytometry if tuft cell numbers are still similar or if absence of HPGDS results in reduced amounts of this particular cell type. As visible in Figure 9, the percentage of tuft cells within Ep-CAM$^+$ IECs stays similar, no matter if HPGDS is deleted or not.

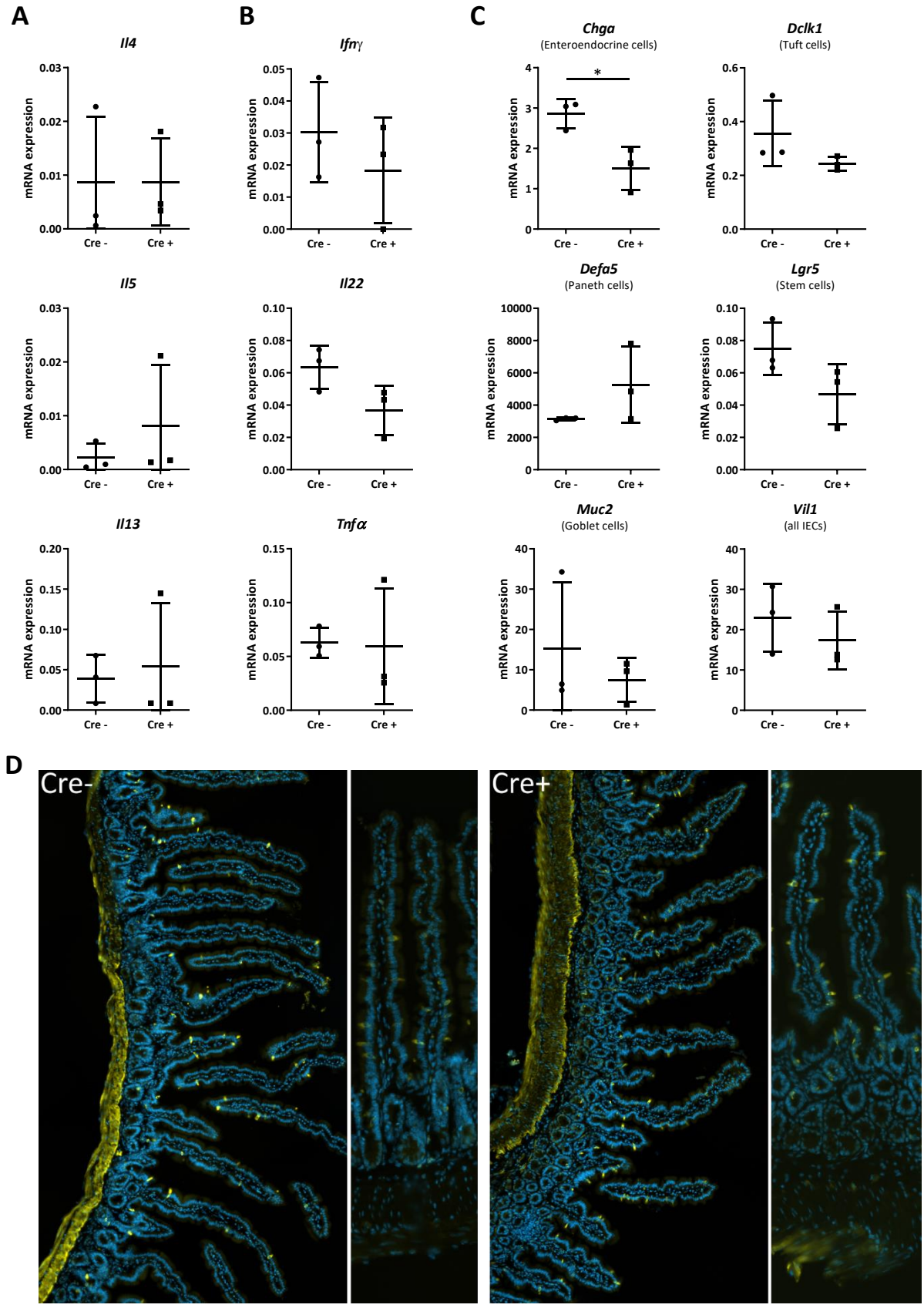

Figure 8: Inflammasome activation in tuft cells of iNLRC4Pou2f3Cre+ and Cre- mice
*Animals were administered succinate in their drinking water and Tamoxifen via oral gavage prior to intravenous injection of PA-LFn-Fla for 6 hours. Whole tissue mRNA was isolated from the small intestine and analyzed for the typical ILC2 (A) and ILC3 (B) cytokines and IEC-specific transcription factors (C). The small intestine was stained for DCLK1 (yellow, tuft cells) and DAPI (blue, cell nuclei) (D). Data represents mean + SD of three mice per group. Statistical significance was evaluated using unpaired t-test. *p<0.05*

One problem that should be taken into consideration is that knocking out only one enzyme of a complex pathway might result in a shift to other synthesis products. To test this, we isolated intestinal crypts of WT and HPGDS$^{-/-}$ mice, stimulated them with PA-LFn-Fla and analyzed supernatants for lipid mediators. In our monolayer experiment we could observe that HPGDS$^{-/-}$ cells are not able to secrete PGD2. Moreover, there was no aberrant expression of other eicosanoids observable (data not shown).

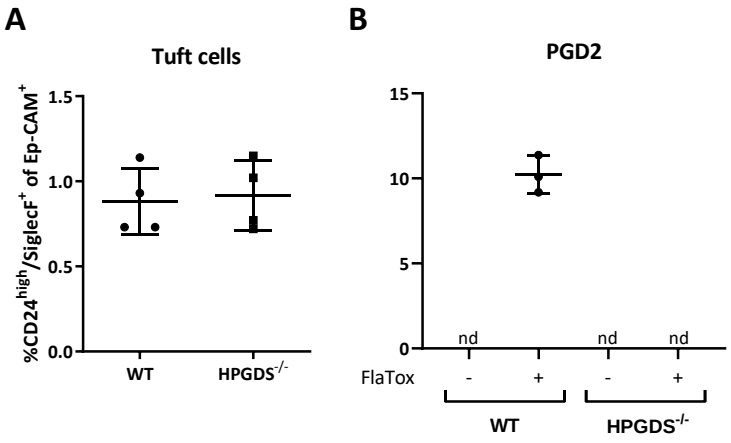

Figure 9: Tuft cell numbers and inflammasome activation in IECs of WT and HPGDS$^{-/-}$ mice
(A*) The percentage of tuft cells within all Ep-CAM^{+} IECs of WT and HPGDS$^{-/-}$ mice was determined using flow cytometry. (B) Crypts of the small intestines were isolated and seeded in matrigel- coated 48- well plates overnight. After stimulation with FlaTox for 30 minutes, supernatants were analyzed for PGD2 release via mass spectrometry.*

Discussion:

One of the main problems in immunological research is the lack of physiologically relevant models for humans due to different behavior of cells *in vitro* compared to *in vivo*. In the past few years, the cultivation of stem cells in spheroid culture became more prominent. Growing cells in 3D cell culture would allow a more physiological condition than normal 2D cell culture. However, using human organoids as a model system still needs to be improved. Our results show, that human intestinal organoids do not react to NAIP/NLRC4 inflammasome activators, while primary immune cells are able to recognize Flagellin, PrgJ and Needle. One possible explanation for these findings might be that human intestinal epithelial cells do not express all required inflammasome components. However, as most of the patients with NLRC4 hypermutations also show intestinal symptoms, this is less likely. Presumably, human cells still miss other factors, which are available *in vivo*. A first step into the right direction might be the usage of special differentiation medium as well as culture of cells in an air-liquid- interphase system. Cells behave different in an optical way but also on mRNA basis. Of course, other evaluation such as analysis of specific transcription factors via qPCR and immunohistochemistry would be required. One major limitation in our experiments is the fact that the different culture media we used for our experiments are optimized for murine organoids. Possibly human cells would behave differently when cultured in media with special human growth factors. Another problem of using human intestinal organoids is the fact that, to our knowledge, there is only one line with a presumably very high passage available so far. After some time in culture, cells change behavior. Therefore, it would be interesting to check inflammasome expression either in earlier passages of this line or preferably in primary human IECs.

In our *in vivo* experiments we wanted to examine the role of inflammasome activation and eicosanoid release of intestinal tuft cells. It is known that tuft cells are important for the activation of ILC2s in presence of parasites. However, their possible function in bacterial or simultaneous co-infections is not investigated yet.

Regions with higher prevalence of worm infections also show a higher death rate due to gastroenteritis [45, 46]. Therefore, we wanted to mimic a co-infection, by administering mice with succinate water for several days and then infecting them with *Salmonella*. As published before by Rauch et al., CFU counts are significantly higher in the cecum of NLRC4$^{-/-}$ animals [30]. We observed that the succinate pre-treatment did not change the number of CFUs in the small intestine and the cecum. Very interestingly, we could see a slightly higher translocation of bacteria to the mesenteric lymph nodes, when tuft cell numbers are increased. As the apical microvilli reach through the mucus into the lumen of the intestine, tuft cells could act as a port of entry for bacteria thereby facilitating tissue invasion. This is also in line with the findings of Lee et al. They infected mice with a murine norovirus, one of the main causes of gastroenteritis worldwide. Very interestingly, absence of tuft cells resulted in resistance whereas under type 2 conditions, virus titers were increased in the presence of intestinal and colonic tuft cells expressing the norovirus receptor CD300LF [50]. These results in addition to lower medical standards in developing countries would explain the high mortality rates due to gastroenteritis with an existing parasite infection. However, it still needs to be confirmed, if there is a higher number of bacteria in tuft cells compared to other IECs and if the bacteria use tuft cells because of their accessibility to invade the tissue. We could also see, that translocation to the lymph nodes is lower in mice expressing inflammasome, therefore activation by bacteria is possible. If activation in tuft cells only would be sufficient to restrict translocation to the lymph nodes still needs to be determined by

repeating this experiment in iNLRC4Pou2f3Cre mice. Moreover, other infection models would be useful, as Salmonella preferably colonize the cecum, but tuft cells are more important in the small intestine.

In our second *in vivo* experiment, we wanted to analyze the effect of inflammasome activation in tuft cells and the subsequent release of eicosanoids on mRNA expression in the surrounding small intestinal tissue. iNLRC4Pou2f3Cre mice were injected intravenously with FlaTox and whole tissue was analyzed for typical ILC2 and ILC3 cytokines and IEC transcription factors. The typical ILC2 cytokines did not change, but we saw slightly lower levels of IL-22 mRNA in mice expressing inflammasome in tuft cells only suggesting an effect on ILC3s. However, whole tissue RNA is difficult to analyze and can vary highly. Therefore, it would be interesting to check cytokines on protein level as well. One other factor that should be addressed in future experiments is the treatment time. Here, we used 6 hours of inflammasome activation whereas longer treatment and/ or repeated injections might allow tissue remodeling. Moreover, in this experiment we only analyzed cytokines in the small intestine but not in distal regions of the gastro-intestinal tract. Tuft cell activation and prostaglandin secretion might for example have a downstream effect on cells in the colon. Additionally, other infection models for example with *Listeria* and *Citrobacter* would be useful, as they infect the intestine as well. The restriction of both bacteria involves recognition by the NAIP/ NLRC4 inflammasome [51, 52]. Moreover, IL-22 is an important factor for the restriction of bacteria in both infection models [53, 54]. Another limitation in this particular experiment setting was the oral tamoxifen gavage. Usually, tamoxifen containing chow is considered as equally effective and reliable but includes less stress for the animals than for example intraperitoneal injection [55]. To address this factor, a comparison of tamoxifen chow and oral gavage on NLRC4-GFP expression in tuft cells would be required.

Taken together, inflammasome activation in tuft cells and subsequent release or secretion of eicosanoids might affect ILCs. There are several possibilities to investigate this *in vitro* such as sorting primary ILCs or use an ILC3 cell line as published by Allan et al. [56]. As there is only one RNAseq dataset of the small intestinal epithelium including ILCs available, these experiments would provide insights into CRTH2 expression of ILCs as well [57]. Additionally, it would be interesting to check if there are differences in eicosanoid release by comparing iNLRC4 VilCre with Pou2f3Cre mice, which were treated with succinate, tamoxifen and FlaTox.

The particular effect of PGD2 can also be analyzed using $HPGDS^{-/-}$ mice. We could already prove that there are similar tuft cell numbers in wild type and HPGDS- deficient mice and that there is no aberrant shift in eicosanoid secretion. Additionally, it needs to be confirmed that there are no intestinal abnormalities or phenotypical differences.

Taken together, our results hint at the possibility of inflammasome activation in tuft cells influencing the immune response in bacterial infections. Using the three described mouse models will give more insight into the particular effects of tuft cells on ILCs.

References:

1. Janeway, C.A., Jr., *Approaching the asymptote? Evolution and revolution in immunology.* Cold Spring Harb Symp Quant Biol, 1989. **54 Pt 1**: p. 1-13.
2. Iwasaki, A. and R. Medzhitov, *Control of adaptive immunity by the innate immune system.* Nat Immunol, 2015. **16**(4): p. 343-53.
3. Hayward, J.A., et al., *Cytosolic Recognition of Microbes and Pathogens: Inflammasomes in Action.* Microbiol Mol Biol Rev, 2018. **82**(4).
4. Bruchard, M., et al., *The receptor NLRP3 is a transcriptional regulator of TH2 differentiation.* Nat Immunol, 2015. **16**(8): p. 859-70.
5. Harton, J.A. and J.P. Ting, *Class II transactivator: mastering the art of major histocompatibility complex expression.* Mol Cell Biol, 2000. **20**(17): p. 6185-94.
6. Meissner, T.B., et al., *NLR family member NLRC5 is a transcriptional regulator of MHC class I genes.* Proc Natl Acad Sci U S A, 2010. **107**(31): p. 13794-9.
7. Xue, Y., et al., *Emerging Activators and Regulators of Inflammasomes and Pyroptosis.* Trends Immunol, 2019. **40**(11): p. 1035-1052.
8. Broz, P., *Recognition of Intracellular Bacteria by Inflammasomes.* Microbiol Spectr, 2019. **7**(2).
9. Bauer, R. and I. Rauch, *The NAIP/NLRC4 inflammasome in infection and pathology.* Mol Aspects Med, 2020: p. 100863.
10. Romanish, M.T., et al., *A novel protein isoform of the multicopy human NAIP gene derives from intragenic Alu SINE promoters.* PLoS One, 2009. **4**(6): p. e5761.
11. Rauch, I., et al., *NAIP proteins are required for cytosolic detection of specific bacterial ligands in vivo.* J Exp Med, 2016. **213**(5): p. 657-65.
12. Zhao, Y., et al., *Genetic functions of the NAIP family of inflammasome receptors for bacterial ligands in mice.* J Exp Med, 2016. **213**(5): p. 647-56.
13. Grandjean, T., et al., *The human NAIP-NLRC4-inflammasome senses the Pseudomonas aeruginosa T3SS inner-rod protein.* Int Immunol, 2017. **29**(8): p. 377-384.
14. Kortmann, J., S.W. Brubaker, and D.M. Monack, *Cutting Edge: Inflammasome Activation in Primary Human Macrophages Is Dependent on Flagellin.* J Immunol, 2015. **195**(3): p. 815-9.
15. Reyes Ruiz, V.M., et al., *Broad detection of bacterial type III secretion system and flagellin proteins by the human NAIP/NLRC4 inflammasome.* Proc Natl Acad Sci U S A, 2017. **114**(50): p. 13242-13247.
16. Kofoed, E.M. and R.E. Vance, *Innate immune recognition of bacterial ligands by NAIPs determines inflammasome specificity.* Nature, 2011. **477**(7366): p. 592-5.
17. Tenthorey, J.L., et al., *The structural basis of flagellin detection by NAIP5: A strategy to limit pathogen immune evasion.* Science, 2017. **358**(6365): p. 888-893.
18. Diebolder, C.A., et al., *Cryoelectron Tomography of the NAIP5/NLRC4 Inflammasome: Implications for NLR Activation.* Structure, 2015. **23**(12): p. 2349-2357.
19. Hu, Z., et al., *Structural and biochemical basis for induced self-propagation of NLRC4.* Science, 2015. **350**(6259): p. 399-404.
20. Zhang, L., et al., *Cryo-EM structure of the activated NAIP2-NLRC4 inflammasome reveals nucleated polymerization.* Science, 2015. **350**(6259): p. 404-9.
21. Broz, P., et al., *Differential requirement for Caspase-1 autoproteolysis in pathogen-induced cell death and cytokine processing.* Cell Host Microbe, 2010. **8**(6): p. 471-83.
22. von Moltke, J., et al., *Recognition of bacteria by inflammasomes.* Annu Rev Immunol, 2013. **31**: p. 73-106.
23. Mitchell, P.S., et al., *NAIP–NLRC4-deficient mice are susceptible to shigellosis.* bioRxiv, 2020: p. 2020.05.16.099929.
24. Semper, R.P., et al., *Helicobacter pylori Exploits the NLRC4 Inflammasome to Dampen Host Defenses.* J Immunol, 2019. **203**(8): p. 2183-2193.

25. von Moltke, J., et al., *Rapid induction of inflammatory lipid mediators by the inflammasome in vivo.* Nature, 2012. **490**(7418): p. 107-11.
26. Wu, C., et al., *Inflammasome Activation Triggers Blood Clotting and Host Death through Pyroptosis.* Immunity, 2019. **50**(6): p. 1401-1411 e4.
27. Duncan, J.A. and S.W. Canna, *The NLRC4 Inflammasome.* Immunol Rev, 2018. **281**(1): p. 115-123.
28. Allam, R., et al., *Epithelial NAIPs protect against colonic tumorigenesis.* J Exp Med, 2015. **212**(3): p. 369-83.
29. Hu, B., et al., *Inflammation-induced tumorigenesis in the colon is regulated by caspase-1 and NLRC4.* Proc Natl Acad Sci U S A, 2010. **107**(50): p. 21635-40.
30. Rauch, I., et al., *NAIP-NLRC4 Inflammasomes Coordinate Intestinal Epithelial Cell Expulsion with Eicosanoid and IL-18 Release via Activation of Caspase-1 and -8.* Immunity, 2017. **46**(4): p. 649-659.
31. Sellin, M.E., et al., *Epithelium-intrinsic NAIP/NLRC4 inflammasome drives infected enterocyte expulsion to restrict Salmonella replication in the intestinal mucosa.* Cell Host Microbe, 2014. **16**(2): p. 237-248.
32. Hausmann, A., et al., *Intestinal epithelial NAIP/NLRC4 restricts systemic dissemination of the adapted pathogen Salmonella Typhimurium due to site-specific bacterial PAMP expression.* Mucosal Immunol, 2020. **13**(3): p. 530-544.
33. Rittchen, S. and A. Heinemann, *Therapeutic Potential of Hematopoietic Prostaglandin D2 Synthase in Allergic Inflammation.* Cells, 2019. **8**(6).
34. Aglas, L., et al., *Context matters: TH2 polarization resulting from pollen composition and not from protein-intrinsic allergenicity.* J Allergy Clin Immunol, 2018. **142**(3): p. 984-987 e6.
35. McKenna, O.E., et al., *Multi-Approach Analysis for the Identification of Proteases within Birch Pollen.* Int J Mol Sci, 2017. **18**(7).
36. Gerbe, F., et al., *Intestinal epithelial tuft cells initiate type 2 mucosal immunity to helminth parasites.* Nature, 2016. **529**(7585): p. 226-30.
37. Howitt, M.R., et al., *Tuft cells, taste-chemosensory cells, orchestrate parasite type 2 immunity in the gut.* Science, 2016. **351**(6279): p. 1329-33.
38. von Moltke, J., et al., *Tuft-cell-derived IL-25 regulates an intestinal ILC2-epithelial response circuit.* Nature, 2016. **529**(7585): p. 221-5.
39. Ting, H.A. and J. von Moltke, *The Immune Function of Tuft Cells at Gut Mucosal Surfaces and Beyond.* J Immunol, 2019. **202**(5): p. 1321-1329.
40. Nadjsombati, M.S., et al., *Detection of Succinate by Intestinal Tuft Cells Triggers a Type 2 Innate Immune Circuit.* Immunity, 2018. **49**(1): p. 33-41 e7.
41. Singh, D., A. Ravi, and T. Southworth, *CRTH2 antagonists in asthma: current perspectives.* Clin Pharmacol, 2017. **9**: p. 165-173.
42. Xu, H., et al., *Transcriptional Atlas of Intestinal Immune Cells Reveals that Neuropeptide alpha-CGRP Modulates Group 2 Innate Lymphoid Cell Responses.* Immunity, 2019. **51**(4): p. 696-708 e9.
43. Klose, C.S.N. and D. Artis, *Innate lymphoid cells control signaling circuits to regulate tissue-specific immunity.* Cell Res, 2020. **30**(6): p. 475-491.
44. Vivier, E., et al., *Innate Lymphoid Cells: 10 Years On.* Cell, 2018. **174**(5): p. 1054-1066.
45. Collaborators, G.B.D.D.D., *Estimates of the global, regional, and national morbidity, mortality, and aetiologies of diarrhoea in 195 countries: a systematic analysis for the Global Burden of Disease Study 2016.* Lancet Infect Dis, 2018. **18**(11): p. 1211-1228.
46. Hotez, P.J., et al., *Hookworm: "the great infection of mankind".* PLoS Med, 2005. **2**(3): p. e67.
47. Miyoshi, H. and T.S. Stappenbeck, *In vitro expansion and genetic modification of gastrointestinal stem cells in spheroid culture.* Nat Protoc, 2013. **8**(12): p. 2471-82.
48. Wang, Y., et al., *Long-Term Culture Captures Injury-Repair Cycles of Colonic Stem Cells.* Cell, 2019. **179**(5): p. 1144-1159 e15.
49. Wilke, G., et al., *A Stem-Cell-Derived Platform Enables Complete Cryptosporidium Development In Vitro and Genetic Tractability.* Cell Host Microbe, 2019. **26**(1): p. 123-134 e8.

50. Lee, S., et al., *Norovirus Cell Tropism Is Determined by Combinatorial Action of a Viral Non-structural Protein and Host Cytokine.* Cell Host Microbe, 2017. **22**(4): p. 449-459 e4.
51. Nordlander, S., J. Pott, and K.J. Maloy, *NLRC4 expression in intestinal epithelial cells mediates protection against an enteric pathogen.* Mucosal Immunol, 2014. **7**(4): p. 775-85.
52. Warren, S.E., et al., *Multiple Nod-like receptors activate caspase 1 during Listeria monocytogenes infection.* J Immunol, 2008. **180**(11): p. 7558-64.
53. Dulson, S.J., et al., *STAT4 Directs a Protective Innate Lymphoid Cell Response to Gastrointestinal Infection.* J Immunol, 2019. **203**(9): p. 2472-2484.
54. Basu, R., et al., *Th22 cells are an important source of IL-22 for host protection against enteropathogenic bacteria.* Immunity, 2012. **37**(6): p. 1061-75.
55. Kiermayer, C., et al., *Optimization of spatiotemporal gene inactivation in mouse heart by oral application of tamoxifen citrate.* Genesis, 2007. **45**(1): p. 11-6.
56. Allan, D.S., et al., *An in vitro model of innate lymphoid cell function and differentiation.* Mucosal Immunol, 2015. **8**(2): p. 340-51.
57. Haber, A.L., et al., *A single-cell survey of the small intestinal epithelium.* Nature, 2017. **551**(7680): p. 333-339.