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TNF-dependent alveolar macrophage and ROS-dependent neutrophil mediated mechanisms control *Legionella pneumophila* lung infection

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Never give up. You only get one life. Go for it!

-Richard E. Grant

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Summary

Legionella pneumophila is a facultative intracellular bacterium that lives in aquatic environments where it parasitizes amoeba. However, upon inhalation of contaminated aerosols it can infect and replicate in macrophages, including human alveolar macrophages (AM), which can result in Legionnaires' disease, a severe form of pneumonia. Upon experimental airway infection of mice, *L. pneumophila* is rapidly controlled by innate immune mechanisms. Here we identified, on a cell-type specific level, the key innate effector functions responsible for rapid control of infection. We show that in addition to the well-characterized NLRC4-NAIP5 flagellin recognition pathway, tumor necrosis factor (TNF) and reactive oxygen species (ROS) are also essential for effective innate immune control of *L. pneumophila*. While ROS are essential for the bactericidal activity of neutrophils, AM rely on neutrophil and monocyte-derived TNF signaling via TNFR1 to restrict bacterial replication. This TNF-mediated antibacterial mechanism depends on cathepsin B, the acidification of lysosomes and their fusion with *L. pneumophila* containing vacuoles (LCVs), and is independent of NLRC4, caspase-1, caspase-11 and ROS. In this thesis, we highlight the differential utilization of innate effector pathways to curtail intracellular bacterial replication in specific host cells upon *L. pneumophila* airway infection. In addition, these studies expand our knowledge of the mechanisms by which TNF contributes to the control of intracellular pathogens.

Résumé

Legionella pneumophila est une bactérie facultative intracellulaire qui vit dans des environnements aquatiques, où elle se reproduit de manière parasitaire dans les amibes. Cependant une fois qu'elle est aspirée dans les poumons d'un humain (ou d'une souris), elle peut infecter et se reproduire dans les macrophages alvéolaires (MA), ce qui peut causer une pneumonie sévère nommée la maladie du légionnaire. Après infection expérimentale des voies respiratoires chez la souris, *L. pneumophila* est rapidement contrôlée par des mécanismes immunitaires innés. Dans nos recherches, nous avons identifié pour plusieurs types cellulaires les principales fonctions effectrices innées permettant le contrôle rapide de cette infection. Nous montrons qu'en plus de la voie immunitaire bien caractérisée qui reconnaît la flagelline grâce à NAIP5-NLRC4, le facteur de nécrose tumorale (TNF) et les espèces réactives de l'oxygène (ROS) sont également essentiels pour le contrôle immunitaire innée de *L. pneumophila*. Alors que les ROS sont essentiels à l'activité bactéricide des neutrophiles, les macrophages alvéolaires dépendent du TNF produit par les neutrophiles et les monocytes, qui transmet un signal dans les MA via le TNFR1 pour empêcher la réplication de *L. pneumophila*. Ce mécanisme antibactérien médié par le TNF dépend de la cathepsine B, de l'acidification des lysosomes et de leur fusion avec avec les vacuoles contenant *L. pneumophila*, et est indépendant du NLRC4, de la caspase-1, de la caspase-11 et des ROS. Dans cette thèse, nous mettons en évidence l'utilisation différentielle des voies effectrices innées pour limiter la réplication bactérienne intracellulaire dans des cellules hôtes spécifiques, dans le contexte d'une infection des voies respiratoires par *L. pneumophila*. En outre, ces études approfondissent nos connaissances sur les mécanismes par lesquels le TNF lutte contre les agents pathogènes intracellulaires.

Abbreviations

a.a.	Amino acid
AEC	Airway epithelial cell
AM	Alveolar macrophage
AMP	Adenosine monophosphate
AP-1	Activator protein-1
Atg5	Autophagy-related 5
BMDM	Bone marrow-derived macrophage
CR1	Type 1 complement receptor
CR3	Type 3 complement receptor
DAMP	Danger associated molecular patters
DC	Dendritic cell
DHE	Dihydroethidium
DT	Diphtheria toxin
ER	Endoplasmic reticulum
FACS	Florescence-activated cell sorting
GAP	GTPase activating protein
GEF	Guanine nucleotide exchange factor
hNAIP	Human NAIP
i.p.	Intraperitoneal
i.v.	Intravenous
IFN	Interferon
IFNAR	Type I interferon receptor
IL	Interleukin
KO	Knockout
LCV	*Legionella* containing vacuole
LPS	Lipopolysaccharide
LRR	Leucine rich repeat
MagA	MIF-associated gene A
MAP	Mitogen-activated protein
MIF	Mature infectious from
NAIP	Neuronal apoptosis inhibitor protein
NFκB	Nuclear factor κB
NK	Natural killer
NLR	Nucleotide-binding domain leucine-rich repeat containing
NLRC4	NLR family, CARD domain containing 4
NLRP3	NLR family, pyrin domain containing 3
PAMP	Pathogen associated molecular pattern
PCR	Polymerase chain reaction
PKR	double-stranded RNA-activated protein kinase
PRR	Pattern recognition receptor
qPCR	Quantitative real time PCR
RIG-1	Retinoic-acid-inducible gene protein 1

RLR	RIG-1-like receptors
ROS	Reactive oxygen species
SP	Surfactant protein
T3SS	Type 3 secretion system
T4SS	Type 4 secretion system
TLR	Toll like receptor
TNF	Tumor necrosis factor
WT	Wild type
v-ATPase	Vacuolar H^+-ATPase
VBNC	Viable but non-culturable

Introduction

Immunity and the lung

In order to efficiently exchange oxygen and carbon dioxide between the air and the blood, the human lung has a large surface area made up of over 300 million alveoli, and these are exposed each day to up to 15 000 L of air [1,2]. Consequently, the lung is also an immunologically relevant microbial entry site, as it is frequently exposed to airborne bacteria and other potential pathogens, and various defense mechanisms have evolved to prevent their local entry and subsequent systemic spread. Perhaps the most important of these defense mechanisms are the tissue inherent mechanical and physical barrier components and the mucosal innate immune system in the lung, which prevent access of microbes to epithelia and the underlying tissue and / or remove contaminants and infectious agents without inducing an overt inflammatory response [3]. These mechanisms include the mucociliary system, which traps foreign particles in mucus and sweeps them towards the laryngopharynx for clearance via the digestive system [4]. In addition, mucus contains many antimicrobial peptides and proteins including antibodies [5]. For example, surfactant proteins such as the collectins can neutralize lipopolysaccharide (LPS), and antimicrobial molecules such as defensins and cathelicidins can bind and kill bacteria [4]. These innate defense systems block chemical and biological threats alike and are essential for maintaining the lungs in an uninflamed state. Genetic disorders such as primary ciliary dyskinesias, in which cilia are dysfunctional, resulting in recurrent bacterial infections and chronic lung inflammation, highlight the critical importance of these systems [3,6].

In addition, the lung is protected by a network of innate immune cells and non-immune cells such as airway epithelial cells that detect pathogens by germline-encoded pattern recognition receptors (PRRs) that recognize microbial molecules and can rapidly initiate pro-inflammatory responses [3]. Pathogen-associated tissue damage and cellular stress can cause the release or upregulation of endogenous molecules, which can also trigger an immune response via detection by PRRs [3,7,8]. Alveolar macrophages (AMs), epithelial cells, natural killer (NK) cells and other lung resident cells can then produce cytokines and chemokines, which amplify inflammation, culminating in the infiltration of neutrophils, monocytes and other immune cells. Concurrently dendritic cells (DCs) initiate the adaptive immune response, by processing foreign antigens and displaying them to T cells and B cells in the draining lymph nodes. The inflammatory environment within the lung is shaped by the type of pathogen involved, and influences the quality of the adaptive immune response that is eventually mounted against the

pathogen [9,10]. For instance, *Streptococcus pneumoniae* is a gram positive bacterium that causes infection in the lungs, and is the most common cause of community acquired pneumonia [11]. Pneumococcal pneumonia is characterized by IL-1β, TNF and IL-6 but not IFNγ, and IFNγ is not protective against this infection in mice [11-13]. In contrast, IFNγ and downstream iNOS are critical for the control of *Mycobacterium tuberculosis*, an intracellular bacterium responsible for respiratory infection in a third of the world's population [14-16].

Innate pattern recognition receptors

A defining feature of innate PRRs is their ability to recognize a wide variety of related molecules with significant molecular differences, which are usually indispensable features common to large groups of microorganisms [17]. For example, Toll-like receptor 4 (TLR4) recognizes LPS from a broad spectrum of bacterial species, despite the fact that these LPS molecules have unique molecular characteristics and variable side chain lengths [3]. In this way, a limited number of germline-encoded PRRs can detect a wide range of microbial molecules.

PRRs can be roughly organized into three general categories, including signaling PRRs, endocytic PRRs and secreted PRRs [1]. Secreted PRRs are primarily opsonins which bind to pathogens and serve to facilitate their ingestion by phagocytes. For instance, surfactant proteins A and D (SP-A and SP-D) are collectins, members of the type C lectin superfamily, that can bind to glycosylated structures in the cell wall of some microorganisms, resulting in their neutralization or opsonization [1]. Elements of the complement system such as C3b and C4b also fall into this category [18].

Endocytic PRRs on the surface of phagocytic cells are important for binding pathogens prior to phagocytosis. For example, neutrophils and macrophages can bind and internalize IgG opsonized particles via Fc receptors, and also express complement receptors with which they can efficiently phagocytose particles opsonized with complement components [19]. In addition, macrophages express many receptors that directly recognize microbial components, such as the mannose receptor and type A scavenger receptors [20]. Mac-1, also known as complement receptor 3, is a heterodimer of CD11b and β2 integrin expressed on macrophages, monocytes and neutrophils which binds iC3b and C4b opsonized bacteria [21]. Finally, non-immune cells such as epithelial cells are equipped with β1 integrins, which also enable them to phagocytose bacteria [22].

The third category of PRR, signaling PRRs, transduce a signal into the cell upon binding to their ligands, resulting in the translocation of transcription factors into the nucleus and the mobilization of inflammatory responses. This can make the cell receiving the signal more restrictive to viral or bacterial pathogens as well as result in the secretion of cytokines and chemokines that can affect neighboring cells and impact the nature of the subsequent adaptive immune response [1]. The three major subfamilies of signaling PRRs that have been characterized to date will be discussed here, including Toll-like receptors (TLRs), nucleotide-binding domain leucine-rich repeat containing (NLR) proteins and retinoic-acid-inducible gene protein 1 (RIG-1)-like receptors (RLRs).

TLRs are transmembrane proteins that evolved in ancestors common to both plants and animals, and are able to detect pathogens both at the cell surface as well as in endosomal compartments [9,23]. There are ten TLRs in humans (TLR 1-10) and twelve in mice (TLR 1-9, 11-13) [8]. They are expressed on many immune cells including macrophages, DCs, B cells, T cells, mast cells and neutrophils, as well as structural cells including endothelial cells, smooth muscle cells and epithelial cells in the skin and mucosal tissues [8,24,25]. All TLRs other than TLR3 signal through the adaptor protein MyD88 resulting in the activation of nuclear factor κB (NFκB) and activator protein-1 (AP-1), leading to the expression of pro-inflammatory cytokines [8,9]. In addition, TLR3 and TLR4 signal via the adaptor protein TRIF which can result in the production of type I interferons (IFNs) [8]. TLRs can respond to both pathogen associated molecular patters (PAMPs) and danger associated molecular patters (DAMPs), alerting the immune system to the presence of pathogens or cellular damage, respectively [9]. TLR 1, 2, 4, 5, 6 and 10 are expressed on the cell surface and mainly recognize ligands derived from bacteria, while TLR 3, 7, 8 and 9 are expressed in endosomes and recognize viral and other nucleic acids [8,9].

NLRs represent another large family of PRRs, with over 20 members identified in humans and over 30 in mice [26]. NLRs are cytosolic sensor proteins, some of which assemble into multiprotein high-molecular-weight complexes termed inflammasomes and recruit and activate proinflammatory proteases such as caspase-1 in response to PAMPs and DAMPs [27]. Surprisingly little is known about how NLRs detect their ligands and become activated, despite intensive research efforts [28]. As an example, NLRP3 mediates inflammasome assembly in response to a host of structurally unrelated PAMPs and DAMPs, and it is generally thought that NLRP3 senses changes in cellular homeostasis, such as redox status or ion concentrations, rather than directly sensing molecular motifs [29]. For instance, there is evidence indicating

that endoplasmic reticulum (ER) stress results in the release of Ca^{2+} from the ER into the cytoplasm, and the subsequent damage done to microchondria upon uptake of this Ca^{2+} triggers an NLRP3-mediated immune response [30]. However, the involvement of mitochondrial reactive oxygen species (ROS) and other ions such as K^{+}, as well as the point of convergence common to all NLRP3 agonists remains to be elucidated [29]. Perhaps the best-characterized member of the NLR family is NLRC4, which upon activation mediates inflammasome activation and inflammasome assembly. It is now understood that NLRC4 is an adaptor protein, which oligomerizes with another set of NLR proteins, the neuronal apoptosis inhibitor proteins (NAIPs), once the latter bind to their cytoplasmic ligands. Mouse NAIP1 detects type 3 secretion system (T3SS) needle protein, NAIP2 detects T3SS rod protein, and NAIP5 and NAIP6 both detect flagellin [31]. The only human NAIP (hNAIP) characterized thus far recognizes T3SS needle protein, and though it is widely believed that it does not additionally recognize flagellin, this is still a point of contention [32-35]. Thus rather than targeting the rapidly evolving T3SS effector proteins, which also differ between bacteria, NAIP proteins target essential, conserved virulence factors which are accidentally injected into the host cell by bacterial secretion systems [29]. By sensing changes in cellular homeostasis in the case of NLRP3, and by integrating the detection of multiple conserved bacterial proteins in the case of NLRC4, both of these NLRs demonstrate an elegant design that helps multicellular organisms win the molecular arms race with rapidly evolving pathogens.

RLRs are cytoplasmic receptors, including three described family members, namely RIG-1, melanoma differentiation-associated gene 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) [36]. These PRRs have been shown to induce NFκB activation and type I interferons (IFN) in response to non-self RNA species generated as a byproduct of viral replication [37]. Indeed, cells deficient in both RIG-I and MDA5 do not produce type I IFN upon viral infection, demonstrating that these PRRs are essential for detecting cytoplasmic viral RNA [37].

TNF and ROS in inflammation and immune defense

TNF is a pleiotropic cytokine that is essential to many inflammatory processes through its role in activating pro-inflammatory transcription factors such as NF-κB and enhancing immune cell survival, but can also have the seemingly opposite effect of triggering cell death, depending on the context of signaling [38]. TNF is synthesized as a 26 kDa transmembrane molecule (memTNF), which can be cleaved by tumor necrosis factor-alpha converting enzyme (TACE)

to yield a 17 kDa soluble molecule (sTNF) [39,40]. It is thought to be produced mainly by macrophages and T cells, but can also be synthesized by many other cell types, including B cells, neutrophils, NK cells, mast cells, smooth muscle cells, cardiomyocytes, fibroblasts, DCs and many others [38]. Both forms are active as homotrimers, and signal through one of two receptors, TNFR1 and TNFR2. TNFR1 is expressed on almost all cell types except erythrocytes, while TNFR2 is expressed mainly on immune and endothelial cells [41]. In contrast to TNFR2, TNFR1 contains a death domain (DD) in its cytoplasmic tail, and has a higher affinity for TNF than TNFR2 [42]. The complexity of TNF regulation is enhanced by the fact that TACE is able to convert both TNFR1 and TNFR2 into soluble molecules, which are released from the cell. The soluble receptors can bind TNF, resulting either in the downregulation of TNF bioactivity, or prolonged TNF bioactivity via its protection from degradation [43].

Upon binding to TNFR1, TNF induces signal transduction that can lead to stimulation of cell survival and the expression of pro-inflammatory genes, through the induction of many transcription factors, the most important of which are NF-κB and AP-1 [38]. Another important pro-inflammatory feature of TNF signaling is the activation of the mitogen-activated protein kinases (MAPK) [44]. In this case the so called signaling complex I is formed, where the silencer of the death domain (SODD) dissociates from the DD, followed by the recruitment of TNFR type 1-associated death domain protein (TRADD), TNF-receptor-associated factor-2 (TRAF-2) and receptor interacting protein-1 (RIP-1) [42,45,46]. TRAF-2 and RIP-1 are required for NF-κB activation via the degradation of IκB kinase (IKK) [47]. The pro-inflammatory genes induced by signaling complex I include iNOS, prostaglandins, leucotrienes, metalloproteinases, adhesion molecules and cytokines, to name a few [38]. Thus TNF can induce vasodilation through iNOS generated nitric oxide (NO) and cyclooxygenase 2 (COX-2) generated prostanoids, which contributes to its ability to increase local inflammation, but also to its ability to induce multiple organ failure during sepsis [48,49].

On the other hand, if NF-κB activation fails, Fas-associated death domain protein (FADD) and procaspase 8 can associate with signaling complex I to form signaling complex II, which drives apoptosis via effector caspases-3,-6 and -7 [50,51]. Depending on the cell type involved, caspase-8 can also induce the intrinsic apoptotic pathway, resulting in the release of cytochrome c from mitochondria and the formation of an apoptosome, resulting in amplification of the process by caspase-9 [38]. Though TNFR2 lacks a DD, TNFR2 can also

induce cell death under some circumstances [52]. However, TNFR signaling remains incompletely understood.

TNF is also a potent stimulator of neutrophil respiratory bursts, which are characterized by the production of bactericidal ROS [38].

ROS are produced by the NADPH oxidases (NOX), which are enzymes that transfer electrons across cell membranes to reduce oxygen to superoxide. NOX2 or $gp91^{phox}$ is the NOX enzyme expressed exclusively in professional phagocytes, and a further 6 homologues, NOX1, NOX3, NOX4, NOX5, DUOX1 and DUOX2, also exist and are expressed in a variety of cell types [53]. As a group, the NOX2 homologues are referred to as the NOX family of NADPH oxidases, and are found in almost all tissues [53]. It is now recognized that the NOX family of NADPH oxidases generate ROS that are required for many reversible regulatory processes essential to cellular function [53].

NOX2 is required for the generation of ROS in phagocytic cells, and can contribute to the killing of bacteria and other microorganisms. ROS are oxygen-derived small molecules, including oxygen radicals (superoxide ($O_2^{\bullet -}$), hydroxyl ($^{\bullet}OH$), peroxyl ($RO_2^{\bullet}$) and alkoxyl ($RO^{\bullet}$)) and certain nonradicals such as hypochlorous acid (HOCl), ozone (O_3), singlet oxygen ($^1O_2$) and hydrogen peroxide (H_2O_2) [53]. These molecules derive their bactericidal properties from the fact that they will readily react with a large number of molecules, including proteins, lipids, carbohydrates and nucleic acids, in the course of which they can irreversibly destroy these molecules [53]. It is therefore perhaps not surprising that ROS have been shown to be essential for the immune defense against a variety of microorganisms, including intracellular bacteria such as *Francisella tularensis* [54,55].

***Legionella pneumophila* in the environment and lung infection**

L. pneumophila is a Gram-negative motile bacterium with global distribution found preferentially in both natural and manmade freshwater environments, where it replicates intracellularly mainly in bacterivorous amoebae such as *Acanthamoeba castellanii* [56-58]. However, *L. pneumophila* is an accomplished generalist and is able to grow in a large number of amoebae and other protozoa, as well as persist in biofilms [59,60]. A recent report has documented that *L. pneumophila* can also establish infection in the intestinal and gonadal tissue of the free-living soil nematode *Caenorhabditis elegans*, potentially establishing a vast alternative environmental reservoir for *L. pneumophila* [61]. In addition, the productive

infection of *C. elegans* by *L. pneumophila* signifies that the bacterium has had evolutionary exposure to at least one multicellular organism, albeit a primitive one.

In order to establish infection in its normally bactericidal host cells, *L. pneumophila* relies on a type IV secretion system (T4SS) to inject over 300 effector proteins into the host cell, reprograming it to support its intracellular replication [62,63]. These effectors block phagosomal maturation and fusion with lysosomes which would result in *L. pneumophila* degradation, and promote the establishment of a *Legionella* containing vacuole (LCV), the intracellular niche in which *L. pneumophila* replicates [64-67].

Using this versatile molecular toolbox, *L. pneumophila* can also replicate in human macrophages [68]. Thus *L. pneumophila* commonly causes community acquired and nosocomial pneumonia, and though normally controlled by the innate immune response, has the potential to cause a severe pneumonia known as Legionnaires' disease with mortality rates of up to 30% if early bacterial replication is not controlled [69-71]. However, human-to-human disease transmission is not observed, and infection is instead contracted by inhaling *L. pneumophila* contaminated water vapor, mostly generated by manmade technologies such as cooling towers, air conditioners or even car windshield wipers [62,72,73]. In the lung, *L. pneumophila* initially exclusively infects alveolar macrophages (AM), paradoxically replicating in the very cells designed to be the first line of defense against bacterial infections in the lung [62,74,75]. This ability makes *L. pneumophila* a clinically relevant human pathogen.

Of the more than 300 experimentally validated T4SS effectors, only 50 have thus far been attributed cellular functions, and in most cases the impact of these on *L. pneumophila*'s life cycle is not fully understood [76]. Their combined effects are vastly complex, manipulating *L. pneumophila*'s uptake, LCV and vesicle trafficking, retrograde trafficking, autophagy, ubiquitination, transcription and mitochondria, to name a few effects [76]. The reductionist approach employed by scientists to dissect the role of individual T4SS effectors is further hampered by the fact that many have redundant roles, and the ablation of a single effector seldom influences *L. pneumophila*'s ability to replicate intracellularly due to functional compensation by other effectors, a fact which probably reflects *L. pneumophila*'s ability to infect an impressive diversity of host cells [77]. What is clear however is that the effectors precisely regulate key host factors and associated pathways both spatially and temporally, in order to achieve an equilibrium which is favorable to bacterial replication.

A repercussion of this complex regulation is that effectors can have seemingly opposing and counterintuitive effects, which only make sense when the timing of expression is taken into account. For example, the small GTPase Rab1, which is recruited to the LCV and is a pivotal regulator of endoplasmic reticulum (ER)-Golgi secretory trafficking, is targeted by at least six T4SS effectors, some of which activate it, and some of which inactivate it [76]. The effector SidM is a Rab1 guanine nucleotide exchange factor (GEF) and can thus activate Rab1, while the effector LepB is a Rab1 GTPase activating protein (GAP), which can switch Rab1 back into its inactive state [78,79]. Further, SidM can covalently attach adenosine monophosphate (AMP) to Rab1, which prolongs its activation by preventing access by GAPs such as LepB, while yet another effector, SidD acts as an AMP-Rab1 deAMPylase, thus having the potential to reverse this chemical modification of Rab1 by SidM [80]. Similarly, the effectors AnkX, Lem3 and LidA can also influence the activation state of Rab1. By controlling the expression of these T4SS effector molecules, *L. pneumophila* can precisely manipulate a key component of the ER-Golgi secretory trafficking pathway to its advantage [76].

As another example, vacuolar acidification is differentially regulated by *L. pneumophila* effectors. The early expression of the effector SidK, which binds to the VatA subunit of the vacuolar H^+-ATPase (v-ATPase), inhibits the early acidification of the LCV [81]. Neutral pH in the LCV until around 6 hours p.i. appears to be crucial for the *L. pneumophila* life cycle [68], however, towards the end of *L. pneumophila*'s replication cycle, LCV acidification is required for optimal intracellular growth [82]. Of note however, SidK appears to be yet another example of functional redundancy among *L. pneumophila* effectors, as it's deletion does not result in a defect in intracellular growth in either BMDM or *D. discoideum* [81].

As a final example of how *L. pneumophila* can push a biological pathway in opposing directions, *L. pneumophila* employs multiple T4SS effector-mediated mechanisms that promote cell survival, but also several others that promote cell death. At least 7 *L. pneumophila* T4SS effectors inhibit host cell translation, having the net effect of promoting NF-κB activation, as IκB is degraded more quickly than NF-κB when translation is blocked [83-85]. Pharmacological inhibition of protein synthesis in the presence of TLR ligands also results in NF-κB activation, demonstrating that this mechanism does not require other bacterial factors [83]. However, other *L. pneumophila* effectors also prevent cell death, such as SidF which neutralizes the proapoptotic factors BNIP3 and Bcl-rambo [86], and LegK1 which directly phosphorylates IκB [87]. Preventing cell death is a logical goal shared by various intracellular pathogens, in order to prevent the destruction of their replication niche [88]. On the other hand,

L. pneumophila also possesses various effectors which have the less intuitive function of promoting cell death. For instance VipD and PlcC destabilize membranes, including those of mitochondria, resulting in the release of cytochrome c into the cytosol, and the subsequent activation of caspase-3 and the induction of apoptosis [89,90]. It is thought that this stimulation of apoptosis by *L. pneumophila* might aide in the release of bacteria from the LCV at the end of the replication cycle [76]. Alternatively, activated caspases might participate in the biogenesis of the LCV independently of their ability to induce apoptosis [88]. In summary, in order to replicate *L. pneumophila* must successfully manipulate key pathways such as lysosomal acidification, cell survival and vesicular trafficking.

As a species, *L. pneumophila* encounters extremely varied conditions as it moves through various environments. Although *L. pneumophila* alternates between a replicative form and a stationary phase form under laboratory culture conditions, it is now established that *L. pneumophila* differentiates along a complex developmental network involving as many as 14 distinct developmental forms [91,92]. Differentiation into the various forms is triggered by environmental and metabolic cues, and allows *L. pneumophila* to adapt to highly disparate conditions [92]. For instance, *L. pneumophila* exists as a free-swimming planktonic form, which is transmissive and resistant to multiple environmental stresses such as nutrient starvation [93], and as a replicative form able to replicate intracellularly in LCVs, even as they mature into acidic lysosomal vacuoles in some cell types [81]. Following this exponential replication phase, *L. pneumophila* can differentiate back into a transmissive state, and even further into a cyst form termed MIF (mature infectious from) during late stages of infection, which has a low metabolic rate, is highly infectious and resistant to environmental stresses [91,92,94]. Interestingly, in contrast to *L. pneumophila* replicating in amoeba, *L. pneumophila* replicating in human macrophages do not fully differentiate into MIFs, and are less infectious as well as less resistant to environmental stresses, and it has been suggested that this might explain why human to human transmission of *L. pneumophila* pneumonia is not observed [95].

The immune response to *L. pneumophila*

Though critical for *L. pneumophila* replication, the T4SS also potently induces the innate immune response by several mechanisms [96]. As mentioned in the previous section, the inhibition of translation by *L. pneumophila* effectors may be beneficial for the bacterium by enhancing host cell survival. However, it also triggers a stress response which potently induces the immune response, by activating mitogen-activated protein (MAP) kinases and inducing the

release of inflammatory mediators [83-85]. AM thus sense the action of the T4SS and respond with the caspase-11-dependent flagellin-independent secretion of IL-1α [97], inducing the secretion of chemokines by airway epithelial cells (AECs), resulting in the rapid recruitment of neutrophils and monocytes to the lung [74,85,98]. Neutrophils are known to be critical for the clearance of *L. pneumophila* lung infection, as evidenced by neutrophil depletion studies [98-100], *in vivo* blockade of CXCR2 [101] and studies examining the role of IL1R signaling [85,98,102]. However, the mechanisms by which neutrophils contribute to the resolution of *L. pneumophila* lung infection remain incompletely understood.

IL-1 is closely linked to the induction of TNF in a broad spectrum of unrelated models of inflammation, and these cytokines are known to have synergistic effects *in vivo* [103-105]. Indeed, anti-TNF therapy is a recognized risk factor for Legionnaire's disease, suggesting that TNF has an important role in the immune response to *L. pneumophila* [106-110]. Previous work has established that TNF is produced in response to *L. pneumophila* in a T4SS-dependent and flagellin-independent manner [83,111] and can limit replication in macrophages [112,113]. Furthermore, it was shown that TNF contributes to immune defense against *L. pneumophila in vivo* [114]. However, the mechanisms by which TNF contributes to innate immune control of *L. pneumophila* and the cells upon which it acts *in vivo* have yet to be elucidated.

In an intravenous (i.v.) mouse infection model examining the innate immune response to *L. pneumophila*, it was shown that IFNγ was the central cytokine required to control bacterial infection, while TNFR1 and IL-1R were dispensable [115]. It was shown that MyD88-dependent NK-derived IFNγ, which has a reciprocal dependency on DC derived IL-12, was required to control i.v. *L. pneumophila* infection [115]. Further, it was shown that while neutrophils in this model did not produce TNF, they were required to produce IL-18, which activated NK cells via MyD88-dependent signaling, and this was required for them to produce IFNγ [100]. However, to what extent these mechanisms participate in the innate immune response to *L. pneumophila* lung infection was not investigated.

Macrophages from C57BL/6 mice are not permissive to *L. pneumophila* replication due to the intracellular sensor NAIP5 which binds cytosolic flagellin and recruits NLRC4, resulting in inflammasome assembly and the activation of Caspase-1 [28,116,117]. Active caspase-1 can initiate a pro-inflammatory form of cell death known as pyroptosis, the secretion of IL-1β and IL-18, as well as activating Caspase-7, which induces the fusion of lysosomes with the LCV, resulting in bacterial degradation [33,118]. Murine macrophages missing key components in

this pathway are permissive to *L. pneumophila* replication, including NAIP5$^{-/-}$, NLRC4$^{-/-}$, Caspase-1$^{-/-}$ and Caspase-7$^{-/-}$ macrophages [33]. Though human NAIP, the orthologue of NAIP5, can mediate inflammasome assembly and *L. pneumophila* restriction when overexpressed in murine macrophages, *L. pneumophila* does not induce Caspase-1 and Caspase-7 activation in human macrophages, which support *L. pneumophila* replication [33,34]. In addition, Caspase-11 has also been shown to restrict *L. pneumophila* growth by promoting lysosomal fusion with the LCVs, though this appears to be redundant in BMDM [67,117].

A/J mice are permissive to *L. pneumophila* replication due to mutations in the NAIP5 gene, resulting in 14 amino acid (a.a.) differences as compared to C57BL/6 mice [119,120]. A/J mice are able to activate caspase-1 in response to *L. pneumophila* infection [121], but fail to activate caspase-7, suggesting that the 14 a.a. are somehow involved in caspase-1 and caspase-7 interactions [33,116]. Other mouse strains also display partial susceptibility to *L. pneumophila* infection, including FvB/N, C3H/HeJ, BALB/c/J and 129S1 mice [119]. Interestingly, in each case this susceptibility was shown to be attributable to mutations in NAIP5, and 6 of the 14 a.a. missense mutations present in A/J mouse NAIP5 (NAIP5$^{A/J}$) are shared by all the permissive strains [119]. None of these mutations occur in the recently defined NAIP5 ligand specificity domain, supporting the hypothesis that these mutations do not impact flagellin binding by NAIP5 but might promote interactions between caspase-1 and caspase-7 [28]. In this thesis we make use of mice with the 129S1 NAIP5 allele (NAIP5^{129S1}) that have a targeted TNF deletion in macrophages, monocytes and neutrophils (MN-TNF NAIP5^{129S1} mice) [122] to examine the role of TNF derived from macrophages, monocytes and neutrophils in *L. pneumophila* lung infection in the absence of strong NAIP5 signaling.

In the case of re-challenge with *L. pneumophila*, the adaptive immune response is known to contribute to the clearance of *L. pneumophila* lung infection. *L. pneumophila* lung infection induces the differentiation of Th1/Th17 effector CD4 T cells which home to the lung [123]. In addition, *L. pneumophila*-specific antibodies can protect against *L. pneumophila* airway infection by engaging Fc receptors, resulting in signal transduction that renders host cells non-permissive to infection and targets the bacteria to lysosomes [124]. Though it was shown that all the IgG subclasses could be protective at high doses, IgG2c and IgG3 were found to be the most prevalent in immunized mice and correlated with reduced bacterial loads in the lung [125]. Of these subclasses, IgG2c proved the most effective at limiting concentrations when administered prophylactically [125].

Aims of the thesis

In this thesis, we investigated the protective role of innate immune factors that have been implicated in the innate immune response during *L. pneumophila* lung infection. We demonstrate that TNF and reactive oxygen species (ROS) are essential for the effective innate immune control of *L. pneumophila* lung infection, and that *in vivo* TNF can compensate for the well characterized NLRC4-NAIP5 flagellin pathway. While ROS are essential for the bactericidal activity of neutrophils, TNF produced by neutrophils and monocytes is required to enhance alveolar macrophage (AM) mediated restriction of *L. pneumophila* via TNFR1 *in vivo*. This TNF mediated antibacterial mechanism is independent of NLRC4 and involves cathepsin B as well as the fusion of LCVs with lysosomes and their acidification. Given the poor NAIP activation in response to *L. pneumophila* infection in humans, the striking susceptibility of MN-TNF NAIP5^{129S1} mice to *L. pneumophila* lung infection suggests that TNF could be a key component of innate immunity to *L. pneumophila* lung infection in humans.

Results

TNF and ROS are important for clearance of *L. pneumophila in vivo*

Many host immune factors have been shown to be involved in *L. pneumophila* control *in vitro*, whereas relatively few studies have assessed their impact *in vivo*. We therefore used an intranasal mouse infection model to identify crucial innate immune effector molecules and pathways that have been implicated in the clearance of *L. pneumophila* lung infection, by assessing their relative impact on bacterial burden in the lung 5 days p.i.. As shown previously, NLRC4$^{-/-}$ mice were moderately susceptible to infection, despite the well-recognized role of NLRC4 in inflammasome activation in response to *L. pneumophila* flagellin, and the high susceptibility of NLRC4$^{-/-}$ macrophages to *L. pneumophila* replication *in vitro* (Figs. 1A and 6B, [126,127]. However, TNF deficiency and ROS deficiency (CYBB$^{-/-}$ mice) resulted in a comparable or even more potent impairment in bacterial control in the BALF 5 days p.i. than did NLRC4 deficiency (Fig. 1A). Interestingly, MN-TNF NAIP5^{129S1} mice, which have a hypofunctional NAIP5 allele and are deficient in TNF in macrophages, monocytes and neutrophils, had a much higher bacterial load than both TNF$^{-/-}$ and NLRC4$^{-/-}$ mice, suggesting that these pathways can compensate for each other *in vivo*. In contrast, IFNγ, IL-12, iNOS, caspase-1/11, IL-17 and TLR5 seem to play a less dominant role in controlling *L. pneumophila* infection. These results indicate that TNF and ROS are key effectors for the innate immune defense against *L. pneumophila* lung infection, and that TNF from macrophages, monocytes or neutrophils can partially compensate for the NLRC4 pathway.

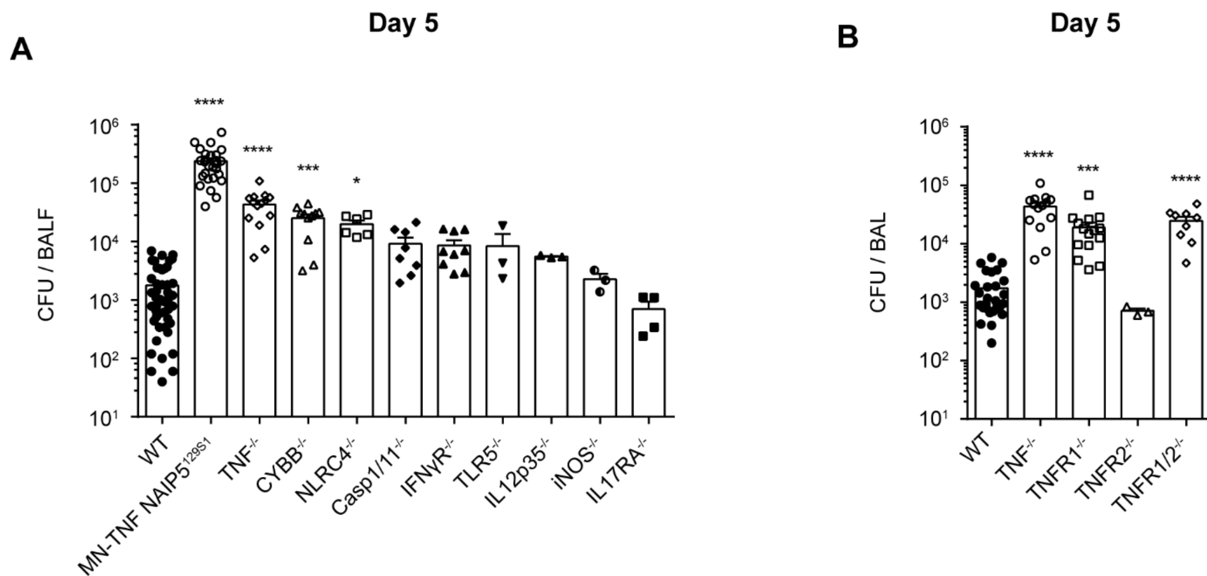

Figure 1. TNF / TNFR1 and ROS are important for clearance of *L. pneumophila in vivo*. **(A-B)** WT or knockout mice were infected intranasally with WT *L. pneumophila*, and 5 days p.i. BALF CFU were quantified on CYE agar plates. Data are from 15 and 8 pooled experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared to WT by Kruskal-Wallis test with Dunn's post test.

MN-TNF NAIP5^{129S1} mice have a hypofunctional NAIP5 allele

In the experiments described above, we originally attributed the phenotype of MN-TNF NAIP5^{129S1} mice solely to the absence of TNF in macrophages, monocytes and neutrophils, as we were not aware of the fact that these mice did not have the C57BL/6 NAIP5 allele. We were surprised to observe that TNF$^{-/-}$ mice had roughly ten fold less CFU in the BALF than MN-TNF NAIP5^{129S1} mice 5 days p.i. (Fig. 1A), which led us to suspect that MN-TNF NAIP5^{129S1} mice had further differences in their genetic background compared with C57BL/6 mice. We were aware of the fact that cre interrupted exon 1 of lysozyme M, and that homozygous MN-TNF NAIP5^{129S1} mice were therefore lysozyme M deficient. However, infection of Lysozyme M$^{-/-}$ mice with WT *L. pneumophila* showed that these mice were not more susceptible than WT mice, ruling out a non-redundant role for Lysozyme M (Fig. 2C). Nevertheless, to rule out interactions between TNF and lysozyme M, and to determine if the genetic background of MN-TNF NAIP5^{129S1} mice differed from that of C57BL/6 mice in genes other than TNF and lysozyme M, we backcrossed them with C57BL/6 mice. We intercrossed the F1 generation to yield F2 progeny with all combinations of either floxed TNF, WT TNF, cre and/or lysozyme M. We found that there were large variations in the bacterial loads in the BALF 5 days p.i. that

could not be explained by either TNF or lysozyme M (Fig. 2A). Further, while $TNF^{-/-}$ BMDM only supported minimal *L. pneumophila* growth (Fig. 2B), MN-TNF $NAIP5^{129S1}$ BMDM supported substantial *L. pneumophila* growth, and neither TNF nor lysozyme M expression could completely predict permissiveness to *L. pneumophila* infection in the BMDM of the F2 offspring (Fig. 2B). Taken together with the observation that susceptibility *in vivo* predicted individual permissiveness of BMDM *in vitro*, these data showed that genetic factors that were neither TNF nor lysozyme M contributed to susceptibility to *L. pneumophila* in MN-TNF $NAIP5^{129S1}$ mice (Fig.2A and B).

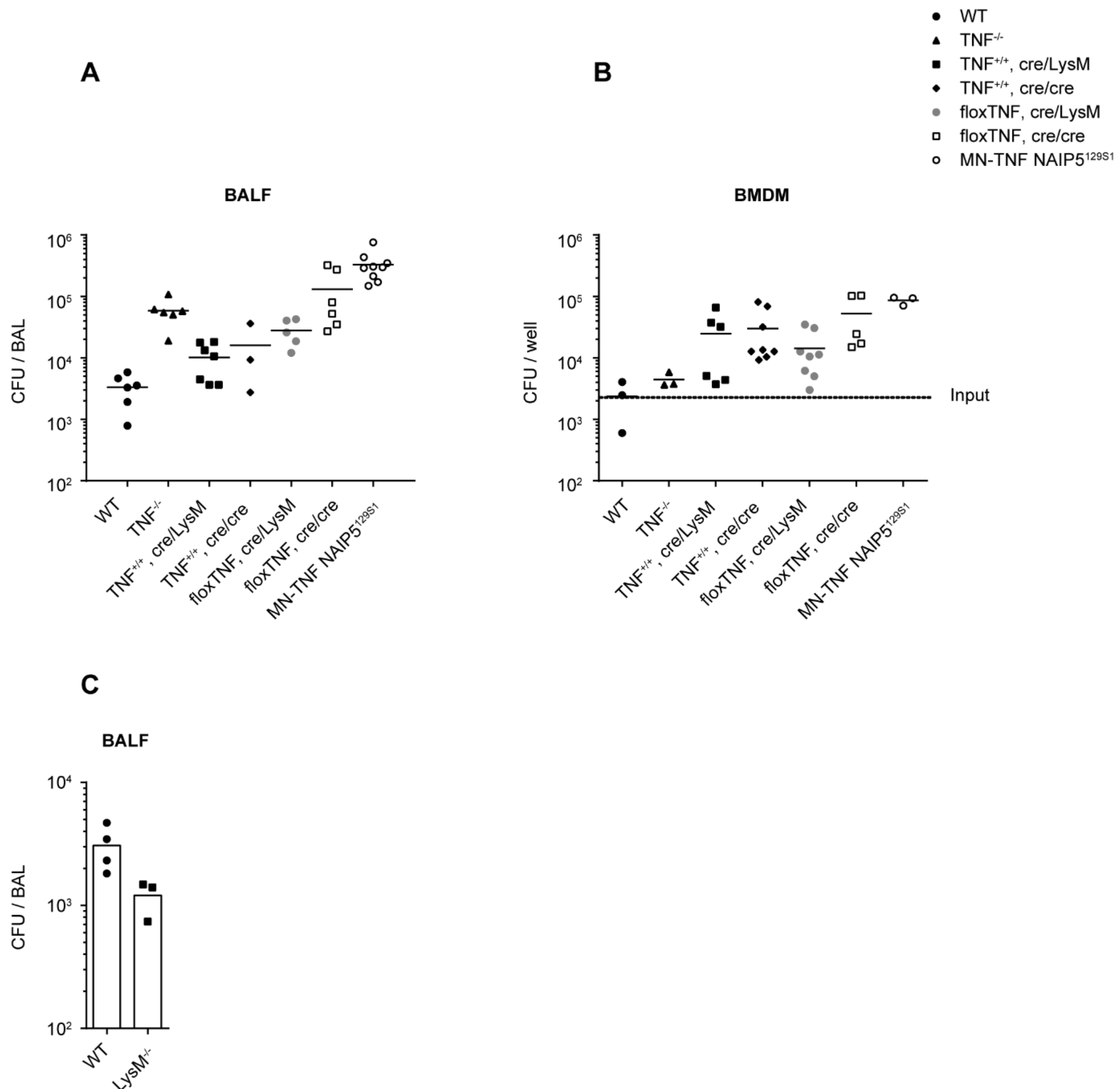

Figure 2. MN-TNF mice appear to differ from C57Bl/6 mice in an undefined gene(s), that are neither TNF nor Lysozyme M. (A) MN-TNF $NAIP5^{129S1}$ mice were crossed with WT, and the F1 were intercrossed to yield F2 progeny with floxed Tnf (floxTNF), WT Tnf ($TNF^{+/+}$)

and either one or two copies of cre. WT, TNF$^{-/-}$, MN-TNF NAIP5^{129S1} and F2 mice were infected intranasally with WT *L. pneumophila*, and 5 days p.i. BALF CFU were quantified on CYE agar plates. Data are from two pooled experiments. **(B)** BMDM were generated from the mice in A, and infected with WT *L. pneumophila* at MOI 0.1. Three days p.i. BMDM were lysed and CFU were quantified on CYE agar plates. Data are representative of two independent experiments. **(C)** WT or LysM$^{-/-}$ mice were infected intranasally with WT *L. pneumophila*, and 5 days p.i. BALF CFU were quantified on CYE agar plates. Data are from one experiment.

The observation that TNFR1/2$^{-/-}$ BMDM were only mildly susceptible to *L. pneumophila* infection, while MN-TNF NAIP5^{129S1} mice were almost as susceptible as NLRC4$^{-/-}$ BMDM led me to suspect that MN-TNF NAIP5^{129S1} mice might be defective in either NLRC4 or NAIP5 (Fig. 6A and B). MN-TNF NAIP5^{129S1} mice were generated using embryonic stem cells from 129S1 mice [122]. 129S1 mice are reported to have mutations in NAIP5 (NAIP5^{129S1}), some of which overlap with those in A/J mouse NAIP5 (table 3 in [119]), and BMDM from 129S1 mice have been shown to be permissive to *L. pneumophila* due to NAIP5^{129S1} [119]. Sequencing of MN-TNF NAIP5^{129S1} mouse NAIP5 confirmed that these mice have the same mutations in NAIP5 as 129S1 mice with the exception of exon 15. We therefore concluded that in addition to ablation of TNF in macrophages, monocytes and neutrophils, MN-TNF NAIP5^{129S1} mice also harbor the NAIP5^{129S1} allele.

TNF acts via TNFR1 to control *L. pneumophila* infection

To identify the receptor through which TNF exerts its protective effect, WT, TNF$^{-/-}$, TNFR1$^{-/-}$, TNFR2$^{-/-}$ and TNFR1/2$^{-/-}$ mice were infected intranasally with WT *L. pneumophila* and CFUs were compared in BALF 5 days p.i.. Bacterial clearance was delayed to a similar extent in TNF$^{-/-}$, TNFR1$^{-/-}$ and TNFR1/2$^{-/-}$ mice compared to WT, but not in TNFR2$^{-/-}$ mice, showing that TNF mediates its anti-bacterial effect via TNFR1 *in vivo* (Fig. 1B).

TNF / TNFR1 signaling contributes to AM but not neutrophil-mediated killing of *L. pneumophila in vivo*

A recent study using a T4SS-based reporter system has demonstrated that AM and neutrophils are the primary targets for *L. pneumophila in vivo*, with *L. pneumophila* replication having been demonstrated in AM [74]. We therefore examined the impact of TNF on AM and neutrophil mediated killing of *L. pneumophila in vivo*. To normalize bacterial burden in WT and TNFR1$^{-/-}$ mice and to compare AM and neutrophil bacterial loads in WT and TNFR1$^{-/-}$ cells in the same mouse, we used a mixed chimera approach in which 50% of hematopoietic cells were of WT

or TNFR1$^{-/-}$ origin. Mixed bone marrow (BM) chimeric mice were generated with a mix of 50% Ly5.1^{+} WT BM and 50% WT Ly5.2^{+} or Ly5.2^{+} TNFR1$^{-/-}$ BM. After 8 weeks of reconstitution, WT:WT and WT:TNFR1$^{-/-}$ mice were inoculated intranasally with WT *L. pneumophila*, and 2 days p.i. Ly5.1^{+} and Ly5.2^{+} AM and neutrophils were sorted from the BALF, and cells were plated on CYE plates to quantify viable *L. pneumophila*. Significantly more CFU / AM were recovered from TNFR1$^{-/-}$ AM than from WT AM, indicating that TNF signaling via TNFR1 promotes the killing of *L. pneumophila* by AM *in vivo* (Fig. 3A). In contrast, there was no difference in the number of viable *L. pneumophila* / neutrophil recovered from WT vs. TNFR1$^{-/-}$ neutrophils, indicating that TNF signaling does not contribute to neutrophil mediated killing of *L. pneumophila* (Fig. 3A). The killing of *L. pneumophila* lacking flagellin was also impaired in TNFR1$^{-/-}$ AM compared to WT AM, demonstrating that the antibacterial mechanism mediated in AM by TNF / TNFR1 is independent of the NAIP5-NLRC4 flagellin recognition pathway (Fig. 3B). These results highlight that TNF / TNFR1 signaling mediates a non-redundant antibacterial mechanism that contributes to *L. pneumophila* killing in AM but not in neutrophils *in vivo*.

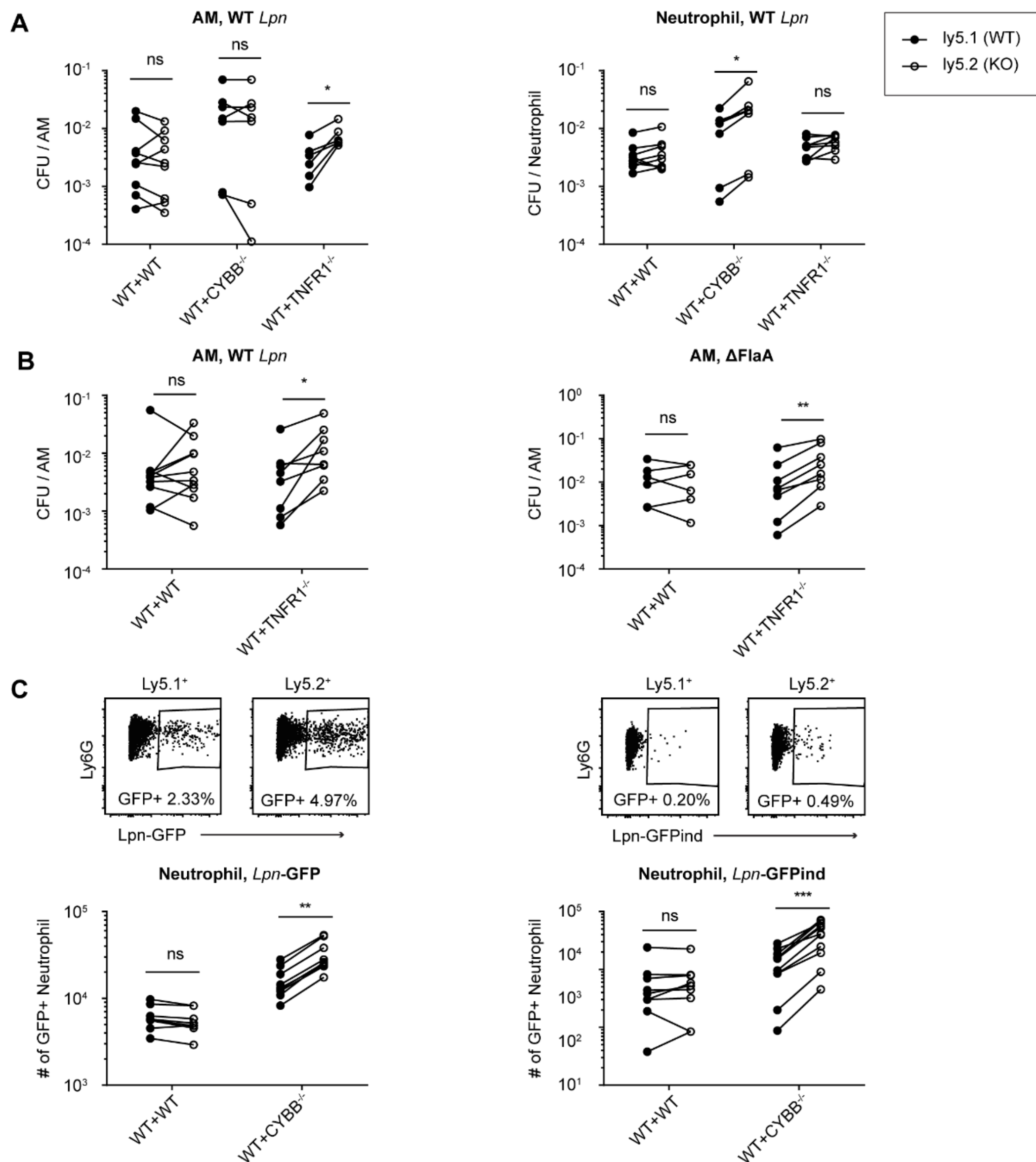

Figure 3. TNF / TNFR1 signaling contributes to AM-mediated killing of *L. pneumophila*, while ROS are required for efficient neutrophil-mediated killing of *L. pneumophila in vivo*. (A-C) Mixed BM chimeric mice reconstituted with 50% Ly5.1^{+} WT BM, and either 50% Ly5.2^{+} WT, TNFR1$^{-/-}$ or CYBB$^{-/-}$ BM were generated. **(A)** Chimeras were infected with WT *L. pneumophila*, and 2 days p.i. BALF was harvested and Ly5.1^{+} and Ly5.2^{+} AM and neutrophils were sorted. Cells were lysed and CFU were quantified on CYE agar plates. **(B)** Chimeras were infected with WT or ΔFlaA *L. pneumophila*, and CFU were quantified in AM as in B). **(C)** Chimeras were infected with *L. pneumophila*-GFP or *L. pneumophila*-GFPind (with IPTG induction) and BALF was analyzed by flow cytometry 38 hr p.i.. GFP^{+} neutrophils were normalized for the number of Ly5.1^{+} and Ly5.2^{+} neutrophils, respectively. Data are from 2-4 pooled experiments. *p<0.05, **p<0.01, ***p<0.001 by Wilcoxon test.

ROS are required for efficient neutrophil but not AM-mediated killing of *L. pneumophila in vivo*

To analyze the impact of ROS on AM and neutrophil mediated killing of *L. pneumophila*, we generated BM chimeric mice with a mix of 50% Ly5.1^{+} WT BM and 50% WT Ly5.2^{+} or Ly5.2^{+} CYBB$^{-/-}$ BM. 2 days p.i. we observed that while sorted CYBB$^{-/-}$ AM did not contain more viable *L. pneumophila* / AM than WT AM, sorted CYBB$^{-/-}$ neutrophils contained more viable *L. pneumophila* / neutrophil than did WT neutrophils from the same mouse (Fig. 3A). This indicates that in contrast to TNF, ROS plays a non-redundant role in neutrophil-mediated killing of *L. pneumophila* but not AM-mediated killing of *L. pneumophila in vivo*.

We performed similar experiments in which WT:WT and WT:CYBB$^{-/-}$ BM chimeric mice were inoculated with either *L. pneumophila* constitutively expressing GFP (*L. pneumophila*-GFP), or with *L. pneumophila* containing a plasmid on which GFP expression can be induced by the addition of IPTG (*L. pneumophila*-GFPind), thereby identifying metabolically active bacteria (Fig. 3C). Neutrophils were analyzed by flow cytometry 38 hours p.i., and in the case of *L. pneumophila*-GFPind infected mice, IPTG was administered intranasally at 35 hours p.i., resulting in the induction of GFP in all viable *L. pneumophila*. In line with the results of the BM chimera sort and plating experiments described above, there were more GFP^{+} CYBB$^{-/-}$ neutrophils than GFP^{+} WT neutrophils in WT:CYBB$^{-/-}$ BM chimeric mice, both with *L. pneumophila*-GFP infection and with *L. pneumophila*-GFPind infection (Fig. 3C). In the case of *L. pneumophila*-GFP infection this indicates that there were more ROS-deficient neutrophils that contained dead or viable *L. pneumophila* than WT neutrophils, and in the case of *L. pneumophila*-GFPind infection it indicates that there were more ROS-deficient neutrophils that contained viable *L. pneumophila* than WT neutrophils in the same mouse. These data support the hypothesis that neutrophils require ROS to kill and degrade *L. pneumophila in vivo*.

In vivo, neutrophils but not AM produce ROS in response to *L. pneumophila* infection

Having established that ROS-dependent mechanisms are involved in neutrophil mediated killing of *L. pneumophila*, we sought to determine if neutrophils actively produce ROS in response to *L. pneumophila*. We infected WT and CYBB$^{-/-}$ mice with WT, T4SS deficient (ΔT) and ΔFlaA *L. pneumophila* and stained neutrophils and AM with a flow cytometry based ROS detection reagent (Dihydroethidium) 24 h p.i.. We observed that neutrophils but not AM produced ROS in response to WT and ΔFlaA *L. pneumophila* 24 h p.i., suggesting that ROS could have direct bactericidal effects in *L. pneumophila* containing neutrophils (Fig. 4A). Since

we did not observe neutrophil ROS production in response to ΔT *L. pneumophila*, our results suggest this ROS production is T4SS-dependent and flagellin independent. Conversely, AM produced ROS only in response to ΔT infection, in line with a publication suggesting that *L. pneumophila* actively inhibits ROS production in macrophages via T4SS-dependent effector molecules [128].

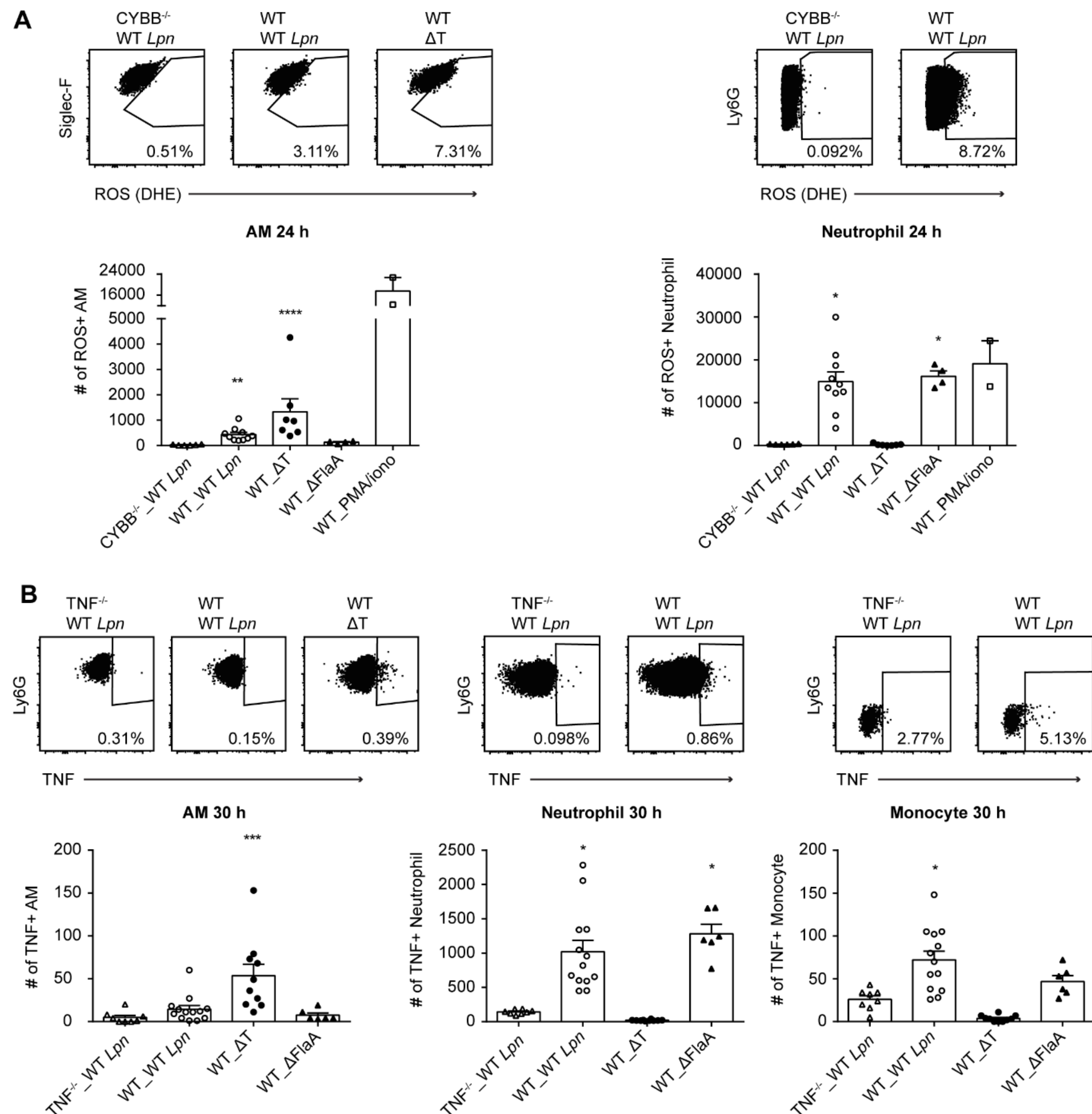

Figure 4. *In vivo*, neutrophils but not AM produce ROS and TNF in response to *L. pneumophila* infection. (A) WT and CYBB$^{-/-}$ mice were infected intranasally with WT, ΔT or ΔFlaA *L. pneumophila* and BALF cells were harvested 24 hr p.i.. AM, neutrophils and monocytes were stained for ROS with Dihydroethidium (DHE) and analyzed by flow cytometry. **(B)** WT and TNF$^{-/-}$ mice were infected intranasally with WT, ΔT or ΔFlaA *L.*

pneumophila and BALF cells were harvested 30 hr p.i.. AM, neutrophils and monocytes were stained for TNF and analyzed by flow cytometry. Data are from 2-3 pooled experiments. $*p<0.05$, $**p<0.01$, $***p<0.001$, $****p<0.0001$ compared to CYBB$^{-/-}$ or TNF$^{-/-}$ mice by Kruskal-Wallis test with Dunn's post test.

The extent to which ROS-deficient neutrophils contribute to *L. pneumophila* clearance *in vivo* remains inconclusive following neutrophil depletion experiments

To verify if ROS but not TNF are required for the contribution of neutrophils to the control *L. pneumophila* infection *in vivo*, we infected WT, TNF$^{-/-}$ and CYBB$^{-/-}$ mice, with or without depletion of neutrophils using three different approaches, and quantified CFU in the BALF 3 days p.i. (Fig. 5A-C). Neutrophils were depleted by injecting either 500 µg α-Ly6G (clone 1A8) i.p. on day -1 and day 1, or by injecting 250 µg α-Ly6G (clone 1A8) i.p. on day -1, and 10 µg α-G-CSF i.p. on day -1, 0, 1 and 2, of finally by injecting 100 µg α-Gr-1 (clone NIMP-R14) i.p. on day -1, 0, 1 and 2. As expected, but with a relatively large margin of error, neutrophil depletion in WT mice resulted in delayed clearance of *L. pneumophila* in the BALF compared to undepleted WT mice, confirming that neutrophils are important for the clearance of *L. pneumophila* from the lung (Fig. 5A-C). However, the results in the other groups varied with the different neutrophil depletion protocols. α-Ly6G depletion of neutrophils in CYBB$^{-/-}$ mice did not increase bacterial loads compared to undepleted CYBB$^{-/-}$ mice, suggesting that the contribution of neutrophils to immune defense in the context of *L. pneumophila* lung infection is mainly ROS-dependent up to and including 3 days p.i. (Fig. 5A). However, this interpretation is hampered by the fact that neutrophils may have been coated with α-Ly6G without actually having been depleted, since a large population of Ly6C-intermediate, MPO^{+}, CD11b^{+} cells remained (data not shown). It is unclear if these are neutrophils, or perhaps a compensatory monocyte population. α-Ly6G depletion of neutrophils in TNF$^{-/-}$ and MN-TNF NAIP5^{129S1} mice tended to increased bacterial titers, indicating that neutrophils have a TNF-independent, or partially TNF-independent role in bacterial clearance. Again this interpretation is hampered by the possibility that neutrophils are not actually depleted.

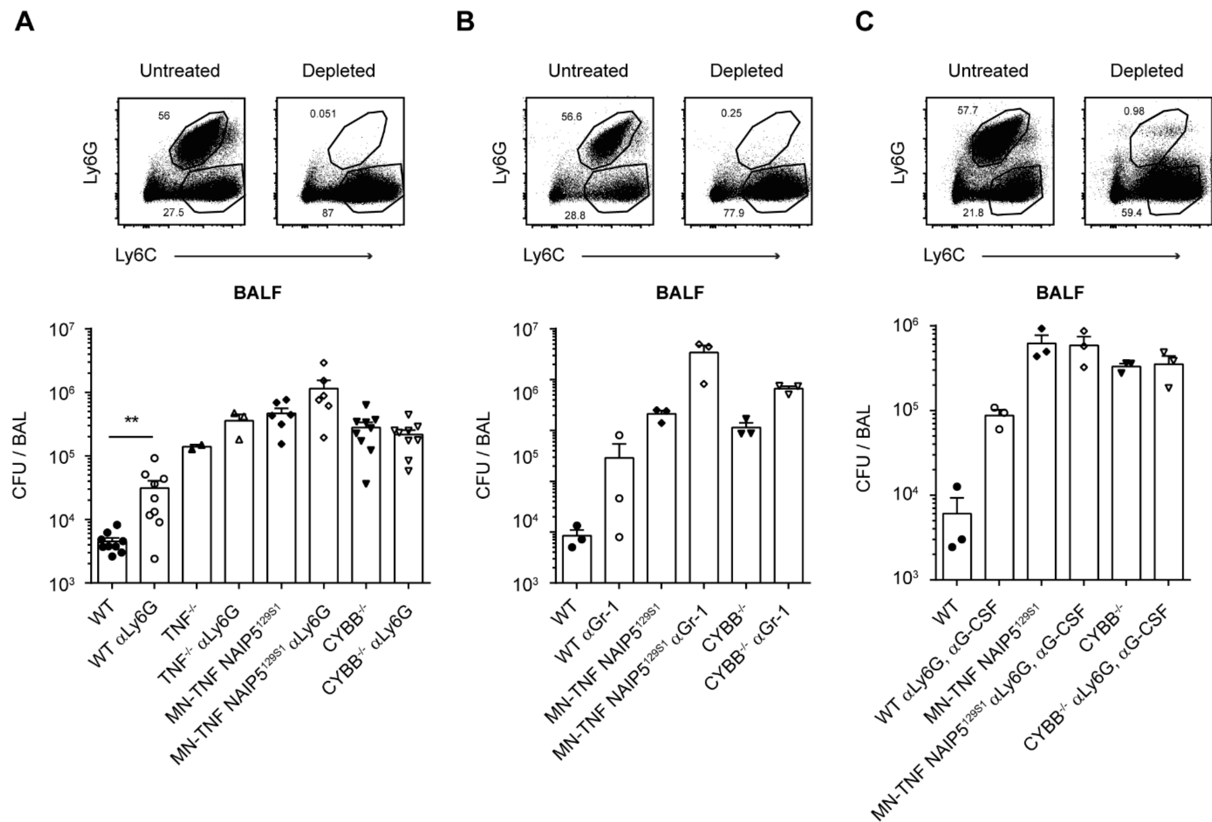

Figure 5. Neutrophil depletion experiments. (A-C) WT, MN-TNF NAIP5^{129S1}, TNF$^{-/-}$ or CYBB$^{-/-}$ mice were infected intranasally with WT *L. pneumophila*, and neutrophils were depleted using various protocols. 3 days p.i. BALF CFU were quantified on CYE agar plates. **(A)** 500µg α-Ly6G (clone 1A8) was administered i.p. day -1 and day 1. Data from 3 pooled experiments. **(B)** 100µg α-Gr-1 (clone NIMP-R14) was administered i.p. day -1, 0, 1 and 2. Data from 1 experiment. **(C)** 250µg α-Ly6G (clone 1A8) was administered i.p. day -1, and 10µg α-G-CSF was administered i.p. day -1, 0, 1 and 2. Data from 1 experiment. **$p<0.01$ by Mann-Whitney test.

Neutrophil depletion using α-Ly6G and α-G-CSF to prevent egress of neutrophils from the BM resulted in a more robust phenotype in WT mice, and also did not further increase bacterial burden in the lungs of CYBB$^{-/-}$ mice (Fig. 5C). However, the Ly6C-intermediate, CD11b^{+} population was again present in depleted mice, and further, G-CSF is known to be required for AM effector functions [129]. It is therefore not possible to distinguish between possible residual neutrophil activity, impaired AM function or ROS deficiency in neutrophils. Neutrophil depletion using α-Ly6G and α-G-CSF in MN-TNF NAIP5^{129S1} mice did not further increase bacterial burden in the BALF, suggesting that neutrophils have a TNF-dependent role in these mice (Fig. 5C) or that a further increase of bacterial titers was not achievable by

neutrophil depletion in these mice, as bacterial titers might already have reached a maximum titer in the absence of TNF and NAIP5 signaling.

Neutrophil depletion using α-Gr-1 lead to an increase in bacterial burden in CYBB$^{-/-}$ mice compared to undepleted CYBB$^{-/-}$ mice, suggesting neutrophils could have a ROS-independent role in the clearance of *L. pneumophila* lung infection. However, also with this neutrophil depletion protocol, a Ly6C-intermediate, MPO^{+}, CD11b^{+} population remained. Also, given that α-Gr-1 has the potential to deplete Ly6C expressing cells, it cannot be ruled out that off target populations such as monocytes could have been affected. However, on the whole the monocyte population was increased in the BALF of α-Gr-1 treated mice, suggesting that a compensatory monocyte population is present, or that α-Gr-1 is also able to mask the Ly6G epitope of neutrophils. Neutrophil depletion using α-Gr-1 in MN-TNF NAIP5^{129S1} mice increase bacterial burden in the BALF, suggesting that neutrophils have a TNF-independent or partially TNF-independent role in these mice, however with the factors listed above standing in the way of any certainty to this conclusion (Fig. 5B).

In summary, none of the neutrophil depletion protocols could be shown to have definitely depleted neutrophils, though their functionality does at least seem to have been partially impaired. A compensatory monocyte population may also infiltrate the BALF in response to neutrophil depletion.

TNF mediates an anti-bacterial effect in macrophages via TNFR1, which is independent of NLRC4 and ROS

The *in vivo* results presented in Figure 3 in combination with the observation that *in vitro*, TNFR1$^{-/-}$ and TNFR1/2$^{-/-}$ but not TNFR2$^{-/-}$ BMDM were more permissive to *L. pneumophila* replication than WT BMDM, suggest that TNF directly inhibits *L. pneumophila* replication in macrophages via signaling through TNFR1 (Fig. 6A). Furthermore, the addition of recombinant TNF (rTNF) to BMDM abrogated growth of *L. pneumophila* in all of the genotypes with a functional TNFR1 gene, including NLRC4$^{-/-}$ and CYBB$^{-/-}$ BMDM. These data show that TNF mediates an antibacterial mechanism in BMDM via TNFR1, which is independent of ROS and the NAIP5-NLRC4 flagellin recognition pathway (Fig. 6A and B). Importantly, 3 day exposure to 100 ng/ml rTNF did not induce BMDM cell death, but instead even slightly enhanced their viability as measured by the Alamar blue assay (Fig. 7) and as observed under the microscope (data not shown), suggesting an active antibacterial mechanism mediated by TNF rather than the induction of cell death. Membrane TNF knock-in (memTNF

KI) BMDM, which are only able to make membrane bound but not secreted TNF, were also more susceptible than WT BMDM, suggesting that TNF signals as a soluble molecule on BMDM *in vitro* (Fig. 6A). To consolidate this observation, we added a neutralizing anti-TNF antibody or TNFR1 fused to the Fc portion of an antibody (TNFR1-Fc) to WT BMDM infected with *L. pneumophila*, in order to neutralize soluble TNF secreted by the BMDM. This resulted in the sensitization of WT BMDM to *L. pneumophila* infection to a similar level as that observed for TNFR1$^{-/-}$ BMDM, suggesting that the difference in susceptibility between WT and TNFR1$^{-/-}$ BMDM is due to endogenously secreted TNF in response to *L. pneumophila* infection (Fig. 6C). Also in line with the conclusion that lack of endogenous TNF results in moderate sensitivity of BMDM to *L. pneumophila* infection is the observation that MYD88$^{-/-}$ BMDM, which fail to secrete TNF in response to *L. pneumophila* infection ([130] and Fig. 8), also have a similar susceptibility to *L. pneumophila* as TNFR1$^{-/-}$ BMDM (Fig. 6A). Taken together, these data suggest that TNF activates an antibacterial mechanism in macrophages via TNFR1 that is independent of NLRC4 and ROS. Furthermore, TNF production by BMDM in response to *L. pneumophila* is downstream of MyD88.

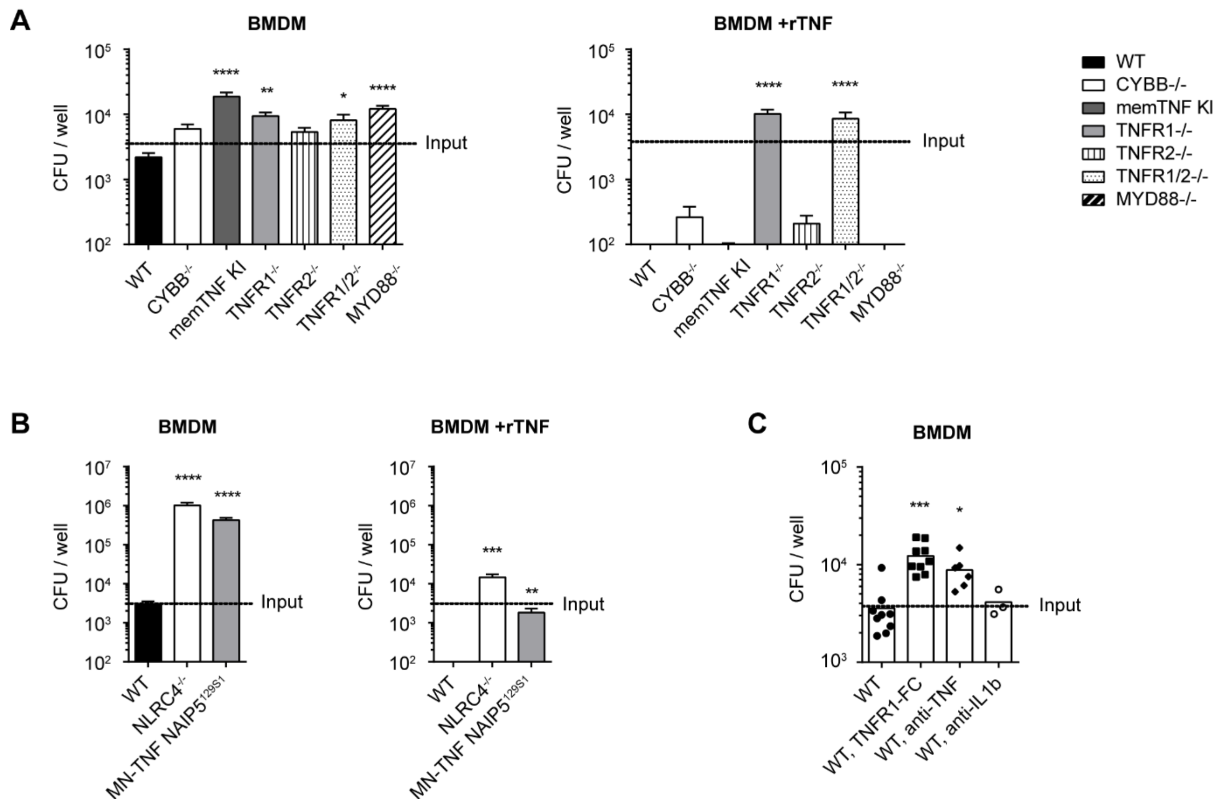

Figure 6. TNF mediates an antibacterial effect in macrophages via TNFR1, which is independent of NLRC4 and ROS. (A-B) WT or knockout BMDM were infected with WT *L.*

pneumophila at MOI 0.1. BMDM were either left untreated (left hand panels) or rTNF was added at the time of infection (right hand pannels). 3 days p.i. BMDM were lysed and CFU were quantified on CYE agar plates. **(C)** WT BMDM were infected with WT *L. pneumophila* MOI 0.1, with or without the addition of TNFR1-Fc, anti-TNF Ab or anti-IL1β Ab. 3 days p.i. BMDM were lysed and CFU were quantified on CYE agar plates. Data are from 3-7 pooled experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001 compared to WT by Kruskal-Wallis test with Dunn's post test.

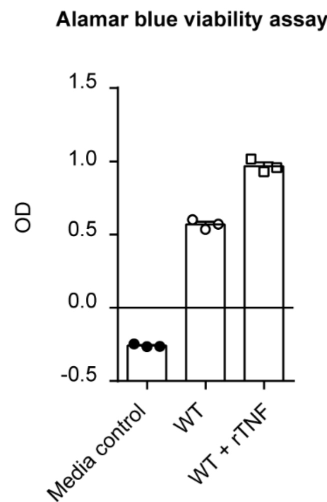

Figure 7. TNF increases the viability of BMDM. WT BMDM were seeded in 96 well plates at $1x10^5$ cells / well. After resting overnight, media was replaced with new media containing 20% L929 conditioned media containing M-CSF, with or without 100 ng/ml rTNF. After 3 days of incubation at 37°C, medium was replaced with 200 µl medium containing 20% L929 conditioned media and 10% alamar blue (Lucerna Chem AG, A1180), and incubated for 6.5 hr at 37°C. Conversion of alamar blue reagent by live cells was then measured with an ELISA plate reader and OD_{570}-OD_{600} was calculated. Results are from one experiment.

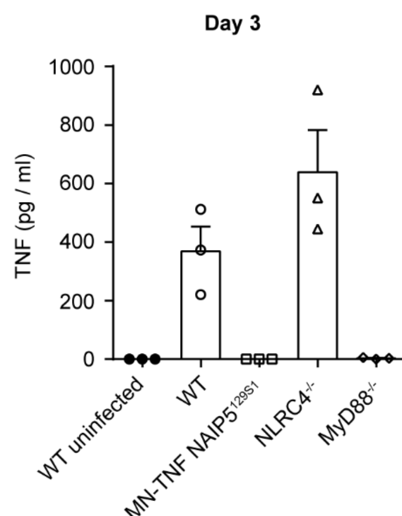

Figure 8. BMDM secrete TNF in response to *L. pneumophila* infection. This secretion is downstream of MyD88 and independent of NLRC4. (A-B) WT, MN-TNF NAIP5^{129S1} or NLRC4$^{-/-}$ BMDM were infected with WT *L. pneumophila* at MOI 0.1 or left untreated. **(A)** 3 days p.i. supernatant was collected and TNF was quantified by cytometric bead array assay. **(B)** 27 and 48 hr p.i. supernatant was collected and TNF was quantified by cytometric bead array assay. Data are each from one experiment.

Endogenous BMDM-derived TNF does not compensate for reduced NAIP5 signaling in BMDM *in vitro*.

Next, we sought to determine if endogenous TNF produced by macrophages in response to *L. pneumophila* infection can signal in a paracrine manner and compensate for reduced NAIP5-NLRC4 signaling. To test this we combined WT and MN-TNF NAIP5^{129S1} BMDM in 96 well plates in various ratios, infected them with WT *L. pneumophila* at MOI 0.1 and quantified CFU on CYE agar plates 3 days p.i. (Fig. 9). As a control, we combined WT and NLRC4$^{-/-}$ BMDM, which are both TNF proficient. Given that the amount of CFU quantified in WT and MN-TNF NAIP5^{129S1} BMDM closely matched the numbers calculated making the assumption that WT and MN-TNF NAIP5^{129S1} BMDM do not interact, this data suggests that endogenous TNF produced by WT BMDM in response to *L. pneumophila* infection is not enough to protect MN-TNF NAIP5^{129S1} BMDM from *L. pneumophila* infection. Note that CFU counts are slightly lower than expected in the 80% WT groups, even in the WT:NLRC4$^{-/-}$ BMDM group, which is probably a reflection of the fact that WT cells start to act as *L. pneumophila* "sinks" that hamper subsequent rounds of infection when they are present in greater numbers than knockout cells (Fig. 9).

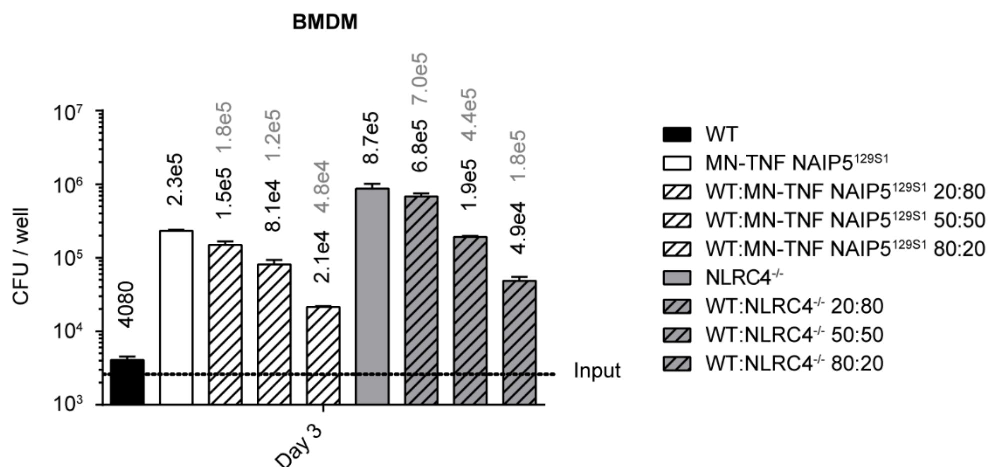

Figure 9. Endogenous BMDM-derived TNF does not compensate for reduced NAIP5 signaling in BMDM *in vitro*. WT and MN-TNF NAIP5^{129S1} or NLRC4$^{-/-}$ BMDM were combined in 96 well plates in the indicated ratios and infected with WT *L. pneumophila* at MOI 0.1. Three days p.i. BMDM were lysed and CFU were quantified on CYE agar plates. The black numbers show the actual CFU counted, and the grey numbers show the CFU calculated making the assumption that WT and knockout cells do not interact. Data are representative of two independent experiments.

In vivo, TNF produced by neutrophils and monocytes enhances AM-mediated killing of *L. pneumophila*, and can compensate for lack of NLRC4

The fact that NLRC4$^{-/-}$ BMDM are highly susceptible to *L. pneumophila* replication *in vitro*, but NLRC4$^{-/-}$ mice are only moderately susceptible *in vivo* suggests that mechanisms that are only present *in vivo* are able to compensate for a lack of NLRC4. To determine if paracrine TNF compensates for reduced NAIP5-NLRC4 mediated signaling *in vivo*, we infected MN-TNF NAIP5^{129S1} mice, which have a hypofunctional NAIP5 allele and are deficient in TNF in macrophages, monocytes and neutrophils, with WT *L. pneumophila*. We found that MN-TNF NAIP5^{129S1} mice were highly susceptible to *L. pneumophila* lung infection, with much greater bacterial burdens in the BALF 5 days p.i. compared to WT, TNF$^{-/-}$ and NLRC4$^{-/-}$ mice (Fig. 1A). This result suggests that TNF produced by macrophages, monocytes and/or neutrophils is essential for *in vivo* control of *L. pneumophila* lung infection. In order to further narrow down the *in vivo* source of TNF, we infected WT mice and TNF$^{-/-}$ mice with WT, ΔT and ΔFlaA *L. pneumophila* and stained BALF cells for TNF 30 h p.i. (Fig. 4B). We found that neutrophils and monocytes, but much less AM, produced TNF in response to *L. pneumophila* lung infection, suggesting that neutrophils and monocytes are the relevant TNF source. In addition, BMDM from MN-TNF NAIP5^{129S1} mice were as susceptible to *L. pneumophila* replication as NLRC4$^{-/-}$ BMDM, and this susceptibility could be abrogated by the addition of rTNF (Fig. 6B). Taken together, these data suggest that neutrophil and monocyte derived TNF enhances AM-mediated *L. pneumophila* killing and partially compensates for reduced NAIP5-NLRC4 signaling in AM *in vivo*.

Caspases do not appear to play a role in TNF-mediated inhibition of *L. pneumophila* replication in BMDM

Given that caspase-1 can activate caspase-7 and induce the fusion of LCVs with lysosomes [33], and caspase-11 can also induce the fusion of LCVs with lysosomes [67], we wished to

determine if the antibacterial effect mediated by TNF is dependent on Caspase-1 or 11. We therefore infected caspase-1/11$^{-/-}$ BMDM with *L. pneumophila*, with or without the addition of rTNF, and CFU were quantified 3 days p.i. (Fig. 10). The addition of rTNF prevented bacterial replication in Caspase-1/11$^{-/-}$ BMDM, demonstrating that the TNF-mediated antibacterial mechanism in BMDM is independent of Caspase 1 and 11 (Fig. 10). To test if other caspases were involved, we infected caspase-1/11$^{-/-}$ BMDM with WT *L. pneumophila* in the presence of the pan-caspase inhibitor Z-VAD-FMK, with or without the addition of rTNF. As Z-VAD-FMK blocked the TNF-mediated effect, it seemed other caspases were involved (Fig. 10). Similar experiments with caspase 3 inhibitor Ac-DEVD-CHO, caspase 3/7 inhibitor Z-DEVD-FMK, caspase 8 inhibitor Z-IETD-FMK but not caspase 9 inhibitor Z-LEHD-FMK, all seemed to demonstrate slight inhibition of TNF-mediated inhibition of *L. pneumophila* replication (Fig. 10). However, it should be noted that in all cases the caspase inhibitors increased the susceptibility of caspase-1/11$^{-/-}$ BMDM to *L. pneumophila* infection (Fig. 10), which may then cause an apparent block of the TNF-mediated inhibition of *L. pneumophila* replication, when this could in fact be independent of TNF. Furthermore, Z-VAD-FMK as well as other caspase inhibitors associated with the FMK (fluoromethylketone) group have been shown to inhibit cathepsins [131], opening the possibility that cathepsins rather than caspases could be responsible for the observed effects. In summary, these experiments did not show that a particular caspase was required for the TNF-mediated inhibition of *L. pneumophila* replication in macrophages.

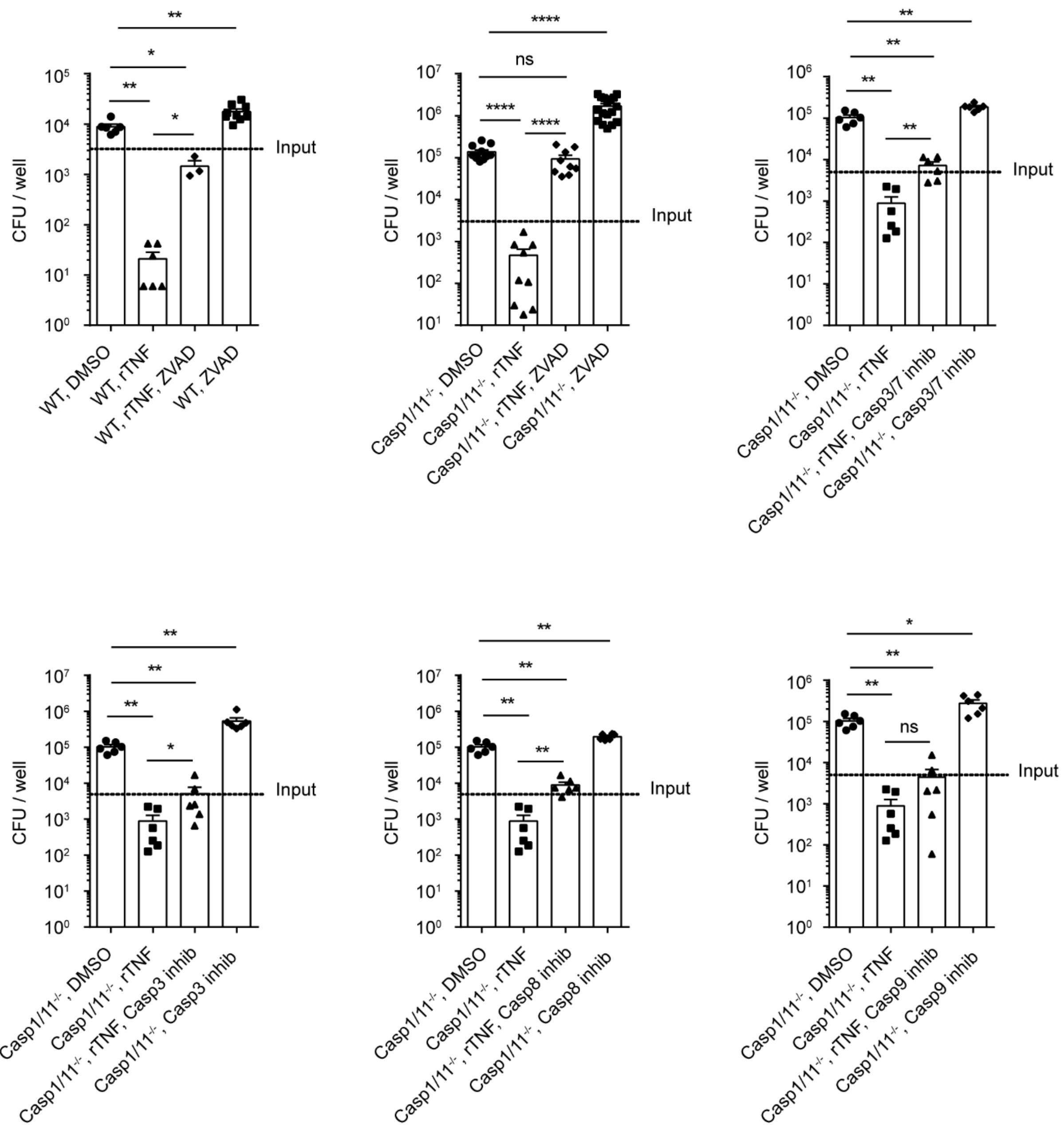

Figure 10. Caspases do not appear to play a role in TNF-mediated inhibition of *L. pneumophila* replication in BMDM. WT or Caspase-1/11$^{-/-}$ BMDM were infected with WT *L. pneumophila* at MOI 0.1. Where indicated rTNF and/or pan caspase inhibitor Z-VAD-FMK, caspase 3 inhibitor Ac-DEVD-CHO, caspase 3/7 inhibitor Z-DEVD-FMK, caspase 8 inhibitor Z-IETD-FMK or caspase 9 inhibitor Z-LEHD-FMK were added at the time of infection. 3 days p.i. BMDM were lysed and CFU were quantified on CYE agar plates. Data are from 2-3 pooled experiments. *p<0.05, **p<0.01, ****p<0.0001 by Mann-Whitney test.

TNF induces the fusion of LCVs with lysosomal compartments in macrophages

To gain further insight into the antibacterial mechanism mediated by TNF in macrophages, we infected MN-TNF NAIP5^{129S1} BMDM with *L. pneumophila*-GFP, in the presence or absence of rTNF or rIFNγ as a positive control [132,133], and examined the fate of the LCV with respect to lysosomal fusion using confocal microscopy (Fig. 11A). By 3 hours p.i. neither 100 ng/ml TNF nor 200 U/ml IFNγ resulted in *L. pneumophila*-GFP co-localization with lysosomal compartments as defined by lysotracker staining (Fig. 11A). However, when MN-TNF NAIP5^{129S1} BMDM were pre-treated with rTNF or rIFNγ overnight, by 1 hour p.i. 50% of *L. pneumophila* in rTNF pre-treated MN-TNF NAIP5^{129S1} BMDM co-localized with lysosomal compartments, but not in rIFNγ pre-treated BMDM. By 3 hours p.i., *L. pneumophila*-GFP co-localization with lysosomal compartments was observed in both rTNF and rIFNγ pre-treated MN-TNF NAIP5^{129S1} BMDM, suggesting that TNF induces the fusion of lysosomes with the LCV, but with different kinetics than IFNγ (Fig. 11A).

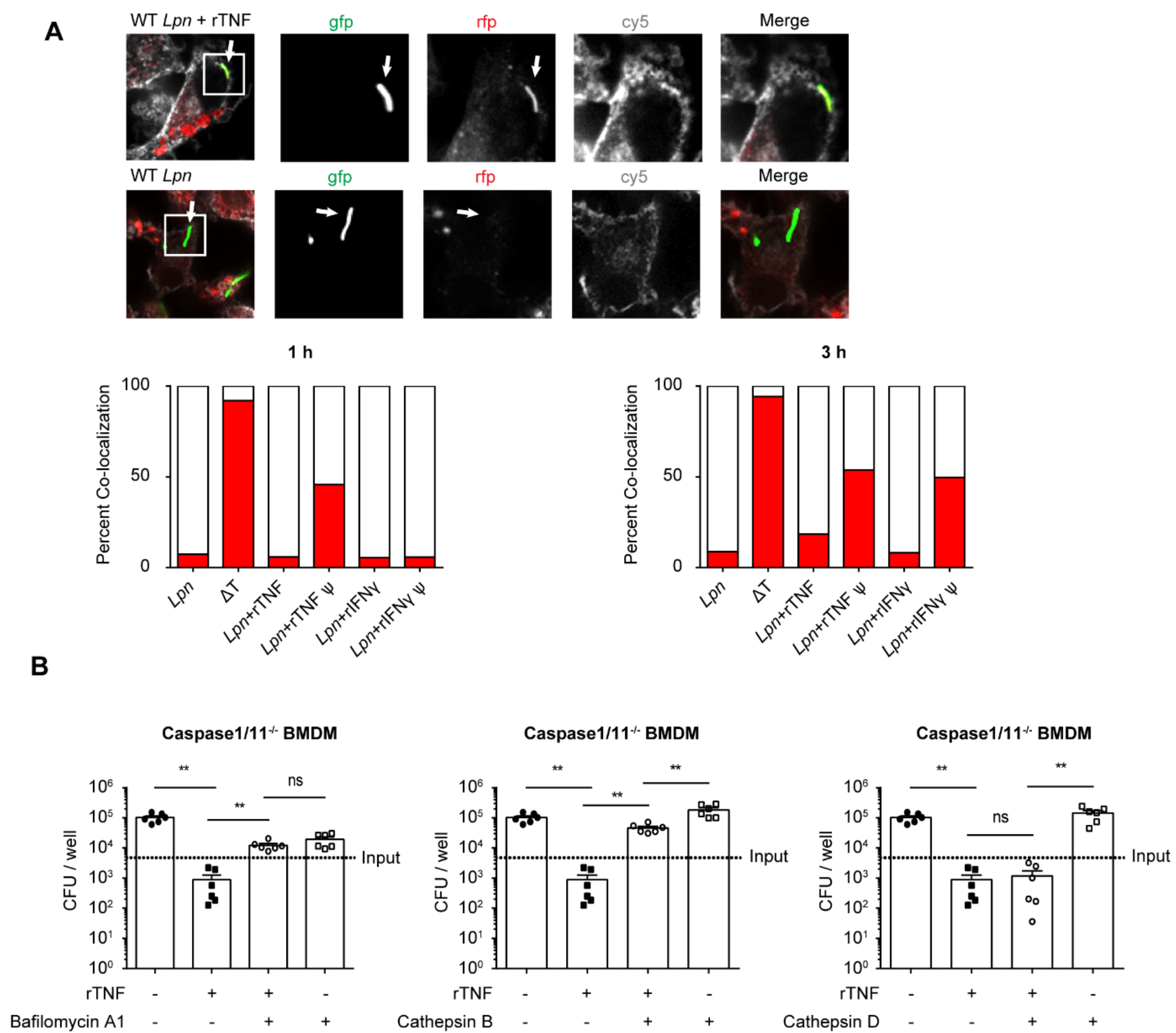

Figure 11. TNF-mediated killing of *L. pneumophila* is dependent on cathepsin B and lysosomal acidification, and is associated with the fusion of LCVs with lysosomal compartments in macrophages. (A) MN-TNF NAIP5^{129S1} BMDM were pre-treated overnight with rTNF (rTNFψ), rIFNγ (rIFNγψ), or were left untreated, and then infected with *L. pneumophila*-GFP at MOI 5 with simultaneous addition of rTNF or rIFNγ where indicated. 1 hr or 3 hr p.i. co-localization of *L. pneumophila*-GFP with lysosomes (stained with lysotracker Red) was analyzed via confocal microscopy, and at least 100 bacteria were counted per group. BMDM cell membranes were stained with Cholera toxin B AF647. Contrast and brightness of images was adjusted. Data are representative of 2 experiments. **(B)** Caspase-1/11$^{-/-}$ BMDM were infected with WT *L. pneumophila* at MOI 0.1. Where indicated rTNF and/or v-ATPase inhibitor bafilomycin A1, cathepsin B inhibitor CA-074-Me or cathepsin D inhibitor pepstatin A were added at the time of infection. 3 days p.i. BMDM were lysed and CFU were quantified on CYE agar plates. Data are from 2 pooled experiments. **$p<0.01$ by Mann-Whitney test.

The antibacterial mechanism mediated by TNF in macrophages is dependent on cathepsin B and lysosomal acidification, and is independent of caspase 1 and 11, and cathepsin D

Since our co-localization experiments suggested that TNF redirected *L. pneumophila* to lysosomal compartments, we sought to determine if lysosomal acidification was required for the TNF-mediated mechanism. To test this we infected Caspase-1/11$^{-/-}$ BMDM with *L. pneumophila* with or without rTNF and in the presence or absence of bafilomycin A1, a vacuolar H^+ ATPase inhibitor that blocks lysosomal acidification (Fig. 11B). We found that bafilomycin A1 abrogates the TNF-mediated inhibition of *L. pneumophila*, suggesting that lysosomal acidification is required downstream of TNF.

Next, we set out to identify the TNF-induced bactericidal mechanisms responsible for *L. pneumophila* degradation in acidic compartments. We tested for an involvement of cathepsins by using the specific cathepsin B inhibitor CA-074-Me and the cathepsin D inhibitor pepstatin A. We found that while inhibition of cathepsin D did not impact TNF-mediated inhibition of *L. pneumophila* replication, inhibition of cathepsin B strongly reduced TNF-mediated inhibition of *L. pneumophila* replication, suggesting that cathepsin B is a key molecule in the TNF-mediated antibacterial mechanism (Fig. 11B). In summary, our data show that the antibacterial mechanism mediated by TNF in BMDM is dependent on cathepsin B and lysosomal acidification, but independent of Caspase-1 and 11, and cathepsin D.

Autophagy and mitochondrial ROS do not appear to play a role in TNF-mediated inhibition of *L. pneumophila* replication in BMDM

Autophagy is a pathway which has been reported to target intracellular pathogens for elimination, including *L. pneumophila* and *M. tuberculosis* [134,135]. We therefore tested if autophagy was involved in TNF-mediated inhibition of *L. pneumophila* replication in macrophages. Autophagy sufficient ($Atg5^+$) and deficient ($Atg5^-$) macrophage cell lines were infected with WT or ΔFlaA *L. pneumophila* at MOI 0.1 in the presence or absence of rTNF, and CFU were quantified 3 days p.i.. We found that both WT and ΔFlaA *L. pneumophila* growth was inhibited by rTNF in autophagy deficient macrophages, suggesting TNF-mediated inhibition of *L. pneumophila* replication is not dependent on autophagy (Fig. 12A). However, the autophagy sufficient cell line was not permissive to WT *L. pneumophila*, and allowed only limited growth of ΔFlaA *L. pneumophila*. Furthermore, the addition of rTNF did not affect *L. pneumophila* growth in either case (Fig. 12A). Perhaps this was because $Atg5^+$ cells have lost TNFR1, or because they produce large amounts of TNF in response to *L. pneumophila* infection. Regardless, the fact that the autophagy deficient cell line was able to respond to TNF suggests that the TNF mediated mechanism is independent of autophagy.

Mitochondrial ROS have been shown to be involved in mediating the activation of the NLRP3 inflammasome [136], and the NLRP3 inflammasome has been shown to be involved in flagellin-independent immunity to *L. pneumophila* [137]. We tested for the involvement of mitochondrial ROS in TNF-mediated inhibition of *L. pneumophila* replication in macrophages by infecting MN-TNF $NAIP5^{129S1}$ BMDM with WT *L. pneumophila* at MOI 0.1 with or without the addition of the mitochondrial superoxide inhibitor mitoTEMPO, in the presence or absence of rTNF. We found that mitoTEMPO did not impart TNF-mediated inhibition of *L. pneumophila* replication in BMDM, suggesting that mitochondrial ROS are not involved in the TNF-mediated mechanism (Fig. 12B).

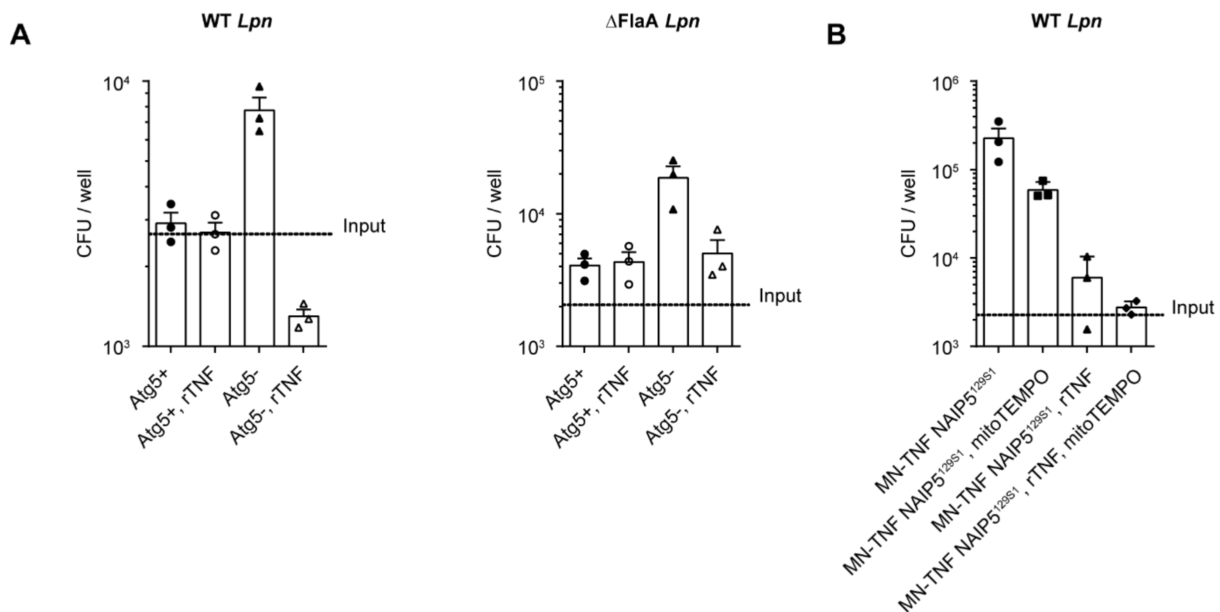

Figure 12. Autophagy does not appear to play a role in TNF-mediated inhibition of *L. pneumophila* replication in BMDM. (A) Autophagy sufficient ($Atg5^+$) and deficient ($Atg5^-$) macrophage cell lines were infected with WT or ΔFlaA *L. pneumophila* at MOI 0.1. Where indicated rTNF was added at the time of infection. 3 days p.i. BMDM were lysed and CFU were quantified on CYE agar plates. Data are representative of two experiments. **(B)** MN-TNF $NAIP5^{129S1}$ BMDM were infected with WT *L. pneumophila* at MOI 0.1. Where indicated rTNF and/or mitochondrial ROS scavenger mitoTEMPO were added at the time of infection. 3 days p.i. BMDM were lysed and CFU were quantified on CYE agar plates. Data are from one experiment.

$CCR2^+$ monocytes might be important for the clearance of *L. pneumophila in vivo*

The major populations that infiltrate the lung upon intranasal *L. pneumophila* infection include neutrophils, macrophages and monocytes. In order to investigate the role of inflammatory monocytes in the immune response against *L. pneumophila*, we infected WT and CCR2-DTR mice, with or without diphtheria toxin (DT) treatment, and measured the bacterial burden in the BALF 5 days p.i. (Fig. 13A). DT treated CCR2-DTR mice had a much higher bacterial load in the BALF than their untreated counterparts, suggesting that monocytes play an important role in the innate immune defense against *L. pneumophila* lung infection (Fig. 13A). However, DT treatment also depleted AM in these mice, leaving open the possibility that monocytes do not play an important role, while AM are essential (Fig. 13B).

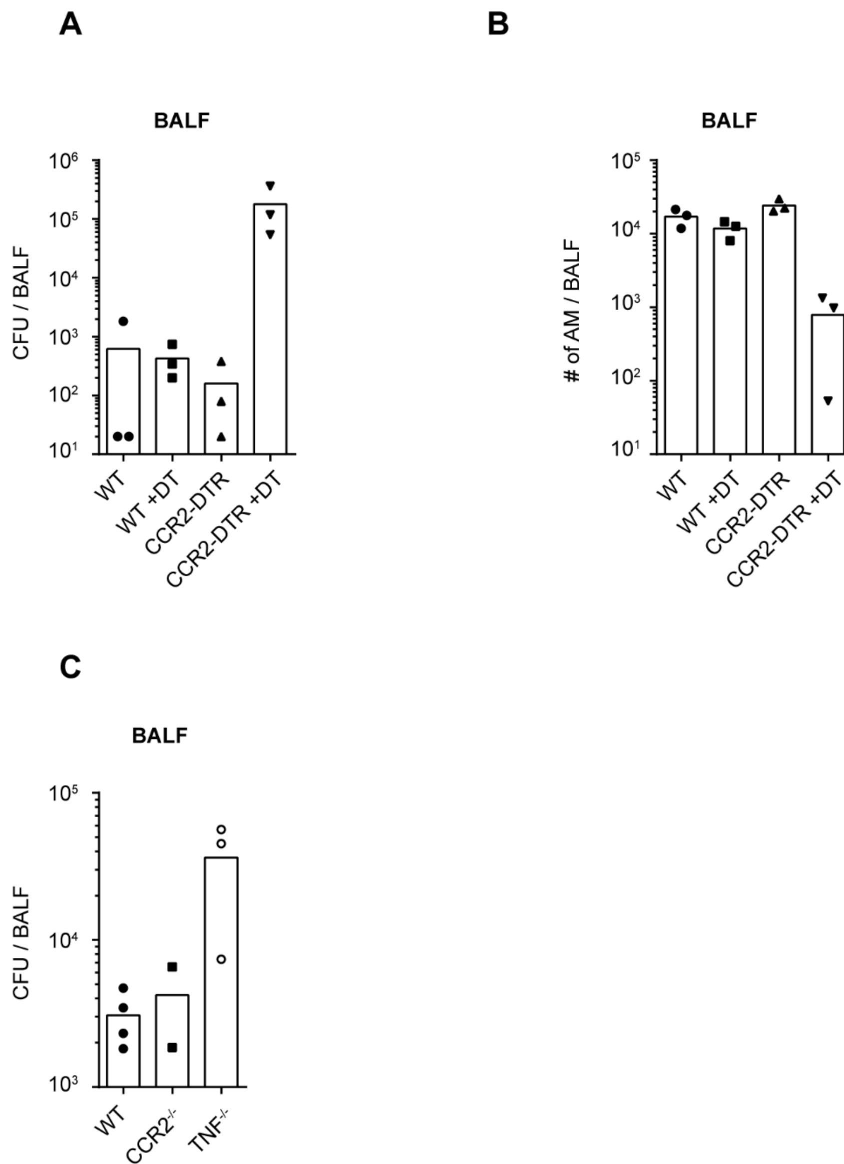

Figure 13. CCR2$^+$ monocytes might be important for the clearance of *L. pneumophila in vivo*. (A-B) WT CCR2-DTR mice were infected intranasally with WT *L. pneumophila*, with or without i.p. DT treatment, and 5 days p.i. BALF CFU were quantified on CYE agar plates. Data is from one experiment. **(C)** WT, CCR2$^{-/-}$ or TNF$^{-/-}$ mice were infected intranasally with WT *L. pneumophila*, and 5 days p.i. BALF CFU were quantified on CYE agar plates. Data are from one experiment.

In an attempt to bypass the possible depletion of AM with DT in CCR2-DTR mice, we also assessed the clearance of *L. pneumophila* in CCR2$^{-/-}$ mice, and TNF$^{-/-}$ mice were included as a reference to control for the quality of the bacteria. Bacterial burden was not increased in CCR2$^{-/-}$ mice in the BALF 5 days p.i. compared to WT mice, suggesting that CCR2$^+$ monocytes are

dispensable for the control of *L. pneumophila* lung infection (Fig. 13C). However, we cannot exclude that compensatory mechanisms are in place in $CCR2^{-/-}$ mice, as has been found with these mice in other models [138]. Such compensatory mechanisms could obscure the contribution of inflammatory monocytes in $CCR2^{-/-}$ mice.

Cytokines in the BALF of *L. pneumophila* infected mice

In order to gain insight into the inflammatory environment in the BALF of WT and MN-TNF $NAIP5^{129S1}$ mice, we measured the levels of TNF, IFNγ, IL-1α, IL-1β, IL-12p70 and IL-6 using a cytometric bead array (CBA) assay. As a reference, rTNF used in the BMDM assays was diluted to 5 ng/ml and 2.5 ng/ml, and measured also. Very little TNF was recovered from the BALF of MN-TNF $NAIP5^{129S1}$ mice, confirming that macrophages, monocytes and/or neutrophils are required to produce TNF in the BALF in response to *L. pneumophila* lung infection (Fig.13). At 16 hours p.i., MN-TNF $NAIP5^{129S1}$ mice had reduced IL-1α, IL-1β and IL-6 compared to WT mice, suggesting that TNF from neutrophils, macrophages and/or monocytes is involved in stimulating the production of these cytokines. IL-12p70 peaked at 36 hours p.i., and very little IFNγ was produced in response to *L. pneumophila* infection in our model (Fig. 14). In summary, TNF from macrophages, monocytes and/or neutrophils had an impact in enhancing IL-1α, IL-1β and IL-6 in the BALF of *L. pneumophila* infected mice.

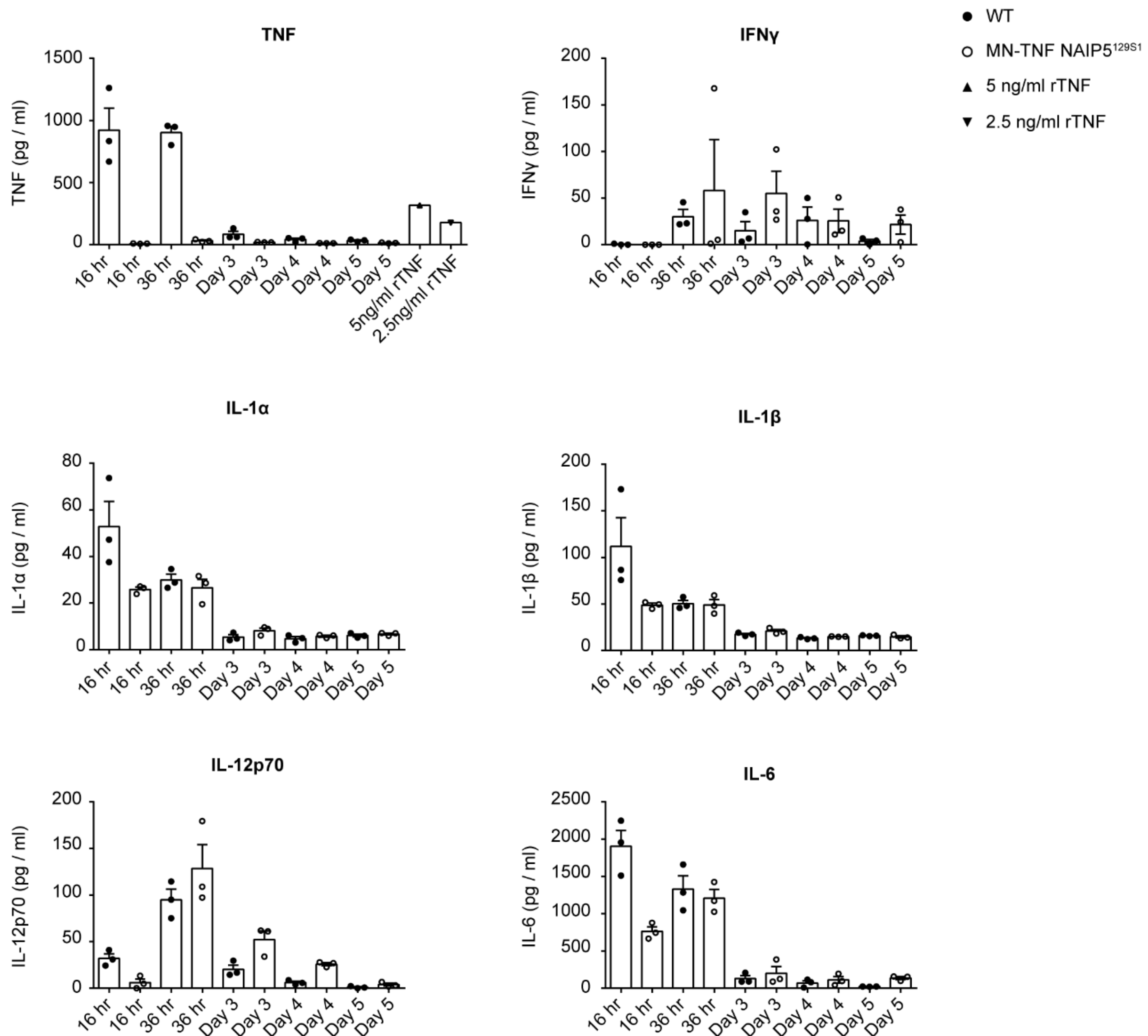

Figure 14. Cytokines in the BALF of *L. pneumophila* infected mice. WT or MN-TNF NAIP5^{129S1} mice were infected intranasally with WT *L. pneumophila*. Mice were sacrificed at the indicated time points, and BALF was cleared by centrifugation and frozen. BALF TNF, IFNγ, IL-1α, IL-1β, IL-12p70 and IL-6 were quantified by cytometric bead array assay (CBA). As a reference, rTNF used in the BMDM assays was diluted to 5 ng/ml and 2.5 ng/ml, and also measured by CBA. Data are from one experiment.

MN-TNF NAIP5^{129S1} BMDM do not produce IL-6 in response to *L. pneumophila* infection

To complement the cytokine data from the BALF, we also measured TNF, IFNγ, IL-1α, IL-1β, IL-12p70 and IL-6 produced by *L. pneumophila* infected WT and MN-TNF NAIP5^{129S1} BMDM. As expected MN-TNF NAIP5^{129S1} BMDM did not produce TNF (Fig. 15). Though the data are only from one experiment and there are not many data points, there is a trend for reduced IL-1α and IL-1β 27 hours p.i., but not at later time points. Interestingly, IL-6 is low in

the supernatants of MN-TNF NAIP5^{129S1} BMDM, suggesting that TNF is important for the induction of this cytokine in response to *L. pneumophila* infection (Fig. 15).

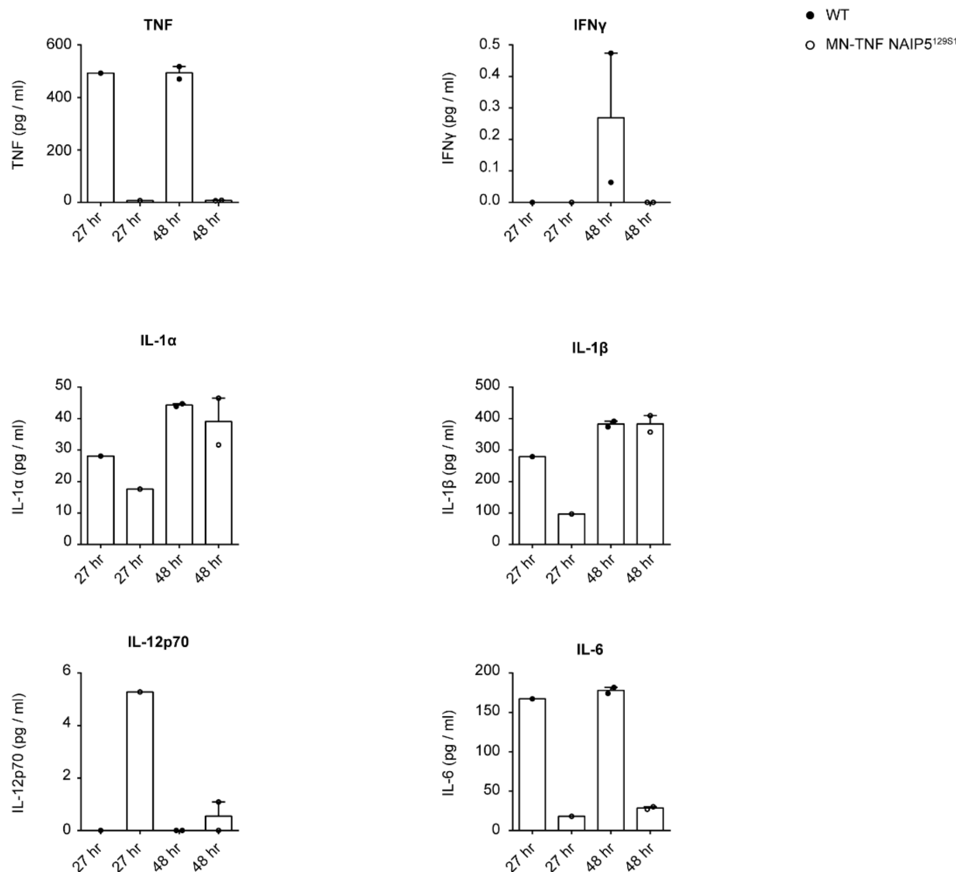

Figure 15. MN-TNF NAIP5^{129S1} BMDM do not produce IL-6 in response to *L. pneumophila* infection. WT or MN-TNF NAIP5^{129S1} BMDM were infected with WT *L. pneumophila* at MOI 0.1 in 24 well plates. At the indicated timepoints, supernatant was collected and frozen. TNF, IFNγ, IL-1α, IL-1β, IL-12p70 and IL-6 were quantified by cytometric bead array assay. Data are from one experiment.

L. pneumophila may differentiate into MIFs in neutrophils but not AM

L. pneumophila replicating in amoeba differentiate into MIF, a highly infectious form of *L. pneumophila* which is resistant to environmental stresses. However, the bacterium does not differente into this form in macrophages [95]. As shown in Figure 16, *L. pneumophila* tended to be more tightly packed in neutrophils than in AM, and appeared to be shorter and rounder, which are characteristics of MIFs [94]. Though this by no means proves that neutrophils contain MIFs, this provides some evidence that the hypothesis might be worth exploring. The

neutrophil in the upper right contains *L. pneumophila* which are nolonger GFP^{+} and stain positive for lysotracker, suggesting that they are in the process of being digested. This suggests that even if the observed *L. pneumophila* are MIFs, neutrophils are able to kill them.

Neutrophil

AM

Figure 16. *L. pneumophila* may differentiate into MIFs in neutrophils but not AM. Mixed BM chimeric mice reconstituted with 50% $Ly5.1^{+}$ WT BM, and either 50% $Ly5.2^{+}$ WT or $TNFR1^{-/-}$ BM were generated. Chimeras were infected with WT *L. pneumophila*-GFP, and 2 days p.i. BALF was harvested and $Ly5.1^{+}$ and $Ly5.2^{+}$ AM and neutrophils were sorted. Cells were stained with lysotracker Red and Cholera toxin B AF647, and analyzed via confocal microscopy.

Discussion

Upon inhalation of contaminated aerosols, *L. pneumophila* initially establishes infection in AM [74,139]. However, the resolution of *L. pneumophila* infection by the innate immune system in the lung requires crosstalk between many cell types, and the coordination of this cross-talk and the mechanisms involved remain poorly understood [106,140]. It has been shown that early inflammasome-independent AM-derived IL-1α complemented by later inflammasome-dependent IL-1β produced by AM and possibly other hematopoietic cells results in IL1R/MyD88 dependent chemokine production by airway epithelial cells [74,85,97,98,102]. These chemokines serve as an amplification of the IL-1 signal produced by hematopoietic cells and are required for the early recruitment of neutrophils to the lung, which are essential in controlling *L. pneumophila* bacterial burden [85,98-101].

Here we show that the neutrophil-mediated mechanisms that lead to *L. pneumophila* clearance *in vivo* are twofold. On the one hand, neutrophils directly kill *L. pneumophila* via ROS mediated mechanisms, and on the other hand neutrophil and monocyte-derived TNF initiates microbicidal mechanisms in AM via TNFR1, which increase their capacity to inhibit *L. pneumophila* replication. The latter involves rerouting the bacteria to lysosomal compartments despite the presence of T4SS effectors, and requires cathepsin B. The importance of TNF and ROS-mediated mechanisms in the control of *L. pneumophila* infection are underscored by the marked susceptibility of TNF$^{-/-}$ and CYBB$^{-/-}$ mice to *L. pneumophila* infection.

The impact of TNF-mediated antimicrobial mechanisms directed against *L. pneumophila* cannot be fully appreciated by the study of macrophages *in vitro*. In accordance with other studies, we observed that TNFR1$^{-/-}$ BMDM only support moderate *L. pneumophila* growth in comparison to NAIP5$^{-/-}$ or NLRC4$^{-/-}$ BMDM, which support several orders of magnitude more growth [126,130] (Fig. 6A and B). However, this difference is not observed when comparing the bacterial burden of TNFR1$^{-/-}$ and NLRC4$^{-/-}$ mice *in vivo*, where there is even a trend for TNF to play a more dominant role (Fig. 1A). A possible explanation for these apparently incongruent results is that paracrine TNF produced *in vivo* by neutrophils and monocytes, rather than autocrine TNF produced by AM, mediates the increased resistance to *L. pneumophila*, and further that this TNF can compensate for a lack of NAIP5-NLRC4 mediated immune defense. Though we and others did observe modest endogenous TNF production by BMDM in response to *L. pneumophila* infection (Figs. 8 and 14, [130]), and found that this TNF accounted for the increased susceptibility of TNFR1$^{-/-}$ BMDMs (Fig. 6A, [130]), this was

not enough to compensate for lack of NAIP5-NLRC4 flagellin sensing, arguing against a dominant role for autocrine TNF production by AM (Fig. 6B, [130]). In fact, NLRC4$^{-/-}$ BMDM secrete more TNF than WT BMDM in response to *L. pneumophila* infection, possibly due to increased bacterial burden or a failure to undergo pyroptosis (Fig. 8, [130]). However, we propose that *in vivo*, AM are exposed to much higher local concentrations of TNF than produced endogenously by isolated BMDMs. *In vitro*, 200 - 600 pg/ml TNF were observed in the supernatant of WT BMDM (Fig. 15, [130]), in comparison to around 20 ng/ml reported in the BALF of A/J mice at peak concentration [141]. Assuming an epithelial lining fluid volume of 100 µl, or a 10 - 20 fold dilution in the BALF of volume 1-2 ml, the actual TNF concentration in the undiluted endothelial lining fluid would be 200 - 400 ng/ml. Indeed, the addition of 100 ng/ml rTNF markedly suppressed *L. pneumophila* replication in NLRC4$^{-/-}$ BMDM and increased cell viability (Fig. 6B). Note that we only measured 1 ng/ml TNF in the BALF at 16 and 36 hours post infection (Fig. 14). However, we may have missed the timepoint at which TNF reached its peak, and the rTNF which was used in the BMDM experiments and which we measured as a reference was also underestimated by around 16 fold, suggesting that 1 ng / ml is an underestimation, or that less than 100 ng/ml rTNF was added in our experiments (Fig. 14). Furthermore, as *in vitro* with NLRC4$^{-/-}$ BMDM, TNF might be increased in the BALF compared to WT mice when NAIP5-NLRC4 signaling is reduced, as would be the case in A/J mice and NLRC4$^{-/-}$ mice. In addition, TNF has been shown to synergize with other cytokines such as IFNγ and type 1 interferons (IFN) in the restriction of *L. pneumophila*, which might also be present at higher concentrations in the epithelial lining fluid [112,130,142].

In order to verify this hypothesis, we made use of MN-TNF NAIP5^{129S1} mice, which lack TNF in macrophages, monocytes and neutrophils and carry the NAIP5^{129S1} allele. BMDM from MN-TNF NAIP5^{129S1} mice were almost as susceptible to *L. pneumophila* infection as BMDM from NLRC4$^{-/-}$ mice, as expected in the absence of proper NAIP5 signaling (Fig. 6B). Strikingly, MN-TNF NAIP5^{129S1} mice were also much more susceptible to *L. pneumophila* infection *in vivo* compared to either NLRC4$^{-/-}$ or TNFR1$^{-/-}$ mice, which in combination with the intracellular staining results showing that monocytes and neutrophils produce TNF in response to *L. pneumophila* (Fig. 4), suggests that neutrophil and monocyte derived TNF compensates to a large degree for weak NAIP5-NLRC4 flagellin sensing *in vivo* (Figs. 1A and B, 4B). Together with the observation that TNF is important for AM but not neutrophil-mediated killing, these experiments highlight the importance of TNF-mediated antibacterial mechanisms in AMs in the context of *L. pneumophila* lung infection (Fig. 3).

In our *in vivo* experiments where we sorted AM and neutrophils from WT:TNFR1$^{-/-}$ and WT:WT chimeric mice, we found that TNF signaling via TNFR1 was important for AM but not neutrophil-mediated restriction of *L. pneumophila* growth (Fig. 3). It is worth noting that we also attempted to show that TNF signaling via TNFR1 in AM restricts *L. pneumophila* replication in AM via FACS analysis, upon infection of chimeric mice with *L. pneumophila*-GFP and *L. pneumophila*-GFPind. However, these attempts proved unsuccessful for various potential reasons, which I will discuss here. Firstly, AM are highly autofluorescent cells, especially in the range of 500-550 nm, making it difficult to discern GFP^{+} cells in this population [143]. In addition, our experiments mixing various ratios of WT and MN-TNF NAIP5^{129S1} BMDM (Fig. 9) led us to believe that TNF acted on BMDM, and presumably AM, almost exclusively in an autocrine manner, as we were not aware at the time that MN-TNF NAIP5^{129S1} mice had a defect in NAIP5. Thus we conducted our experiments with WT:MN-TNF NAIP5^{129S1} and WT:TNF$^{-/-}$ chimeric mice rather than WT:TNFR1$^{-/-}$ mice, as we initially did not consider the effects of paracrine TNF. The fact that we did not see differences in CFU / cell between MN-TNF NAIP5^{129S1} or TNF$^{-/-}$ AM and WT AM was expected, given that we hypothesized that paracrine TNF from neutrophils and monocytes yielded the observed *L. pneumophila* restriction, and both MN-TNF NAIP5^{129S1} and TNF$^{-/-}$ AM express functional TNFR1.

After discovering that MN-TNF NAIP5^{129S1} mice are not simply conditional TNF knockouts, we revised our logic and conducted an experiment in which WT:TNFR1/2$^{-/-}$ mice were infected with *L. pneumophila*-GFP. However, only two WT:TNFR1/2$^{-/-}$ mice were available, and though both mice tended to have more GFP^{+} TNFR1/2$^{-/-}$ AM than GFP^{+} WT AM, (after having normalized for the total number of TNFR1/2$^{-/-}$ and WT AM), the variability was high and this did not tell us whether or not the *L. pneumophila* contained in the AM were alive (data not shown). Furthermore, experiments with *L. pneumophila*-GFPind infected chimeric mice showed us that GFP^{+} AM containing *L. pneumophila*-GFPind were very difficult if not impossible to tell apart from uninfected AM, as *L. pneumophila*-GFPind are much less bright than *L. pneumophila*-GFP (data not shown). Thus, we did not expect this approach to have the potential to accurately quantify live *L. pneumophila* within AM. In parallel, we obtained reproducible results by sorting and plating AM on CYE plates indicating that WT:TNFR1$^{-/-}$ mice contained more CFU within TNFR1$^{-/-}$ AM than within WT AM, and thus we abandoned the FACS analysis based approach. As a final consideration, I would like to note that we were only able to detect differences in the number of CFU contained within TNFR1$^{-/-}$ AM vs. WT

AM using the cell sorting and plating approach after introducing a cell lysis procedure, in which AM were lysed using 0.7% Tween 20 in PBS. This observation, and the observation made using the confocal microscope that TNFR1$^{-/-}$ AM tended to contain more *L. pneumophila* than WT AM within the same mouse, suggested that differences in numbers of CFU between TNFR1$^{-/-}$ and WT AM might only be detectable if the total number of CFU rather than the total number of infected AM are taken into account. Thus, this leaves open the possibility that the FACS based analysis of GFP^{+} AM cannot even in theory detect a difference in the number of *L. pneumophila* in TNFR1$^{-/-}$ and WT AM in *L. pneumophila* infected chimeric mice.

Though it is known that A/J mice are permissive to *L. pneumophila* replication due to allelic variations in the NAIP5 gene, the molecular basis for the interactions between NAIP5, flagellin, NLRC4 and other inflammasome components are still being unraveled [28]. The permissiveness to *L. pneumophila* of A/J, FvB/N, C3H/HeJ, BALBcJ and 129S1 mice was shown to be attributable to mutations in NAIP5, and 6 of the 14 amino acid (aa) missense mutations present in A/J mouse NAIP5 (NAIP5$^{A/J}$) are shared by all the permissive strains (Exon 11 aa 472, 533, 538 and Exon 12 aa 1092, 1116, 1123), suggesting that these aa are important for NAIP5 function [119]. We made the observation that none of these 6 mutations occur in the recently defined NAIP5 ligand specificity domain [28], suggesting that they are involved in other interactions that contribute to the restriction of *L. pneumophila* growth in macrophages. Since NAIP5$^{A/J}$ is able to activate caspase-1 but not downstream caspase-7 in response to *L. pneumophila* flagellin, these 6 aa may promote interactions between caspase-1 and caspase-7 [33,121]. Of the 14 aa differences between NAIP5$^{A/J}$ and C57BL/6 NAIP5 (NAIP5^{B6}), only two occur in the ligand specificity domain (Exon 11 aa 647 and 755) [28,119], and may account for the observed increase in susceptibility of A/J mice compared to 129S1 mice.

In addition there are 5 aa in NAIP5^{129S1} that differ from NAIP5^{B6} (Exon 3 aa 92 and 144, Exon 5 aa 242 and Exon 11 aa 516 and 521) but also from NAIP5$^{A/J}$ and the NAIP5 alleles of all the permissive mouse strains listed above, and these are also not located in the ligand specificity domain. We therefore consider these mutations less likely candidates to be involved in the hypofunctionality of NAIP5^{129S1}, though this will need to be verified. The last two out of the 14 aa (position 1241 and 1275) mutated in NAIP5$^{A/J}$ and also in NAIP5^{129S1} as published by Wright et al. are not present in our mice, as Exon 15 was replaced by WT exon 15 in our mice, ruling out an effect mediated by mutations in these 2 aa in our experiments. These two aa are located in the leucine rich repeat (LRR) domain, which has been shown to be involved

in the autoinhibition of NAIP5, predicting that mutations in this domain could result in the constitutive activation of NAIP5 [28]. Our study validates the hypofunctionality of NAIP5^{129S1} in addition to the better characterized NAIP5$^{A/J}$, and will hopefully contribute to future studies investigating the molecular basis for NAIP5 function.

One caveat in our experiments involving MN-TNF NAIP5^{129S1} mice is that the mice were backcrossed onto the C57BL/6 background only four times, and the possibility remains open that further genetic elements other than NAIP5^{129S1} from the 129S1 background might contribute to the susceptibility of these mice and their macrophages to *L. pneumophila* infection. However this seems unlikely, since the susceptibility of 129S1 mice and other permissive strains mapped to NAIP5 [119,120], and since MN-TNF NAIP5^{129S1} BMDM are similarly susceptible to NLRC4$^{-/-}$ BMDM, and no other pathway has been shown to cause as much susceptibility to *L. pneumophila* replication.

There is discrepancy in the literature as to whether other mouse strains besides A/J mice are susceptible to *L. pneumophila* infection, and the statement is often made that only A/J mice are susceptible, or that they represent a "notable exception" to other mouse strains [117,120,144,145]. Furthermore, the study published in 2003 in Nature Genetics by Diez et al. [120], which along with Wright et al. [119] originally identified NAIP5 as the gene responsible for the susceptibility of A/J mice to *L. pneumophila* infection, found that 129X1 mice were not susceptible to *L. pneumophila* infection, and that NAIP5^{129X1} could functionally complement NAIP5$^{A/J}$. However, it was noted in 1977 that what is widely referred to as 129 mice actually represents a collection of mice with varying genetic backgrounds, due to incomplete documentation of various genetic backgrounds that were intercrossed with the original 129 mouse strain [146,147]. This recognition prompted the renaming of 129 mice in an attempt to classify them into groups with similar genetic backgrounds (Table 1). Notably, 129S1 and 129X1 mice are different subgroups of 129Sv, and might not have the same genetic background (Table 1), and thus these mice might not have the same NAIP5 allele. The apparent lack of awareness of the genetic variability present in 129Sv mice has led to confusion, as evidenced by a recent study in which 129Sv mice are simply referred to as 129 mice, and it is assumed that the only gene of relevance lacking as compared to C57BL/6 mice is caspase-11, despite Wright et al.'s publication regarding NAIP5^{129S1} [117]. Thus, discrepancies in the literature regarding the status of the susceptibility of 129S1 mice to *L. pneumophila* infection are at least in part the result of a lack of awareness of the genetic variability present in 129Sv mice.

Table 1. Nomenclature for the 129 inbred mouse strains

Strain Designation (Former Designation)	Abbreviated Designation	Genotype	Phenotype
129P1/ReJ (129/ReJ)	129P1	$A^w/A^w\ Oca2^p\ Tyr^{c-ch}/Oca2^p\ Tyr^{c-ch}$	white-bellied, pink-eyed, light chinchilla (light tan)
129P2/OlaHsd (129/OlaHsd)	129P2	$A^w/A^w\ Oca2^p\ Tyr^{c-ch}/Oca2^p\ Tyr^{c-ch}$	white-bellied, pink-eyed, light chinchilla (light tan)
129P3/J (129/J)	129P3	$A^w/A^w\ Oca2^p\ Tyr^{c-ch}/Oca2^p\ Tyr^{c}$ or $A^w/A^w\ Oca2^p\ Tyr^{c}/Oca2^p\ Tyr^{c}$ or $A^w/A^w\ Oca2^p\ Tyr^{c-ch}/Oca2^p\ Tyr^{c-ch}$	white-bellied, pink-eyed, light chinchilla (off-white) or albino or white-bellied, pink-eyed, light chinchilla (light tan)
129X1/SvJ (129/SvJ)	129X1	$A^w/A^w\ Oca2^p\ Tyr^{c-ch}/Oca2^p\ Tyr^{c}$ or $A^w/A^w\ Oca2^p\ Tyr^{c}/Oca2^p\ Tyr^{c}$ or $A^w/A^w\ Oca2^p\ Tyr^{c-ch}/Oca2^p\ Tyr^{c-ch}$	white-bellied, pink-eyed, light chinchilla (off-white) or albino or white-bellied, pink-eyed, light chinchilla (light tan)
129S1/Sv-$Oca2^+\ Tyr^+\ Kitl^{Sl-J}$ (129/Sv-$p^+\ Tyr^+\ Kitl^{Sl-J}$/+) (129/Sv-$+^p\ +^{Tyr-c}Mgf^{Sl-J}$/+)	129S1	$A^w/A^w\ Kitl^{Sl-J}/Kitl^+$	white (or light)-bellied agouti, coat color dilution, variable white spotting
129S1/SvImJ (129S3/SvImJ) (129/SvImJ) (129/Sv-$p^+\ Tyr^+\ Kitl^+$/J) (129Sv-$+^p\ +^{Tyr-c}\ +^{Mgf-SlJ}$/J)	129S1	A^w/A^w	white (or light)-bellied agouti
129S2/SvPas (129/SvPas)	129S2	A^w/A^w	white (or light)-bellied agouti

129S4/SvJae (129/SvJae)	129S4	A^w/A^w	white (or light)-bellied agouti
129S5/SvEvBrd (129/SvEvBrd)	129S5	A^w/A^w	white (or light)-bellied agouti
129S9/SvEvH (129/SvEv)		A^w/A^w	white (or light)-bellied agouti
129S6/SvEvTac (129/SvEvTac)	129S6	A^w/A^w	white (or light)-bellied agouti
129S7/SvEvBrd-$Hprt1^{b\text{-}m2}$ (129/SvEvBrd-$Hprt^{b\text{-}m2}$)	129S7	A^w/A^w	white (or light)-bellied agouti
129S8/SvEv-$Gpi1^c$ $Hprt1^{b\text{-}m2}$/J (129/SvEv-$Gpi1^c$ $Hprt^{b\text{-}m2}$@J)	129S8	A^w/A^w	white (or light)-bellied agouti
129T1/Sv-$Oca2^+$ $Tyr^{c\text{-}ch}$ $Dnd1^{Ter}$/+ (129/Sv-p^+ $Tyr^{c\text{-}ch}$ *Ter*/+@Na) (129/Sv-$+^p$ $Tyr^{c\text{-}ch}$ *Ter*/+@Na)	129T1	A^w/A^w $Tyr^{c\text{-}ch}/Tyr^{c\text{-}ch}$	white (or light)-bellied chinchilla
129T2/SvEms (129/SvEms-Ter^+?)	129T2	A^w/A^w $Tyr^{c\text{-}ch}/Tyr^{c\text{-}ch}$	white (or light)-bellied chinchilla
129T2/SvEmsJ (129/SvEms-Ter^+?/J)	129T2	A^w/A^w $Tyr^{c\text{-}ch}/Tyr^{c\text{-}ch}$	white (or light)-bellied chinchilla

URL: http://www.informatics.jax.org/mgihome/nomen/strain_129.shtml

Using various neutrophil depletion protocols, we attempted to tease apart ROS-dependent and ROS-independent neutrophil functions during the innate immune response to *L. pneumophila* lung infection. All three depletion approaches, namely the i.p. injection of either α-Ly6G, or α-Gr-1, or α-Ly6G and α-G-CSF, resulted in a delay in the clearance of *L. pneumophila* from the BALF of WT mice, suggesting that each approach was able to reduce if not eliminate neutrophil function. However these differences, especially with the first two approaches, were relatively small and inconsistent, which as previously mentioned might indicate that depletion might not have been entirely effective. This may be due to residual Ly6G$^-$ Ly6C-intermediate, MPO$^+$, CD11b$^+$ cells, which could represent neutrophils that have been coated with Ly6G, or

due to compensatory cells. However, it could also be a reflection of the fact that day 3 may be too early an endpoint for neutrophil depletion experiments. It has been observed by other groups that clearance of *L. pneumophila* in the lung by neutrophils seems to take effect as of 12-24 hours with WT *L. pneumophila* [85] or 2 days p.i. with ΔFlaA [102], and 2 days p.i. with WT *L. pneumophila* in A/J mice [101], though there is no proven mechanistic explanation for why this might be the case. Thus by day 4 or 5 p.i., perhaps a larger difference would have been observed in our experiments. In addition, α-Gr-1 treatment might also damage off target cells such as monocytes, which also express Ly6C, and α-G-CSF might inhibit AM function [129], reducing the specificity of these experimental approaches.

Our data suggest that neutrophils have a dual role in *L. pneumophila* clearance, in that they kill ingested bacteria via ROS-dependent killing, and that they prime AM via TNF. We would thus have expected that the depletion of neutrophils would delay bacterial clearance in $TNF^{-/-}$, MN-TNF $NAIP5^{129S1}$ and $CYBB^{-/-}$ mice, with the greatest relative difference expected between WT depleted and undepleted mice, since both neutrophil functions should be present in WT mice. To some extent, that is what we see with the α-Gr-1 treatment, though we are lacking $TNF^{-/-}$ mice in that experiment (Fig. 5B).

Other groups have also observed the presence of "compensatory" cells in *L. pneumophila* infected $IL\text{-}1R^{-/-}$ mice, which resembled AM or were CD45-negative/low [85]. They speculated that these cells might be damaged cells, which are normally phagocytosed and eliminated by neutrophils. The fact that they used $IL\text{-}1R^{-/-}$ mice rather than Ab depletion demonstrates that "additional" cells arise in the BALF in the absence of neutrophils during *L. pneumophila* infection. Thus it is possible that the additional cells observed in our depletion experiments represent damaged cells normally eliminated by neutrophils, neutrophils masked by α-Ly6G or α-Gr-1, or compensatory monocytes or other cells. In summary, our neutrophil depletion studies remain inconclusive as to the relative contribution of ROS and TNF to neutrophil-dependent control of *L. pneumophila* lung infection.

In light of the finding that neutrophil and monocyte-derived TNF mediates an essential AM-driven immune response that can compensate for weak NAIP5-NLRC4-mediated immunity, it is interesting to note that ΔFlaA *L. pneumophila* is able to replicate in WT AM within the first 2 days p.i. [102], as is WT *L. pneumophila* in A/J AM [101], after which bacteria are cleared. These kinetics fit with the observations that TNF peaks in the BALF 2 days p.i. (Fig. 14, [141]), that macrophages require pre-activation of around 20 hours with TNF before they become

restrictive for *L. pneumophila* replication (Fig. 11A, [130]), and that failure to recruit neutrophils to the lung from 12 hours up to around 2 days p.i. does not greatly impact bacterial burden, though these kinetics may vary with the size of the inoculum [85,102]. Also consistent with a need for neutrophil-derived TNF is the observation that clearance of ΔFlaA *L. pneumophila* is delayed to 72 hours p.i. in IL1R$^{-/-}$ mice, in which neutrophil recruitment is delayed, and that in MyD88$^{-/-}$ mice clearance is postponed to 6 days p.i., or even abrogated [102]. Since MyD88$^{-/-}$ BMDM fail to secrete TNF in response to *L. pneumophila* (Fig.8, [130,148], and neutrophils secrete TNF in a flagellin-independent manner (Fig. 4B, [74]), it seems highly likely that impaired TNF production by neutrophils and monocytes contributes to the striking susceptibility of MyD88$^{-/-}$ mice to *L. pneumophila* lung infection.

The fact that AM do not produce much TNF in response to *L. pneumophila* infection but instead rely mostly on neutrophils and monocytes, which must first be recruited to the airways to produce TNF, likely reflects a mechanism which limits overzealous lung inflammation. Indeed, TNF is a very potent cytokine, and it's leakage from the airspace to the circulation can on its own strongly contribute to anaphylactic shock, as shown by systemic anti-TNF treatment in a rabbit model of *Pseudomonas aeruginosa* pneumonia and other infection models [149-151]. Congruent with this idea, though neutrophils are essential for the resolution of *L. pneumophila* lung infection they are also associated with lung pathology in Legionnaires' disease [152,153]. This may in part be due to their role in TNF secretion.

Our CBA data measuring cytokines in the BALF of WT and MN-TNF NAIP5^{129S1} mice suggest that TNF from macrophages, monocytes and / or neutrophils has an impact in enhancing IL-1α, IL-1β and IL-6 in the BALF of *L. pneumophila* infected mice (Fig. 14). Since MN-TNF NAIP5^{129S1} BMDM also produce very little IL-6 in response to *L. pneumophila* infection, it appears that BMDM have a cell intrinsic requirement for TNF in order to secrete IL-6 in response to *L. pneumophila* (Fig. 15). However, these data should be interpreted cautiously, as it is possible that hypofunctional NAIP5 signaling as well as differences in the genetic background of MN-TNF NAIP5^{129S1} and C57BL/6 mice might account for these differences. In order to confirm that TNF from macrophages, monocytes and / or neutrophils is required for normal levels of IL-6 in response to *L. pneumophila* lung infection, experiments could be conducted using conditional TNF knockout mice on a pure C57BL/6 background, or one could use anti-TNF antibodies. By 36 hours p.i. MN-TNF NAIP5^{129S1} mice appear to have normal levels of IL-6, indicating that this defect is transient, at least until 5 days p.i.. However, given that MN-TNF NAIP5^{129S1} mice have higher bacterial loads than WT mice, IL-6 levels equal to

those of WT mice might still be indicative of impairment. The early reduction of IL-6 correlates well with the early reduction of IL-1α and IL-1β, which fits with recently published data showing that IL-1α and IL-1β induce TNF and IL-6 in non-infected cells in response to *L. pneumophila* infection [154]. Thus it is possible that both NAIP5 and TNF contribute to the induction of IL-1α and IL-1β, and in their absence or impairment, IL-6 production in response to *L. pneumophila* is reduced.

IL-6 has been implicated in immune defense against Legionnaires' disease. There has been a case study in which a patient receiving anti-IL-6 therapy for rheumatoid arthritis succumbed to Legionnaires' disease [155], and both mice and humans produce IL-6 in response to *L. pneumophila* lung infection [154,156-158]. Furthermore, evidence from experiments with IL-6 deficient mice suggest that IL-6 is important for immune defense against *S. pneumoniae* pneumonia [159]. However the extent of involvement of IL-6 in *L. pneumophila* lung infection is not yet known, and neither is the mechanism by which it acts.

We also show that neutrophils kill *L. pneumophila* in the lung directly by ROS-dependent mechanisms. Interestingly, AM do not produce ROS in response to WT *L. pneumophila*. This is in line with a study demonstrating that *L. pneumophila* actively represses ROS in AM by a T4SS-dependent mechanism [128] and our observation that AM produce ROS in response to ΔT but not much ROS in response to WT *L. pneumophila* (Fig. 4B). Why this mechanism is not active in neutrophils remains unclear, given that both neutrophils and AM are targeted by the T4SS and harbor live *L. pneumophila in vivo* (Fig. 3A, [74]). In fact, for neutrophils the opposite is true, as our results show that ROS induction in neutrophils is T4SS-dependent. On a similar note, a recent study has shown differential responses between macrophages and neutrophils to *Salmonella* flagellin, in that NAIP5-NLRC4 triggered pyroptosis in macrophages but not neutrophils [160]. How *L. pneumophila* adapts to these two different intracellular environments also remains unknown. The study of the differential activation of neutrophils and AM by *L. pneumophila* will likely yield interesting insights into these cell specific host-pathogen interactions in future investigations.

It is intriguing that *L. pneumophila* has at least 7 effectors which block host cell translation, and yet T4SS effectors still trigger a robust proinflammatory response via the host cell's detection of "pathogen associated activity" [83-85,97]. However this paradoxical situation has recently become more clear in light of a study which has shown that in spite of host translation blockage, IL-1α and IL-1β are still released by infected cells via an MyD88-dependent

mechanism [154]. IL-1 then acts on uninfected bystander cells which release pro-inflammatory cytokines including TNF, initiating a potent innate immune response [154]. This turn of events is perhaps a reflection of the fact that though *L. pneumophila* is well equipped to manipulate single cell organisms, it is not well adapted to complex metazoans with advanced immune systems. In line with this thinking, we have shown that TNF contributes to the control of *L. pneumophila* infection both *in vitro* and *in vivo*.

Legionella is able to survive or even replicate in a multitude of diverse host cells, including amoeba, ciliates, nematode cells and human phagocytes [61,91]. In the course of my studies of *L. pneumophila* in macrophages and neutrophils, I made observations consistent with the hypothesis that *L. pneumophila* might differentiate into motile cyste-like MIFs in neutrophils, even though it has been shown not to do so in macrophages [95]. I observed that triple distilled H_2O-mediated lysis of AM seemed to reduce the number of *L. pneumophila* that were culturable on CYE plates more strongly than triple distilled H_2O-mediated lysis of neutrophils, consistent with a more environmentally stable form of *L. pneumophila* present in neutrophils than in AM (data not shown). However, this difference was not observed when AM and neutrophils were lysed with 0.7% Tween-20 in PBS, which presumably does not trigger the viable but non-culturable (VBNC) state in *L. pneumophila*. This may be because PBS is less hypotonic than triple distilled H_2O, and therefore less of a stress. Furthermore, *L. pneumophila* in neutrophils were densely packed into shorter rounder shapes than observed AM, again consistent with MIFs being present in neutrophils (Fig. 16, [94]). Finally, the observation that neutrophils require ROS to kill *L. pneumophila* is also indicative that a robust form of the bacterium is present in these cells (Fig. 3). In order to test this hypothesis, one could use real time quantitative PCR (Q-PCR) to test AM and neutrophils infected with *L. pneumophila* for the expression of genes which are highly upregulated in MIFs, such as MIF-associated gene A (MagA) or superoxide dismutase (SodC) [91,161]. Knowledge of the form *L. pneumophila* assumes in different cells types could provide insight into the biology of the bacterium. In addition, different forms of *L. pneumophila* could potentially have antigenic differences from one another, and might differ in the way they interact with PRRs and antibodies, and differ in susceptibility to various antimicrobial pathways.

In contrast to intranasal *L. pneumophila* infection, TNFR1 and IL1R do not appear to be important for i.v. *L. pneumophila* infection, but instead neutrophil derived-IL18 and NK cell-derived IFNγ play a central role [100,115]. This observation is congruent with the hypothesis that TNF is important for priming AM, and IL-1 is important for recruiting neutrophils to the

lungs, both of which are not required for killing *L. pneumophila* delivered i.v.. However, it is interesting to note that neutrophil depletion (with α-Gr-1 clone NimpR14 or RB6-8C5) did not impact TNF levels in the serum in response to i.v. *L. pneumophila* infection, and that splenic monocytes stained positive for TNF in this model [100]. This suggests that monocytes rather than neutrophils secrete TNF in the blood in response to *L. pneumophila*. The question thus arises as to why neutrophils secrete TNF in the lungs / BALF but not in the blood in response to *L. pneumophila*. This could for instance be due to activating factors present in the BALF but not in the blood, or conversely, due to inhibitory factors present in the blood but not the BALF. Alternatively, physical changes in the neutrophil's environment from circulation to BALF might influence neutrophil behavior, as it was shown that adherent neutrophils signal via JNK in response to LPS, while neutrophils in suspension do not [162]. In addition, *L. pneumophila* is known to be relatively resistant to neutrophil-mediated killing as shown in an *in vitro* system [163], in spite of the fact that we have demonstrated that neutrophils kill *L. pneumophila* in the lung via ROS-dependent mechanisms. Though it is not known, to my knowledge, if neutrophils kill *L. pneumophila* in the circulation, it would also be interesting to determine if certain factors prevent neutrophils from killing *L. pneumophila* in the blood, or license them to kill *L. pneumophila* in the BALF. In summary, consideration of intranasal and i.v. infection data suggest that mechanisms that boost TNF production and neutrophil-mediated killing in the lung could be beneficial to treat patients with *L. pneumophila* pneumonia, while patients who progress into septic stages of Legionaires' disease could benefit from additional measures to augment NK cell-derived IFNγ.

Besides neutrophils, monocytes are also recruited to the lungs and airspaces in significant numbers upon *L. pneumophila* lung infection [98]. Human monocytes have been shown to phagocytose *L. pneumophila* via type 1 complement receptor 1 (CR1) and type 3 complement receptor (CR3) [164], and can be activated to kill *L. pneumophila* via IFNγ [165], indicating that monocytes have the potential to limit *L. pneumophila* infection. As mentioned in the results section, our experiment with CCR2-DTR mice potentially implicates inflammatory monocytes in the innate immune defense against *L. pneumophila* lung infection, while our experiment with CCR2$^{-/-}$ mice does not (Fig. 13). However, since DT might deplete AM in CCR2-DTR mice, and CCR2$^{-/-}$ mice might have compensatory mechanisms, it is difficult to draw conclusions from these results at this time. It would therefore be beneficial to determine if DT does in fact deplete AM in CCR2-DTR mice, by repeating the intranasal infection experiment with WT and CCR2-DTR mice, with or without treatment with DT, and with or without infection with

WT *L. pneumophila*. If uninfected CCR2-DTR mice treated with DT are depleted of AM, it will be clear that AM depletion is a direct effect of DT in these mice. If this is in fact the case, perhaps the dose of DT could be titrated down in order to deplete monocytes but not AM. In any case it would be beneficial to establish if AM are depleted upon DT treatment of these mice since they are used to determine the involvement of monocytes also in other infection models [166]. As an alternative to CCR2-DTR mice, DT treatment of CD11b-DTR mice, systemic liposomal clodronate administration and antibody-dependent ablation of $CCR2^{hi}$ cells could be employed to evaluate the involvement of monocytes in *L. pneumophila* lung infection, and reportedly do not affect AM [166].

In this thesis, we show that the TNF-mediated antibacterial mechanism in AM is dependent on cathepsin B and the rerouting of *L. pneumophila* to lysosomal compartments, where they are degraded via processes that involve acidification. This acidification likely occurs early in the infection cycle, since fusion of LCVs and lysosomes can be observed within an hour of infection in BMDM pre-treated with TNF. Consistent with this idea, a previous study found that the T4SS effector SidK inhibits the v-ATPase, and is highly induced upon dilution of stationary bacteria in fresh medium [81]. The observation that bafilomycin A1 alone reduced *L. pneumophila* replication in BMDM is expected, since *L. pneumophila* require acidification of the LCV in late stages of infection for proper LCV maturation [82]. However, how cathepsin B contributes to the control of *L. pneumophila* remains unclear. It is possible that cathepsin B directly degrades *L. pneumophila* in lysosomes, or has an indirect effect such as the activation of other effector molecules [167]. In fact, cathepsin B has been implicated in mechanisms as diverse as the degradation of Rip1 kinase and TLR signaling [167,168]. Interestingly, a recent study with *Chlamydia muridarum*, another pathogen that blocks phagosomal maturation and replicates in macrophages, also found a bactericidal role for cathepsin B [169]. Though no connection to TNF was made, this suggests that this mechanism might be broadly applicable to intracellular pathogens that block phagosomal acidification. Since inhibition of cathepsin B did not completely block the TNF-mediated restriction of *L. pneumophila* growth, inhibition was either incomplete, or part of this restriction is independent of cathepsin B. Of note, given that Z-VAD-FMK caused a block in TNF-mediated suppression of *L. pneumophila* replication in BMDM, further experiments will be required to determine if other caspases are involved in this TNF-mediated effect. For instance, the pan caspase inhibitor Q-VD-OPh could be used in a growth curve assay, with or without rTNF, since Q-VD-OPh does not inhibit cathepsins

[131]. Further study of this TNF-induced bactericidal mechanism is needed to assess its impact on the control of other intracellular pathogens.

It is well recognized that in murine models of *L. pneumophila* infection, bacterial replication is heavily restricted by flagellin-NAIP5-NLRC4-mediated mechanisms in AM, while in humans this mechanism is absent due to poor inflammasome induction by hNAIP, which instead detects type 3 secretion system (T3SS) needle proteins [32,170]. In this work, we highlight that neutrophil and monocyte-derived TNF in combination with ROS are essential effectors in the innate immune response to *L. pneumophila* lung infection, especially in the absence of inflammasome induction. This suggests that TNF and ROS are the key effectors mediating clearance of *L. pneumophila* in the human lung, and perhaps also of other intracellular bacteria lacking a T3SS.

Materials and Methods

Mice and *L. pneumophila* infections

All mice used in this study were bred at the Swiss Federal Institute of Technology Zürich or purchased (Janvier Labs, Le Genest Saint Isle, France) and used at 6–20 weeks of age (age- and sex-matched within experiments). All mice were backcrossed on the C57BL/6 background. This study was conducted in accordance to the guidelines of the animal experimentation law (SR 455.163; TVV) of the Swiss Federal Government. The protocols were approved by the Cantonal Veterinary Office of the canton Zurich, Switzerland (permit number 125/2012). MemTNF KI mice and MN-TNF $NAIP5^{129S1}$ mice have been previously described [122,171]. Sequencing revealed the same mutations in 129S1 NAIP5 ($NAIP5^{129S1}$) as previously described [119], with the exception of the two mutations in exon 15, which matched the C57Bl/6 DNA sequence. Bone marrow chimeric mice were generated as described previously [98], reconstituting with a total of 5 x 10^6 bone marrow cells and allowing at least 8 weeks for reconstitution of lethally irradiated $Ly5.1^+$ WT recipient mice.

The *L. pneumophila* strains used in this study were the wildtype strain JR32 (Philadelphia-1) [172], as well as modifications of JR32 including an aflagellated mutant (ΔFlaA) [173], JR32-GFP [174], JR32-GFPind (pGS-GFP-04) [175], a deletion mutant lacking a functional Icm/Dot T4SS (ΔT) [176], and ΔT-GFP [174]. *L. pneumophila* was grown for 3 days at 37°C on charcoal yeast extract (CYE) agar plates before use, with chloramphenicol (5 mg/ml) added for selection of strains containing GFP-encoding plasmids.

For intranasal (i.n.) infections mice were anesthetized with an i.p. injection of 5 mg xylazine/100 mg ketamine per gram body weight, and 5 x 10^6 CFU *L. pneumophila* (unless otherwise specified) resuspended in 20 µl PBS were directly applied to one nostril using a Gilson pipette. Bacterial titers in bronchoalveolar lavage fluid (BALF) were determined by plating serial dilutions in PBS on CYE plates. For quantification of CFU from sorted AM and neutrophils, cells were lysed to release viable *L. pneumophila* by vortexing 30 seconds in 1 ml PBS with 0.7% Tween 20 prior to plating serial dilutions in PBS on CYE plates.

In vitro L. pneumophila infection of BMDM

Bone marrow-derived macrophages (BMDM) were generated by plating bone marrow in L929 conditioned medium containing M-CSF in 5 cm diameter non-cell culture treated Petri dishes as described previously [98]. On day 7, BMDM were harvested in ice cold PBS, 5% FBS, 2.5 mM EDTA by incubating 12 min in the fridge and resuspending by pipetting. The cells were

then seeded at 1 x 10^5 cells/well in 96-well plates and rested overnight prior to infection. *L. pneumophila* used for infection was grown for 3 days at 37°C on CYE agar plates, then inoculated in ACES yeast extract medium at an OD600 of 0.1 and grown for 21 h at 37°C before use, with chloramphenicol (5 mg/ml) added to maintain plasmids. BMDM were infected at MOI 0.1, infection was synchronized by centrifugation, and the cells were incubated for 3 days at 37°C, 5% CO_2. Intra- and extracellular CFU were quantified on day 3 by plating on CYE plates after a 10 min incubation of BMDM in dH_2O to lyse them. Where indicated, 100 ng/ml rTNF, 20 nM V-ATPase inhibitor bafilomycin A1 (Enzo Life Sciences, BML-CM110-0100), 25 µM cathepsin B inhibitor CA-074-Me (Enzo Life Sciences, BML-PI126-0001), 25 µM cathepsin D inhibitor pepstatin A (Enzo Life Sciences, ALX-260-085-M005), 50 µM caspase 3 inhibitor Ac-DEVD-CHO (Enzo Life Sciences, ALX-260-030-M001), 50 µM caspase 3/7 inhibitor Z-DEVD-FMK (Enzo Life Sciences, ALX-260-141-R020), 50 µM caspase 8 inhibitor Z-IETD-FMK (Enzo Life Sciences, ALX-260-144-R020), 50 µM caspase 9 inhibitor Z-LEHD-FMK (Enzo Life Sciences, ALX-260-145-R020) or 20 µM pan-caspase inhibitor Z-VAD-FMK (Enzo Life Sciences, ALX-260-020-M001) were added 15 min prior to infection.

***In vitro L. pneumophila* infection of autophagy deficient cell line**

Immortalized autophagy deficient macrophage cell line M^- was previously generated by Eicke Latz (generously provided by Prof. Dr. Christian Münz) from Atg5fl/fl mice treated with TAT-cre to eliminate Atg5. An untreated Atg5 sufficient control cell line (M^+) was also generated. Cells were cultured in high glucose DMEM with 10% FBS and 100 mM sodium pyruvate. On day -1, M^+ and M^- cells were trypsinated to remove them from T75 flasks, and were seeded in 96-well plates at 1x10^4 cell/well. On day 0 they were infected with WT *L. pneumophila* or ΔFlaA *L. pneumophila* at MOI 1, infection was synchronized by centrifugation, and the cells were incubated for 3 days at 37°C, 5% CO_2. Intra- and extracellular CFU were quantified on day 3 by plating on CYE plates after a 10 min incubation of M^+/M^- cells in dH_2O to lyse them. Where indicated, 100 ng/ml rTNF were added 15 min prior to infection.

Neutrophil depletion experiments

Neutrophils were depleted in WT, $TNF^{-/-}$, MN-TNF $NAIP5^{129S1}$ and $CYBB^{-/-}$ mice, infected with 5x10^6 CFU WT *L. pneumophila*, by 3 different methods. In the first, 500 µg α-Ly6G (clone 1A8, Bio X cell, BE0075.1) were injected i.p. on day -1 and day 1, where day 0 is defined as the day of infection. In the second, 100 µg α-Gr-1 (clone NIMP-R14, Lucerna Chem

AG, ab2557) were injected i.p. on day -1, 0, 1 and 2. In the third, 250 µg α-Ly6G (clone 1A8) i.p. on day -1, and 10 µg α-G-CSF (clone 67604, Bio- Techne AG, MAB414) i.p. on day -1, 0, 1 and 2. CFU in the BALF were quantified on CYE plates 3 days p.i..

DT treatment of CCR2-DTR mice

10 ng/g DT was injected i.p. in WT or CCR2-DTR mice, infected with $5x10^6$ CFU WT *L. pneumophila*, on day -1, 1 and 3 (diluted from 1 µg/µl frozen stock). CFU in the BALF were quantified on CYE plates 5 days p.i..

Microscopy experiments

BMDM were seeded in 24-well plates containing 0.01% polylysine solution (Sigma P4707) coated 12 mm cover glasses (Faust 6080181) at 2.5 x 10^5 cells/well and rested overnight. Where indicated 100 ng/ml TNF (Peprotech 315-01A) or 200 U/ml IFNγ was added to pre-activate the BMDM. Cells were infected with *L. pneumophila*-GFP as described above at MOI 5 for 1 or 4 hours at 37°C, 5% CO_2. For the final 30 minutes of incubation 1µM lysotracker Red DND-99 (Life Technologies, L7528) and 0.5 µg/ml Cholera toxin B AF647 (CTB-AF647, Life Technologies, C34778) were added to the cells. Cells were then washed with 1 ml PBS, and cover glasses were then placed on parafilm, and fixed 5-10 min at RT with 200 µl 4% PFA in PBS. Cells were washed 3 times with 200 µl PBS, incubating 2 min after applying each wash. Cover glasses were dipped in dH_2O, blotted on paper towel to remove excess water and mounted on glass slides with cells facing downwards with 6 µl Mowiol (VWR, 475904-100). BMDM were then analyzed by confocal microscopy.

Antibodies and Flow cytometry

BALF was recovered from mice at the specified timepoint in 1 ml sterile PBS containing 5 mM EDTA as previously described (23150417). Cells were surface stained 30 min in cold FACS buffer (PBS with 2.5% FBS, 5 mM EDTA) with Siglec-F (clone E50-2440, Biolegend), CD11c (clone N418, Biolegend), Ly6G (clone 1A8, BD Biosciences), Ly6C (clone AL-21, BD Biosciences, Allschwil, Switzerland), CD11b (clone M1/70, Biolegend), CD45.1 (clone A20, BD Biosciences), CD45.2 (clone 104, BD Biosciences).

For intracellular staining of TNF (clone MP6-XT22, Biolegend), mice were injected i.p. with 50 µl of 5 mg/ml Brefeldin A in EtOH (diluted with 100 µl PBS) 3 hours prior to taking BALF. Lavage was performed with PBS containing 5 µg/ml Brefeldin A, and was immediately placed on ice. After surface stain, cells were washed with FACS buffer and fixed, permeabilized and

stained using the BD Biosciences Cytofix/Cytoperm Kit according to the manufacturer's instructions. Data were acquired on an LSRII (BD Biosciences) and analyzed with FlowJo software (TreeStar, Ashland, OR). An Aria III instrument (BD Biosciences) was used for cell sorting.

ROS assay

ROS was stained in BALF cells by collecting BALF as usual in 1 ml PBS 5 mM EDTA, washing with 2 ml RPMI 10% FBS at RT, and staining with 60 µM Dihydroethidium (Sigma, D7008) for 1 hour at 37°C, 5% CO_2. For a po145sitive control, cells were stimulated with PMA/ionomycin. Cells were then washed in 2 ml cold FACS buffer and stained as usual with fluorescence-labeled Abs. Data were acquired on an LSRII (BD Biosciences), Dihydroethidium was measured in the FITC channel.

CBA assay

BD Bioscience CBA kit reagents were used to detect TNF (558299), IFNγ (558296), IL-1α (560157), IL-1β (560232), IL-12p70 (558303) and IL-6 (558301) in BMDM supernatant and BALF fluid. Lyophilized standards were diluted in assay diluent, so that the top standard had a concentration of 5 ng/ml. The standards were titrated by two-fold serial dilution including an 11th tube with assay diluent only as a blank. 25 µl of each bead mix were added to a V-bottom plate (0.5 µl / bead type). Diluent volumes were calculated as per kit instructions. 25 µl sample or standard was added to bead mix, and incubated 1 hr at room temperature. 25 µl of detection reagent was added to each well and incubated for 1 hr at room temperature. After washing twice with 150 µl wash buffer, the samples were analyzed using an LSRII (BD Biosciences) as per manufacturer's instructions, and analyzed with FlowJo software (TreeStar, Ashland, OR).

Statistical analysis

Non-parametric tests, including the Kruskal-Wallis test with Dunn's post test, the Mann-Whitney test, or in the case of paired samples, the Wilcoxon test, were applied for statistical analysis using Prism GraphPad software (La Jolla, CA).

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