Innate immune sensing of *Streptococcus pyogenes* extracellular vesicles

Introduction

Streptococcus pyogenes (group A Streptococcus, GAS) is a strict human pathogen, that causes a variety of clinical manifestations ranging from mild, superficial infections such as pharyngitis to toxin-mediated or severe invasive diseases (e.g., toxic-shock syndrome, necrotizing fasciitis, and endocarditis) with a high mortality rate[1]. In addition, post-infectious autoimmune sequelae, such as acute rheumatic fever, rheumatic heart disease, and glomerulonephritis, especially in developing countries, where access to healthcare and antibiotics is limited, are a major concern. *S. pyogenes* expresses a wide array of virulence components that facilitate its survival within the host by hijacking the activity of immune cells [2–4]. Factors associated with bacterial virulence can be classified depending on their location as membrane-bound, membrane-anchored or cytosolic. Interestingly, several virulence mediators are present in so-called *S. pyogenes* extracellular vesicles (Spy EVs) including the Streptococcal inhibitor of the complement (Sic)[5] and streptolysin O (slo)[6] shown to promote monocyte activation and evasion from lysosomal degradation[7], respectively. Notably, increased EV production occurs in invasive strains carrying natural inactivating mutations in the two-component system CovRS, which controls ~ 15% of the genes in *S. pyogenes.*

EVs are nanoparticles consisting of a lipid bilayer shell encapsulating proteins and nucleic acids derived from their parental cells. The release of EVs is a process, conserved in all domains of life, that coordinates the intra- and interspecies transport of cellular components[8–10]. In Gram-negative bacteria, the composition, biogenesis and functions in bacterial physiology of outer membrane vesicles (OMVs) have been extensively characterized[11,12]. Several reports have demonstrated that Gram-positive model organisms and pathogens also release EVs [13,14]. However, only a few of these studies have addressed the impact of Gram-positive EVs on the host [15–17]. Due to their small size and resistance to degradation, bacterial EVs are able to diffuse and travel long distances in the human body, reaching locations that their parental cells might not access [18]. In addition, microbial EVs can be internalized and/or recognized through pathogen recognition receptors (PRRs), which may differ from the PRRs participating in the recognition of their parental cells [19].

The delivery of Gram-negative bacterial components to the cytosol of host cells via OMVs was shown to promote inflammasome assembly leading to caspase-1-dependent cleavage and subsequent release of the pro-inflammatory cytokines IL-1α, IL-1β, and IL-18 as well as the induction of pyroptotic cell death[20–22]. Typically, the activation of this large multiprotein complex is a two-step process: PRR-engagement on the cell surface stimulates NFκB-dependent gene expression of inactive precursor molecules for the aforementioned cytokines (priming). Then, the presence of bacterial products within the cytosol is sensed by various NOD-like receptors (NLRs) thereby initiating oligomerization and caspase-1 (CASP1) recruitment (activation). However, for human monocytes LPS was shown to trigger an alternative one-step inflammasome activation pathway that is dependent on TLR4 signaling and seemingly does not require LPS internalization [23,24].

The interaction of *S. pyogenes* with host cells was shown to activate innate immune pathways through Toll-like receptors[25], type I interferon signaling[26], the NLRP3 inflammasome[27], as well as autophagy[28]. However, while the composition of *S. pyogenes* EVs has been thoroughly characterized[6], it remains elusive whether these EVs are sensed by innate immune cells through the same pathways that participate in the sensing of intact *S. pyogenes*. Therefore, we aimed to understand the innate immune responses against hypervirulent *S. pyogenes* and their secreted EVs to potentially identify new targets for therapeutic intervention.

Results

<u>Transcriptome Analysis of Human Monocytes Reveals a Unique Set of Genes that are Upregulated upon encountering Spy EVs</u>

Our laboratory has previously characterized the content of Spy EVs by proteomic, RNA sequencing and lipidomic analysis[6]. Spy EVs and their parental cells show an asymmetrical distribution in their composition (e.g. of virulence factors), supporting the idea that EVs might trigger distinct responses than *S. pyogenes* cells. To characterize and compare the response of innate immune cells when encountering Spy EVs or intact bacteria, we performed RNA sequencing on human primary monocytes and B-cell Leukemia C/EBPα Estrogen Receptor clone 1 (BLaER1)-derived monocytes after EV stimulation or *S. pyogenes* infection (Fig.1).

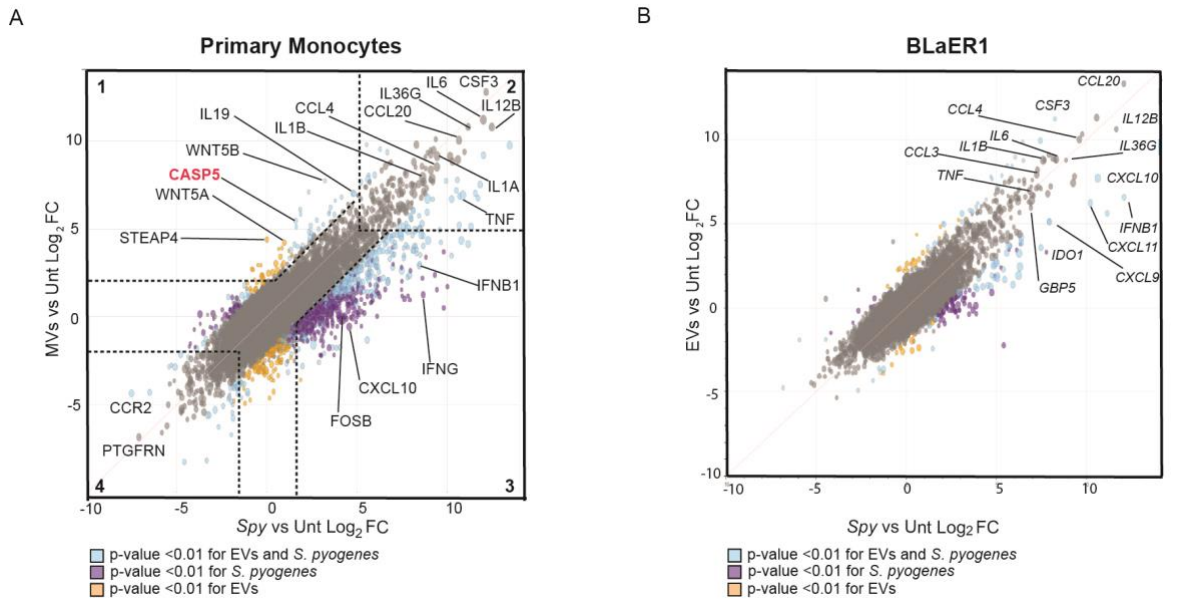

Fig. 1: *S. pyogenes* EVs Induce a Distinct Expression Profile in Monocytes. Log2 fold change of transcription for EV-treated versus untreated cells is plotted against the log2 fold change of transcription for *S. pyogenes*-infected versus untreated cells in **(A)** primary human monocytes or **(B)** BLaER1 cells at 4h upon MV or *S. pyogenes* stimulation. Dashed areas indicate four distinct gene groups that share (2 and 4) or display unique (1 and 3) transcription patterns for Spy or Spy EVs. Representative genes for each group are indicated. Genes were defined as differentially transcribed when the log2 expression was ≤ -2 or ≥ 2 and the adjusted p-value was <0.01 after Benjamini-Hochberg correction for multiple comparisons. Data are representative of **(A)** eight or **(B)** four biological replicates.

Our transcriptomic datasets showed that 83% and 69% of genes are upregulated in response to both stimuli in monocytes and BLaER1 cells, respectively. Among the commonly upregulated genes, we found cytokines (e.g. *IL1A, IL1B* and *IL6*), chemokines (e.g. *CCL3*, *CCL4* and *CXCL8*), and growth factors (e.g. *CSF3*). In contrast, several immune receptors (e.g. *TLR1, TLR6* and *CCR2*) and adhesion factors (e.g. PECAM1 and VCAN) were downregulated for both stimuli. We then analysed the differentially expressed genes (DEGs) belonging specifically to one of the treatments. DEGs upon challenge with Spy EVs included cytokines (e.g. IL19), components of the Wnt signaling pathway (*WNT5A* and *WNT5B*), and, strikingly, the immune sensor caspase-5 (*CASP5)*. Infection with *S. pyogenes* specifically upregulated an additional 1119 genes, including interferon and interferon-related genes (IRGs, e.g. *IFNG, IFNB1* and *CXCL10*), growth factors (e.g. *CSF1*), and chemokines (e.g. *CCL8*), indicating that the sensing of whole bacteria induces distinct responses compared to EVs.

CASP4/5 are Required for the Sensing of Spy EVs

In monocytes, IL-1β is secreted upon activation of the canonical, the non-canonical and/or the alternative inflammasome [24,29–31]. Notably, we observed that Spy EV treatment induced the expression of the non-canonical inflammasome component CASP5, compared to untreated cells (log2:5.45 after 4 h incubation). CASP4/5 and their murine homolog, CASP11, known to recognize intracellular LPS during Gram-negative infection[32], are also involved in the sensing of the Gram-positive species *Staphylococcus aureus* (*S. aureus*) and *Listeria monocytogenes*

(*L. monocytogenes*)[29,30]. Upregulation of *CASP5* upon EV treatment prompted us to investigate whether IL-1β might be induced differently by Spy EVs than by their parental cells. To determine the role of CASP4/5 in the sensing of Spy EVs, we used the chemical inhibitor Ac-LEVD-CHO, which blocks the action of these two caspases. Incubation of monocytes with the CASP4/5 inhibitor before adding Spy EVs or *S. pyogenes* decreased IL-1β secretion (Fig.2), although with a greater reduction in IL- 1β in response to EVs. In contrast, the levels of caspase-independent cytokines such as IL-6 and IL-1α remained unchanged (Figure 2A-2B). Activation of the non-canonical inflammasome can lead to pyroptotic cell death[35]. However, cell death measured by secretion of lactate dehydrogenase (LDH) was negligible upon Spy EV stimulation and did not vary upon inhibition of CASP4/5 (Figure 2A-2B). Collectively, our data suggest that IL-1β release in response to is Spy EVs and *S. pyogenes* requires CASP4/5 without inducing cell death.

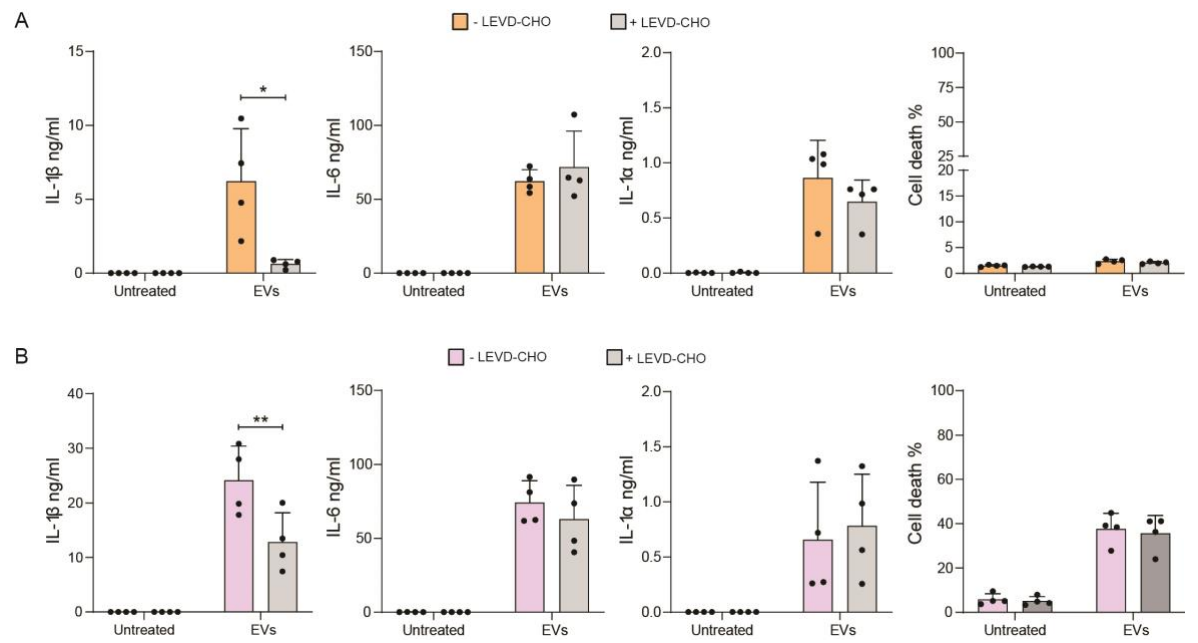

Fig. 2: *S. pyogenes* EVs are Recognized by CASP4 and CASP5. IL-1β, IL-6, IL-1α, and LDH released by monocytes after stimulation with either Spy EVs (A) or intact S. pyogenes (B) for 18 h. Monocytes were either pre-incubated with the CASP4/5 inhibitor Ac-LEVD-CHO (grey) or left untreated (orange for EVs and purple for *S. pyogenes*). Bars represent the mean ± standard deviation (SD) of four biological replicates. Two-way ANOVA with Holm-Šídák correction for multiple comparisons was applied for statistical analyses. * p≤0.05, ** p≤0.01

<u>CASP4/5-mediated Secretion of IL-1β is partially triggered by Lipoteichoic Acid (LTA)</u>

LTA is a major component of the cell membrane of Gram-positive bacteria that is sensed by TLR2 [36,37]. Since LTA has been shown to induce CASP11-mediated secretion of IL-1β in mice[33], we hypothesized that LTA might be the bacterial ligand triggering CASP4/5 signaling. Because LTA mutants of Gram-positive bacteria display severe growth defects[38], we used purified *S. pyogenes* LTA to test its role in monocyte activation. Consistent with our hypothesis, pharmacological inhibition of CASP4/5 followed by treatment of *S. pyogenes* LTA in monocytes (Fig.3A) and BLaER1 cells (Fig.3B) reduced IL-1β secretion compared to cells without inhibitor, whereas the release of IL-6 and LDH in the supernatants remained unaffected by CASP4/5 inhibition. In contrast, transfection of LTA into monocytes did not result in further increased IL-1β production, suggesting that the CASP4/5-mediated signaling triggered by LTA most likely begins at the cell surface, in line with the role of TLR2 in the induction of IL-1β (Fig3C).

Since the synthesis of teichoic acids is moderately conserved among Gram-positive bacteria[39,40], we asked whether EVs derived from other Gram-positive bacteria could trigger IL-1β production through CASP4/5. Cells treated with CASP4/5 inhibitor and then incubated

with EVs from *S. aureus*, *Streptococcus agalactiae,* or *Bacillus subtilis* released less IL-1β but equal amounts of LDH and IL-6 than monocytes without inhibitor (Fig.), demonstrating that CASP4/5 is also involved in the sensing of other Gram-positive-derived EVs.

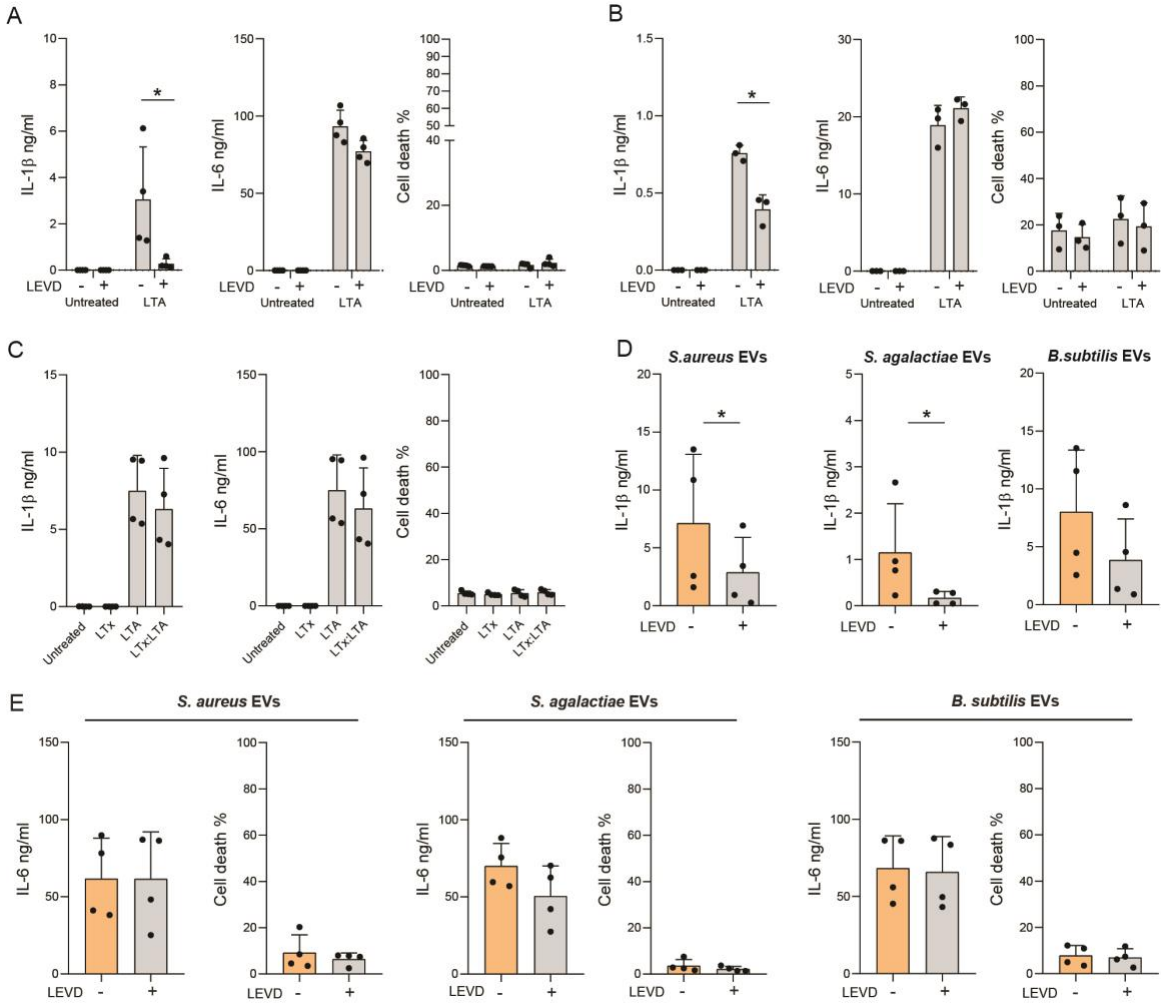

Fig. 3: LTA Conserved in Gram-positive Bacteria Partially Recapitulates CASP4 and CASP5 Activation. IL-1β, IL-6, and LDH released by **(A)** monocytes or **(B)** BLaER1 cells after stimulation with *S. pyogenes* LTA at 18 h. **(C)** IL-1β, IL-6, and LDH released by monocytes transfected with *S. pyogenes* LTA using Lipofectamine LTX. **(D)** IL-1β released by monocytes in response to *S. aureus* (left), *S. agalactiae* (middle), or *B. subtilis* (right) EVs at 18 h. **(E)** IL-6 and LDH released by monocytes in response to *S. aureus* (left), *S. agalactiae* (middle), or *B. subtilis* (right) EVs at 18 h. Cells were left untreated or preincubated with Ac-LEVD-CHO prior to stimulation. Bars represent the mean ± standard deviation (SD) of 3-4 biological replicates. Two-way ANOVA with Holm-Šídák correction for multiple comparisons was applied for statistical analyses. * p≤0.05

Discussion

In the present study, we explored the response of human phagocytes to *S. pyogenes* and its EVs and identified mediators of these recognition events. Strikingly, only one report using murine macrophages has so far addressed the cellular response during *S. pyogenes* infection on a genome-wide level[41]. Here, RNA sequencing revealed that responses triggered by *S. pyogenes* and its EVs strongly overlap, which is consistent with the observation that Spy EVs contain at least half of the proteins of the total proteome of their parental cells[6]. However, *S. pyogenes* and its EVs also regulate the expression of distinct sets of genes, indicating that unique components in the intact bacteria or EVs contribute to triggering a differential immune response.

In the wide range of clinical manifestations caused by *S. pyogenes*, inflammation plays a dual role in preventing *S. pyogenes* invasion and limiting an excessive and potentially harmful immune response[42]. Notably, treatment of patients suffering from autoimmune diseases with IL-1β inhibitors increases their risk of developing invasive *S. pyogenes* infections by 330-fold, suggesting a central role for IL-1β in controlling *S. pyogenes* infection in humans[43]. While it has been already demonstrated, that NLRP3 is a key sensor of intracellular *S. pyogenes* leading to IL-1β production[27], we also identified components of the non-canonical inflammasome, CASP4 and CASP5, contributing to IL-1β secretion in response to *S. pyogenes* EVs and to a lesser extent their parental cells in human monocytes. In support of this finding, CASP4/5 in THP-1 cells have been shown to be required for *L. monocytogenes* and *S. aureus* sensing[33,44]. In addition, a recent report suggests that *S. pyogenes* infection induces CASP4 expression in human neutrophils, indicating that this system might participate in *S. pyogenes* sensing in other cell types[45].

Our study provides evidence that LTA decorating the surface of *S. pyogenes* and its EVs modulates CASP4/5-dependent responses. Along with these observations, we have shown that CASP4/5-dependent sensing of EVs is conserved for different Gram-positive species. Nevertheless, it has been shown that CASP4/5/11 do not bind directly to any component present in lysates of several Gram-positive species[31], suggesting that other sensors act upstream of these enzymes. In agreement with this hypothesis, CASP11 coordinates the sensing of cytosolic LTA from *L. monocytogenes* as a component of the NLRP6 inflammasome in mice[33]. Additionally, NLRP7, a newly identified sensor that recognizes acylated Gram-positive lipoproteins, has been described in humans[46]. It remains to be elucidated whether any of these sensors act upstream of CASP4/5 in human monocytes during the sensing of Gram-positive bacteria. However, given that in our experimental set up LTA internalization was not required to trigger CASP4/5-dependent IL-1β production, we expect that the upstream receptor(s) of this cascade are located on the cell surface. Since LPS stimulation of human monocytes was reported to activate a one-step inflammasome pathway, which is linked to TLR4 signaling, it seems plausible that a similar mechanism might exist for LTA that potentially also involves CASP4/5. Furthermore, it is conceivable that different surface ligands besides LTA are sensed upstream of CASP4/5, or in the endosomal or cytosolic compartments, as suggested by the observation that LTA only partially recapitulates the CASP4/5-dependent effect that we observed with Spy EVs.

Overall, our study highlights a new role for CASP4/5 in the recognition of Gram-positive EVs. However, given the complexity of the immune response against bacterial pathogens, it is difficult to establish whether Spy EVs have an overall protective role during *S. pyogenes* infection or, on the contrary, exacerbate disease. For Gram-negative bacteria, OMVs were already shown to induce sepsis-like symptoms in mice[47,48]. Thus, future experiments should explore the sensing of Spy EVs using in vivo models.

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