UMEÅ UNIVERSITY

Role of extracellular ATP in immune mechanisms against infection

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Change the strategy not the goal when you fail to achieve it

Bhagavad Gita

To my father with love and inspiration

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Abstract

Inflammation is driven either by infection with pathogens or sterile stimuli, such as tissue damage or autoimmune diseases. Upon tissue damage, ATP is released passively from the dead or compromised cells. During stress, ATP can be secreted from the cells. Extracellular ATP (eATP) acts as a endogenous danger signal. An increase in eATP is sensed by cell surface purinergic receptors and regulates the onset and resolution of inflammation. Extracellular ATP is an important inflammatory mediator during sterile inflammation. On the other hand, the role of eATP is poorly studied during infection, both bacterial and viral. In this thesis, I present the molecular mechanisms underlying ATP secretion during bacterial infections and the role of eATP in human hantaviral infections.

During infection with certain enteropathogenic Gram-negative bacteria, intestinal epithelial cells secrete ATP via connexin hemichannels as an alert signal to activate the immune system, which triggers acute inflammation in the gut. However, neither what triggers ATP secretion nor the molecular mechanisms of ATP secretion were known. Pharmacological, genetic and microscopy-based evidence shows that during invasive bacterial infections, the plasma membrane ruffles act as mechanical immune stimuli and activate the inherently mechanosensitive plasma membrane channel PIEZO1. Mechanically activated PIEZO1 leads to the influx of Ca^{2+} ions and concurrent ATP secretion. In addition, PIEZO1 also activates protective transcriptional responses. Thus, PIEZO1 acts as a sensor for invasive infection using mechanical stimuli, unlike the so far described immune sensors of infection, which all recognize microbial components by chemical interaction.

During human hantavirus infection, the humoral immune responses is poorly studied. Our collaborators found that atypical B cells, which do not have the surface marker CD27, show increased frequency in a cohort of hantavirus-infected patients. CD27 shedding in murine lymphocytes had been previously linked to eATP-dependent activation of a purinergic receptor$_7$. To test whether ATP levels in the circulation of hantavirus-infected patients are elevated, an approach to perform same-day eATP quantifications in human plasma was developed. This assay was used to establish the normal eATP concentration in plasma in a cohort of healthy volunteers and to show that eATP levels are elevated in the acute and convalescent stages of hantavirus infection. Further, addition of ATP to isolated human B cells recapitulated the observed CD27 shedding via a metallomatrix proteinase-8-dependent (MMP8) mechanism. Together, these projects provide an evidence for the importance of eATP in bacterial and viral infectious diseases.

Abbreviations

PAMPs: pathogen associated molecular patterns

eATP: extracellular ATP

InlA: internalin A

IECs: intestinal epithelial cells

PRRs: pattern recognition receptors

MAMPs: microbial associated molecular patterns

TLRs: Toll-like receptors

NLRs: NOD-like receptors

RLRs: RIG-I-like receptors

MAPK: mitogen activated protein kinase

DAMPs: danger associated molecular patterns

MDA5: melanoma differentiation-associated protein 5

LGP2: laboratory of genetics and physiology 2

CTD: carboxy-terminal domain

ds-RNA: double standard RNA

MAVS: mitochondrial antiviral-signaling proteins

TBK1: TANK-binding protein 1

IKKε: IκB kinase-ε

NF-kB: nuclear transcriptional factor

cGAS: cyclic GMP–AMP synthase

STING: stimulator of interferon genes

ER: endoplasmic reticulum

GI: gastrointestinal

NO: nitric oxide

ATP: adenosine triphosphate

Cx: connexin

Ado: adenosine

P2XRs: P2X receptors

P2YRs: P2Y receptors

PAIs: pathogenicity islands

PMNs: polymorphonuclear neutrophils

T3SS: Type III secretion system

LLO: listeriolysin

InlB: internalin B

HLY: hemolysin

CytoD: cytochalasin D

LatB: latrunculin B

PBMCs: peripheral blood mononuclear cells

MMP8: metallomatrix proteinase-8-dependent mechanism

HFRS: hemorrhagic fever with renal syndrome

Gn: glycoprotein

N: nucleocapsid

MyD88: Myeloid differentiation primary response 88

TRIF: TIR-domain-containing adapter-inducing interferon-β

IRAK-1: Interleukin-1 receptor-associated kinase 1

IRAK-4: Interleukin-1 receptor-associated kinase 4

TRAF6: TNF receptor-associated factor 6

TAK1: Transforming growth factor β-activated kinase 1 TAK1

Sammanfattning på svenska

Inflammation orsakas antingen av infektion med patogener eller av sterila stimuli, såsom vävnadsskador eller autoimmuna sjukdomar. Vid vävnadsskada frisätts ATP passivt från de döda eller funktionsnedsatta cellerna. och under stress kan ATP utsöndras från cellerna. Extracellulär ATP (eATP) fungerar som en endogen varningssignal. En ökning av eATP detekteras av purinerga receptorer och reglerar uppkomsten och upphävandet av inflammation. Extracellulärt ATP är en viktig inflammatorisk mediator vid steril inflammation. Vilken roll eATP har under infektion , både bakteriell och viral, är dock dåligt studerad. I denna avhandling presenterar jag de molekylära mekanismerna bakom ATP-utsöndring under bakteriella infektioner och rollen av eATP i humana hantavirusinfektioner.

Vid infektion med vissa enteropatogena Gram-negativa bakterier utsöndrar tarmepitelceller ATP via connexin-hemikanaler som en varningssignal för att aktivera immunförsvaret, vilket utlöser akut inflammation i tarmen. Men varken vad som utlöser ATP-utsöndring eller de molekylära mekanismerna för ATP-utsöndring har varit kända. Farmakologiska, genetiska och mikroskopibaserade bevis visar nu att under invasiva bakterieinfektioner, fungerar plasmamembranruggningar som mekaniska immunstimuli och aktiverar den inneboende mekanokänsliga plasmamembrankanalen PIEZO1. Mekaniskt aktiverad PIEZO1 leder till inflöde av Ca^{2+}-joner och samtidig ATP-sekretion. Dessutom aktiverar PIEZO1 också skyddande transkriptionssvar. Således fungerar PIEZO1 som en sensor för invasiv infektion med hjälp av mekaniska stimuli, till skillnad från de hittills beskrivna immunsensorerna för infektion, som alla känner igen mikrobiella komponenter genom kemisk interaktion.

Under human hantavirusinfektion är de humorala immunsvaren dåligt studerade. Våra medarbetare fann att en ökad frekvens av atypiska B-celler, som inte har ytmarkören CD27, i en kohort av hantavirusinfekterade patienter. CD27-utsöndring i murina lymfocyter har tidigare kopplats till eATP-beroende aktivering av en purinerg receptor 7 (P2X7). För att testa om ATP-nivåerna i cirkulationen hos hantavirus infekterade patienter är förhöjda, utvecklades ett tillvägagångssätt för att utföra eATP-kvantifieringar samma dag i human plasma. Denna metod användes för att fastställa den normala eATP-koncentrationen i plasma i en grupp av friska frivilliga försökspersoner och för att visa att eATP-nivåerna är förhöjda i de akuta och konvalescenta

stadierna av hantavirusinfektion. Vidare rekapitulerade dessutom, genom tillförsel av ATP till isolerade humana B-celler kunde den observerade CD27-utsöndringen via en metallomatrix-proteinas-8-beroende (MMP8) mekanism återskapas. Tillsammans ger dessa projekt bevis för betydelsen av eATP vid bakteriella och virala infektionssjukdomar.

List of papers

1. **Infection-induced membrane ruffling initiates danger and immune signaling via the mechanosensor PIEZO1.**
   Tadala L, Langenbach D, Dannborg M, Cervantes-Rivera R, Sharma A, Vieth K, Rieckmann LM, Wanders A, Cisneros DA, Puhar A.
   Cell Rep. 2022

2. **Generation of plasma cells and CD27-IgD- B cells during hantavirus infection is associated with distinct pathological findings.**
   Kerkman PF, Dernstedt A, Tadala L, Mittler E, Dannborg M, Sundling C, Maleki KT, Tauriainen J, Tuiskunen-Bäck A, Wigren Byström J, Ocaya P, Thunberg T, Jangra RK, Román-Sosa G, Guardado-Calvo P, Rey FA, Klingström J, Chandran K, Puhar A, Ahlm C, Forsell MN.
   Clin Transl Immunology. 2021

3. **PIEZO1 activation during invasive infection triggers broad changes in the transcriptional landscape**
   Tadala L, Cervantes-Rivera R, Puhar A.
   Manuscript.

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Introduction

Intestinal epithelium

The gastrointestinal epithelium is the largest interface between the tissue and microbes (Pott and Hornef, 2012). It serves as a physical and immunological barrier with heterogeneous, dynamic cell populations to maintain gut homeostasis during health (Okumura and Takeda, 2017).

The intestinal epithelium consists of various types of cells, such as intestinal epithelial stem cells, enterocytes, goblet cells, endocrine cells, tuft cells, paneth cells, and M cells (Figure 1). Enterocytes act as a physical barrier and absorb nutrients. The goblet cells secrete mucus and antimicrobial peptides to clear the bacteria from the epithelium. Paneth cells secrete antimicrobial peptides and maintain the epithelial stem cell niche. Endocrine cells and tuft cells release hormones and IL-25, respectively. M cells help to carry live bacteria and intact antigens to immune cells. The intestinal epithelium is covered by a thick layer of mucus. The mucus layer acts as a primary physical barrier against commensal bacteria and invading pathogens into the mucosa. Professional immune cells such as T cells, B cells, dendritic cells, and macrophages are also present (Figure 1) (Burgueño and Abreu, 2020; Kong et al., 2018; Peterson and Artis, 2014).

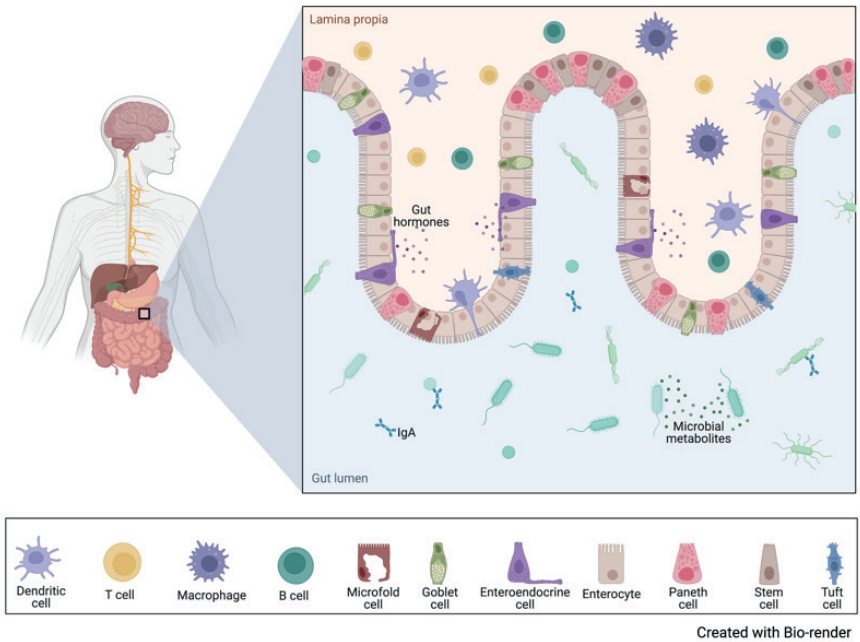

Figure 1 Composition of the intestinal epithelium

Professional immune cells express pattern recognition receptors (PRRs). These receptors detect the molecules that come from pathogens such as bacteria, viruses, parasites, and fungi, which are called pattern-associated molecular patterns (PAMPs) or microbial-associated molecular patterns (MAMPs) and induce immunological responses (Li and Wu, 2021) . IECs act as sentinels to protect the host from invading harmful pathogens by expressing some PRRs similar to immune cells, such as Toll-like receptors (TLRs), NOD-like receptors (NLRs), and RIG-like receptors (RLRs) (Allaire et al., 2018; Peterson and Artis, 2014).

Toll Like Receptors (TLRs)

These are plasma membrane and endosomal-associated receptors, which detect molecules from microorganisms on the cell surface and inside endosomes. There are ten specific receptors to detect specific molecules that come from microbes in mammals. TLRs are synthesized in the endoplasmic reticulum and transported into specific locations according to their specificity. Some of them are transported to endosomes (TLR3, 7, 8, and 9), and rest transported to the plasma membrane (TLR1, 2, 4, 5, 6, and 10). For example, LPS from Gram-negative pathogens is detected by TLR4, and lipoproteins from Gram-positive pathogens is detected by TLR2. Viral DNA particles are sensed by TLR9 and RNA by TLR7 and TLR8 (Fitzgerald and Kagan, 2020).

TLRs have an ectodomain that recognizes PAMPs and a cytoplasmic domain that recruits major adaptor proteins such as Myeloid differentiation primary response 88 (MyD88) and *TIR-domain-containing adapter-inducing interferon-β* (TRIF) to activate transcription factors and cellular functions (Takeda and Akira, 2015).

In the MyD88-dependent pathway, after recruitment MyD88 forms a complex called Myddosome, which contains IRAK1 and IRAK4. IRAK1 activates TRAF6 via K63-linked polyubiquitination of TAK1 and TRAF6 itself. TAK1 is a member of the mitogen-activated protein kinase family (MAPK). Activated TAK1 leads to the activation of the IKK complex-NF-κB pathway and the MAPK pathway to regulate inflammatory responses (Figure 2). In TRIF-dependent pathways, activated TRIF recruits TRAF6 and TRAF3. TRAF6 recruits RIP-1 kinase, which is ubiquitinated and activates the TAK1 complex. The activated TAK1 complex leads to NF-κB and MAPK signaling. On the other hand, TRAF3 interacts with TBK1, NEMO, and IKK kinases to phosphorylate IRF3. The activated IRF3

translocates to the nucleus and induces the expression of type I interferons (Figure 2) (Abreu et al., 2005; Buchholz and Bauer, 2010; Dahan et al., 2007; Kawasaki and Kawai, 2014).

NOD Like Receptors (NLRs)

These are cytosolic receptors, which recognize PAMPs or danger-associated molecular patterns (DAMPs) inside cells. These proteins are divided into four subfamilies, NLRA, NLRB, NLRP, and NLRC, depending on their domain structures. The activation of NLRs leads to downstream signaling, which results in the activation of inflammatory responses, inflammasomes, and NF-κB. NLRP and NLRC are larger subfamilies that constitute the NLR family. The NLRC subfamily contains six receptors, namely, NLRC1 (NOD1), NLRC2 (NOD2), NLRC3 (NOD3), NLRC4 (IPAF), NLRC5, and NLRX1 (Motta et al., 2015).

The NODs contain C-terminal ligand-binding LRRs, a central NACHT domain, and a single (NOD1) or tandem (NOD2) N-terminal CARD domain. Cytosolic peptidoglycan is recognized by the NOD1 and NOD2 receptors. Upon recognition, NODs form a complex with RIP kinase through CARD domains, resulting in the activation of NF-κB and MAPK (Figure 2). NOD receptors are expressed in the gut to maintain homeostasis by preventing inflammatory responses from commensal bacteria while protecting against pathogenic bacteria (Brubaker et al., 2015; Motta *et al.*, 2015; Pott and Hornef, 2012).

The NLRP subfamily encompasses NLRP1-14 proteins, which share a common domain called pyrin domain (PYD). In addition NLRP1 contains other domains called function to find domain (FIIND) and CARD domain (Corridoni et al., 2014). The activation of NLRP proteins results in the formation of an inflammasome. The inflammasome is a multimeric

protein complex, which recruits adaptor proteins and caspases depending on the type of NLRP proteins (Ranson et al., 2017).

NLRP1 does not need ASC adaptor protein to activate caspase-1 because it has its own CARD domain, which activates the caspase-1 (Faustin et al., 2007). The other NLRP proteins need ASC adaptor protein to cleave or activate the caspases (Lu et al., 2014). The activated caspase-1 cleaves the pro-inflammatory cytokines such as IL-1β and IL-18 to induce a type of cell death called pyroptosis (Figure 2) (Guo et al., 2015; Lamkanfi and Dixit, 2012).

It has been shown that ATP activates the NLRP2 inflammasome complex in human astrocytes (Minkiewicz et al., 2013). The activation of NLRP3 inflammasome protects from dextran sodium sulfate (DSS)-induced colitis and maintains the gut homeostasis by epithelial integrity (Zaki et al., 2010)

RIG-I Like Receptors (RLRs)

These receptors are localized in the cytoplasm, where the entry or replication of the virus occurs. They recognize foreign RNA molecules such as 5'-triphosphate RNA, long double-stranded RNA, and sequences specific to pathogens different from endogenous RNA molecules. These receptors are necessary to protect against RNA viral infections and some DNA viruses and bacterial pathogens (Brubaker *et al.*, 2015; Chiu et al., 2009).

RLRs family consists of 3 members, namely, RIG-I, melanoma differentiation-associated protein 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2) (Rehwinkel and Gack, 2020). All RLR family proteins have a common central helicase domain and carboxy-terminal

domain (CTD). RIG-I and MDA5 have CARD domains, which are called Caspase recruitment domains that lead to downstream signal transduction and induce type I interferons (Gitlin et al., 2006; Kato et al., 2006).

When foreign RNA is bound to the RIG-I receptor, the helicase domain wraps tightly around the RNA and leads to conformational changes in the receptor to expose CARD domains for downstream signaling. MDA5 receptors activated by double standard RNA (ds-RNA) and DNA viruses undergo conformational changes and interact with mitochondrial antiviral-signaling proteins (MAVS) and form a complex. The signaling complex activates TANK-binding protein 1 (TBK1) and IκB kinase-ε (IKKε), which activate the downstream signaling transcription factors IRF3 and IRF7. Eventually, NF-κB is activated and induces an antiviral response and type I interferons (Figure 2) (Rehwinkel and Gack, 2020).

STING-Pathway

Cytosolic DNA is sensed by cyclic GMP–AMP synthase (cGAS). Upon binding, cGAS undergoes a conformational change and generates the second messenger cyclic GMP–AMP (cGAMP), which binds to the adaptor protein STING (stimulator of interferon genes) in the endoplasmic reticulum (ER). The activated complex translocates from the ER to the Golgi for downstream signaling, where it recruits TBK1 and KKε, resulting in the induction of interferons (Figure 2) and immunological responses such as antibacterial and antiviral immunity, cellular differentiation, and regulation of barrier function (Wottawa et al., 2021).

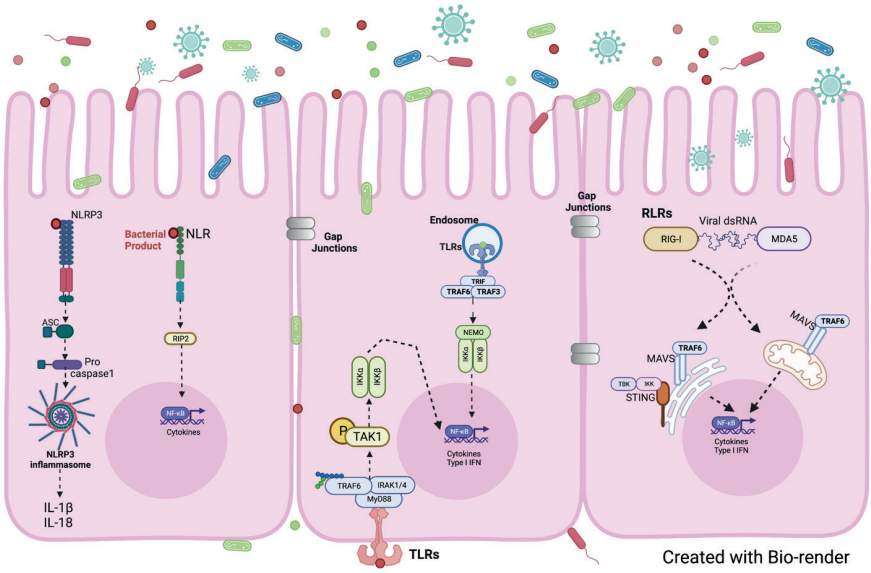

Figure 2 Schematic representation of PRRs in intestinal epithelial cells

Mechanosensitive ion channels

IECs express ion channels, which help in electrolyte absorption and secretion to maintain the physiology of the gut. Mechanical forces play an important role in the gastrointestinal (GI) tract. During mechanotransduction, mechanical energy is converted into either electrical or chemical energy in biological processes. The impairment of mechanotransduction leads to diseases such as obesity, constipation, and colon cancer. The majority of mechanosensitive channels in GI are unknown (Alcaino et al., 2017; Ghazi et al., 1998).

From *in vitro* functional studies two general models have been proposed for the opening of mechanosensitive channels. One of them is the 'force-from-lipids' model: when mechanical force is applied to the membrane, the tension creates a disturbance in the lipid-protein interaction, and the ion channel opens. The second model is the 'force-from-filaments' model, in which the extracellular matrix or cytoskeletal proteins are involved in opening the channel when force is applied (Coste et al., 2012; Murthy et al., 2017; Syeda et al., 2016).

The first mechanically activated ion channels discovered in vertebrates are Piezos (Coste et al., 2010; Murthy *et al.*, 2017). These are the largest mechanosensitive and pore-forming multimeric cation channels present in mammalian cells. These multimeric subunits are arranged as trimers. Each subunit contains 2,500 amino acids. In humans, there are two types of PIEZO channels, identified as PIEZO1 and PIEZO2. They share ~50% identity at the amino acid level (Murthy *et al.*, 2017).

PIEZO1 is present in non-neuronal cells such as erythrocytes, vascular endothelial cells, bladder urothelial cells, immune cells, and chondrocytes, whereas PIEZO2 is mainly present in sensory neuronal cells and some specialized mechanosensory tissues (Fang et al., 2021). The mechanosensitive cationic channels are important for many biological processes such as vascular and lymphatic development (Li et al., 2014; Nonomura et al., 2018; Ranade et al., 2014a), touch (Ranade et al., 2014b) and pain (Murthy et al., 2018).

The PIEZO channels are activated by changes in the membrane tension in lipid bilayers, allowing their opening and influx of cations (Figure 3) (Syeda *et al.*, 2016).

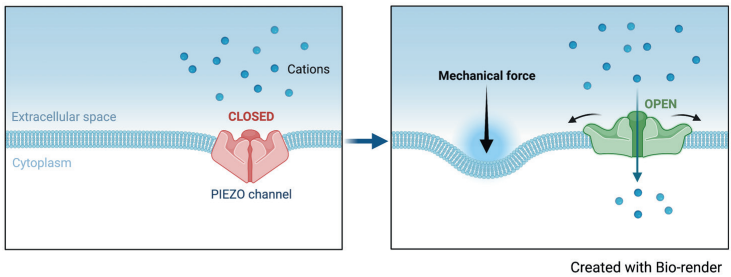

Figure 3 Illustration of PIEZO channel opening

PIEZO channels are blocked by peptide blockers, such as GsMTX4 (Bae et al., 2011). Both human (PIEZO1) and mouse (Piezo1) are specifically activated by the chemical agonist Yoda1, but not Piezo2. Yoda1 directly binds and activates PIEZO1, which allows an influx of Ca^{2+} ions. This activation does not require any cellular components other than the cell membrane (Syeda et al., 2015).

Some gastrointestinal and infectious diseases have been linked to mutations in PIEZO1 and several physiological processes require PIEZO1. During health, dying cells are removed by a process called epithelial apoptosis to maintain a healthy gut. The dying cells send signals to the adjacent cells to expel from the epithelium, and the live cells contract and facilitate the process. Mechanical forces are crucial to maintaining a healthy gut because abnormalities lead to diseases such as cancer (Eisenhoffer et al., 2012). Piezo1 promotes migration in gastric cancer cell lines (Yang et al., 2014). Patients who suffer from colorectal polyposis have mutations in the *PIEZO1* gene (Spier et al., 2016). In humans, the gain of function in the *PIEZO1* gene showed resistance to malaria due to red blood cell dehydration (Ma et al., 2018). Piezo1 is involved in gut and bone homeostasis through serotonin production. Deletion of the

intestinal epithelial *Piezo1* gene in mice blocked gut motility and increased bone formation (Sugisawa et al., 2020).

Murine urothelial cells express Piezo1. Under mechanical stimuli, the Piezo1 channel opens, which allows the influx of Ca^{2+} ions, leading to ATP release. In vitro studies have shown that deletion of *Piezo1* in bladder and kidney epithelial cells ablates stretch-activated channel activity (Miyamoto et al., 2014; Peyronnet et al., 2013). Piezo1 is expressed in lung immune cells such as myeloid cells to sense their external force and pressure to regulate the proinflammatory immune response (Solis et al., 2019).

Under fluid shear stress exerted by blood flow, the vascular endothelial mechanosensitive channel Piezo1 is activated and releases ATP. Piezo1-dependent ATP release is mediated by pannexin channels and activates subsequent Gq/G11-coupled purinergic $P2Y_2$ receptors to induce vasodilatory factors such as nitric oxide (NO) and vasodilation. Endothelial cells distinguish flow patterns such as laminar flow into atheroprotective signals and disturbed flow in atherosclerosis via the mechanosensitive channel Piezo1. Both flow patterns initially activate Piezo1, the purinergic $P2Y_2$ receptor, and Gq/G11-mediated signaling. However, only disturbed flow activates the downstream signaling pathway NF-κB by integrin activation (Albarran-Juarez et al., 2018; Wang et al., 2016).

Adenosine Triphosphate (ATP)

Inside cells, ATP acts as an energy source, building block of nucleic acids, and substrate for kinases that regulate biological processes such as ion transport, muscle contraction, nerve impulse propagation, and substrate phosphorylation (Dunn and Grider, 2022). ATP is released passively from

dying or damaged cells and actively from stressed cells or infected cells via membrane-associated channels such as pannexins or connexin (Cx) hemichannels. When ATP is extracellular, it acts as signaling molecule (Evans et al., 2006; Puhar et al., 2013; Venereau et al., 2015)

Connexin hemichannels are hexamers consisting of connexin protein subunits. These channels form gap junctions between adjacent cells by pairing with hemichannels from each cell, which help to communicate between the cytosol of neighboring cells. When hemichannels face the extracellular side, they allow to interchange molecules between the cytosol and the extracellular medium (Bai and Wang, 2014). There are 21 different connexin hemichannels known to date and they are named according to their molecular weight. The expression of connexins is tissue specific. Many connexin channel variants have been characterized in the intestine (Fiori et al., 2014; Maes et al., 2015). TC7 intestinal epithelial cells specifically express Cx26, Cx32, and Cx43 (Clair et al., 2008).

Pannexin and connexin hemichannels do not share any sequence homology, but they share a similar membrane topology. The pannexin subunits oligomerize and form a single membrane channel. These channels do not form any gap junctions between the cells and are always facing the extracellular medium. Loss of pannexin and connexin hemichannel functions are associated with pathological and inflammatory diseases (Maes *et al.*, 2015).

The ATP that comes out of the cells is called extracellular ATP (eATP). It acts as a signaling molecule during physiological processes, especially during neurotransmission in astrocytes. During pathological conditions, eATP is considered a Danger Associated Molecular Pattern (DAMP). The eATP is short-lived and degraded by ectoenzymes into ADP, AMP, adenosine (Ado), inosine (Ino), and uric acid. Under homeostatic

conditions, the intracellular ATP concentration varies from 3-10 mM, and the eATP concentrations are very low in range. The concentration in plasma varies from 400-700 nM. The adenosine concentration in plasma is usually 10-fold lower than the ATP concentration in plasma (Bours et al., 2006).

The eATP and its breakdown products play a crucial role in many biological processes, such as muscle contraction, cardiac function, vasodilatation, bone metabolism, liver glycogen metabolism, and inflammation (Agteresch et al., 1999; Bours *et al.*, 2006).

eATP acts as a signaling molecule by activating nucleotide receptors called purinergic receptors. There are two types of receptors, P1 and P2. The P1 receptor is activated by adenosine or inosine and subdivided into four receptor families: A1, A_2A, A_2B, and A_3. These receptors are activated depending on the concentration of the ligands. The P2 receptors are activated by ATP or other nucleotides. P2 receptors are further divided into two subfamilies: P2X receptors (P2XRs) and P2Y receptors (P2YRs). P2XRs are nucleotide-gated ion channels that allow the influx of Ca^{2+} and Na^{+} and efflux of K^{+} ions. P2YRs are G-protein coupled proteins (Junger, 2011; Venereau *et al.*, 2015).

The P2X and P2Y receptors are expressed on immune cells such as mast cells, macrophages, and dendritic cells as well as on IECs and are involved in the regulation of immune responses (Bours *et al.*, 2006; Coutinho-Silva et al., 2005).

Immune cells express P1 and P2, which shows the dual activity of purinergic signaling. The result of the signaling depends on the receptor expression and the immune responses by the receptors. eATP acts as a proinflammatory and immune modulatory signaling molecule. When

there is a high concentration of ATP outside the cells, the purinergic receptor P2 is activated and sometimes up-expressed by immune cells, which results in acute inflammation. At the same time, high levels of eATP activate ectoenzymes, which degrade ATP into Ado. The signaling molecule Ado initiates the anti-inflammatory pathways that contribute to the downregulation of immune responses. Thus, eATP and its breakdown products act in both ways (Bours *et al.*, 2006).

During infection of IEC with Gram-negative bacteria such as *Shigella flexneri*, ATP is secreted through connexin (Cx) hemichannels from the cytoplasm into the extracellular space (Puhar *et al.*, 2013). However, what triggers the opening of hemichannels is not known.

Shigella

Shigella is a rod-shaped, nonmotile, Gram-negative pathogen that causes bloody mucoid diarrhea, which can be life-threatening in children, elderly individuals, and immunocompromised people. There are four species, namely, *S. dysenteriae, S. boydii, S. flexneri,* and *S. sonnei.* Each of them is divided into different serotypes based on the structure of lipopolysaccharide O-antigen repeats. *S. dysenteriae*: 15 serotypes, *S. boydii*: 20 serotypes, *S. flexneri*: 14 serotypes and *S. sonnei* has a single serotype (Marteyn et al., 2012).

Very few bacteria are enough to cause disease, and in severe cases, they cause dysentery or shigellosis. These species are closely related to *E.coli* and have more than 90% homologous genes. *Shigella* acquired a virulence plasmid and two pathogenicity islands (PAIs), namely, SHI-1 and SHI-2. The SHI-1 encodes enterotoxin, protease, and mucinase, while SHI-2 encodes the aerobactin iron transport system (Schroeder and Hilbi, 2008).

Shigella infection occurs through contaminated food and water, the fecal-oral route, and person-to-person transmission, especially in developing countries, which have very poor sanitation facilities. Improving water sanitation decreased the infections of *S. flexneri* but not *S. sonnei*. For this reason, only improving sanitation is not efficient to eliminate *Shigella* infection but also requires vaccines. Vaccine development is a great challenge due to different serotypes and the absence of cross-protective antigens. Shigellosis is diagnosed by the presence of erythrocytes, polymorphonuclear neutrophils (PMNs), and mucus in patient stools and by doing PCR or ELISA for the virulence plasmid genes (Anderson et al., 2016; Hale and Keusch, 1996; Marteyn *et al.*, 2012; Maurelli, 2007; Sidik et al., 2014).

Upon ingestion, *Shigella* crosses the colonic epithelium with the help of microfold (M) cells and enters macrophages, which induce apoptosis. Next, it invades intestinal epithelial cells (IECs) from the basolateral side with the help of effector proteins (Zaidi and Estrada-García, 2014). However, recent studies indicate that *Shigella* can invade both the basolateral side and the apical side of IECs (Grassart et al., 2019).

Invasion of host cells by *Shigella*

The bacterium has a very large virulence plasmid (232 kb), which encodes a Type III secretion system (T3SS) as well as its effectors and regulators (Cervantes-Rivera et al., 2020). The activity of T3SS can be tested on Congo red agar plates at 37°C by streaking the bacteria. The dye highlights red colonies in which the T3SS is active, but the mechanism is not known. For *Shigella* infections, the T3SS is very important for invading nonphagocytic cells, such as intestinal epithelial cells (Bahrani et al., 1997; Sasakawa et al., 1986).

Shigella can invade nonprofessional phagocytes, such as intestinal epithelial cells, through a process called the trigger mechanism (Figure 4). In professional phagocytes, the bacterium plays a passive role in the invasion process, whereas in nonprofessional phagocytes, the bacterium is key and actively participates in the invasion process (Finlay and Cossart, 1997).

In the trigger mechanism, there are four stages involved for a successful interaction of the bacteria with the epithelial cell. The first stage is the pre-interaction stage, in which all effector proteins are stored in the cytoplasm and the T3SS is properly assembled. The T3SS is a needle-like structure spanning the bacterial double membrane, which injects effector proteins directly into the host cell cytosol once the bacterium makes contact with the host cell. Before the attachment of the bacterium to the cell surface, it is captured by the finger-like protrusions from the cells called filopodia, which retracts the bacterium to the cell surface (Blocker et al., 2003; Parsot et al., 2003).

Second stage is the interaction stage, in which *Shigella* are in contact with the host cell plasma membrane, the effector translocon complex is activated with the help of IpaB and IpaC with guided IpaD proteins in the needle tip. The activated needle tip injects effector proteins into the host cell cytosol (Figure 4) (Lafont et al., 2002).

Third stage is the formation of macropinocytic cup. The injected effectors make actin membrane rearrangements or ruffles to engulf the bacteria (Figure 4). At the early stage of entry the effector protein VirA, depolymerize the microtubules. IpaC mediates actin polymerization during entry, which leads to the formation of ruffles polymerization and disruption of epithelial cell tight junctions, which facilitates the

dissemination of bacteria to neighboring cells (Carayol and Tran Van Nhieu, 2013; Yoshida et al., 2002).

The final stage or fourth stage of interaction is actin depolymerization and closing of the macropinocytic cup. In this stage, IpaA binds to vinculin, a cytoskeletal protein that helps to depolymerize actin and close the cup (Carayol and Tran Van Nhieu, 2013; Cossart and Sansonetti, 2004; Parsot, 2005). After entering the IECs, the bacterium disrupts the vacuole membranes and gains access to the cytoplasm, where it divides and disseminates into the neighboring cells with the help of host actin protrusions (Ashida et al., 2015; Ogawa et al., 2008).

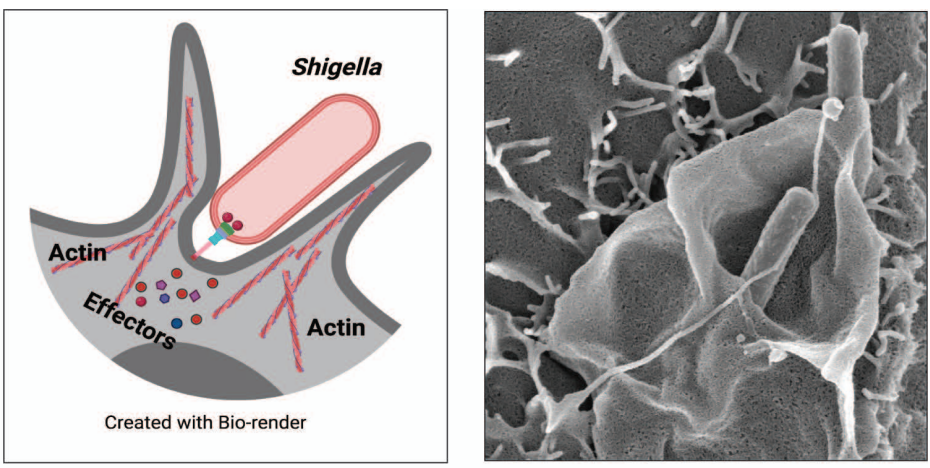

Figure 4 Schematic illustration of nonphagocytic cell invasion by *Shigella*

Shigella delivers a subset of effectors to evade immune responses from the host cell to promote its colonization and dissemination. It manipulates host inflammatory responses by effector proteins, including IpgD, OspI,

OspF, OspG, and IpaH. The effector OspG binds to ubiquitin proteins and prevents the activation of IκBα, and OspI prevents the ubiquitination of TRAF6 by deaminating UBC13, which is essential for the activation of the NF-κB pathway. IpaH dampens the acute inflammatory response by ubiquitinating TRAF2 protein, which is required for NF-κB activation. The effector protein IpgD acts as a phosphatase and generates PI5P to block ATP secretion through hemichannels from IECs upon infection to dampen ATP-mediated inflammation (Ashida *et al.*, 2015; Romero et al., 2011; Sasakawa *et al.*, 1986).

Listeria:

Listeria is a foodborne Gram-positive rod-shaped bacterium in soil that causes listeriosis in immunocompromised people. Currently, *Listeria* has 17 different species, of which two are considered pathogens, specifically *L. monocytogenes* and *L. ivanovii* (Milillo et al., 2012; Scallan et al., 2011).

The pathogenic *Listeria* strains can breach the intestinal, placental, and blood–brain barriers, leading to abortions in pregnant women and conditions such as gastroenteritis, inflammation in the central nervous system, and maternofetal infections. Unlike *Shigella* infections, which rely on the T3SS, the entry of *Listeria* into intestinal epithelial cells is mediated by either surface invasive protein called internalin A (InlA) with its host receptor E-cadherin (terminal 35 amino acids) or internalin B (InlB) with its host receptor Met through a zipper mechanism (Figure 5). Upon adhesion of bacteria, internalins induce actin polymerization in a Rac-dependent manner, which is mediated by the Arp2/3 complex and membrane extensions to form a phagocytic cup around the bacteria. After cup formation, retraction of bacteria into epithelial cells occurs (Cossart and Sansonetti, 2004).

During entry of the bacterium into epithelial cells, *Listeria* utilizes the *hly* encoded pore-forming toxin along with bacterial lipases PlcA and PlcB to lyse the vacuole and thereby reach the cytoplasm for replication, which is the next step in the pathogenesis of *Listeria*. Once in the cytosol, the bacteria polymerize the host cell actin with the help of ActA and disseminate it into neighboring cells (Lecuit, 2005; Pizarro-Cerda et al., 2012; Radoshevich and Cossart, 2018).

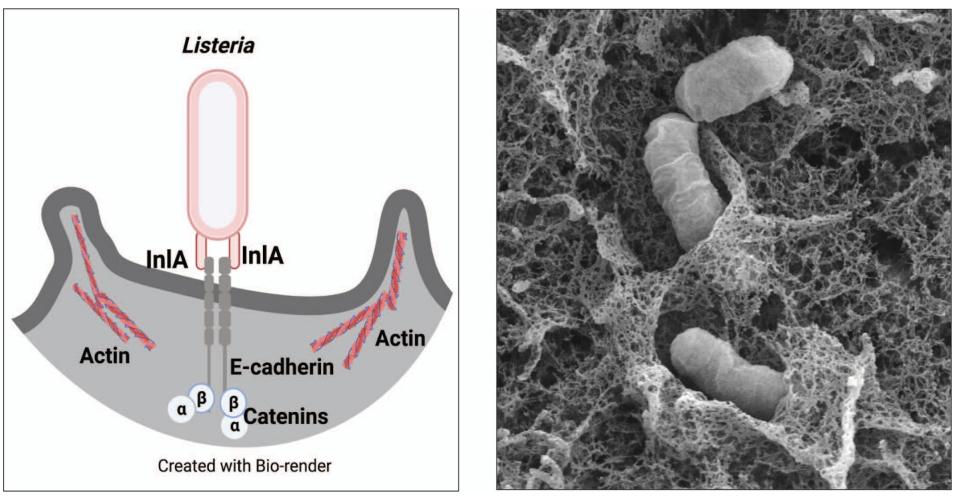

Figure 5 Schematic illustration of nonphagocytic cell invasion by *Listeria*

The significant difference between the pathogenic *L. monocytogenes* and nonpathogenic *L. innocua* is the presence of a virulence gene cluster. The gene cluster consists of *prfA*, *plcA*, *hly*, *mpl*, *actA*, and *plcB*, which is absent in *L. innocua* (Glaser et al., 2001). The pathogenic *Listeria* does not induce acute inflammation in the gut as other enteropathogens. The IECs secrete antimicrobial peptides, which kill the bacteria in the mucus

layer before reaching the epithelium. In immune-compromised people, *Listeria* can enter into the gut epithelium and result in the activation of TLRs mainly from bacterial compounds. There are MyD88-dependent pathways in the phagosome, STING/IRF3-dependent pathways in the cytosol, and AIM2-dependent pathways from the DNA released after lysis are activated by pathogenic *Listeria* infections (Witte et al., 2012).

Aims of the thesis

1. To identify the cues and molecular mechanisms triggering ATP secretion during invasive infection (Paper I)

2. To describe the role of extracellular ATP in human hantaviral infections (Paper II)

3. To investigate PIEZO1-dependent changes in the transcriptional landscape during invasive infection (Manuscript)

Results and Discussion

Paper I

In paper I, I found that not only Gram-negative bacteria such as *Shigella flexneri*, *Salmonella enterica* and enteropathogenic *Escherichia coli* (Puhar et al., 2013), but also Gram-positive invasive bacteria, such as *Listeria monocytogenes* and *Listeria innocua* expressing InlA, induce ATP secretion through connexin hemichannels in infected epithelial cells. During infection, actin and plasma membrane rearrangements play an important role in intestinal epithelial (nonphagocytic) cells. To test whether uptake of bacteria is necessary for ATP secretion, cytochalasin D (CytoD) and latrunculin B (LatB) were used to block actin cytoskeleton rearrangements and membrane ruffling, which resulted in reduced ATP secretion from intestinal epithelial cells during infection. Blocking the tubulin cytoskeleton with colchicine did not affect ATP secretion during infection.

To test whether ATP secretion is triggered by the transfer of bacterial molecules to the target cell cytosol or by cellular movements during bacterial entry, I used sterile latex beads of similar size to bacteria. Uptake of sterile latex beads induced ATP secretion through connexin hemichannel. Similarly to invasive infection, blocking the uptake of beads with CytoD or LatB abolished ATP secretion. These data indicated that mechanosensation of particle uptake is sufficient to induce ATP secretion, independent of the well-studied PRRs.

To test the expression of mechanosensitive channels in the intestinal epithelial cells, RNA sequence was used and showed that PIEZO1, but not PIEZO2, was highly expressed. To confirm the expression of PIEZO1 in the gut, human colonic sections were stained and showed the distribution of PIEZO1 throughout the plasma membrane. Immunofluorescence

showed that PIEZO1 is present in plasma membrane ruffles during invasive infection.

To check the activity of PIEZO1 in invasive infection, Yoda1, which is a PIEZO1 activator, was used, along with CytoD to block the invasion of bacteria or the uptake of beads, and I found that chemical activation of PIEZO1 can restore ATP secretion from intestinal epithelial cells. Furthermore, we tested the involvement of PIEZO1 by blocking the channel with the toxin GsMTx4 and found that there is no ATP secretion from the epithelial cells during invasive infection and uptake of sterile latex beads.

PIEZO1 mutant cell lines were made using enhanced CRISPR/Cas9 mutagenesis. I tested ATP secretion from mutant cell lines during stimulations by invasive infection and the uptake of sterile latex beads, and found no ATP secretion, indicating that PIEZO1 is necessary and sufficient for mechanically induced ATP secretion.

The opening of the PIEZO1 channel allows the influx of cations. To determine whether the influx of Ca^{2+} ions alone triggers ATP secretion, intestinal epithelial cells were stimulated with the calcium ionophore calcimycin, and the influx of Ca^{2+} ions was found to be sufficient to trigger ATP secretion.

Calcium chelation experiments were performed with the intracellular calcium chelator BAPTA-AM during invasive infection and uptake of beads to confirm the role of calcium during ATP secretion. I found that there was no ATP secretion in the presence of BAPTA-AM.

To confirm that the influx of Ca^{2+} ions occurs via PIEZO1 during membrane rearrangements, live-cell imaging was performed on the parental epithelial cells in the presence or absence of GsMTx4 and on the *PIEZO1* mutant cell line during invasive infection and uptake of sterile latex beads with the help of the fluorescent dye Fluo-4AM, which turns green during the influx of Ca^{2+} ions. These experiments showed that during invasive infection or the uptake of beads, the influx of Ca^{2+} ions through the PIEZO1 channel coincides with plasma membrane ruffling. From these results, we concluded that during invasive infection and the uptake of sterile latex beads, PIEZO1 is sufficient for the influx of Ca^{2+} ions and concurrent ATP secretion.

To determine whether activation of PIEZO1 induces gene expression, similarly to the downstream effects of activation of PRRs, a nanoluc-luciferase reporter assay was used to check the activation of the transcription factor NF-κB during challenge with Yoda1, beads, and Gram-positive and Gram-negative bacterial infections. The reporter assay showed that NF-κB is activated during all the stimulations at an early stage in the case of infections and for more extended times during chemical and mechanical stimulations with beads. Chemical or genetic inhibition of PIEZO1 reduced NF-κB activation at early time points.

To confirm that PIEZO1 induces gene expression and to investigate which pathways are activated upon mechanical stimulation, RNA sequence analysis was performed on intestinal epithelial cells during stimulation with Yoda1 and beads. Sequencing analysis showed that PIEZO1 activation regulates the expression of genes participating in innate immune pathways, epithelial barrier function, lipid and cholesterol biosynthesis, cell growth and survival.

To strengthen the findings that PIEZO1 activation leads to the activation of immune genes, an *in vitro* assay was performed, where the amount of the proinflammatory cytokine IL-8 was measured. The experiment was performed during chemical (Yoda1) and mechanical (beads) stimulation in the parental and *PIEZO1* mutant cell lines and in the presence of the PIEZO1 inhibitor GsMTx4. A significant amount of IL-8 production at 8 h upon Yoda1 and bead stimulation was observed and was repressed in the *PIEZO1* mutant cell line or in the presence of GsMTx4.

These results showed that PIEZO1 acts as a mechanosensor at the initial stages of infection, during the stage involving plasma membrane rearrangements, and triggers an immune response via the influx of Ca^{2+} ions, independently of PRRs.

Paper II

This paper is a collaborative work to understand the pathogenic mechanisms and humoral immune responses to human hantavirus infections. This is a clinical cohort study of hantaviral patients. The correlation between the typical features of diseases caused by Pumuula hantavirus infection, such as thrombocytopenia and transient kidney dysfunction, was investigated. During hantaviral infection, our collaborators found low expression of CD27 on the surface of B cells. It has been shown that extracellular ATP (eATP) induces shedding of CD27 on mouse lymphocytes.

From this knowledge, I set up a quantitative assay to measure ATP concentrations in fresh plasma, but not from frozen biobanked samples because of the short life and instability of eATP. I have also optimized the assay to measure breakdown products in the plasma of patients who are infected with hantavirus as an indirect measure of eATP in frozen samples. These experiments showed that during acute hantavirus infection and up to 3 months of convalescence, eATP levels in patient plasma are elevated with respect to a healthy cohort that I also characterized for reference.

To identify the mechanisms of the B cell surface marker CD27 shedding, in vitro assays were performed on isolated peripheral blood mononuclear cells (PBMCs). The PBMCs were incubated with different concentrations of eATP. We found decreased expression of CD27 and CD23 surface markers on B cells via a metallomatrix proteinase-8-dependent mechanism (MMP8).

Paper III

In this manuscript, the idea is to identify the genes, that are regulated during PIEZO1 activation in invasive infections. I have shown that the mechanosensitive channel PIEZO1 is activated during chemical and mechanical stimulation and results in gene expression in IECs. The activated PIEZO1 allowed the influx of calcium ions and concurrent ATP secretion. However, which signaling pathways lead to gene expression downstream of PIEZO1 activation at the post-translational level is not known. I performed immunoblot on the samples which were treated with PIEZO1 activator Yoda1 and looked for the phosphorylation of MAPK kinases and found phosphorylation of JNK and c-Jun during the chemical activation of PIEZO1.

RNA sequence was performed to determine the genes, that are regulated during both Gram-negative and Gram-positive bacterial invasive infections. This analysis showed immune pathways are upregulated during infection. To find the regulation of PIEZO1 in immune responses, PIEZO1 inhibitor GsMTx4 was used during infections.

The pathway analysis predicted that PIEZO1 potentiates immune responses during infections by regulating the NF-κB pathway and proinflammatory cytokines IL-8. To strengthen transcriptomic analysis, I performed an in vitro assay where I measured proinflammatory cytokine IL-8 using ELISA. I found that IL-8 production was reduced in the PIEZO1 mutant cells infected with invasive pathogens compared to the parental cells infected with invasive pathogens.

Conclusions

Paper I

- I have shown that not only Gram-negative bacterial infections but also Gram-positive bacterial infections induce ATP secretion via connexin hemichannels from intestinal epithelial cells.

- Plasma membrane rearrangements caused by invasion induce ATP secretion and serve as a mechanical immune signal.

- Mechanical immune signals are detected by the mechanosensitive channel PIEZO1.

- PIEZO1 triggers ATP secretion and protective gene expression programs via Ca^{2+} influx independently of PRRs.

- PIEZO1 acts as a sensor that detects invasive pathogens but not non-invasive pathogens or commensal bacteria.

Paper II

- There are two hallmark symptoms of haemorrhagic fever with renal syndrome (HFRS), which is caused by hantavirus: thrombocytopenia and kidney dysfunction.

- These symptoms are associated with antiviral B cell responses.

- During hantaviral infections, an increased number of plasma blasts or plasma cells and atypical B cells $CD27^{-}$ IgD^{-} is correlated with kidney dysfunction.

- The hantaviral particles are neutralized by the direct binding of envelope glycoprotein Gn (antigenic component) but not with nucleocapsid (N) protein.

- The elevated ATP and its breakdown products during hantaviral infection lead to shedding of the surface marker CD27 on the surface of B cells via a metallomatrix proteinase-8-dependent (MMP8) mechanism.

Paper III

- The chemical activation of PIEZO1 induces MAPK signaling.

- PIEZO1 potentiates immune responses by regulating NF-κB transcriptional factors during invasive infections.

- PIEZO1 activation leads to the release of proinflammatory cytokine IL-8 during both Gram-negative and Gram-positive infections.

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