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ROLES IN INNATE IMMUNE RECEPTORS: HOST - PATHOGEN INTERACTIONS AND STRENGTHENING VACCINES

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ABSTRACT

During the past decades our knowledge of innate immunology has increased drastically. This has improved our understanding about how innate immune cells can distinguish self from non-self, commensal bacteria from pathogens, and how it affects later adaptive immune responses. This knowledge can lead to new approaches for treatment of diseases and autoimmunity, and the development of new vaccines.

Vaccines are among the greatest inventions in medical history. However traditional vaccine approaches, such as live attenuated or inactivated viruses, have failed as vaccine candidates to address certain diseases including HIV/AIDS. Non-living non-replicating DNA vaccines represent an alternative approach, capable of inducing broad cell-mediated and humoral responses, while being safe and fast to produce. Still, despite its efficacy in animal models, DNA vaccines have not yet succeed to induce effective immune responses in human. To enhance the immunogenicity, a combination of more optimized vectors, delivery methods and adjuvants will be required. Skin electroporation (EP) is a promising method known to elicit robust humoral and $CD8^+$ T cell responses. However, the data on $CD4^+$ T cell responses has been limited. In **paper I** we compare immunization by skin EP with intramuscular injection, and find that EP increases both the magnitude and the polyfunctionality of the $CD4^+$ T cell responses to the HIV antigen Gag.

In **paper II** we show that plasmid encoding a secreted flagellin (pFliC) adjuvant promotes both humoral and MHC Class I-dependent cellular immunity when delivered through different routes representing dermal, systemic, and mucosal tissues. Additionally, it enhances mucosal humoral and MHC Class II-dependent cellular immunity when delivered mucosally. With *in vitro* studies we could show that secreted pFliC has the ability to activate macrophages through Toll-like receptor 5 (TLR5), but also cytoplasmic Nod-like receptor C4 (NLRC4), leading to inflammasome dependent cell death (pyroptosis).

In **paper III** we continue to study NLRC4 activation upon recognition of flagellin. We have established a system that allows for inducible expression of a NLRC4 agonist in a macrophage cell line, without additional stimuli. Using this system we have shown that NLRC4 induced caspase-1-dependent pyroptosis is independent of LPS priming, reactive oxygen species, or classical mitochondrial involvement. Nevertheless, pyroptotic macrophages release the alarmin high mobility group box 1 (HMGB1). Importantly, the functional isoform of HMGB1 is affected by the priming event and unprimed pyroptotic cells release a chemotactic form of HMGB1. However, priming during pyroptosis causes oxidation of the protein thereby changing it to a TLR4-agonist.

Combined, these studies will contribute to the understanding of the regulation of inflammasome activity, and how to deliver the next generation of DNA vaccines in combination with adjuvants.

POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Den här avhandlingen fokuserar dels på studier av det medfödda immunförsvaret och dels på olika sätt att öka effekten av DNA-vacciner.

Under de senaste årtionderna har vår förståelse för hur immunförsvaret fungerar ökat drastiskt. Inte minst gäller detta hur det medfödda immunförsvaret känner igen patogena bakterier och skiljer dem från den vanliga normalfloran. Celler i immunförsvaret uttrycker en uppsättning av olika receptorer som känner igen specifika ytstrukturer som är unika för patogena bakterier. En av dessa strukturer är flagellin, proteinet som bygger upp de flageller som bakterier använder för att röra sig.
Vaccin är en av de viktigaste uppfinningarna i medicinhistorien och räknat oräkneliga liv. Däremot har traditionella vaccinstrategier så som levande försvagat virus eller avdödat virus inte varit tillräckligt effektiva för sjukdomar som HIV/AIDS. Nya vaccintyper har tagits fram, så som DNA-vaccin vilket är plasmider som bär på gener som kodar för delar av viruset, så kallade antigen. Efter vaccineringen tas plasmiden upp av kroppens egna celler som skriver av DNA-koden och producerar antigenet. Antigenet kan då frisättas från cellerna och aktivera alla delar av immunförsvaret för att därigenom utveckla ett försvar mot viruset. DNA-vaccin utgör en säkrare, snabbare och potentiellt mer effektiv metod än traditionella vaccin. Dock har metoden inte visat sig vara effektiv i människa, trots framgångsrika djurstudier, och behöver optimeras. I **artikel I** har vi jämfört två olika sätt att administrera DNA-vaccin; standardmetoden intramuskulär och den lovande metoden intradermal elektroporering. Vi har visat att elektroporering ger ett både kraftigare och mer kvalitativt immunsvar.

Ett ytterligare sätt att optimera vaccin är tillsatts av adjuvans, en tillsats som förstärker och förbättrar immunsvaret. I **artikel II** studerade vi adjuvanseffekten av DNA-kodat flagellin när vaccinet administrerades på tre olika sätt: via nässlemhinna, via intramuskulär injektion och via huden. Vi visade att flagellin förstärkte effekten av vaccinet via alla tre administrationsvägarna. När vaccinet administrerades via nässlemhinnan förstärkte flagellin dessutom försvaret i övriga slemhinnor vilket är fördelaktigt eftersom slemhinnorna är infektionsvägen för de flesta virus.

För att förstå mekanismerna bakom adjuvanseffekten studerade vi hur makrofager reagerar på flagellin. Makrofager är en celltyp idet medfödda immunförsvaret vars roll är att hitta patogener och visa upp dem för övriga celler i immunförsvaret för att på så sätt dra igång ett försvar som kan hantera infektionen. Makrofager känner igen flagellin med hjälp av två olika receptorer; Toll-like receptor 5 (TLR5) som uttrycks på cellytan och Nod-like receptor C4 (NLRC4) inuti cellen. Aktivering av makrofagen genom TLR5 gör att cellen producerar signalsubstanser som startar valda delar av immunsystemet. Aktivering av NLRC4 medför en hopsamling av ett proteinkomplex kallat inflammasom i cellen. Inflammasomen klyver de kraftfulla signalsubstanserna IL-1β och IL-18 vilket gör att de kan frisättas från cellen. Dessutom inleder inflammasomen en självmordsprocess i cellen som gör att cellen dör i så kallad pyroptos. Den här mekanismen gör att bakterier inte kan uppehålla sig och föröka sig i cellen. I kastad de ut och kan dödas av andra immunceller som lockats till området av de cellstrukturer som frisatts under celldöden. I **artikel II** kunde vi visa att den formen av flagellin som vi använt som

adjuvant hade kapaciteten att aktivera makrofager via både TLR5 och NRLC4, vilket kan förklara den breda aktivering av immunförsvaret.

I **artikel III** fortsatte vi att studera NLRC4-inflammasomen i cellkulturer. Vi kunde visa att flagellin kan inducera pyroptos oberoende av andra stimuli. Vidare kunde vi visa NLRC4-inflammasomen själv inte gav upphov till eller påverkades av syreradikaler eller involverade cellens mitokondrier. Det enda som påverkades av andra ytterligare stimuli simultant med aktivering av NLRC4-inflammasomen var i vilken form varningsmolekylen HMGB1 frisattes i. Eftersom HMGB1 har olika funktioner beroende på dess form innebär det celler kan reagera olika på celler som dör i pyroptos beroende på vilka stimuli cellen får samtidigt som NRLC4-inflammasomen aktiveras. Det kan vara värdefull kunskap för att förstå både hur immunförsvaret fungerar vid sjukdom och autoinflammatoriska sjukdomar.

LIST OF PUBLICATIONS

I. Bråve, S., **Nyström, S**., Roos, AK., Applequist, SE. 2011. Plasmid DNA Vaccination using skin electroporation promotes poly-functional CD4 T-cell responses. *Immunol Cell Biol.* Mar:89(3):492-6

II. **Nyström S**, Bråve A, Falkborn T, Devito C, Rissiek B, Johansson DX, Uematsu S, Akira S, Hinkula J, Applequist SE. DNA-encoded flagellin activates TLR5, NLRC4, and acts as an epidermal, systemic and mucosal adjuvant. Submitted

III. **Nyström S**, Antoine DJ, Lundbäck P, Lock JG, Nita AF, Högstrand K, Grandien A, Erlandsson-Harris H, Andersson U, Applequist SE. 2013. TLR activation regulates damage-associated molecular pattern isoforms released during pyroptosis. *EMBO J*. Jan 9;32(1):86-99

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LIST OF ABBREVIATIONS

HIV	Human immunodeficiency virus
AIDS	Acquired immunodeficiency syndrome
DNA	Deoxyribonucleic acid
EP	Electroporation
CD	Cluster of differentiation
pFliC	Plasmid encoded flagellin
MHC	Major histocompatibility complex
TLR	Toll-like receptor
NOD	Nucleotide oligomerization domain
CARD	Caspase activation and recruitment domains
NLRC4	NLR family CARD domain-containing protein 4
LPS	Lipopolysaccharid
HMGB1	High-mobility group box 1
PRR	Pattern recognition receptors
APC	Antigen-presenting cells
DC	Dendritic cell
PAMP	Pathogen associated molecular patterns
DAMP	Danger-associated molecular pattern
ATP	Adenosine triphosphate
CLR	C-type lectin family
RIG-I	Retinoic acid inducible gene I
RLR	RIG-I)-like receptors
Th	T helper
RNA	Ribonucleic acid
IRF	Interferon response factor
IFN	Interferon
LRR	Leucin-rich repeats
TIR	Toll/IL-1 receptor
MyD88	Myeloid differentiation primary response gene (88)
TRIF	TIR-domain-containing adapter-inducing interferon-β
RIP	receptor-interacting protein
PYD	Pyrin
IAP	Inhibitor of apoptosis
BIR	Baculovirus IAP repeat
ASC	Apoptosis associated speck-like protein containing a CARD
NLRP	NACHT, LRR and PYD domains-containing protein
NAIP5	NLR family, apotosis inhibitory protein
AIM2	Absent in melanoma 2
LCMV	Lymphocytic Choriomeningitis Virus
IL	Interleukin
ER	Endoplasmic reticulum
NO	Nitric oxide
ROS	Reactive oxygen species
PKR	Protein Kinase R
TXNIP	Thioredoxin-interacting protein
PKCδ	Protein kinase C delta
MDP	Muramyl dipeptide

XIAP	X-linked inhibitor of apoptosis
T3SS	Type III secretion system
T4SS	Type IV secretion system
EHEC	Enterohemorrhagic *Escherichia coli*
EPEC	Enteropathogenic *Escherichia coli*
Mtb	*Mycobacterium tuberculosis*
PI-9	Protease inhibitor 9
CrmA	Cytokine response modifier A
IBD	Inflammatory bowel disease
CAD	Caspase-activated DNase
ICAD	Inhibitor of caspase-activated DNase
MOMP	Mitochondrial outer membrane permeabilization
Apaf-1	Apoptotic protease activating factor 1
TNF	Tumor necrosis factor
TRAIL	TNF-related apoptosis inducing ligand
DISC	Death-inducing signaling complex'
DD	Death domain
FADD	FAS-associated protein with a DD
BID	BH3-interacting domain death
PARP	Poly (ADP-ribose) polymerase 1
S1P	Spingosine 1-phosphate
PtdSer	Phosphatidylserine
Treg	Regulatory T cells
Ig	Immunoglobulin
CTL	Cytolytic T lymphocyte
NK cell	Natural killer cell
MPLA	Monophosphoryl lipid A
CpG	Cysteine poly-Guanosine
TBK-1	TANK-binding kinase-1
STING	Stimulator of interferon genes

1. INTRODUCTION

The immune system can be subdivided into the innate and the adaptive immune systems. Innate immunity is genetically predetermined and a rapid first line of defense, but is limited in its ability to adapt to a changing microbe. As it genomically static it does not carry a large memory of pathogenic structures. Responses by the adaptive immune system on the other hand develop when a person is exposed to pathogens. Although the responses develop slower relative to innate immune responses, the adaptive response has the capacity to form immunological memory to prevent future reinfection with the same pathogen. Both of these arms are important for efficient host defense, and to develop protective vaccines.

The focus of this thesis is pathogen recognition by the innate immune system. Specifically, recognition of bacteria by inflammasome complexes that induces pyroptosis and cytokine release. The second focus is improvement of plasmid DNA vaccines, by adjuvants used to activate the innate immune system and enhanced delivery methods. To provide a background, this first part will be an introduction to the immune system, with the main focus on innate immunity. This will be followed by a presentation of the materials and methods used in this work, and the results together with discussion. Also included are the three papers on which this thesis is based.

1.1 THE INNATE IMMUNE SYSTEM

Traditionally, innate immunity has been viewed as a fundamental first defense-line, pivotal for discriminating self from non-self and friend from foe, but not as specific as the adaptive immune system. However, it is a sophisticated system that also has a role in orchestrating the adaptive responses. Innate immune responses can be elicited in nearly all known cell types. Physical barriers such as the skin and mucosa, as well as anti-microbial molecules such as anti-microbial peptides and the complement system are also parts of the innate immunity.(1)

To rapidly recognize and identify different classes of invading pathogens and initiate responses accordingly, a variety of germ line-encoded receptors specific for conserved structures associated with pathogens and general danger molecules have evolved. These pattern recognition receptors (PRRs) are expressed by most cell types but within the immune system, particularly by the antigen presenting cells (APCs), namely DCs and macrophages. PPRs identify pathogen associated molecular patterns (PAMPs) that are highly conserved molecular structures shared by large groups of microbes, but also danger-associated molecular patterns (DAMPs), typically signs of damaged cells, such as extracellular ATP, or a variety of intracellular proteins. Various classes of PRRs have been discovered including the Toll-like receptors (TLRs), members of the C-type lectin family (CLRs), cytosolic retinoic acid inducible gene I (RIG-I)-like receptors (RLRs) and nucleotide oligomerization domain (NOD)-like receptors (NLRs).

The CLRs consist of around one thousand members although all of them are not involved in recognition of PAMPs. These receptors typically recognize carbohydrates that are rich in mannose, fucose and glycan. The downstream result of binding to CLRs is often classified as activation of adaptive Th17 or Th1 responses.(2)

However, with so many family members, it is likely that they will be found to perform other functions as well.

RLRs, expressed by most cell types, respond to viral double-stranded RNA (dsRNA) in the cytosol. RLR binding of dsRNA from virus such as Flavi viruses, influenza and Epstein-Barr. Generally, they have been observed to initiate signaling through NF-kB and Interferon Response Factor 3 (IRF3) leading to the production of pro-inflammatory cytokines and Type-I and III IFNs.(3)

This thesis is built around the recognition of the PAMP flagellin by NLRs and TLR5, the introduction will therefore focus on these receptors and how they affect immune responses.

1.1.1 TLRs

The most well studied PRRs are the TLRs, first discovered in 1985. Today, there are 10 known TLRs in human beings and 13 in mice (4). The TLRs are class I transmembrane proteins found both on the cell surface (TLR1, -2, -4, -5, and -6) to detect various PAMPs and others are found in the endosome (TLR3, -7, -8, and -9) where they can detect viral PAMPs or bacterial nucleic acids.

The receptors consist of extra-cellular ligand binding leucine-rich repeat domains (LRRs) and cytoplasmic TLR signal domains known as Toll/IL-1 receptor (TIR) domains that transduce the signals through interactions with cytoplasmic adaptor proteins.(5) TLRs are expressed as dimers in a low-affinity complex and can form both homodimers and heterodimers, for example TLR1/TLR2. Once the ligand binds, a conformational change brings the two TIR domains closer together, allowing for interaction with adapter proteins.(5) The adapter protein is in most cases MyD88, which activates a signaling cascade ending with activation of transcription factors (NF-κB and/or AP-1) and phosphorylation of for example interferon regulatory factors (IRFs), which activates the type I IFN promoters. The MyD88 independent exceptions are TLR3, which recruits only TRIF, and TLR4 that can utilize both MyD88 and TRIF as adaptors. TRIF signaling and RIP kinases leading to up-regulation of NF-kB-inducible cytokines and phosphorylation of IRF-3. The TRIF-signaling pathway is activation production of both NF-kB induced release of cytokines and type I and type III IFNs.(4, 6)

1.1.2 NLRs, caspases and inflammasomes

The nucleotide-binding domain leucine-rich repeat containing (NLR) family represents the largest group of intracellular PRRs. To date it is compromised of 22 human genes and 34 mouse genes.(7) Similar to TLRs, the NLRs contain three domains. All NLRs share a common central nucleotide-binding and oligomerization (NACHT) motif, enabling for activation of the signaling complex through ATP-dependent oligomerization. This domain is typically flanked by C-terminal LRRs, thought to function in ligand sensing and to modulate NLR activity through auto-repression (8). The N-terminus contains either a caspase recruitment (CARD) domain, a pyrin (PYD) domain or a baculovirus IAP repeat (BIR) allowing for recruitment of adaptor proteins, typically caspase-1 and apoptosis associated speck-like protein containing a CARD (ASC).
The NLRs can be divided into three phylogenetic subfamilies (9). Members of the nucleotide-binding oligomerization domain (NOD) subfamily (NOD1-2,

NOD3/NLRC3, NOD4/NLRC5, NOD5/NLRX1, CIITA) have diverse functions including acting as co-activators and NF-kB activating platforms. The remaining two groups consist of NLRs known to be involved in inflammasome formation. The NLRP (NACHT, LRR and PYD domains-containing protein) subfamily include NLRP1-14, while the IPAF subfamily consists of NLR family CARD domain-containing protein 4 (NLRC4) and Neuronal apoptosis inhibitory protein.

Shortly after the discovery of the NLRs, they were hypothesized to recruit caspases, because of their CARD/PYD domains. Caspases are cystein proteases that are most known to have essential roles in apoptosis, but they can also induce inflammation. Dogmatically speaking, caspases are categorized into proapoptotic (caspase-2, -3, -7, -8, -9 and -10) and proinflammatory (caspase-1, and -11 in mouse and caspase-1, -4, and -5 in human)(10). The best characterized proinflammatory caspase is caspase-1. It is a rather promiscuous protease, highly expressed in leukocytes, monocytes and epithelial cells (11) which is best known to process IL-1β and to induce a form of regulated cell death called pyroptosis (8). Similar to most other caspases, caspase-1 is synthesized as an inactive monomeric zymogen and acquires proteolytic activity upon dimerization and autoproteolytic processing into the subunits p20 and p10 (12, 13). Notably, and important to remember in the context of this thesis, this processing step is not required for the induction of pyroptosis (14).

In 2002 Martinon et al demonstrated that NALP1 recruited and activated caspase-1 and 5, leading to release of active IL-1β and IL-18 as well as initiation of pyroptosis (8). The caspase-1 activation was dependent on assembly of a large protein complex (700 kDa), containing caspase-1, NALP1 and ASC. The protein complex was termed inflammasome because of its similarities with the apoptosome and the term was subsequently broadened to describe further caspase-1 recruitment platforms. So far, the NLR family members reported to be part of inflammasomes are: NLRP1b, NLRP3, NLRC4/NAIP5 (10) and more recently NLRP6 (15) and NLRP1a (16). In addition to these, certain non-NLRs can also induce inflammasome assembly, namely the DNA sensor absent in melanoma 2 (Aim2)(17) and the RLR receptor retinoic acid inducible gene I (RIG-I)(15) (18). (Figure 1) Other candidates for inflammasome formation based pilot studies are NLRC5(19) and NLRP7 (20). Inflammasome function has been observed mainly in myeloid cells, but also in nonmyeloid cells such as keratinocytes and astrocytes.(21, 22) Importantly, numerous other cell types also express inflammasome components but their role in immunity and disease is poorly studied.

Upon detection of their respective ligands, ASC is recruited to the NLR through PYD or CARD interactions. Because ASC contains both a PYD and a CARD, it can oligomerize with certain NLRs to form a cytosolic structure, and also recruit caspase-1. The CARD containing NLRs can recruit caspase-1 directly whereas the PYD containing NLRs are totally dependent on ASC. This does not mean that ASC may not have affects on CARD containing NLRs. It has been observed that the presence of ASC could influence the function of CARD containing NLRs(23). However, currently how this could occur is poorly understood.

The NLRP1a assembles in response to LCMV and 5-fluorouracil, NLRP1b to inflammasome anthrax lethal toxin (24), Aim2 is activated by dsDNA (17) and RIG-I responds to certain RNA viruses such as VSV and serves as a dual sensor that can trigger both NF-kB-dependent expression of pro- and inflammasome activation(18). The ligands and signaling pathways for NLRP6 are less clear. It contributes to

protection against colitit, colorectal tumorigenesis and non-alcoholic steatosis by regulating the gut epithelia and microflora.(25,26) However, mice with NLRP6 are less resistant to bacteria such as *S. typhimurium* and *Listeria monocytogenes*.(27) It does appear that various agonist are able to activate the same, or similar NLR family members which makes it difficult to understand the precise molecular mechanisms of how these systems work. Nevertheless, these systems may play a key role in the sensing of infection and cell stress and further work is warranted.

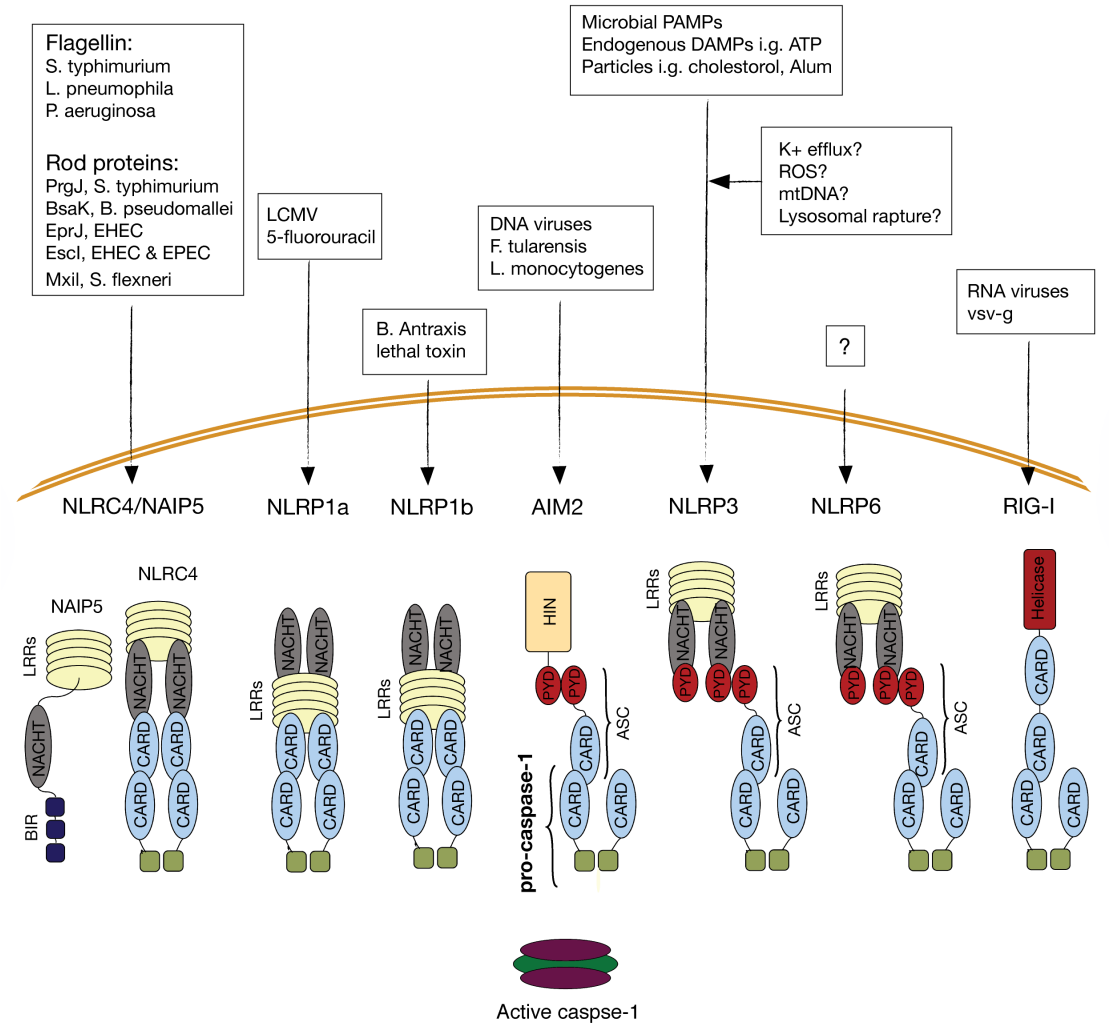

Figure 1: The currently established inflammasomes.

IL-1β is a powerful inflammatory mediator contributing to acute systemic and local inflammation by inducing fever, recruitment of leukocytes and promoting adaptive immune responses.(28) However, IL-1β also contributes to several chronic conditions and must be rigorously controlled at several levels. First, triggering of TLRs is required for expression of the precursor form, which accumulates in the cytosol until it, secondly, is cleaved into its mature release form. The mature IL-1β lacks a conventional secretion signal(29), and it is not secreted through the standard Golgi-ER pathway, but its release is controlled by a poorly understood mechanism.(Further discussed in section 4.4) Additionally, an endogenously produced and secreted IL-1 receptor antagonist (IL-RA) can also regulate IL-1ß action after release indicating an important biological need to control this inflammatory mediator.

In contrast to pro-IL-1β, pro-IL-18 is constitutively expressed and apparently does not rely on priming. Its function after release depends on the cytokine environment. In presence of IL-12p40, IL-18 induces production of INF-γ by T and NK cells, thereby promoting Th1-like responses. In the absence of IL-12, it instead induces production of IL-4, IL-5 and IL-10, thereby promoting a Th2-like response. It is also known to synergize with IL-23 to enhance the IL-17 production from Th1 cells. Additionally, IL-18 can stimulate the synthesis of nitric oxide (NO) and reactive oxygen species (ROS) as well as induce expression of other cytokines, chemokines, and cell adhesion molecules.(30-32). Importantly, there are reports of the importance of caspase-1-dependent IL-18 production in protective immunity against pathogen infection. For example, protection against *S. flexneri* infection is controlled by IL-18, not IL-1β (33).

In addition to caspase-1, its homologue caspase-11 (in human caspase-4) has been presumed to have a role in inflammasome function. Studies of the impact of caspase-11 have been difficult since caspase-1 and -11 are too close in the genome to be segregated by homologous recombination and furthermore, caspase-11 is dysfunctional in strain 129 mice. A strain of mouse commonly used to generate genetic deficiencies. Hence, the Casp1-/- developed using 129 mouse embryonic stem cells used in many publications also lack caspase-11 (34). Lately new mice have been generated and caspase-11 has been reported to have a crucial role in alternative inflammasome activation called the non-canonical inflammasome pathway. Activation of this non-canonical pathway is essential for inflammasome response to *Escherichia coli, Citrobacter rodentium, Vibrio cholerae*, cholera toxin B(34) as well as *S. typhimurium* (35) and its defense against cytosolic, but not vacuolar bacteria.(36). Cell death induced by this pathway is independent of NLRC4/NLRP3 and caspase-1. IL-1β processing on the other hand requires NLRC4/NLRP3, and is impaired in the absence of caspase-1 (34, 35). Furthermore, LPS toxicity has been shown to be dependent on caspase-11 rather than caspase-1, suggesting that caspase-4 in human may be better targets than caspase-1 in sepsis treatment (34, 37).

Additional molecules have been reported to modulate inflammasome activation. NLRC4 activity requires phosphorylation by the kinase PKCδ between the NACHT and LRR domain in response to *S. typhimurium* (38). NLRP3 activity, and possibly also other inflammasomes, is dependent on Protein Kinase R (PKR). PKR interacts with all known parts of the inflammasome complex and mutant PKR carrying a Lys296Arg mutation lacking kinase activity, fails to activate the NLRP3 inflammasome (39). This finding has however been contradicted in a later publication (40). The thioredoxin-interacting protein (TXNIP), a protein linked to insulin resistance, has been identified as a redox-sensitive ligand of NLRP3 (41). NLRP3 inflammasome activators trigger binding of TXNIP to NLRP3 and its activation is impaired in mice deficient in TXNIP (41). However, these results have not yet been confirmed by other groups (42).

1.1.2.1 NLRP3

NLRP3 is the most well characterized inflammasome, activated by a broad range of stimuli utilizing a two-step mechanism. As NLRP3 is not expressed in resting cell, it requires a NF-kB-dependent priming event as a first signal (43). The second signal can be mediated by a great variety of stimuli, such as ATP, bacterial pore-forming

toxins (44), urate crystals (45), asbestos and silica (46), muramyl dipeptide (MDP) (47, 48), bacterial DNA and RNA, viral RNA and imidazoquinolone antiviral compounds(49,50), components of fungal cell wall such as zymosan and mannan (51), the adjuvant Alum (52), and cholesterol (53) LPS alone has also been used to activate the NLRP3 inflammasome but IL-1β is then processed very inefficiently (54, 55). It can also be discussed if the activation is due to LPS itself or ATP/mtDNA release caused by LPS stimulation or in some cases contaminating agonists such as MDP (47). However, LPS-priming alone can induce capase-1 activation in macrophages genetically deficient in XIAP, cIAP1 and cIAP2 (56) and it is possible that variations in their expression or function may affect inflammasome activation.

Even with all these different activators, no direct ligand binding has been observed and how NLRP3 detects the ligands is incompletely understood. Although the precise mechanism of NLRP3 activation is still debated, it is suggested to involve K^+ efflux (57,58), reactive oxygen species production (9,59), lysosomal disruption (60), mitochondria dysfunction (61, 62) and to be dependent on intrinsic apoptosis-mediated loss of the mitochondrial inner membrane potential, leading to release of oxidized mitochondrial DNA that activates the NLRP3 inflammasome (63). It may also be dependent on a combination of these factors. As NLRP3 has a PYD domain it is dependent on ASC for recruitment of caspse-1. Furthermore, the activation is thought to be controlled by ASC, microRNA, ubiqutination, phosphorylation and calcium.(64)

In conclusion, activation of the NLRP3 inflammasome is a potent response to various stimuli of both bacterial, endogenous, and particle origin. Because of the diversity of the agonists, the need for a priming signal may be of importance to avoid auto-inflammatory states. Although the NLRP3 inflammasome has been extensively studied, there are still new agonists being reported and many questions remain. For example, the mechanisms for recognition of ligands and how they induce the inflammasome assembly is generally unknown. Furthermore, even though "the inflammasomes" are being grouped together as caspase-1-activating platforms, new knowledge regularly reveals their functional differences.

1.1.2.2 NLRC4

NLRC4 is expressed in myeloid cells, with exceptions including neutrophils, but also additional cell types including intestinal endothelial cells (65, 66). It is activated by cytosolic flagellin, the monomer of the bacterial swimming device flagella, present in most Gram-negative and Gram-positive bacteria.(67, 68) Additionally it responds to components of the bacterial type III and type IV secretion systems (T3SS and T4SS). These systems are major virulence factors in most Gram-negative bacteria and function to transfer effector proteins from the bacteria into the host's cytosol. The secretion apparatus attaches to the bacteria with basal rod proteins and forms a hollow needle complex that spans the bacterial membrane. Mouse NLRC4 recognize rod proteins from bacteria including *S. typhimurium* (PrgJ), *Burkholderia pseudomallei* (BsaK), *E. coli* (EprJ and EscI), *S. flexneri* (MxiI), and *Pseudomonas aeruginosa* (PscI).(69, 70) All of these components share sequence and structural similarity(69, 71). Flagellin is typically transferred into the cytoplasm through the bacterial secretion system. For example activation of NLRC4 by *S. typhimurium* does not only require flagellin, but also a functional T3SS (68, 72-74). However, purified flagellin

monomers can also be sensed by NLRC4, suggesting that at least some cells have the means of cytosolic uptake of free flagellin(75).

In contrast to NLRP3, NLRC4 is constitutively expressed and can be activated by flagellin without the need of a priming signal.(59) (**paper III**). Furthermore, it has a CARD domain and can recruit caspase-1 and initiate pyroptosis independently of ASC (76), although the IL-1β production is severely impaired in the absence of ASC (77).

NLRC4 agonists are not sensed directly by NLRC4 but instead bind members of the BIR domain containing NLR family apotosis inhibitory protein (NAIP) family members. In mice, NAIP5 and probably also the closely related NAIP6, can directly bind flagellin (70, 78) while the rod proteins are recognized by NAIP2 (70). Ligand binding promotes a physical association between NAIP5/6 and NLRC4 resulting in assembly and activation of the inflammasome complex (70, 78, 79).

The mouse genome encodes seven NAIP proteins while the human only harbors one, a fact that did concern researches for some time. Recently, the human NAIP has been shown to also recognize components from bacterial secretion system, to subsequently induce NLRC4 inflammasome activation. Needle subunits from the T3SS of *S. typhimurium* as well as EHEC, *B. thalandensis*, *P. aeruginosa*, *S. flexneri* can interact with the human NAIP indicating that it can function in a similar way as the mouse NAIP5(6)/2. There appears to be a broad conservation between the human and mouse NAIPs in terms of structural recognition and biological function, even though the exact ligands and receptors differ (70, 78).

In conclusion, NLRC4/Naip5 activation is clearly a rapid and important defense against intracellular bacteria. It is in some ways better understood than the NLRP3 inflammasome, since there is a defined ligand binding triggering assembly. However, there are still many questions remaining regarding its regulation and the induction of pyroptosis. For example, how caspase-1 functions to execute pyroptosis without being proteolytic processed.

1.1.3 Flagellin

Flagellin has been known to induce innate immune responses since 1998, when it was reported that it was a potent inducer of cytokines from human monocytes (80). Later on it was established that flagellin rapidly induces activation of IL-1R-associated kinase 1 and the receptor for extracellular flagellin was found to be TLR5(81, 82). Intracellular localized flagellin signals via NLRC4/NAIP5 (67, 68, 71) and the distinct domains required for these interactions are completely separate from those of TLR5. Most flagellin monomers have four domains (D0, D1, D2 and D3) and are shaped as a bent hairpin, with the D3 domain forming the looped end of the hairpin and the D1 and D2 regions comprising the arms. The D0 and D1 domains are conserved and while the D2 and D3 domains are highly variable in primary sequence and length can be used to discriminate different bacteria. The D1 portion of the conserved C- and N-terminal domains of flagellin is required for binding to TLR5 (83, 84). This binding can be abolished by an mutation at residue I411A but conformational changes can also abolish binding (85). The interaction of flagellin with NLRC4/Naip5 instead requires the 34-35 amino acid long tail at the C-terminal end of the D1 portion (71) (**paper III**). Specific residues within this tail are also required for activity (86).

Properties of flagellin are studied in two of the papers in this thesis. In paper II we evaluated the adjuvant properties of DNA-encoded flagellin, and in paper III we focused on flagellin-induced activation of the NLRC4 inflammasome.

1.1.4 Microbial inflammasome evasion

To survive in the host, pathogens have acquired sophisticated mechanisms to interfere with the host responses to infection. Pathogen-mediated control of inflammasome recognition can allow for intracellular replication and reduced influx of immune cells. For such evasion strategies, all stages of the inflammasome function can be targeted. *S. typhimurium* encodes two distinct T3SS that promote different virulence aspects. During the infection phase it expresses the *Salmonella* pathogen island 1 (SPI1) T3SS including the rod protein PrgJ, which is recognized by NLRC4. To avoid recognition during the systemic phase and promote intracellular replication, this T3SS is later repressed and replaced by the SPI2 system including the rod protein Ssal, which is not recognized by NLRC4 (68, 87, 88). Similarly, *Yersinia enterocolitica* shuts down its flagellin expression in temperatures above 37°C (89). Other bacteria such as EPEC, *C. violaceum* and *B. thailandensis* express flagellin unable to bind NAIP5 and can thereby evade detection (70). These escape mechanisms may be one reason to the slightly redundant recognition of bacterial structures by the inflammasomes (90).

Yersinia also produces a set of T3SS effector molecules called Yop proteins. The Yops can hijack intracellular machinery in order to interfere with the inflammatory response and phagocytosis. YopE and YopT prevent oligomerization of caspase-1 by interfering with certain Rho GTPase proteins, thereby disassembling the actin skeleton. Rho GTPases regulate the intracellular actin dynamics, which somehow is involved in caspase-1 activation. YopE acts as a GTPase-activating protein, switching off Rho GTPases by accelerating GTP hydrolysis, while YopT acts as a cystine protease and inactivates Rho GTPases by cleaving of their C-terminal prenyl membrane anchor (91). More recently, YopK of *Y. pseudotuberculosis* was shown to associate with the T3SS translocon to mask its detection by the inflammasome (92).

Infections with *Mycobacterium tuberculosis* (Mtb) or the vaccine strain Mycobacterium bovis (BCG) are associated with low levels of IL-1β. One reason may be that BCG produces a Zn^{2+} metalloprotease that inhibits processing of IL-1β by a currently unknown mechanism (93).

In contrast, *S. flexneri and S. typhimurium* can also activate the inflammasome and utilize the ensuing inflammation for invasion and disease progression. Caspase-1-deficient mice confer resistance to these bacteria (94-96).

Viruses also have strategies to evade detection by the inflammasome. Common viral strategies are to mimic the host suppression of the inflammasomes, to target especially ASC and caspase-1. Certain Pox-viruses have a PYD containing protein analogous to the host's cPOP proteins (97) that bind ASC to prevent inflammasome IL-1β processing. Additionally Poxviruses produce viral serpins analogous to the mammalian protease inhibitor 9 (PI-9) protein. PI-9 is constitutively expressed in vascular smooth muscle cells and prevents processing of pro-IL-1β and pro-IL-18 by blocking the active site of caspase-1 (98). The best known inflammasome inhibitor is the cowpox gene CrmA, which is a potent inhibitor of Caspase-1 and -8 (99). Other viral caspase-1 inhibitors are NS1 from influenza A viruses which inhibits PKR and type I interferon response, and the apoptotic protein pR35 from baculovirus (100).

Microbes have evolved to escape the inflammasomes in many different ways. Some use strategies to avoid recognition or inhibit involved proteins while other take advantage of the system. An improved understanding of their evasion mechanisms and hijacking is necessary for both better prevention and treatment of infections. Mimicking the mechanisms may also be an option to control inflammasome regulation.

1.1.5 Deregulation of inflammasomes

Inflammasome activity is important for clearance of bacteria and viral defense, but when dysregulated, it can cause autoimmune diseases. Gain of function mutations in the gene coding for NLRP3 are associated with a group of autoinflammatory diseases collectively termed cryopyrin-associated periodic syndrome (CAPS), characterized by cyclical periodic fever and inflammation due to increased NF-kB signaling, caspase-1 activation and production of IL-1β (101, 102). More than 70 inherited and disease-associated mutations have been identified so far, most of them are situated within and around the NLRP3 NACHT domain (103). Fortunately, CAPS patients with excessive IL-1β production have benefited from IL-1 neutralizing therapies(104, 105). Another mutation in NLRP3 has been associated with increased susceptibility for Crohn's disease and inflammatory bowel disease (IBD)(106). Mice lacking NLRP3 and ASC are protected from EAE and this is associated with reduced Th1 and Th17 responses (107,108). Similarly, mutation in NLRP1, reducing the threshold for inflammasome assembly, increases the incident of vitiligo and Addison's disease (109). There are currently no reported cases of mutations in NLRC4.

Dysregulation of IL-1β is also implicated in the pathophysiology of several common diseases including atherosclerosis, osteoartritis, metabolic syndrome, gout and type 2 diabetes. This is partially because NLRP3 causes inflammation when activated by crystals such as cholesterol crystals, involved in atherosclerosis, urate crystals, known to cause gout, and islet amyloid polypeptide, a hormone secreted together with insulin. Additionally, TXNIP, a protein upregulated by glucose and linked to insulin resistance, has been suggested to activate NLRP3. IL-1β is further involved in impairment of both insulin signaling and promotes beta-cell dysfunction and cell death (110-112).

Also other diseases with an inflammation aspect involve inflammasome activity. Both acute brain injuries such as stroke and chronic neurodegenerative diseases, including Alzheimer's, are exacerbated by inflammation and IL-1. Mice deficient in caspase-1 show reduced infarcts after stroke, and reduced brain injury. These mice also get reduced endotoxemic acute real failure, although neutralization of IL-1β and IL-18 has little protective effect (11).

In conclusion, dysregulation of not only cytokine secretion but also cell death, can be devastating. Overactive NLRP3 inflammasome is involved in various autoimmune diseases, however also other inflammasomes can cause harm.

1.2 CELL DEATH

For many years the death of mammalian cells was based on a simple dichomoty: death by programmed apoptosis or uncontrolled necrosis. This two-sided view of cell

death no longer holds true and there are many defined mechanisms able to activate cell death. The first descriptions of programed cell death mechanisms were done in the mid 1960s and based on morphology. Today morphology is not the only parameter used to classify cell death but also biochemical and molecular techniques are required. Using new techniques there is also a new understanding of the complexities of cell death leading to newer and more diverse classifications. In 2009, the Nomenclature Committee on Cell Death (NCCD) proposed recommendations for classifications for the following forms of death: Anoikis, autophagic cell death, caspase-dependent intrinsic apoptosis, caspase-independent intrinsic apoptosis, cornification, entosis, extrinsic apoptosis by death receptors, extrinsic apoptosis by dependence receptors, mitotic catastrophe, necroptosis, netosis, partanatos, and pyroptosis (113). The work of this thesis addresses mainly pyroptosis. However, to be able to draw distinctions between pyroptosis relative to apoptosis and necroptosis, a brief introduction to all three forms of cell death is needed.

1.2.1 Apoptosis

Apoptosis is a form of regulated cell death used for organ shaping during embryonic development and homeostasis in adults, as well as a suicide program for damaged cells. Apoptosis is dependent on caspase cascades but the induction can be divided into extrinsic and intrinsic apoptosis. Generally, the apoptotic caspases are divided into "initiator caspases" (caspase-2, -8, -9, and -10) and ‘‘executioner caspases’’ (caspases-3, -6, and -7). Morphologically, apoptosis is characterized by blebbing of the plasma membrane, and the formation of 'apoptotic bodies'. The chromatin in the nucleus undergo an irreversible condensation, after which the nucleus breaks up into fragments (114, 115). The chromosomal DNA is cleaved into characteristic fragments pattern by caspase-activated DNase (CAD), which is activated when its inhibitor ICAD is cleaved off by caspases (116, 117).

1.2.1.1 Intrinsic apoptosis

Intrinsic apoptosis is induced by signals such as DNA damage, high oxidative stress, cytosolic Ca^{2+} overload, ER stress and other diverse triggers. As the triggers are quite variable, the signaling cascades also appear to be highly heterogenous. However, all these responses eventually end up affecting the mitochondria. Both pro-apoptotic and anti-apoptotic signals converge at the mitochondrial membrane. When the pro-apoptotic signals dominate, mitochondrial outer membrane permeabilization (MOMP) occurs due to the activation of pore-forming pro-apoptotic members of the Bcl-2 family, or through opening of the permeability transition pore complex in the inner mitochondrial membrane. MOMP has multiple lethal consequences, such as dissipation of the inner mitochondrial transmembrane potential, inhibition of the respiratory chain and release of toxic proteins such as AIF, SMAC/DIABLO and Cytochrome C. Cytosolic Apaf-1 senses the release of its ligand, cytochrome C, which binding triggers oligomerization. This complex, "the apoptosome", recruits caspase-9, which initiates the caspase cascade to apoptosis.(118)

1.2.1.2 Extrinsic apoptosis

Extrinsic apoptosis is induced by extracellular signals. It is triggered by binding of ligands such as FAS/CD95 ligand, tumor necrosis factor a (TNFa) and TNF (ligand) superfamily TNF-related apoptosis inducing ligand (TRAIL) to their respective so-called death receptors. A signal can also be dispatched by the 'dependence receptors' including netrin receptors, which can exert lethal functions when their ligands fall below a threshold level. When FAS binds its ligand FASL, the assembly of FAS subunit trimers are stabilized leading to a conformational change that allows for formation of a multiprotein complex at the cytosolic tail of the receptor. This 'death-inducing signaling complex' (DISC) consists of the death domain (DD), shared by all death receptors, and proteins such as receptor-interacting protein kinase 1 (RIP1), different forms of apoptosis inhibitors, E3 ubiquitin, and FAS-associated protein with a DD (FADD). The DISC complex serves as a platform for recruitment and activation of pro-caspase-8 and -10 as well as degradation of RIP1 and RIP3.(117-119)

Cell types that perform extrinsic apoptosis can be divided into 'type I and II cells'. Type I cells, which includes lymphocytes, can activate caspase-8 directly after DISC formation to catalyze maturation of caspase-3, thereby triggering the execution phase of apoptosis in a mitochondria-independent manner. In type II cells, including hepatocytes and B cells, caspase-8 instead mediates cleavage of BH3-interacting domain death agonist (BID) after DISC formation to generate the mitochondrion-permeabilizing fragment truncated BID (tBID). Hence, extrinsic apoptosis in type I cells is mitochondria-independent, even though tBID and MOMP can occur, while in type II cells it is associated with MOMP including the dissipation of mitochondrial transmembrane potential.(118, 119)

1.2.3 Pyroptosis

Pyroptosis was first described in macrophages infected with *S. flexneri* and soon thereafter also with *S. thyphimurium* (120-122). Pyro, the Greek word for fire, refers to the heat caused by IL-1β induced fever and -ptosis to its similarities with apoptosis (123). Even though several features of pyroptosis appear to overlap with apoptosis, this is a distinct form of regulated death with distinct mechanisms and morphology. So far, pyroptosis has mainly been studied in macrophages and dendritic cells. However, it has also been observed in for example epithelial cells (124, 125) and as also other cell types express inflammasome components, pyroptosis may occur in some capacity in additional cell types.

Pyroptotic cell death is dependent on caspase-1, but appears not to involve apoptotic caspases (121, 126, 127). Caspase-1 has the ability to process the apoptotic caspase-3 and -7 and has been observed to do so during pyroptosis (126, 128, 129). However, the pyroptotic events are not affected by the lack of these caspases(126, 128) even though caspase-7 is important in the defense to *Legionella pneumophil a*(129). A possible explanation for the lack of involvement of caspase-3 and -7 is that their processing is a late event that gets preceded by the more rapid death program.

While the classic morphological features apoptotic cells are cell shrinkage and blebbing, the pyroptotic events are proposed to lead to the formation undefined pore formation in the plasma membrane causing a net increased osmotic pressure, water influx and cell swelling (116, 130). This process can be delayed by extracellular osmoprotectants such as high extracellular K^+ or by extracellular glycine(121, 130). Similar to apoptosis, chromosomal DNA is cleaved into fragments during pyroptosis

(131) and the cells are positive using TUNEL assay (120, 121) although, the DNA is not laddered in the same pattern as in apoptosis (116). The DNA degradation is followed by nuclear condensation but in contrast to apoptosis, the nuclear integrity remains intact (116). The mechanisms of DNA degradation are unclear, and cleavage of certain target proteins characteristic for apoptosis is still discussed. For example, poly (ADP-ribose) polymerase 1 (PARP1) and ICAD are cleaved in some studies but remain intact in other (116, 128, 130, 132).

Pyroptotic cell death is implicated in clearance of pathogens by four mechanisms. It
1) eliminates the intracellular niche required for bacterial replication 2) leads to release of intracellular content that function as DAMPs recruiting immune cells
3) releases surviving bacteria for destruction by recruited neutrophils (87) and
4) releases mature IL-1β and IL-18. The importance of pyroptosis as a host-defense mechanism is highlighted by observations that IL-1β and IL-18 are not required for caspase-1-dependent clearance of several bacteria such as *Legionella pneumophila, Burkholderia thalandensis*, and *S. typhimurium* that are modified to constitutively express flagellin (87). Caspase-1 deficient mice are also more susceptible to *Francisella tularensis* than mice deficient in both IL-1β and IL-18 (133). Furthermore, mice lacking both IL-1β and IL-18 are still susceptible to endotoxic shock, whereas mice deficient in caspase-1 are protected. This indicates that the endotoxic shock is mediated by other DAMPs released from the lysed cells, such as high-mobility group box 1 (HMGB1) (134). Alternatively, the endotoxic shock may be mainly dependent on caspase-11, which does not release much IL-1β/IL-18 (34).

Pyroptosis is not only involved in immune responses during bacterial infection. NLRP1a-induced pyroptosis plays a role regulating hematopoietic progenitor cells, by restricting their proliferation and enable self-destruction of infected cells to limit dissemination of infection during their proliferation and maturation. On this recent work, mice with mutated hyper-responsive NLRP1a develop leukopenia in the steady state and LCMV infection or chemotherapy causes prolonged cytopenia, bone marrow hypoplasia, and immunosuppression. These reactions are independent of IL-1β but could be caused by NLRP1a triggered pyroptosis and depletion of hematopoietic stem cells in the bone-marrow stroma.(16)

In summary, inflammasome induced pyroptosis is a rapid caspase-1-dependent type of cell death, accompanied by release of IL-1β, IL-18 and putative mix of other DAMPs. The different inflammasome can together recognize a diversity of stimuli and initiate pyroptosis. There are still many unanswered questions about how inflammasomes and pyroptosis are regulated and when their responses are beneficial or not.

1.2.4 Regulated necrosis/necroptosis

Necrosis was for a long time considered as an accidental fully uncontrolled death mode. This paradigm started to be challenged in 1988 when it was discovered that cells can respond to TNF-α by dying in two different ways, either showing typical apoptotic or a more necrotic morphology. It is now clear that necrosis can occur in regulated manner and have both physiological and pathological functions. The cells have morphological features of necrosis, but the cell death can be regulated by a signaling pathway associated with activation of receptor interacting protein (RIP)

kinases.(135, 136) Necrosis can be triggered by stimuli such as DNA damage, physical damage, PRRs and hypoxic conditions but also ligation to death receptors including TNFR1, TNFR2, Fas and TRAIL (135, 136). When necrosis is induced by ligand binding to death receptors it is termed 'necroptosis'. This ligand binding is best known to initiate extrinsic apoptosis but if the apoptosis pathway is inhibited, the response can be shifted to necroptosis (135, 137). In the final stage of initiation of apoptosis, caspase-8 is activated to inactivate RIP1 and RIP2 and to initiate the caspase cascade. However, if caspase-8 is inhibited, the so-called necrosome complex is formed instead. This phosphorylation complex contains FADD, RIP1 but also RIP3 and initiate the necroptosis signaling cascade.(135, 138, 139)

Necroptosis is now considered to be a major cell death pathway regulating both development and immunity(140). RIP-1 deficient mice display extensive apoptosis and die soon after birth (141). Even though the necroptotic morphology appears similar to uncontrolled necrosis, this is clearly a form of regulated cell death since necroptosis is 1) induced by defined ligands 2) can be inhibited by inhibiting proteins in the signaling cascade, for example RIP1 or RIP3 3) involves degradation of intracellular content. It is an important exit strategy when apoptosis caspases are inhibited, by for example *S. typhimurium*, known to down-regulate caspase-8 (142) but necroptosis likely also has other functions and more is to be revealed.

1.2.5 Coping with dying cells - Find them and eat them

The body has a turnover of over one billion cells every day. These cells fall into different classes depending on type of cell death and cell type and need to be handled at the time. Apoptosis is generally a non-inflammatory type of cell death since the cytoplasmic content is contained/shielded by packing in 'apoptotic bodies' and cleared up by macrophages. However, apoptotic cells need to be found and engulfed before they go into immunostimulatory secondary necrosis, and therefore need to advertise their death already in the early stages of apoptosis by sending out 'find-me signals'. These signals can be lipid lysophosphatidyl-choline, spingosine 1-phosphate (S1P), the fractalkines, and the nucleotides including ATP.(143, 144). To avoid recruitment of neutrophils, additional keep-out signals are released, for example lactoferrin(145). ATP is also released in very low concentration, as long as the cell membrane is intact (146). The uptake is important in maintenance of self-tolerance (147) and DC phagocytosing antigen-loaded apoptotic cells are seen to induce tolerance(148, 149) unless the DC is receiving an activation/maturation signal during uptake of the apoptotic cell (150, 151).

Once they find the dying cells, the apoptotic cells encourage the macrophages to phagocytosis, by expression of eat-me signals. The most classic one is the "flipping-out" of phosphatidylserine (PtdSer), which normally is present on the inner leaflet of the plasma membrane. Other eat-me signals include changes in charge and glycosylation patterns, alteration in intracellular adhesion molecule-1 epitopes on the cell surface and exposure or the ER-protein calreticulin.(147, 152, 153)

Since these signals are released early in apoptosis, they should be released while the cell membrane is still intact. The mechanisms may involve S1P kinases, binding to PtdSer while it is flipped out (154) and Pannexin-1 pore formation, induced by cleavage by caspase-3 and -7 (155).

Necrotic cells, caused by either by physical cell destruction or necroptosis, are highly inflammatory and can cause neutrophil influx and sterile inflammation through

the release of various DAMPs (156). Pyroptosis is also seen as an inflammatory form of death. However, unlike necrosis it is specifically caspase-1-dependent. It also releases various DAMPs (134) but its inflammatory signatures are still under investigation.

In summary, these different forms of cell death are well controlled and have the ability to regulate each other in sophisticated manners. The type(s), of cell death that occur in various cell types and how it is achieved is not always easy to predict. However, as a whole it is likely to be strongly influenced by the trigger(s), environment, and the time frame in which it occurs.

1.3 THE ADAPTIVE IMMUNE SYSTEM

Generally, the adaptive immune system does not play a role in defense until days/weeks after infection occurs. Since development of adaptive immunity also involves the development of memory lymphocytes, repeated exposure to a pathogen, alternatively a vaccination program, results in a faster and more prominent adaptive immune response. Generally, adaptive immunity can be divided into two parts: the cellular responses mediated by T cells, and the humoral responses mediated by B cells. T cells can be divided into numerous subsets including CD4+ T cells, CD8+ T cells and CD4+ regulatory T cells (Tregs). Standard CD4+ T cells generally function as T helper (Th) cells producing cytokines able to support B cell and CD8+ T cell development and differentiation. CD8+ T cells, with the help of CD4+ T cells, develop into cytotoxic T cells (Tc) with the ability to kill infected target cells.(1)

Normally, adaptive immune responses are promoted by cells expressing PRRs. Antigen-presenting cells (APCs), namely macrophages, DC and B cells, recognize foreign particles, phagocytose and degrade it. Phagocytosed particles are processed into peptides and presented on MHC class I or class II complexes to activate T cells in secondary lymphoid tissue. Exogenous antigens are presented by MHC class II molecules where they activate CD4+ T cells, whereas both exogenous and endogenous peptide antigens such as bacteria, viruses or tumor cells can end up in MHC class I molecules to activate CD8+ T cells. Normally peptides from general protein turnover is displayed in MHC class I, but in the case of intracellular pathogens or mutated proteins, these proteins will be exposed and activate CTLs previously activated primed and licensed by APCs. The activation of the CTLs triggers them to kill the antigen-presenting cell.(157) Presentation of exogenous antigens on MHC class I requires cross-presentation, which mainly is performed by specific DC subsets by mechanisms not fully understood (158, 159). Generally, it has been observed that antigens from intracellular bacteria residing inside phagosomes and presented on MHC class II while intracellular bacteria escaping the phagosomes into the cytosol are presented on MHC class I molecules in a method analogus to viral infection.(157)

While APCs such as macrophages and DCs recognize pathogens by recognition of PAMPs and engulf antigens, the B cells express membrane-bound antibodies that can bind to and promote the engulfment of antigens. Endocytosed antigens are processed into peptides and presented on MHC class II molecules. When a $CD4^+$ T cell detects presented antigen together with the proper co-stimulatory molecules, the $CD4^+$ T cell produce cytokines that allow the B cell to differentiate into an antibody-secreting plasma cell. It also stimulates the B cell to class switch antibody isotype from the

standard IgM or IgD to IgG, IgA, or IgE as well as form memory populations. Antibodies secreted by plasma cells can neutralize and/or target microbes for degradation by numerous mechanisms.(1)

The $CD8^+$ cytolytic T lymphocytes (CTLs) are the main cytolytic cell of the adaptive immune system. Upon recognition of antigens presented on MHC class I molecules by APCs, $CD8^+$ T cells develop into antigen-specific memory CTL, effector CTL or both. This process is more efficient if supported by $CD4^+$ T cells producing cytokines in responses to antigens also presented on class II MHC(160). Cytotoxic T effector cells can kill virus-infected cell, thereby controlling viral replication. The killing is either mediated by release of granules containing granzymes and perforin or by induction of apoptosis by Fas-Fas ligand interactions between the infected cells and the T cell. Additionally, the CTLs also produce cytokines such as INF-γ. INF-γ induces direct anti-microbial and anti-tumor mechanisms, recruits leucocytes, promotes maturation and differentiation of many cell types, enhances natural killer cell (NK) activity, regulates B cell function and antibody class switching (161).

The type of effector function that dominate the adaptive immune response depends on the pathogen and is can be influenced by the types of cytokines secreted by various cells upon infection. Simplified, the control and expansion of Tc and B cells is controlled by $CD4^+$ Th cell sub-types and the responses they make after detection of presented antigen (1). Type 1 Th (Th1) cells produce a cytokine profile, typically INF-γ and IL-2, which supports inflammatory-style responses and activates macrophages and cytolytic T cells, leading to a predominantly cellular immunity. It also induces antibody-mediated responses in certain subclasses of the IgG isotype - IgG2a. Type 2 Th (Th2) cells mainly activate B cells and antibody production through production of cytokines such as IL-4, IL-5, and IL-13. The response is associated with IgG1 and sometimes IgE antibody isotype. Generally, regulatory T cells (CD4+ Tregs) restrain and control the activity of responding lymphocytes to prevent autoimmunity and tissue damage. Th17 cells producing IL-17A, IL-17F, and IL-22 can rapidly initiate immune responses dominated by neutrophils. The defense is essential in the defense against extracellular bacteria, fungi and opportunistic bacteria, especially in mucosal tissue, but also involved in viral defense. IL-17A stimulates epithelial cells and fibroblasts to produce inflammatory mediators, thus promoting granulopoiesis and neutrophil recruitment. Further the Th17 cytokines also induce production of cytokines and antimicrobial peptides from both immune and non-immune cells such as mucosal and epithelial cells. IL-17 can also enhance the capacity of CD4+ T cells to produce IL-2, as well as enhance the proliferation of both conventional T cells and Treg cells. Th17 cells are associated with autoinflammatory diseases and gastric inflammations.(162, 163)

1.4 VACCINES

The goal of a vaccine is to educate the immune system to recognize a pathogen in the absence of infection and thereby create long-lasting immune-based protection against said pathogen. In 1796, Edward Jenner performed the first well-documented vaccinations. During a time when smallpox spread over the world, Jenner had observed that the women who where milking cows seemed to be protected against the disease. Jenner inoculated people with the smallpox related cowpox and could observe that they were less likely to get infected by the dangerous smallpox. Now

smallpox is in principle eradicated, and vaccines are known to be one of the most life-saving inventions ever.

Historically, vaccine efficacy has been based on empirical data, and there are still no detailed mechanisms understanding the correlates to efficacy. Often, the vaccine "take" is measured by induction of an antibody response and the titer induced is observed to be a correlate of protection (164).

There is a great need for research and insight into how vaccines work in order to design more efficient vaccines and more specific immune-boosting additives, so-called adjuvants. In the work presented in this thesis we are trying to evaluate the adjuvant properties of flagellin and to decipher the mechanisms behind it.

1.4.1 Traditional vaccines

The majority of classic vaccines, such as smallpox, measles, mumps, rubella, polio (Sabin) and yellow fever, are live attenuated vaccine. In these formulations, the vaccine-microbe has lost its pathogenicity through several passages in cell cultures or has been grown in a way (cold passage) that promotes adaptation to a non-optimal environment. Alternatively, a closely related virus can be used instead of the primary pathogen, as in the example of smallpox vaccine. These vaccines cause a mild infection with less pathology that induces cross-protective adaptive immune memory against the major pathogen. This infection may however cause disease in rare cases and it is not advisable to be administrated to immunocompromised individuals.

A similar but safer strategy to vaccine development is to use inactivated non-replicating vaccines. Common vaccines such as influenza virus, polio virus (Salk) and hepatitis A are inactivated virus vaccines. However these vaccines in their current forms have a tendency to skew the immune responses towards predominantly humoral responses. In many cases this is not necessarily a barrier to successful vaccine development. However, it may be that a mixed cellular-antibody response may provide even greater protection than a response dominated by one arm of adaptive immunity. The predominantly humoral responses elicited by current influenza virus vaccines are sufficient to confer seasonal influenza protection. However, the current vaccination strategies require yearly production of a new vaccine as the influenza vaccines fails to provide strong cross-protection against emerging viral strains. Hence, we need new flu vaccines for every season. Rapidly mutating viruses, such as influenza and HIV-1 can evade humoral immunity by antigenic drift and shift (165). The immune system preferable recognizes variable rather than constant parts of the virus. A current goal in flu vaccine research is therefore to make a universal influenza vaccine that is efficacious and one strategy is to targets constant parts of the virus that it cannot afford to mutate.

An alternative to whole virion vaccine is subunit vaccine, which is the use of specific viral proteins, usually surface proteins since these are primary targets for neutralizing antibodies. This strategy is currently used in vaccines against hepatitis B and human papilloma virus. However, also this strategy mainly elicits antibody response towards variable parts and furthermore subunit vaccines do not contain any natural PAMPs, in contrast to whole virions, and addition of adjuvants is therefore required.

Also even smaller parts of the virus can be employed. Peptides are short stretches of 8 to 30 amino acids and the ones used in vaccines represent epitopes known to induce strong immune responses. This possesses advantages such as easy

production and the possibility to rapidly alter the amino composition however, peptide vaccines are generally less immunogenic than whole proteins and require strong adjuvants.

1.4.2 Genetic vaccines

Genetic vaccines, either as plasmid DNA or recombinant viral vectors, are a promising alternative approach to traditional protein vaccines for the prevention and treatment of infectious diseases, cancer and allergy. These vaccines work by producing the antigen of interest within the cells of the recipient from the genetic construct.

Viral vaccine delivery vectors such as Modified Vaccinia Virus Ankara (MVA), based on Vaccinia virus, and ALVAC, based on a Poxvirus, have been extensively passaged to attenuate the virus and are now considered to be safe enough to be used as vectors in human vaccines. However, one problem with the use of viral vectors to deliver antigen-producing genes is the risk for pre-existing anti-vector immunity. In this scenario, neutralization of the vaccine occurs before successful "infection" and production of protective antigen due to the recipient harboring neutralizing antibodies to the viral delivery system. Additionally, the use of viral vaccine delivery vectors may have limited utility because previous vaccinations with the same technology may promote neutralizing antibodies affecting efficient boosting or responses to new antigens.

1.4.2.1 Plasmid DNA vaccines

Non-living/replicating plasmid-based DNA vaccines are composed of a bacterial plasmid that encodes the antigen of interest under the control of a strong eukaryotic promoter. This approach provides a range of advantages over the conventional virus or protein based vaccines as it is a safe, rapid and cost effective, and does not suffer problems from improper folding of a protein antigen. The stability of DNA simplifies administration and it is also very flexible as the plasmid vaccine sequences can easily be changed to adapt to changes in the antigenic target. For example antigenic drift and shift of pandemic mutated influenza virus.

In the end of the 1980s and early 1990s it was discovered that naked DNA when injected intra-muscularly generated *in vivo* expression of the plasmid encoded reporter gene, and could be used as vaccine vectors. Short thereafter, experiments showed that genes encoding influenza virus could protect mice from subsequent influenza inflection.(166, 167)

During DNA vaccination, the plasmid encoding the antigen is delivered to the skin, muscle or mucosal tissue through one of several delivery methods. Using a poorly identified host cellular machinery, the plasmid enters the nucleus of transfected local cells including myocytes, keratinocytes and/or APCs. Once in the nucleus, the plasmid-encoded genes are expressed and transcribed into antigen proteins, which are seen by the immune system as foreign. Directly transfected somatic cells can secrete the synthesized protein to resident APCs or degrade it and display it on MHC class I. Resident APCs can present the endogenous peptide on MHC class I either after direct transfection or cross-presentation after uptake of dying transfected cells (158). Additionally, APCs can present the peptides on MHC class II after uptake of secreted protein antigens. The activated antigen-loaded APCs migrate to the draining lymph

nodes where they present the antigenic peptide-MHC complexes to naive T cells. This step promotes the activation and expansion of T cells and/or activation of B cells and antibody production. In this way, both humoral and cellular immune responses are generated in response to a plasmid DNA vaccine.(Figure 2) Furthermore, such an approach is not hampered by the anti-vector immunity observed for the attenuated viruses or bacterial vaccine vectors (168).

An early safety concern of DNA vaccines was the risk of integration (partial or complete) of the plasmid DNA into the host genome. Fortunately, this has been shown not to be the case and indeed, the rate of plasmid integration is negligible and much lower than the spontaneous mutation rate of DNA (169). Another safety concern was the risk of producing anti-DNA or anti-nuclear antibodies in response to the vaccine resulting in autoimmunity. However, to date there are numerous studies indicating that there is no evidence of autoimmunity associated with DNA-vaccination (170-174).

In animal studies, plasmid DNA vaccines have been shown to provoke protective immunity to infections including HIV, malaria and influenza (175). In 2005 the first DNA vaccine for veterinary use obtained licensure. The vaccine was for prevention of disease caused by infectious hematopoietic necrosis virus in farm raised Atlantic salmon and it has been followed by prevention of West Nile virus in horse and also therapeutic DNA vaccine to treat melanoma in dogs.

In spite of these breakthroughs in animal studies, in human the non-living/replicating DNA vaccines have suboptimal immunogenicity when compared with traditional protein-based vaccines. So far, no plasmid DNA-vaccines have been able to elicit protective immunity in humans. It is due to these reasons that many researchers are actively pursuing techniques and technologies to improve the immunogenicity of non-living/non-replicating DNA vaccines.

1.4.2.1.1 Features of the vaccine plasmid

To function as an expression vector and DNA vaccine, the plasmid is required to contain certain basic elements described here. Additionally, through optimization of the plasmid backbone the uptake and expression of plasmid-encoded protein can enhance the built-in adjuvant function of the plasmid itself and a list of examples is provided below;

1. Basic elements: An origin of replication that allows for replication of the plasmid in bacteria and affordable production of the vaccine.
2. Antibiotic resistance gene: for selective growth in bacteria. Can be removed for safety concerns if necessary.
3. A promoter to initiate transcription of the encoded protein. This requires a strong eukaryotic promoter since microbial promoters not necessarily are optimized to drive mammalian gene expression. This promoter is designed not be silenced despite expression in different cell types.
4. A polyadenylation (poly-A) signal in the 3' end of the gene. This is required for proper termination of transcription and stabilizing the mRNA during the export from the nucleus.
5. Additional modifications including a translational enhancing sequence called a Kozak sequence upstream of the antigen genes start site.

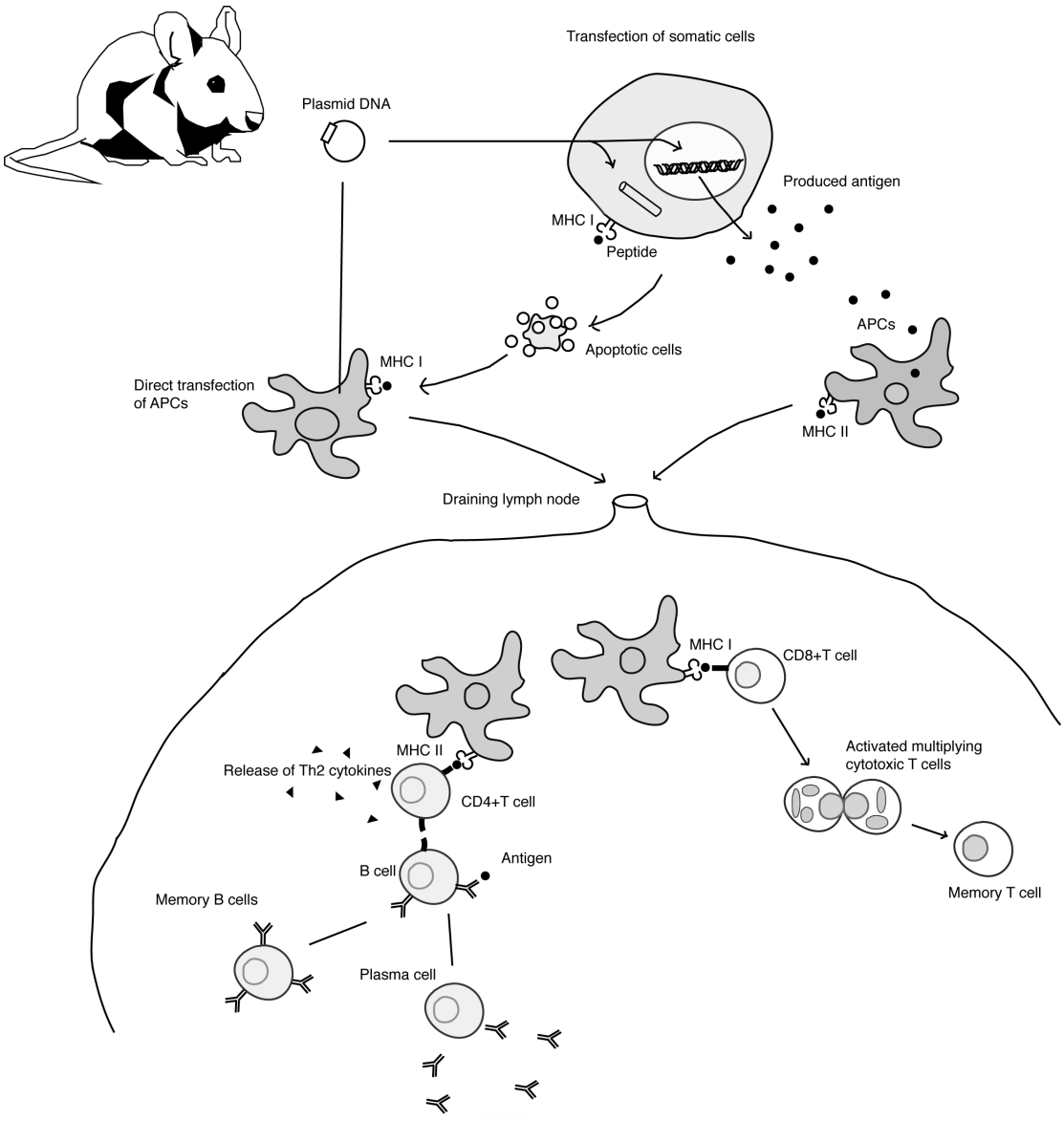

Figure 2: Immune response after plasma DNA vaccination. *Adapted from Kutzler 2008.*

1.4.2.1.2 Increasing the immunogenicity of plasmid DNA vaccines

The cellular uptake of injected DNA plasmid is inefficient. Transfection of cells in culture often results in high levels of produced protein. However, delivery of plasmid DNA to cells as part of a larger system such as the body is complicated. The ideal gene delivery system should protect the DNA from degradation by extracellular enzymes, e.g. endonucleases, and protect DNA from the presence of serum and interstitial fluids, as well as deliver the DNA mainly to the target cell of interest. Once inside the cell, ideal delivery technology should also prevent the DNA from getting degraded in endosome-lysosome compartments if it should happen to enter a cell by

endocytic mechanisms. Additionally, the correct/appropriate adjuvant signals have to be provided in order to dictate the type of immune response to be induced.

There are many different strategies that can be used to enhance the immunogenicity of DNA vaccines. These can be techniques to optimize the cellular uptake of plasmids by various delivery methods. Special plasmids allow co-expression of antigen and adjuvant in the same plasmid. Certain technologies make use of different viral vectors and antigenic gene codon optimization to maximize the antigen expression. Prime-boost schedules may be optimized for the type of vaccine recipient as well as the use of different adjuvant technologies.

To induce an efficient immune response, not only the strength of the response but also its quality is of importance. The most standard way to evaluate T cell responses is the magnitude, commonly represented by the frequency of antigen-specific T cells producing a specific cytokine, such as IFN-γ. However, this only reflects one parameter of the T cell function and does not always correlate with protection. The T cells function is not only cytokine production but also includes the ability to proliferate or secrete growth factors, secrete chemoattractants to organize the immune response, and, for the CD8+ T cells, perform direct killing through cytolytic mechanism or secretion of cytokines. The optimal combination of delivery method and adjuvant needs to provoke these multifunctional immune responses.

1.4.2.1.2.1 Adjuvants

The inclusion of adjuvants in a vaccine can improve both the strength and quality of the response by providing a depot effect, protect the vaccine from degradation, facilitate uptake by APCs and modulate the immune response to tilt it to a more favorable Th1/Th2 ratio. They also have the ability to improve the immune responses in poorly responding individuals and reduce the amount of antigen.

Over the past two decades, our increased understanding of innate immunity and antigen presenting cells allows us to rationally design adjuvants. Indeed, bacterial cell walls, bacterial DNA and viral RNA, present in vaccines for nearly a century are now known to engage distinct TLRs and activate TLR expressing antigen-presenting cells.

Many of the traditional adjuvants are still among the most widely used, and tested also in conjunction with DNA vaccines. These include oil-in-water emulsions, bacterial components and aluminum salt (Alum) (176). Aluminum salts, commonly referred to as alum, are currently the most used adjuvants in human vaccines. They result in a gel-like matrix that creates a depot of antigen, and additionally it activates the NLRP3 inflammasome (177). Oil–water emulsions also work by sequestering the antigen and slowly releasing it. The most classic water-in-oil emulsion, incomplete Freund's adjuvant, is widely used together with livestock vaccines, but not in human because of its tendency to induce granulomas at the injection site (176). However, MF59, a variant of the biodegradable oil squalene is suitable in human (178). Other adjuvant approaches in clinical trials are PRR agonists such as flagellin, MPLA (an non-toxic LPS-derivate) and Imiquimod (TLR-7 ligand), as well as cytokines such as IL-12, IL-2, IL-15 and GM-CSF (176). Also chemical adjuvans including liposomes, polymers and microparticles have been shown to enhance the immunogenicity of DNA vaccines, mainly by facilitating delivery and uptake of DNA (179).

The DNA vaccination plasmid itself has adjuvant properties and may function in

numerous ways. Cysteine poly-Guanosine (CpG) motifs in the backbone can be recognized by TLR9 and trigger proinflammatory response through MyD88 (180-182) The double-stranded DNA of the plasmid can activate cytoplasmic DNA sensors and signaling pathways such as TBK-1 and STING, thereby inducing type 1 interferons (183-186). RIG-I (187) and Aim2 (187, 188) have also been shown to sense cytosolic DNA and could plausibly be involved in sensing a DNA vaccine.

1.4.2.1.2.2 Delivery methods

The gene-gun technique was the first delivery device used to increase the transfection rate during DNA vaccination. With this technique, the plasmid is coated on gold particles and shot directly into the outer living layers of the skin. In this way the DNA is delivered directly into the cell cytoplasm or nucleus instead of into the extra-cellular space as with injections, which drastically increases plasmid uptake and require much smaller amounts of plasmid to elicit immune responses compared to needle injections. It induces cellular and humoral immunity in mouse and humans (189-192) but as it skews the response towards Th2 (193, 194) its use is sometimes limited.

Electroporation is currently the most favored and potent technology for delivery of naked DNA vaccines. The DNA is injected intra-dermally or intra-muscularly followed by a brief electrical stimulation at opposite sides of the injection site. This technology is thought to permeabilize cell membranes near the DNA injection site and improve plasmid delivery to surrounding cells (195). This, in turn, leads to greater antigen production. Additionally, it causes a mild inflammation inducing proinflammatory cytokines and migration of immune cells (196, 197). It was first used for cancer immunotherapy but is now known to enhance DNA vaccine efficacy and induce robust immune responses comparable to those with protein vaccines with the added advantage that it efficiently promotes Tc responses (198). This technology has been shown to increase the immunogenicity of numerous vaccines antigens as well as enhancing vaccine responses in larger animals that previously have demonstrated poor responses to plasmid DNA vaccination (199). EP can be applied both intradermal and intramuscular depending on the length of the needles and electrodes used.

Electroporation vaccination is currently in several clinical trials against both infectious diseases and cancer (clinicaltrials.gov) and is utilized for the administration of a DNA-encoded growth hormone veterinary product for pigs. The main downside for electroporation is the brief discomfort, which may prohibit it from being used in prophylactic vaccines.

Mucosal surfaces have major advantages as immunization sites since they are easily accessible, rich in immune cells and the site of entry for many pathogens. Mucosal delivery is also associated with the ability to elicit higher mucosal immune responses, including IgA antibodies(200).However, most antigens are poorly immunogenic when administrated at mucosal surfaces and require appropriate delivery systems and adjuvants in order to induce potent immune responses. Numerous delivery systems and adjuvants have been evaluated for mucosal administration, including liposomes, immunostimulatory complexes, CpG oligodeoxynucleotides, bacterial toxins, nanoparticles and microparticles (200). Most studies focus on intranasal or oral administration as these are clinically easy to access. There are currently licensed

mucosal vaccines based on killed or live-attenuated microorganisms. These include oral vaccines against poliovirus, rotavirus, *S. typhimurium* and V. cholerae and the intranasal influenza vaccine FluMist (201). The live seasonal flu vaccine FluMist contains live-attenuated intranasal influenza vaccine from three live influenza viruses and has been approved since 2003. It is administered as a nasal spray, and it has proven to be well tolerated and efficient. The virus is cold-adapted and can only reside in cooler parts of the nose and not infect the lungs (202, 203).

There are a variety of additional delivery methods being evaluated including the non-invasive devices where the DNA is propelled into the skin or muscle, and invasive using conventional tattoo machines. All methods have their pros and cons and optimization of the delivery method can clearly enhance the efficacy of DNA vaccines.

1.4.2.1.2.3 Heterologous immunization

Vaccination strategies based on a combination of more than one vaccine modality has been shown to enhance and broaden responses against pathogens such as HIV or malaria, and most of the current clinical HIV studies utilize this strategy (clinicaltrials.gov). These strategies typically involve a priming using plasmid DNA, followed by a boost with a similar but not identical antigen in a viral vector. The advantages are that the prime induces a highly specific priming but weak response which is boosted by the viral vector, allowing for an efficient expansion of the antigen-specific immune response. Additionally, this strategy circumvents the risk for vector-specific immune responses that can lower the effect of repeated immunizations.

Another alternative is priming with plasmid/viral vector DNA and boost with protein. This is a highly promising strategy as it combines the best of two worlds by providing the possibility to prime a strong T cell response using the DNA vaccine then maximizing the antibody responses with the protein boost.(204, 205) Additionally, this method does not use an attenuated pathogen or infectious delivery system which would broaden its use in immunocompromised individuals.

1.4.3 Safety issues of vaccines and adjuvants

The safety standard of prophylactic vaccines and adjuvants need to be extremely high as they are products intended for healthy individuals. The safety of routinely used vaccines has over the past several decades been examined in both randomized-controlled trials and observational studies with great safety records. However, it would be incorrect to paint vaccines as being totally safe. With the great genetic variance among humans, there may always be individuals with unpredictable reactions. The risks with vaccinations must be put in perspective to risks of possible adverse effects of the vaccine with severity of the health effects associated with the diseases. The risk populations, such as young children, elderly and people with chronic diseases, must be taken into consideration. Their impaired immune system both put them at higher risk for infection and decreases the vaccination efficacy. The inability of these populations to receive vaccines highlights the importance of vaccines to promote herd immunity. In the western world the children vaccination program have more or less eradicated many diseases, and is it easy to forget how deadly several vaccine-preventable childhood diseases can be and instead question the

need and safety of vaccines. This has further increased the public demand for evidence of vaccine safety.

Addition of adjuvants triggers the immune system and may make the response harder to predict and in the worst cases too strong. However, adjuvants are often necessary for the efficacy of the vaccine, especially in elderly people. Addition of adjuvants can also make the vaccine much more cost effective. For safety it's important to thoroughly test the new vaccine formulas in phase III studies and also established have post-marketing surveillance program and follow up reports of possible adverse effects.

Most adverse effects after vaccinations are common local reactions including pain, swelling, and redness at the injection site. Systemic reactions, including fever, irritability, drowsiness, and rash, may also occur. Allergies to any of the components used in vaccine development, such as gelatin, egg or traces of neomycin, should be taken into consideration.

Many diseases have been suggested to occur in correlation with vaccine use without significant scientific evidence. Since the onset of diseases, miscarriage etc. can coincide with vaccination without causality, it is important to have information of background levels of different conditions. Sadly, the idea that the triple-vaccine for measles, mumps and rubella would cause autism is still widely spread after the falsified study by Andrew Wakefield in 1998, which was withdrawn from Lancet when scientific data manipulations were revealed (206, 207). Vaccinations are also not associated with an increased risk of Crohn's disease, as previously suggested (208).

Severe adverse effects such as increased risk for certain diseases have occasionally been correlated to vaccination, such as encephalitis after measles vaccination (209), febrile seizures in young children after the seasonal influenza vaccine Fluvax (210), asthma after immunization with live attenuated influenza vaccines (211, 212). The risk for the neurological diseases GBS was significant increased during the swine influenza immunization program in USA 1976 (213) but this has not been observed since (214). Another drawback for vaccinology was the STEP trial, a HIV-vaccine study enrolling 300 individuals at high risk of HIV-infection, which was discontinued due to futility (215, 216). The vaccine based on the Adenovirus type 5 (Ad5) vector, increased the incidence of HIV-infection rather than protected, and pre-existing Ad5 immunity was subsequently shown to increase the risk of acquiring HIV (215).

The worst case of vaccine related events lately is the association between narcolepsy and H1N1 vaccine. In 2009, the pandemic influenza A (H1N1) virus spread rapidly, resulting in millions of cases and over 18,000 deaths in over 200 countries (217). Vaccines ware licensed using fast track procedures and many countries decided to mass-vaccinate the population, on the recommendation of the world health orgination (WHO). The USA, Australia and parts of Europe offered only non-adjuvanted vaccines. In the rest of Europe, the offered vaccines were mainly two versions with adjuvants based on the oil-in water adjuvant squalene: Foceteria (Novartis) with MF59 and Pandemrix (GlaxoSmithKline) with AS03. AS03 is squalene in combination with α-tocopherol (a form of vitamin E) (217). The Pandemrix vaccine has been associated with cases of narcolepsy. Sweden, Finland, Norway, Ireland and England have reported that Pandremix induced a 3- to 14-fold increased risk for children and adolescents below the age of 20 (217-222). Narcolepsy

is a rare conditions; for Sweden this entailed an increase from 0.97 to 4.06 cases per 100,000 inhabitants (221).

Narcolepsy is a chronic neurological disorder of excessive daytime sleepiness, often accompanied by cataplexy; sudden muscle weakness triggered by strong emotion. It is tightly associated with the human leukocyte antigen (HLA)-DQB1*0602 haplotype and the loss of the neurons normally producing hypocretin, a neurotransmitter important for regulation of sleep and wakefulness, possibly by autoimmune reactions. These associations also hold true also for the vaccine-associated cases. How the vaccination triggered the onset of disease is unknown but narcolepsy has also been correlated to upper airway infections such as *Streptococcus pyogenes* (223). In China the occurrence of narcolepsy is seasonal and correlates with upper airway infections including influenza. The number of narcolepsy cases increased 3 fold during the winter 2009-2010 to return to baseline levels two years late. Since only 5.6 % of the patients were vaccinated (224, 225), the trigger is suggested to be the infection itself, and that the vaccine and the infection may trigger the same reaction.

In retrospect one can say that the decision to mass vaccinate and include children, however the severity if the influenza was hard to predict. The induction of narcolepsy was an unpredicted adverse effect, and too rare to catch in clinical studies. What should be discussed is whether it was correct to continue with the vaccination program when the cases of narcolepsy were reported, and what to learn for the next pandemic. These incidents are not only disastrous for the affected children, but also for the attitude to vaccines as well as the trust in scientists with subsequent decrease of vaccine coverage.

2 AIMS

1) To perform DNA vaccinations and compare immunization by skin electroporation to intramuscular injection with special focus on multifunctional CD4+ T cell responses (**paper I**).

2) To evaluate the adjuvant properties of plasmid encoded secreted flagellin when delivered by different routes representing mucosal, systemic, and dermal tissue compartments (**paper II**).

3) To study responses to unprimed NLRC4 inflammasome activation and inflammatory properties of pyroptosis (**paper III**).

4) To study the effect of various priming pathways and additional stimuli on NLRC4 inflammasome activity (**paper III**).

5) To see if metabolic changes activated by TLR agonism correlated with post-translational modifications of HMGB1 released during pyroptosis (**paper III**).

3 METODOLOGY

Presented below is a brief overview and discussion about some of the methods used in the studies included in this thesis. For more comprehensive descriptions, please see respective paper.

Immunizations (**paper I and II**)
Electroporation: The mice were gently anesthetized using Isofluran. The flanks were shaved and the vaccine mixture (10µl) injected intra-cutaneously in both flanks forming a small bubble of fluid. Immediately after injection a needle array electrode was placed over the injection site and a brief pulse of low voltage currant is applied to over the area.
Intradermal injection: The injection procedure was the same as describe for above without the electroporation step.
Intramuscular injection: The mice were restrained and the vaccine mixture injected into the quadriceps. Total amount of antigen was 10ug/mouse of pOVA and 10 ug/mouse HIV-1 in a 50µl volume.
Intranasal immunization: The mice were gently anesthetized using Isofluran and laid with the dorsal side up. The vaccine mixture was dropped is each nostril with the mice breathing it in. The mice were supported until they regained consciousness. Total amount of pOVA was 5ug/mouse in a 10µl volume.
Gene gun immunization: The mice were anesthetized using Isofluran. The abdominal skin is shaved and the g.g. is held directly against the abdomen and discharged. 0.5 mg of gold particles was coated with 0.5ug pOVA/mouse.

The immunization schedules are indicated in **paper I** and **paper II**.

*Immunization antigens **(paper I and II)***
The antigens used in the vaccine studies in this thesis are plasmid DNA encoding Ovalbumin (pOVA) (**paper II**) and the HIV-1 antigens pGag (the whole 55kDa antigen) (**paper I**), pGag24 (a smaller 24kDa region) (**paper II**), and pgp160 (full-length gp160) (**paper II**).
pOVA encodes an egg white protein commonly used as a model antigen in mice due to its robust immunogenicity and known class I and II MHC peptide epitopes. pGag encodes a human codon optimized HIV clade C Gag (p55). pGag24 and pgp160 encode codon optimized HIV clade B p24Gag and gp160, respectively. All antigens reside in the Invitrogen expression plasmid pcDNA3.1/Zeo. As an empty vector control the same plasmid is used. The pcDNA3.1/Zeo plasmid has a cytomegalovirus (CMV) enhancer-promoter, commonly used for DNA vaccines because its abilty to drive high constitutive expression levels of transcripts in most cells.

*Studies of functional immunity **(paper I and II)***
The immunity elicited by immunization was evaluated using the methods multicolor flow cytometry, ELISA, and ELISPOT. ELISA and ELISPOT are sensitive and reliable methods. Flow cytometry provides the ability to distinguish cell types and detect multiple cytokines from single cells in mixed populations. The FLUOROspot technique (not used here) also has the ability to detect two cytokines produced by the same cell. However, this is still only two cytokines and without the ability to directly observe the cell type producing it.

Plasmid preparations ***(paper I-III)***
All plasmid preparations were made using Qiagen EndoFree Plasmid Maxi Kit.

Western blot analysis ***(paper II-III)***
Western blot analysis (also called immunoblot) was used to confirm that the plasmid FliC versions were transcribed into proteins of the predicted size. It was also used to confirm processing of pro-IL-1β to IL-1β (17 kDa) and release of HMGB1.

Establishing the retroviral inducible system ***(paper III)***
We have established a tetracycline-on based system to achieve inducible expression of C34-EGFP and C19-EGFP gene products in a mouse macrophage cell line. The gene induction is activated using a tetracycline analogue called doxacycline (Dox) and is indirectly determined by detection of EGFP expression in living cells by flow cytometry. The establishment of this system is further explained in 4.3 and **paper III**.

The cell line B10R ***(paper III)***
Since we wanted to stably transduce cells and have the possibility to genetically modify them in additional downstream experiments, the use of cell lines were more appropriate than use of primary cells. Primary cells can be more relevant to physiological function, however there are functional differences between mouse strains and housing conditions that may account for different priming of immune cells derived *ex vivo*. Furthermore, primary cells are less well characterized, have limited life-span, will differ from time to time and will change over time in culture with the number of passages. However, cell lines are also affected by passaging and variations in culture conditions such as growth density. Indeed, we have noticed that our C34s drift in their ability to respond to expressed C34-EGFP over time. To avoid this, we limit the time the cells are cultured and thaw out fresh cells if they start to drift. Additionally the C34s have been single cell clone selected repeatedly. Most of the studies are done with 400,000 cells/700ul medium/well on a 24-well plate, as this is a cell density that allows for the harvesting of concentrated supernatants without culture overgrowth and decreases in cell viability during 24 hours. We have observed no apparent differences in pyroptosis when altering the cellular density before seeding or during Dox induction. Additionally the C34s have been single cell clone selected repeatedly with similar behavioral phenotypes (pyroptosis) observed.

The cell line used in **paper III** is B10R, also known as Bcg^r. This is a murine macrophage cell line established to study susceptibility to Mycobacterial infection. Bcg is a single, autosomal dominant gene that controls susceptibility or resistance species of Mycobacteria including Mycobacterium bovis (BCG), but also *S. typhimurium* and *Leishmania donovani*. There are numerous functional differences associated with the *Bcg* locus, however the candidate gene associated with resistance has been identified to be the gene natural resistance associated macrophage protein (Nramp1) encoding an endsomal resident protein. The resistance can be abrogated by a single amino acid substitution.(226, 227) The pleiotropic effects of Nramp1 are not fully understood but proposed to involve increased bactericidal activity, respiratory burst activity, responses to IFN-γ, NO production, iron transport and antigen-presenting ability (226, 227). The human homologue NRAMP1 appears to have the same function and mutations in human NRAMP1 is associated with genetically predisposition to diseases including tuberculosis (228).

There is no such thing as the perfect cell line or mouse strain since and they all

have some differences. Inbred mouse strains have either the resistant (Bcg^r) or the susceptible (Bcg^s) allele and the common laboratory mouse strains C57BL/6 and BALB/c both have the Bcg^s allele(94). Knockout mouse lines are most frequently constructed using 129-derived embryonic stem (ES) cells, which also have a Bcg^r (226). The commonly used macrophage-like cell line RAW264.7 on the other hand lacks functional Nramp1 (229).

Congenic B10A.Bcgr mice have been constructed by transferring the Bcg^r allele of the A/J strain into the C57BL/10.A background using Nx backcross system. From these mice, bone-marrow derived macrophages have been immortalized by infection with J2 virus.(230) We choose to work with this cell line since it is derived from bone-marrow known to express all the necessary components for inflammasome activity. Furthermore, the C57BL/6 mice that B10R is based on is the most commonly used mouse strain for inflammasome studies. In contrast, the widely used RAW 264.7 cells derive from BALB/c mice. We have phenotyped the cells by screening for surface markers using flow cytometry (S1, **paper III**) and gene expression using RT-qPCR (Figure 1B, **paper III**). From the markers and genes we have studied, these cells appear to be typical macrophages.

*Monitoring TLR5 activation (**paper II**)*
293T cells were transfected with pFliC(-gly), pFliC(-gly)D34 or empty vector. The supernatants were confirmed to contain FliC proteins of the predicted size and used to stimulate mouse alveolar macrophages from TLR5-/- or C57BL6 mice, with recombinant flagellin and LPS used as positive control. Macrophage responses were evaluated by measuring release of TNFa by ELISA. The alveolar macrophages were obtained from bronchioaveolar lavage (BAL) washes harvested by flushing lungs of sacrificed mice with PBS. Alveolar macrophages were used because mouse, unlike human, bone-marrow derived macrophages do not express TLR5 (231, 232).

*Retroviral lethality screen (**paper II**)*
The retroviral lethality screen was based on a developed by Miao et al.(87). pFliC(-gly), pcFliC(-gly), pFliC(-gly)**Δ**34, or pcFliC(-gly)**Δ**34 ORFs were inserted into the packaging vector pMSCV-IRES-GFP/neo and retrovirally packaged using Phoenix amphotropic cells. The supernatants containing viral particles were concentrated by centrifugation and transduced to B10R and 293T cells using spinduction. Three days after transduction the B10R cells were analyzed for viability and GFP expression. The ability for a certain flagellin version to induce pyroptosis in B10R cells were defined an absence of GFP signal relative to control constructs and GFP signals seen in 293T cells (non-responders).

*Monitoring cell death (**paper III**)*
Thus, the Nomenclature Committee on Cell Death (NCCD) has proposed that a cell should be regarded as 'dead' when 1) the cell has lost the integrity of its plasma membrane and/ or 2) the cell, including its nucleus, has undergone complete disintegration, and/or 3) its corpse has been engulfed by a neighboring cell in vivo.(113) Our methods to monitor cell death has focused on the first criteria. The assay used throughout **paper III** monitoring up-take of the membrane-impermeant fluorochrome propidium iodide (PI) by flow cytometry. The exception is the TMRE mitochondrial membrane potential assay where PI could not be used because of conflicts in the use of filter settings by the Fortessa. In this instance viability was

detected using the amine-reactive Viability Dye eFluor 450. In the PI assay, PI is passivly able to cross membranes and binds DNA in cells that have lost their plasma membrane integrity. This is a fast, easy assay with relatively low toxicity to living cells. Since pyroptosis is associated with the formation of a hypothetical plasma membrane "pore" during early stage of pyroptosis that could account for slight up-take of PI, we have confirmed death by additional assays. We have also studied release of lactate dehydrogenase (LDH) a cytoplasmic protein that is released from cells during plasma membrane breakdown. Additionally, the dead cells have a forward-side shatter localization that is dramatically different from living cells. We find that the results using these different methods correlate with each other. As such, we are quite certain that the cells defined in our experiments as dead are indeed dead.

To initiate the production of pro-IL-1ß substrate for NLRC4 activity we prime the cells with LPS (100 ng/ml) and add Doxacyclin (500 ng/ml) to express the NLRC4 agonist. Apoptotic cell death in our assays is initiated by the standard apoptosis inducer etoposide (50 μM). In additional studies, NLRP3 activation has been induced by LPS priming and treatment with ATP (500 μM) alt. Nigericin (40mM).

*Monitoring responses to NLRC4 activation (**paper III**)*
There is no gold standard of how to estimate inflammasome activation. We are using pyroptosis, confirmed to be caspase-1 dependent, as our read-out. Other groups measure death, release of IL-1β or study cleavage of IL-1β or caspase-1. We control for caspase-1 activation is indirectly by detection of cleaved IL-1β. Release of IL-1β was detected by ELISA and cleavage by western blot.

*Protein over-expression (**paper III**)*
Protein over-expression is conducted by retroviral transduction of the cells with plasmids encoding for CrmA, cFLIP-L, cFLIP-S and Bcl-xL. The functional assays were performed as soon as possible post transduction and selection (around one week) to avoid drifting of the cells. We feel that genetically-encoded inhibitors are beneficial over chemical inhibitors as chemicals often have poorly characterized off-targets effects.

*Assays to evaluate mitochondrial functionality (**paper III**)*
Whole cell intracellular ROS was detected using CellROX Deep Red (Invitrogen Life Technologies). In this assay, the cells are stained with fluorogenic probes that are non-fluorescent in reduced form but when oxidized (by various forms of ROS) it exhibit strong fluorogenic signal that can be detected by flow cytometry.

To determine changes in mitochondrial membrane potential, the cells were loaded with TMRE for 30 min, to washed and resuspended add it in a lower maintenance concentration of TMRE. The cells were activated with Dox +/- CsA or the positive control CCCP to activate a collapse of the potential. When applicable, CsA was added to cultures 1 h prior to TMRE loading. Decreases in membrane potential were defined by decreases in TMRE staining detected by flow cytometry relative to loaded, washed, and incubated but unstimulated cells. Since TMRE and PI overlap in fluoresent emission, Viability Dye eFluor 450 was used as an alternative to assess cell viability after activation.

*Confocal microscopy (**paper III**)*
The plasma membrane (PM) of C34 and C19 cells were labeled with Cell Mask Orange and the cells imaged every 10 min for 22.9 h. The PM labeling, C19 and C34 signals were detected and separated by spectral unmixing.

*Quantitative RT-PCR (**paper III**)*
Confirmation of gene expression in B10R/C34/C19 cells was done using RT-qPCR with SYBR Green primers. The expression was normalized with the house-keeping genes HPRT1 and GAPDH as endogenous controls and up- or down-regulation of genes calculated using the ΔΔCt method. The ΔΔCt method is an approximate method and only valid if the amplification efficiencies of the target and the endogenous control are approximately equal. However, all used primers have been titered to confirm that their efficacy is similar to the controls. To confirm size of the predicted amplification products they were visualized on agarose gels stained with Gel Green Nucleic Acid Stain. In additional studies, RT-qPCR was also used to detect the presence of mtDNA.

*LC-MS/MS (**paper III**)*
Liquid-chromatography mass spectrometry (LC-MS/MS) was used to define the redox and acetylation state of HMGB1. HMGB1 was immunoprecipitated from pre-cleaned supernatants using a polyclonal antibody. Free thiol groups were alkylated with iodoacetamide. The cysteins residues in disulphide bonds were then reduced with dithiothreitol (DTT) and the newly exposed thiol groups were labeled with NEM. The samples were digested with trypsin or GluC, desalted and individual HMGB1 peptides were characterized using an AB Sciex TripleTOF 5600.

Statistics
Data were analyzed using Graphpad Prism v5.0d software and is shown as mean +/- standard error of the mean (s.e.m.). For statistical analysis two-tailed Student t-test was used and $p \leq 0.05$ was considered significant.

4 RESULTS AND DISCUSSION

4.1 Increased immunogenicity using intradermal electroporation

Even though HIV-1 was discovered 30 years ago (233), there is still no efficient HIV-1 vaccine. The vaccine field currently put more weight on mechanistic understanding and less on empirical observations than it used to, still, there is not yet clearly defined immune correlate of protection for diseases such as HIV-1. To understand the correlations is complicated, as the natural immune response to HIV-1 in general is unable to clear the infection. There are however exceptions with individuals that remain persistently uninfected with HIV-1 despite frequent exposure. One such example is the well-studied cohort of sex workers in Nairobi, whose protection were associated with strong HIV-specific $CD8^+$ T cells (234). Another exception are the so-called elite controllers, who are infected with HIV-1 but capable to suppress the virus to undetectable viral load (235). There is not a simple explanation to how these individuals manage to control HIV-1 infection, however Gag-specific $CD8^+$ T cells are implied to be important Characteristic of the controllers is not only the quantity of $CD8^+$ T cells but also their polyfunctional capacity, or their ability to produce high levels of multiple cytokines and chemokines simultaneously (236, 237). However, this immune profile may correlate to control of infection rather than to protection. To generate hypotheses about the immune responses necessary for protection against HIV-1 infection, successful immunizations or studies in monkeys are needed. Additionally, clues can be obtained from other vaccines.

Antibody titers are commonly considered to correlate with protection of vaccines. However, eliciting neutralizing antibodies to HIV-1 has been proven difficult with complicating features such as the rapid mutation rate of the virus and its heavy glycosylation. In the individuals developing neutralizing antibodies, these arise first weeks after infection and tend to be strain-specific (238, 239). Even though around 30% of the infected individuals can produce broadly neutralizing antibodies, only 1%, the so-called elite-neutralizers develop extremely broad neutralizing antibodies capable of neutralizing 95 % of circulating HIV-1 strains (240-242). Moreover, passive transfer of broadly neutralizing antibodies to macaques has shown that these antibodies can protect against infection. There are still many unanswered questions on how to design antigens and optimize immunization schedules in order to be able to induce such broad and robust neutralizing antibodies by vaccination.(243, 244)

Protection against HIV-1 probably requires a combination of humoral and multi-functional cellular immunity as well as innate responses. Moreover, this will require durable T cell responses of sufficient magnitude and quality. In addition, the $CD4^+$ T cells engaged in the response should not be provided as targets for HIV-1 infection and thereby fuel an infection. Great effort has been put into the development of $CD8^+$ T cell inducing vaccines. These responses have been beneficial in macaque studies but their importance was questioned with the failure of the STEP trial (215, 216). However, the large, more recent RV144 trial conducted in Thailand, showed more encouraging results as it was the first HIV vaccine candidate to show significant, although moderate (31,2%, $p=0.04$), protection against infection (245). The vaccine was a combination of two vaccines, prime with multiple-antigen viral-vector (ALVAC-HIV) and boost with recombinant gp120 protein (AIDSVAX), both previously evaluated as safe but insufficiently protective on their own(246).

Numerous immunologic assays on samples from the study have been performed and are still being followed up. The vaccine induced antibodies directed to the V2 loop of gp120, which may block from binding $CD4^+$ T cells (247).

The use of optimized delivery devices can enhance immunization potency and preferably also the quality of the response. In **paper I**, we studied immune responses induced by immunization of mice with the plasmid-encoded HIV antigen Gag (pGag) comparing homologous prime-boost intradermal (i.d.) electroporation (EP) and intramuscular (i.m.) injection. As mentioned, (section 1.4.2.3.2) i.m. injection is known to induce broad but rather weak immune responses in mice, while electroporation induces very robust responses. To date, studies of skin EP have mainly focused on antigen expression, antigen specific humoral immunity, induction of IFN-γ-producing $CD8^+$ T cells, and protective efficacy to infection (248-253). There has been a lack of knowledge of $CD4^+$ T cell responses, especially their ability to produce combinations of cytokines. Therefore, in **paper I** we wanted to demonstrate if skin EP enhances not only the magnitude of cytokine-producing antigen-specific $CD4^+$ T cells but also their diversity. As $CD4^+$ T cells are critical for inducing functional $CD8^+$ T memory cells and long-lived antibody responses, they are most likely required for protective vaccines.

$CD8^+$ T cells producing multiple cytokines appear to correlate with control of viral replication. $CD8^+$ T cells in patients with progressive HIV-1 disease secrete only IFN-γ, while they in elite controllers are multi-functional, secrete multiple cytokines typically in combinations of IFN-γ/TNF/IL-2 (238, 254-258). IFN-γ is necessary for an efficient response, however not always sufficient to predict control of viral replication used as a single parameter. Also TNF can mediate control of intracellular infections and synergizes with IFN-γ to induce killing of pathogens. IL-2 on the other hand has little direct effector function but enhances the expansion of CD4+ and CD8+ T cells.(259)

Similar correlations have also been shown for $CD4^+$ T cells. Highly-exposed seronegative individuals such as commercial sex-workers appear to be in a state of HIV-specific immune activation, with HIV-specific $CD4^+$ T cells that may correlate to protection (260-262). Hence, it is possible to induce these protective $CD4^+$ T cells without infection. Also elite controllers are associated with proliferating, highly functional HIV-specific $CD4^+$ T cells producing IFN-γ, IL-2 and/or TNF. In contrast, progressors are associated with HIV-specific $CD4^+$ T cells that secrete only IFN-γ and fail to proliferate (239, 263-266). Furthermore, vaccine efficacy to other diseases than HIV-1 has been observed to correlate with their induction of polyfunctional T cells (259, 267).

To evaluate the $CD4^+$ T cell responses, we stimulated splenocytes from immunized mice with Gag peptide pool, OVA peptides (negative control) or media alone, for 6 hours. We then stained the cells and used multicolor flow cytometry and applied a boolean gating strategy to detect single $CD3^+CD4^+$ cells producing combinations of IFN-γ, TNF, and IL-2. Additionally, in parallel assays we evaluated the quantity of produced IFN-γ by ELISPOT. Immunization with pGag by EP, compared to i.m. injection, significantly increased both the quantity of IFN-γ (Figure 1B, **paper I**) and the number of $CD4^+$ T cells being double or triple cytokine producers.(Figure 1C, **paper I**) The results were consistent in both inbred BALB/c and C57Bl/6 mice. These results imply that EP not only enhances the magnitude but also the polyfunctional

quality of the $CD4^+$ T cell responses.

Additionally, to study $CD8^+$ T cell responses, we stimulated splenocytes for 6 hours with a library of clade C Gag peptides (a p55 Gag peptide pool with 121 overlapping 15-mers) and for the BALB/c mice immunodominant Gag peptide (AMQMLKDTI) to a defined H2-Kd epitope. Responses were evaluated using IFN-γ ELISPOT. In BALB/c mice, we could observe increased IFN-γ production by $CD8^+$ T cells in EP group compared to the i.m. group in response to both clade C Gag peptides and AMQMLKDTI (Figure 2A, **paper I**). However we could not observe IFN-γ production above background levels in the C57Bl/6 mice (data not shown). The lack of detectable response is probably due to an inability for the cells to process the 15-mer peptides in the peptide pool and present them in the MHC class I complex during the restimulation. Shorter peptides, such as 9-mers, can bind directly to the MHC class I complex (248), which could have facilitated the stimulation. BALB/c mice have been observed to respond to the immunodominant clade C gag peptide AMQMLKDTI, which binds H2-Kd. Stimulation with this peptide triggered $CD8^+$ T cell responses from BALB/c mice also in this study. However, there is no such defined epitope for C57Bl/6 mice.

Finally, antigen-specific total IgG antibodies were detected by anti-p24Gag ELISA. We could observe that EP immunization induced two-fold higher IgG anti-p24 titers compared to i.m. injection. The results were consistent in both inbred BALB/c and C57Bl/6 mice.(Figure 2B, **paper I**)

In conclusion, our data showed that homologous prime-boost skin electroporation immunization induces robust and diverse immune responses. It provokes both humoral and cellular immunity with highly functional $CD4^+$ T cells. As shown by others, it is also likely that these $CD4^+$ T cells support $CD8^+$ T cell functionality.

These results further strengthen the advantages that electroporation has over standard needle-based injections. It induces more robust and diverse immune responses using smaller volumes and lower doses of antigen. This is thought to, at least partly, be due to better cellular up-take of the DNA. This advantage may be even more pronounced in larger animals than mice, as the efficacy of i.m. injection is not only dependent on the vaccine concentration but also the injected volume. The standard injection volume in the mouse tibialis anterior muscle in DNA vaccination is 50µl. This volume exceeds the fluid capacity of the muscle, leading to swelling and pressure that facilitates the cellular uptake of the DNA vaccine. When this the concentration of the DNA vaccine is kept the same but the volume is decreased to 5µl, the cellular uptake of DNA is diminished and both the antibody titers and T cell responses are substantially decreased (268). Similarly, the immunogenicity is higher when the vaccine is delivered into a small muscle such as the tibialis anterior rather than a larger muscle such as quadriceps femoris. Simplistic calculations comparing the size of the mouse calf with the human upper arm, show that the cross section of the human arm is about 900 times larger than the mouse leg. That implies that we would need to inject almost 45 liters into the human deltoideus to reach the same effect. It is highly unlikely that humans would approve of such a scaled up version.

4.2 Adjuvant properties of plasmid-encoded flagellin

TLR ligands are now included as adjuvants in various vaccination strategies. There are also adjuvants and vaccines that are empirically successful, which only recently have been shown to function through TLRs. For example; the so-called Coley's toxin, a mixture of bacterial cell lysate used since 1893, has it response mediated through TLR9. Commonly used vaccines such as BCG-SSI, Influvac, and Typhim contain TLR ligands inducing a Th1 response. The live attenuated viral yellow-fever vaccine 17D activates DC subsets via TLR2, TLR7, TLR8 and TLR9 and elicits response involving IL-12p40, IL-6 and IFN-α, resulting in a mix of Th1/Th2 cytokine profile and antigen-specific $CD8^+$ T cells (188, 269). The TLR5 agonist flagellin is a well-established adjuvant in protein-based vaccines, used since 1998 (270, 271). As a polypeptide adjuvant it elicits a robust humoral immunity also when used in very low concentrations and prior immunity to flagellin does not impair the response (271, 272). Since flagellin is one of the few TLR agonists possible to use in a DNA-encoded form, it is a very appealing adjuvant in plasmid DNA vaccination.

In **paper II** we evaluate the adjuvant properties of plasmid-encoded flagellin (pFliC) when administrated through three routes representing different tissue compartments: mucosal (intranasal, i.na.), systemic (i.m. injection), and dermal (gene gun, g.g.). Even though i.m. injection is a well studied and efficient route of administration, it may not always be the most adequate, especially not when a mucosal immunity is requested.

In this study we can conclude that pFliC(-gly) increases both humoral and MHC Class I-dependent cellular immunity when co-delivered with the model antigen pOVA through all three routes (Figure 2 and 4A, **paper II**). Furthermore, it also induces mucosal antibody responses and MHC Class II-dependent cellular responses when delivered mucosally (Figure 3 and 4B, **paper II**).

Since we could show that pFliC promoted both cellular and humoral mucosal immunity we decided to test its effectiveness when delivered together with the clinical plasmid antigen gp160 (pgp160Lfai/pRev) in a heterologous plasmid prime-protein boost setting. This approach was chosen because it has been observed that heterologous prime-boost vaccine regimes can be more immunogenic than homologous prime-boost(273). Here we could again show that intranasal (i.na.) immunization is dependent on the delivery lipid N3, which we believe protects the DNA vaccine from degradation. However adding pFliC further enhanced the production of total IgG in serum (Figure 5B, **paper II**) and both enhance the production of IgG1 and IgG2a and sustained it to later time points (Figure 5C, **paper II**). Similarly, addition of pFliC enhanced the titers of IgA in nasal washes (Figure 6A, **paper II**). The IgA was additionally shown to be reactive to clade A and B and slightly to clade C (Figure 6B, **paper II**). To further study the quality of the antibody we did B cell epitope mapping against a gp160 peptide epitope library. The groups immunized with plasmid gp160 with N3 and boosted with recombinant gp160 with L3B exhibited clear reactivity against gp160 epitopes. Again, addition of pFliC improved the response by expanding the detectable populations by five, after excluding one new reactive site because of slight similarities between FliC and the region of increased reaction.

The adjuvant effect of polypeptide flagellin is mainly due to its high affinity for TLR5 (274, 275). However soluble flagellin can also elicit NLRC4 mediated generation of inflammasome cytokines. Vijay-Kumar et al. have shown that soluble flagellin enhances production of anti-OVA antibodies in both TLR5 KO and NLRC4 KO mice following i.p. immunization. In mice deficient of both TLR5 and NLRC4 however, the antibody production is totally abrogated. This indicates that either receptor is sufficient for humoral responses. In the TLR5 KO mice, flagellin failed to induce release of cytokines other than IL-18, indicating that IL-18 plays an important role in antibody induction in these mice. In contrast to pro-IL-1β, pro-IL-18 is stored intracellularely and is not in need of a priming signal. NLRC4 KO mice on the other hand produced normal levels of all studied cytokines except IL-1β and IL-18 (75, 276). As both receptors can be sufficient for humoral responses, it is possible that the involvement of TLR5 and NLRC4, respectively, can vary depending on the immunization site and cell type, as the expression pattern of these receptors differs between tissues. Additionally, not all cells respond in the same way to NLRC4 activation. The immunogenicity can for example differ depending on whether the cell undergoes pyroptosis or not. How soluble flagellin is getting into the cytoplasm is unknown. It is likely to occur in limited rate, and NLRC4 is possibly activated to a higher degree using DNA vaccination. The plasmid-DNA encoded FliC used in **paper II** is transcribed into a protein equipped with a leader sequence and should be secreted. However, on its way out of the cell some of it may retrotranslocate from the ER into the cytosol where is could activate NLRC4/Naip5. It is therefor possible that its adjuvancy is due to a combination of TLR5 and NLRC4 signaling, and it may differ from the result of immunization with peptide flagellin. Flagellin has previously been shown to induce different responses depending on in which form it is administered. The response to soluble recombinant flagellin following systemic administration has been shown to be predominantly Th2, while in contrast, when FliC is administered during subsequent infection with/bound to S. *typhimurium* or produced by a plasmid DNA as an adjuvant, it enhances Th1 responses (192, 277, 278).

To determine if secreted pFliC(-gly) protein produced from pFliC vectors could activate TLR5, we applied culture supernatants from pFliC(-gly), pFliC(-gly)**Δ**34 transfected 293 cells, or recombinant FliC protein to alveolar macrophages from B57BL/6 or TLR5 deficient mice. Both full-length and D34 secreted FliC(-gly) polypeptides were able to activate B57BL/6 macrophages to produce TNF, but not macrophages from TLR5 deficient mice, indicating that it can signal through TLR5 independently of NLRC4. To evaluate NLRC4 activation we used an *in vitro* retroviral lethality screen assay. This assay detects the ability of macrophages, B10R cells, virally transduced with genes expressing various flagellin constructs as well as GFP to undergo pyroptosis in response to our secreted form of pFliC. GFP positive cells are taken as evidence of a lack of NLRC4 activation while GFP negative cells, relative to controls, are taken as evidence of NLRC4 activation. As positive control we used cytoplasmic flagellin (pcFliC(-gly)), which lack the leader sequence and remains in the cytosol. In this simple set-up, pFliC(-gly) seems to kill the cells as good as pcFliC(-gly). As negative control we used both secreted and cytoplasmic FliC with the NLRC4-activating tail deleted (Δ34c). These do not kill the cells, indicating that the cell death is NLRC4 dependent.

The transduction rate of is low in 293T cells however it is even lower in B10R cells, which is most likely because of poor packaging efficacy of the plasmids when

they contain flagellin inserts. Some plasmids do not package easily, an IRES sequence for example is known to drastically reduce packaging efficacy.((279)) However, we have repeated this experiment multiple times with the same results and have confidence in these results.

We do not know if our *in vitro* observations also hold true *in vivo*. However, if they did it would suggest that the some of the pFliC(-gly) in the immunization remains in the cytosol after transcription instead of being secreted by the Golgi-ER pathway. If so, it may activate the NLRC4 inflammasome, thereby affecting the immune response and the adjuvant properties of flagellin. This occurs when using peptide flagellin (276), and is likely to happen also with plasmid flagellin. The question is what impact the inflammasome activity and subsequence secretion of cytokines such as IL-1β and IL-18 has on the adjuvancy of pFliC and how it skews the immunity. It has been observed that IL-1-family cytokines (IL-1α, IL-1β, IL-18 and IL-33) have strong mucosal adjuvant potential and a propensity to promote Th1-like responses (280). Inflammasome dependent IL-1β, is further known to favor Th17 polarization in infections such as *Legionella pneumophila* and *Schistosoma mansoni* and absence of IL-1 often leads to decreased numbers of cells producing IL-17 and IFN-γ (281, 282). Furthermore, IL-1β can promote T cell proliferation and contribute to maturation of dendritic cells (281). Supporting the theory of inflammasome induced Th17 responses, NLRP3 activation by uric acids induces Th17 responses(52). IL-17 plays an important role against infection especially in mucosal tissues, promoting epithelial regeneration, recruiting neutrophils, promoting macrophage recruitment and survival, stimulating production of proinflammatory cytokines and antibacterial peptides from both immune and non-immune cells. Furthermore it enhances the capacity for $CD4^+$ T cells to produce IL-2 and enhance proliferation of both T cells and Tregs (163). Because of this Th17 cells are likely to be important for defense against for example HIV-1, and HIV-1 long-term non-progressors have also been reported to have greater numbers of Th17 than progressors. IL-18, as mentioned (section 1.1.2) can amplify different cytokines depending on its cytokine milieu and hence favor different responses. NLRC4 activity can mediate non-cognate IFN-γ release during *Salmonella typhimurium* infection. Released IL-18 drives IFN-γ -production by memory $CD8^+$ T cells, as well as NK and NKT cells (283). This could affect the frequency of IFN-γ observed in **paper II**.

Unexpectedly, the well-studied NLRP3 inflammasome activating adjuvant Alum induces strong Th2 responses. It is unknown why, but can of course be due to a differently balanced immune response. Additionally, the Th2 skewing has been seen to by independent of inflammasome activation (284, 285). Furthermore, Alum has successfully been combined with certain TLR agonists. When Alum is combined with the TLR4 ligand monophosphoryl lipid A (MPL) in the licensed adjuvant AS04 (GlaxoSmithKline), it results in a predominantly Th1 response(286). MPL is a compound, derived from *Salmonella minnesota* LPS, that is less toxic than LPS, possibly because it only signal through the TRIF-TRAM pathway while LPS signal through both MyD88-TIRAP and TRIF-TRAM (287).

In **paper II** we did not have the possibility to assay IL-17, however both the production of IFN-γ following immunization through all three routes as well as the $CD4^+$ T cell response profile indicates that pFliC(-gly) promotes a Th1 response rather than a Th2 response. Apparently pFliC does not entail the same problem with Th2 skewing as Alum. We cannot say how much of the adjuvant effect that is due to signaling through TLR5 vs. NLRC4 even though we can conclude that both systems

can be activated by pFliC(-gly). Some of these questions could be answered by immunizing with a plasmid DNA-vaccine combined with full-length pFliC and pFliC Δ34C adjuvants in wild type, TLR5-, NLRC4-, Caspase-1-, IL-1β-, IL-18-, IL-1/IL-18-, IL-1βR-deficient mice.

It is interesting that we can observe IgG and IgA in mucosal compartments following i.na., but not i.m. or g.g immunization. Mucosal immunization is well known to elicit stronger mucosal immune responses than systemic immunization, also at different mucosal sites than the immunization site. This is thanks to the mucosal-associated lymphoid tissue (MALT), a highly compartmentalized immunological system that is anatomically separated from the systemic immune apparatus. I.na. immunization is however known to induce more widely disseminated mucosal IgA and systemic IgG compared to other mucosal immunization routes e.g. oral, rectal and vaginal(288). What is more difficult to explain is how flagellin has these special adjuvant effects only when delivered mucosally. One mechanism of how flagellin could act as a mucosal adjuvant involves the $CD103^+$ dendritic cells in the lamina propria in the small intestine. These cells express high levels of TLR5 and respond directly to flagellin. Immunization with soluble FliC induces TLR5 dependent selective recruitment in of $CD103^+$ DCs to the mesenteric lymph nodes, where it primes for Treg induction and promotes switch of naive B cells from IgM IgD to IgA (289, 290). How flagellin drives the migration of $CD103^+$ DCs is unknown, but it probably involves binding of TLR5, since these cells express very high levels of TLR5 compared to macrophages and DCs in other tissue compartments (231). However, this recruitment $CD103^+$ DCs follows both i.p. and s.c. flagellin immunization, indicating that this phenomenon is not restricted to mucosal immunization (290). The discovery of the NLRC4/Naip5 inflammasome system in sensing flagellin indicates that these previous observations may also be influenced by these innate immune receptors.

4.3 NLRC4 induced pyroptosis

The previous two papers were focused on pre-clinical vaccine studies. In **paper II** we realized that activation of the NLRC4 inflammasome can contribute to the adjuvant effect of pFliC. Therefore, in **paper III** the focus was shifted to studies on flagellin-induced inflammasome activation.

Most inflammasome studies have focused on the involvement of the NLRP3 inflammasome. Mainly because it is activated by a large array of stimuli and is involved in many different conditions which allows medical researchers from diverse fields to find a tie-in. However, there are still more inflammasome complexes being discovered and the individual importance of the NLRP3 inflammasome in inflammatory responses and pathologies remains to be seen.

To activate the NLRC4 inflammasome, it is possible to infect macrophages with whole bacteria expressing agonist(s), transduce macrophages with retroviral vectors expressing flagellin, or transfect macrophages with recombinant flagellin protein. However, all of these techniques activate other cell signaling pathways in addition to NLRC4 inflammasome activation. The aim of **paper III** was to establish a system to induce expression of C34, the NLRC4 activating region of flagellin, without triggering other cell signaling pathways. We wanted to be able to activate the NLRC4 inflammasome alone, to then add back different forms of stimuli to understand how they affect the system. To establish such a system, a mouse

macrophage cell line, B10R, was stably transduced with a Tet-ON based retroviral vector expressing the C-terminal 34 or 19 amino acids of *S. typhimurium* (FliC) fused to EGFP. Expression of C34-EGFP or control C19-EGFP was controlled by the addition of the tetracycline analogue Doxycycline (Dox).

It has previously been shown that the expression of NLRC4 is independent of a priming signal (59). Using our Dox-inducible system we can now further conclude that a biological function of NLRC4 activation, pyroptosis, can be activated independently of LPS priming. Additionally, LPS priming does not affect the degree of pyroptosis (for 3 and 18 hours).(Figure 1C-E, I-J **paper III**) However, when priming is applied, this allows for the expression of pro-IL-1ß which is then processed and released as processed IL-1β with NLRC4 inflammasome activation. (Figure 1G-H, **paper III**).

Interestingly, the fact that pyroptosis is unaffected by TLR priming and associated pro-survival signals activated through NF-κB, suggests that this type of cell death is "separate from" or "above" NF-κB in a signal hierarchy. The pyroptosis system appears to function in this way and may be a last resort for an infected cell to take if an infection is 'perceived' to be present in the cytoplasm. Pyroptotic independence from the NF-kB system is likely of great importance since a variety of pathogens attempt to interfere with immune defense by promoting NF-κB-dependent pro-survival signals. Furthermore, our observations of pyroptosis occurring independently of LPS priming would ensure pathogen recognition and successful pyroptosis also in cells expressing TLR4 but unresponsive to LPS stimuli. This would be of special importance in hyporesponsive compartments as the gut, where the cells are anergic to TLRs (see section 4.6) Indeed, it has been observed that prolonged LPS exposure of macrophages and dendritic cells leads to LPS tolerance and downregulation of TLR4 (291). We, as well as others have observed that a LPS priming signal simultaneous with, as well as too long prior to (18 hour prime) inflammasome stimuli in our immortalized BMMs fails to induce IL-1β release (data not shown). Additionally, a requirement for an inflammasome priming signal during infection could make delayed activation of host-defense a handicap to successful defense.

The ability to respond without priming is however especially important in the intestine. Resident intestinal mononuclear phagocytes (iMPs) are anergic to TLRs (292-294) and have down-regulated levels of both mRNA and protein for multiple innate response molecules as well as adapter molecules such as MyD88 and TIR.(295) In contrast to monocytes and BMDMs, unstimulated iMPs constitutively express pro-IL-1β, and have been observed to undergo NLRC4 dependent pyroptosis and in fact also release IL-1β independent of priming in response to *S. typhimurium*. Induction of the NLRP3 inflammasome using LPS and ATP on the other hand, does not induce IL-1β processing, consistent with the lack of NF-κB-mediated upregulation of NLRP3 (294). Since NLRC4 is thought to exclusively recognize components from the Type III secretion system and flagellin from pathogenic bacteria, this system ensures that pathogens are discriminated from the commensal flora and can respond rapidly also in a "tolerant" environment.

To confirm that our observed cell death is pyroptosis, defined as caspase-1 dependent cell death, we transduced C34 cells with the cowpox virus serpin CrmA, inhibiting caspase-1 and -8. Expression of CrmA inhibited the cell death to base line levels (Figure 1F, **paper III**). However the capase-8 specific inhibitors cFLIP-long and

cFLIP-short did not affect the cell death indicating that the cell death is dependent on caspase-1. Occasionally, cell death correlating with NLRP3-dependent processing of IL-1β is independent of caspase-1 (63). We feel this form of cell death is not to be defined as pyroptosis but rather apoptosis in a primed setting with parallel inflammasome activity.

The outcome of NLRC4 activation is most likely affected by the strength and length of stimulation. Activation of a stress response pathway called autophagy is probably among the first pathways activated upon infection. Stress management, if successful would allow cell survival upon NLRC4 activation. However, in situations where activation is dominant these pathways, at best, would only slow down inevitable pyroptotic death. In our model pyroptosis occurs rapidly and a significant increase in cell death is seen already after two hours with a maximum after 24-32 hours. In comparison, etoposide-induced apoptosis in C34 cells is first observed at around eight hours. Some studies report that IL-1β secretion precedes pyroptosis (296, 297), however in our system these two responses occur simultaneously (Figure 1C-E, G-J, **paper III**). Additionally, both the IL-1β release and the pyroptosis are dose-dependent to the amount of NLRC4 agonist (data not shown) For studies such as ours, a functioning inducible system is very beneficial, since we can control the time point of NLRC4 activation, agonist dose, and general persistence of the stimuli. Additionally we can study the outcome of cells after removing the stimuli. To remove whole bacteria after initial macrophage infection requires antibiotics, commonly gentamicin, which could possibly affect the inflammasome/pyroptosis system. To study the recovery of C34 cells after NLRC4 activation we Dox-induced cells followed by Dox removal by washing cells at various time points in a kinetic study over 24 hours. This data is not present in paper III but is presented here in this thesis. Agonist induction and cell death were examined at the indicated times after Dox removal and at 24 hours after Dox addition. When examining cell death at the indicated times, we found that it increased and correlated with agonist expression over time with a maximum at 24 hours. However, when Dox was removed at different time points after initial induction, we found that the amount of EGFP signal did not continue to increase compared to cells continually incubated in Dox (Figure 3A). Nevertheless, the cell death continued to increase also after Dox removal. Even the cultures that had only one hour of NRLC4 activation followed by Dox removal also reached almost maximum pyroptosis after 24 hours.(Figure 3B) These results could indicate that even a brief NLRC4 inflammasome activation could be a point of no return. In our system, after the agonist is detected in the cytoplasm, some cells die rapidly, and others slower. However, in cells only briefly Dox activated, the presence of persistent but low levels of agonist is still able to instruct nearly all responding cells (EGFP+) to undergo pyroptosis at the same frequency by 24 hours. To extrapolate these findings to an infectious model system, these results suggest that also lower doses of bacteria expressing NLRC4 agonists could induce pyroptosis in a susceptible cell if the infection and agonists persist.

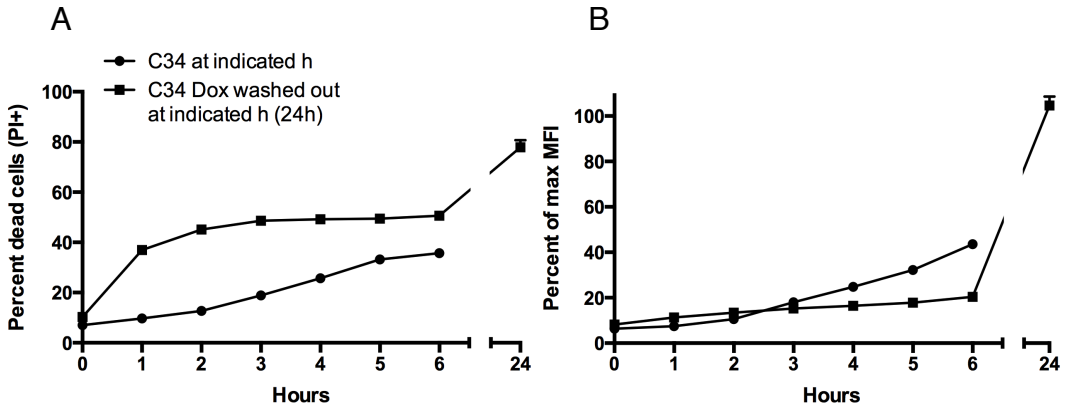

Figure 3. C34 cells eventually die even though the Dox is washed out at different time points. A. Viability. B. EGFP induction.

4.4 Release of IL-1β

As mentioned, IL-1β lacks a conventional secretion signal and is not released by the ER-Golgi secretory pathway. Generally speaking, when IL-1ß is released other leaderless cytokines and DAMPs, such as HMGB1 can be secreted simultaneously. Different release mechanisms have been reported and they are probably not mutually exclusive but influenced by the cell type, stimuli, and time. Proposed mechanisms are exocytosis of secretory lysosomes, release of membrane-delimited microvesicles as plasma membrane blebs (298-300), release of membrane-delimited exosomes formed by recycling endosomes (301), exocytosis of autophagosomes or autophagolysosomes (29,302-305). Pannexin-1 pore formation (306-308) or direct release by cell death (116, 130).

Autophagy, the stress response responsible for degradation of dysfunctional cellular components, can affect IL-1β release in two ways. In a context of the NLRP3 inflammasome, baseline autophagy decreases the release of IL-1β. This is mainly due to the NLRP3 inflammasome's dependence on mitochondrial DNA and ROS, which during normal conditions is cleared by autophagy, thereby hampering or delaying NLRP3 activation. Impaired or inhibition of autophagy leads to an accumulation of dysfunctional mitochondria leading to generation of ROS and release of mitochondrial DNA which augments NLRP3 inflammasome activity (302, 305, 309). Furthermore autophagosomes can control IL-1ß release by sequestering and degrading pro-IL-1β polypeptide and inflammasome components needed to process pro-IL-1ß (303, 309, 310). In contrast, autophagy induced by starvation or inhibition of mTOR (rapamycin) can act as a mechanism for IL-1ß secretion. Most produced pro-IL-1β is thought reside in the cytosol, however some of it is protected inside vesicles suggested to be autophagosomes (302-305). LPS treatment induces recruitment of IL-1β to autophagosomes and the autophagosome can either fuse with a lysosome and the IL-1β will be degraded, or it can be transported to the cell surface and the IL-1β released (303, 305). NLRC4 and NLRP4 negatively regulate autophagy by binding the autophagy component Beclin-1 which may be a way that activation of certain NLRs could release the brakes on IL-1ß production. Furthermore, NLRP4 inhibits maturation of autophagosomes and thereby the fusion with lysosomes and hence degradation of content. In contrast, NOD1 and NOD2 directly induce

autophagy by recruiting the autophagy regulator Atg16L1 to phagosomes containing bacteria. Atg16L1 activation may be a specific pathway linking bacterial recognition and autophagy. Cells from Atg161 deficient mice undergo standard physiological activated autophagy but not bacteria-induced autophagy and produce more IL-1β in response to stimuli such as LPS. Not surprisingly, a consequence of Atg16L1 deficiency is associated with the hyper-inflammatory condition Crohn's disease (304).

In our system, the release of IL-1β correlates with pyroptosis (Figure 1G-J, **paper III**), indicating that cell lysis could be a mechanism of release. However the IL-1β ELISA that we used does not discriminate between the unprocessed and the active form of IL-1β. By using immunoblotting techniques to detect IL-1β in supernatants and lysates, we observe the presence of cleaved p17 IL-1β, but also remaining uncut pro-IL-1β.(Figure 1H, **paper III**) This indicates that the release of processed IL-1ß does not necessarily increase proportionally to pyroptosis. Interestingly, unprocessed pro-IL-1β released cells can also be inflammatory. It is not exclusively cleaved by caspase-1 enzymatic activity but can also be processed by neutrophil-derived proteases (311).

Hypothetically, it could be possible that in our system we would observe more processed IL-1β released if the cells went through a "slower" death. Unlike NLRP3, the absolute requirement for NLRC4 inflammasome function is not dependent on the inflammasome adaptor ASC. However, it has been observed that IL-1β processing is strongly diminished in its absence (312). The presence of ASC can also function in a negative feed-back loop to inhibit pyroptosis and favor IL-1β processing (313). Since most of our cells die with rather low levels of processed IL-1β, around 150 pg/ml, it is possible to speculate that the ASC levels are detectable, but low, and the death/IL-1β ratio may be possible to skew by over-expression of ASC.

Arguing against the idea of lysis-dependent release of IL-1β are studies where glycine is used to inhibit/slow NLRC4 and NLRP3-induced cell death but still allows cytokine release. However, researchers observing these phenomena have suggested that IL-1β could leak out through proposed pore-like formations in the cell membrane (130, 314). In experiments ongoing in our lab we are working to inhibit pyroptosis, without inhibiting caspase-1 enzymatic activity and thereby possibly influencing the release of processed IL-1β.

However, as mentioned, the mechanisms of IL-1ß release and pyroptosis are probably not mutually exclusive and may be strongly influenced by cell type, kinetics of activation, cytokine environment and type of infection.

4.5 Involvement of mitochondria and ROS in NLRC4 inflammasome function.

The activation of the NLRP3 inflammasome has been observed to be dependent on disrupted mitochondrial function, mitochondrial ROS production, K^+ efflux and release of Ca2+ from the endoplasmatic reticulum (59, 62, 63, 302, 315). The study by Shimada et al. (63) established a working model by linking these three events. They could demonstrate that many NLRP3 activators disrupt mitochondrial function, as revealed by dissipation of the inner mitochondrial membrane potential, possibly as a consequence of K^+ efflux and Ca^{2+} mobilization (315) upstream of inflammasome assembly. The disruption leads to mitochondrial ROS production and, furthermore, release of oxidized mitochondrial DNA (mtDNA) into the cytosol, where it acts as an agonist to promote NLRP3 inflammasome assembly (63).

Since we have the ability to study responses to NLRC4 inflammasome activation in absence of TLR activation or other additional stimuli, we wanted to study the role of mitochondria in this activation process and whether ROS is produced.

From our first experiments we could conclude that NLRC4-induced pyroptosis is unaffected by ROS production since it was unaffected by ROS generating LPS priming. Our ongoing observations were confirmed along the way by Zhou and colleagues (62). Furthermore, we observed that pretreatment of C34 cells with the general antioxidant N-acetyl-cysteine (NAC) or the organelle-specific antioxidants MitoQ (mitochondrial) or the NADPH oxidase (Nox) inhibitor VAS-2870 prior to Dox addition does not affect pyroptosis.(Figure 4) We have not yet studied release of IL-1β during inhibition of ROS. However, its processing could possibly be diminished with antioxidant use since ROS may be required for recruitment of ASC (62).

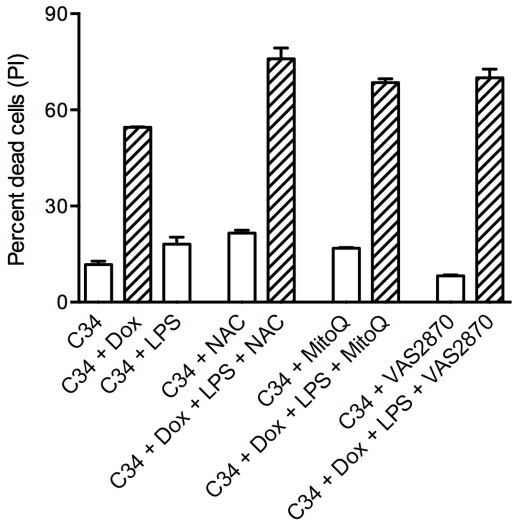

Figure 4: Pre-treatment of C34s with antioxidants NAC, MitoQ or VAS-2870 does not affect NLRC4 induced pyroptosis

When Dox-activating unprimed C34 cells, we did not observe production of ROS or nitric oxide (NO), although the cells were capable of such production after LPS stimuli.(Figure 3B-C, **paper III**) In macrophages, mitochondria and NADPH oxidases are thought to be the major sub-cellular sources of ROS production. In our experiments we used an assay that detects whole-cell ROS. It is conceivable that the source of ROS after LPS activation is the mitochondria as shown by West et al. (62, 316). The mitochondria, rather than NADPH oxidases, have been observed to be the source of ROS upon activation of the NLRP3 inflammasome, as macrophages lacking functional NOX1, NOX2 and NOX4 respond normally to NLRP3 stimulation(317, 318). Here we use the term ROS to describe numerous species of reactive oxygen intermediates. In reality, several types of ROS species are formed during the process of oxidative phosphorylation used to generate ATP. In this process, O_2 is reduced to H_2O by a series of protein complexes (I to IV) to generate an H^+ gradient which powers ATP synthesis by protein complex V. If complex I is inhibited, by for example the chemical rotenone**,** this "short-circuits" the process and leads to

increased ROS production. Our observed lack of ROS production during NLRC4 activation and pyroptosis indicated to us that NLRC4 agonists apparently do not cause disruption of the mitochondria as NLRP3 agonists do. However, being curious we decided to investigate mitochondrial involvement in greater detail.

To inhibit mitochondrial outer membrane permeabilization (MOMP) involvement in pyroptosis, we overexpressed the Bcl-2-like protein Bcl-xL in C34 cells. MOMP occurs during standard intrinsic apoptosis (section 1.2.1.1) and overexpression of Bcl-2 and family members lead to partial closure of the voltage-dependent anion channels (VDAC) in the outer membrane of the mitochondria. This closure decreases mitochondrial membrane permeability and ROS production upon apoptotic challenge. Overexpression of Bcl-2 inhibits apoptosis associated with NLRP3 activation (63) and decreased IL-1β release after NLRP3 stimuli (62). However, in our system, over-expression of Bcl-xL does not inhibit NLRC4-induced pyroptosis although it inhibited etoposide-induced apoptosis.(Figure 4A-B, **paper III**)

To evaluate the stability of the inner mitochondrial membrane potential during NLRC4 activation we loaded the cells with the fluorescent dye TMRE which is specifically taken up by mitochondria and held inside dependent on a stable inner membrane potential. Decreases in inner membrane potential can be measured as a decrease in TMRE fluresence ($\Delta\psi_m$) using flow cytometry. Dox-induction of the cells led to a slight, but still significant and reproducible decrease in $\Delta\psi_m$. We then questioned whether this decrease was associated with pyroptosis. When inhibiting this slight decrease by suppressing inner membrane permeability with the compound Cyclosporin A (CsA), we could prevent the slight decrease in membrane potential but observed no difference in the degree or speed of cell death, indicating that NLRC4 induced pyroptosis occurs without a need to dissipate the inner mitochondrial membrane potential.(Figure 4C-G, **paper III**)

It has been shown that the mitochondrial disruption observed after NLRP3-triggering stimuli leads to release of oxidized mtDNA into the cytosol where it promotes NLRP3 activation (62, 63, 302). In unpublished experiments I performed quantitative RT-PCR to detect release of mtDNA from Dox-induced C34 cells, but the levels we observed were not above background (Figure 5). These results further support (indirectly) our observations that mitochondria likely remain intact during NLRC4 activation.

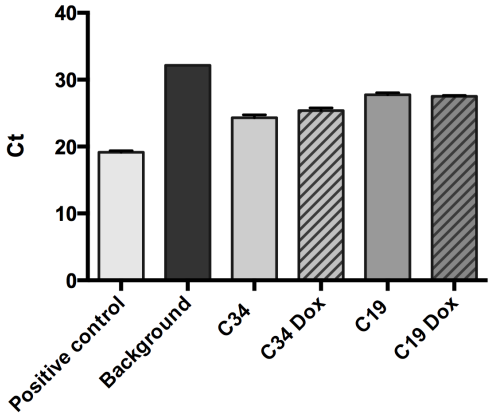

Figure 5: There is no increased release of mitochondrial DNA from Dox-induced C34s compared to unstimulated cells.

While performing our experiments indicating that NLRC4 activation and pyroptosis occurred independently of standard mitochondria involvement, we tested whether we could observe cutting of the pro-apoptotic protein Bid. Generally, when Bid is cut during the activation of cell death pathways into a truncated form called tBid, this small form acts to promote MOMP opening and trigger apoptotic cell death(319). Analysis of Bid by immunoblot revealed an intact form in unactivated cells but we observed a cleaved tBid form upon triggering pyroptosis (Figure 6). However, while searching the extensive cell death literature we found that this phenomenon has been observed before in situations of regulation of NOD1 and NOD2 signaling and does not absolutely implicate mitochondria involvement in cell death pathways (320). Indeed, this observation may mean that macrophage pyroptosis is more of 'type I' cell death (section 1.2.1.2).

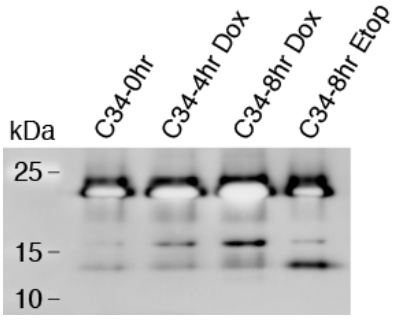

Figure 6: NLRC4 activation induces Bid cleavage.

Potassium (K^+) efflux from cells is associated with, and may be a necessary factor to activate the NLRP3 and NLRP1 inflammasomes. Indeed, lower intracellular K^+ is thought to be what triggers decrease in $\Delta\psi_m$ in mitochondria and facilitates release of oxidized mtDNA. The involvement of K^+ in NLRC4 activation is less clear. We have shown that the NLRC4-induced pyroptosis is independent of decreases in $\Delta\psi_m$, and could thereby speculate that it would be independent of K^+ efflux. However, pyroptosis and IL-ß processing may not be absolutely linked. It may be possible that IL-1β processing could be diminished by inhibiting K^+ efflux during inflammasome activation as physiological levels of K^+ inhibits ASC assembly. ASC can be co-immunoprecipited together with NLRP3 (and NLRC4) in normal K^+ conditions but not when K^+ efflux is inhibited by high extracellular K^+ indicating that K+ could influence the NLRC4 inflammasome (58).

I have attempted to experimentally address these issues by inducing NLRC4 activation in C34 cells in various concentrations of extracellular K^+. However, I have seen no difference in the degree of pyroptosis using various K^+ concentrations (data not shown). However, our C34 cells cannot cope with as high concentrations of K^+ as is used in other studies.

In conclusion, activation of the NLRC4 inflammasome clearly differs from the NLRP3 inflammasome as it does not require ROS production or involvement of the mitochondria in the same fashion. Furthermore, activation of the NLRC4 inflammasome is not associated with generation of ROS, NO or release of mtDNA. These findings illustrate the numerous differences between the NLRC4 and the

NLRP3 inflammasomes, and can partly explain why both these inflammasomes are activated while recognizing the same pathogen in the same cell.

4.6 When would cells find enough NLRC4 stimuli without activation by other PAMPs?

We have shown that NLRC4 activation is independent of TLR priming and ROS. But would such a situation arise *in vivo*? In all proposed systems NLRC4 activation would nearly always happen with surface TLR activation. So when would a cell find enough flagellin to activate the NLRC4 inflammasome, without additional TLR stimuli or ROS production through TLR activation?

In commonly used cell systems using in vitro differentiated macrophages activation of the NLRC4 system likely occurs at the same time as stimulation through TLRs which are still expressed by these cell types such as TLR4. In this context these models we would predict that NLRC4 and TLR signaling would always occur hand in hand.

However, we could imagine certain scenarios in which NLRC4 activation may occur with TLR engagement but cells have a "tolerized" TLR signaling pathway. Alternatively, TLRs are not engaged due to different cellular compartmentalization, lack of expression. Or maybe even standard expression and signaling however the cells may have altered metabolic functions and do not respond to produce ROS in the same fashion. Here, I discuss these possibilities in more detail.

The cells in tissues such as the intestine need to prevent harmful responses to commensal bacteria. The epithelia in the gut, as well as in the kidney and airways, is polarized with the apical side towards the lumen, exposed to microbiota and nutrients, and the basolateral side protected from such stimuli. Most TLRs expressed by epithelial cells, including TLR5, are located on the basolateral side. Hence, TLR5 is only activated if the epithelial is disrupted (231, 321, 322). As most chronic infectious bacteria, such as H. pylori, is known to be non-invasive, it will not activate TLR5. Because of this polarization, it is possible that non-invasive bacteria could activate NLRC4 in epithelial cells without TLR5 recognition. Furthermore, the epithelial TLR expression pattern is altered in different parts of the gut. TLR2 and TLR4 are expressed in low levels by intestinal epithelial cells, TLR3 is abundantly expressed in small intestine and colon while TLR5 is predominantly expressed in the colon (322). In this way the selective expression of TLRs may create scenarios in which an enteric pathogen could possibly activate NLRC4 without activating standard TLR responses.

Also the intestinal phagocytes have altered TLR expression. The already mentioned (section 4.3) resident iMPs in the gut are anergic to TLRs and may encounter NLRC4 inflammasome stimuli without the ability for TLR signaling.
However, a subpopulation of CD11c+ DC in the small intestine express high levels of TLR5, but not TLR4, and respond to flagellin even though they are unable to respond to TLR4 (231).

If ROS production in iMPs is dependent only on TLR signaling, a scenario of TLR-independent NLRC4 activation is likely to occur without generation of ROS, as mimicked by our system. If so, we could hypothesize that if pyroptosis occurs in an iMP that released HMGB1 should be in chemotactic all-thiol form. It therefore may be possible that pyroptotic iMPs cells could recruit more neutrophils to the site of infection than circulating macrophages during inflammasome activity. NLRC4 and

neutrophil influx in the intestine is an essential part in the defense against *S. typhimurium*. Even though IL-1β accounts for this influx to a great extent (294, 323), chemotactic HMGB1 may also play an unstudied role.

To avoid inflammatory overreactions, TLRs are downregulated upon prolonged stimuli. Endotoxin tolerance is a well known phenomenon where prolonged LPS stimuli induces downregulation of TLR4 at mRNA level as well as internalization of the receptor (291, 324). Similarly, prolonged flagellin exposure to epithelial cells blocks activation of NF-kB and MAPK signaling pathways and results in internalization of TLR5. This flagellin tolerance is a rapid process that occurs within 1-2 hours after flagellin stimuli, and the cells require more than 24 hours to recover (74). The TLR5 mRNA expression is also downregulated by other TLR agonists such as LPS and CpG (325). This form of tolarization may lead to establishment of the pathogen and chronic infection. In those cases, the ability to induce NLRC4 response in TLR hyporesponsive cells could be of importance.

Cells ability to respond to TLR stimuli can also be affected by surrounding cytokines. Th2 cytokines such as IL-4 and IL-13 downregulate TLR3 and TLR4 on mRNA level leading to functional impairment (326). IL-10 has also been suggested to decrease TLR expression since mice lacking IL-10 are hyperreactive to LPS (327) and blocking IL-10 leads to severe colitis in mice (323). Additionally, autocrine IL-10 appears to be involved in the "exhaustion" of DCs and blocking IL-10 enables DC to remain in mature state rather than get exhausted (328). We have induced pyroptosis in C34 cells stimulated with IL-10 and could observe no decrease in death, indicating that IL-10 does not affect NLRC4 activation (data not shown). However, it is possible that in a context of LPS priming together with IL-10 that less ROS could be produced affecting HMGB1 isoforms released by pyroptosis. This remains to be tested.

The ability of cells to undergo pyroptosis without any requirement for priming can also be important to sidestep bacterial evasion mechanisms. There are situations where bacterial evasion mechanisms could affect TLR signaling and ROS production. As the flagellin polypeptide activates TLR5 and NLRC4/Naip5 through different functional domains bacteria could possibly have a mutated flagellin to confer TLR5 activation while the inflammasome activating region is still intact. Indeed, point mutations of flagellin at I411A abolish motility and TLR5 activation, however it can still activate cells through NLRC4(329). Alternatively, NLRC4 can recognize non-flagellated bacteria through components of the T3SS/T4SS (330, 331).

Additionally, some bacteria avoid clearance by the host by interfering with ROS by neutralization or inhibiting the generation. However, they usually target the NADPH oxidation chain rather than mitochondrial ROS production.(332)

Very low levels of flagellin are required to activate cells through TLR5. The described mechanisms to avoid TLR signaling and generation of ROS do not completely abrogate ROS production but rather decrease is. However, this decrease may still have an effect on the isoform of HMGB1. Low levels of stimuli may induce levels of ROS that is low enough to be absorbed by stress-induced adaptation programs such as autophagy. As mentioned, the ROS dependent NLRP3 inflammasome can be hampered or delayed by basal autophagy clearing out low levels of ROS. In the same way, it is possible that autophagic activity inhibits the oxidation of HMGB1 at low levels of ROS (section 4.4).

Admittedly, these are only speculations and there is yet not enough known about the expression and involvement of the NLRC4 inflammasome in different situations of infection to know when ROS is produced, where ROS is produced, and how much ROS is produced.

CONCLUDING

The aim with this thesis was to study approaches to improve the efficacy of DNA vaccines. We have evaluated different delivery routes and methods as well as the adjuvant properties of plasmid encoded flagellin. As it turned out, the molecular mechanisms behind the adjuvancy of flagellin were too exciting not to study and this curiosity grew into a project in itself. By establishing our inducible system we also developed a great tool to study the effect of different additional stimuli on the NLRC4 inflammasome activity.

Our main findings demonstrate:

- Homologous prime/boost DNA vaccinations using skin EP enhances not only the magnitude of cytokine-producing antigen-specific CD4+ T cells, but also increases the functional heterogeneity (**paper I**).
- Plasmid-encoded flagellin enhances both cellular an humoral immunity when delivered i.na., i.m. or g.g. with additional mucosal immunity when delivered i.na.(**paper II**).
- NLRC4-induced pyroptosis is unaffected by LPS priming, ROS production, and standard mitochondrial involvement (**paper III**).
- The functional isoform of HMGB1 released during pyroptosis is affected by priming and correlates with ROS production (**paper III**).

To improve the strength of DNA vaccinations, a combination of an appropriate adjuvant customized to the antigen, recipient, immunization site and an effective delivery method will be required. Furthermore, the delivery route must be chosen to induce desired immunity at the infection site. Plasmid-encoded flagellin is a good adjuvant candidate, especially when mucosal immunity is desired. Its ability to induce a broad immune response through activation of both TLR5 and NLRC4 may mimic a "natural" inflammatory environment which could have advantages over other adjuvants to induce protective immunity. We have further shown the benefits of skin electroporation as a delivery method inducing both robust and diverse immune responses. However, mucosal immunization may be required for sufficient mucosal immunity.

Our studies, along with others, highlight the differences between inflammasome platforms. Differences in how inflammasomes are triggered can explain why a pathogen may have the ability to activate multiple inflammasomes. Furthermore, with our increased understanding of the cellular differences associated with how inflammasomes work, we may be able to better understand differences in inflammasome outcomes.

Although it has been previously shown that pyroptosis is inflammatory beyond IL-1β and IL-18 release, our results reveal an increased understanding of the metabolic state of pyroptosis and how changes induced by priming likely alter the body's perception of pyroptotic cell death. Although experimentally challenging, we look forward to a better understanding of the relationship between other innate immune receptors and metabolism in the inflammasomes roll in host-defense and pathology.

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