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DISSERTATION

Titel der Dissertation

The role of Interferons and their signal transducers in *Listeria monocytogenes* infection of mice

Verfasserin

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Abstract

Interferons (IFN) are important cytokines for the immune response to many pathogens. The signal transducer and activator of transcription (Stat1) is the central mediator of IFN responses downstream of the interferon gamma receptor (IFNGR) and type I interferon receptor (Ifnar). Its deficiency is associated with increased susceptibility to bacterial and viral infections in humans and mice. Due to the remarkable sensitivity of Stat1-/- animals to infection with viral and nonviral pathogens many aspects of Stat1 function in the immune system are not readily amenable to experimental studies.

Listeria monocytogenes is a human pathogen causing mild to life threatening disease, due to its ability to cross several host barriers including epithelial layers and to infect a variety of cells.

In the first part of my PhD thesis, cell type specific ablation of the Stat1 gene was used to determine effects of Stat1 during innate and adaptive immune responses. We report an essential role of Stat1 for macrophage activation and increased resistance during the innate immune response to *Listeria monocytogenes*. Conversely, Stat1 signaling in T cells reduces innate resistance against Listeria monocytogenes. During secondary, acquired immune responses both T cell and dendritic cell Stat1 play protective roles, whereas macrophage Stat1 is dispensable. Stat1 in dendritic cells shapes the adaptive immune response not only by activating effector T cells, but also by influencing the ability of DC to control the proliferation of regulatory T cells. Our study further reveals important functions of Stat1 in the regulation of chemokine production, hence of cellular influxes to local inflammatory reactions..

The second part of the PhD thesis provides clear evidence that the route of infection with *Listeria monocytogenes* determines the effect of type I interferons (IFN-I) on innate resistance to *Listeria monocytogenes* infection. Whereas after intraperitoneal infection IFN-I exhibit detrimental activity towards the host, this effect was not observed after intragastric administration of *Listeria monocytogenes*, which represents the natural infection route. Intragastric infections with natural *Listeria monocytogenes* isolates are extremely inefficient because of the incompatibility of the receptor-ligand system important for uptake to epithelial cells. To overcome this problem we constructed and used a murinized *Listeria monocytogenes* strain expressing mutant Internalin A that interacts with high affinity with its E-cadherin receptor on epithelial cell surfaces, thus

promoting robust infection. Using this strain for intragastric administration of *Listeria monocytogenes* we observed a protective effect of IFN-I reflected in an enhanced susceptibility of Ifnar1-/- animals. Concomitantly, lack of the Ifnar resulted in a delayed proinflammatory immune response, which might contribute to the enhanced susceptibility of Ifnar1-/- mice to intragastric *Listeria monocytogenes* infections.

In the third part of my PhD thesis the cell type responsible for IFN-I production during *Listeria monocytogenes* infection of mice was identified. Several gene-targeted mouse strains were used to identify the transcription factors important for IFN-I production during *Listeria monocytogenes* infection. We could show that myeloid cells residing in the spleen are responsible for the majority of IFN-I produced during innate responses to *Listeria monocytogenes*.

Taken together the results presented in my thesis elucidate the delicate role of IFNs and their signal transducer Stat1 in different cell populations and in different stages of infection of mice with the pathogen *Listeria monocytogenes*. They provide clear evidence that important facets of the innate response are critically influenced by the route of infection, i.e. the initial confrontation of host and pathogen.

Zusammenfassung

Interferone sind wichtige Zytokine für die antivirale und antibakterielle Antwort des Immunsystems. Interferone binden an spezifische Zellrezeptoren, wie den Interferon alpha/beta oder gamma Rezeptor und lösen dabei Stat1 vermittelte Signaltransduktionsprozesse aus. Bei Abwesenheit von Stat1 in folge von Mutationen erhöht sich bei Mäusen und Menschen in gleicher Weise die Empfindlichkeit gegen virale und bakterielle Infektionen.

Listeria monocytogenes ist ein Gram positives, fakultativ intrazelluläres Bakterium, welches leichte bis schwere Erkrankungen in Menschen auslösen kann. Mehrere Virulenzfaktoren ermöglichen die Invasion und die Verbreitung von *Listeria monocytogenes* im Wirtsorganismus.

Im ersten Teil der vorliegenden Doktorarbeit wurde der Einfluss von Stat1 auf verschiedene Zellen des Immunsystems während unterschiedlicher Phasen der Immunreaktion gegen das Pathogen untersucht. Dafür wurden Mäuse mit Stat1 Defizienz in Makrophagen, dendritischen Zellen oder T Zellen verwendet. Durch die durchgeführten Experimente konnte festgestellt werden, dass Interferon aktivierte Makrophagen außerordentlich wichtig für das angeborene Immunsystem sind. Im Kontrast dazu haben sie aber eine vernachlässigbare Rolle während der adaptiven Phase der Immunreaktion gegen *Listeria monocytogenes*. Differenzierter ist hingegen das Bild für Dendritische Zellen. Diese haben keinen Einfluss auf die Immunreaktion während der frühen Infektionsphase, sind aber wichtig für die Entstehung einer effizienten adaptiven Antwort, unter anderem da sie die Proliferation von regulatorischen T Zellen beeinflussen. Ein ambivalentes Bild bieten T-Zellen. Während Mäuse ohne T Zell Stat1 resistenter gegenüber *Listeria monocytogenes* Infektionen sind, können nur Mäuse mit intaktem Stat1 in T Zellen eine protektive Immunantwort entwickeln. Des weiteren beeinflusst Stat1 andere infektionsassozierte Prozesse, wie zum Beispiel die Zusammensetzung von zellulären Infiltraten zu Entzündungreaktionen.

Im zweiten Teil der vorliegenden Dissertation wurde der Einfluss von Typ I Interferonen auf unterschiedliche Infektionsrouten getestet. Die bevorzugte Infektionsroute von *Listeria monocytogenes* auf den Menschen erfolgt über den intragastrische Infektionsweg. Das Mausmodell weist allerdings einige Schwächen auf, diese Schwierigkeit konnte aber mit einem an die Maus adaptierten Stamm überwunden

werden. Dabei wurde das Internalin A der Listerien dahingehend mutiert, dass es besser mit dem Maus E-cadherin an Epithelzellen interagieren kann, wobei eine robuste Infektion der Versuchsmäuse erzielt wurde. Die Ergebnisse der Studie zeigen, dass die gut dokumentierte schädliche Wirkung von Typ I Interferonen nach intraperitonealer Infektion nicht rekapituliert werden. Im Gegensatz zu den bisherigen Ergebnissen scheinen Typ I Interferone eine protektive Wirkung nach dieser Art der Infektion zu besitzen, besonders zwei Tage nach Inokulation der Mäuse. Der Vergleich zwischen intragastrischer und intraperitonealer Infektion zeigt darüber hinaus, dass die Zytokinproduktion der Ifnar1-/- Mäuse nach intragastrischer Infektion unterschiedlich zu jenem nach intraperitonealer Infektion war. Als Schlussfolgerung für die Studie wurde demnach gezogen, dass die Infektionsroute entscheidend für die Rolle der Typ I Interferone während einer *Listeria monocytogenes* Infektion ist.

Ziel des dritten Teils der PhD Thesis war es die Interferon produzierende Zelle *in vivo* nach Infektion mit *Listeria monocytogenes* zu identifizieren. Dafür wurden Mausstämme verwendet, welche defizient für unterschiedliche Proteine sind, die eine Rolle für die Induktion und Produktion von Typ I Interferonen besitzen. Die gewonnen Resultate zeigen, dass myeloide Zellen in der Milz als Hauptproduzenten für Typ I Interferone dienen.

Zusammengefasst konnten wir mit den drei vorliegenden Studien wichtige Aspekte der Interferonbiologie in *Listeria monocytogenes* Infektionen beleuchten. Erstens konnten wir individuellen interferonaktivierten Zellpopulationen eine Rolle in unterschiedlichen Phasen der Immunantwort zu *Listeria monocytogenes* zuordnen. Zweitens konnten wir den Einfluss von dem Infektionsweg auf die Immunantwort auf *Listeria monocytogenes* bestimmen. Und schließlich konnte jener Zelltyp identifiziert werden, welcher *in vivo* für die Interferonproduktion zuständig ist.

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Introduction

1. *Listeria monocytogenes*

Listeria monocytogenes is a Gram positive facultative intracellular rod shaped bacterium of the Firmicutes phylum [1]. It is ubiquitously present due to its ability to live under different environmental conditions, like temperature, acid or salt stress [2]. Listeria colonises humans, animals and decaying vegetation in soil [3] and can cause mild to life threatening disease in humans. Its capacity to withstand most procedures used to limit bacterial growth on food products makes Lm a potential problem in the food industry [2].

Seven family members of the genus Listeria are described at present, *L. monocytogenes, L. ivanovii, L. welshimeri, L. seeligeri, L. innocua, L. grayi, L. marthii, L. recourtiae* [4]. The pathogenic species for humans and ruminants are *L. monocytogenes* and *L. ivanovii,* respectively [5].

L. monocytogenes (Lm) is usually transmitted to humans via ingestion of contaminated food. In immunocompetent individuals a mild, non-invasive form of listeriosis can develop which manifests as a febrile gastroenteritis or remains asymptomatic. However, in case of immunocompromised individuals Lm can cause invasive listeriosis. Lm can gain access to the brain causing meningoencephalitis and it may cause sepsis which ultimately leads to death [6]. In case these severe forms of disease manifest, listeriosis has high mortality rates. Additionaly, Lm has the ability to cross the feto-placental barrier and infects the fetus which may cause abortion. Newborns may also be infected during traversion of a colonised birth canal [6, 7].

Treatment of listeriosis requires antibiotics which penetrate cells and are stable intracellularly. Penicillin and Ampicillin are often chosen as no resistance against those antibiotics have been reported [7].

1.1 Listeria monocytogenes life cycle

During infection of hosts Lm encounters various different environmental conditions and the pathogen possesses the ability to respond to these challenges with the help of different virulence factors.

After ingestion with contaminated food Lm arrives at a first barrier in the gastrointestinal tract. There, Lm has to adapt to low pH, bile and osmotic stress. Under these stress conditions a stress response transcription factor sigma factor B (σ^B) has to be activated [5]. σ^B turns on transcription of stress response genes and virulence genes, which enable Lm to withstand the hostile environment of the gastrointestinal tract. The next step in the infectious process is the attachment to epithelial cells and mucus. Another transcription factor, prfA (see 1.2.1) is responsible for the activation of virulence genes required for host invasion and intracellular replication. Attachment to epithelia is accomplished with the help of different internalins (see 1.2.2). Interaction of internalins with their respective receptor on epithelial cells leads to the uptake of the pathogen into cells. Lm uses the “zippering” uptake mechanism which employs actin rearrangments and receptor clustering [8]. Recently also clathrin-mediated endocytosis and septins were described to be involved in the ingestion of Lm into host cells [9]. With the help of different virulence factors Lm can rapidly evade the endocytic vacuole, which would otherwise become a hostile environment (see 1.2.3). Once in the cytoplasm, Lm replicates and moves randomly using its ability to induce the polymerisation of actin. With the help of actin comets Lm pushes into neighbouring cells where it can lyse the double-walled vacuole and escape to the cytoplasm (see figure 1).

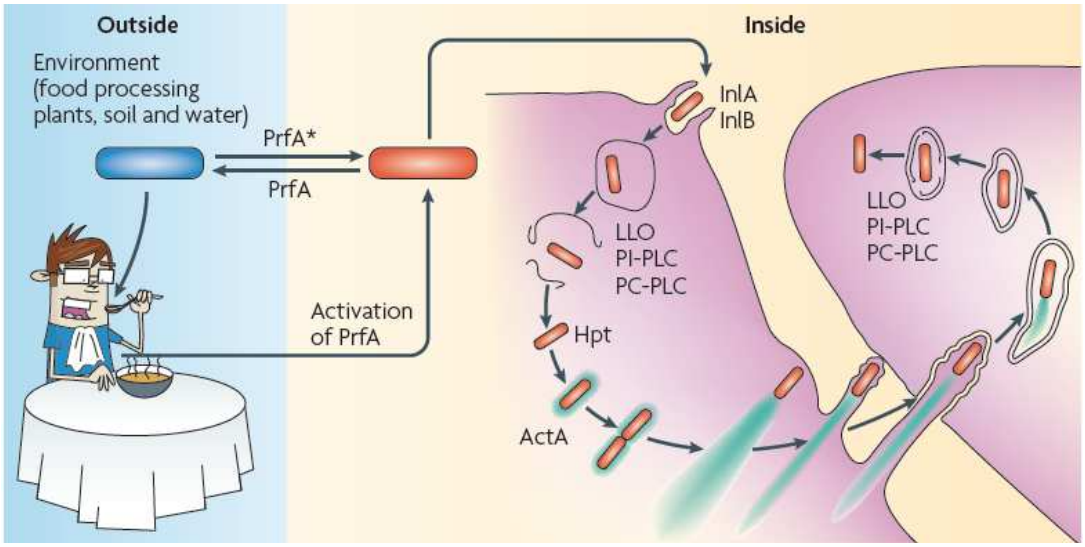

Figure 1: Upon ingestion with contaminated food Lm has to activate a set of virulence genes to adapt to the intracellular life style. Lm has to attach to cells and force its uptake in case of non-phagocytic cells. The pathogen has to escape the formed vacuole by forming pores. In the cytoplasm Lm can replicate and form actin comets which help to push into neighbouring cells. There the lysis of the vacuole and movement using actin tails begins again. From: [2]

The traversion of the first epithelial barrier in the gastrointestinal tract happens either by direct uptake of Lm by epithelial cells or through specialized cells, the M cells. M cells sample the gut lumen for antigens and deliver them to underlying immune structures like Peyer`s patches [10]. The role of M cells for Lm uptake has been investigated by several groups and it has been shown that although Lm can interact with M cells and replicates preferably within Peyer`s patches [11, 12], other uptake mechanisms play an important role for epithelial invasion [13, 14].

Lm colonises the host further by disseminating to liver, spleen and at later stages by infecting also the brain. This can take place by passive spread within immune cells, for example dendritic cells [15] or passage via blood and or lymphatics [1]. In the target organ liver, Lm rapidly infects hepatocytes where it can replicate and which have been shown to be a major niche for Lm in the body [16]. Immune reactions towards Lm in the liver lead to cell influx and in turn to microabscesses [17]. A further niche for Lm is the spleen where entry has been described to be dependent on dendritic cells [18]. Within the spleen however, different phagocytes take up Lm and the cellular localisation changes over time [19] to ensure development of potent immune reactions. In healthy individuals Lm is eradicated with the help of innate and adaptive immune responses (see part 2). In case of a compromised immune system Lm can infect either actively the brain by crossing the blood

brain barrier or passively by travelling within leukocytes. Another way of brain invasion is the retrograde migration along cranial nerves [6] (see figure 2).

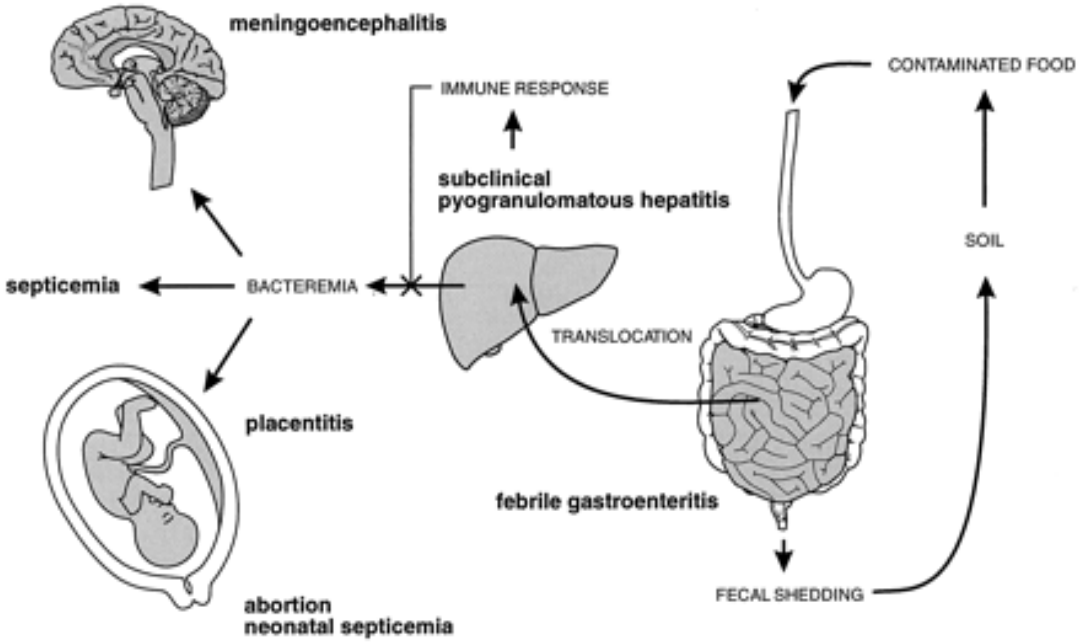

Figure 2: Lm is taken up with contaminated food and has the ability to cross the epithelium of the gastrointestinal tract. In healthy individuals Lm can spread to liver and spleen where a robust immune reaction can stop Lm from further colonisation. Immunocompromised individuals cannot cope with Lm and the pathogen can infect the central nervous system, causing meningoencephalitis, sepsis may develop. In pregnant women Lm can cause fetal abortion. From: [1]

1.2 *Listeria monocytogenes virulence factors*

Lm is armed with a set of virulence factors to achieve the robust infection of various animals and adaptation to different environmental conditions. The virulence factors of Lm are clustered in distinct chromosomal locations, the Listeria pathogenicity islands (LIP) [20]. In the following section the most important virulence factors are described.

1.2.1 PrfA

PrfA is the major transcription factor regulating Lm`s virulence gene expression upon infection of animals and cells. Thereby it switches the lifestyle of Lm from saprophytic to pathogenic [21]. Dimers of PrfA activate transcription of genes involved in uptake into cells (inlA, B), polymerisation of actin (actA), evasion of endosomes and cell to cell spread (hly, plcA, plcB, mpl) and intracellular growth (hpt). PrfA directly activates ten genes but indirectly changes expression of up to 145 genes [2]. PrfA is regulated to be active only upon infection of hosts. This is accomplished by a temperature sensor, a RNA thermoswitch which enables activity at 37°C [22]. Availability of carbon has also been implicated to affect activity of PrfA [23]. Other methods of regulation are riboswitches which bind directly to PrfA (sreA, B) [24] and posttranslational modifications [2].

1.2.2 Internalins

The Lm genome comprises 25 internalins, proteins which are characterised by the presence of leucine-rich repeats (LRRs). Although inlA and inlB are the only family members implicated in the uptake into host cells [25], the whole family was named after this function. InlA and inlB are clustered in one operon and are regulated by prfA.

InlA mediates uptake into epithelial cells via interaction with the host protein E-cadherin [26]. E-cadherin is a transmembrane protein, which mediates calcium dependent cell- cell contact at adherence junctions. It is present at the basolateral side of epithelial cells and not readily accessible for luminal bacteria. A recent report demonstrated that the interaction between InlA and E-cadherin happens at sites of cell exclusion during turnover of epithelial cells [27]. InlA dependent uptake involves action of β-catenin, which links E-cadherin to the actin cytoskeleton and α-catenin, which regulates the dynamics of actin polymerisation. Involved in actin rearrangements is Arp2/3 which is activated through signals coming from the GTPases Cdc42 and Rac. ARHGAP10 and myosin VIIa have

also been shown to be important for InlA mediated entry into epithelial cells [25] (see figure 4).

InlA binds with high affinity to human E-cadherin but only with low affinity to murine E-cadherin due to a single amino acid change. This problem made the mouse a poor experimental system for intragastric infections with Lm and initiated studies in guinea pigs which posses the same amino acid structure of E-cadherin as humans. The mouse, however is one of the best model organisms, therefore mice were created with humanized E-cadherin [28] and Lm were constructed with murinized InlA [29] (see figure 4).

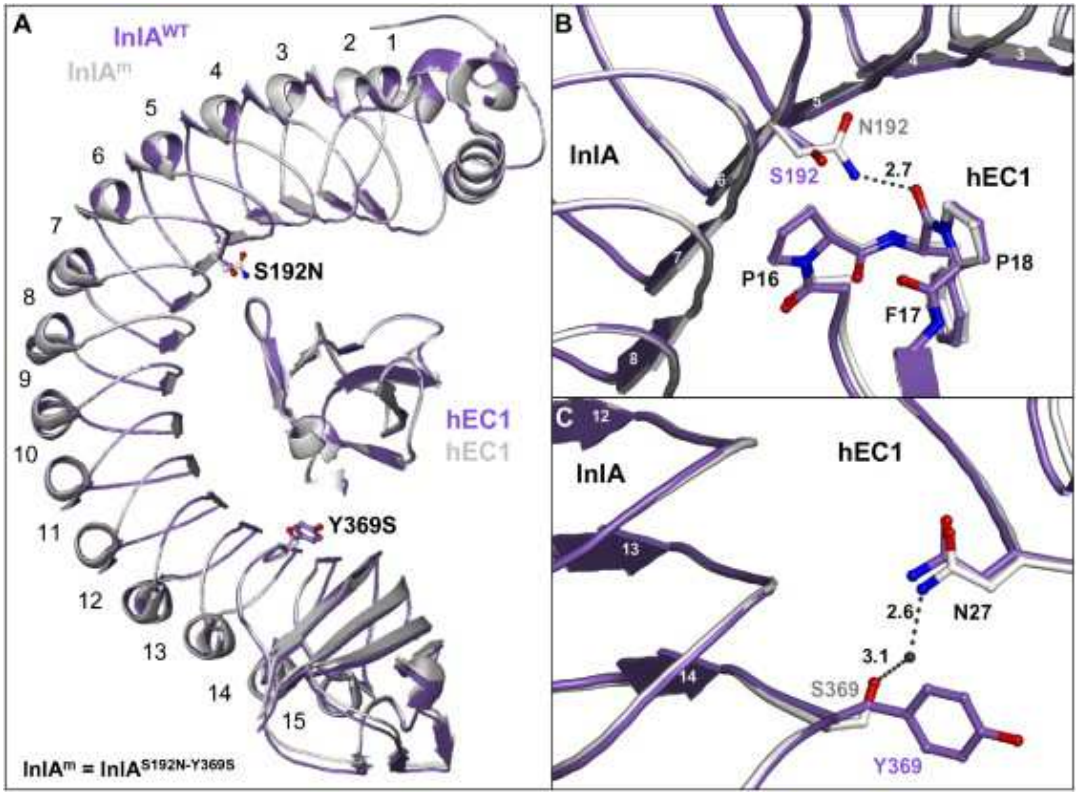

Figure 3: Structure of the interaction between the mutated thereby murinized Internalin A and the human E-cadherin. The point mutations in the mutated Internalin A are highlighted in the right panels. From: {Wollert, 2007 #1305

InlB mediates entry of Lm not only into epithelial cells but also in hepatocytes and endothelial cells. InlB interacts with HGF-R/Met, proteoglycans and gC1q-R and activates thereby signalling events leading to phagocytosis of Lm and several signal transduction pathways including the NFκB, PKC and Ras-MAPK pathways {Bierne, 2007 #1397}. InlB mediated entry is also actin dependent and involves Arp2/3, Rac and WAVE [8]. InlB has been shown to accelerate binding of Lm to intestinal epithelial cells via activation of the Met receptor [30](see figure 4).

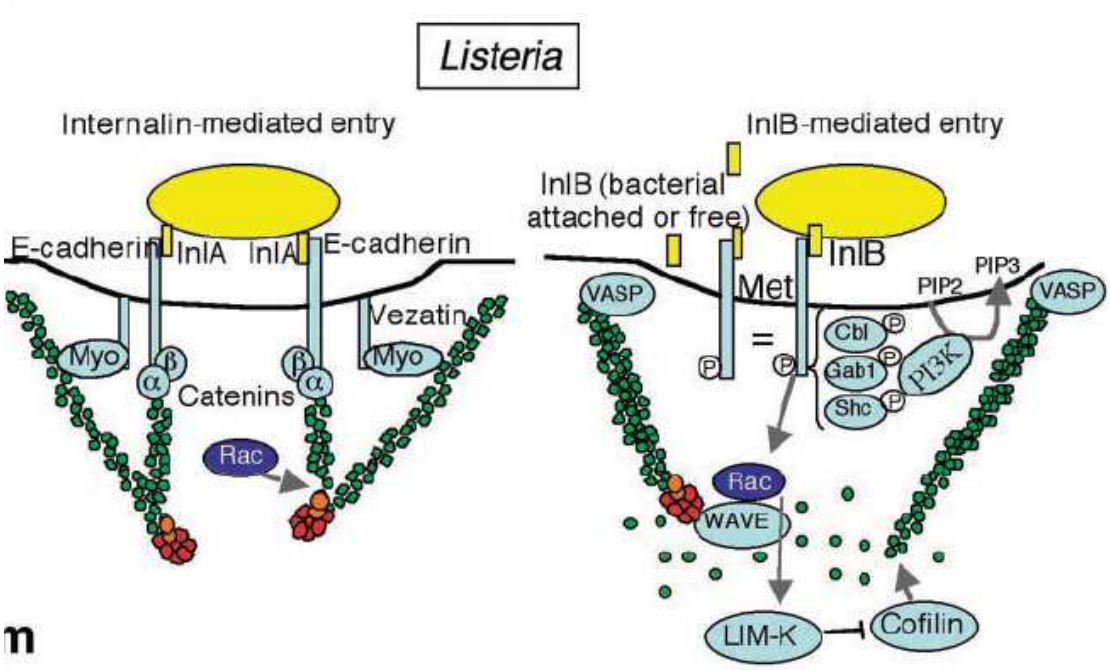

Figure 4: Zipper mechanism of Lm entry into epithelial cells. Upon interaction of InlA with E-cadherin α– and β– catenin link the actin cytoskeleton to the receptor. Signalling via Rac activates actin polymerisation. InlB not only activates Rac mediated actin polymerisation but also leads to activity of PI3 kinase. From: [8]

The functions of other members of the internalin family are not fully elucidated and have only been described recently and not in great detail.

InlC was shown to enhance virulence [31] by disturbing apical cell contacts and promoting cell to cell spread by forming protrusions [32].

InlH is needed to downregulate inflammatory reactions *in vivo*, as infection of mice with Lm lacking InlH are less virulent and mice have higher amounts of interleukin 6 [33]. The cellular source of interleukin 6 (IL6) however needs to be determined as no direct interaction of InlH with IL 6 could be assessed.

InlJ was shown to be robustly expressed *in vivo* during infections but not under steady state conditions, it is believed to act as an adhesin [34].

InlK helps Lm to evade autophagy through its function in the decoration the bacterial surface with a host protein [35].

1.2.3 Listeriolysin

Listeriolysin O (LLO) is the gene product of the hly gene and a major virulence factor of Lm. Lack of LLO makes Lm completely avirulent [36]. LLO is a cholesterol-dependent pore-forming toxin with haemolytic activity [37] which has to oligomerize in order to be able to insert in host membranes and form pores [38]. LLO is essential for Lm to lyse phagosomes as well as secondary vacuoles formed after cell-to-cell spread. LLO is most active at a pH of 5.5 and bacterial escape requires acidification of the vacuoles [39]. Not only the activity of LLO is regulated, transcription of the hly gene is also induced upon intracellular infection [40]. Furthermore LLO has been recognized as a signalling molecule, which stimulates NFκB, calcium signalling, MAPK, interferon γ (IFNγ) and IL1 production in different cell types. Interestingly, LLO itself can cause apoptosis in T cells, dendritic cells and macrophages [39, 41, 42].

Recently listeriolysin S, similar to the known streptolysin S of group A streptococcus, has been shown to be present in Lm strains which are involved in epidemic outbreaks [43]. This second hemolysin of some Lm strains is only active under oxidative stress and contributes to virulence in murine experimental models.

1.2.4 ActA

ActA is essential for intracellular movement of Lm [44, 45]. This molecule resides polarly on the surface of the bacterium and recruits actin filaments [46]. Lm lacking actA are highly attenuated in murine infections. ActA mimics the host cell WAVE protein

which recruits the Arp2/3 complex. A tail of short cross-linked actin filaments is formed which pushes Lm within one cell and also into neighbouring cells [47].

1.2.5 Phospholipases

Lm possesses two phospholipases a phosphatidylinositol-specific phospholipase C (PI-PLC) and a broad-range phosphatidylcholine-preferring PLC (PC-PLC) encoded by plcA and plcB, respectively. These two enzymes support LLO in lysing vacuoles and their concerted action is especially required for lysis of double-walled vacuoles. The exact mode of action is still under investigation [48]. Lm lacking these two partially redundant phospholipases are highly attenuated and are defective in cell-to-cell spread [49].

1.2.6 p60

p60 is the product of the *iap* gene, which is not under control of prfA [1]. Lm abundantly secretes p60, which was demonstrated to be important for septum formation and virulence *in vivo*. Recently it was shown that p60 stimulates natural killer (NK) cell activity [50].

1.2.7 Mpl

Mpl is a zinc-dependent metalloenzyme required for modification of PC-PLC into its active form [51]. Mpl autocatalyse its own processing outside of the bacterium and this event is pH dependent [52].

1.2.8 More virulence factors

More factors like hpt, a hexose phosphate transporter, which mediates the uptake of glucose [53], phosphatases (LipA, STP) [54, 55] and superoxyde dismutases (SOD) [56] have also been shown to be important for virulence of Lm.

Although Lm has been studied for many years the exact role of many virulence factors has not been elucidated. Manipulation of the host via induction of miRNAs [57, 58], changes of SUMOylation of host cells upon infection [59] and changes in mitochondrial dynamics [60] have been discovered only recently and make the infectious process of Lm even more complex.

2. Immune responses to *Listeria monocytogenes*

2.1 Recognition of *Listeria monocytogenes* by the immune system

Lm presents several ligands for pattern recognition receptors (PRR) of the innate immune system [61]. PRRs sense common pathogen-associated molecular patterns (PAMPs) and can be either located at the cell surface sensing extracellular PAMPs, in intracellular compartments like endosomes or in the cytosol [62]. They can be grouped into major families, the toll like receptors (TLR), the Nod like receptors (NLR), the retinoic acid (RIG) like receptors (RLR) and the members of the inflammasome [63]. All TLRs, except TLR3, utilize MyD88 as an adaptor molecule which links the receptor to downstream signalling molecules, which ultimately activate proinflammatory genes, in the case of endosomal TLRs IFN-I, and chemokines via transcription factors NFκB and AP1 [64].

Lipoteichoic acid, peptidoglycan and lipoproteins of Lm are recognised by toll like receptor (TLR) 2. The role of TLR2 in host protection against Lm is controversial. One group has shown that TLR2-/- mice were more susceptible to Lm infection compared to WT animals [65] however, other studies could not determine an important role for TLR2 in host protection [66]. Lipoproteins have been identified as the main TLR2 ligand [67].

Flagellin of Lm is recognised by TLR5, the role of TLR5 recognition *in vivo*, however, is unclear, as Lm deficient in flagellin do not show reduced virulence [68, 69].

The adaptor protein MyD88 which is downstream of most TLRs has been shown to be central to establish protective immunity to Lm. MyD88-/- mice are highly susceptible to Lm infection and are unable to clear the bacteria due to the inability to produce the important cytokines TNFα and IFNγ [66, 70].

DNA of Lm is exposed in endosomes to TLR9 and activates MyD88 dependent gene activation *in vitro*, the importance *in vivo* for host protection however remains to be clarified [71].

Cytosolic receptors like Nod1, Nod2 and proteins of the inflammasome family (Nalp3, Ipaf, Aim2) which recognize peptidoglycan related molecules, muramyl dipeptide flagellin and cytosolic DNA, respectively have also been implicated to be important for recognition of Lm *in vivo* [72, 73]. Ligation of these receptors leads to activation of caspase 1 and subsequent IL1 and IL18 release [74].

Receptors and molecules implicated in the induction of IFN-I are discussed below (see 3.3).

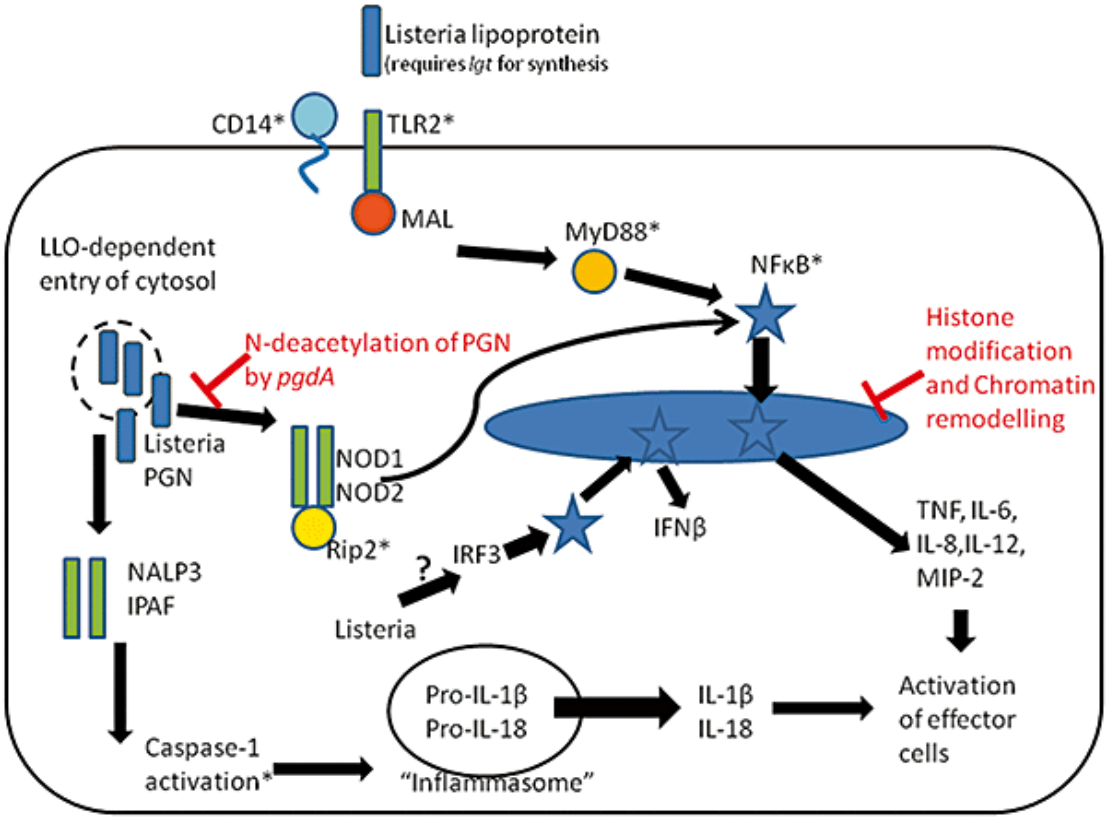

Figure 5: Recognition of Lm by PRRs. Ligation of respective receptors by Lm leads to production of proinflammatory cytokines, chemokines, or IFN-I. From:[75]

2.2 Innate immune reactions to *Listeria monocytogenes*

The innate immune system is important for the clearance of Lm as a first line of defence against invading pathogens. In the following section the most important aspects and cells are described.

The first cells recruited to sites of infection by chemoattractants released by infected cells like hepatocytes are neutrophils. Neutrophils have the ability to produce various proinflammatory cytokines and chemokines to activate surrounding cells participating in the innate response such as macrophages. For a long time neutrophils were described to be important cells of the innate immune defence against Lm, because studies using depleting antibodies against GR1 showed increased susceptibility to Lm infections [76]. The importance of neutrophils for Lm infections has been challenged recently, as a more specific depleting antibody for Ly6G+ neutrophils became available [77Carr, #1593]. The effect seen in earlier studies could be attributed to inflammatory monocytes which express GR1 and which are indeed crucial for innate defence.

Natural killer cells have the potential to recognize and lyse tumours, stressed or infected cells. Additionally, NK cells can produce large amounts of IFNγ. These properties make NK cells important in the immune response against Lm. During the early stage of Lm infection NK cells are the major source of IFNγ [78].

Professional phagocytic cells like macrophages are equipped with a large array of antimicrobial mechanisms. Upon infection these cells try to kill ingested pathogens using reactive oxygen species, reactive nitrogen species and antimicrobial enzymes or peptides, which are produced within phagolysosomes [79]. Detection of Lm by macrophages through PRRs leads to the production of proinflammatory cytokines, most importantly IL12 and TNFα. These cytokines in turn can induce IFNγ production by NK cells, which is extremely important for host protection [80]. Kupffer cells, which are resident macrophages in the liver are responsible for clearance of Lm from the blood stream and for production of IL6, IL12, IL1 and TNFα which in turn activates other cells of the immune system [81]. Monocytes from the blood are attracted to sites of infection through the action of chemokines, mainly CCL2 (MCP1) [82], which are produced by neutrophils. Recruitment of monocytes through CD11b was demonstrated to play a key role in defence against Lm [83]. Resident macrophages of the spleen have been show to be essential for trapping microorganisms from the blood, thereby initiating early immunity to pathogens.

Marginal zone macrophages or marginal zone metallophilic macrophages are, however not implicated in initiation of antigen dependent T cell responses [84].

Dendritic cells are professional antigen-presenting cells and a potent link between innate and adaptive immunity. In Lm infections CD11c+ dendritic cells were demonstrated to be crucial for priming naïve CD8+ T cells for mounting an adaptive immune response [85]. In addition to this important role in adaptive immunity a subset of dendritic cells, named TipDCs were shown capable of producing large amounts of TNFα and iNOS in the spleen during the innate immune response [86]. Dendritic cells located in the spleen are important for clearance of Lm from the blood stream, as several studies showed that the entrance of Lm to the spleen is dependent on the presence of CD8α+ dendritic cells [18, 87].

2.3 Adaptive immune reactions to *Listeria monocytogenes*

Mice lacking cells of the adaptive immune system (SCID mice) are not able to mount sterilizing immunity against Lm. Although the innate immune system can control the infection for the first few days, the adaptive immune system is needed to eradicate the bacteria [88]. CD4+ and CD8+ T cells are pivotal for adaptive immunity to Lm whereas B cells play only a minor role.

CD8+ T cells are potent in killing cells infected with Lm as the most important aspect of the adaptive anti-listeria response [89]. As mentioned above, dendritic cells are the most efficient professional antigen presenting cells to prime naïve CD8+ T cells. Priming of T cells occurs early during Lm infection at day 1-3 [90] and proliferation of antigen specific T cells can also occur independently of presented antigen at later stages of infection [91].

CD4+ T cells also undergo proliferation after Lm infection [92]. The most important role for these cells is the production of Th1 cytokines in response to IL12 [93] and thereby helping to shape the adaptive immune response.

Th1 cells with the signature cytokine IFNγ are the crucial helper T cell lineage in anti Lm immunity. Some reports however, also assign important function to the newly described Th17 cells [94]. Early after Lm infection IL17 producing γδT cells in the liver have been identified which control Lm growth and granuloma formation [95, 96].

Regulatory T cells (Tregs) positive for the transcription factor Foxp3 control the behaviour of autoreactive T cells and try to suppress detrimental T cell responses. In some infection models Tregs were shown to be protective, in Lm infection, however high abundance of Tregs go in line with increased susceptibility to infection. A recent report correlated the increased susceptibility to Lm during pregnancy with increased numbers of Tregs [97, 98].

B cells were thought not to matter for immunity against Lm, a few reports however describe the presence of natural antibodies against Lm [99] and the effectiveness of anti-LLO antibodies during short term infection [100]. The abundance of anti Lm antibodies is very low and the overall importance for immunity is debatable [101].

Complement is responsible for opsonisation of extracellular bacteria and has been shown to increase phagocytosis of Lm by macrophages [102]. However, it is not important for the fight against intracellular Lm. A recent report demonstrates the importance for complement C3 at early timepoints after infection when Lm travels in the blood. There, platelets together with complement direct Lm to splenic CD8α+ dendritic cells and thereby facilitating the uptake of Lm into the immunological organ spleen [103].

3. Interferons

In 1957 Isaacs and Lindenmann identified a factor interfering with virus replication and termed it interferon (IFN) [104]. Since then, a large family of IFNs have been described and different functions are now assigned to the IFNs, which go beyond antiviral activity [105]. IFNs belong to the larger class of type II cytokines, together with IL10, IL19, IL20, IL22, IL24, and IL26 [106].

Three major types of IFNs have been classified, which are discussed in the following section.

3.1 Types of interferon

3.1.1 Type I interferon and activation of interferon stimulated genes

Type I interferons (IFN-I) comprise of IFNα, IFNβ, IFNε, IFNκ and IFNω. All type I IFN exert anti-viral, anti-proliferative activity and stimulate NK and T cells *in vitro* [107], but only IFNα and IFNβ are employed by the immune system for antimicrobial defence. 13 individual IFNαs have been identified, which show distinct activity profiles after infection as a result of different potency of action and different timing and intensity of synthesis. All cells of the body possess the ability to produce and respond to IFN-I as an important anti-pathogenic process [105]. The importance of the IFN-I signalling for host protection could be demonstrated in various viral infections models. Mice deficient for IFNβ or parts of the signal transduction cascade after IFN-I cannot tolerate viral and some bacterial infections [105]. In humans defects of the IFN-I system could similarly be linked to higher susceptibility of viral infections like herpes simplex encephalitis [108].

IFN-I signal via the type I IFN receptor (IFNAR), composed of IFNAR1 1 and IFNAR 2 chains which are associated with, respectively, Jak 1 and Tyk 2, Janus tyrosine kinases (Jaks) for signal transduction [109]. Jak 1 and Tyk 2 phosphorylate receptor docking sites for signal transducer and activator of transcription 1 (Stat) and Stat2. Receptor-bound Stats are phosphorylated by the Jaks on a single tyrosine residue. An activated heterodimer of Stat 1 and 2 translocates to the nucleus where it binds the interferon regulatory factor 9 (IRF9). This heterotrimeric complex is called the interferon stimulating gene factor 3 (ISGF3), which binds to interferon response elements (ISRE) to activate transcription of interferon stimulated genes (ISGs) at [110] (see figure 5).

Over 300 factors belong to the group of ISGs, which interfere with different stages of viral replication within host cells. Important members are the 2`, 5` oligoadenylate syntethase (OAS) and ribonuclease L (RNAseL) which degrade viral and host RNA [111]. The dsRNA protein kinase R (PKR) inhibits the protein synthesis by phosphorylation of the translation initiation factor eIF2 [112]. Other ISGs, p56 and p54 from the IFIT family inhibit the eIF3 by direct binding to its subunits [113]. Members of the MX family interfere with intracellular trafficking of viral polymerases by incorporation into viral nucleocapsids [114]. Other ISGs like TRAIL promote apoptosis of infected cells [115]. Many proteins of the IFN signal transduction pathway are also themselves ISG to amplify the IFN response.

Taken together, IFN-I exert pleitropic functions in the immune system, as they act against viral and bacterial pathogens, and, due to their antiproliferative and sometimes proapoptotic activity, they also contribute to immunesurveillance against tumors. Because of these potential implications in the aforementioned processes, type I IFNs are used in clinical trials as antivirals and anti-cancer drugs and IFNβ is used in treatment of multiple sclerosis [105].

3.1.2 Type II interferon

IFNγ is the sole member of the IFN-II family. This IFN is produced only by specialized cells of the immune system, like NK cells and T cells. By contrast, its receptor is ubiquitously expressed, although at different levels [116]. The IFNγ receptor, consisting of the IFNGR 1 and IFNGR 2 subunits, is associated with the kinases Jak 1 and Jak 2, which phosphorylate Stat 1 homodimers upon activation. Tyrosine phosphorylated Stat 1 homodimers can translocate to the nucleus and bind there to interferon gamma response elements (GAS) and activate transcription (see figure 5). The role of IFNγ in the immune system is rather to establish a proinflammatory status than to act antivirally [117].

The main described target cells of IFNγ are macrophages and T cells. IFNγ activates macrophages to be potent antimicrobial and antitumour effector cells. The cytokine is also responsible for the upregulation of antigen processing and presentation pathways [118]. IFNγ is the central cytokine in Th1 responses of the adaptive immune system, as it represses the development of Th2 or Th17 T cell responses and is therefore crucial for effective cell-mediated immunity [118]. Another effect of IFNγ is the upregulation of chemokines and adhesion molecules, thereby directing cells to sites of

inflammation. IFNγ acts directly on activated B cells to promote class switching from IgG2 to IgG3 [119].

IFNγ is induced by action of cytokines like IL12 or IL18 produced by antigen presenting cells. IFNγ therefore serves as a link between innate and adaptive immunity. Cytokines like IL4, IL10, TGFβ or glucocorticoids were demonstrated to negatively regulate IFNγ induction.

Humans or mice with mutated or no IFNγ or its receptor are highly susceptible to infections, particularly with intracellular bacteria. Furthermore a role for IFNγ in autoimmunity has also been demonstrated. In addition, tumour rejection in mouse models is enhanced by IFNγ [118].

3.1.3 Type III interferon

IFN-IIIs are the newest members of the IFN family, called IFNλ1, IFNλ2, IFNλ3 or IL28a, IL28b and IL29, respectively. These molecules are very similar to IFN-I in their structure and signal transduction. IFNλ can be produced by all cell types, but the receptor is only present on epithelial cells. IFN-III use the IL28R (ILλR) and IL10R2, however the ISGF3 complex is also activated via activation of Jak1, Tyk2 and Stat1/ Stat2 heterodimers [120] (see figure 5). Therefore, the induced genes are very similar to the IFN-I induced genes. In infected cells IFN-I and IFN-III are mostly coexpressed, however IFN-III are present to a lesser extent [121]. The biological activities of IFN-III are highly similar to those of IFN-I.

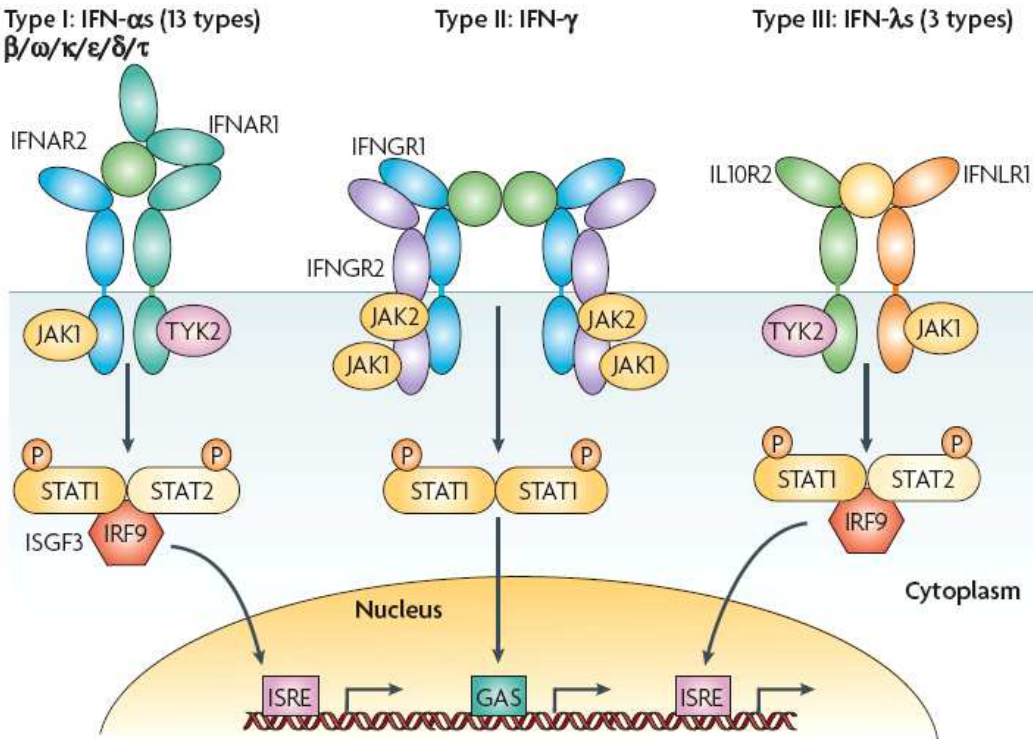

Figure 6: Signal transduction by the IFN receptors. Ligation of receptor chains by the respective IFN causes receptor and STAT phosphorylation by receptor-associated tyrosine kinases JAK1, TYK2 or JAK2, respectively. STAT1 homodimers translocate to the nucleus where they bind directly to GAS DNA sequences to activate transcription. STAT1/STAT2 heterodimers associate with a third subunit, IRF9, to form the ISGF3 complex. ISGF3 binds to ISRE sequences to activate transcription of interferon-stimulated genes. From: [105]

3.2 Signal transducer and activator of transcripton 1 in the immune system

Seven members belong to the Stat family (Stat1, Stat2, Stat3, Stat4, Stat5A, Stat5B, Stat6), which are highly homologous with regard to their structure (see figure 6). STAT molecules possess a SH2 domain for receptor linkage, a DNA-binding domain and a transactivation domain [122]. All members of the Stat family transmit signals from cytokine or growth factor receptors.

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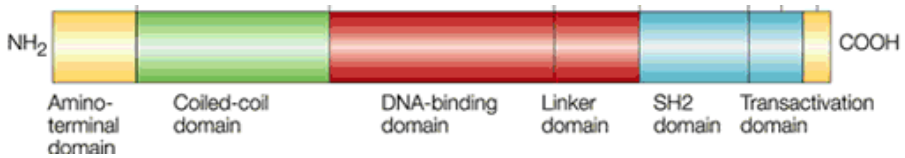

Figure 7: Structure of STAT molecules. From: [123]

Here, I focus on Stat1 as the central molecule in the interferon response.

Upon receptor ligation Stat1 homodimers or Stat1/ Stat2 heterodimers are recruited to tyrosine-phosphorylated docking sites at the IFN receptors. There Stat1 is phosphorylated at tyrosine 701 by the receptor-associated Jaks. Stat1 dimers then change their conformation to an antiparallel form and acquire the ability for nuclear translocation by exposing an important signal [123, 124]. After translocation to the nucleus Stat1 is further phosphorylated by an unknown kinase at serine residue 727. This serine phosphorylation is important for optimal transcriptional activity [125, 126].

Stat1 exists in two splice variants, Stat1α and Stat1β, with Stat1β lacking parts of the carboxyterminus where the serine 727 resides. Growing evidence suggests the presence of unphosphorylated Stat1 in the nucleus and a function in the regulation of gene expression [127].

Stat1 is activated mainly after IFN signalling, but other stimuli like cytokines (IL2, IL6) or growth factors (PDGF, EGF) [128] also lead to Stat1 phosphorylation. In Stat1-/- cells and animals no major contribution of these signalling events could be determined [129]. The phenotype of Stat1 deficiency mirrors in large parts the effects seen when IFN-I or IFNγ are missing. However, there are reports which show Stat 1 independent IFNγ signalling and gene induction, particularly for induction of antiproliferative events [130,

131]. On the other hand Stat1 deficiency causes a more severe phenotype compared to deficiency in IFNs alone in NK cell function [132].

Stat1 deficiencies or mutations in patients have been linked to increased susceptibility to viral and bacterial infections [133-135]. Its role in tumour biology has shown divergent effects depending on the experimental setting used, with Stat1 being either a tumour promoter or suppressor [136 30, 137, 138].

Stat1 has been demonstrated to be important in various cell types of the immune system to establish a proper immune response. Stat1 dependent activation of macrophages and the induction of antiviral and antibacterial pathways within these phagocytes is one of the most important events. However, Stat1 has been shown to be important for IFNγ induced T-bet expression in CD4 cells and thereby for the commitment of these cells to the Th1 lineage [139]. Due to its role in the interferon response Stat1 is important for various steps in the antigen processing and presentation pathways by major histocompatibility complexes (MHC) I and II, as well as for the induction of CIITA (class II transactivator), the major regulator of MHC II [128]. In B cells Stat1 is essential for class switching during B cell activation.

Stat1 mediates the antiproliferative effects of IFNα and IFNγ by activation of inhibitors of cyclin dependent kinases (CDKs) and repression of CDKs [140]. Procaspases are induced by Stat1 in response to various stimuli thereby regulating rates of cell death [128].

3.3 Induction of type I interferons

IFN-I are induced following bacterial, viral and fungal infection. Their expression is stimulated by multiple receptors belonging to different PRR families. Foreign RNA derived from viruses or bacteria can be detected by RLRs like MDA5 and RIG-I in the cytoplasm or by TLR3 and TLR7 located in the endosome [141]. DNA can be recognized in the endosome by TLR9 or in the cytoplasm by DAI and other members from the group of DNA receptors. Sensors like IFI16 or AIM2 have been recently described to take part in DNA recognition; however experimental data suggests the presence of more, yet uncharacterised DNA receptors [142, 143]. A more indirect way of DNA to stimulate innate responses is provided by RNA polymerase III-mediated transcription to RNA and subsequent interaction with RLR [144]. Bacterial products like cyclic-di-GMP can also be recognized by a newly identified molecule, STING, which triggers IFN-I synthesis [145, 146].

All these receptors except TLR7 and TLR9 will associate with various adapter molecules and thus initiate signal transduction that results in the activation of a pair of S/T kinases, TANK binding kinase 1 (TBK1) and IKKi. The kinases, in turn, phosphorylate the interferon regulatory factor 3 (IRF3), which serves as a transcription factor for the IFNβ gene [141]. IFNβ is secreted by the infected cell and induces signalling via IFNAR and ISGF3. IFNAR signalling amplifies type I IFN synthesis by upregulation of another interferon regulatory factor, IRF7. Both IRF3 and IRF7 stimulate expression of IFN-I and, indirectly, ISGs (see figure 7). In specialized interferon producing cells, plasmacytoid dendritic cells, TLR9 and TLR7 can directly activate IRF7 via MyD88 making them fast responders to pathogens with a strong IFN production. The adaptor molecule TRIF, employed by TLR4 and TLR3, is also a potent activator of a pathway stimulating TBK1.

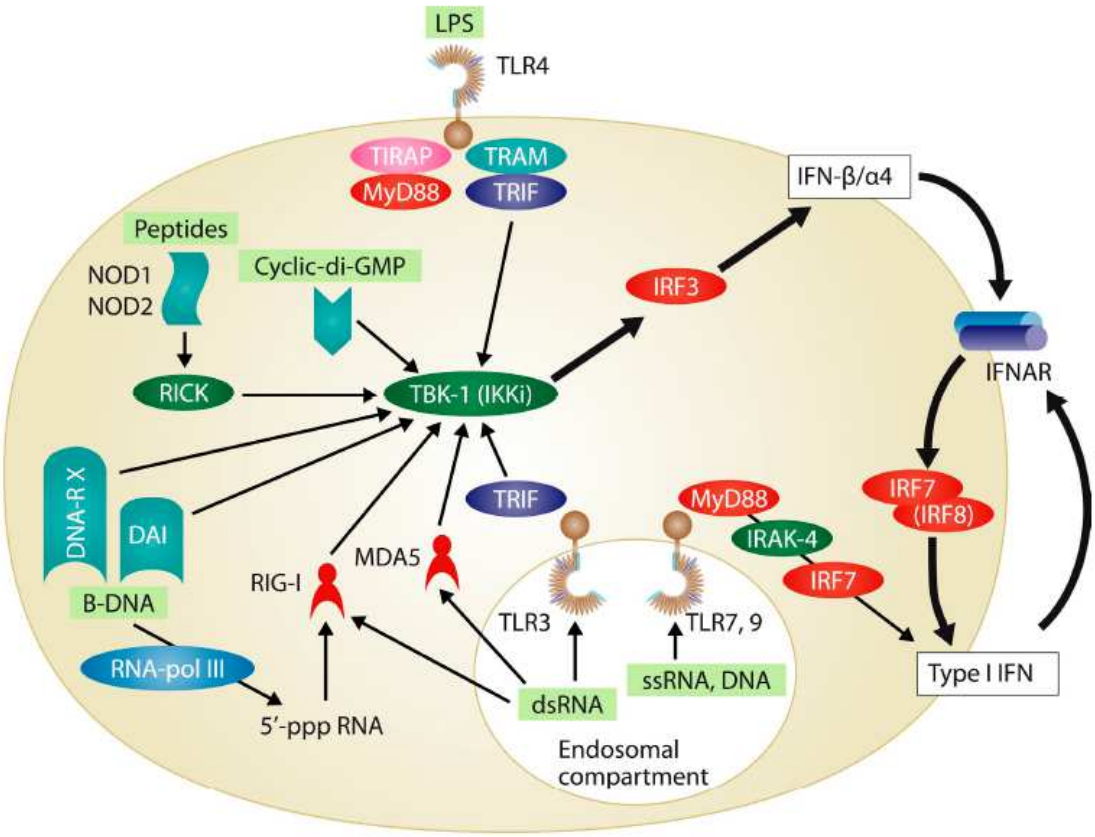

Figure 8: Induction of IFN-I. Upon ligation of various pattern recognition receptors including TLRs, NLRs and others located in the cytosol the kinase(s) TBK1(IKKi) are activated. Phosphorylation of IRF3 and its binding to the IFNb promoter produces a first wave of IFN-I. By activation of the IFN-I receptor and upregulation of the Irf7 gene, a feed forward loop is activated which induces the transcription of more IFN-I and ISGs. From: [141]

3.4 Role of type I interferons in *Listeria monocytogenes* infections

Lm induces a strong IFN-I response in cells and mice. The receptor responsible for the induction of IFN-I, however is not known. TLRs and Nod receptors could be excluded to be responsible for IFN-I induction [147]. Additional studies revealed that the relevant receptor resides in the cytoplasm, as only Lm able to escape the phagosome induce IFN-Is. It is speculated that DNA might be the trigger for IFN-I induction, however no receptor up to date has been found to be responsible for the IFN-I production. New members of the DNA receptor family like DAI/ZBP1 or AIM2 could be excluded to be involved in major IFN induction by Lm in cells [148, 149]. By contrast, the recently identified Ifi16 is a strong candidate [150]. Other studies linked the induction of IFN-I to the activation of STING by the release of cyclic-di-GMP and cyclic-di-AMP, as bacteria lacking the transport system for this molecule could not induce IFN-I [145, 151].

IFN-I have been shown to be detrimental to cells and mice during infections with Lm [152-155]. Different explanations have been put forward to describe the increased survival of Ifnar1-/- animals lacking the type I IFN receptor. For example, IFN-I were shown to enhance apoptosis of T cells and macrophages after contact with the bacterial virulence factor LLO [41, 153]. Due to an IFN-I dependent increase of apoptotic cells more IL10 is produced by phagocytes which clear these cells [156]. High abundance of IL10 is associated with bad outcome of Lm infections, which can also be seen by the fact that IL10-/- mice are more resistant to infection compared to WT animals [157]. The IFN-I dependent death of infected macrophages seems to be a major event which contributes to the detrimental effects of the cytokines.

Another group has shown that IFN-I cause downregulation of the IFNGR on cells which makes them less responsive to IFNγ [158]. Portnoy and colleagues demonstrated that IFN-I are repressing TNFα producing cells in the spleen thereby suppressing inflammatory reactions [152]. Most probably a combination of all these effects lead to the increased survival of Ifnar1-/- mice.

Results

1. Conditional Stat1 ablation reveals the importance of interferon signaling for immunity to *Listeria monocytogenes* infection

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Running title: Cell type restricted antibacterial Stat1 activity

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Author contribution: EK 100% of experiments (CS helped with adoptive transfer experiments)

- animal infection
- adoptive transfer experiment
- bacterial load and survival assays
- Peritoneal cell isolation
- cytokine analysis
- RNA analysis
- FACS analysis

Abstract

Signal transducer and activator of transcription 1 (Stat1) is a key player in responses to interferons (IFN). Mutations of Stat1 cause severe immune deficiencies in humans and mice. Here we investigate the importance of Stat1 signaling for the innate and secondary immune response to the intracellular bacterial pathogen *Listeria monocytogenes* (Lm). Cell type-restricted ablation of the Stat1 gene in naïve animals revealed unique roles in three cell types: macrophage Stat1 signaling protected against lethal Lm infection, whereas Stat1 ablation in dendritic cells (DC) did not affect survival. T lymphocyte Stat1 reduced survival. Type I IFN (IFN-I) signaling in T lymphocytes reportedly weakens innate resistance to Lm. Surprisingly, the effect of Stat1 signaling was much more pronounced, indicating a contribution of Stat1 to pathways other than the IFN-I pathway. In stark contrast, Stat1 activity in both DC and T cells contributed positively to secondary immune responses against Lm in immunized animals, while macrophage Stat1 was dispensable. Our findings provide the first genetic evidence that Stat1 signaling in different cell types produces antagonistic effects on innate protection against Lm that are obscured in mice with complete Stat1 deficiency. They further demonstrate a drastic change in the cell type-dependent Stat1 requirement for memory responses to Lm infection.

Author summary

Signal transducer and activator of transcription 1 (Stat1) is an indispensable component of the cellular response to interferons (IFN) during immune reactions to pathogens. Stat1 deficiency leads to severe immune defects in humans and mice. The sensitivity of animals with complete Stat1 ablation to microbial pathogens prevented determining its contribution to various effector systems of the immune response. By way of tissue-restricted Stat1 ablation we now decipher the impact of Stat1 signaling in different cell populations on the innate and adaptive immune response to the intracellular pathogen *Listeria monocytogenes*. Our data highlight the importance of and requirement for IFNγ-activated macrophages for clearance of the pathogen during early phases of infection, and show a yet unanticipated detrimental role for T cell Stat1. During secondary responses the picture changes and Stat1 in T cells is crucial for proper clearance of *L. monocytogenes*. Likewise, Stat1 signaling in dendritic cells plays a fundamental role for adaptive immunity to *L. monocytogenes*.

Exploring the local response to *L. monocytogenes* infection we reveal a role of Stat1 in shaping the cellular composition of inflammatory infiltrates. Furthermore, Stat1 increases the proliferation of regulatory T cells, an effect likely to dampen the antibacterial response.

Introduction

Signal transducer and activator of transcription (Stat1) is a central mediator of interferon responses in the immune system. Signals from type I (IFNα/IFNβ; IFN-I), type II (IFNγ; IFN-II) and type III (IFNλ, IFN-III) interferons employ receptor-associated Janus kinases (Jaks) to activate Stat by tyrosine phosphorylation [105, 159]. Gene transcription is induced and leads to a range of cellular changes, including anti-viral properties, growth inhibition, apoptosis and differentiation. Depending on the cellular context Stat1 can act as either a tumour-suppressor or promote oncogenesis [136-138]. The central character of Stat1 in signal transduction by the IFN receptors results from the importance of Stat1 homodimers for transcriptional regulation by IFNγ. Moreover, Stat1 forms the ISGF3 complex together with Stat2 and interferon regulatory factor 9 (Irf9). ISGF3 is the main player in transcriptional responses to both IFN-I and IFN-III. Consistent with its central role, Stat1 deficiency in mice recapitulates the lack of IFN-I, IFN-III and IFNγ responses and leads to high susceptibility to viral and bacterial infections [123, 160, 161]. The critical importance of Stat1 for resistance to infection is emphasized by mutations of the Stat1 gene in humans. Patients with various degrees of Stat1 loss-of-function present clinically with recurrent and often lethal mycobacterial and viral infections [108, 134, 162].

Listeria monocytogenes (Lm) is the causative agent of human listeriosis and a serious threat for the health of immunocompromised individuals. It is also a well-studied model organism to analyse cell-mediated immunity to intracellular pathogens. Innate protection critically depends on the activities of the cytokines interleukin (IL) 12 and IFNγ [93, 163]. This most likely reflects NK cell activation, IFNγ production and subsequent clearance of the bacteria by activated macrophages. Sterile immunity and immunological memory result from the development of CD8+ T cells [61, 164]. Stat1-deficient mice succumb to

Lm during the early, innate phase of infection, strongly suggesting a dominant role for Stat1 in IFNγ-mediated macrophage activation [161]. As even very low numbers of Lm, even if attenuated, rapidly kill Stat1-/- mice it is difficult to study attributes of the innate response. For example, Lm replicates in a variety of non-hematopoietic cell types such as epithelial cells or hepatocytes and the contribution of Stat1 to bacterial clearance in these cell types is not known. Moreover, the impact of Stat1 on the generation of adaptive immunity and immunological memory is unclear [165-169]. In this regard the potential role of both IFN-I and IFNγ in the maturation and activation of dendritic cells [170-172] and the impact of both IFN types on the development of effector and memory CTL is of particular interest. Moreover, it has not been possible to investigate a potential contribution of macrophage activation to Lm clearance in secondary immune responses of mice lacking Stat1 in all tissues.

Further interest in cell type-specific Stat1 activities derives from the opposing effects of IFNγ and IFN-I on innate resistance to Lm. IFNγ-deficient mice show a similar susceptibility as Stat1-/- mice [173]. By contrast IFN-I receptor (Ifnar) deficient mice are protected from lethal Lm infections [152-154]. Suppression of protective innate immunity by IFN-I was suggested to result from increased T lymphocyte apoptosis and subsequent IL10-mediated immunosuppression [156]. Furthermore, infection of macrophages and DCs with Lm causes IFN-I dependent downregulation of the IFNγ-receptor, hence unresponsiveness to IFNγ [158]. IFN-I also sensitize infected macrophages *in vitro* to die from infection with Lm [41, 155].

To overcome the limitations posed by the exquisite sensitivity of Stat1-/- mice to infections with Lm or other pathogens we generated mice with floxed Stat1 alleles. Here we report that cell type-restricted Stat1 ablation reveals a striking dichotomy of immunological effects. Macrophage Stat1 produces protective innate immunity whereas the opposite is true for T lymphocytes. In secondary immune responses to Lm T

lymphocyte and dendritic cell Stat1 signaling becomes protective, but Stat1 in macrophages does not contribute to clearance of bacteria.

Results

STAT1 in the hematopoietic compartment is crucial for host protection to *Listeria monocytogenes* infection. To decipher the importance of Stat1 signaling for protective immunity to Lm in the hematopoietic and non-hematopoietic cell compartments we conducted adoptive transfer experiments. WT and Stat1-/- mice were lethally irradiated and bone marrow of either Stat1-/- or WT mice was implanted in these mice. After 8 weeks the chimerism was examined in blood, spleen and liver showing an efficient implantation of the transferred bone marrow (supplemental S1). Bone marrow-chimeric mice were subjected to intraperitoneal infections with sublethal doses of Lm and 72hrs later the bacterial burden in spleen and liver was determined (fig 1A, B). Compared to WT mice reconstituted with WT bone marrow, mice lacking Stat1 in non-hematopoietic cells showed a minor reduction of bacterial clearance, hence minor contribution of non-hematopoietic Stat1 to innate resistance. This suggests that hepatocytes, although representing an important niche for Lm multiplication [16, 174], are not protected by Stat1 signaling. By contrast mice lacking Stat1 in bone marrow-derived cells displayed a clear loss of resistance.

In addition to pathogen clearance we tested the impact of Stat1 deficiency on the systemic cytokine response. WT mice which received Stat1-/- bone marrow responded to infection with a cytokine storm, i.e elevated serum levels of almost all measured cytokines and chemokines (IL6, IL22, TNFα, MCP1, IL10, Rantes, IP10 and MCP3; figure 1C and data not shown). This is likely to reflect the increase in bacterial burden, hence a higher intensity of the innate response. Intriguingly however, the highest levels of IL12p70 were determined in the group of Stat1-/- mice which received protective WT bone marrow and

had very similar bacterial loads as WT mice. This suggests that Stat1 of non-hematopoietic cells participates in the negative regulation of IL12 synthesis. IFNγ production was increased compared to WT. Likewise IFNγ was increased in mice with Stat1-/- bone marrow although IL12 levels were normal. Therefore, IL12 and IFNγ levels are not strictly correlated.

Stat1 signaling in myeloid cells is essential for innate resistance to *Listeria monocytogenes* infection. To further study the contribution of individual immunecompetent cells for the innate phase of Lm infection we analysed resistance to lethal infection and bacterial clearance after tissue-restricted Stat1 ablation. To determine the importance of Stat1 signaling in myeloid cells we used LysMCreStat1flfl mice, which delete predominantly in macrophages and neutrophils [175, 176]. These mice display a significantly reduced ability to clear even a low dose of Lm (figure 2A) and hence succumbed to infection, whereas all WT mice survived the intraperitoneal infection (figure 2B).

To test the involvement of Stat1 to the immune response to Lm in other cell types of the immune system, mice with Stat1 deficiency in CD11c-positive cells, predominantly dendritic cells (CD11cCreStat1flfl, Fig S2), and in T cells (LckCreStat1flfl; Fig S2) were similarly subjected to a sublethal dose of Lm. These mice did not show elevated numbers of Lm in spleen (figure 2C) and liver (data not shown) or a significantly altered susceptibility to sublethal infection (2B). Interestingly, mice with Stat1 deficiency in T cells showed an increased resistance following intraperitoneal infection with a higher than LD50 inoculum of Lm. This lack of activity of T cell Stat1 resulted in both increased survival of the animals and an enhanced clearance of the bacteria (2D, 2E).

IFN-I signaling in T cells was previously shown to reduce the clearance of Lm upon infection in the spleen [153]. To examine whether the enhanced survival in LckCre Stat1flfl mice was due to a lack of IFN-I signaling, we analysed the bacterial load and

survival in mice with IFN-I receptor deficiency in T cells (LckCreIfnarflfl). We noted a significantly lower number of splenic Lm in LckCreIfnarflfl mice compared to WT mice at high doses of infection (figure 2F). However, mice with complete Ifnar1 deficiency showed significantly stronger Lm clearance than mice lacking Ifnar1 only in T cells. The increased ability of LckCreIfnarflfl mice to clear bacteria in the spleen did not result in a higher rate of survival compared to WT mice, whereas complete Ifnar1 deficiency did (2G). This result suggests that the increase in resistance produced by the absence of T cell Stat1 cannot be entirely explained on the basis of the lack of IFN-I signaling in T cells.

Systemic cytokine levels in mice with cell specific Stat1 ablation after *Listeria monocytogenes* infection. Examination of systemic cytokine/chemokine levels demonstrated that mice lacking myeloid Stat1 signaling show increased levels of IL6, IL12p70, MCP1, MCP3, IL22, MIP1α, MIP1β, Rantes and IFNγ in their serum, similar to but not as dramatic as complete Stat1 deficiency (3A and data not shown). As these mice have strongly elevated numbers of pathogens in their organs the increase in inflammatory cytokines may again reflect an increased activity of the innate immune system. However, Stat1 could also exert direct negative regulation on cytokine expression as reported for IL6 [177].

Stat1 signaling in CD11c+ cells had a very selective impact on the levels of systemic cytokines, showing elevated levels of MCP1 compared to WT mice but interestingly, lower amounts of IL12p70. By contrast, higher levels of IL12p70 were detected in the serum of mice lacking Stat1 signaling in T cells, despite an equal bacterial load. The levels of TNFα and IL10 were too low to be detectable at this dose of infection.

Organ damage in mice with cell type-restricted ablation of STAT1 signaling after *Listeria monocytogenes* infection. Livers of infected animals with conditional Stat1 gene ablation were analysed using H&E staining to determine the severity of inflammation two days post-infection. In the case of myeloid cell ablation of Stat1 the radius of the infiltrate area in the liver, classified as microabscess [17], correlated with the increase of bacteria found in this organ (figure 4A, 4B). In addition, the numbers of micro-abscesses correlated with bacterial burden, as lack of Stat1 in myeloid cells increased the area of infiltrates in the liver. Whereas CD11c+ cell-specific ablation of Stat1 led to significantly bigger areas of infiltrates compared to WT (figure 4B), the amount of bacteria in the liver was not significantly enhanced (data not shown). Mice with T cell-restricted Stat1 gene deletion showed smaller infiltrate areas compared to the WT, again reflecting the protection of these mice to infection.

Liver failure may significantly contribute to the lethality of Lm infection [178]. To assess liver damage, we measured the amount of circulating amino aspartate transferase (AST; figure 4C). LysMCreStat1flfl mice displayed strongly elevated levels of AST, indicating massive liver damage. No significant differences in AST were found in all other animals/genotypes compared to WT animals.

In addition to organ damage we examined the immune status of cell restricted Stat1 ablated mice by analysing the composition of blood leukocytes. Mice lacking Stat1 in T cells had the highest numbers of circulating immune cells in their blood 72h after infection (figure 4D, 4E, 4F). This is consistent with the notion that the reduced number of Lm in organs, coupled with reduced numbers of apoptotic cells led to a diminished recruitment of blood leukocytes. Additionally this result may indicate a defect in Stat1 regulated synthesis of T cell-derived chemokines.

STAT1 signaling regulates cellular influx to local sites of inflammation. The results shown in figures 1 and 2 emphasize the importance of Stat1 mediated macrophage activation. In spite of this, mice completely devoid of Stat1 cleared Lm less well than LysMCreStat1flfl animals. This result could be explained by incomplete deletion of the Stat1 gene in macrophages. Our recent inspection of macrophages demonstrates deletion of the Stat1 gene with very high efficiency [176]. Alternatively therefore the difference between LysMCreStat1flfl and Stat1-/- mice may reflect shaping of the innate immune response by Stat1 signaling in several different leukocyte populations. In our infection model the peritoneum is the site of immediate exposure of innate cells to the bacterial pathogen which initiates a local inflammatory response. To determine the degree to which cell type-specific Stat1 signaling determines this local immune response, we first analyzed the local chemokine/cytokine milieu (Fig 5A). Absence of Stat1 in myeloid cells increased MIP1α amounts. MCP1 and RANTES were decreased upon CD11c-Cre-mediated Stat1 ablation. Examination of the pro- and anti-inflammatory cytokine gene expression patterns of adherent peritoneal macrophages isolated from infected mice indicated a small but significant role of myeloid cell Stat1 in the negative regulation of IL12 (Fig 5B). Remarkably, Stat1 signaling in T cells was required for full IL12p40 expression. A more profound effect was noted with regard to IL6 production which was markedly upregulated upon STAT1 deficiency in either myeloid cells or the CD11c+ population. The CD11cCreStat1flfl genotype was unique in producing an adherent cell population with reduced IL10 production. Together with the systemic analyses shown in figure 1 and 2 our data suggest that peritoneal macrophages are major producers of IL6 and IL12. Lack of Stat1 signaling in CD11c+ dendritic cells or inflammatory monocytes may stimulate macrophages to produce excess amounts of IL6 and decreased amounts of IL10.

To determine whether the altered peritoneal chemokine/cytokine altered cell recruitment we isolated peritoneal exudate cells two days after intraperitoneal Lm infection and

analysed the cell composition by Wright-Giemsa-stained cytospins and flow cytometry (figure 5C-E). Myeloid cells together constitute >95% of the peritoneal exudates in WT mice. In animals lacking Stat1 in DC reduced numbers of leukocytes were recruited, however neutrophils were increased at the expense of macrophages (figures 5A-C). Thus, Stat1 signaling in CD11c+ DC regulates monocyte/macrophage migration to the inflamed peritoneum. Mice with myeloid Stat1 ablation showed an increased influx of total peritoneal leukocytes with a similar tendency to reduce monocytes/macrophages and increase neutrophils. Finally, the absence of Stat1 signaling in T cells caused a strong increase in the amount of immune cells travelling to the peritoneum without altering their composition.

Together the data characterizing the peritoneal inflammatory response suggest a profound impact of Stat1 in different cell types on the cytokine milieu and on leukocyte composition. This may explain why myeloid cell restricted Stat1 ablation does not fully reproduce the loss of bacterial clearance observed upon complete Stat1 gene deletion.

Stat1 expression in DC and T cells regulates adaptive immunity to *Listeria monocytogenes*. To analyse the impact of Stat1 signaling in different cell populations on establishing adaptive immunity against Lm, we applied an immunisation and challenge protocol to the respective conditional knockout mice. Under these conditions mice lacking Stat1 signaling in T cells failed to clear Lm from the spleen (figure 6A). Accordingly, an increased percentage of LckCreStat1flfl mice succumbed to infection compared to WT mice (figure 6B). Immunized mice lacking Stat1 in CD11c+ cells showed a slight impairment in clearing splenic Lm, yet the impact on survival was almost as pronounced as in mice lacking Stat1 in T cells. Myeloid Stat1 did not contribute to the establishment of adaptive immunity to Lm as bacterial clearance after immunisation is as strong as in WT mice.

Overall systemic cytokine levels were generally lower than those found after infection of naïve mice (data not shown). Stat1 deficiency in CD11c+ cells caused a selective reduction of systemic IFN-γ that may contribute to the reduced ability to raise adaptive immunity to Lm (Fig 6C). The levels of IFN-γ in mice lacking Stat1 signaling in T cells were equally high as in naïve mice. This may reflect the lack of negative regulation of T cell IFNγ production imposed by Stat1 [179].

To further analyse the immunisation defects in CD11cCre Stat1flfl mice, we investigated T cell responses after immunisation. Proliferation of splenic CD3+ T cells showed no significant differences (figure 7A). However, examination of the Treg population (Cd4+Foxp3+) revealed an enhanced proliferative response in the spleens of mice with CD11c+-restricted Stat1 ablation (figure 7B). As regulatory T cells represent only a minor percentage of total splenic T cells it is not surprising that the difference in proliferation went unnoticed when analyzed in the context of total CD3+ T cell cells. The data suggest a contribution of DC Stat1 to the proliferation of a small proportion of antigen-specific Treg.

Discussion

Studies in gene-modified mice and with cells from human patients suffering from recurrent infectious disease have unequivocally established the central importance of Stat1 for the establishment of protective innate immunity to viral and nonviral pathogens [133, 160, 161]. This includes Lm, the bacterial pathogen studied here. Conditional gene targeting allowed us to examine whether there is a uniform immunological impact of Stat1 across different cell types. Furthermore, we were able to investigate the importance of Stat1 signaling in the same cell types for the development of acquired antibacterial immunity.

Clearance of intracellular bacterial pathogens is caused either by a microbicidal effector mechanism of the infected cell or indirectly through $CD8^+$ T cell-mediated cytolysis. Lm infects a variety of different cell types *in vitro*, either by active invasion or phagocytosis [180, 181]. In infected mice the pathogen replicates in both hematopoietic cells, predominantly macrophages, and non-hematopoietic cells amongst which the hepatocytes form a major niche [16, 174]. To our surprise Stat1 signaling provides non-hematopoietic cells with little effector potential, posing the question how Listeria are killed in these cell compartments particularly before the influx of antigen-specific CTL. One possibility is the death of infected hepatocytes and the subsequent phagocytosis of the cell contents including bacterial cargo by phagocytic cells of the innate immune system [16]. Subsequent sterile clearance most likely requires the development of CTL and active lysis of infected cells [89, 182, 183]. The importance of clearing Lm in the liver is underscored by our findings that the death of mice with different Stat1 genotypes correlated well with the inflammatory infiltrate in this organ and with the hepatotoxicity caused by infection.

LysMCre mediated gene deletion occurs predominantly in macrophages and granulocytes [175]. A recent report shows that the contribution of neutrophils to the immune response against Lm is surprisingly small [77] and, given the short half life of granulocytes, Stat1-

dependent transcriptional response to IFN is unlikely to enhance their microbicidal activity. Therefore our data are consistent with the previous notion that IFNγ and Stat1 cause macrophage activation and that activated macrophages represent a dominant innate anti-listericidal effector mechanism of the innate immune response [184, 185]. Indeed, our data reveal the essential role of this cell type for clearance of Lm. That said the clearance deficit of mice lacking macrophage Stat1 was significantly lower than that of Stat1-/- mice. In this regard inspection of the local immune reaction elicited by intraperitoneal infection with Lm. showed that besides macrophages T cells and particularly CD11c+ cells shape the inflammatory environment by regulating chemokine production and cell influx. CD11c+ cells in this situation are likely to represent inflammatory DC that arise from inflammatory monocytes [186]. Although lack of Stat1 signaling in DC or in T cells does not per se affect survival and clearance of infection, it may synergize with the Stat1 deficiencies of other cell types to produce the more severe outcome of the complete Stat1 knockout. Increased Lm replication in Stat1-/- compared to LysMCreStat1flfl mice caused a more severe cytokine storm that is likely to be one cause of their accelerated death.

While the amount of serum cytokines generally followed the severity of infection in different Stat1 genotypes, IL12 was the exception because it was strongly increased in mice with Stat1-deficiency outside the hematopoietic compartment that were able to cope with infection nearly as well as WT mice. This finding reveals negative regulation of IL12 synthesis by a non-hematopoietic cell. IL10 is a negative regulator of IL12 production [187] and its synthesis can be suppressed by IFNγ [188]. However, IL10 is considered to be a product of hematopoietic cells [189]. The nature of the suppressive cell type and the mechanism of IL12 suppression will require further investigation.

The exacerbation of infection by Stat1 in T lymphocytes is particularly intriguing. Unanue and colleagues demonstrated the T cell response to IFN-I as a mechanism underlying their adverse effect [153, 156]. While our data confirm that the IFN-I response of T cells indeed

reduces bacterial clearance, Ifnar1-deficiency in this cell type alone does not reproduce the consequences of the complete Ifnar1 knockout and it does not cause the robust effect of T cell-specific Stat1 deletion. Hence, T cells indeed inhibit protective innate immunity to Lm, but the effect of Stat1 goes beyond IFN-I signaling. Furthermore, additional cell compartments must contribute to the suppressive effect of IFN-I, as suggested by the comparison between infected Ifnar-/- and LckCreIfnar1flfl mice. At present we do not understand how Stat1 signaling in T cells reduces innate immunity to Lm. Systemic cytokine profiles of LckCreStat1flfl animals showed little change with respect to WT mice. IL10 levels were below the detection limit which leads us to assume that reduced clearance does not result from global immunosuppression. Annexin V staining of splenic leukocytes did not reveal an increase of apoptotic CD3+ T cells or CD11b+ myeloid cells beyond the levels seen in control mice (data not shown). However, we cannot rule out that cell apoptosis specifically in infected areas are decisive although it does not cause measurable changes in the total leukocyte population. In addition, bacterial multiplication might be enhanced by the reported suppressive activity of IFN-I on IFNγ receptor expression [158].

Infection of immunized mice with Lm caused a drastic change in the consequences of Stat1 signaling in cell types of the immune system. Most importantly Stat1 activity in T cells was now required for protective immunity. Antigen-specific CTL are critical effectors for adaptive immunity to Lm [61, 89]. Our data provide the first genetic proof that Stat1 signaling in both T cells and DC is required for acquired resistance, in addition to showing that IFNγ-activated macrophages are dispensable once memory lymphocytes have been produced. The essential stimulus for Stat1 signaling in T cells is unclear. IFN-I appear to be dispensable for both CD4+ and CD8+ T cell development during Lm infection [190, 191]. Furthermore, a study analyzing CTL development in IFNγ-deficient

animals infected with very low numbers of Lm shows that IFNγ is not essential for protective CTL-mediated immunity [183]. Possibly IFNγ contributes to protective CTL memory when infection occurs with a high infectious dose. Therefore, Stat1 may increase the efficacy of memory CD8+ T cell responses.

Defective Stat1 signaling in CD11c+ DC also reduced protection by the adaptive immune system upon secondary challenge with Lm. This is consistent with our recent finding that immunization with Stat1-/- DC caused a strongly diminished CTL response to Ova peptide [125] and with reports by others that Stat1-/- DC fail to elicit protective immunity to *Leishmania major* [192]. IFN-I and Stat1 reportedly support DC maturation and activation [193, 194]. Data in the literature thus suggest a defect of Stat1 deficient DC to present antigen to T cells. In line with this IFN-I were reported to stimulate the ability of DC to cross-present antigen [172]. In our experiments the lack of DC Stat1 affected survival of mice more than splenic clearance of bacteria, which argues against a general defect in generating effector CTL. Moreover, activation of both CD4+ and CD8+ T cells to the levels found with wt cells occurred *in vitro* when Stat1-/- DC were used as antigen presenters (data not shown). In a mouse model of graft versus host disease Ma and colleagues noted an increased proliferation of FoxP3+ regulatory T cells upon transfer of Stat1-/- splenocytes into irradiated hosts [195]. Prompted by this finding we tested whether Stat1 deficiency in DC might similarly cause increased Treg proliferation in Listeria-infected mice. Indeed we noted that the proportion of proliferating FoxP3+ cells was about two-fold higher in spleens from infected mice with a CD11cCreStat1flfl genotype. It is therefore possible that an increased number of antigen-specific regulatory T cells suppresses effector T cells and thus reduces the immune response to Lm.

In summary, cell type-restricted ablation reveals a fascinating complexity of Stat1's regulatory power for the development of both innate and adaptive immune responses to Lm.

Material and Methods

Mice and bacteria

Animal experiments were discussed and approved by the University of Veterinary Medicine Vienna institutional ethics committee and carried out in accordance with protocols approved by the Austrian law (BMWF-68.205/0204-C/GT/2007; BMWF-68.205/0210-II/10b/2009, BMWF-68.205/0243-II/3b/2011). Bacteria were prepared for infection as described previously [71]. For infection, Lm LO28 were washed with PBS and injected intraperitoneally (i.p) or intravenously (i.v) of 8- to 10-week-old sex and age matched C57BL/6N (WT), Stat1flfl (B6.129P2-Stat1^{tmBiat} ,[176]), LysMCre Stat1flfl (B6.129P2-Lyz2$^{tm1(cre)Ifo}$/J-Stat1^{tmBiat}[175]), CD11cCre Stat1flfl (B6.Cg-Tg(CD11c-Cre)-Stat1^{tmBiat} [196]), LckCre Stat1flfl (B6.129P2-Tg(LckCre)-Stat1^{tmBiat} LckCre[197]), Ifnar1-/- (B6.129P2-IfnaR1^{tm1})[198], Stat^-/- (B6.129P2-Stat1tm1), Ifnar1flflLckCre (B6.129P2-IfnaR1^{tm1}-Tg (LckCre) [199] mice at the respective dose. The infectious dose was controlled by plating serial dilutions on Oxford agar plates. The survival of mice was monitored for 10 days, and data were displayed as Kaplan-Meier plots. For determination of bacterial loads of liver and spleen mice were killed at the indicated time points. The respective organs were isolated and homogenized in PBS. Serial dilutions of the homogenates were plated on BHI plates and incubated at 37 °C for 24 h. For immunisation mice were injected i.p with 1x10^6 attenuated Listeria (ΔActA). After 2-3 weeks mice were infected i.v with 1x10^5 Lm.

Cytokine analysis

For cytokine analysis mice were bled via the retro-orbital sinuses and serum was collected and stored at -80 °C. Using the FlowCytomix system (ebioscience) concentrations of

indicated cytokines (IFNγ, IL6, IL10, IL12p70, Mcp1, Mcp3, Rantes, GMCSF, Mip1α, Mip1β, IP10, IL22, TNFα) were measured.

Peritoneal exudate cell isolation

For isolation of peritoneal macrophages mice were infected for 48 h with 5x10^6 LO28 i.p. Mice were sacrificed, the peritoneum was flushed with two times 10 ml of DMEM and cells were harvested by centrifugation. After 2 h adherent cells were washed and RNA was prepared. The composition of peritoneal exudate cells was determined by flowcytometric analysis and Wright-Giemsa stain of cytospins. Composition of adherent cells were analysed by flowcytometric analysis. Chemokines and cytokines were measured using the FlowCytomix system after flushing the peritoneum with 1 ml of DMEM.

RNA isolation, cDNA synthesis and Real time PCR

RNA was isolated using the Nucleospin II kit (Macherey and Nagel) according to protocol. Reverse transcription was accomplished using RevertAid (Fermentas). The realtime PCRs were run on an Eppendorf cycler. After correction for the housekeeping gene Gapdh, every sample was calculated to the mean of WT mRNA levels.

Flowcytometric analysis

Spleens were isolated after indicated timepoints and single cell suspensions were prepared using a 80 µm cell strainer. After red blood cell lysis cells were stained for CD3-PE, CD4-FITC, CD8-APC, CD11b-PerCP, Gr1-PE, FOXp3-APC, Ki67-PerCP (all BD bioscience).

Histology

Mouse organs were fixed with 4% paraformaldehyde over night, paraffin embedded and sections were prepared using a microtome. Hematoxyline and eosin staining (H&E) were

performed using standard protocols. The radius of the infiltrate was measured using the Zeiss Axioplan software.

AST measurement

Aspartate amino transferase concentrations were measured in mouse serum using a COBASc11 analyzer (Roche).

Adoptive transfer

WT (Ly5.1 and C57BL/6) and Stat1-/- animals were lethally irradiated with 8,2 Gy for 17 minutes and engrafted with 5x10^6 bonemarrow cells of respective genotypes. After 6 weeks engraftment was analysed in blood, spleen and liver by flowcytometry using the antibodies Ly5.1 Fitc, Ly5.2 PE, CD11b PerCP (BD biosciences).

Supplemental method for S2

Western Blot analysis of MACS purified splenic DC and T cells was performed following standard protocols. Fluorophore-linked secondary antibodies (LI-COR) were used for detection on an Odyssey infrared imaging system (LI-COR). Antibodies against Stat1N (Cell Signalling) and against pan-erk (BD biosciences) was used.

References:

1. Borden, E.C., et al., *Interferons at age 50: past, current and future impact on biomedicine.* Nat Rev Drug Discov, 2007. 6(12): p. 975-90.
2. Stark, G.R., et al., *How cells respond to interferons.* Annu Rev Biochem, 1998. 67: p. 227-64.
3. Kaplan, D.H., et al., *Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice.* Proc Natl Acad Sci U S A, 1998. 95(13): p. 7556-61.
4. Kovacic, B., et al., *STAT1 acts as a tumor promoter for leukemia development.* Cancer Cell, 2006. 10(1): p. 77-87.
5. Schneckenleithner, C., et al., *Putting the brakes on mammary tumorigenesis: Loss of STAT1 predisposes to intraepithelial neoplasias.* Oncotarget.
6. Durbin, J.E., et al., *Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease.* Cell, 1996. 84(3): p. 443-50.
7. Meraz, M.A., et al., *Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway.* Cell, 1996. 84(3): p. 431-42.
8. Levy, D.E. and J.E. Darnell, Jr., *Stats: transcriptional control and biological impact.* Nat Rev Mol Cell Biol, 2002. 3(9): p. 651-62.
9. Chapgier, A., et al., *A partial form of recessive STAT1 deficiency in humans.* J Clin Invest, 2009. 119(6): p. 1502-14.
10. Kristensen, I.A., et al., *Novel STAT1 alleles in a patient with impaired resistance to mycobacteria.* J Clin Immunol. 31(2): p. 265-71.
11. Zhang, S.Y., et al., *Inborn errors of interferon (IFN)-mediated immunity in humans: insights into the respective roles of IFN-alpha/beta, IFN-gamma, and IFN-lambda in host defense.* Immunol Rev, 2008. 226: p. 29-40.
12. Hsieh, C.S., et al., *Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages.* Science, 1993. 260(5107): p. 547-9.
13. Lu, B., et al., *Targeted disruption of the interferon-gamma receptor 2 gene results in severe immune defects in mice.* Proc Natl Acad Sci U S A, 1998. 95(14): p. 8233-8.
14. Kaufmann, S.H., *Immunity to intracellular bacteria.* Annu Rev Immunol, 1993. 11: p. 129-63.

15. Pamer, E.G., *Immune responses to Listeria monocytogenes.* Nat Rev Immunol, 2004. 4(10): p. 812-23.
16. Badovinac, V.P. and J.T. Harty, *Adaptive immunity and enhanced CD8+ T cell response to Listeria monocytogenes in the absence of perforin and IFN-gamma.* J Immunol, 2000. 164(12): p. 6444-52.
17. Durbin, J.E., et al., *Type I IFN modulates innate and specific antiviral immunity.* J Immunol, 2000. 164(8): p. 4220-8.
18. Fallarino, F. and T.F. Gajewski, *Cutting edge: differentiation of antitumor CTL in vivo requires host expression of Stat1*. J Immunol, 1999. 163(8): p. 4109-13.
19. Gil, M.P., et al., *Modulation of STAT1 protein levels: a mechanism shaping CD8 T-cell responses in vivo.* Blood, 2006. 107(3): p. 987-93.
20. Messingham, K.A., et al., *A role for IFN-gamma from antigen-specific CD8+ T cells in protective immunity to Listeria monocytogenes.* J Immunol, 2007. 179(4): p. 2457-66.
21. Akbar, S.M., et al., *Unique features of dendritic cells in IFN-gamma transgenic mice: relevance to cancer development and therapeutic implications.* Biochem Biophys Res Commun, 1999. 259(2): p. 294-9.
22. Banyer, J.L., et al., *Combinations of IFN-gamma and IL-4 induce distinct profiles of dendritic cell-associated immunoregulatory properties.* Genes Immun, 2003. 4(6): p. 427-40.
23. Le Bon, A., et al., *Cross-priming of CD8+ T cells stimulated by virus-induced type I interferon.* Nat Immunol, 2003. 4(10): p. 1009-15.
24. Huang, S., et al., *Immune response in mice that lack the interferon-gamma receptor.* Science, 1993. 259(5102): p. 1742-5.
25. Auerbuch, V., et al., *Mice lacking the type I interferon receptor are resistant to Listeria monocytogenes.* J Exp Med, 2004. 200(4): p. 527-33.
26. Carrero, J.A., B. Calderon, and E.R. Unanue, *Type I interferon sensitizes lymphocytes to apoptosis and reduces resistance to Listeria infection.* J Exp Med, 2004. 200(4): p. 535-40.
27. O'Connell, R.M., et al., *Type I interferon production enhances susceptibility to Listeria monocytogenes infection.* J Exp Med, 2004. 200(4): p. 437-45.
28. Carrero, J.A., B. Calderon, and E.R. Unanue, *Lymphocytes are detrimental during the early innate immune response against Listeria monocytogenes.* J Exp Med, 2006. 203(4): p. 933-40.

29. Rayamajhi, M., et al., *Induction of IFN-alphabeta enables Listeria monocytogenes to suppress macrophage activation by IFN-gamma.* J Exp Med, 2010. 207(2): p. 327-37.
30. Stockinger, S., et al., *Production of type I IFN sensitizes macrophages to cell death induced by Listeria monocytogenes.* J Immunol, 2002. 169(11): p. 6522-9.
31. Zwaferink, H., et al., *IFN-beta increases listeriolysin O-induced membrane permeabilization and death of macrophages.* J Immunol, 2008. 180(6): p. 4116-23.
32. Rogers, H.W., et al., *Listeria monocytogenes induces apoptosis of infected hepatocytes.* J Immunol, 1996. 156(2): p. 679-84.
33. Gregory, S.H., A.J. Sagnimeni, and E.J. Wing, *Internalin B promotes the replication of Listeria monocytogenes in mouse hepatocytes.* Infect Immun, 1997. 65(12): p. 5137-41.
34. Clausen, B.E., et al., *Conditional gene targeting in macrophages and granulocytes using LysMcre mice.* Transgenic Res, 1999. 8(4): p. 265-77.
35. Wallner, B., et al., *Generation of mice with a conditional Stat1 null allele.* Transgenic Res.
36. Kimura, A., et al., *Aryl hydrocarbon receptor in combination with Stat1 regulates LPS-induced inflammatory responses.* J Exp Med, 2009. 206(9): p. 2027-35.
37. Dancygier, H., *Bacterial Liver Abscess and Other Bacterial Infections*, in *Clinical Hepatology*. 2010. p. 831-842.
38. Sasaki, T., et al., *Roles of CD4+ and CD8+ cells, and the effect of administration of recombinant murine interferon gamma in listerial infection.* J Exp Med, 1990. 171(4): p. 1141-54.
39. Nguyen, K.B., et al., *Critical role for STAT4 activation by type 1 interferons in the interferon-gamma response to viral infection.* Science, 2002. 297(5589): p. 2063-6.
40. Chapgier, A., et al., *Novel STAT1 alleles in otherwise healthy patients with mycobacterial disease.* PLoS Genet, 2006. 2(8): p. e131.
41. Portnoy, D.A., V. Auerbuch, and I.J. Glomski, *The cell biology of Listeria monocytogenes infection: the intersection of bacterial pathogenesis and cell-mediated immunity.* J Cell Biol, 2002. 158(3): p. 409-14.
42. Hamon, M., H. Bierne, and P. Cossart, *Listeria monocytogenes: a multifaceted model.* Nat Rev Microbiol, 2006. 4(6): p. 423-34.

43. Gregory, S.H. and C.C. Liu, *CD8+ T-cell-mediated response to Listeria monocytogenes taken up in the liver and replicating within hepatocytes.* Immunol Rev, 2000. 174: p. 112-22.
44. Harty, J.T. and M.J. Bevan, *Specific immunity to Listeria monocytogenes in the absence of IFN gamma.* Immunity, 1995. 3(1): p. 109-17.
45. Lara-Tejero, M. and E.G. Pamer, *T cell responses to Listeria monocytogenes.* Curr Opin Microbiol, 2004. 7(1): p. 45-50.
46. Shi, C., et al., *Ly6G+ Neutrophils Are Dispensable for Defense against Systemic Listeria monocytogenes Infection.* J Immunol. 187(10): p. 5293-8.
47. Mackaness, G.B., *Cellular resistance to infection.* J Exp Med, 1962. 116: p. 381-406.
48. Shaughnessy, L.M. and J.A. Swanson, *The role of the activated macrophage in clearing Listeria monocytogenes infection.* Front Biosci, 2007. 12: p. 2683-92.
49. Shi, C. and E.G. Pamer, *Monocyte recruitment during infection and inflammation.* Nat Rev Immunol. 11(11): p. 762-74.
50. Zhou, L., A.A. Nazarian, and S.T. Smale, *Interleukin-10 inhibits interleukin-12 p40 gene transcription by targeting a late event in the activation pathway.* Mol Cell Biol, 2004. 24(6): p. 2385-96.
51. Hu, X., et al., *IFN-gamma suppresses IL-10 production and synergizes with TLR2 by regulating GSK3 and CREB/AP-1 proteins.* Immunity, 2006. 24(5): p. 563-74.
52. Saraiva, M. and A. O'Garra, *The regulation of IL-10 production by immune cells.* Nat Rev Immunol. 10(3): p. 170-81.
53. Havenar-Daughton, C., G.A. Kolumam, and K. Murali-Krishna, *Cutting Edge: The direct action of type I IFN on CD4 T cells is critical for sustaining clonal expansion in response to a viral but not a bacterial infection.* J Immunol, 2006. 176(6): p. 3315-9.
54. Thompson, L.J., et al., *Innate inflammatory signals induced by various pathogens differentially dictate the IFN-I dependence of CD8 T cells for clonal expansion and memory formation.* J Immunol, 2006. 177(3): p. 1746-54.
55. Pilz, A., et al., *Dendritic cells require STAT-1 phosphorylated at its transactivating domain for the induction of peptide-specific CTL.* J Immunol, 2009. 183(4): p. 2286-93.
56. Johnson, L.M. and P. Scott, *STAT1 expression in dendritic cells, but not T cells, is required for immunity to Leishmania major.* J Immunol, 2007. 178(11): p. 7259-66.

57. Jackson, S.H., et al., *Dendritic cell maturation requires STAT1 and is under feedback regulation by suppressors of cytokine signaling.* J Immunol, 2004. 172(4): p. 2307-15.
58. Le Bon, A., et al., *Type i interferons potently enhance humoral immunity and can promote isotype switching by stimulating dendritic cells in vivo.* Immunity, 2001. 14(4): p. 461-70.
59. Ma, H., et al., *Absence of Stat1 in donor CD4 T cells promotes the expansion of Tregs and reduces graft-versus-host disease in mice.* J Clin Invest. 121(7): p. 2554-69.
60. Stockinger, S., et al., *Characterization of the interferon-producing cell in mice infected with Listeria monocytogenes.* PLoS Pathog, 2009. 5(3): p. e1000355.
61. Caton, M.L., M.R. Smith-Raska, and B. Reizis, *Notch-RBP-J signaling controls the homeostasis of CD8- dendritic cells in the spleen.* J Exp Med, 2007. 204(7): p. 1653-64.
62. Orban, P.C., D. Chui, and J.D. Marth, *Tissue- and site-specific DNA recombination in transgenic mice.* Proc Natl Acad Sci U S A, 1992. 89(15): p. 6861-5.
63. Muller, U., et al., *Functional role of type I and type II interferons in antiviral defense.* Science, 1994. 264(5167): p. 1918-21.
64. Kamphuis, E., et al., *Type I interferons directly regulate lymphocyte recirculation and cause transient blood lymphopenia.* Blood, 2006. 108(10): p. 3253-61.

Figure legends

Fig 1: **Role of STAT1 in the hematopoietic compartment.**

C57BL/6N (WT) and Stat1-/- mice were lethally irradiated and bonemarrow of the respective donor mice was transferred into recipient mice. After engraftment mice were infected i.p with 1x10^5 Lm and the bacterial load of spleen (1A) and liver (1B) was determined and medians were calculated (n=5). Statistical significance was determined using the Mann-Whitney Test. Serum of infected mice was collected and cytokines were determined (1C). Means and standard deviations are shown.

Fig 2: **Bacterial load and survival in cell-type specific Stat1 ablated mice.**

Stat1flfl, Stat1-/- and LysMCreStat1flfl mice were infected for 72 h with 1x10^5 Lm i.p and bacterial load of spleen was determined after 72 h (2A) or survival was monitored over 10 days (n=11-14) (2B). Stat1flfl, Stat1-/- and CD11cCreStat1flfl, LckCreStat1flfl mice were infected with 1x10^5 Lm i.p and bacterial load was determined after 72 h (2C). Stat1flfl and LckCreStat1flfl mice were infected with 1x10^6 and survival was monitored over 10 days (n=11-13) (2D). Using 1x10^6 Lm LckCreStat1flfl and control mice (2E) and LckCreIfnar1flfl and respective control mice (2F) were infected and the CFU after 72 h was determined or survival was monitored for 10 days (n=6-15) after 5x10^6 i.p infection, respectively (2G). Representative results of at least two independent experiments are shown.

Fig 3: **Cytokine analysis of 72h infected cell-type specific Stat1 ablated mice.**

72 h after infection of respective mice with 1x10^5 Lm serum was collected and indicated cytokines were analysed (3A). Mean values of a representative experiment (n=5) out of two independently performed experiments are depicted with standard deviations.

Fig 4: **Liver damage and blood cell populations after 72h of infection.**

Liver sections of 72h infected animals were stained with H&E, arrows point to infiltrates (4A). The radius of infiltrated area was measured in µm of three individual mice in three eyefields (10x) using the Zeiss AxiVision LE software and depicted in (4B). One representative picture is shown. Amino aspartate transferase levels were analysed in the serum of indicated mice (n=6) infected with Lm for 72h (4C). In collected blood the amount of lymphocytes (4D), monocytes (4E) and granulocytes (4F) was determined of uninfected (Ni), 24h and 72h infected mice (n=3-6). Data from two independent experiments are shown.

Fig 5: **Peritoneal inflammatory reaction 48h after infection.**

Cytokines were determined in the peritoneal lavage fluid of 48 h infected mice (n=6) (5A). RNA of of adherent cells of peritoneal exudates was prepared and subjected to qPCR (5B). Data from 3-5 experiments (n=10-15) are pooled and means with standard deviation are shown. 48 h after i.p infection peritoneal exudate cells were isolated and Wright-Giemsa stained after cytospin. The amount of cells (5C), the percentage of macrophages (5D) and the percentage of neutrophils (5E) were counted and depicted (n=9).

Fig 6: **Adaptive immunity to *Listeria monocytogenes* in tissue restricted Stat1 ablated mice.**

Immunised and non immunised mice were infected i.v with 1x10^5 Lm and bacterial load was determined 48 h after infection (n=4- 5) (6A) or survival was monitored of 7 mice of each genotype (6B). Serum of immunised and naive mice was subjected to cytokine analyses (6C). One representative result of at least two independently performed experiments is shown (n=4-5).

Fig 7: **CD11c Stat1 regulates adaptive immunity.**

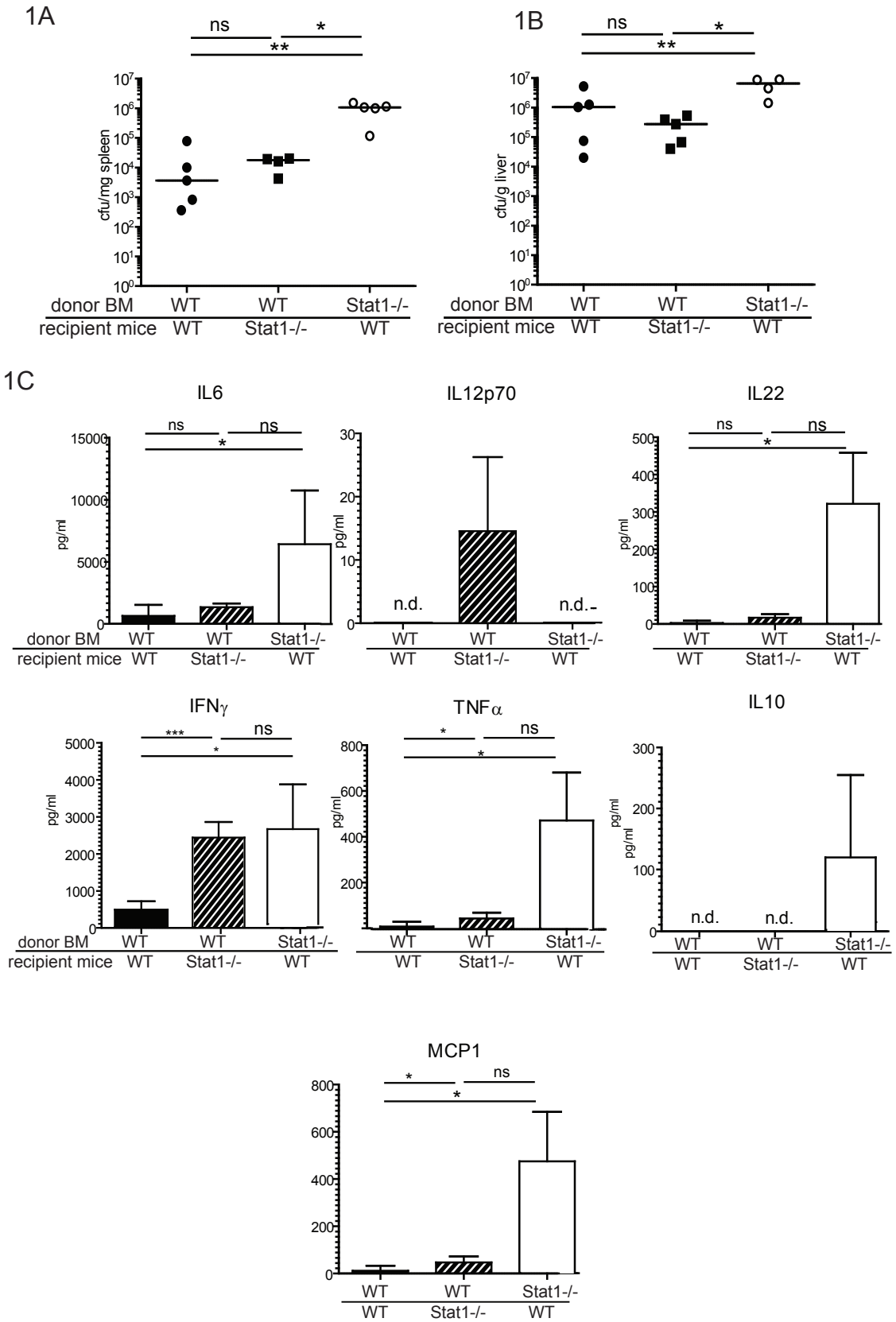
Splenocytes were isolated of immunised and rechallenged Stat1flfl and CD11cCreStat1flfl mice and evaluated for proliferating CD3+ cells (7A) and Tregs (CD3+, CD4+, FoxP3+, Ki67+) (7B). Means and standard deviations of one representative experiment out of two independently performed experiments is shown with 5 mice per group.

Fig S1: **Bone marrow chimerism and blood lineage analysis.**

Representative blots of a Stat1-/- mouse which received WT (Ly5.1) bone marrow are shown: Percentage of Ly5.1 positive leukocytes in the blood and Ly5.1+ and CD11b+ cells in spleen and liver. For bone marrow Ly5.2 positive cells are shown due to a large proportion of Ly5.1 and Ly5.2 negative cells. Percentage of positive cells are indicated in the figure (S1).

Fig S2: **Deletion efficiency of CD11c and LckCre in splenic DC and T cells.**

Splenic dendritic cells (S2a) and splenic T cells (S2b) were isolated out of CD11cCreStat1flfl and LckCreStat1flfl, respectively and control mice (S2b) and subjected to Western blot analysis for Stat1N and pan-erk.


1A
ns
*
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recipient mice WT Stat1-/- WT
1B
ns
*
**
cfu/g liver
donor BM WT WT Stat1-/-
recipient mice WT Stat1-/- WT
1C
IL6
pg/ml
ns
ns
*
n.d.
IL12p70
pg/ml
n.d.
IL22
pg/ml
ns
ns
*
donor BM WT WT Stat1-/-
recipient mice WT Stat1-/- WT
IFNγ
pg/ml
***
ns
*
TNFα
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ns
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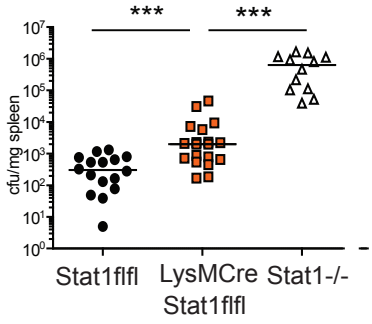
IL10
pg/ml
n.d.
n.d.
MCP1
pg/ml
*
ns
*

2A



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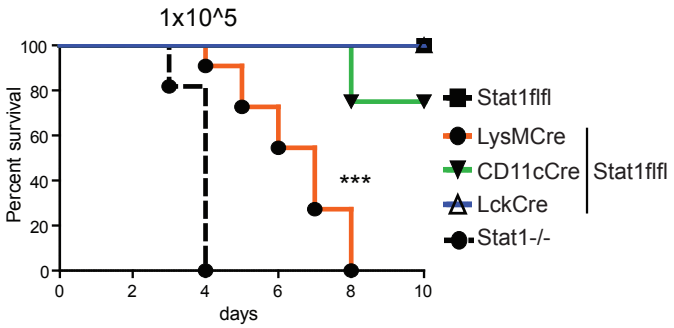

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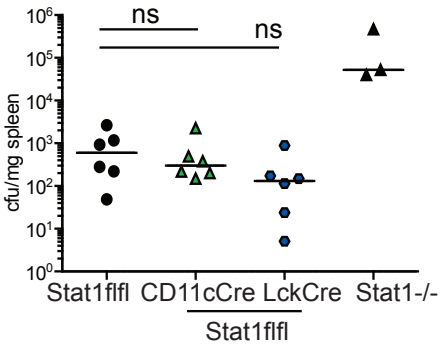

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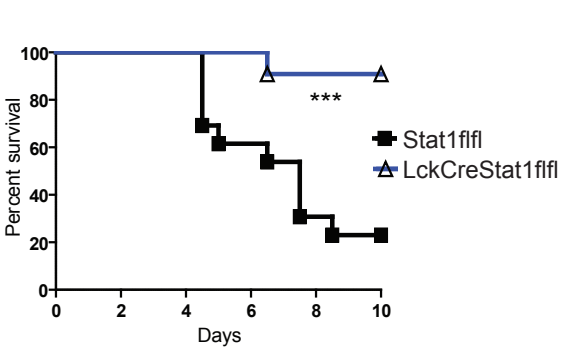

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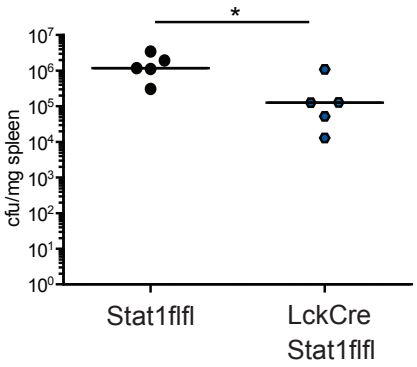

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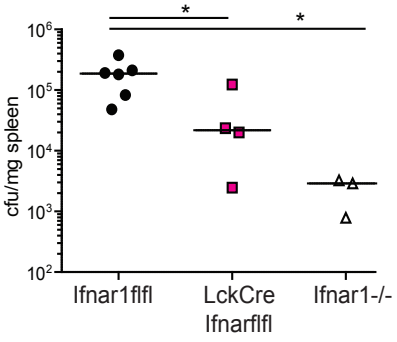

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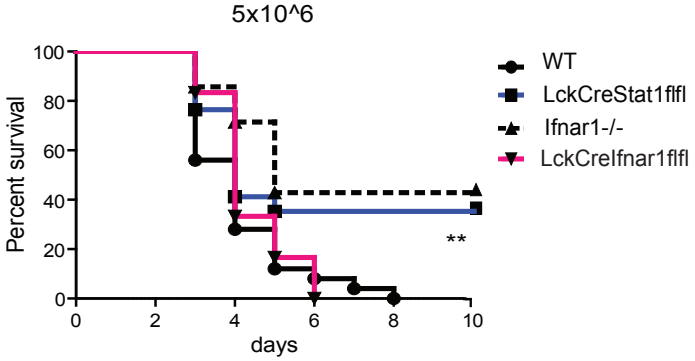

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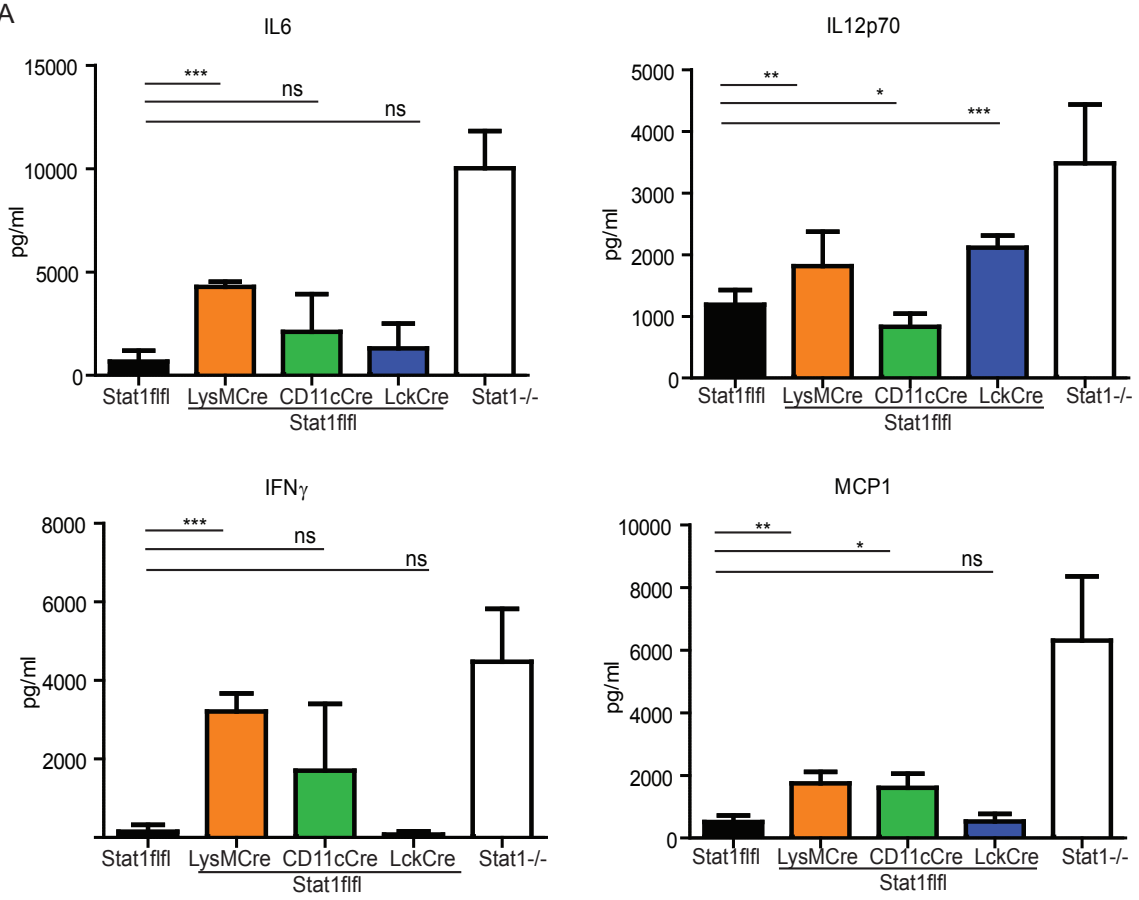
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pg/ml
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Stat1-/-
IFNγ
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pg/ml
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Stat1flfl
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CD11cCre
LckCre
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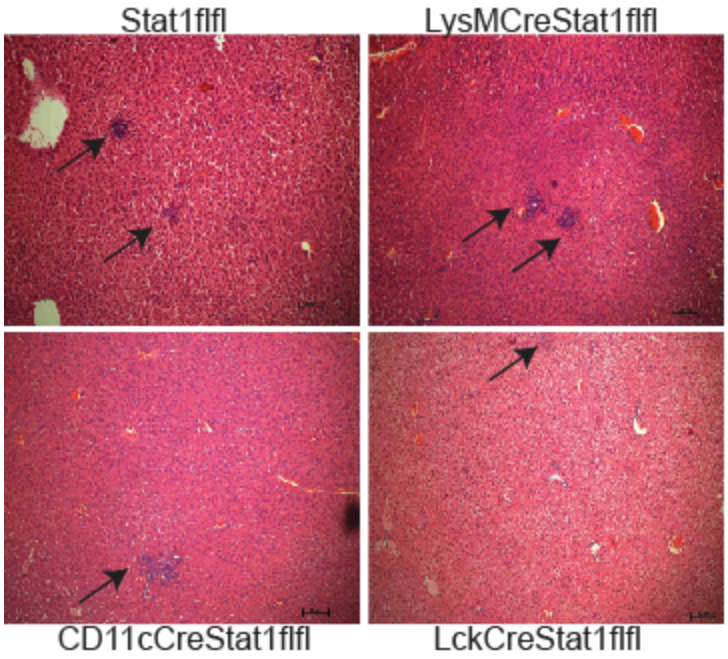
Stat1-/-

4A



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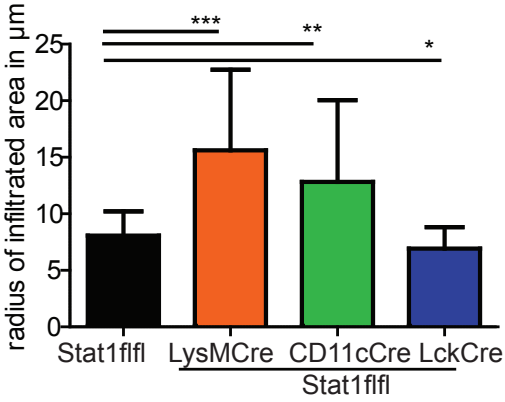

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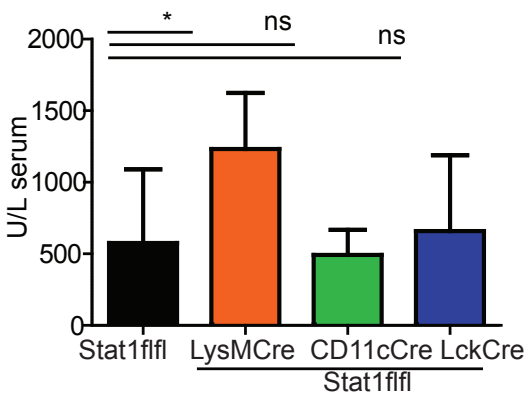

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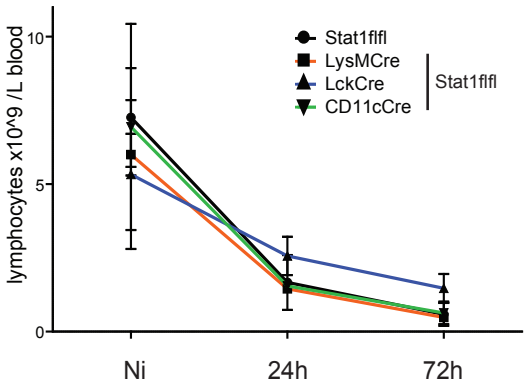

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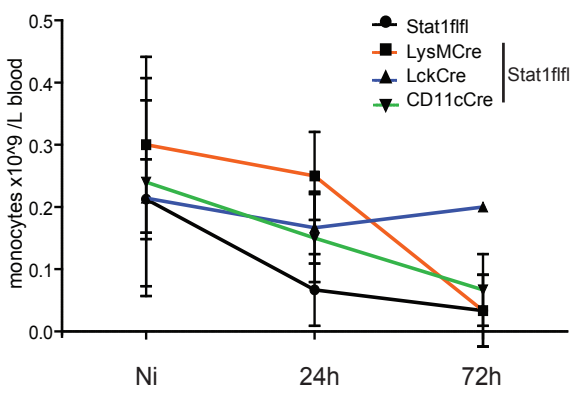

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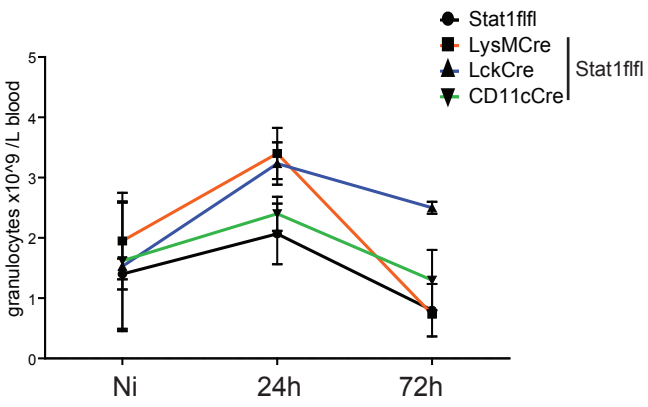

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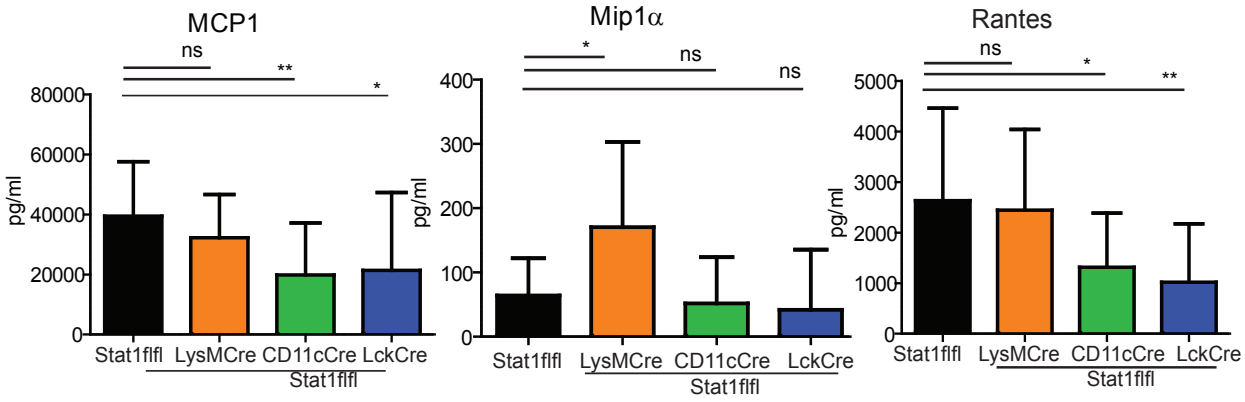
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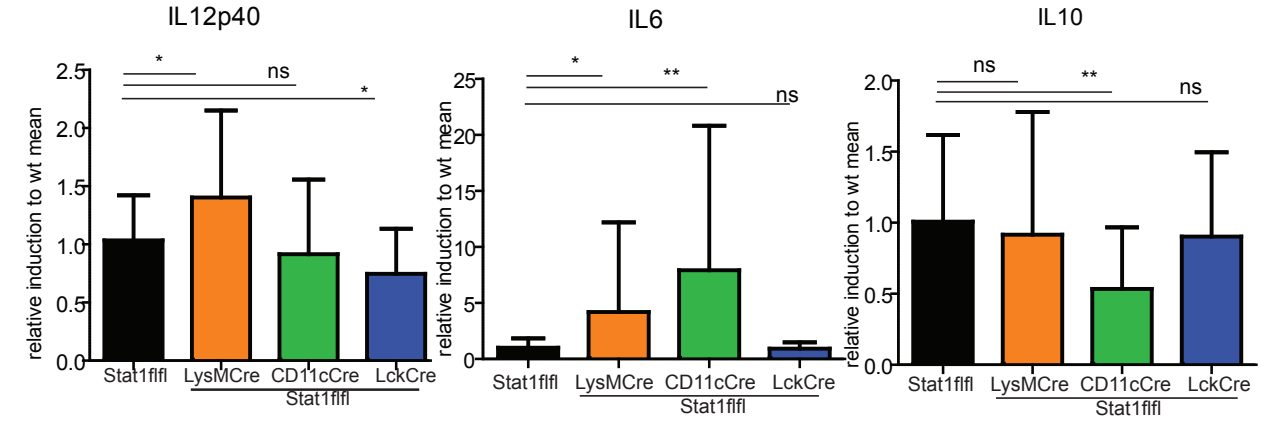
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LysMCre
CD11cCre
LckCre
Stat1flfl
IL6
*
**
ns
25
20
15
10
5
0
relative induction to wt mean
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LysMCre
CD11cCre
LckCre
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IL10
ns
**
ns
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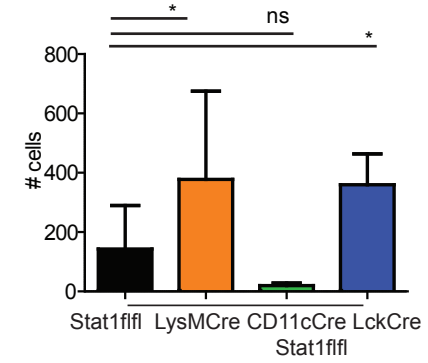
5C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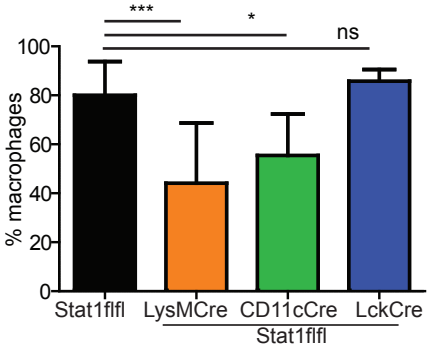
5D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CD11cCre
LckCre
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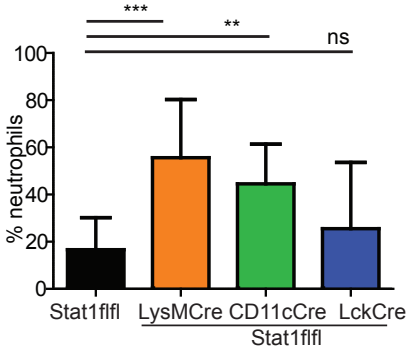
5E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40
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% neutrophils
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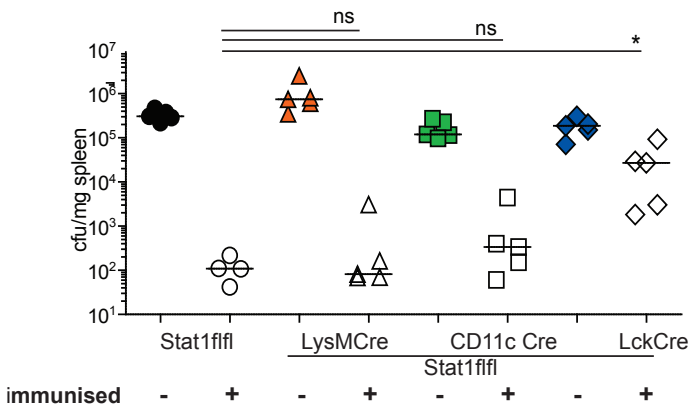
LysMCre
CD11cCre
LckCre
Stat1flfl

6A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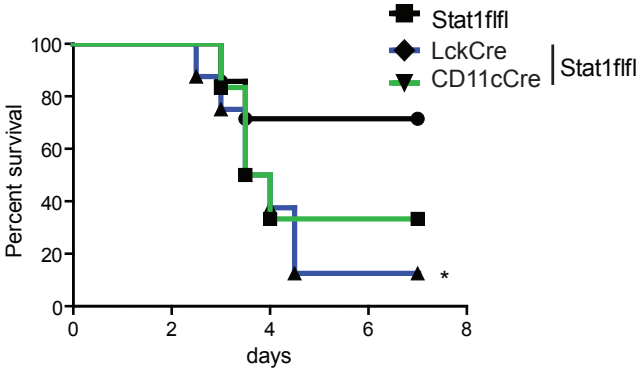
CD11c Cre
LckCre
Stat1flfl
immunised - + - + - + - +

6B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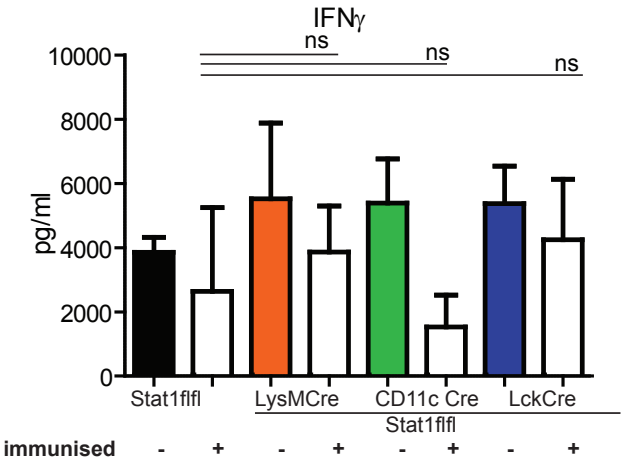
LckCre
CD11cCre
Stat1flfl
Percent survival
days
*

6C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ns
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pg/ml
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CD11c Cre
LckCre
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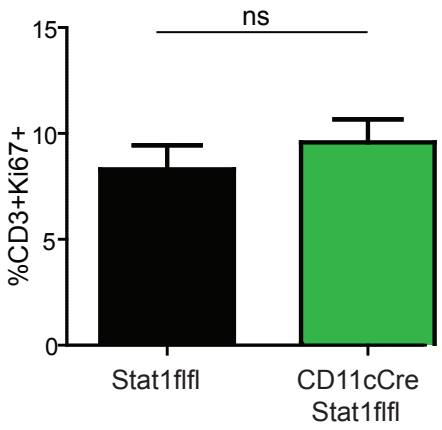
immunised - + - + - + - +

7A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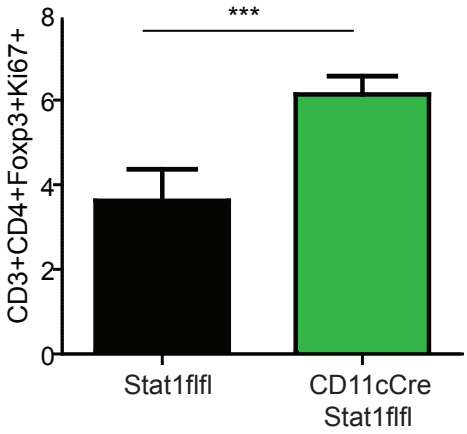
5
0
%CD3+Ki67+
Stat1flfl
CD11cCre
Stat1flfl

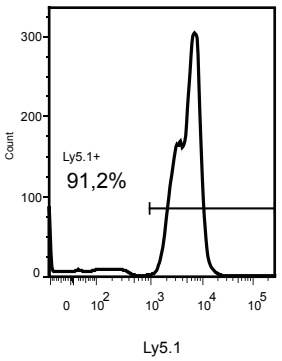
7B


***
8
6
4
2
0
CD3+CD4+Foxp3+Ki67+
Stat1flfl
CD11cCre
Stat1flfl

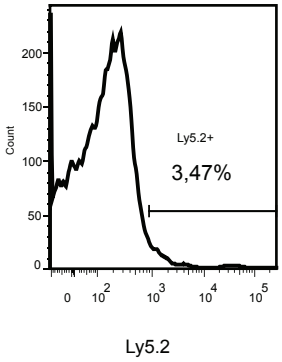
S1

WT (Ly5.1) bonemarrow implanted in Stat1-/- mice

Blood

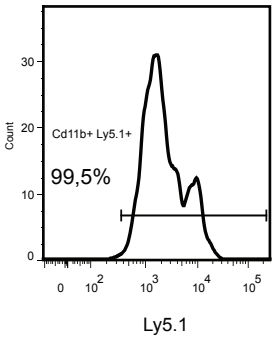


Bonemarrow

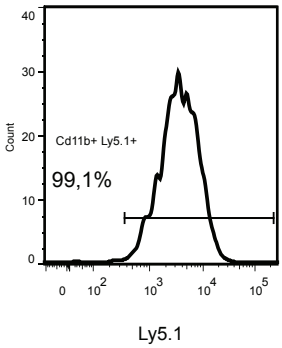


WT (Ly5.1) bonemarrow implanted in Stat1-/- mice

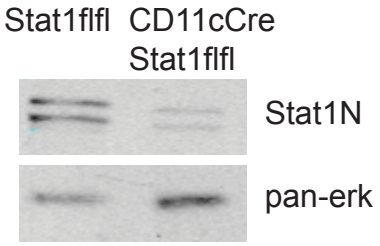
Spleen CD11b+



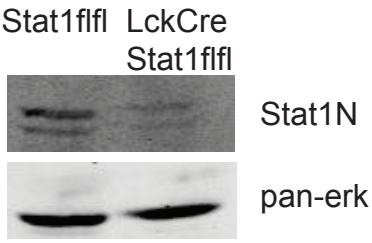
Liver CD11b+



S2a


Stat1flfl
CD11cCre
Stat1flfl
Stat1N
pan-erk

S2b


Stat1flfl
LckCre
Stat1flfl
Stat1N
pan-erk

2. Route of infection determines impact of type I Interferons on immunity to *Listeria monocytogenes*

Elisabeth Kernbauer[1], Verena Maier[1], Mathias Müller [2], Thomas Decker[1]

[1] Max F. Perutz Laboratories, University of Vienna, Dr.Bohrgasse 9/4, 1030 Vienna Austria

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Running title: Intragastric Listeria infections

Status: Manuscript in preparation for submission to Infection and Immunity

Experimental contribution: EK: 70%
VM: 30%

- mouse infections
- bacterial load assays together with VM
- cytokine analysis together with VM

Abstract

Listeria monocytogenes is a Gram positive facultative intracellular pathogen which can cause mild to life threatening disease in humans. Infection occurs after ingestion of contaminated food. The pathogen has the ability to cross epithelial barriers and to spread from the gastrointestinal tract to liver, brain and placenta. Type I Interferons (IFN-I), best known for their antiviral properties, are produced during infection and decrease host resistance after intraperitoneal or intravenous delivery of *L. monocytogenes*. Here we show that intragastric infection abrogates the detrimental effects of IFN-I. 48h after intragastric infection *L. monocytogenes* replicated to high numbers in livers and spleens of Ifnar1-/- animals lacking the IFN-I receptor and more Ifnar1-/- animals died after intragastric infection compared to WT mice. The differential impact of IFN-I on the course of intragastric versus intraperitoneal infection corresponded with altered cytokine production in Ifnar1-/- mice, especially that of IL10 and IFNγ. Taken together, our data provide the first evidence that the adverse or beneficial role of a cytokine varies with the route of infection and that IFN-I are not harmful when infection with *L. monocytogenes* occurs via the natural route.

Introduction:

Listeria monocytogenes (Lm) is a foodborne pathogen which can cause potentially life threatening disease [1]. It has the ability to cross several host barriers including the intestinal epithelium, the placental and blood brain barrier. Lm can cause severe disease in immunocompromised individuals leading to encephalitis and sepsis. It is also a major threat for pregnant woman as Lm can lead to abortion. Humans are infected with Lm via ingestion of contaminated food and although the course of infection is usually very mild, manifested disease has one of the highest mortality rates among foodborne diseases. Lm uses a set of virulence factors to successfully invade hosts. Amongst them the internalins are expressed by Lm to achieve attachment of and invasion in various different cell types. Internalin A and B have been demonstrated to be important for colonisation of epithelial cells and hepatocytes [181].

Type I interferons (IFN-I) are cytokines important for the establishment of innate antiviral immunity. Their role in bacterial infections can be either protective or detrimental, depending on the pathogen [110]. For Lm IFN-I were shown to decrease the ability of mice to combat infection, as Ifnar1-/- are more resistant compared to WT mice [152-154]. The harmful effects of IFN-I have been assigned to different aspects of the immune response. The death of effector cells such as T cells and macrophages was shown to increase as a consequence of IFN-I signalling during Lm infection [41, 154]. As a consequence of the increased uptake of apoptotic cells more Interleukin 10 (IL10) is produced in WT compared to Ifnar1-/- animals [156]. This IL10 production has been linked to higher susceptibility of WT mice to infection. In line with that IL10-/- mice are more resistant to Lm infection compared to WT mice [157]. In addition to increasing IL10, IFN-I may also interfere with macrophage activation by IFNγ by decreasing cell surface expression of the IFNGR [158].

The data describing the adverse effect of IFN-I on the course of Lm infection stem from systemic and high dose infection models, like intravenous or intraperitoneal infection. Immune reactions to intragastric Lm infections, the most natural route of infection, has not been studied intensively due to the limitations of the murine system, namely low efficiency and receptor incompatibility. Receptor incompatibility results mainly from the inability of murine E-Cadherin on epithelial surfaces to interact with the bacterial InlA [4, 200].

Here we show for the first time the effect of IFN-I during intragastric infection using a murinized Lm strain, which allows robust and efficient i.g. infection of mice. We clearly demonstrate that the route of infection matters for the impact of IFN-I on innate resistance, as mice benefit from IFN-I after intragastric infection. By contrast, cytokine profiles suggest that IFN seem to dampen a protective proinflammatory response at early stages of i.g. infection.

Results

Infection via the intragastric route changes the dependence of IFN-I for innate immune response. We infected WT and Ifnar1-/- with Lm i.g. and i.p. to determine the influence of IFN-I during different routes of infection. In keeping with previous reports, a drastic difference in the bacterial load was observed in livers and spleens of WT and Ifnar-/- mice after i.p. infection (1D, 1E). When mice where infected via the i.g. route of infection bacterial loads at d3 were similar or even slightly elevated in the liver (Fig 1A-1C). To monitor survival we infected i.g. Ifnar1-/- mice and control mice with a high dose of Lm (Fig 1F). IFN-I signalling protected mice from lethal infection with Lm, as Ifnar1-/- mice are the only animals to succumb to infection. This is in striking contrast to the results reported after i.p. or i.v. infections [154].

Bacterial loads of Lm-infected animals are routinely assessed at d3 after inoculation. However, the differences in bacterial loads at d3 after i.g. infection, insufficiently explain the increased lethality and poorer condition of Ifnar1-/- mice. In a timecourse experiment, performed to determine the time point at which the protective effects of IFN-I develop, we detected equal or reduced amounts of bacteria in livers, spleens and MLN of Ifnar1-/- mice compared to WT mice 24h after infection (Fig 2A-2C). At 48h however, Ifnar1-/- mice showed dramatically higher numbers of bacteria in these organs, especially in the liver. At day three after infection the bacterial loads in Ifnar1-/- mice were comparable to Wt levels. In striking contrast, i.p. infection did not lead to an increase in the bacterial load at 48h (Fig. 2D) or 72h (Fig. 1D, E) after infection. Likewise, Lm replication 24h after i.p. infection is not increased in Ifnar1-/- compared to Wt mice [71]. Together the result shows that IFN-I are beneficial in preventing vigorous Lm replication 48hrs after i.g. infection.

IFN-I accelerate and increase proinflammatory cytokine activity after intragastric *Listeria monocytogenes* infection. The impact of IFN-I on anti-Listeria immunity is likely to be reflected by the overall pattern of cytokine production in infected mice. To address this hypothesis we first examined IFN-I production in various target organs after i.g. infection with Lm. In spleen IFN-b mRNA was expressed up to 72 hrs after i.g. application of Lm. (Fig 3A). In accordance with expectations, the IFN-I induced gene Mx2 was expressed in infected spleens up to 72 hrs in Wt, but dramatically reduced in Ifnar1-/- mice (Fig. 3B). Serum levels of IFN-a were much lower in Ifnar1-/- mice, consistent with the role of Ifnar signalling in the amplification of IFN-a production (Fig 3C).

To determine the consequences of IFN-I on cytokine production we analysed expression of pro- and anti-inflammatory genes in spleens of animals infected by the intragastric route. Interestingly, we found a delayed onset of the expression of the genes encoding TNFa, IL6, IL10, MCP1 and IFNg in Ifnar1-/- animals (Fig 3D-3G). This may explain the increase in bacterial loads at 48h after infection. At later stages of infection expression of these cytokines increases in Ifnar1-/- mice, eventually reaching higher expression levels than in Wt controls. This finding is in line with an accelerated clearance of the bacteria from the organs of surviving Ifnar1-/- mice at d3 (fig. 2). Most impressively IFNg levels are highly increased at day 2 after infection in the serum of Ifnar1-/- mice (Fig 3H).

The detrimental or beneficial effect of IFN-I is not determined by the size of the inoculum. I.p. or i.v. injections of bacteria deliver the entire inoculum directly to internal organs. By contrast, i.g. infection might result in a more gradual release of bacteria from the intestinal tract, hence a lower primary infectious dose for internal organs. To examine whether the size of the primary inoculum deliverd to target organs influence the effect of IFN-I we infected Ifnar1-/- and control animals with 10^2 Lm i.v. Fig. 4A shows that even

at this very low dose Ifnar1-/- mice showed an increased ability to prevent Lm replication, similar to mice infected with a 100-fold higher dose. Therefore, low doses of infectious Lm alone cannot be responsible for the different impact of IFN-I. A different explanation for the difference between i.p. and i.g. infection might be provided by the mode of dissemination. Lm traversing the intestinal epithelium are thought to be taken up and spread throughout the host organism by phagocytic cells residing in mucosal lymphoid organs such as macrophages or DC [15]. We tried to mimic this situation by injecting various amounts of Lm-infected macrophages or dendritic cells i.v. to WT and Ifnar1-/- mice. Fig 4B demonstrates that intracellular delivery of Lm resulted in better clearance by cell Ifnar1-/- compared to Wt mice. Therefore intracellular dissemination cannot per se explain how the infection route shapes the impact of IFN-I on antibacterial innate immunity.

Discussion

Lm is a widely studied pathogen that has been instrumental in advancing the knowledge about innate responses to intracellular bacteria. However, the vast majority of data about immunity to Lm stems from animals infected via the intraperitoneal or intravenous routes. Comparably little knowledge has been obtained about gastrointestinal infection which represents the natural route of infection in humans. This is largely due to the incompatibility of the InlA/ E-Cadherin interaction in murine hosts, required for efficient invasion of epithelial cells. To overcome this limitation, Wollert and colleagues constructed a Lm strain expressing a mutant, 'murinized' InlA gene which interacts with mouse E-cadherin and therefore improves the invasive capacity of Lm [29]. In our study the same approach was applied to a different Lm strain, LO28, a potent inducer of IFN-I [201]. As an alternative to the mutation of Lm InlA, an improved murine infection model was generated by knock-in of the human E-cadherin to the mouse genome [28].

E-cadherin is located at the basolateral side of epithelial cells, making it less accessible to bacteria in the gut lumen. Pentecost and colleagues have shown that at sites of cell exclusion E-cadherin becomes accessible to Lm [27]. InlB also contributes to the initial uptake into host epithelia [30, 202]. Finally, the major virulence factor Listeriolysin O (LLO), a member of the bacterial hemolysin family, promotes the uptake of Lm into hepatocytes [203].

The results of our study clearly show that the route of uptake, hence the initial interactions of Lm with the host, determine the innate antibacterial response. Strikingly, the impact of IFN-I synthesis changes from being adverse to being beneficial when i.p. and i.g. routes of infection are compared. IFN-I exert pleiotropic functions ranging from anti-viral to anti-proliferative capacity. Their role in bacterial infections varies from protective to detrimental depending on the particular pathogen [110]. Their role in Lm infections has been studied in great detail showing a clear detrimental effect for the host of IFN-I during

systemic infections [152-154]. The possible mechanisms of these detrimental actions are increased cell death upon IFN-I signalling of lymphocytes and macrophages and unresponsiveness to IFNγ [204, 205]. IFN-I increase production of immunesuppressive IL10 and restrain TNFα producing cells in the spleen, limiting thereby a protective inflammatory response [152, 156] after i.p. infections. We could show that after i.g. infections a different temporal pattern of cytokine synthesis is established. TNFα, IL6 or IFNγ were expressed at lower levels in Ifnar1-/- mice early after infection, but caught up or even exceeded levels in Wt animals at later stages of infection. The delayed onset of an inflammatory response might explain the replication advantage of Lm in Ifnar1-/- mice two days after i.g. infection, whereas the increase of proinflammatory cytokines later during infection explains why mice surviving the early infection stage proceed to clear bacteria with equal efficiency as their Wt counterparts. The data show that IFN-I protect mice infected via the gastrointestinal tract at an early infection stage, but are dispensable, if this early stage is survived.

Future studies will have to clarify the reasons underlying the different immune responses after systemic or i.g. infection with Lm. Our studies rule out the possibility that the infectious dose determines the input of IFN-I because i.v. infection with very low doses of Lm did not reproduce the beneficial effect of IFN-I observed after i.g. infection. Systemic delivery of infected cells instead of suspended bacteria similarly revealed an adverse effect of IFN-I. This suggests that the difference between i.g. and systemic infection does not result from dissemination via infected cells in one situation, but not the other. However, we cannot rule out the possibility that different results would be obtained with macrophages or DC from Peyer's patches or mesenteric lymph nodes, instead of the *in vitro* differentiated cells used in our studies. The scarcity of intestinal DC and macrophages prohibits this superior experimental approach.

In summary our experiments provide clear evidence for the route of infection as a determinant of the innate response to Lm, emphasizing the importance of the immediate contact between host and pathogen for the course of infection.

Material and methods

Mice and bacteria

Animal experiments were discussed and approved by the University of Veterinary Medicine Vienna institutional ethics committee and carried out in accordance with protocols approved by the Austrian law (BMWF-68.205/0204-C/GT/2007; BMWF-68.205/0210-II/10b/2009, BMWF-68.205/0243-II/3b/2011). Bacteria were prepared for infection as described previously [71]. For infection, Lm LO28InlAmut were washed with PBS and injected intraperitoneally (i.p) or intravenously (i.v) of 8- to 10-week-old sex and age matched C57BL/6N (WT) and Ifnar1-/- (B6.129P2-IfnaR1^{tm1} [198]) mice at the respective dose. For intragastric infection mice were infected as described [206]. Briefly, mice were starved over night and orally gavaged with CaCO3 succeeded by the respective dose of Lm. The infectious dose was controlled by plating serial dilutions on Oxford agar plates. The survival of mice was monitored for 10 days, and data were displayed as Kaplan-Meier plots. For determination of bacterial loads of liver, spleen and mesenteric lymph nodes (MLN) mice were killed at the indicated time points, organs isolated and homogenized in PBS. Serial dilutions of the homogenates were plated on BHI plates and incubated at 37°C for 24 h.

Construction of Lm LO28InlAmut

To be able to construct a LO28Inlamut we prepared an InlA knockout in LO28 using the pMAD [207] vector and the following primers for amplification of the upstream and downstream region of the InlA gene of genomic DNA of Lm LO28: InlA_A 5` CATGGTCGACGGCAGTCCGCGATTTAATGGAAGT 3`, InlA_B 5` CATGGGATCC CCTAATCTATCCGCCTGAAGCGTTGT 3` InlA_C 5` CATGGGATCCGGGAATTCAGCCAGCACAACAAGT 3´ and InlAD 5`

CTGCCATGGAGGTTTAGGTGCAGTTATCCGCGT 3`. For genomic integration we used the described protocol. We transformed the LO28 Inla knockout strain with the pAUL-A InlA-inlB construct kindly provided by Wollert [29] and obtained genomic integration according to protocol [208]. Here the abbreviation Lm for experiments always refers to the murinized InlA in LO28.

RNA isolation, cDNA synthesis and Real time PCR

Organs of respective animals were isolated and stored at -80°C until further use. For RNA preparation organs were homogenized in RA1 buffer of the NucleoSpin II RNA isolation kit (Macherey and Nagel) and processed according to protocol. cDNA was prepared as described.

The realtime PCRs were run on an Eppendorf cycler. After correction for the housekeeping gene Gapdh, every sample was calculated to the mean of WT mRNA levels.

Cytokine analysis

For cytokine analysis mice were bled via the retro-orbital sinuses and serum was isolated and stored at -80°C. The indicated cytokines and chemokines were measured using the FlowCytomix kits by ebioscience in 25µl of serum (IFNγ, IL6, IL10, IL12p70, Mcp1, Mcp3, Rantes, GMCSF, Mip1α, Mip1β, IP10, IL22, TNFα).

Cell culture

Bone marrow derived cells were isolated and grown as described [71]. (Briefly, bone marrow was isolated from femurs of 6–8 week old mice. For differentiation of BMM, cells were grown in DMEM (Gibco, Invitrogen) in the presence of 10% FCS (Gibco, Invitrogen) and L-cell derived CSF-1 as described. The cultures contained >99% F4/80+ cells. mDCs were obtained by culture of bone marrow in DMEM (Gibco, Invitrogen)

supplemented with 10% FCS (Gibco, Invitrogen) and X-6310 derived GM-CSF as described. mDC cultures contained virtually no F4/80+ cells and the purity of CD11c+/CD11b+ cells was between 60 and 70%.)

Statistical analysis

Bacterial loads of organs were compared using the Mann-Whitney test and mRNA expression data was analysed with the Students t test. For both the GraphPad Software was used.

References

1. Vazquez-Boland, J.A., et al., *Listeria pathogenesis and molecular virulence determinants.* Clin Microbiol Rev, 2001. **14**(3): p. 584-640.
2. Hamon, M., H. Bierne, and P. Cossart, *Listeria monocytogenes: a multifaceted model.* Nat Rev Microbiol, 2006. **4**(6): p. 423-34.
3. Decker, T., M. Muller, and S. Stockinger, *The yin and yang of type I interferon activity in bacterial infection.* Nat Rev Immunol, 2005. **5**(9): p. 675-87.
4. Auerbuch, V., et al., *Mice lacking the type I interferon receptor are resistant to Listeria monocytogenes.* J Exp Med, 2004. **200**(4): p. 527-33.
5. Carrero, J.A., B. Calderon, and E.R. Unanue, *Type I interferon sensitizes lymphocytes to apoptosis and reduces resistance to Listeria infection.* J Exp Med, 2004. **200**(4): p. 535-40.
6. O'Connell, R.M., et al., *Type I interferon production enhances susceptibility to Listeria monocytogenes infection.* J Exp Med, 2004. **200**(4): p. 437-45.
7. Zwaferink, H., et al., *IFN-beta increases listeriolysin O-induced membrane permeabilization and death of macrophages.* J Immunol, 2008. **180**(6): p. 4116-23.
8. Carrero, J.A., B. Calderon, and E.R. Unanue, *Lymphocytes are detrimental during the early innate immune response against Listeria monocytogenes.* J Exp Med, 2006. **203**(4): p. 933-40.
9. Dai, W.J., G. Kohler, and F. Brombacher, *Both innate and acquired immunity to Listeria monocytogenes infection are increased in IL-10-deficient mice.* J Immunol, 1997. **158**(5): p. 2259-67.
10. Rayamajhi, M., et al., *Induction of IFN-alphabeta enables Listeria monocytogenes to suppress macrophage activation by IFN-gamma.* J Exp Med, 2010. **207**(2): p. 327-37.
11. Czuprynski, C.J., N.G. Faith, and H. Steinberg, *A/J mice are susceptible and C57BL/6 mice are resistant to Listeria monocytogenes infection by intragastric inoculation.* Infect Immun, 2003. **71**(2): p. 682-9.
12. Cossart, P., *Illuminating the landscape of host-pathogen interactions with the bacterium Listeria monocytogenes.* Proc Natl Acad Sci U S A. **108**(49): p. 19484-91.
13. Stockinger, S., et al., *Characterization of the interferon-producing cell in mice infected with Listeria monocytogenes.* PLoS Pathog, 2009. **5**(3): p. e1000355.

14. Pron, B., et al., *Dendritic cells are early cellular targets of Listeria monocytogenes after intestinal delivery and are involved in bacterial spread in the host.* Cell Microbiol, 2001. **3**(5): p. 331-40.
15. Wollert, T., et al., *Extending the host range of Listeria monocytogenes by rational protein design.* Cell, 2007. **129**(5): p. 891-902.
16. Reutterer, B., et al., *Type I IFN are host modulators of strain-specific Listeria monocytogenes virulence.* Cell Microbiol, 2008. **10**(5): p. 1116-29.
17. Disson, O., et al., *Modeling human listeriosis in natural and genetically engineered animals.* Nat Protoc, 2009. **4**(6): p. 799-810.
18. Pentecost, M., et al., *Listeria monocytogenes invades the epithelial junctions at sites of cell extrusion.* PLoS Pathog, 2006. **2**(1): p. e3.
19. Chiba, S., et al., *Listerial invasion protein internalin B promotes entry into ileal Peyer's patches in vivo.* Microbiol Immunol. **55**(2): p. 123-9.
20. Pentecost, M., et al., *Listeria monocytogenes internalin B activates junctional endocytosis to accelerate intestinal invasion.* PLoS Pathog. **6**(5): p. e1000900.
21. Vadia, S., et al., *The Pore-Forming Toxin Listeriolysin O Mediates a Novel Entry Pathway of L. monocytogenes into Human Hepatocytes.* PLoS Pathog. **7**(11): p. e1002356.
22. Rayamajhi, M., et al., *Antagonistic crosstalk between type I and II interferons and increased host susceptibility to bacterial infections.* Virulence. **1**(5): p. 418-22.
23. Carrero, J.A. and E.R. Unanue, *Impact of lymphocyte apoptosis on the innate immune stages of infection.* Immunol Res, 2007. **38**(1-3): p. 333-41.
24. Muller, U., et al., *Functional role of type I and type II interferons in antiviral defense.* Science, 1994. **264**(5167): p. 1918-21.
25. Cabanes, D., M. Lecuit, and P. Cossart, *Animal models of Listeria infection.* Curr Protoc Microbiol, 2008. **Chapter 9**: p. Unit9B 1.
26. Arnaud, M., A. Chastanet, and M. Debarbouille, *New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, gram-positive bacteria.* Appl Environ Microbiol, 2004. **70**(11): p. 6887-91.
27. Lingnau, A., et al., *Expression of the Listeria monocytogenes EGD inlA and inlB genes, whose products mediate bacterial entry into tissue culture cell lines, by PrfA-dependent and -independent mechanisms.* Infect Immun, 1995. **63**(10): p. 3896-903.

Acknowlegments

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Figure legends

Figure 1: **IFN-I increase host resistance after intragastric infection with Lm.** C57BL/6 and Ifnar1-/- mice were infected i.g. with 5x10^9 Lm and bacterial load was examined 72h after infection of liver (1A), spleen (1B) and MLN (1C). Bacterial loads 72h after i.p. infections were determined 72h after infection with 1x10^6 Lm of livers (1D) and spleens (1E). 7 mice per group were infected i.g. with 7x10^9 Lm and survival was monitored over ten days.

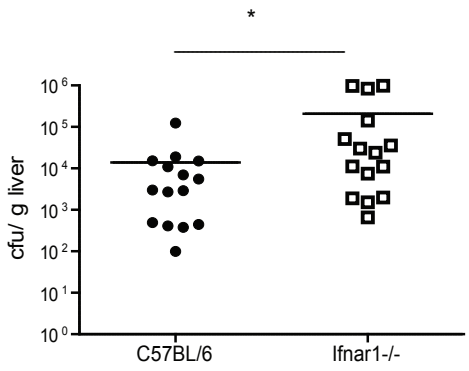
Figure 2: **Bacterial burden after various periods of intragastric infections of Wt and Ifnar1-/- mice with Lm.** C57BL/6 and Ifnar1-/- mice were infected with 5x10^9 i.g. and bacterial loads of livers (2A), spleens (2B) and MLN (2C) were monitored over four days. The bacterial load after 48h was determined of spleen (2D) and liver after i.p. infection of C57BL/6 and Ifnar1-/- mice (2E).

Figure 3: **Delayed cytokine response in Ifnar1-/- compared to Wt mice after i.g. infection with Lm.** Cytokine expression after i.g. infection was determined 24h, 48h and 72h after infection. IFNβ and the IFN-I inducible gene MX2 were expressed in spleens upon infection. Relative inductions compared to non infected (n.i.) are shown (3A, 3B). Serum levels of IFNα were determined after three days after i.g. infection (3C). TNFα

(3D), IL6 (3E), IL10 (3F), MCP1 (3G) and IFNγ (3H) mRNA levels were measured after extracting RNA from spleens of infected animals. IFNγ levels were determined in the serum of C57BL/6 and Ifnar1-/- mice infected i.g. for 24h, 48h or 72h, respectively (3I).

Figure 4: **Low infectious doses of infection or dissemination via infected cells do not alter the adverse effect of IFN-I after systemic infection. Doses of** 10^2 and 10^4 Lm were injected i.v. into C57BL/6 and Ifnar1-/- mice and bacterial loads in the spleen were monitored 72h after infection (4A). BMDM and BMDC were infected *in vitro* with a MOI of 10 for 1h and were then injected i.v. into C57BL/6 and Ifnar1-/- mice. Bacterial loads in the spleen were measured 72h after infection (4B).

1A



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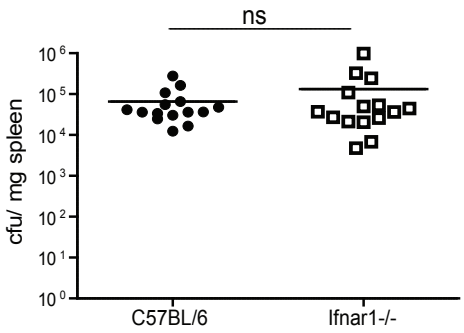

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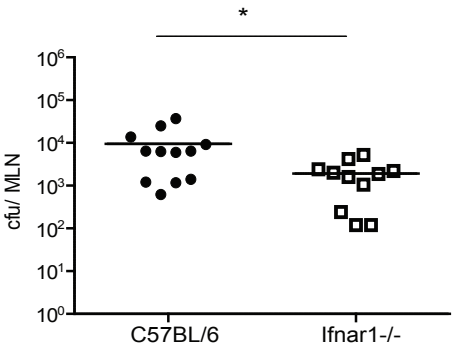

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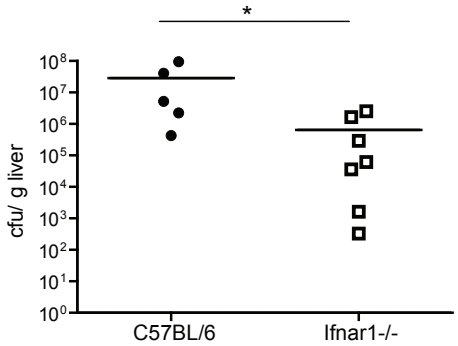

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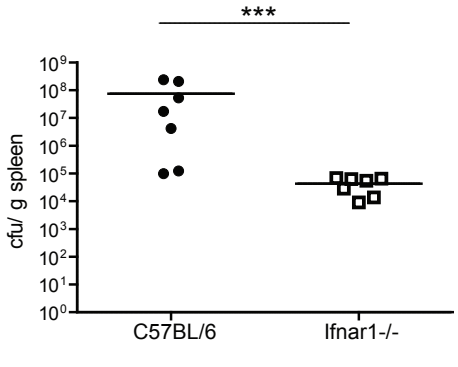

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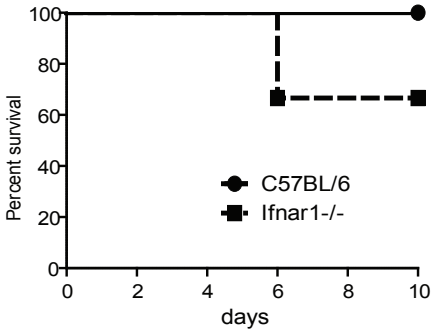

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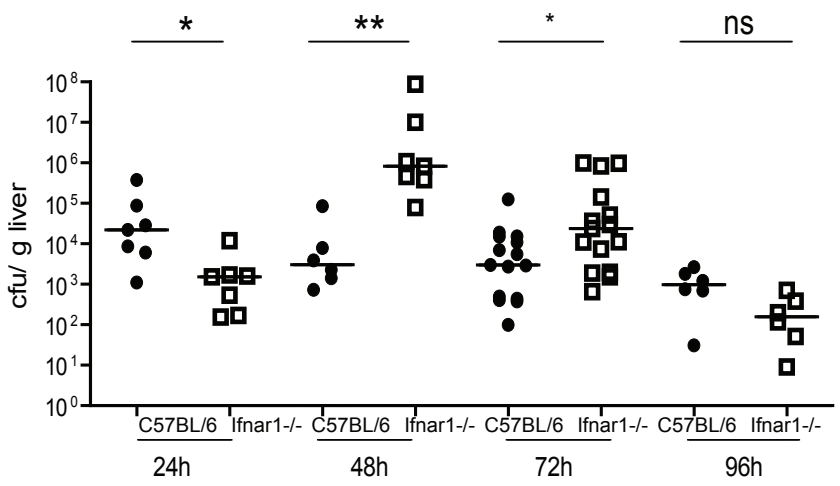
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Ifnar1-/-
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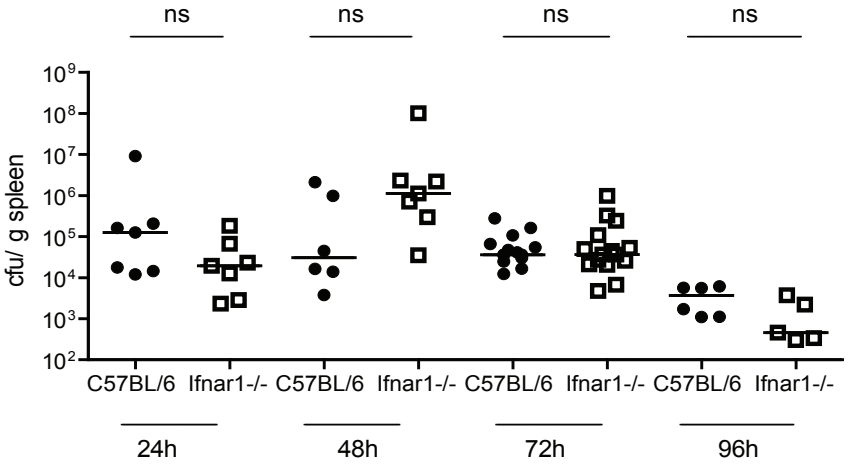
72h
96h

2B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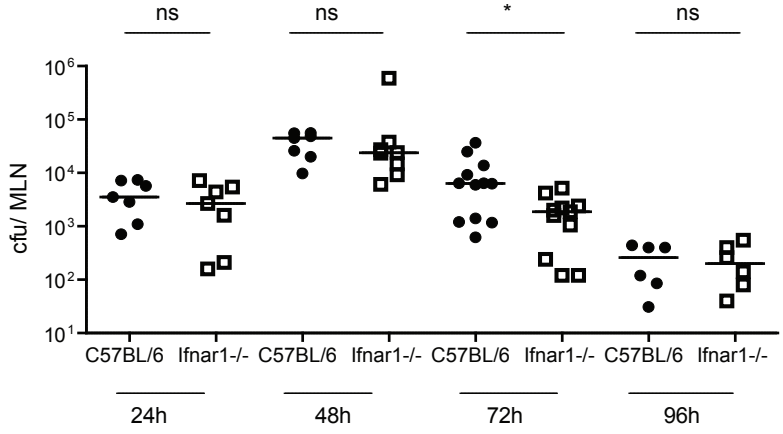
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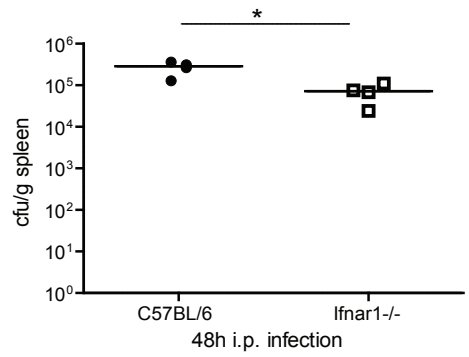
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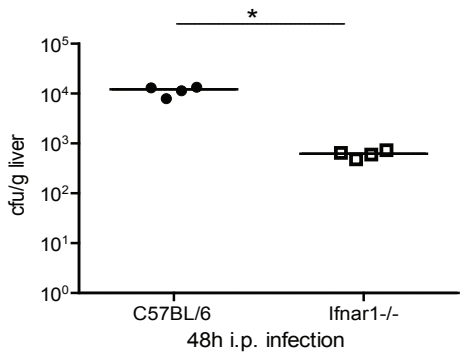
2E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Ifnar1-/-
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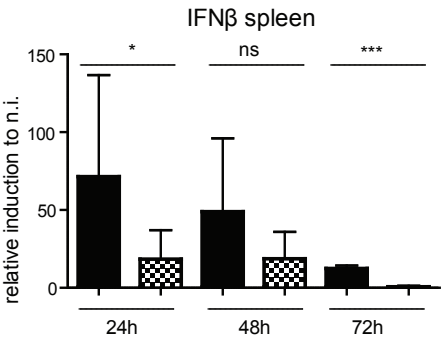
3A



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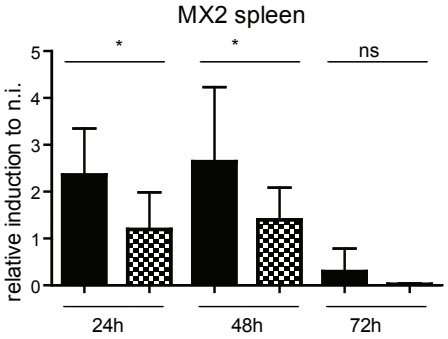

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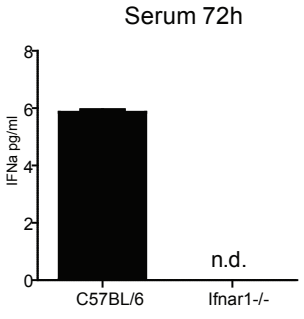

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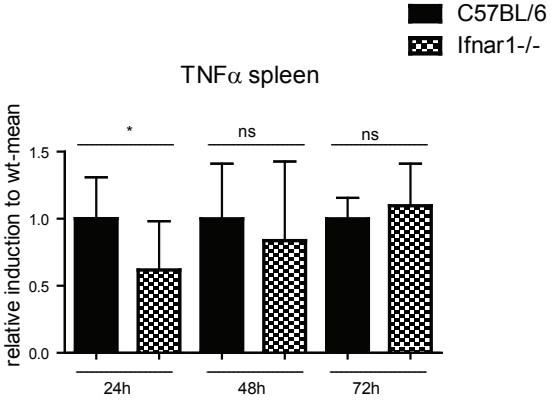

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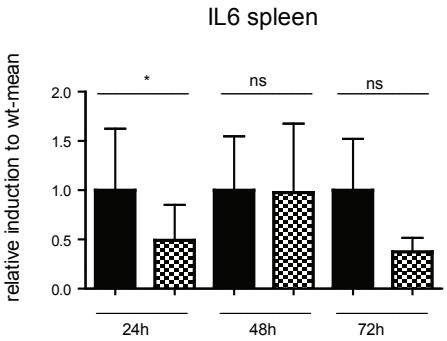

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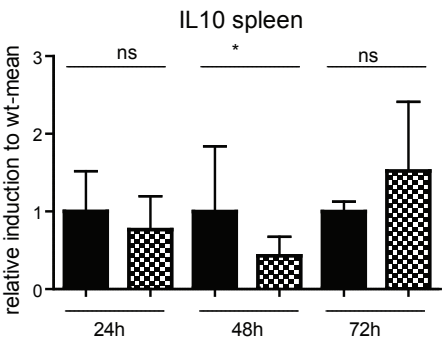

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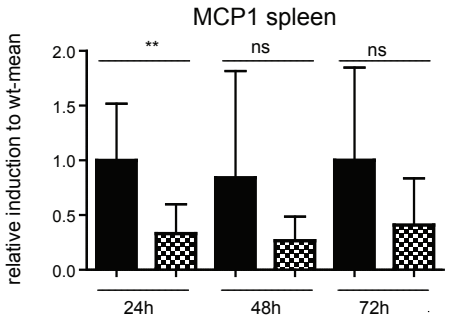
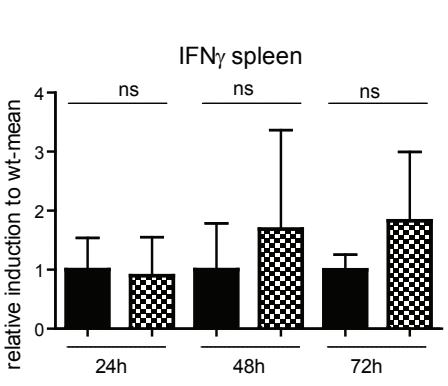


3H



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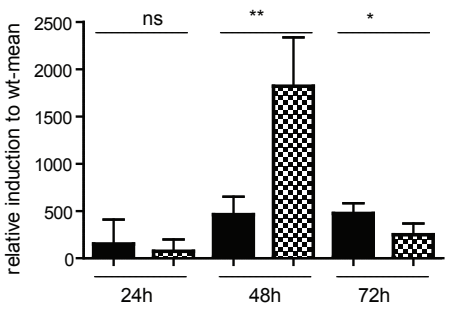

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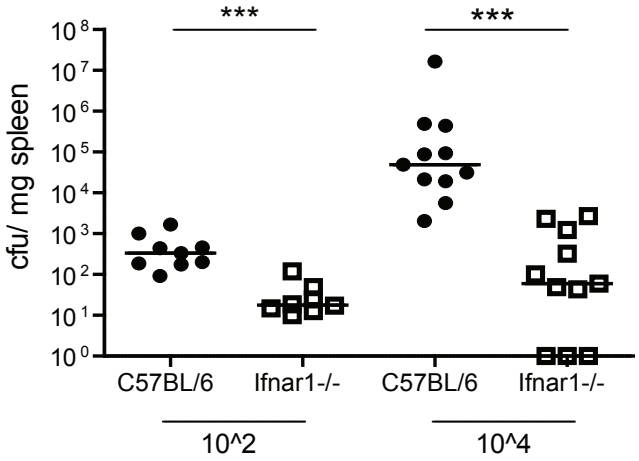
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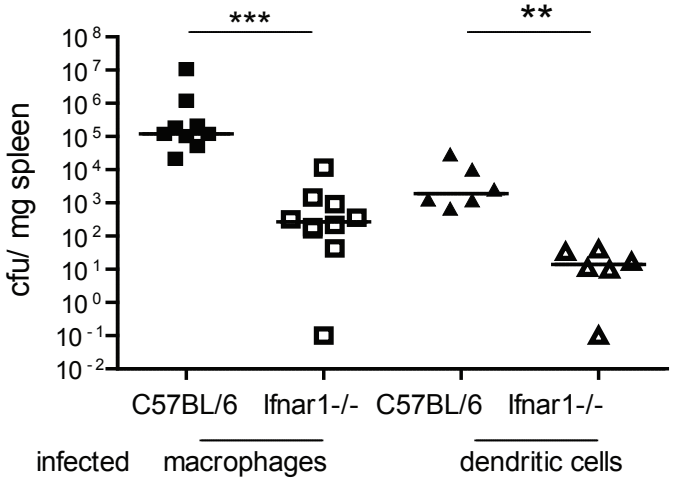
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Ifnar1-/-
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macrophages
dendritic cells

3. Characterization of the Interferon-Producing Cell in Mice Infected with *Listeria monocytogenes*

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Experimental contribution: EK 30%

- Cytokine measurements
- FACS Sort of cells
- RNA expression analysis
- Analysis in bone marrow derived macrophages

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Characterization of the Interferon-Producing Cell in Mice Infected with *Listeria monocytogenes*

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Abstract

Production of type I interferons (IFN-I, mainly IFNα and IFNβ) is a hallmark of innate immune responses to all classes of pathogens. When viral infection spreads to lymphoid organs, the majority of systemic IFN-I is produced by a specialized "interferon-producing cell" (IPC) that has been shown to belong to the lineage of plasmacytoid dendritic cells (pDC). It is unclear whether production of systemic IFN-I is generally attributable to pDC irrespective of the nature of the infecting pathogen. We have addressed this question by studying infections of mice with the intracellular bacterium *Listeria monocytogenes*. Protective innate immunity against this pathogen is weakened by IFN-I activity. In mice infected with *L. monocytogenes*, systemic IFN-I was amplified via IFN-β, the IFN-I receptor (IFNAR), and transcription factor interferon regulatory factor 7 (IRF7), a molecular circuitry usually characteristic of non-pDC producers. Synthesis of serum IFN-I did not require TLR9. In contrast, *in vitro*–differentiated pDC infected with *L. monocytogenes* needed TLR9 to transcribe IFN-I mRNA. Consistent with the assumption that pDC are not the producers of systemic IFN-I, conditional ablation of the IFN-I receptor in mice showed that most systemic IFN-I is produced by myeloid cells. Furthermore, results obtained with FACS-purified splenic cell populations from infected mice confirmed the assumption that a cell type with surface antigens characteristic of macrophages and not of pDC is responsible for bulk IFN-I synthesis. The amount of IFN-I produced in the investigated mouse lines was inversely correlated to the resistance to lethal infection. Based on these data, we propose that the engagement of pDC, the mode of IFN-I mobilization, as well as the shaping of the antimicrobial innate immune response by IFN-I differ between intracellular pathogens.

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☯ RK and EK contributed equally to this study.

Introduction

Type I interferons (IFN-I) comprise a family of around 20 members that bind a common receptor, the type I IFN receptor (IFNAR) [1]. Immunologically most relevant are IFNβ, with only one member in humans and mice, and the IFNα family with more than ten members. Pattern recognition receptors (PRR) for all classes of microbes are able to stimulate transcription of the IFN-I genes, establishing IFN-I production as a hallmark of innate immune responses to a vast number of viral and nonviral pathogens [2–8]. A common property of these receptors is to stimulate the activation of one or more transcription factors of the interferon regulatory factor (IRF) family, most importantly IRF3 and IRF7 [9].

Cells producing IFN-I employ different molecular strategies to induce synthesis of the cytokines. A feed-forward amplification mode is characterized by IRF3-dependent IFNβ production that predominates the early phase of infection. In a subsequent phase signaling of the IFNβ through the IFNAR stimulates activation of the transcription factor ISGF3 consisting of a STAT1/STAT2 heterodimer in conjunction with IRF9 [10]. This transcriptional complex activates the IRF7 gene promoter and initiates expression of the IRF7 mRNA. Finally, IRF7 is instrumental in transcribing multiple IFNα genes in addition to IFNβ [11]. An alternative way of stimulating IFN-I was described for virus-infected or toll-like receptor (TLR) ligand-stimulated plasmacytoid dendritic cells and, under certain conditions, myeloid dendritic cells (pDC and mDC, respectively) [12–14]. In these cells, the IFN-I genes are targeted by a pathway originating from endosomal TLRs (TLR 7 and TLR 9) that assemble a signalosome including MyD88 and IRF7 together with other IRFs such as IRF1 or IRF5 [15–20]. In pDC the TLR signal is rapidly relayed to IRF7, which is available for immediate stimulation of all IFN-I promoters. Thus, pDC produce vast quantities of IFN-I in response to infection with viruses and are generally referred to as interferon-producing cells (IPC).

Author Summary

Type I Interferons (IFN-I) are cytokines produced by the innate immune system immediately after intrusion of a pathogen. To produce large quantities of IFN-I once an infection is starting to spread throughout the body, the innate immune system employs a specialized "interferon-producing cell" (IPC). In the case of viral infections, IFN-I protect the host organism from rapid viral replication and spread. Conversely, organisms that cannot produce IFN-I are exquisitely sensitive to viral infections. Intriguingly, the opposite has been reported for the pathogen *Listeria monocytogenes*. Like virus, this bacterium replicates within cells of the host organism and stimulates IFN-I synthesis. Unlike virus, however, IFN-I sensitize the infected host to lethal pathology resulting from *L. monocytogenes* infection. In this article, we show that all tested molecules contributing to IFN-I production in Listeria-infected mice are responsible for a corresponding increase in mortality. We address the question of which cell type is responsible for producing vast quantities of IFN-I that can be measured in the serum of mice infected with Listeria. We show that these are not IPC, but rather macrophages, cells specialized to ingest and kill bacteria. We conclude that the engagement of cells for IFN-I production and also the effect of IFN-I on innate immunity is determined by the tropism and lifestyle of a particular pathogen.

Listeria monocytogenes is a Gram-positive, food-borne bacterial pathogen [21]. Major sites of Listeria replication during systemic infection of mammals are liver and spleen. Infection is exacerbated by the activity of IFN-I, shown convincingly by the increased resistance of IFNAR or IRF3-deficient mice to lethal infection [22–24]. Several explanations for this IFN-I effect have been provided, including decreases in TNF production or an enhancement of macrophage death [22–25]. Moreover, IFN-I were shown to sensitize cytotoxic T lymphocytes to the lytic action of LLO [24]. Enhanced lymphocyte killing and IL-10-dependent suppression of innate immunity as a result of phagocytic uptake of apoptotic cells increase the severity of infection-borne pathological effects [26].

L. monocytogenes is endowed with the ability to infect the cytoplasm of cells in the host organism [27]. This ability results from the endo/phagosome-disrupting activity of the major *L. monocytogenes* virulence factor, the Listeriolysin O (LLO), a bacterial cholesterol-dependent hemolysin [28]. A common property of *L. monocytogenes* and viruses is that recognition and signaling occur through both membrane-associated and cytosolic PRR [3]. In contrast to viruses, the cytosolic *L. monocytogenes* receptors mediating IFN-I induction have not been identified. In *L. monocytogenes*-infected macrophages, IFN-I synthesis occurs via feed-forward amplification [29]. The exact pathway and cell type responsible for IFN-I production during infection of mice with *L. monocytogenes* have not been examined. Considering the fact that IFN-I enhance the adverse effects of the early, innate immune response to *L. monocytogenes* [22–24], it is of major importance to understand their mobilization and mode of action.

In this study we sought to identify the essential IFN-I-producing cells during infection with *L. monocytogenes*. We used sorted splenic cell populations and multiple mouse models with defects in the IFN-I production machinery or with tissue-specific disruption of the *ifnar1* gene to decipher the pathways and cells required for both IFNβ and IFNα production. Our results show that in contrast to viral infection models, pDC are not the source of IFN-I in response to *L. monocytogenes*. Rather, the cytokines are produced via feed-forward amplification by a splenic cell with cell surface markers characteristic of macrophages. We also show for the first time that IFNβ and IRF7 make strong contributions to the harmful action of IFN-I in *L. monocytogenes* infection because the lower levels of IFN-I production in these mouse lines were correlated with decreased susceptibility to lethal infection.

Results

Prerequisites for IFN-I production in mice and cells infected with *L. monocytogenes*

Knock-out mice were used to study pathways contributing to increase of IFNβ and IFNα in the serum of mice infected with *L. monocytogenes*. We established 24 h post intraperitoneal (i.p.) injection of bacteria as the point of maximum IFN-I induction in preliminary experiments (data not shown); therefore, IFNβ and IFNα were measured 24 h after infection with *L. monocytogenes* in subsequent experiments. Serum levels of IFNβ were generally at the detection limit of the ELISA and differences between wt and the IRF3−/− or IRF7−/− genotypes could not be reliably determined. By contrast, statistically significant differences were observed comparing wt and IFNAR1−/− animals (Figure 1A). This finding most likely reflects the importance of the IFNAR for IFNβ clearance. Increased accumulation of the cytokine in the blood of IFNAR−/− mice is therefore not in contradiction to subsequent findings showing that a fraction of IFNβ production occurs via feed-forward amplification through the IFNAR (e.g. Figures 2 and 3). Serum of infected wt mice contained higher levels of IFNα than IFNβ. IFNα species were reduced in absence of IFNβ, IRF3 or IRF7 (Figure 1B). Serum IFNα was virtually absent in mice lacking IFNAR1. Collectively the data show that the levels of serum IFNα critically depend on 'early' IFNβ and on IFN-I signaling, hence feed-forward amplification. Bacterial loads in liver and spleen after 24 h of infection were virtually identical between wt and IFNAR−/− mice (Figure S1). Therefore, differences in IFN-I production did not result from lower numbers of infecting bacteria.

The spleen is a major target organ of *L. monocytogenes*, irrespective of the route of infection by intragastric, intraperitoneal or intravenous application [21,30]. Therefore, we tested both IFN-I production and response in spleens following infection with *L. monocytogenes* (Figure 2A and 2B, and Figure S2A). Production of both IFNβ and IFNα mRNA, assayed by RT-PCR (Figure 2A and 2B), was observed in wt animals. IFNβ mRNA synthesis required both IRF3 and IRF7 and, surprisingly, the IFNAR. Splenic IFNα production was reduced in absence of IRF3 or IFNβ and even more affected by the lack of IRF7 or the IFNAR. A representative analysis of four mice/genotype is shown in Figure 2A, whereas Figure 2B summarizes all experiments to show how many mice of each genotype produced IFN-I and account for individual responses to Listeria infection.

STAT2 is a subunit of the ISGF3 transcription factor complex and its tyrosine phosphorylation is a hallmark of IFN-I-treated cells [10]. Phosphorylated STAT2 was absent in IFNAR1−/− spleens, reduced slightly in absence of IFNβ and IRF3, and reduced strongly in absence of IRF7 (Figure S2A). STAT1 tyrosine phosphorylation occurs in response to both IFN-I and IFNγ [10]. Likewise, the STAT target gene Gbp2 (Figure 2A) responds to both IFN types. Both STAT1 tyrosine phosphorylation and Gbp2 expression occurred at levels similar to wt in all genotypes including IFNAR1−/− (Figure S2A). In agreement with this, IFNγ mRNA levels were equal to wildtype in all investigated genotypes (data not shown). Together the data show that the molecular requirements for splenic IFN-I production are

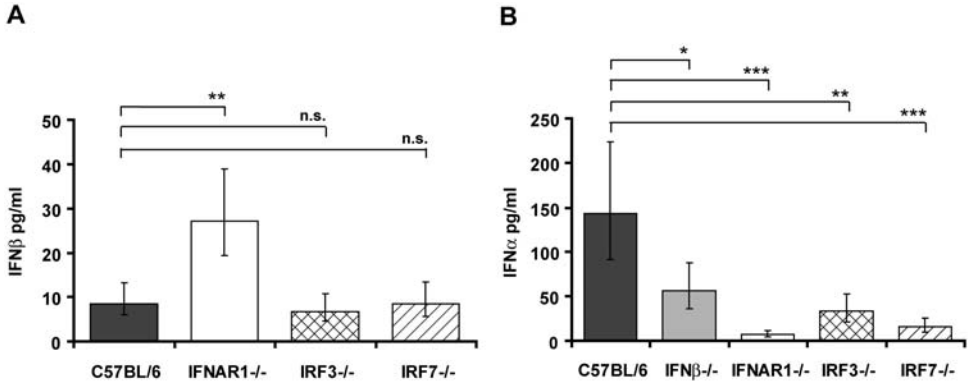

Figure 1. Serum levels of IFN-I in mice deficient in components of the IFN-I signaling pathway. Mice of the indicated genotypes were injected intraperitoneally (i.p.) with 5×10^6 *L. monocytogenes* (C57BL/6 n = 25, IFNβ−/− n = 25, IFNAR1−/− n = 24, IRF3−/− n = 25, IRF7−/− n = 20) or PBS as a control (data not shown). After 24 h, serum was collected and ELISAs for IFNβ (A) and IFNα (B) were performed. The data presented here are a summary of several individual experiments with groups of four to five infected mice per genotype. Data representing IFNβ (A) and IFNα (B) concentrations were log-transformed (after adding one) to achieve approximate normality. Linear models with genotype and experiment as fixed effects were fitted using SPSS. Means plus/minus standard errors of wt and mutant genotypes are plotted (after back-transformation). Significant values are indicated by: n.s. not significant $p>0.05$, * $p\leq0.05$, ** $p\leq0.01$, *** $p\leq0.001$.
doi:10.1371/journal.ppat.1000355.g001

very similar to those found for serum IFNα production. In both cases signaling through IFNAR and upregulation of IRF7 expression are of critical importance and indicate feed-forward amplification.

In bone-marrow-derived macrophages (BMM), *L. monocytogenes* stimulates IFNβ synthesis through the IRF3 pathway independently of TLR9 and MyD88 [29,31]. Requirements for IFN-I production were further investigated in BMM and mDC infected with *L. monocytogenes*. IRF7 or IFNAR-deficiency had no effect on BMM IFNβ production (Figure 2C). Unlike IFNβ, IFNα production by infected BMM was dependent on IRF3, IRF7, IFNβ and the IFNAR (Figure 2D). In case of IRF7-deficiency, residual IFN-I still caused unimpaired tyrosine phosphorylation of STAT2 (Figure S2B). In mDC, surprisingly, both IFNβ and IFNα production strictly required IRF3, IRF7 and IFNAR1 (Figure 2E and 2F).

Prerequisites for the increase in resistance to lethal infection caused by IFN-I

To investigate the contribution of IFNβ and IRF7 to the IFN-I-dependent increase in mortality, we monitored the survival of animals infected with *L. monocytogenes*. In accordance with previous findings [22–24], IFNAR deficiency caused a strong resistance to lethal infection, particularly during the innate phase of the anti-Listeria immune response (up to day 6, Figure 4A). By comparison, the increase caused by IFNβ deficiency was less pronounced. Lack of IRF7 had a greater impact on survival than absence of IRF3. This difference became smaller if survival was monitored beyond the period of the innate immune response (up to day 10). Lower pathogen burdens in liver and spleens from IRF7 or IFNβ-deficient mice reflected the increase in survival on day 3 (Figure 4B). Similar findings have been reported for IFNAR1 and IRF3-deficient mice [22–24]. Since differences in the bacterial load between wt animals and those with defects in IFN-I synthesis and/or response are not present 24 h after infection (Figure S1) the inhibitory effect of IFN-I on bacterial clearance must develop between day 1 and 3 post infection. Together, the data emphasize the importance of the early IFNAR, IFNβ and IRF7-mediated amplification of IFN-I production for adverse IFN-I action during the early, innate immune response.

Myeloid cells contribute to IFN-I production and the IFN-I–dependent sensitization of mice to lethal infection with *L. monocytogenes*

To examine the cell type(s) important for IFN-I production and response during *L. monocytogenes* infection, we made use of tissue-specific IFNAR ablation. The contribution of myeloid cells to IFN-I effects was tested by mating mice with a floxed *ifnar1* allele [32] with LysM-Cre mice [33]. Using this technology, a high degree of floxed allele conversion is obtained in macrophages and neutrophils, whereas it is inefficient in splenic DC [33]. In accordance with these observations, IFNAR1 expression in $IFNAR^{fl/fl}$-LysM-Cre mice was strongly reduced in $CD11b^+$ cells that include macrophages, but similar to wt on $CD11c^+$ cells that include myeloid and plasmacytoid DC (Figure S3). Absence of the IFNAR in myeloid cells caused a highly significant increase in the survival of Listeria-infected mice during the innate phase of the immune response up to day 6 (Figure 3A). In this period we observed 100% survival in mice with complete IFNAR1 deletion and 80% survival in mice with myeloid IFNAR1 deletion. At later times the survival curves diverged further, most likely due to a contribution of non-myeloid cells to the lethal outcome of infection. The increase in survival compared to wt mice could result from an important role of myeloid cells in IFN-I production. Alternatively, the myeloid response to IFN-I might directly contribute to the cytokines' detrimental action. To distinguish between these possibilities, serum IFN–I was compared in wt mice and mice lacking the IFNAR either on all or only on myeloid cells. This experiment revealed a major contribution of myeloid cells to serum IFNα by IFNAR-dependent feed-forward amplification (Figure 3B). Differences in the low level of serum IFNβ were not statistically significant between wt mice and those disrupted for the myeloid *ifnar1* gene (data not shown).

Analysis of splenic IFN-I production and response in mice lacking the myeloid IFNAR showed that IFNα mRNA was completely absent (Figure 3C). Residual IFNβ mRNA was present, but the induction was reduced and more transient (compare 24 h and 48 h time points). No difference between genotypes was observed for the induction of Gbp2.

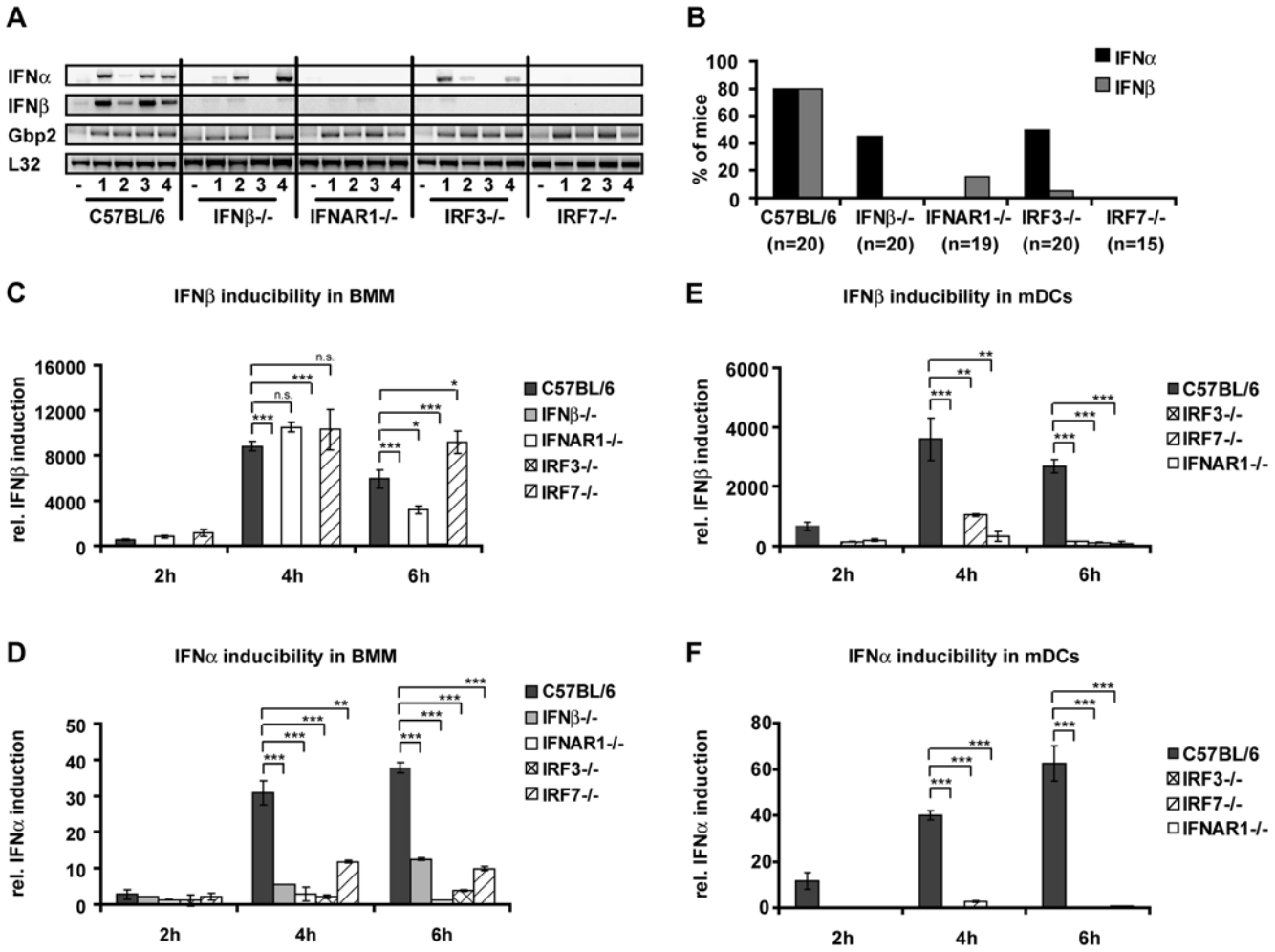

Figure 2. Prerequisites for IFN-I production in infected spleens and *in vitro*–generated BMM and mDCs. Mice of the indicated genotypes were injected i.p. with 5×10^6 *L. monocytogenes* or PBS as a control. After 24 h of infection, mice were killed and spleens and livers isolated. (A) RNA was extracted from spleens of four infected mice per genotype (1–4) or from mice injected with PBS (−), and RT-PCR for the indicated genes was performed. As a normalization control, the housekeeping gene L32 was measured. (B) Summary of experiments performed with mice of the indicated genotypes. In total, 20 C57BL/6, 20 IFNβ−/−, 19 IFNAR1−/−, 20 IRF3−/−, and 15 IRF7−/− mice were analysed for IFN-I expression and expression of Gbp2. The graph shows the percentage of mice of the respective genotypes showing detectable IFNα (black) or IFNβ (grey) expression after infection with 5×10^6 *L. monocytogenes* for 24 h. (C–F) BMM (C,D) or mDC (E,F) of the indicated genotypes were infected with *L. monocytogenes* at a MOI of 10. At the indicated time points, total RNA was prepared. The isolated RNA was reverse-transcribed and induction of the IFNβ (C,E) or IFNα genes (D,F) was measured by real-time PCR. For normalization to a housekeeping gene, GAPDH was measured.
doi:10.1371/journal.ppat.1000355.g002

IFN-I production and response in *L. monocytogenes*–infected hosts is independent of the TLR9 pathway

The data collected so far suggest that the majority of IFN-I is produced by a splenic cell type that employs an IFNβ/IFNAR/IRF7 pathway for feed-forward amplification of IFNα synthesis. This profile does not match IFN-I production by pDC where expression of IFN-I genes is regulated by a pathway originating from endosomal TLRs [34]. To further exclude the relevance of this pathway for IFN-I production during Listeria infection, TLR9-deficient mice were analyzed. The data summarized in Figure 5 show that neither serum IFNβ or IFNα (Figure 5A and 5B), nor splenic IFN-I mRNA synthesis or response were reduced in the absence of TLR9 (Figure 5C, Figure S4A). By contrast, production of IFN-I mRNA in bone-marrow-derived pDCs exposed to *L. monocytogenes* showed strong (IFNβ) or absolute (IFNα) dependence on TLR9 and/or MyD88 (Figure 5D–5F). Both IFNβ and IFNα mRNA synthesis were strongly affected by IRF7 deficiency and, surprisingly, also the absence of IRF3 (Figure 5G and 5H). Unlike pDC, mDC produced IFNβ independently of the TLR9 pathway, resembling macrophages in this regard (Figure S4B). Consistent with IFN-I production (Figure 5A–5C), the absence of TLR9 did not increase survival following infection with *L. monocytogenes* (Figure 5I). Rather, TLR9 deficiency slightly enhanced the lethality of *L. monocytogenes* infection, suggesting that the TLR9 pathway plays a protective role.

Cells with a surface profile characteristic of macrophages are the IFNα producers in *L. monocytogenes*–infected spleens

To further rule out pDC as major producers of IFN-I during Listeria infection of mice and to confirm a prominent role of myeloid cells, we purified cell populations from *L. monocytogenes* infected spleens by FACS and measured IFN-I production by RT-

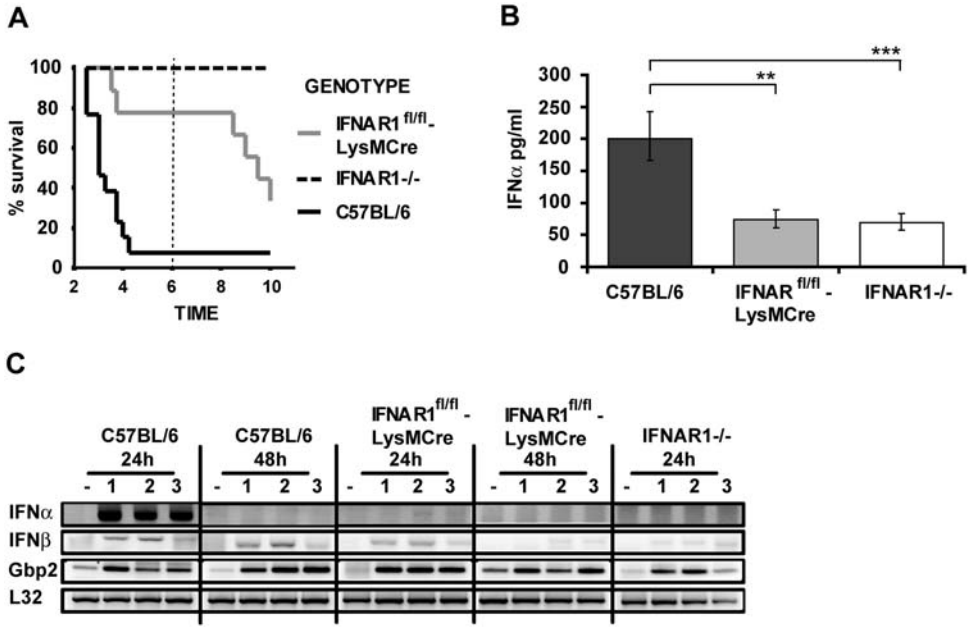

Figure 3. Myeloid cells strongly contribute to IFN-I production. (A) C57BL/6 wt (n = 13), IFNAR1−/− (n = 4), and mice with LysM-Cre-mediated deletion of IFNAR1 (LysMCre-IFNAR1$^{fl/fl}$, n = 9) were infected with 5×10^6 *L. monocytogenes*, and survival was monitored for ten days. (B) Mice of the indicated genotypes were infected with 5×10^6 *L. monocytogenes* (seven mice per group) or injected with PBS (two mice per group). After 24 h, serum was collected and ELISA was performed for IFNβ (data not shown) and IFNα levels. Data representing IFNα concentrations were log-transformed to achieve approximate normality. Linear models with genotype as fixed effect were fitted using SPSS. Means plus/minus standard errors of wt and mutant genotypes are plotted (after back-transformation). Significant values are indicated by ** $p\leq0.01$, *** $p\leq0.001$. (C) Mice of the indicated genotypes were infected with 5×10^6 *L. monocytogenes* (1–3) or injected with PBS (−). After 24 h and 48 h, spleens were isolated and RNA was extracted. The RNA was reverse-transcribed and subjected to PCR for the indicated genes. As a normalization control, the housekeeping gene L32 was measured.

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PCR. Figure 6 clearly shows that cells with a phenotypic profile of pDC (PDCA1$^+$B220$^+$CD11c^{dim}CD11b$^-$) fail to produce IFN-I in response to *L. monocytogenes* infection. The CD11c^{hi} population (either CD11b$^+$PDCA1$^-$B220$^-$ or CD11b$^-$PDCA1$^-$B220$^-$) including typical mDCs did not express detectable IFNα mRNA, whereas these cells produced minor amounts of IFNβ mRNA. In contrast, CD11b$^+$ cells not expressing CD11c, PDCA1 or B220, a profile characterizing macrophages, expressed high levels of both IFNα and IFNβ mRNA. Macrophages, therefore, are most likely the predominant IFN-I producers in Listeria-infected mice.

Discussion

Using mice and cells ablated for genes involved in IFN-I synthesis, we established a molecular profile of the cell type(s) producing IFN-I upon infection with *L. monocytogenes* and correlated

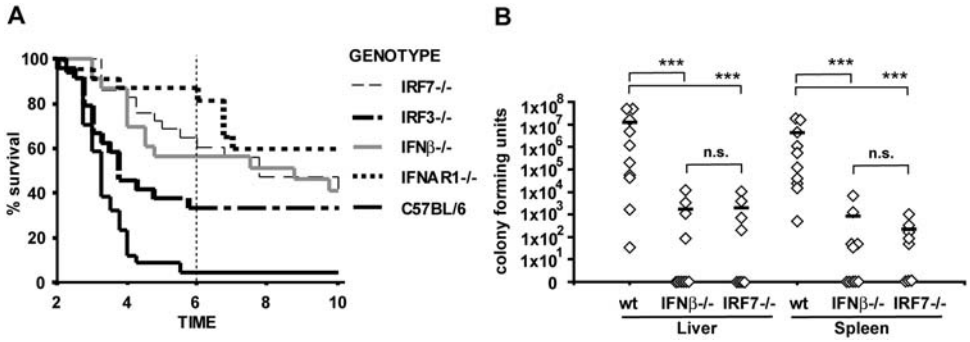

Figure 4. Prerequisites for the increase in resistance to lethal infection caused by IFN-I. (A) Mice of the indicated genotypes were infected with 5×10^6 *L. monocytogenes*, and survival was monitored for ten days. The data presented here are a summary of several individual experiments with groups of 4–6 mice per genotype and treatment. Total number of mice analysed are: C57BL/6 wt n = 34, IFNAR1−/− n = 23, IFNβ−/− n = 23, IRF3−/− n = 24, IRF7−/− n = 29. (B) Groups of ten (C57BL/6 wt and IFNβ−/−) or eight (IRF7−/−) mice were infected with 1×10^6 *L. monocytogenes*. After three days, mice were killed and the *L. monocytogenes* titre was determined in the liver and spleen and presented as CFU. Data were log-transformed to achieve approximate normality. Linear models with genotype as fixed effect were fitted using SPSS. Significant values are indicated by: n.s. not significant $p>0.05$, *** $p\leq0.001$.

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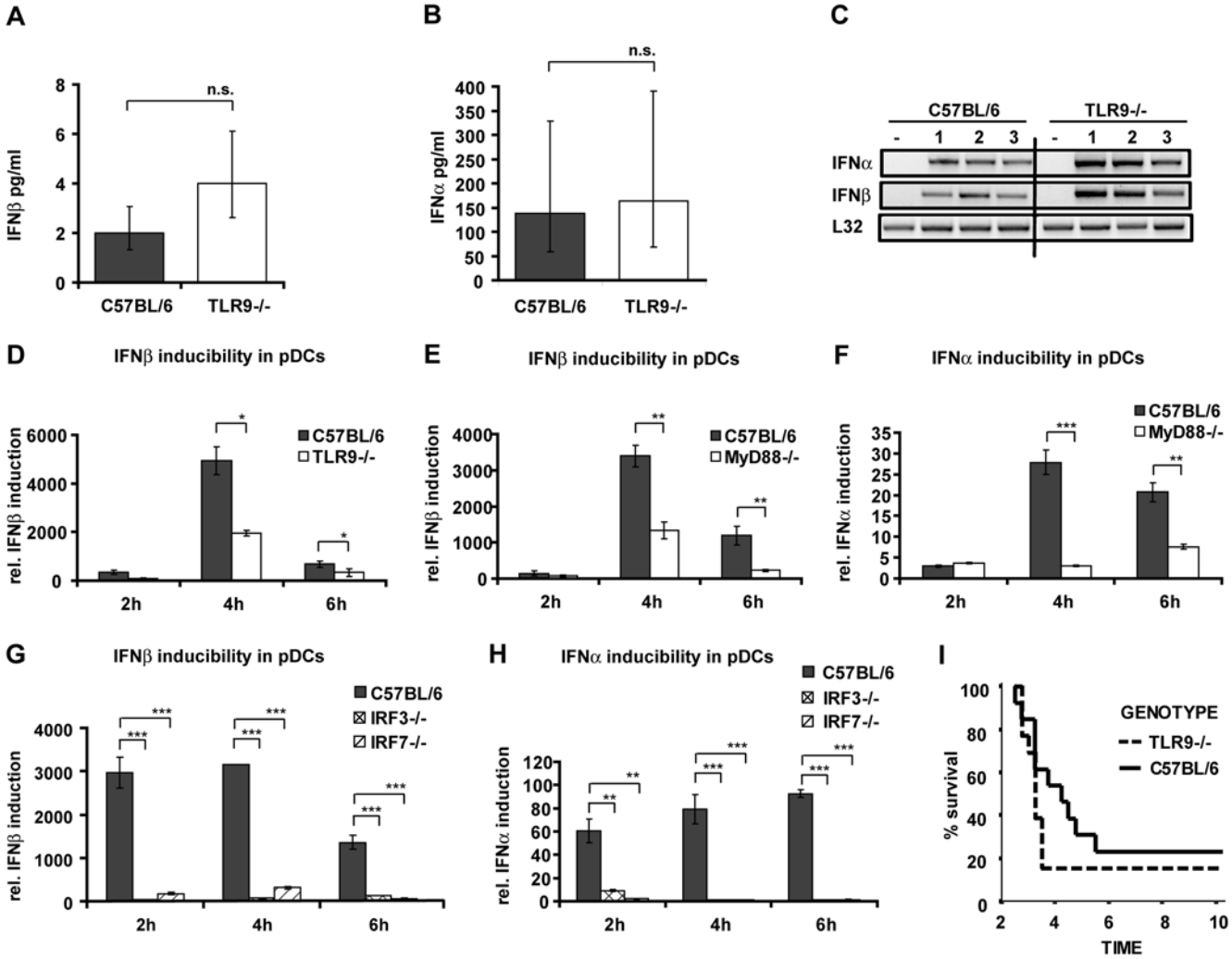

Figure 5. The role of TLR9 in mice and cells infected with *L. monocytogenes*. (A–C) C57BL/6 wt or TLR9−/− mice were injected i.p. with 5×10^6 *L. monocytogenes* (four mice per genotype) or PBS as a control (data not shown). After 24 h of infection, serum was collected and ELISAs for IFNβ (A) and IFNα (B) were performed. Data representing IFNβ (A) and IFNα (B) concentrations were log-transformed (after adding one) to achieve approximate normality. Linear models with genotype as fixed effect were fitted using SPSS. Means plus/minus standard errors of wt and mutant genotypes are plotted (after back-transformation), n.s.: not significant ($p>0.05$). (C) After 24 h of infection, mice were killed and spleens isolated. RNA was extracted from spleens of three infected mice per genotype (1–3) or from mice injected with PBS (−), and RT-PCR for the indicated genes was performed. As a normalization control, the housekeeping gene L32 was measured. (D–H) pDCs of the indicated genotypes were infected with *L. monocytogenes* at a MOI of 10. At the indicated time points, total RNA was prepared. The isolated RNA was reverse-transcribed, and induction of the IFNβ (D,E,G) or IFNα (F,H) genes was measured by real-time PCR. For normalization to a housekeeping gene, GAPDH was measured. (I) C57BL/6 wt or TLR9−/− mice (13 mice per genotype, data represent a summary of two experiments) were injected i.p. with 5×10^6 *L. monocytogenes*. Survival was monitored for ten days.

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IFN-I production in the spleen and serum with the survival of infected hosts. Clear results emerging from our studies are that I) the TLR9/MyD88 pathway, a hallmark of IFN-I synthesis by pDC, does not significantly contribute to splenic (local) or systemic IFN-I production; II) consistently, feed-forward amplification requiring signaling through the IFNAR is of great importance and III) the main IFN-I producers are cells with characteristics typical of splenic macrophages. Finally and IV), IFNβ and IRF7 contribute to the enhancement of lethal infection by IFN-I. Reduction in IFN-I production that resulted from IRF3, IRF7, IFNβ or IFNAR deficiency by and large reflected the increase in resistance to lethal infection. Examination of pathogen organ loads revealed that the bacterial growth-promoting, hence lethality-enhancing activity of IFN-I develops between day 1 and 3 post infection.

Our studies focused on the early, innate phase of infection up to about day 6. IFN-I may additionally influence the ensuing adaptive immune response and, as suggested by recent studies, this effect may not be primarily adverse as in the innate response [35–37]. Diverse IFN-I effects on adaptive and innate immunity most likely explain that the relative increases in resistance to infection caused by the knock-out mice used in our study differ to some degree when analyzed at day 6 or day 10 after infection (Figure 4). Furthermore, the survival curve of mice with myeloid cell-specific IFNAR1 ablation suggests that cells outside this cell compartment influence the survival of Listeria infection particularly after the innate phase of the immune response.

Two recent studies closely examining the innate response to Listeria in infected spleens revealed a highly complex process

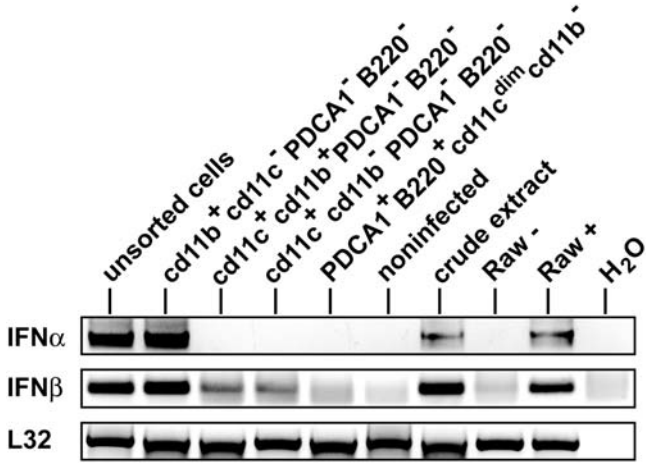

Figure 6. Macrophages and not pDC are the IFN-I–producing cells after *L. monocytogenes* infection. C57Bl/6 wt mice (n = 15) were infected with 5×10^6 *L. monocytogenes*. After 24 h, mice were killed, splenic cells were isolated, labelled with antibodies against CD11b (FITC-conjugated), CD11c (APC), PDCA1 (PE-Cy5), and B220 (PE-Cy7), and subjected to FACS sorting. RNA of the isolated cell populations was extracted, reverse-transcribed, and subjected to PCR for the indicated genes. Unsorted cells and whole spleen extracts from infected spleens (crude extract) as well as uninfected spleens were used as controls. As a positive control for the PCR reactions, Raw264.7 macrophages were infected (+) or not (−) with *L. monocytogenes* for 8 h at a MOI of 10. As a negative control, H_2O was used instead of template cDNA. As a normalization control, the housekeeping gene L32 was measured.
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requiring $CD11c^+$ DC for the movement of Listeria to the white pulp and to initiate a concerted response involving macrophages, TIP-DC, NK cells and neutrophils [38,39]. Our study shows that the TLR9 pathway was not engaged by *L. monocytogenes* for IFN-I synthesis in mice. By contrast, data with pDC-enriched bone marrow cultures show that Listeria can present a TLR9 ligand and stimulate this cell type for IFN-I production. This finding strongly implies that pDC do not contribute to IFN-I production during *L. monocytogenes* infection of mice, in spite of high bacterial loads in lymphoid organs that harbor this cell type. Although TLR9/MyD88-independent IFN-I synthesis has also been observed in mice infected with viruses [40–42], several reports suggest that the engagement of the pDC/TLR system for IFN-I production occurs when viruses gain access to lymphoid tissue either directly or because the pathogen breaks the initial barrier of infection formed by macrophages and/or epithelial cells [34,41–43]. For example, lung infection with Newcastle disease virus led to IFNα production by alveolar macrophages, whereas systemic infection engaged both mDC and pDC for IFNα production [41]. Splenic mDC are reportedly major IFN-I producers during infection with Adenovirus, underscoring pathogen specificity in the choice of IPC and defying a general role of pDCs as producers of systemic IFN-I upon viral infection [44]. Under steady state conditions, pDC are mainly found in peripheral blood and lymphoid organs [13]. A recent report by Tam and Wick demonstrated expansion and activation of cells phenotypically resembling pDC in lymphoid tissue infected with *L. monocytogenes* [45]. The cells expressed activation antigens, but, consistent with our results, did not produce cytokines. Together, the data suggest that the ability of *L. monocytogenes* to stimulate pDC expansion occurs without direct contact and may be caused by cytokines released from infected cells. Unlike virus, *L. monocytogenes* appears to be unable to efficiently gain access to pDC even at lethal doses of infection when lymphoid organs are heavily infected. Direct contact may be prevented by an efficient containment of the pathogen in cells that are more readily infected than pDC. Owing to their phagocytic potential, splenic macrophages activated by NK cell-derived IFNγ, might clear Listeria before they reach pDC. It will be interesting to determine whether the choice of IPC determines the IFN-I effect on infection and whether macrophages as IFN-I producers are related to fatal outcome as in the case of *L. monocytogenes*. In this regard it will be important to examine IPCs in murine infection models such as *S. pneumoniae*, where the host benefits from the production of IFN-I [46].

IFNα production in BMM via feed-forward amplification required IRF3-dependent IFNβ production, signaling through IFNAR1 and IRF7. This resembled the molecular mechanism observed for splenic or systemic IFNα production after *in vivo* infection. To confirm the assumption that cells different from pDC are important IFNα producers *in vivo* we used $IFNAR^{fl/fl}$-LysM-Cre mice and demonstrated that IFNα synthesis critically depends on the presence of IFNAR1 on myeloid cells. Consistently, cells expressing surface markers characteristic of macrophages express both IFNβ and IFNα mRNA when purified from infected spleens. By contrast, this was not observed for the cell populations that include mDC and pDC. Thus, macrophages are the most likely IFNα producer cells after *L. monocytogenes* infection *in vivo*. An additional contribution of neutrophils cannot be ruled out entirely. However, little is known about the presence and abundance of this cell type in the spleen early after Listeria infection and it is similarly unclear whether neutrophils produce IFN-I in response to infection.

Whereas IRF3 was also critically involved in IFNβ production by bone marrow-derived macrophages, the IFNAR or IRF7 were not required. This contrasts the more important role of IRF7 for IFNβ synthesis in the *L. monocytogenes* infected spleen. Unlike bone marrow macrophages, much of splenic IFNβ resulted from an IFNAR/IRF7-dependent amplification loop because IFNAR−/− spleens expressed strongly reduced amounts of IFNβ mRNA. This may reflect a contribution of splenic mDC to IFNβ production. This cell type required IRF3 as well as IFNAR/IRF7 for IFNβ synthesis also when grown from mouse bone marrow. We assume that mDC produce low amounts of IFNβ immediately after Listeria infection in an IRF3-dependent manner. This initiates the feed-forward amplification loop causing synthesis of the majority of both IFNβ and of IFNα. This model, while consistent with the data, fails to provide a satisfactory explanation for the obvious difference between bone marrow-derived macrophages and mDC regarding the significance of IFNAR1/IRF7 for IFNβ synthesis. Possibly the much larger rate of cytoplasmic infection of macrophages drives IRF3-dependent IFNβ synthesis much more efficiently. A deeper understanding of cell type differences in the mode of IFN-I production is needed to provide ultimate clarity regarding this point. The assumption that splenic mDC participate in IFNβ production via IFNAR and IRF7 in infected mice is supported by the presence of mRNA for the cytokine in $CD11c^+$/$CD11b^+$ cells purified from infected mice. Nonetheless, a larger amount of IFNβ mRNA was detected in purified splenic macrophages, suggesting a major contribution of these cells to total IFNβ production. At the same time, a large fraction of splenic IFNβ requires feed-forward amplification and, judging from our findings with BMM, this appears to contrast the assumption of macrophages being major producers. A simple explanation for this discrepancy might be an intrinsic difference between splenic macrophages and BMM regarding feed-forward amplification of IFNβ production. Alternatively, the IFNAR/IRF7 requirement of

splenic macrophages may derive from the fact that feed-forward amplification results from paracrine, not autocrine IFN-I priming. Under this assumption IFN-I is released by infected macrophages within or outside the spleen to prime uninfected splenic macrophages. Signaling through the IFNAR will result in IRF7 expression and the transcription factor is available for both IFNβ and IFNα production once primed cells become infected by Listeria. By contrast BMM are synchronously infected in culture and will immediately produce IFNβ in absence of IRF7 expression, employing exclusively IRF3 instead.

In summary our results provide answers to the question how the IFN-I system is deployed by an intracellular bacterial pathogen. Both, the observations made with cultured cells and our studies in mice, emphasize that molecular mechanisms governing IFN-I synthesis and response strongly depend on the pathogen as well as the host cell type. Deciphering the importance of these differences for various routes of infection and for diverse types of pathogens remains a challenging task for future research.

Materials and Methods

Ethics Statement

All animal experiments were discussed and approved by the University of Veterinary Medicine, Vienna institutional ethics committee and carried out in accordance with protocols approved by the Austrian law (GZ 680 205/67-BrGt/2003).

Bacteria

Listeria monocytogenes LO28 [47] were grown in brain heart infusion (BHI) (Difco) broth. Concentrations of bacteria were determined by measurement at OD_{600} and confirmed by plating serial dilutions onto BHI or Oxford agar (Merck) plates.

Mice

All mice were on a C57BL/6 background and housed under specific pathogen-free conditions according to FELASA guidelines [48].

Cells

Raw264.7 macrophages were cultured in DMEM (Gibco, Invitrogen) supplemented with 10% FCS (Gibco, Invitrogen). Bone marrow was isolated from femurs of 6–8 week old mice. For differentiation of BMM, cells were grown in DMEM (Gibco, Invitrogen) in the presence of 10% FCS (Gibco, Invitrogen) and L-cell derived CSF-1 as described [49]. The cultures contained >99% $F4/80^+$ cells. mDCs were obtained by culture of bone marrow in DMEM (Gibco, Invitrogen) supplemented with 10% FCS (Gibco, Invitrogen) and X-6310 derived GM-CSF as described [50]. mDC cultures contained virtually no F4/80+ cells and the purity of $CD11c^+/CD11b^+$ cells was between 60 and 70%. pDCs were obtained by culture of bone marrow in DMEM (Gibco, Invitrogen) supplemented with 50 ng/ml of Flt-3-L for 6 days. The purity of $CD11c^{dim}/B220^+$ pDCs was between 60 and 70%. pDC cultures contained no $F4/80^+$ cells.

Infection of cells

Raw264.7 macrophages, primary BMM, mDCs and pDCs were infected with *L. monocytogenes* (derived from overnight culture) at a multiplicity of infection (MOI) of 10 and incubated for 60 min at 37°C in a humidified CO_2 atmosphere. Extracellular bacteria were subsequently killed by exchanging medium to gentamicin-containing medium (final concentration 50 μg/ml). After another 60 min, medium was changed to medium containing 10 μg/ml gentamicin.

Infection of mice

Listeria monocytogenes was grown in BHI broth (Difco) to late logarithmic phase (OD_{600} 0.8), pelleted and resuspended in BHI containing 20% glycerol. Aliquots were shock-frozen and stored at −80°C. The concentration of *L. monocytogenes* aliquots was quantified by plating serial dilutions onto Oxford agar plates (Merck). For infection, the bacteria were thawn on ice, washed with PBS for three times and diluted in PBS (endotoxin-free, Sigma) to the appropriate concentrations. 500 μl of the bacterial suspension were injected into the peritoneum of 10- to 12-week-old mice. For the experiments shown, survival of infected mice was monitored for 10 days. For determination of the bacterial load, mice were killed at the indicated time points after infection and spleens and livers were homogenized in PBS. Serial dilutions of homogenates were plated on Oxford agar plates and colonies were counted after growth at 37°C for 24–36 h. For RNA and protein extraction from spleen and for sorting of splenic cells, mice were killed after 24 h of infection and spleens were isolated.

ELISA

Mice were infected intraperitoneally with *L. monocytogenes* or injected with PBS (Sigma). Sera were collected after 24 h of infection and levels of IFNβ and IFNα were determined by ELISA according to the manufacturer's instructions (PBL InterferonSource).

Fluorescence-activated cell sorting (FACS)

Spleens were digested in 1 mg/ml collagenase (Roche) for 10 min and homogenized using a cell strainer. The cell suspension was labelled with the following antibodies: CD11b−FITC (Becton Dickinson), CD11c-APC (Becton Dickinson), B220-PE-Cy7 (Beckton Dickinson) and PDCA1-PE-Cy5 (Miltenyi Biotec). Four different cell populations were sorted by FACS: $PCDA1^+B220^+CD11c^{dim}$ (pDC), $CD11b^+CD11c^-B220^-PDCA1^-$ (macrophages), $CD11b^+CD11c^+B220^-PDCA1^-$ and $CD11b^-CD11c^+B220^-PDCA1^-$ (myeloid dendritic cells).

RNA extraction and PCR

RNA extraction from cells, reverse transcription and real-time PCR was performed as already described [29]. Data obtained by real-time PCR were analysed using SPSS and a Student's t-Test. Significant values are indicated by: n.s. not significant $p>0.05$, * $p\leq0.05$, ** $p\leq0.01$, *** $p\leq0.001$.

Primers for real-time PCR were as described, except for IFNα: Probe: panIFNα: 5′-(6-Fam) AG+AA+GAA+A+C+AC+AG+CC (BHQ1)-3′ (+indicating LNA (Locked Nucleic Acid) nucleotides; Proligo); panIFNα-for: 5′-CCACAGGATCACTGTGTACCT-GAGA-3′; panIFNα-rev: 5′-CTGATCACCTCCCAGGCA-CAG-3′

Spleens were isolated from mice infected with *L. monocytogenes* or injected with PBS for 24 h, shock-frozen in liquid nitrogen and stored at −80°C. For RNA extraction, 20 mg of frozen tissue was homogenized in lysis buffer RA1 (NucleoSpin RNAII kit, Macherey-Nagel) with the Precellys 24 homogenizer (Peqlab) at 6000 rpm for 30 sec. Subsequent isolation of total RNA and cDNA synthesis was performed as described [29]. Primers used for PCR were as follows: panIFNα-for-5′-ATGGCTAG(A/G)CTC-TGTGCTTTCCT-3′; panIFNα-rev-5′-AGGGCTCTCCAGA(T/C)TTCTGCTCTG-3′; IFNβ-for 5′-CATCAACTATAAGCAG-CTCCA-3′; IFNβ-rev 5′-TTCAAGTGGAGAGCAGTTGAG-3′; Gbp2-for-5′-TGCTAAACTTCGGGAACAGG-3′; Gbp2-rev-5′-GAGCTTGGCAGAGAGGTTTG-3′; L32-for 5′-ATTAAGC-

GAAACTGGCGGAAACCC-3′; L32-rev 5′- TTTCTTCGCT-GCGTAGCCTGG-3′.

Supplementary Materials and Methods are presented in Text S1.

Supporting Information

Figure S1 Groups of 9 C57BL/6 wt or IFNAR1−/− mice were infected with 5×10^6 *L. monocytogenes*. After 24 h of infection, mice were killed and the *L. monocytogenes* titre was determined in the liver and spleen and presented as CFU. Data were log transformed to achieve approximate normality. Linear models with genotype as fixed effect were fitted using SPSS. No significant differences between genotypes were observed (n.s. not significant $p>0.05$).
Found at: doi:10.1371/journal.ppat.1000355.s001 (0.11 MB TIF)

Figure S2 A) Mice of the indicated genotypes were injected i.p. with 5×10^6 *L. monocytogenes* or PBS as a control. After 24 h of infection, mice were killed and spleens isolated. Protein was extracted from spleens of four infected mice per genotype (1–4) or from mice injected with PBS (−), and Western blot for the indicated proteins was performed. STAT1 and STAT2 activation was detected using antibodies recognizing phosphorylated tyrosine 701 and 689, respectively. As a positive control for STAT phosphorylation, Raw264.7 macrophages were infected (+) or not (−) with *L. monocytogenes* for 4 h at a MOI of 10, and protein extracts were loaded onto the same gel. B) BMM derived from mice of the indicated genotypes were infected with *L. monocytogenes* at a MOI of 10. At the indicated time points, protein extracts were prepared and subjected to Western blotting. STAT2 activation was detected using an antibody recognizing phosphorylated tyrosine 689. A+B) For control of equal loading, the blots were reprobed with an antibody specific for the Erk kinases Erk1 and Erk2 (panErk).
Found at: doi:10.1371/journal.ppat.1000355.s002 (1.23 MB TIF)

Figure S3 A–C) Splenocytes were isolated from C57BL/6, IFNAR1fl/fl-LysMCre, and IFNAR1−/− mice and CD11b+ cells were enriched by magnetic activated cell sorting (MACS). CD11b-enriched cell fraction was stained for CD11c and IFNAR1 and percentages of CD11c+IFNAR+ and CD11c+IFNAR− cells were determined by flow cytometry. D–F) CD11b-enriched splenocytes were stained for CD11b and IFNAR1. IFNAR1 expression by cells gated for coexpression of CD11b was analysed by flow cytometry. G) Peritoneal exudate cells were isolated from wt C57/BL6 mice (solid line), IFNAR1fl/fl-LysMcre mice (dashed line), and IFNAR1−/− mice (gray shaded curve). IFNAR1 expression by cells gated for coexpression of F4/80 (left diagram) was determined by flow cytometry. As a control IFNAR1 expression on total lymphocytes (gated on lymphocytes in FSC/SSC plot (not shown) was analysed.
Found at: doi:10.1371/journal.ppat.1000355.s003 (1.38 MB TIF)

Figure S4 A) C57BL/6 wt or TLR9−/− mice were injected i.p. with 5×10^6 *L. monocytogenes* or PBS as a control. After 24 h of infection, mice were killed and spleens isolated. Protein was extracted from spleens of three infected mice per genotype (1–3) or from mice injected with PBS (−), and Western blot for the indicated proteins was performed. STAT1 and STAT2 activation was detected using antibodies recognizing phosphorylated tyrosine 701 and 689, respectively. As a positive control for STAT phosphorylation, Raw264.7 macrophages were infected (+) or not (−) with *L. monocytogenes* for 4 h at a MOI of 10, and protein extracts were loaded onto the same gel. For control of equal loading, the blots were reprobed with an antibody specific for the Erk kinases Erk1 and Erk2 (panErk). B) mDC of the indicated genotypes were infected with *L. monocytogenes* at a MOI of 10. At the indicated time points, total RNA was prepared. The isolated RNA was reverse-transcribed and induction of the IFNβ gene was measured by Real-time PCR. For normalization to a house-keeping gene, GAPDH was measured.
Found at: doi:10.1371/journal.ppat.1000355.s004 (0.28 MB TIF)

Text S1 Supplementary Materials and Methods
Found at: doi:10.1371/journal.ppat.1000355.s005 (0.03 MB DOC)

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Author Contributions

Conceived and designed the experiments: SS TD. Performed the experiments: SS RK EK AP SW BR DS GS TF ZW. Analyzed the data: SS RK EK CV TD. Contributed reagents/materials/analysis tools: TT TR UK MM TD. Wrote the paper: TD.

References

1. Pestka S, Krause CD, Walter MR (2004) Interferons, interferon-like cytokines, and their receptors. Immunol Rev 202: 8–32.
2. Akira S, Takeda K (2004) Toll-like receptor signalling. Nat Rev Immunol 4: 499–511.
3. Decker T, Muller M, Stockinger S (2005) The yin and yang of type I interferon activity in bacterial infection. Nat Rev Immunol 5: 675–687.
4. Meylan E, Tschopp J, Karin M (2006) Intracellular pattern recognition receptors in the host response. Nature 442: 39–44.
5. Jefferies CA, Fitzgerald KA (2005) Interferon gene regulation: not all roads lead to Tolls. Trends Mol Med 11: 403–411.
6. Carpenter S, O'Neill LA (2007) How important are Toll-like receptors for antimicrobial responses? Cell Microbiol 9: 1891–1901.
7. Noppert SJ, Fitzgerald KA, Hertzog PJ (2007) The role of type I interferons in TLR responses. Immunol Cell Biol 85: 446–457.
8. Takaoka A, Wang Z, Choi MK, Yanai H, Negishi H, et al. (2007) DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response. Nature 448: 501–505.
9. Honda K, Taniguchi T (2006) IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors. Nat Rev Immunol 6: 644–658.
10. Levy DE, Darnell JE Jr (2002) Stats: transcriptional control and biological impact. Nat Rev Mol Cell Biol 3: 651–662.
11. Levy DE, Marie I, Prakash A (2003) Ringing the interferon alarm: differential regulation of gene expression at the interface between innate and adaptive immunity. Curr Opin Immunol 15: 52–58.
12. Reis e Sousa C (2006) Dendritic cells in a mature age. Nat Rev Immunol 6: 476–483.
13. Barchet W, Cella M, Colonna M (2005) Plasmacytoid dendritic cells—virus experts of innate immunity. Semin Immunol 17: 253–261.
14. Matsui K, Kumagai Y, Kato H, Sato S, Kawagoe T, et al. (2006) Cutting edge: Role of TANK-binding kinase 1 and inducible IkappaB kinase in IFN responses against viruses in innate immune cells. J Immunol 177: 5785–5789.
15. Uematsu S, Akira S (2007) Toll-like receptors and Type I interferons. J Biol Chem 282: 15319–15323.
16. Schoenemeyer A, Barnes BJ, Mancl ME, Latz E, Goutagny N, et al. (2005) The interferon regulatory factor, IRF5, is a central mediator of toll-like receptor 7 signaling. J Biol Chem 280: 17005–17012.
17. Takaoka A, Yanai H, Kondo S, Duncan G, Negishi H, et al. (2005) Integral role of IRF-5 in the gene induction programme activated by Toll-like receptors. Nature 434: 243–249.
18. Negishi H, Fujita Y, Yanai H, Sakaguchi S, Ouyang X, et al. (2006) Evidence for licensing of IFN-gamma-induced IFN regulatory factor 1 transcription factor by MyD88 in Toll-like receptor-dependent gene induction program. Proc Natl Acad Sci U S A 103: 15136–15141.

19. Colonna M (2007) TLR pathways and IFN-regulatory factors: to each its own. Eur J Immunol 37: 306–309.
20. Schmitz F, Heit A, Guggemoos S, Krug A, Mages J, et al. (2007) Interferon-regulatory-factor 1 controls Toll-like receptor 9-mediated IFN-beta production in myeloid dendritic cells. Eur J Immunol 37: 315–327.
21. Vazquez-Boland JA, Kuhn M, Berche P, Chakraborty T, Dominguez-Bernal G, et al. (2001) Listeria pathogenesis and molecular virulence determinants. Clin Microbiol Rev 14: 584–640.
22. O'Connell RM, Saha SK, Vaidya SA, Bruhn KW, Miranda GA, et al. (2004) Type I interferon production enhances susceptibility to Listeria monocytogenes infection. J Exp Med 200: 437–445.
23. Auerbuch V, Brockstedt DG, Meyer-Morse N, O'Riordan M, Portnoy DA (2004) Mice lacking the type I interferon receptor are resistant to Listeria monocytogenes. J Exp Med 200: 527–533.
24. Carrero JA, Calderon B, Unanue ER (2004) Type I interferon sensitizes lymphocytes to apoptosis and reduces resistance to Listeria infection. J Exp Med 200: 535–540.
25. Stockinger S, Decker T (2008) Novel functions of type I interferons revealed by infection studies with Listeria monocytogenes. Immunobiology 213: 889–897.
26. Carrero JA, Unanue ER (2006) Lymphocyte apoptosis as an immune subversion strategy of microbial pathogens. Trends Immunol 27: 497–503.
27. Hamon M, Bierne H, Cossart P (2006) Listeria monocytogenes: a multifaceted model. Nat Rev Microbiol 4: 423–434.
28. Schnupf P, Portnoy DA (2007) Listeriolysin O: a phagosome-specific lysin. Microbes Infect 9: 1176–1187.
29. Stockinger S, Reutterer B, Schaljo B, Schellack C, Brunner S, et al. (2004) IFN Regulatory Factor 3-Dependent Induction of Type I IFNs by Intracellular Bacteria Is Mediated by a TLR- and Nod2-Independent Mechanism. J Immunol 173: 7416–7425.
30. Gregory SH, Wing EJ (1998) Neutrophil-Kupffer cell interaction in host defenses to systemic infections. Immunol Today 19: 507–510.
31. Stockinger S, Materna T, Stoiber D, Bayr L, Steinborn R, et al. (2002) Production of type I IFN sensitizes macrophages to cell death induced by Listeria monocytogenes. J Immunol 169: 6522–6529.
32. Prinz M, Schmidt H, Mildner A, Knobeloch KP, Hanisch UK, et al. (2008) Distinct and nonredundant in vivo functions of IFNAR on myeloid cells limit autoimmunity in the central nervous system. Immunity 28: 675–686.
33. Clausen BE, Burkhardt C, Reith W, Renkawitz R, Forster I (1999) Conditional gene targeting in macrophages and granulocytes using LysMcre mice. Transgenic Res 8: 265–277.
34. Barchet W, Cella M, Odermatt B, Asselin-Paturel C, Colonna M, et al. (2002) Virus-induced interferon alpha production by a dendritic cell subset in the absence of feedback signaling in vivo. J Exp Med 195: 507–516.
35. Le Bon A, Schiavoni G, D'Agostino G, Gresser I, Belardelli F, et al. (2001) Type i interferons potently enhance humoral immunity and can promote isotype switching by stimulating dendritic cells in vivo. Immunity 14: 461–470.
36. Le Bon A, Etchart N, Rossmann C, Ashton M, Hou S, et al. (2003) Cross-priming of CD8+ T cells stimulated by virus-induced type I interferon. Nat Immunol 4: 1009–1015.
37. Freudenberg MA, Merlin T, Kalis C, Chvatchko Y, Stubig H, et al. (2002) Cutting edge: a murine, IL-12-independent pathway of IFN-gamma induction by gram-negative bacteria based on STAT4 activation by Type I IFN and IL-18 signaling. J Immunol 169: 1665–1668.
38. Kang SJ, Liang HE, Reizis B, Locksley RM (2008) Regulation of hierarchical clustering and activation of innate immune cells by dendritic cells. Immunity 29: 819–833.
39. Aoshi T, Zinselmeyer BH, Konjufca V, Lynch JN, Zhang X, et al. (2008) Bacterial entry to the splenic white pulp initiates antigen presentation to CD8+ T cells. Immunity 29: 476–486.
40. Honda K, Yanai H, Negishi H, Asagiri M, Sato M, et al. (2005) IRF-7 is the master regulator of type-I interferon-dependent immune responses. Nature 434: 772–777.
41. Kumagai Y, Takeuchi O, Kato H, Kumar H, Matsui K, et al. (2007) Alveolar macrophages are the primary interferon-alpha producer in pulmonary infection with RNA viruses. Immunity 27: 240–252.
42. Prakash A, Smith E, Lee CK, Levy DE (2005) Tissue-specific positive feedback requirements for production of type I interferon following virus infection. J Biol Chem 280: 18651–18657.
43. Scheu S, Dresing P, Locksley RM (2008) Visualization of IFNbeta production by plasmacytoid versus conventional dendritic cells under specific stimulation conditions in vivo. Proc Natl Acad Sci U S A 105: 20416–20421.
44. Fejer G, Drechsel L, Liese J, Schleicher U, Ruzsics Z, et al. (2008) Key role of splenic myeloid DCs in the IFN-alphabeta response to adenoviruses in vivo. PLoS Pathog 4: e1000208. doi:10.1371/journal.ppat.1000208.
45. Tam MA, Wick MJ (2006) Differential expansion, activation and effector functions of conventional and plasmacytoid dendritic cells in mouse tissues transiently infected with Listeria monocytogenes. Cell Microbiol 8: 1172–1187.
46. Mancuso G, Midiri A, Biondo C, Beninati C, Zummo S, et al. (2007) Type I IFN signaling is crucial for host resistance against different species of pathogenic bacteria. J Immunol 178: 3126–3133.
47. Kocks C, Gouin E, Tabouret M, Berche P, Ohayon H, et al. (1992) L. monocytogenes–induced actin assembly requires the actA gene product, a surface protein. Cell 68: 521–531.
48. Nicklas W, Baneux P, Boot R, Decelle T, Deeny AA, et al. (2002) Recommendations for the health monitoring of rodent and rabbit colonies in breeding and experimental units. Lab Anim 36: 20–42.
49. Baccarini M, Bistoni F, Lohmann Matthes ML (1985) In vitro natural cell-mediated cytotoxicity against Candida albicans: macrophage precursors as effector cells. J Immunol 134: 2658–2665.
50. Reutterer B, Stockinger S, Pilz A, Soulat D, Kastner R, et al. (2008) Type I IFN are host modulators of strain-specific Listeria monocytogenes virulence. Cell Microbiol 10: 1116–1129.

Discussion

IFNs are potent cytokines for innate and adaptive immunity to pathogens. IFN-I are important for host protection against a wide variety of viruses as they have the potential to induce antiviral responses in host cells as well as shaping the adaptive immune response. IFNγ protects against many bacterial infections and some viral infections mostly due to its activating effects on immune cells. Whereas the impact of IFN-I on viral infections is generally protective, their role in bacterial infections can be either beneficial or detrimental for the host, depending on the particular pathogen [152-154, 209-211]. Adverse effects of IFN-I reportedly occur also after systemic infection with the fungal pathogen Candida albicans [212]. In Lm infections IFN-I are described to enhance lethality and bacterial burden in mice. These effects have been attributed to several aspects of IFN-I biology, like increased cell death upon infection of macrophages and T cells, decreased sensitivity to IFNγ and decreased TNFα production.

Stat1 is the signal transducer downstream of IFN-I and IFNγ receptors and mice deficient in Stat1 are hypersusceptible to a range of pathogens, including Lm. This sensitivity to infection does not allow studying Stat1 dependent immune responses beyond very early phases. Furthermore, the cytokine storm initiated by the high bacterial load in Stat1-/- animals obscures many aspects of IFN signalling after Lm infection. Therefore it was imperative to analyse tissue-restricted Stat1 ablation. In this way we were able to decipher which cell types act at various stages of the IFN-dependent immune response to Lm.

Many different cell types produce IFN-I when exposed to Lm *in vitro*. The cell type responsible for production of IFN-I *in vivo*, however had not been characterised. With the help of mice ablated in various genes involved in either initiation of IFN-I or in the feed-forward amplification loop of IFN-I we could determine which pathway of IFN-I induction is predominant in Lm infected mice. Furthermore we could isolate the cell type responsible for major production of IFN-I after *in vivo* infection of mice. Together, the studies clearly establish splenic myeloid cells, most likely splenic macrophages, as the major IFN-I producers after Lm infection. Studies by other groups also showed the importance for myeloid cells to IFN-I production after Lm infection [213] however, also a specialised kind of TipDCs have been reported to produce IFN-I after Lm infection in a fluorescence reporter mouse [214]. According to our data dendritic cells, especially

plasmacytoid dendritic cells, make no or minor contributions to IFN-I production after Lm infection and the conditional ablation of Stat1 shows that their IFN response has little impact on host resistance. In other infection models, especially viral infections dendritic cells have been identified as the main IFN-I producers [215, 216].

The picture changes during the adaptive response as now IFN signalling in dendritic cells becomes important for protective immunity. Given that we identified the IFN-I producing cell in the innate response after intraperitoneal infection, it will be interesting to expand these studies and pinpoint the cell type responsible for IFN-I after different routes of infection and during various phases of the immune response against Lm. Another interesting aspect would be the effect of different doses of Lm on the cellular source of IFN-I.

IFN responses in T lymphocytes are of particular interest because of reports attributing the adverse effects of IFN-I to this cell type [156]. Indeed, we observed a major contribution of IFN signalling in T cells for both innate and adaptive immunity; however going in two different directions. Whereas IFN signalling during early stages was detrimental to the host, at later stages it was absolutely required for the initiation of sterilizing adaptive immunity.

The adverse effects of IFN-I for the host during Lm infections were determined after intraperitoneal or intravenous infections in previous reports. Here we show the effects of the IFN system on the natural, gastrointestinal route of infection. Intragastric infection adds complexity to the immune response to Lm as more host barriers need to be overcome and a very specialized immune system in the gastrointestinal tract is involved in the initiation of the immune response. We speculated that these additional factors might produce an altered influence of IFN-I on the course of infection. Using the murinized Lm strain constructed as part of my work we could show that the route of infection indeed changes impact of IFN-I. IFN-I become protective for early responses to the pathogen and this effect is most drastically seen in the liver. The cytokine profile after intragastric infection is also very different from that after other infection routes. Conceptually two different explanations can be put forward for the dependence of IFN-I effects on the course of infection: either the protective effect after intragastric infection overrides the negative effect dominating after intraperitoneal infection, or, alternatively, the detrimental event is restricted to intraperitoneal infection and does not occur when Lm enters the host via the gastrointestinal tract.

The nature and mechanisms of the decrease in host resistance caused by IFN-I are an area of intensive research. In line with Unanue and colleagues [156] we show that IFN signalling in T cells increases the bacterial burden of mice after intraperitoneal infection with Lm. Importantly, however, our studies comparing T cell-restricted ablation of Ifnar1 or Stat1 reveal that the adverse action of T cells is not caused by IFN-I signalling alone. Unanue and colleagues suggest IL-10 production to be a major factor in the IFN-I-dependent decrease of host resistance [156]. Immunesuppressive IL10 is thought to be released by phagocytic cells after incorporation of T cell-derived apoptotic bodies. This has to be debated, as after intragastric delivery of Lm IL10 can be detected in Ifnar1-/- animals at similar or higher amounts than in WT mice. Clearly mechanisms other than ingestion of apoptotic bodies have to account for IL10 production in these animals. In light of the findings with intragastric infection the mechanisms proposed for the detrimental effects of IFN-I have to be revisited and analysed for their general applicability. The rate of apoptosis or other kinds of cell death after infection with Lm via different routes should be compared to pinpoint direct IFN-I effects. Another striking aspect in the comparison of infection routes results from our finding that the difference between Wt and Ifnar1-/-genotypes is most prominent in the liver and to a lesser extent in the spleen which, in terms of bacterial burden, are similarly affected after i.p. or i.g. administration of Lm. If effector cell apoptosis corresponded to bacterial burden, IFN-I dependent cell death would be expected to occur at similar rates after both infection routes.

A major difference between intragastric and intraperitoneal infection is most likely the delivery of Lm to internal organs. The delivery of Lm from the intestine to inner organs is not well described however a report implicates dendritic cells in this process [15]. This might be very different after intraperitoneal infection where Lm can readily infect target organs. Assuming that Lm is taken up by mucosal cells after crossing the intestinal epithelium we tried to mimic the spread via cells by injecting infected *in vitro* differentiated macrophages or dendritic cells intravenously into mice. Using this rather artificial system the detrimental effects of IFN-I persisted. The cells Lm targets after passage of the gastrointestinal tract are not fully identified and their isolation might be very inefficient. We cannot rule out that different cells either of the lamina propria or other immune structures of the gastrointestinal tract would influence the immune reaction in a different way. Unfortunately, the limitations of the experimental system do not allow this kind of analysis at the moment.

Another explanation for the different infection outcome after changing the infection route may be the expression of a different set of virulence genes by the bacterium itself. Lm has the ability to adapt to many different environmental cues and also to various situations within the host [217]. Hypothetically, the passage through the gastrointestinal tract might alter an immunologically relevant property of Lm. This question could be addressed by transcriptional profiling of Lm after administration via the two infection routes. Another way of testing this hypothesis could be to administer Lm intragastrically, isolate the bacteria after initial colonisation of the host and reinject them intraperitoneally. If the initial colonisation changed the phenotype of Lm the negative impact of IFN-I should be abrogated despite the intraperitoneal infection route. To determine differences in bacterial dissemination improved experimental systems, such as traceable Lm would be of great advantage.

Reportedly, IFN exert considerable influence on dendritic cells. Most of these effects are associated with their maturation and the subsequent ability to present and crosspresent antigen to T lymphocytes for the initiation of adaptive immunity [172, 193]. Our studies did not reveal a general defect of Stat1-/- dendritic cells in their ability to stimulate T cell responses *in vitro* or *in vivo*. However, a significant change in the proliferation of regulatory T cells was observed in immunized mice lacking dendritic cell Stat1. The hyperproliferation of Tregs goes in line with the enhanced susceptibility of these animals to secondary infections with Lm. In studies addressing tolerance to bone marrow transplantation another group similarly reported a greater proliferation of Tregs when Stat1 was missing [195]. An enhanced number of Tregs was also implicated in the higher susceptibility of pregnant woman to infections with Lm [98].

Stat1 signalling regulates the composition of the cellular influx to sites of inflammation. Consistently, we found considerable influence of Stat1 signalling on chemokine production after intraperitoneal infection with Lm. In line with this, others established a role for Ifnar1-/- signalling in the regulation of cellular influx to the peritoneum in a mouse model of listeriosis [218]. Type I IFN were also shown to be involved in regulating the migration of macrophages and NK cells to the liver in mice infected with murine cytomegalovirus [219]. Based on these findings it will be interesting to assess the impact of IFN/Stat-mediated chemokine synthesis in other experimentally tractable settings of local inflammation such as that of the skin.

A potential weakness of conditional ablation of the Stat1 gene as with any other gene is the cell type and tissue specificity of the Cre gene used for deletion. There is hardly a marker gene, which is exclusively expressed by only one cell type. This has to be kept in mind when interpreting results. The LysM-driven ablation of Stat1 does not only take place in macrophages but also in neutrophils and to a minor degree also in myeloid dendritic cells [175]. However, we attributed most effects seen in these animals to macrophages as the influence of IFN on neutrophils seems to be limited due to their short life span. The heterogeneity of macrophages also calls for better systems for gene ablation. The CD11c marker used for deletion in dendritic cells is also present on inflammatory monocytes and some resident macrophage populations such as alveolar macrophages in the lungs of mice. Recently a marker only present in CD8α+ dendritic cells has been found, Batf3, which will be interesting to use for Stat1 deletion [220].

In conclusion our studies shed some light on the influence and importance of IFNs in infections with non-viral pathogens. Immune responses to bacterial infections presented very different concerning their requirement for IFN signalling. It became clear that the way of entering a host shapes the immune response in general and, concomitantly, the role of IFN. This phenomenon will be interesting to study also with other bacteria infecting their hosts via the gastrointestinal route, for example *Salmonella typhimurium*. Lm are sometimes considered a model system for the more pathogenic mycobacterial infections. The effect of IFN-I on infections with *Mycobacterium tuberculosis* is also not clearly established at the moment, with some experiments showing detrimental and others revealing no influence of IFN-I on host protection [211, 221, 222]. Based on our results the timing, dose and localization of the initial contact with the pathogen should be included as important parameters for further studies. Analysing this and other bacterial or fungal infections with regard to the route of infection might help to better establish the importance of IFN and other cytokines for the immune response to human pathogens.

References

1. Vazquez-Boland, J.A., et al., *Listeria pathogenesis and molecular virulence determinants.* Clin Microbiol Rev, 2001. **14**(3): p. 584-640.
2. Freitag, N.E., G.C. Port, and M.D. Miner, *Listeria monocytogenes - from saprophyte to intracellular pathogen.* Nat Rev Microbiol, 2009. **7**(9): p. 623-8.
3. Allerberger, F. and M. Wagner, *Listeriosis: a resurgent foodborne infection.* Clin Microbiol Infect. **16**(1): p. 16-23.
4. Cossart, P., *Illuminating the landscape of host-pathogen interactions with the bacterium Listeria monocytogenes.* Proc Natl Acad Sci U S A. **108**(49): p. 19484-91.
5. Sleator, R.D., et al., *The interaction between Listeria monocytogenes and the host gastrointestinal tract.* Microbiology, 2009. **155**(Pt 8): p. 2463-75.
6. Drevets, D.A. and M.S. Bronze, *Listeria monocytogenes: epidemiology, human disease, and mechanisms of brain invasion.* FEMS Immunol Med Microbiol, 2008. **53**(2): p. 151-65.
7. Lamont, R.F., et al., *Listeriosis in human pregnancy: a systematic review.* J Perinat Med. **39**(3): p. 227-36.
8. Cossart, P. and P.J. Sansonetti, *Bacterial invasion: the paradigms of enteroinvasive pathogens.* Science, 2004. **304**(5668): p. 242-8.
9. Mostowy, S. and P. Cossart, *Cytoskeleton rearrangements during Listeria infection: clathrin and septins as new players in the game.* Cell Motil Cytoskeleton, 2009. **66**(10): p. 816-23.
10. Kraehenbuhl, J.P. and M.R. Neutra, *Epithelial M cells: differentiation and function.* Annu Rev Cell Dev Biol, 2000. **16**: p. 301-32.
11. Pron, B., et al., *Comprehensive study of the intestinal stage of listeriosis in a rat ligated ileal loop system.* Infect Immun, 1998. **66**(2): p. 747-55.
12. Marco, A.J., et al., *A microbiological, histopathological and immunohistological study of the intragastric inoculation of Listeria monocytogenes in mice.* J Comp Pathol, 1992. **107**(1): p. 1-9.
13. Jensen, V.B., J.T. Harty, and B.D. Jones, *Interactions of the invasive pathogens Salmonella typhimurium, Listeria monocytogenes, and Shigella flexneri with M cells and murine Peyer's patches.* Infect Immun, 1998. **66**(8): p. 3758-66.

14. Daniels, J.J., I.B. Autenrieth, and W. Goebel, *Interaction of Listeria monocytogenes with the intestinal epithelium.* FEMS Microbiol Lett, 2000. **190**(2): p. 323-8.
15. Pron, B., et al., *Dendritic cells are early cellular targets of Listeria monocytogenes after intestinal delivery and are involved in bacterial spread in the host.* Cell Microbiol, 2001. **3**(5): p. 331-40.
16. Rogers, H.W., et al., *Listeria monocytogenes induces apoptosis of infected hepatocytes.* J Immunol, 1996. **156**(2): p. 679-84.
17. Dancygier, H., *Bacterial Liver Abscess and Other Bacterial Infections,* in *Clinical Hepatology*. 2010. p. 831-842.
18. Edelson, B.T., et al., *CD8alpha(+) dendritic cells are an obligate cellular entry point for productive infection by Listeria monocytogenes.* Immunity. **35**(2): p. 236-48.
19. Aoshi, T., et al., *The cellular niche of Listeria monocytogenes infection changes rapidly in the spleen.* Eur J Immunol, 2009. **39**(2): p. 417-25.
20. Vazquez-Boland, J.A., et al., *Pathogenicity islands and virulence evolution in Listeria.* Microbes Infect, 2001. **3**(7): p. 571-84.
21. de las Heras, A., et al., *Regulation of Listeria virulence: PrfA master and commander.* Curr Opin Microbiol. **14**(2): p. 118-27.
22. Johansson, J., et al., *An RNA thermosensor controls expression of virulence genes in Listeria monocytogenes.* Cell, 2002. **110**(5): p. 551-61.
23. Milenbachs, A.A., et al., *Carbon-source regulation of virulence gene expression in Listeria monocytogenes.* Mol Microbiol, 1997. **23**(5): p. 1075-85.
24. Loh, E., et al., *A trans-acting riboswitch controls expression of the virulence regulator PrfA in Listeria monocytogenes.* Cell, 2009. **139**(4): p. 770-9.
25. Bierne, H., et al., *Internalins: a complex family of leucine-rich repeat-containing proteins in Listeria monocytogenes.* Microbes Infect, 2007. **9**(10): p. 1156-66.
26. Ireton, K., *Entry of the bacterial pathogen Listeria monocytogenes into mammalian cells.* Cell Microbiol, 2007. **9**(6): p. 1365-75.
27. Pentecost, M., et al., *Listeria monocytogenes invades the epithelial junctions at sites of cell extrusion.* PLoS Pathog, 2006. **2**(1): p. e3.
28. Disson, O., et al., *Modeling human listeriosis in natural and genetically engineered animals.* Nat Protoc, 2009. **4**(6): p. 799-810.

29. Wollert, T., et al., *Extending the host range of Listeria monocytogenes by rational protein design.* Cell, 2007. **129**(5): p. 891-902.
30. Pentecost, M., et al., *Listeria monocytogenes internalin B activates junctional endocytosis to accelerate intestinal invasion.* PLoS Pathog. **6**(5): p. e1000900.
31. Engelbrecht, F., et al., *A new PrfA-regulated gene of Listeria monocytogenes encoding a small, secreted protein which belongs to the family of internalins.* Mol Microbiol, 1996. **21**(4): p. 823-37.
32. Rajabian, T., et al., *The bacterial virulence factor InlC perturbs apical cell junctions and promotes cell-to-cell spread of Listeria.* Nat Cell Biol, 2009. **11**(10): p. 1212-8.
33. Personnic, N., et al., *The stress-induced virulence protein InlH controls interleukin-6 production during murine listeriosis.* Infect Immun. **78**(5): p. 1979-89.
34. Sabet, C., et al., *The Listeria monocytogenes virulence factor InlJ is specifically expressed in vivo and behaves as an adhesin.* Infect Immun, 2008. **76**(4): p. 1368-78.
35. Dortet, L., S. Mostowy, and P. Cossart, *Listeria and autophagy escape: Involvement of InlK, an internalin-like protein.* Autophagy. **8**(1).
36. Gaillard, J.L., P. Berche, and P. Sansonetti, *Transposon mutagenesis as a tool to study the role of hemolysin in the virulence of Listeria monocytogenes.* Infect Immun, 1986. **52**(1): p. 50-5.
37. Gilbert, R.J., *Pore-forming toxins.* Cell Mol Life Sci, 2002. **59**(5): p. 832-44.
38. Kayal, S. and A. Charbit, *Listeriolysin O: a key protein of Listeria monocytogenes with multiple functions.* FEMS Microbiol Rev, 2006. **30**(4): p. 514-29.
39. Schnupf, P. and D.A. Portnoy, *Listeriolysin O: a phagosome-specific lysin.* Microbes Infect, 2007. **9**(10): p. 1176-87.
40. Moors, M.A., et al., *Expression of listeriolysin O and ActA by intracellular and extracellular Listeria monocytogenes.* Infect Immun, 1999. **67**(1): p. 131-9.
41. Zwaferink, H., et al., *IFN-beta increases listeriolysin O-induced membrane permeabilization and death of macrophages.* J Immunol, 2008. **180**(6): p. 4116-23.
42. Carrero, J.A., B. Calderon, and E.R. Unanue, *Listeriolysin O from Listeria monocytogenes is a lymphocyte apoptogenic molecule.* J Immunol, 2004. **172**(8): p. 4866-74.

43. Cotter, P.D., et al., *Listeriolysin S, a novel peptide haemolysin associated with a subset of lineage I Listeria monocytogenes.* PLoS Pathog, 2008. **4**(9): p. e1000144.
44. Domann, E., et al., *A novel bacterial virulence gene in Listeria monocytogenes required for host cell microfilament interaction with homology to the proline-rich region of vinculin.* EMBO J, 1992. **11**(5): p. 1981-90.
45. Kocks, C., et al., *L. monocytogenes-induced actin assembly requires the actA gene product, a surface protein.* Cell, 1992. **68**(3): p. 521-31.
46. Cossart, P. and H. Bierne, *The use of host cell machinery in the pathogenesis of Listeria monocytogenes.* Curr Opin Immunol, 2001. **13**(1): p. 96-103.
47. Lambrechts, A., et al., *Listeria comet tails: the actin-based motility machinery at work.* Trends Cell Biol, 2008. **18**(5): p. 220-7.
48. Hybiske, K. and R.S. Stephens, *Exit strategies of intracellular pathogens.* Nat Rev Microbiol, 2008. **6**(2): p. 99-110.
49. Smith, G.A., et al., *The two distinct phospholipases C of Listeria monocytogenes have overlapping roles in escape from a vacuole and cell-to-cell spread.* Infect Immun, 1995. **63**(11): p. 4231-7.
50. Schmidt, R.L., et al., *A LysM and SH3-domain containing region of the Listeria monocytogenes p60 protein stimulates accessory cells to promote activation of host NK cells.* PLoS Pathog. **7**(11): p. e1002368.
51. Poyart, C., et al., *The zinc metalloprotease of Listeria monocytogenes is required for maturation of phosphatidylcholine phospholipase C: direct evidence obtained by gene complementation.* Infect Immun, 1993. **61**(4): p. 1576-80.
52. Forster, B.M., et al., *The metalloprotease of Listeria monocytogenes is regulated by pH.* J Bacteriol. **193**(19): p. 5090-7.
53. Chico-Calero, I., et al., *Hpt, a bacterial homolog of the microsomal glucose- 6-phosphate translocase, mediates rapid intracellular proliferation in Listeria.* Proc Natl Acad Sci U S A, 2002. **99**(1): p. 431-6.
54. Kastner, R., et al., *LipA, a tyrosine and lipid phosphatase involved in the virulence of Listeria monocytogenes.* Infect Immun. **79**(6): p. 2489-98.
55. Archambaud, C., et al., *Translation elongation factor EF-Tu is a target for Stp, a serine-threonine phosphatase involved in virulence of Listeria monocytogenes.* Mol Microbiol, 2005. **56**(2): p. 383-96.
56. Vasconcelos, J.A. and H.G. Deneer, *Expression of superoxide dismutase in Listeria monocytogenes.* Appl Environ Microbiol, 1994. **60**(7): p. 2360-6.

57. Ma, F., et al., *The microRNA miR-29 controls innate and adaptive immune responses to intracellular bacterial infection by targeting interferon-gamma.* Nat Immunol. **12**(9): p. 861-9.
58. Schnitger, A.K., et al., *Listeria monocytogenes Infection in Macrophages Induces Vacuolar-Dependent Host miRNA Response.* PLoS One. **6**(11): p. e27435.
59. Ribet, D., et al., *Listeria monocytogenes impairs SUMOylation for efficient infection.* Nature. **464**(7292): p. 1192-5.
60. Stavru, F., et al., *Listeria monocytogenes transiently alters mitochondrial dynamics during infection.* Proc Natl Acad Sci U S A. **108**(9): p. 3612-7.
61. Pamer, E.G., *Immune responses to Listeria monocytogenes.* Nat Rev Immunol, 2004. **4**(10): p. 812-23.
62. Akira, S., S. Uematsu, and O. Takeuchi, *Pathogen recognition and innate immunity.* Cell, 2006. **124**(4): p. 783-801.
63. Kawai, T. and S. Akira, *Toll-like receptors and their crosstalk with other innate receptors in infection and immunity.* Immunity. **34**(5): p. 637-50.
64. Kawai, T. and S. Akira, *The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors.* Nat Immunol. **11**(5): p. 373-84.
65. Torres, D., et al., *Toll-like receptor 2 is required for optimal control of Listeria monocytogenes infection.* Infect Immun, 2004. **72**(4): p. 2131-9.
66. Edelson, B.T. and E.R. Unanue, *MyD88-dependent but Toll-like receptor 2-independent innate immunity to Listeria: no role for either in macrophage listericidal activity.* J Immunol, 2002. **169**(7): p. 3869-75.
67. Machata, S., et al., *Lipoproteins of Listeria monocytogenes are critical for virulence and TLR2-mediated immune activation.* J Immunol, 2008. **181**(3): p. 2028-35.
68. Way, S.S., et al., *Characterization of flagellin expression and its role in Listeria monocytogenes infection and immunity.* Cell Microbiol, 2004. **6**(3): p. 235-42.
69. Hayashi, F., et al., *The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5.* Nature, 2001. **410**(6832): p. 1099-103.
70. Seki, E., et al., *Critical roles of myeloid differentiation factor 88-dependent proinflammatory cytokine release in early phase clearance of Listeria monocytogenes in mice.* J Immunol, 2002. **169**(7): p. 3863-8.
71. Stockinger, S., et al., *Characterization of the interferon-producing cell in mice infected with Listeria monocytogenes.* PLoS Pathog, 2009. **5**(3): p. e1000355.

72. Kim, Y.G., et al., *The cytosolic sensors Nod1 and Nod2 are critical for bacterial recognition and host defense after exposure to Toll-like receptor ligands.* Immunity, 2008. **28**(2): p. 246-57.
73. Kim, S., et al., *Listeria monocytogenes is sensed by the NLRP3 and AIM2 inflammasome.* Eur J Immunol. **40**(6): p. 1545-51.
74. Warren, S.E., et al., *Multiple Nod-like receptors activate caspase 1 during Listeria monocytogenes infection.* J Immunol, 2008. **180**(11): p. 7558-64.
75. Corr, S.C. and L.A. O'Neill, *Listeria monocytogenes infection in the face of innate immunity.* Cell Microbiol, 2009. **11**(5): p. 703-9.
76. Conlan, J.W. and R.J. North, *Neutrophils are essential for early anti-Listeria defense in the liver, but not in the spleen or peritoneal cavity, as revealed by a granulocyte-depleting monoclonal antibody.* J Exp Med, 1994. **179**(1): p. 259-68.
77. Shi, C., et al., *Ly6G+ Neutrophils Are Dispensable for Defense against Systemic Listeria monocytogenes Infection.* J Immunol. **187**(10): p. 5293-8.
78. Thale, C. and A.F. Kiderlen, *Sources of interferon-gamma (IFN-gamma) in early immune response to Listeria monocytogenes.* Immunobiology, 2005. **210**(9): p. 673-83.
79. Flannagan, R.S., G. Cosio, and S. Grinstein, *Antimicrobial mechanisms of phagocytes and bacterial evasion strategies.* Nat Rev Microbiol, 2009. **7**(5): p. 355-66.
80. Unanue, E.R., *Inter-relationship among macrophages, natural killer cells and neutrophils in early stages of Listeria resistance.* Curr Opin Immunol, 1997. **9**(1): p. 35-43.
81. Cousens, L.P. and E.J. Wing, *Innate defenses in the liver during Listeria infection.* Immunol Rev, 2000. **174**: p. 150-9.
82. Serbina, N.V., et al., *Monocyte-mediated defense against microbial pathogens.* Annu Rev Immunol, 2008. **26**: p. 421-52.
83. Rosen, H., S. Gordon, and R.J. North, *Exacerbation of murine listeriosis by a monoclonal antibody specific for the type 3 complement receptor of myelomonocytic cells. Absence of monocytes at infective foci allows Listeria to multiply in nonphagocytic cells.* J Exp Med, 1989. **170**(1): p. 27-37.
84. Aichele, P., et al., *Macrophages of the splenic marginal zone are essential for trapping of blood-borne particulate antigen but dispensable for induction of specific T cell responses.* J Immunol, 2003. **171**(3): p. 1148-55.

85. Jung, S., et al., *In vivo depletion of CD11c+ dendritic cells abrogates priming of CD8+ T cells by exogenous cell-associated antigens.* Immunity, 2002. **17**(2): p. 211-20.
86. Serbina, N.V., et al., *TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection.* Immunity, 2003. **19**(1): p. 59-70.
87. Neuenhahn, M., et al., *CD8alpha+ dendritic cells are required for efficient entry of Listeria monocytogenes into the spleen.* Immunity, 2006. **25**(4): p. 619-30.
88. Bhardwaj, V., et al., *Chronic Listeria infection in SCID mice: requirements for the carrier state and the dual role of T cells in transferring protection or suppression.* J Immunol, 1998. **160**(1): p. 376-84.
89. Lara-Tejero, M. and E.G. Pamer, *T cell responses to Listeria monocytogenes.* Curr Opin Microbiol, 2004. **7**(1): p. 45-50.
90. Mercado, R., et al., *Early programming of T cell populations responding to bacterial infection.* J Immunol, 2000. **165**(12): p. 6833-9.
91. Wong, P. and E.G. Pamer, *Cutting edge: antigen-independent CD8 T cell proliferation.* J Immunol, 2001. **166**(10): p. 5864-8.
92. Mittrucker, H.W., A. Kohler, and S.H. Kaufmann, *Substantial in vivo proliferation of CD4(+) and CD8(+) T lymphocytes during secondary Listeria monocytogenes infection.* Eur J Immunol, 2000. **30**(4): p. 1053-9.
93. Hsieh, C.S., et al., *Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages.* Science, 1993. **260**(5107): p. 547-9.
94. Curtis, M.M. and S.S. Way, *Interleukin-17 in host defence against bacterial, mycobacterial and fungal pathogens.* Immunology, 2009. **126**(2): p. 177-85.
95. Hamada, S., et al., *IL-17A produced by gammadelta T cells plays a critical role in innate immunity against listeria monocytogenes infection in the liver.* J Immunol, 2008. **181**(5): p. 3456-63.
96. Miyamoto, M., et al., *Neutrophilia in LFA-1-deficient mice confers resistance to listeriosis: possible contribution of granulocyte-colony-stimulating factor and IL-17.* J Immunol, 2003. **170**(10): p. 5228-34.
97. Rowe, J.H., et al., *Foxp3(+) regulatory T cell expansion required for sustaining pregnancy compromises host defense against prenatal bacterial pathogens.* Cell Host Microbe. **10**(1): p. 54-64.
98. Rowe, J.H., J.M. Ertelt, and S.S. Way, *Foxp3(+) Regulatory T cells, Immune Stimulation and Host Defense against Infection.* Immunology.

99. Ochsenbein, A.F., et al., *Control of early viral and bacterial distribution and disease by natural antibodies.* Science, 1999. **286**(5447): p. 2156-9.
100. Edelson, B.T., P. Cossart, and E.R. Unanue, *Cutting edge: paradigm revisited: antibody provides resistance to Listeria infection.* J Immunol, 1999. **163**(8): p. 4087-90.
101. Edelson, B.T. and E.R. Unanue, *Immunity to Listeria infection.* Curr Opin Immunol, 2000. **12**(4): p. 425-31.
102. Drevets, D.A. and P.A. Campbell, *Roles of complement and complement receptor type 3 in phagocytosis of Listeria monocytogenes by inflammatory mouse peritoneal macrophages.* Infect Immun, 1991. **59**(8): p. 2645-52.
103. Verschoor, A., et al., *A platelet-mediated system for shuttling blood-borne bacteria to CD8alpha+ dendritic cells depends on glycoprotein GPIb and complement C3.* Nat Immunol. **12**(12): p. 1194-201.
104. Isaacs, A. and J. Lindenmann, *Virus interference. I. The interferon.* Proc R Soc Lond B Biol Sci, 1957. **147**(927): p. 258-67.
105. Borden, E.C., et al., *Interferons at age 50: past, current and future impact on biomedicine.* Nat Rev Drug Discov, 2007. **6**(12): p. 975-90.
106. Renauld, J.C., *Class II cytokine receptors and their ligands: key antiviral and inflammatory modulators.* Nat Rev Immunol, 2003. **3**(8): p. 667-76.
107. Pestka, S., C.D. Krause, and M.R. Walter, *Interferons, interferon-like cytokines, and their receptors.* Immunol Rev, 2004. **202**: p. 8-32.
108. Zhang, S.Y., et al., *Inborn errors of interferon (IFN)-mediated immunity in humans: insights into the respective roles of IFN-alpha/beta, IFN-gamma, and IFN-lambda in host defense.* Immunol Rev, 2008. **226**: p. 29-40.
109. Uze, G., et al., *The receptor of the type I interferon family.* Curr Top Microbiol Immunol, 2007. **316**: p. 71-95.
110. Decker, T., M. Muller, and S. Stockinger, *The yin and yang of type I interferon activity in bacterial infection.* Nat Rev Immunol, 2005. **5**(9): p. 675-87.
111. Silverman, R.H., *Viral encounters with 2',5'-oligoadenylate synthetase and RNase L during the interferon antiviral response.* J Virol, 2007. **81**(23): p. 12720-9.
112. Williams, B.R., *Signal integration via PKR.* Sci STKE, 2001. **2001**(89): p. re2.
113. Sarkar, S.N. and G.C. Sen, *Novel functions of proteins encoded by viral stress-inducible genes.* Pharmacol Ther, 2004. **103**(3): p. 245-59.

114. Haller, O., P. Staeheli, and G. Kochs, *Interferon-induced Mx proteins in antiviral host defense.* Biochimie, 2007. **89**(6-7): p. 812-8.
115. Kayagaki, N., et al., *Type I interferons (IFNs) regulate tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) expression on human T cells: A novel mechanism for the antitumor effects of type I IFNs.* J Exp Med, 1999. **189**(9): p. 1451-60.
116. Young, H.A. and J.H. Bream, *IFN-gamma: recent advances in understanding regulation of expression, biological functions, and clinical applications.* Curr Top Microbiol Immunol, 2007. **316**: p. 97-117.
117. Goodbourn, S., L. Didcock, and R.E. Randall, *Interferons: cell signalling, immune modulation, antiviral response and virus countermeasures.* J Gen Virol, 2000. **81**(Pt 10): p. 2341-64.
118. Schroder, K., et al., *Interferon-gamma: an overview of signals, mechanisms and functions.* J Leukoc Biol, 2004. **75**(2): p. 163-89.
119. Boehm, U., et al., *Cellular responses to interferon-gamma.* Annu Rev Immunol, 1997. **15**: p. 749-95.
120. Uze, G. and D. Monneron, *IL-28 and IL-29: newcomers to the interferon family.* Biochimie, 2007. **89**(6-7): p. 729-34.
121. Donnelly, R.P. and S.V. Kotenko, *Interferon-lambda: a new addition to an old family.* J Interferon Cytokine Res. **30**(8): p. 555-64.
122. Schindler, C., D.E. Levy, and T. Decker, *JAK-STAT signaling: from interferons to cytokines.* J Biol Chem, 2007. **282**(28): p. 20059-63.
123. Levy, D.E. and J.E. Darnell, Jr., *Stats: transcriptional control and biological impact.* Nat Rev Mol Cell Biol, 2002. **3**(9): p. 651-62.
124. Mertens, C., et al., *Dephosphorylation of phosphotyrosine on STAT1 dimers requires extensive spatial reorientation of the monomers facilitated by the N-terminal domain.* Genes Dev, 2006. **20**(24): p. 3372-81.
125. Pilz, A., et al., *Dendritic cells require STAT-1 phosphorylated at its transactivating domain for the induction of peptide-specific CTL.* J Immunol, 2009. **183**(4): p. 2286-93.
126. Varinou, L., et al., *Phosphorylation of the Stat1 transactivation domain is required for full-fledged IFN-gamma-dependent innate immunity.* Immunity, 2003. **19**(6): p. 793-802.

127. Yang, J. and G.R. Stark, *Roles of unphosphorylated STATs in signaling.* Cell Res, 2008. **18**(4): p. 443-51.
128. Najjar, I. and R. Fagard, *STAT1 and pathogens, not a friendly relationship.* Biochimie. **92**(5): p. 425-44.
129. O'Shea, J.J., M. Gadina, and R.D. Schreiber, *Cytokine signaling in 2002: new surprises in the Jak/Stat pathway.* Cell, 2002. **109 Suppl**: p. S121-31.
130. Ramana, C.V., et al., *Stat1-independent regulation of gene expression in response to IFN-gamma.* Proc Natl Acad Sci U S A, 2001. **98**(12): p. 6674-9.
131. Gil, M.P., et al., *Biologic consequences of Stat1-independent IFN signaling.* Proc Natl Acad Sci U S A, 2001. **98**(12): p. 6680-5.
132. Lee, C.K., et al., *Distinct requirements for IFNs and STAT1 in NK cell function.* J Immunol, 2000. **165**(7): p. 3571-7.
133. Chapgier, A., et al., *Novel STAT1 alleles in otherwise healthy patients with mycobacterial disease.* PLoS Genet, 2006. **2**(8): p. e131.
134. Chapgier, A., et al., *A partial form of recessive STAT1 deficiency in humans.* J Clin Invest, 2009. **119**(6): p. 1502-14.
135. Chapgier, A., et al., *Human complete Stat-1 deficiency is associated with defective type I and II IFN responses in vitro but immunity to some low virulence viruses in vivo.* J Immunol, 2006. **176**(8): p. 5078-83.
136. Kaplan, D.H., et al., *Demonstration of an interferon gamma-dependent tumor surveillance system in immunocompetent mice.* Proc Natl Acad Sci U S A, 1998. **95**(13): p. 7556-61.
137. Kovacic, B., et al., *STAT1 acts as a tumor promoter for leukemia development.* Cancer Cell, 2006. **10**(1): p. 77-87.
138. Schneckenleithner, C., et al., *Putting the brakes on mammary tumorigenesis: Loss of STAT1 predisposes to intraepithelial neoplasias.* Oncotarget.
139. Lighvani, A.A., et al., *T-bet is rapidly induced by interferon-gamma in lymphoid and myeloid cells.* Proc Natl Acad Sci U S A, 2001. **98**(26): p. 15137-42.
140. Chin, Y.E., et al., *Cell growth arrest and induction of cyclin-dependent kinase inhibitor p21 WAF1/CIP1 mediated by STAT1.* Science, 1996. **272**(5262): p. 719-22.
141. Trinchieri, G., *Type I interferon: friend or foe?* J Exp Med. **207**(10): p. 2053-63.
142. Hornung, V. and E. Latz, *Intracellular DNA recognition.* Nat Rev Immunol. **10**(2): p. 123-30.

143. Schattgen, S.A. and K.A. Fitzgerald, *The PYHIN protein family as mediators of host defenses.* Immunol Rev. **243**(1): p. 109-18.
144. Barber, G.N., *Cytoplasmic DNA innate immune pathways.* Immunol Rev. **243**(1): p. 99-108.
145. Sauer, J.D., et al., *The N-ethyl-N-nitrosourea-induced Goldenticket mouse mutant reveals an essential function of Sting in the in vivo interferon response to Listeria monocytogenes and cyclic dinucleotides.* Infect Immun. **79**(2): p. 688-94.
146. Eitel, J., N. Suttorp, and B. Opitz, *Innate immune recognition and inflammasome activation in listeria monocytogenes infection.* Front Microbiol. **1**: p. 149.
147. Stockinger, S., et al., *IFN regulatory factor 3-dependent induction of type I IFNs by intracellular bacteria is mediated by a TLR- and Nod2-independent mechanism.* J Immunol, 2004. **173**(12): p. 7416-25.
148. Lippmann, J., et al., *IFNbeta responses induced by intracellular bacteria or cytosolic DNA in different human cells do not require ZBP1 (DLM-1/DAI).* Cell Microbiol, 2008. **10**(12): p. 2579-88.
149. Rathinam, V.A., et al., *The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses.* Nat Immunol. **11**(5): p. 395-402.
150. Unterholzner, L., et al., *IFI16 is an innate immune sensor for intracellular DNA.* Nat Immunol. **11**(11): p. 997-1004.
151. Woodward, J.J., A.T. Iavarone, and D.A. Portnoy, *c-di-AMP secreted by intracellular Listeria monocytogenes activates a host type I interferon response.* Science. **328**(5986): p. 1703-5.
152. Auerbuch, V., et al., *Mice lacking the type I interferon receptor are resistant to Listeria monocytogenes.* J Exp Med, 2004. **200**(4): p. 527-33.
153. Carrero, J.A., B. Calderon, and E.R. Unanue, *Type I interferon sensitizes lymphocytes to apoptosis and reduces resistance to Listeria infection.* J Exp Med, 2004. **200**(4): p. 535-40.
154. O'Connell, R.M., et al., *Type I interferon production enhances susceptibility to Listeria monocytogenes infection.* J Exp Med, 2004. **200**(4): p. 437-45.
155. Stockinger, S., et al., *Production of type I IFN sensitizes macrophages to cell death induced by Listeria monocytogenes.* J Immunol, 2002. **169**(11): p. 6522-9.
156. Carrero, J.A., B. Calderon, and E.R. Unanue, *Lymphocytes are detrimental during the early innate immune response against Listeria monocytogenes.* J Exp Med, 2006. **203**(4): p. 933-40.

157. Dai, W.J., G. Kohler, and F. Brombacher, *Both innate and acquired immunity to Listeria monocytogenes infection are increased in IL-10-deficient mice.* J Immunol, 1997. **158**(5): p. 2259-67.
158. Rayamajhi, M., et al., *Induction of IFN-alphabeta enables Listeria monocytogenes to suppress macrophage activation by IFN-gamma.* J Exp Med, 2010. **207**(2): p. 327-37.
159. Stark, G.R., et al., *How cells respond to interferons.* Annu Rev Biochem, 1998. **67**: p. 227-64.
160. Durbin, J.E., et al., *Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease.* Cell, 1996. **84**(3): p. 443-50.
161. Meraz, M.A., et al., *Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway.* Cell, 1996. **84**(3): p. 431-42.
162. Kristensen, I.A., et al., *Novel STAT1 alleles in a patient with impaired resistance to mycobacteria.* J Clin Immunol. **31**(2): p. 265-71.
163. Lu, B., et al., *Targeted disruption of the interferon-gamma receptor 2 gene results in severe immune defects in mice.* Proc Natl Acad Sci U S A, 1998. **95**(14): p. 8233-8.
164. Kaufmann, S.H., *Immunity to intracellular bacteria.* Annu Rev Immunol, 1993. **11**: p. 129-63.
165. Badovinac, V.P. and J.T. Harty, *Adaptive immunity and enhanced CD8+ T cell response to Listeria monocytogenes in the absence of perforin and IFN-gamma.* J Immunol, 2000. **164**(12): p. 6444-52.
166. Durbin, J.E., et al., *Type I IFN modulates innate and specific antiviral immunity.* J Immunol, 2000. **164**(8): p. 4220-8.
167. Fallarino, F. and T.F. Gajewski, *Cutting edge: differentiation of antitumor CTL in vivo requires host expression of Stat1.* J Immunol, 1999. **163**(8): p. 4109-13.
168. Gil, M.P., et al., *Modulation of STAT1 protein levels: a mechanism shaping CD8 T-cell responses in vivo.* Blood, 2006. **107**(3): p. 987-93.
169. Messingham, K.A., et al., *A role for IFN-gamma from antigen-specific CD8+ T cells in protective immunity to Listeria monocytogenes.* J Immunol, 2007. **179**(4): p. 2457-66.

170. Akbar, S.M., et al., *Unique features of dendritic cells in IFN-gamma transgenic mice: relevance to cancer development and therapeutic implications.* Biochem Biophys Res Commun, 1999. **259**(2): p. 294-9.
171. Banyer, J.L., et al., *Combinations of IFN-gamma and IL-4 induce distinct profiles of dendritic cell-associated immunoregulatory properties.* Genes Immun, 2003. **4**(6): p. 427-40.
172. Le Bon, A., et al., *Cross-priming of CD8+ T cells stimulated by virus-induced type I interferon.* Nat Immunol, 2003. **4**(10): p. 1009-15.
173. Huang, S., et al., *Immune response in mice that lack the interferon-gamma receptor.* Science, 1993. **259**(5102): p. 1742-5.
174. Gregory, S.H., A.J. Sagnimeni, and E.J. Wing, *Internalin B promotes the replication of Listeria monocytogenes in mouse hepatocytes.* Infect Immun, 1997. **65**(12): p. 5137-41.
175. Clausen, B.E., et al., *Conditional gene targeting in macrophages and granulocytes using LysMcre mice.* Transgenic Res, 1999. **8**(4): p. 265-77.
176. Wallner, B., et al., *Generation of mice with a conditional Stat1 null allele.* Transgenic Res.
177. Kimura, A., et al., *Aryl hydrocarbon receptor in combination with Stat1 regulates LPS-induced inflammatory responses.* J Exp Med, 2009. **206**(9): p. 2027-35.
178. Sasaki, T., et al., *Roles of CD4+ and CD8+ cells, and the effect of administration of recombinant murine interferon gamma in listerial infection.* J Exp Med, 1990. **171**(4): p. 1141-54.
179. Nguyen, K.B., et al., *Critical role for STAT4 activation by type 1 interferons in the interferon-gamma response to viral infection.* Science, 2002. **297**(5589): p. 2063-6.
180. Portnoy, D.A., V. Auerbuch, and I.J. Glomski, *The cell biology of Listeria monocytogenes infection: the intersection of bacterial pathogenesis and cell-mediated immunity.* J Cell Biol, 2002. **158**(3): p. 409-14.
181. Hamon, M., H. Bierne, and P. Cossart, *Listeria monocytogenes: a multifaceted model.* Nat Rev Microbiol, 2006. **4**(6): p. 423-34.
182. Gregory, S.H. and C.C. Liu, *CD8+ T-cell-mediated response to Listeria monocytogenes taken up in the liver and replicating within hepatocytes.* Immunol Rev, 2000. **174**: p. 112-22.
183. Harty, J.T. and M.J. Bevan, *Specific immunity to Listeria monocytogenes in the absence of IFN gamma.* Immunity, 1995. **3**(1): p. 109-17.

184. Mackaness, G.B., *Cellular resistance to infection.* J Exp Med, 1962. **116**: p. 381-406.
185. Shaughnessy, L.M. and J.A. Swanson, *The role of the activated macrophage in clearing Listeria monocytogenes infection.* Front Biosci, 2007. **12**: p. 2683-92.
186. Shi, C. and E.G. Pamer, *Monocyte recruitment during infection and inflammation.* Nat Rev Immunol. **11**(11): p. 762-74.
187. Zhou, L., A.A. Nazarian, and S.T. Smale, *Interleukin-10 inhibits interleukin-12 p40 gene transcription by targeting a late event in the activation pathway.* Mol Cell Biol, 2004. **24**(6): p. 2385-96.
188. Hu, X., et al., *IFN-gamma suppresses IL-10 production and synergizes with TLR2 by regulating GSK3 and CREB/AP-1 proteins.* Immunity, 2006. **24**(5): p. 563-74.
189. Saraiva, M. and A. O'Garra, *The regulation of IL-10 production by immune cells.* Nat Rev Immunol. **10**(3): p. 170-81.
190. Havenar-Daughton, C., G.A. Kolumam, and K. Murali-Krishna, *Cutting Edge: The direct action of type I IFN on CD4 T cells is critical for sustaining clonal expansion in response to a viral but not a bacterial infection.* J Immunol, 2006. **176**(6): p. 3315-9.
191. Thompson, L.J., et al., *Innate inflammatory signals induced by various pathogens differentially dictate the IFN-I dependence of CD8 T cells for clonal expansion and memory formation.* J Immunol, 2006. **177**(3): p. 1746-54.
192. Johnson, L.M. and P. Scott, *STAT1 expression in dendritic cells, but not T cells, is required for immunity to Leishmania major.* J Immunol, 2007. **178**(11): p. 7259-66.
193. Jackson, S.H., et al., *Dendritic cell maturation requires STAT1 and is under feedback regulation by suppressors of cytokine signaling.* J Immunol, 2004. **172**(4): p. 2307-15.
194. Le Bon, A., et al., *Type i interferons potently enhance humoral immunity and can promote isotype switching by stimulating dendritic cells in vivo.* Immunity, 2001. **14**(4): p. 461-70.
195. Ma, H., et al., *Absence of Stat1 in donor CD4 T cells promotes the expansion of Tregs and reduces graft-versus-host disease in mice.* J Clin Invest. **121**(7): p. 2554-69.
196. Caton, M.L., M.R. Smith-Raska, and B. Reizis, *Notch-RBP-J signaling controls the homeostasis of CD8- dendritic cells in the spleen.* J Exp Med, 2007. **204**(7): p. 1653-64.

197. Orban, P.C., D. Chui, and J.D. Marth, *Tissue- and site-specific DNA recombination in transgenic mice.* Proc Natl Acad Sci U S A, 1992. **89**(15): p. 6861-5.
198. Muller, U., et al., *Functional role of type I and type II interferons in antiviral defense.* Science, 1994. **264**(5167): p. 1918-21.
199. Kamphuis, E., et al., *Type I interferons directly regulate lymphocyte recirculation and cause transient blood lymphopenia.* Blood, 2006. **108**(10): p. 3253-61.
200. Czuprynski, C.J., N.G. Faith, and H. Steinberg, *A/J mice are susceptible and C57BL/6 mice are resistant to Listeria monocytogenes infection by intragastric inoculation.* Infect Immun, 2003. **71**(2): p. 682-9.
201. Reutterer, B., et al., *Type I IFN are host modulators of strain-specific Listeria monocytogenes virulence.* Cell Microbiol, 2008. **10**(5): p. 1116-29.
202. Chiba, S., et al., *Listerial invasion protein internalin B promotes entry into ileal Peyer's patches in vivo.* Microbiol Immunol. **55**(2): p. 123-9.
203. Vadia, S., et al., *The Pore-Forming Toxin Listeriolysin O Mediates a Novel Entry Pathway of L. monocytogenes into Human Hepatocytes.* PLoS Pathog. **7**(11): p. e1002356.
204. Rayamajhi, M., et al., *Antagonistic crosstalk between type I and II interferons and increased host susceptibility to bacterial infections.* Virulence. **1**(5): p. 418-22.
205. Carrero, J.A. and E.R. Unanue, *Impact of lymphocyte apoptosis on the innate immune stages of infection.* Immunol Res, 2007. **38**(1-3): p. 333-41.
206. Cabanes, D., M. Lecuit, and P. Cossart, *Animal models of Listeria infection.* Curr Protoc Microbiol, 2008. **Chapter 9**: p. Unit9B 1.
207. Arnaud, M., A. Chastanet, and M. Debarbouille, *New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, gram-positive bacteria.* Appl Environ Microbiol, 2004. **70**(11): p. 6887-91.
208. Lingnau, A., et al., *Expression of the Listeria monocytogenes EGD inlA and inlB genes, whose products mediate bacterial entry into tissue culture cell lines, by PrfA-dependent and -independent mechanisms.* Infect Immun, 1995. **63**(10): p. 3896-903.
209. Weigent, D.A., et al., *Role of interferon in streptococcal infection in the mouse.* Microb Pathog, 1986. **1**(4): p. 399-407.

210. Freudenberg, M.A., et al., *Cutting edge: a murine, IL-12-independent pathway of IFN-gamma induction by gram-negative bacteria based on STAT4 activation by Type I IFN and IL-18 signaling.* J Immunol, 2002. **169**(4): p. 1665-8.
211. Manca, C., et al., *Virulence of a Mycobacterium tuberculosis clinical isolate in mice is determined by failure to induce Th1 type immunity and is associated with induction of IFN-alpha /beta.* Proc Natl Acad Sci U S A, 2001. **98**(10): p. 5752-7.
212. Bourgeois, C., et al., *Conventional dendritic cells mount a type I IFN response against Candida spp. requiring novel phagosomal TLR7-mediated IFN-beta signaling.* J Immunol. **186**(5): p. 3104-12.
213. Solodova, E., et al., *Production of IFN-beta during Listeria monocytogenes infection is restricted to monocyte/macrophage lineage.* PLoS One. **6**(4): p. e18543.
214. Dresing, P., et al., *A fluorescence reporter model defines "Tip-DCs" as the cellular source of interferon beta in murine listeriosis.* PLoS One. **5**(12): p. e15567.
215. Barchet, W., et al., *Virus-induced interferon alpha production by a dendritic cell subset in the absence of feedback signaling in vivo.* J Exp Med, 2002. **195**(4): p. 507-16.
216. Fejer, G., et al., *Key role of splenic myeloid DCs in the IFN-alphabeta response to adenoviruses in vivo.* PLoS Pathog, 2008. **4**(11): p. e1000208.
217. Camejo, A., et al., *In vivo transcriptional profiling of Listeria monocytogenes and mutagenesis identify new virulence factors involved in infection.* PLoS Pathog, 2009. **5**(5): p. e1000449.
218. Brzoza-Lewis, K.L., J. Jason Hoth, and E.M. Hiltbold, *Type I interferon signaling regulates the composition of inflammatory infiltrates upon infection with Listeria monocytogenes.* Cell Immunol. **273**(1): p. 41-51.
219. Salazar-Mather, T.P., C.A. Lewis, and C.A. Biron, *Type I interferons regulate inflammatory cell trafficking and macrophage inflammatory protein 1alpha delivery to the liver.* J Clin Invest, 2002. **110**(3): p. 321-30.
220. Hildner, K., et al., *Batf3 deficiency reveals a critical role for CD8alpha+ dendritic cells in cytotoxic T cell immunity.* Science, 2008. **322**(5904): p. 1097-100.
221. Bouchonnet, F., et al., *Alpha/beta interferon impairs the ability of human macrophages to control growth of Mycobacterium bovis BCG.* Infect Immun, 2002. **70**(6): p. 3020-5.

222. Cooper, A.M., et al., *Expression of the nitric oxide synthase 2 gene is not essential for early control of Mycobacterium tuberculosis in the murine lung.* Infect Immun, 2000. **68**(12): p. 6879-82.

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University Studies

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Publications

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***Characterization of the interferon-producing cell in mice infected with* Listeria monocytogenes**,

*PLoS Pathog. 2009 March;5(3) (**R.K. and E.K. contributed equally to this study)

Kastner R, Dussurget O, Archambaud C, **Kernbauer E**, Soulat D, Cossart P, Decker T.

LipA, a tyrosine and lipid phosphatase involved in the virulence of Listeria monocytogenes.,

Infect Immun. 2011 June;79(6):2489-98.

Wallner B, Leitner NR, Vielnascher RM, **Kernbauer E**, Kolbe T, Karaghiosoff M, Rülicke T, Decker T, Müller M.

Generation of mice with a conditional Stat1 null allele.

Transgenic Res. 2011 May 7

Pending publication

E. Kernbauer, B. Strobl, M. Müller and T. Decker

Conditional Stat1 ablation reveals the importance of interferon signalling for innate and adaptive immunity to *Listeria monocytogenes* infection

Paper submitted

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