Tartrate resistant acid phosphatase: the entangled enzyme in viral toll-like receptor signaling

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Abstract

Acute exacerbations are impactful events characterized by a rapid decline in lung function and are mainly caused by viral infections, ultimately driving a poor outcome of COPD patients. Exacerbating patients have shown to react differently upon viral infections compared to healthy subjects, but the mechanism remains elusive. We previously showed tartrate resistant acid phosphatase (TRAP) to be implicated in toll-like receptor signaling which is responsible for functional viral clearance and pro-inflammatory balance. TRAP is an enzyme that is expressed in osteoblasts and alveolar macrophages. In bone, the role of TRAP is well defined but its function in the lung has yet to be determined. Therefore, we set out to establish the effect of this enzyme on anti-viral and pro-inflammatory cytokine excretion via endosomal viral toll-like receptors TLR7 and 8 by using genetic analysis and cytokine quantifying techniques. We found TLR7 signaling to be linked with ACP5 (TRAP encoding gene), and despite being species and cell-type specific, we discovered that TRAP can selectively stimulate both anti-viral and pro-inflammatory cytokine production in murine as well as human immune cells. Where and how TRAP interacts is still unknown, but it may be involved in TLR trafficking to the endosomal compartments, link with the endosomal receptor, or interact with downstream regulators in the cytoplasm. Overall, these findings clearly show a link between ACP5/TRAP and viral immunity. Therefore, determining the exact role of ACP5/TRAP in the lung could be crucial to increase viral clearance and decrease excessive inflammation in exacerbating patients, as there are still limited treatment options to prevent or treat these severe and pathologically complex episodes.

Introduction

Acute exacerbations in patients with chronic pulmonary diseases are impactful events characterized by acute deterioration of lung function and more symptoms including increased dyspnea, cough and sputum production[1,2]. These episodes remain hard to control with corticosteroids and greatly contribute to high mortality, morbidity and exorbitant healthcare costs for both COPD and asthma patients[3]. Viral infections like influenza virus and rhinoviruses are the major causes of exacerbations, and exacerbating patients react different to viral

infections than healthy individuals, including having excessive inflammation and worsening of the underlying disease[2,4,5]. Although the exact pathogenic mechanism is still unclear, this indicates defects in the host innate immune responses and implies tissue environment changes induced by the underlying disease which impairs the ability of the immune system to clear viral infections.

When clearing a viral infection, type I interferons play a crucial role by directly inhibiting viral replication in cells and indirectly by stimulating the adaptive immune response. Its anti-viral mechanisms include control of viral transcription, blocking translation, RNA cleavage and inducing apoptosis[3]. The inability to induce an interferon response after a viral infection can result in unconstrained viral replication and increased inflammation[4]. This could be proposed as an underlying defective mechanism for virus-induced exacerbations in patients suffering from chronic lung diseases. In fact, type I interferon deficiency has been found in stable COPD patients and asthmatic patients with rhinovirus-induced exacerbations[6,7]. Knowing this, we set out to elucidate the differences and similarities of anti-viral responses in murine and human immune cells of the lung by focusing on type I interferons in more detail. This to get a better insight in why exacerbating patients suffering from asthma or COPD react differently to viral pathogens compared to healthy individuals.

In the quest for elucidating changes in lung environment of chronic lung diseases that can influence inflammation and anti-viral responses, the enzyme tartrate-resistant acid phosphatase (TRAP, also mentioned by name of the encoding gene ACP5) emerges. TRAP is an endosomal metallo-enzyme that exist in two isoforms which facilitates hydrolysis of phosphate esters and generation of reactive oxygen species (ROS) in different cell types[8]. It is involved in numerous biological processes and found in myeloid cells of the bone and immune system[9]. TRAP is associated with osteoporosis, obesity and autoimmune disorders[10–12]. Observations in patients with deficient ACP5 protein activity, due to biallelic null mutations in the ACP5 gene, show skeletal dysplasia and features of immune dysfunction caused by upregulation of interferon-stimulated genes and elevated serum levels of type I interferons[10]. This suggests that ACP5 may have a suppressive effect on type I interferon production. Interestingly, alveolar macrophages, which play a crucial role in innate immune responses against viruses, show by far the highest ACP5 expression in both healthy controls and COPD patients compared to other immune cells (COPDcellatlas.com). In addition, when compared to controls, TRAP is more highly expressed in the lung tissue of COPD, asthma, and pulmonary fibrosis patients compared to controls[8]. According to these findings, TRAP may be implicated in antiviral immune responses, which could explain why patients with chronic lung disorders are unable to fight infections during an exacerbation. However, its exact role in the lungs has yet to be determined.

To get a better insight into the role of ACP5/TRAP in lung tissue, we previously performed RNA sequencing analysis on lung tissue of ACP5 knockout mice and wildtype mice. From this, toll-like receptor 7 (TLR7) was one of the genes most downregulated in lung tissue of knockout

mice compared to wildtype mice, whereas mRNA expression of the highly similar TLR8 remained unaltered[13]. Both TLR receptors can recognize phagocytosed single stranded RNA viruses (e.g. rhinovirus, influenza virus) and are intracellularly expressed in the endosome where activation will lead to pro-inflammatory and anti-viral responses including type I interferon production[14–16]. In addition, we discovered that influenza-infected mice exhibited higher levels of ACP5 and TLR7 in lung tissue than healthy controls, with their expression being substantially correlated[13]. TLR8 expression, on the other hand, was unaffected in these mice. Combined, these data clearly indicated a relationship between ACP5 and TLR7 that may be involved in the aberrant type I interferon anti-viral responses in patients with COPD or asthma.

The first aim of this project was therefore to determine pro-inflammatory and anti-viral protein secretion in monocytes and alveolar macrophages after TLR7 and TLR8 stimulation and asses if TRAP/ACP5 is involved in this. This is relevant because recent studies discovered that both human and murine TLR 7 and 8 receptors cross-regulate their signaling pathways [14,17–19]. These findings demonstrated that TLR7-dependent pro-inflammatory cytokine production could be inhibited by TLR8 activation. In contrast, TLR8-dependent type I interferon production was found to be inhibited by TLR7 activation. However, these studies also showed this was cell type and organism dependent. Since TLR7 and TLR8 cross regulation has not been studied extensively in alveolar macrophages yet, our second aim was to elucidate the effects of such cross-regulatory mechanisms on type I interferon excretion and find out what modulatory role TRAP/ACP5 has in this process.

In our studies we hypothesized that excessive ACP5 expression is involved in inhibition of TLR7 signaling, possibly modulated through cross-regulation with TLR8. This then causes lower anti-viral type I interferon production and higher pro-inflammatory cytokine production in chronic lung disease patients suffering from exacerbations. To answer this question, (genetic) interactions between ACP5 on TLR7/8 and corresponding downstream mechanisms were investigated by analyzing microarray data of ACP5-deficient patients and healthy subjects. Furthermore, we determined TLR7/8-stimulated pro-inflammatory and anti-viral cytokine levels using receptor-specific agonists with or without a TRAP inhibitor. Altogether, the intention of this project was to expand our knowledge about anti-viral responses in lung tissue and lung macrophages, as this is essential to create new therapeutic options that prevent or improve treatment of exacerbations in the future.

Material and methods

Cell culture

Different human and murine cell types were incubated at 37°C with 5% CO2. Murine alveolar-like macrophages (a kind gift from Dr G. Fejer, Plymouth Fejer et al.) were cultured in RPMI 1640 + Glutamax medium (21875034, Gibco, The Netherlands) supplemented with 10% FBS, GM-CSF (10 ug/ml, 215-GM, R&D systems, The Netherlands) and gentamycin (10 mg/ml; 15710, Gibco). RAW 264.7 macrophages (TIB-71, ATCC) were cultured in DMEM medium

(11960044, Gibco) supplemented with 10% FBS, 2mM L-glutamine, and Gentamycin (10 mg/ml). THP-1 monocytes (TIB-202, ATCC) were cultured in RPMI 1640 + Glutamax medium supplemented with 10% FBS. RAWblue-ISG cells (InvivoGen) were cultured in DMEM medium supplemented with 10% FBS, L-glutamine (2 mM), Normocin (50mg/ml, ant-nr-1, InvivoGen) and selection antibiotic Zeocin (100mg/ml, ant-zn-1, InvivoGen). Two different batches of MPI cells were used due to logistical reasons but cultured under identical circumstances.

Cells (200.000 cells/well) were treated with TLR7 agonist gardiquimod (GDQ), TLR7/8 agonist resiquimod (RSQ), TLR8 agonist TL8-506 (TL8), TLR8 antagonist CU-CPT9a (CUC) (all InvivoGen, tlrl-gdqs; tlrl-r848; tlrl-tl8506; inh-cc9a), lipopolysaccharide (LPS, L, 880 Merck Mellipore) and TRAP inhibitor AubipyOme (SML2210, Sigma). All TLR ligands were used in concentrations ranging from 10 ng/ml-2.5 ug/ml and 0.1 uM-10 uM for AubipyOme.

ELISA

Enzyme-linked immunosorbent assay (ELISA) was performed according to manufacturer protocol to quantify murine and human pro-inflammatory cytokine levels of TNF-α and IL-1β (DY410-05; DY210-05; DY401-05; DY201-05, R&D systems) on collected cell-free culture medium. Absorbance was measured with a Biotek Synergy H1 Microplate Reader at wavelength 450 nm and corrected for impurities measured at 570 nm. To compare cytokine levels from different experiments, we normalized cytokine production in medium to untreated control conditions and expressed this as a percentage of total excreted protein.

SEAP QUANTI-Blue assay

SEAP QUANTI-blue assay (rep-qbs, InvivoGen) was performed according to manufacturer protocol by measuring absorbance of secreted embryonic alkaline phosphatase (SEAP) by RAWblue cells. Cell culture supernatant was collected (t=2h, 4h, 24h) and incubated for 3.5 h at 37 °C after which optimal absorbance was measured at 620-655 nm using a Biotek Synergy H1 Microplate Reader Microplate reader. To compare cytokine levels from different experiments or new cell culture batch, we normalized cytokine production in medium to no-cell control conditions and expressed this as a percentage of total excreted protein.

Microarray

Previously collected microarray data by Briggs et al. which compared 3 healthy individuals with 3 SPENCD patients was analyzed with RStudio software version 2022.02.1 (RStudio: Integrated Development for R. RStudio, PBC, Boston, MA) to compare expression levels of TLR7, TLR8 and related downstream pathway regulators[10]. By pairing and adding up multiple probe intensities encoding for a single gene, cumulative gene expression was obtained as variable and used to display gene expression differences.

Software & Statistical Analysis

GraphPad Prism 9 Version 9.2.0 (GraphPad Prism Software Inc., La Jolla, CA, USA) was used for graphical presentation and means set as standard to display multiple measurements. Statistical analysis was not applicable due to the limited number of replicates per experiment.

Results

ACP5 deficiency associates with upregulation of TLR7 and downstream signaling regulators

To gain insight into the (genetic) interaction of ACP5 on TLR7/8 and corresponding downstream mechanisms, we first analyzed an existing microarray data set from Briggs et al. which compared whole blood samples from healthy control and ACP5-deficient patients[10]. Compared to healthy controls, we found that ACP5 deficient patients express more TLR7 but equal TLR8. On top of this, TLR7 and TLR8-related downstream pathway regulators in these patients showed altered expression including upregulation of NF-kB, IRF7 and IRF5 which are essential for type-1 interferon and pro-inflammatory cytokine production (Fig. 1). Gene expression of other TLR7 and TLR8 downstream pathway regulators is not graphically represented, but results can be found in the supplemental information (Sup. Fig. 1).

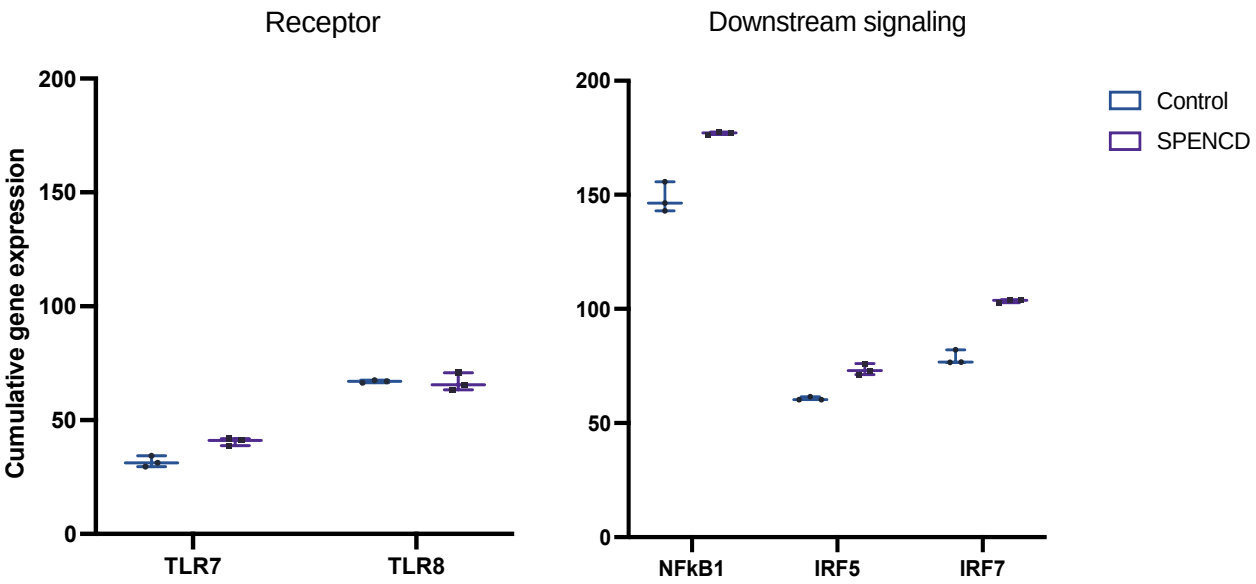

Figure 1: ***Influence of ACP5 on gene expression of TLR 7/8 and related downstream regulators in ACP5 deficient patients versus healthy individuals.*** *Cumulative gene expression of genes TLR7, TLR8, NFkB1, IRF5 and IRF7 of whole blood samples from healthy controls (n=3) and ACP5-deficient patients (SPENCD, n=3)).*

AubipyOme inhibits type I interferon production in murine macrophages

To assess the influence of TRAP on murine type I interferon expression, we first performed a QUANTI-blue assay on RAWblue cells in order to measure secreted embryonic alkaline

phosphatase (SEAP) production, which indirectly corresponds to type I interferon levels, after stimulation with gardiquimod, resiquimod and lipopolysacharide in combination with TRAP inhibitor AubipyOme (Fig. 2). Untreated, these cells show little sign of activation of type I interferon stimulation, suggesting basal levels of type I interferon to be low. However, after 24 h of incubation, all agonists showed higher type I interferon production compared to untreated control, which was inhibited by the highest concentration of inhibitor AubipyOme (10 uM). Treatment with AubipyOme-only (any concentration) did not result in stimulation of cytokine production and is therefore not graphically represented, but can be found in the supplementary information (Sup. Fig. 2).

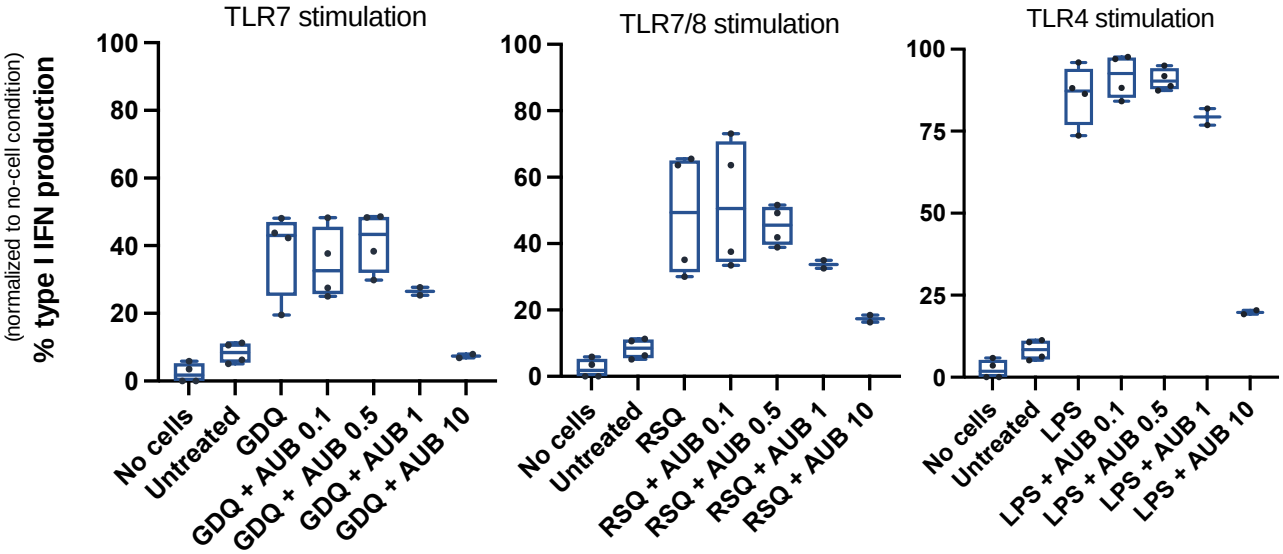

Figure 2: ***Type-1 interferon production in TLR stimulated RAWblue cells co-treated with TRAP inhibitor****. Percentage of type I interferon (IFN) production in medium (normalized to no-cell condition) by RAWblue cells after TLR7 stimulation with gardiquimod (GDQ; 1 ug/ml), TLR7/8 stimulation with resiquimod (RSQ; 2.5 ug/ml), TLR4 stimulation with lipopolysacharide (LPS; 10 ng/ml), and combinations of those with AubipyOme (AUB; 0.1-10 uM)(N=2).*

AubipyOme inhibits pro-inflammatory TLR signaling pathways in murine monocytes/macrophages

Because AubipyOme inhibited type I interferon production at a concentration starting from 10 uM and downstream regulators of TLR pathways are intertwined, we questioned whether AubipyOme affects production of other cytokines as well[15]. To investigate this, we tested if stimulation with TLR agonists and possible inhibition with AubipyOme resulted in changes in production of two pro-inflammatory cytokines IL-1β (NF-κB/inflammasome) and TNF-α (NF-κB)[20,21]. Treatment with AubipyOme-only (any concentration) did not result in stimulation of cytokine production and is therefore not graphically represented, but can be found in the supplementary information (Sup. Fig. 3).

With respect to inflammasome activation, we measured cytokine production of RAWblue cells after stimulation with gardiquimod, resiquimod, liposaccharide, in combination with TRAP

inhibitor AubipyOme (0.1-10 uM) (Fig. 3). Treatment with any of the agonists resulted in higher IL-1β production compared to untreated controls. In addition, both TLR7 stimulation with gardiquimod and TLR4 stimulation with lipopolysaccharide were inhibited by the highest concentration of inhibitor AubipyOme (10 uM), but TLR7/8 stimulation with resiquimod stimulation was not affected by AubipyOme treatment.

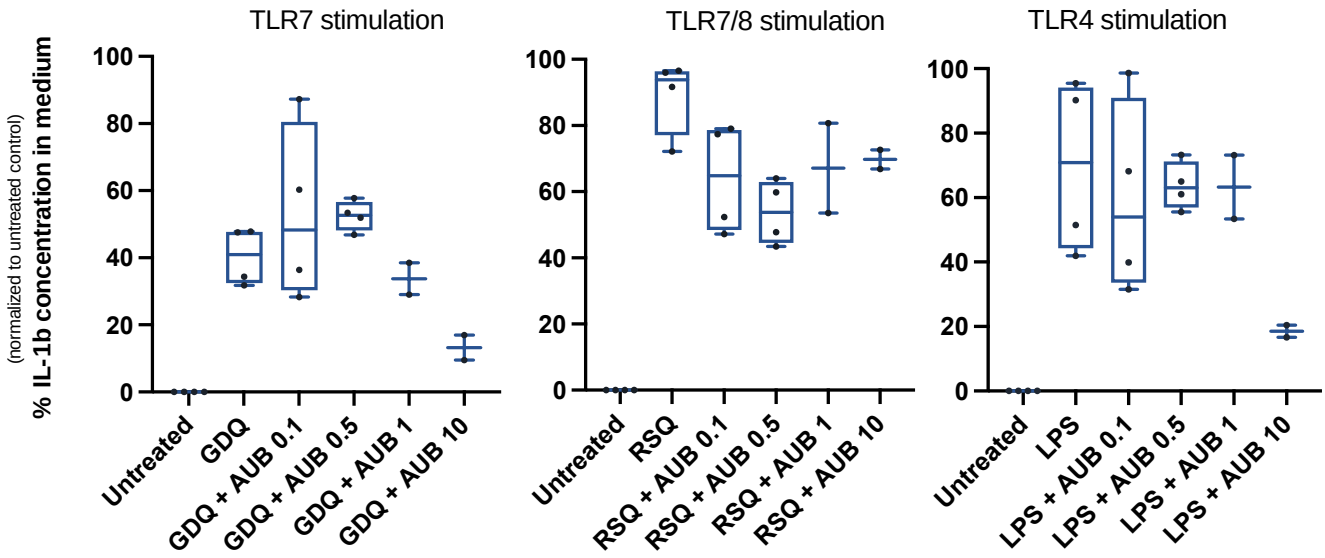

Figure 3: ***IL-1β production in TLR stimulated RAWblue cells co-treated with TRAP inhibitor.*** *Percentage of IL-1β production in medium (normalized to untreated control) by RAWblue cells after TLR7 stimulation with gardiquimod (GDQ; 1 ug/ml), TLR7/8 stimulation resiquimod (RSQ; 2.5 ug/ml), TLR4 stimulation lipopolysacharide (LPS; 10 ng/ml) and combinations with AubipyOme (AUB; 0.1-10 uM) (N=2).*

With respect to NF-κB activation, we also measured cytokine production in medium of RAWblue cells after stimulation with gardiquimod, resiquimod, lipopolysacharide, in combination with TRAP inhibitor AubipyOme (0.1-10 uM). All TLR agonists greatly induced TNF-α production compared to untreated controls, resulting in levels beyond detection range and therefore this experiment needs to be repeated in the future. However, we also investigated TNF-α production in normal RAW264.7 macrophages and MPI macrophages that were identically stimulated but treated with lower inhibitory concentrations (0.1 and 0.5 uM). In RAW264.7 macrophages, TLR7 stimulation with gardiquimod resulted in more TNF-α production compared to untreated cells, which was reduced one fourth by co-treatment with AubipyOme (Fig 4A). Similarly, in MPI macrophages TLR7 stimulation with gardiquimod resulted in more TNF-α production compared to untreated controls, but these cells were unresponsive to inhibition by AubipyOme. Combined stimulation of TLR7 and TLR8 with resiquimod, or TLR4 stimulation with lipopolysacharide did induce TNF-α production by MPI macrophages. However, only lipopolysaccharide-induced TNF-α production was inhibited by the highest concentration of AubipyOme (0.5 uM).

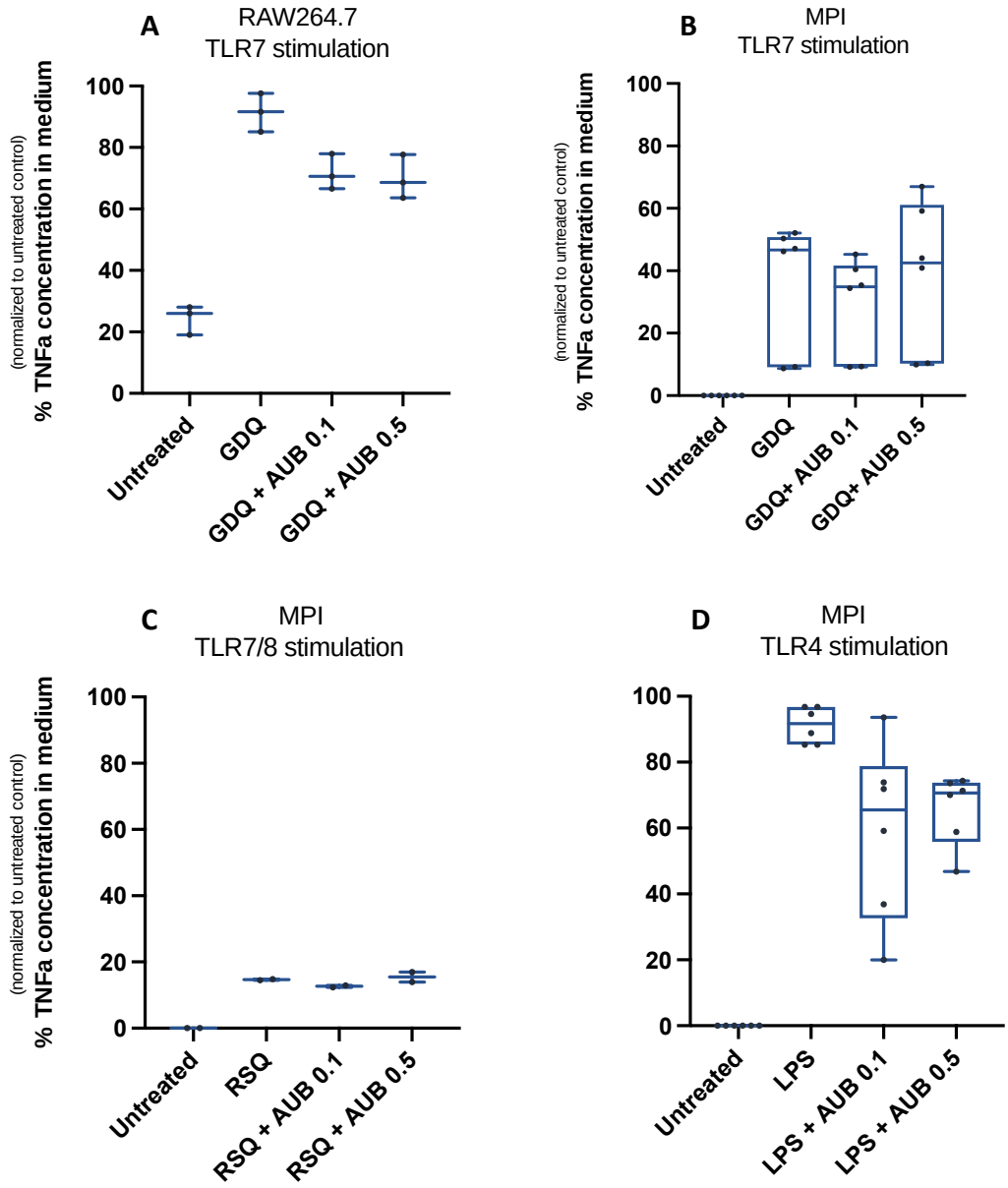

Figure 4: ***TNF-α production of TLR stimulated RAW264.7/MPI cells co-treated with TRAP inhibitor.*** ***A)*** *Percentage of TNF-α production in medium (normalized to untreated control) by RAW264.7 cells after stimulation with gardiquimod (GDQ; 1 ug/ml) and combinations with AubipyOme (AUB; 0.1 and 0.5 uM)(N=1)* ***B-D)*** *Percentage of TNF-α production in medium (normalized to untreated control) by MPI cells after stimulation with gardiquimod (GDQ; 1 ug/ml; n=2), resiquimod (RSQ; 2.5 ug/ml; n=1), lipopolysacharide (LPS (10 ng/ml; n=2), or combined with AubipyOme(AUB; 0.1 and 0.5 uM).*

AubipyOme inhibits pro-inflammatory TLR7/8 induced pro-inflammatory cytokine production in human monocytes

Because TLR4/7/8 downstream signaling is species and cell-type specific, we also wanted to explore the influence of TRAP on TNF-α and IL-1β production in the human monocytic cell line THP-1 after stimulation with gardiquimod, resiquimod, TL8-506, lipopolysacharide or combinations with AubipyOme (0.5 uM). Stimulating TLR7 with gardiquimod did not lead to

production of TNF-α in THP-1 cells, whereas TLR8 stimulation with TL8-506, TLR7/8 stimulation with resiquimod, and TLR4 stimulation with lipopolysacharide did result in TNF-α production compared to untreated controls (Fig. 5A). Only resiquimod co-treated with AubipyOme inhibited TNF-α production, whereas other stimulations were not affected. Compared to untreated controls, IL-1β production was induced by all stimulations except TLR7 with gardiquimod which showed no production (Fig. 5B). In addition, TLR7/8 stimulation with resiquimod resulted in higher cytokine levels than TLR8 stimulation only, despite absence of effect of TLR7 stimulation. Only IL-1β levels induced by resiquimod were inhibited by combinations with AubipyOme (0.5 uM).

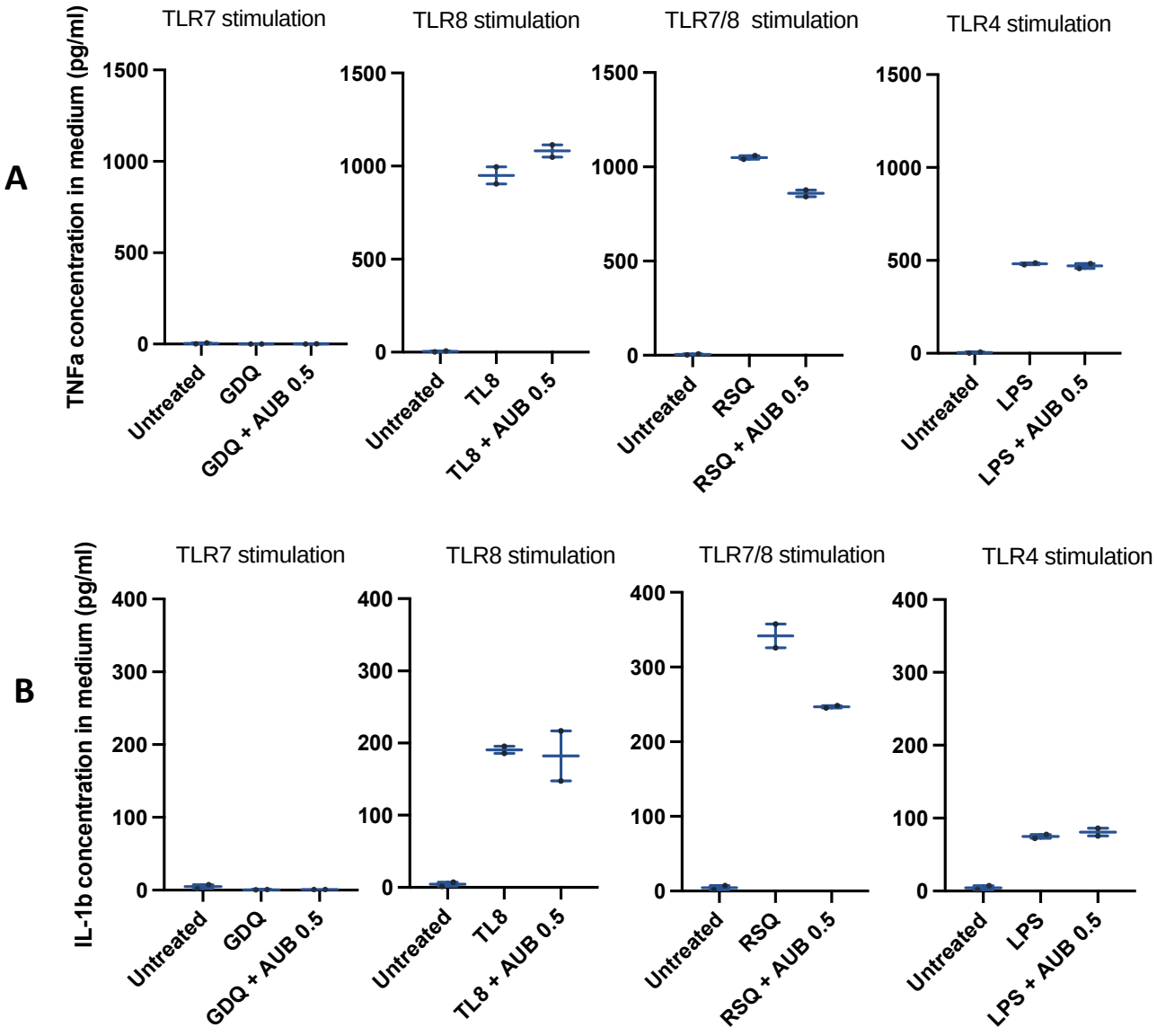

Figure 5: ***TNF-α and IL-1β production of TLR stimulated THP-1 cells co-treated with TRAP inhibitor.*** *Concentration of TNF-α (**A**) and IL-1β (**B**) production (pg/ml) in medium of THP-1 cells after TLR7 stimulation with gardiquimod (GDQ; 1 ug/ml), TLR8 stimulation with TL8-506 (TL8; 100ng/ml), TLR7/8 stimulation with resiquimod (RSQ; 2.5 ug/ml), TLR4 stimulation with lipopolysaccharide (LPS; 10 ng/ml) and combinations with AubipyOme (AUB; 0.5 uM) (N=1).*

Discussion

As the function of ACP5/TRAP in bone has been studied extensively, its role in lung tissue remains unclear and is to our best knowledge never studied before in relation to anti-viral and pro-inflammatory responses. Based on its high expression in alveolar macrophages (especially of chronic lung disease patients), excessive type I interferon excretion in ACP5 deficient patients, and involvement in TLR7 related genetic alterations, we set out to determine the influence of ACP5 on TLR7/8 and related downstream regulators. Next, we questioned what the effects were after TLR7 and TLR8 stimulation of monocytes and alveolar macrophages on pro-inflammatory and anti-viral protein secretion, and if TRAP inhibition could alter this. Finally, we wanted to elucidate whether TLR7/8 cross-regulate their signaling mechanisms for type I interferon excretion and if so, find out what modulatory role ACP5/TRAP has in this process. Although previous work on this subject was limited, we believed ACP5/TRAP to genetically repress or enzymatically inhibit TLR7 signaling, possibly regulated through cross-regulation with TLR8. We found genes related to TLR7 and its downstream signaling proteins to be upregulated in ACP5 deficient patients. In addition, we illustrated that TRAP may exert a stimulatory effect on type I interferon and pro-inflammatory cytokine production via TLR7 and TLR4 signaling. Signs of cross-regulation between TLR7 and 8 were observed, but like the stimulatory effect of TRAP, the exact mechanism remains inconclusive.

Previous unpublished work from us indicated that TLR7 was one of the top downregulated genes in ACP5 knockout mice compared to wildtype mice. This is the exact opposite of gene expression analysis that we performed ourselves by comparing healthy individuals and ACP5-deficient patients. This showed upregulation of TLR7 and related downstream signalling proteins NF-κB, IRF5, and IRF7. Therefore, in humans, TLR7 may be inhibited by ACP5 on a genetic level or by TRAP (encoded by ACP5) on a protein level. The latter could be caused by inhibiting trafficking of transcribed TLR7 from endoplasmic reticulum to endosome. After transcription in the nucleus, TLR7 is translocated to the Golgi apparatus from where it is delivered to the endosome by adaptor proteins[16]. As TRAP showed to be present in the Golgi apparatus, it is possible that it exerts an inhibitory effect here[22]. Consequently, this may lead to lower expression levels needed of downstream signaling proteins, as there is less activation due to reduced TLR7 in the endosome. On the other hand, absence or deficiency of ACP5/TRAP may lift this genetic repression or trafficking inhibition of TLR7, leading to upregulation of TLR7 and/or downstream regulatory genes NF-κB, IRF5, and IRF7, like our data displayed. These specific genes are involved in cytokine production via pro-inflammatory (NF-κB, IRF5) and anti-viral (IRF5, IRF7) pathways[15,23,24]. Excessive production of anti-viral type I interferons, as observed in ACP5-deficient patients, can therefore be seen as a logical consequence of increased expression of TLR7 and anti-viral downstream regulator IRF7. Interestingly, all three upregulated genes that encode for their corresponding downstream signalling protein have adaptor proteins IRAK4-IRAK1/2 (complex) and TRAF6 in common in their signalling cascade. This could indicate a shared point of interaction where ACP5/ TRAP interferes which may subsequently lead to compensatory upregulation of these downstream

signaling proteins. Previous work showed TRAF6 knockout mice to develop osteopetrosis hinting towards possible interactions with TRAP, knowing it is highly involved bone homeostasis[25]. However, for the gene analysis we performed, whole blood samples were used which may not fully represent the cell-types that we want to investigate here. Analysing samples of alveolar macrophages originating from (exacerbating) COPD patients may better reflect the pulmonary environment and give a more representative image of anti-viral and pro-inflammatory responses. Though, it remains to be seen if this solves the observed contradiction in TLR7 gene expression between humans and mice. In addition, upregulated gene expression does not directly relate to increased protein synthesis and should therefore be studied in further detail to ultimately determine possible downstream interactions with for instance co-immunoprecipitating techniques. Despite these limitations, a previous study which investigated TRAP-knockout in human plasmacytoid dendritic cells after stimulation with TLR9, also showed comparable regulation of pro-inflammatory and anti-viral gene expression and cytokine production, displaying higher expression of interferon stimulated genes (ISGs) and elevated levels of type I interferon alpha and TNF-α compared to wild-type cells[22]. This elevation was proposed to originate from TRAP phosphorylating osteopontin and therefore preventing it from complex formation with downstream regulator MYD88. This protein regulates TLR4/7/8 downstream signalling, leading to anti-viral and pro-inflammatory cytokine production. Collectively, these findings clearly indicate a regulatory role of TRAP on pro-inflammatory and anti-viral TLR signaling, however the exact interactive or regulatory mechanism has yet to be determined.

It remains uncertain whether ACP5 may be genetically involved or TRAP interacts through possible enzymatic inhibition. TRAP has been shown to be present intracellularly in the Golgi apparatus and vesicles like endosomes of human macrophages[11,22,26–28]. With the knowledge of TLR7/8 also being present in endosomes, we focused on production of anti-viral and pro-inflammatory cytokines in the context of TRAP inhibition and stimulation of different TLR receptors. TLR4 is localized on the plasma membrane of cells but can be internalized into the endosome where it induces type I interferon production by activating transcription factor IRF3 through mediators TRAM and TRIF[29,30]. TLR7/8 are expressed on endosomal and lysosomal membranes which activate IRF3 like TLR4 and IRF7 through MYD88. Stimulating TLR4, 7, or 8 in RAWblue cells all resulted in type I interferon production, which confirms successful induction of signalling via IRF3 and IRF7. No differences in cytokine levels were observed between TLR7-only or TLR7+8, possibly indicating absence of TLR8-induced interferon production. All types of stimulations were lower when TRAP was inhibited, implying a stimulatory role of TRAP on type I interferon production via TLR7/8/4 signaling. This is in line with our previous work on ACP5 knockout mice which displayed expression of ACP5 to also have a stimulatory role on TLR7. Furthermore, all three receptors are localized in the endosome and share downstream protein TRAF3 in their signaling cascades for type I interferon production[21,31,32]. Based on our findings in RAWblue macrophages, TRAP may therefore directly interact with an endosomal receptor or indirectly with TRAF3 in the cytosol

to exert a stimulatory effect that leads to higher type I interferon production via both signaling pathways. However, if and how such interactions may take place needs to be studied in further detail.

With respect to effects on the inflammasome pathway, we tested IL-1β excretion by RAWblue macrophages. TLR7/8/4 signalling can all activate transcription of pro-IL-1β mRNA through AP-1 and NF-κB which needs further activation by a stimulated inflammasome via TLR4 (e.g. with LPS) to obtain mature IL-1β[20,29,33–35]. In RAWblue macrophages, all three stimulations gave IL-1β production, confirming successful activation of NF-κB and AP-1. This also indicates that IL-1β production can originate not only from TLR4-dependent inflammasome activation, but also from an alternative source via TLR7/8. Higher IL-1β levels were observed after TLR7/8 stimulation compared to TLR7 stimulation-only, which could point at amplification of IL-1β excretion by TLR8 on top of TLR7. This also confirms absence of inhibitory cross-regulation by TLR8 contradicting findings in other cell-types[14,17–19]. In addition, IL-1β levels induced by TLR7 and TLR4 stimulation were inhibited by TRAP inhibition, but TLR7/8 activation remained unaffected. Because of this unresponsiveness of TLR7/8, the inhibitory effect could therefore be considered as selective for TLR4 and TLR7. However, if TLR7 is selectively affected by the TRAP inhibitor, TLR7/8 stimulation should also have been inhibited which it did not. This could possibly be explained by TLR8 signaling compensating for less TLR7 activation, resulting in no net change in measured cytokine concentrations. Absence of murine TLR8 agonists limited further investigation of these hypotheses. Combined, we can conclude that stimulation of TLR7/8/4 induced IL-1β production and we can confirm absence of inhibitory or stimulatory cross-regulation by either TLR7 or TLR8 in RAWblue cells. On top of this, TRAP exerted a stimulatory role on TLR7 and TLR4 dependent IL-1β excretion.

In contrast to the murine system, agonists are available for human TLR8. We therefore tested a TLR8-only agonist in addition to all previously used stimulants on human THP-1 monocytes. After TLR7 stimulation, IL-1β levels were absent, whereas TLR7/8, TLR8, and TLR4 stimulation did result in cytokine excretion. Because TLR7 signals via NF-κB and AP-1 like TLR8, we also expected IL-1β excretion. However, this was not observed and remains a point of discussion, as comparable studies also presented large variability in IL-1β excretion after TLR7 stimulation[14,36]. Furthermore, higher production of IL-1β after TLR7/8 stimulation compared to TLR8-only indicates TLR7 signaling can stimulate TLR8 in a cross-regulatory manner but does not induce IL-1β on its own. How this interaction may take place is still an open question. Additionally, inhibition of TRAP only inhibited TLR7/8 stimulation which may confirm selective stimulation of TLR7 signaling by TRAP but rejects the assumption of selective TLR4 inhibition. Absence of TLR4 inhibition may be caused by differences in cell-type used or could be explained by lower inhibitory concentrations used, as earlier experiments showed reduction starting from higher concentrations (10 uM).

We also tested effects of TRAP on NF-kB signalling by investigating TNF-α levels. RAW264.7 and MPI macrophages both had higher TNF-α production after TLR7 stimulation suggesting activated NF-κB signalling. This response was lower in RAW264.7 macrophages after inhibiting TRAP (0.5 uM) and confirmed our previous assumption of selective targeting of TLR7 signaling by the TRAP inhibitor. Despite having high expression of TRAP, MPI cells show a similar response. This could be normal response for alveolar macrophage-like cells, but may also be explained by MPI cells needing higher inhibitory concentrations to obtain the same effect, as there is more TRAP. We also showed that TLR4-induced TNF-α was inhibited by AubipyOme, verifying our previous assumption of the TRAP also targeting TLR4 signaling. Surprisingly, TLR7/8 stimulation of MPI cells resulted in minimal TNF-α excretion, which may be caused by inhibitory cross-regulation of TLR8 on TLR7, as this receptor shows highest expression in alveolar macrophages compared to other TLRs[36]. However, this theory contradicts our previous assumption of no inhibitory cross-regulation by TLR8 and therefore needs further investigation by for instance using selective TLR8 agonist or inhibitors of TLR7 and TLR8.

Because these ligands are not available for murine cell-lines, we used THP-1 monocytes for further analysis. These cells showed TNF-α excretion after TLR8, TLR7/8, and TLR4 stimulation, but not after TLR7 stimulation. This may indicate TNF-α production to be regulated via a signaling pathway independent of TLR7, as only this stimulation showed no induction of TNF-α production. Previous work on primary human monocytes also demonstrated this, by showing evidence of TLR7 preferentially signaling via MAPK and TLR8 via NF-kb. Our results therefore suggest TLR4 to signal similarly via NF-kb and exclude the possibility of MAPK dependent TNF-α production. However, this assumption does not take possible cross-regulatory or enzymatic interactions into account[14,36]. In addition, TLR7/8 stimulation gave similar TNF-α levels compared to TLR8 stimulation, indicating no inhibitory or stimulatory cross-regulation by TLR7 in this pathway. With respect to TRAP, combinations with TRAP inhibitor only lowered TLR7/8 induced TNF-α levels, indicating specificity for TLR7 signaling. Although, how this is regulated remains unclear as it does not induce cytokine expression itself. Overall, our results showed TLR7/8/4 agonists to successfully induce TNF-α production, but this was highly dependent on cell type and receptor preferential signaling pathway. Cross-regulatory mechanisms were absent in RAW264 macrophages and THP-1 monocytes, whereas in MPI macrophages TLR7 possibly inhibited TLR8 signaling. Furthermore, we found that TRAP specifically exerted a stimulatory effect on at least TLR7 signaling in all cell-types and on TLR4 signaling in MPI cells.

To conclude, acute exacerbations are invasive events mainly caused by viruses and drive a poor outcome of patients suffering from chronic pulmonary diseases due to excessive inflammation and impaired anti-viral response leading to a rapid decline in lung function. Previous work from us and others showed ACP5/TRAP to be implicated in TLR-signaling responsible for functional viral clearance and pro-inflammatory balance. Therefore, the main goal of this study was to establish the effect of this gene and enzyme on anti-viral and pro-

inflammatory cytokine excretion via viral toll-like receptor signaling by using genetic analysis and cytokine quantifying techniques. Genetic analysis confirmed TLR7 signaling to be linked to ACP5, giving reason to investigate this on protein level by looking at TRAP. Despite being species and cell-type specific, we discovered that TRAP can selectively stimulate both anti-viral and pro-inflammatory cytokines production in murine as well as human immune cells. Although where and how TRAP interacts is still unknown, it may be involved in TLR trafficking to the endosomal compartments, link with the endosomal receptor, or interact with downstream regulators in the cytoplasm. TLR7 and 8 cross-regulation did not appear evident but should not be excluded in future studies, as this was hard to demonstrate based on limited ligand possibilities. By looking directly at protein expression, our results gave a first glimpse on the effect of ACP5/TRAP on viral signaling through TLR7/8/4, as previous studies only indicated differences in gene expression and did not focus on an immunological role in the lung. Unraveling the exact role of ACP5/TRAP in the lung requires further investigation but could be crucial to increase viral clearance and decrease excessive inflammation in exacerbating patients, because there are still very limited options available to prevent or treat these severe and pathologically complex events.

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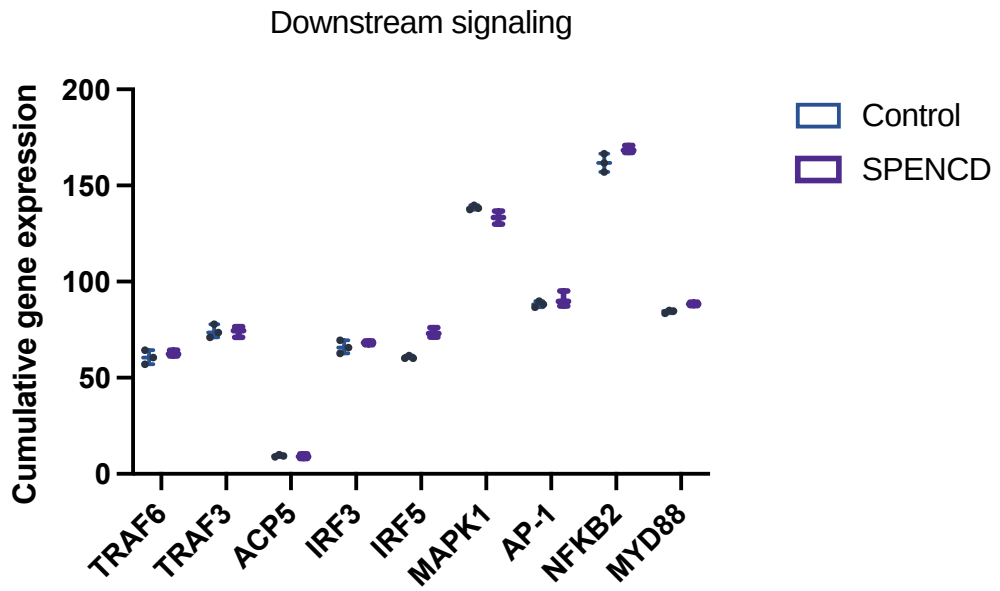
Supplemental information



Supplementary figure 1: ***Influence of ACP5 on gene expression of TLR 7/8-related downstream signaling proteins in ACP5 deficient patients versus healthy individuals.*** *Cumulative gene expression of TLR7/8-related downstream genes TRAF6, TRAF3, ACP5, IRF3, IRF5, MAPK1, AP-1, NFKB2, MYD88 of whole blood samples from healthy controls (n=3) and ACP5-deficient patients (SPENCD, n=3))*.

	TYPE I IFN: RAWBLUE	IL-1B RAWBLUE	TNFA : RAW264.7	TNFA: MPI	TNFA: THP-1	TNFA THP-1
AUB 0.1	10.234	11.034	2.334	7.754	15.498	12.121
AUB 0.5	5.094	8.939	2.432	9.285	10.092	1.056

Supplementary figure 2: ***Effect of TRAP inhibitor-only on anti-viral and pro-inflammatory cytokine production in different murine and human immune cells.*** Type I interferon, IL-1b and TNFa excretion levels (pg/ml) after TRAP inhibitor-only additions with AubipyOme (0.1-0.5 uM) on RAWblue, RAW264.7, MPI and THP-1 cells.