Summer 8-15-2015

DNA Damage Responses Regulate Macrophage Function During Innate Immune Responses

Abigail Rodriguez Morales
Washington University in St. Louis

WASHINGTON UNIVERSITY IN ST. LOUIS

Division of Biology and Biomedical Sciences
Immunology

Dissertation Examination Committee:

Barry Sleckman, Chair
Marco Colonna
Michael Diamond
Brian Edelson
Eugene Oltz
Emil Unanue

DNA Damage Responses Regulate Macrophage Function During Innate Immune Responses
by
Abigail Rodriguez Morales

A dissertation presented to the
Graduate School of Arts & Sciences
of Washington University in
partial fulfillment of the
requirements for the degree
of Doctor of Philosophy

August 2015
St. Louis, Missouri

Table of Contents

List of Figures

<u>Acknowledgments</u>

First and foremost, I would like to thank my thesis mentor, Dr. Barry Sleckman. Barry's enthusiasm for science and in particular for quality research knows no bounds. He encourages all of his trainees to plan carefully constructed and controlled experiments, and expects us to evaluate the data with a rational and unbiased eye. His brilliance is perhaps most evident in the way he approaches data presentation. His reductionist approach places a premium on clarity. I hope I will be able to take all of what I have learned from him forward as I embark on my own career. He is also incredibly supportive of his trainees and truly wants to do anything and everything he can to help us succeed.

Second, I would like to thank Dr. Emil Unanue for allowing me to spend so much time in his lab and for the invaluable input over the years. I would also like to thank Drs. Javier Carrero and Boris Calderon. Javier provided years of intellectual support and was incredibly patient and helpful with what was at times a very difficult project. Boris taught me some very useful techniques that I will likely use in my post-doctoral training.

I would like to thank the members of my thesis committee for years of providing helpful scientific insight and reagents over the years. I also thank the members of the Oltz, Payton, and Bednarski labs for many useful discussions within our joint lab meeting forum. I extend all the appreciation in the world to the members of the Sleckman lab for the years of scientific discourse and for countless fun times and laughs. In particular, I would like to thank Dr. Jeffery Bednarski for training me when I first joined the lab and for being endlessly supportive throughout my graduate training. I would like to thank my long-time baymate, Dr. Anthony Tubbs, for years of

ABSTRACT OF THE DISSERTATION

DNA Damage Responses Regulate Macrophage Function

During Innate Immune Responses

by

Abigail Rodriguez Morales

Doctor of Philosophy in Biology and Biomedical Sciences

Immunology

Washington University in St. Louis, 2015

Professor Barry P. Sleckman, Chair

Activated macrophages produce genotoxins such as reactive oxygen and nitrogen intermediates that are critical for the eradication of pathogens. Here we show that one of these agents, nitric oxide (NO), damages macrophage genomic DNA, resulting in the activation of DNA damage responses (DDR). The DDR is primarily initiated through DNA double-strand break (DSB) intermediates and depends on the PI3-like kinases ATM and DNA-PKcs. In response to *Listeria monocytogenes* infection, ATM and DNA-PKcs regulate a tissue-specific genetic program that includes the expression of inflammatory cytokines, chemokines and cell surface receptors, several of which are critical for cell migration during immune responses to bacterial infection. These kinases also regulate inflammasome activation and production of the inflammatory cytokines IL-1β and IL-18. Due to the near-complete block in IL-18 production by DNA-PKcs-deficient macrophages, these cells are unable to optimally stimulate NK cells to produce IFN-γ, which is important for controlling early *L. monocytogenes* infection. These findings establish

DNA damage, and the initiation of DDR by this damage, as important signaling intermediates in the innate immune responses mediated by macrophages.

Chapter 1: Introduction and Background

The information that is required to build a cell or organism is contained in its genes, which are hereditary units that control identifiable traits of an organism. A genome is composed of an organism's complete set of genes, which are encoded in stable molecular units called deoxyribonucleic acids, or DNA. The faithful transmission of genetic information from a parent cell to a daughter cell is critical for the generation and maintenance of multicellular organisms. Within a species, the exact duplication of this information from one generation to the next ensures genetic continuity within that species.

Cells must be able to accurately replicate their DNA before passing it along to their daughter cells. Errors in this process can result in alterations in genetic information, which will be transmitted to daughter cells as mutations. Mutations can also arise from spontaneous chemical changes in DNA constituents, or can be introduced by environmental agents that inflict damage upon the DNA. In some cases, the mutation is innocuous or may even be beneficial to the cell or organism. Mutations that improve cellular fitness are, in fact, critical for the evolution of the species. However, mutations can also render the cell unresponsive to cues that normally would keep the cell from undergoing cell division or would initiate a programmed cell death. These abnormally-growing cells make up a neoplasia, which can form a mass called a tumor. Many tumors are malignant, meaning they are not self-limiting in their growth. These tumors are usually characterized by genome instability, and thus they are able to rapidly accumulate additional mutations that are then selected for by the tumor to favor rapidly-growing cells that out-compete other cells for space and nutrients.

One of the biggest challenges to genome stability arises from DNA damaging agents. These agents can be exogenous (i.e. they are derived from the environment) or they can be formed during normal physiologic processes that all or some cell types undergo. In order to maintain genome stability, cells must initiate a rapid response to DNA damage, which is orchestrated by a complex signal transduction network known as the DNA damage response. The *de facto* response is generally to repair the damage, which generally involves repair or removal of the specific lesion. In cases where the extent of damage overwhelms the survival response machinery, however, a programmed cell death is initiated. The mechanisms underlying the choice between repair and cell death are not entirely clear. Indeed, these choices are likely governed by the integration of complex cellular and environmental cues. However, as a single genomic alteration can contribute to the onset of malignancy, a more complete understanding of how cells sense and respond to DNA damage is critical for the future prevention and treatment of cancer.

1.1) Exogenous and endogenous agents induce DNA damage.

Genomic DNA damage can be induced by environmental agents and as byproducts of normal physiologic processes that cells undergo, such as transcription and DNA replication. Environmental DNA damage can be introduced by genotoxins such as ionizing radiation (IR) or ultraviolet (UV) light. IR induces oxidation of DNA bases and generates both single-strand and double-strand DNA breaks (SSBs and DSBs), while UV light generates pyrimidine dimers and photoproducts (Ciccia and Elledge 2010). Cancer therapeutic agents can also induce a variety of different DNA lesions. Chemical agents such as camptothecin (CPT) and etoposide induce SSBs and DSBs via covalent complex formation with DNA and topoisomerase I and II, respectively. Agents such as cisplatin or mitomycin C introduce covalent links between bases of DNA strands. Finally, alkylating agents such as methyl methanesulfonate (MMS) attach alkyl groups to DNA bases (Ciccia and Elledge 2010). Other damage lesions can be generated spontaneously during metabolic processes, such as mismatched DNA base pairs introduced during DNA replication (Jackson and Bartek 2009). Moreover, reactive oxygen species and reactive nitrogen intermediates (ROS and RNI, respectively) generated during both the immune response to infection and during metabolic processes oxidize DNA bases and induce DNA breaks (Lindahl and Barnes 2000, Kawanishi, Hiraku et al. 2006, Nathan and Cunningham-Bussel 2013).

Given that DNA damaging agents generate a wide range of lesions, many different repair mechanisms have evolved to resolve each type of damage. Chemical DNA base alterations are excised by base excision repair (BER), whereas mismatch repair (MMR) inserts correct DNA bases in place of mispairings. Complex lesions such as intrastrand crosslinks and pyrimidine

dimers are corrected by nucleotide excision repair (NER) through removal of a small oligonucleotide containing the lesion. Single-strand DNA breaks are repaired by single-strand break repair (SSBR), while DSBs can be processed either by the non-homologous end joining pathway (NHEJ) or homologous recombination (HR) (Caldecott 2008, Ciccia and Elledge 2010). NHEJ is the predominant repair pathway in G1-phase cells, whereas HR is primarily active in post-replicative stages of the cell cycle. Unlike NHEJ, which can join two unrelated DNA ends, HR restores the genomic sequence of the broken DNA by using sister chromatids as a repair template (Chapman, Taylor et al. 2012).

If incorrectly repaired, DNA DSBs can lead to chromosomal rearrangements and genomic instability, making them a particularly dangerous lesion (Jackson and Bartek 2009). In addition to the sources mentioned previously, DNA DSBs are introduced by the RAG endonuclease as essential intermediates in lymphoid cells undergoing V(D)J recombination, the process by which developing lymphocytes rearrange their antigen receptor loci to generate functional antigen receptor genes (Helmink and Sleckman 2012). In this somatic recombination process, RAG introduces DSBs at specific recognition sequences flanking the DNA segments to be joined. The programmed DSBs are subsequently resolved by NHEJ (Fugmann, Lee et al. 2000, Rooney, Chaudhuri et al. 2004). In contrast to RAG expression, which is confined mainly to cells of lymphoid origin, NHEJ proteins are expressed in most tissues, as they function in general DSB repair (Rooney, Chaudhuri et al. 2004, Lieber 2010). Site-specific DNA DSBs are also introduced in activated B cells undergoing class switch recombination (CSR). These breaks are introduced in switch (S)-region repeat elements by a series of enzymes including the cytidine

deaminase AID, uracil glycosylase, and apyrimidic/apurinic (AP)-endonucleases and are subsequently repaired by NHEJ factors (Chaudhuri and Alt 2004).

1.2) The canonical response to DNA DSBs.

Regardless of the source of the damage, all DNA breaks activate the DNA damage response (DDR), a complex signal transduction network that senses the damage and orchestrates an appropriate response (Jackson and Bartek 2009). This response can include the activation of DNA repair pathways, cell cycle checkpoints, senescence, and cell death pathways (Shiloh 2003, Ciccia and Elledge 2010). The DDR is primarily initiated by proteins of the PI3-like kinase family, which include the ataxia telangiectasia mutated (ATM) kinase, the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and the ATM- and Rad3-related (ATR) kinase. These serine-threonine kinases activate target proteins via phosphorylation of SQ/TQ (serine or threonine residues followed by a glutamine) motifs. ATM and DNA-PKcs are activated by DSBs and can phosphorylate downstream targets in all stages of the cell cycle. In contrast, ATR is activated following recruitment to ssDNA regions that are generated at stalled replication forks, and thus is generally active in post-replicative cell cycle stages. ATR is also recruited to ssDNA generated via DSB processing; however, optimal ATR activation in response to IR-induced DSBs requires ATM (Myers and Cortez 2006, Ciccia and Elledge 2010).

One of the major DDR substrates is the histone H2A variant H2AX. Immediately following a DNA double- strand break, H2AX is phosphorylated at an SQ motif (serine 139) in its C- terminal tail by ATM, ATR, or DNA-PKcs. This modification, known as γ-H2AX, creates

a binding site for the adaptor MDC1, which propagates the spread of γ-H2AX in chromatin for up to 500 kilobases surrounding the DSB (Stucki, Clapperton et al. 2005). This chromatin-based platform thereby allows for DNA repair factors to aggregate around the DNA break, forming repair foci that can be visualized by immunofluorescence (Paull, Rogakou et al. 2000, Savic, Yin et al. 2009). Another established DDR substrate is the transcriptional repressor KAP-1, which is transiently recruited to DNA breaks induced by ionizing radiation or laser-induced micro-irradiation and induces genome-wide chromatin relaxation after ATM phosphorylates serine 824 in its C-terminus (Ziv, Bielopolski et al. 2006). Though neither substrate has any known role in macrophage function specifically, the DDR-specific activation of both H2AX and KAP-1 can be readily detected using phospho-specific antibodies, allowing the activation of DDR to be reliably read out after a cell has been exposed to DNA damaging agents.

In response to IR-induced genotoxic stress, ATM and ATR were found to phosphorylate a broad network of over 700 proteins that they participate in diverse biological processes, including (but not limited to) DNA replication and recombination, DNA repair, cellular assembly and morphology, nucleic acid metabolism, cellular growth and proliferation, cell cycle, and cell death (Matsuoka, Ballif et al. 2007). To date, the full range of targets activated by DNA-PKcs is not as well-characterized. However, it shares at least a subset of targets with ATM. One of the ways in which both ATM and DNA-PKcs modulate downstream DNA damage responses is via activation of CHK2, a serine-threonine kinase that amplifies the damage response via activation of a broad set of targets. Phosphorylation of threonine 68 in the SQ cluster domain (SCD) promotes the dimerization and activation of CHK2 monomers. Catalytically active CHK2 promotes the ubiquitin-mediated proteasomal degradation of Cdc25a, preventing G1-phase cells

from transitioning into S phase. In response to DNA damage, CHK2 also regulates the transcription factor p53, which in turn activates downstream targets that influence the overall response (Zhou and Elledge 2000). p53 induces cell-cycle arrest or senescence in cells that have sustained DNA damage via the transcriptional regulation of CDK inhibitor p21, which enforces the G1-S checkpoint. In some contexts, p53 initiates a cell death program in response to DNA damage through the transcriptional up-regulation of target genes *Bax* (Bcl2-associated X protein), Fas ligand *Puma* (p53-up-regulated modulator of apoptosis), and *Noxa* (Ciccia and Elledge 2010). ATM and DNA-PKcs also activate p53 directly via phosphorylation at serine 15. Following DSB induction, p53 expression is cyclically activated and shut off by the E3 ubiquitin ligase MDM2 and the WIP1 phosphatase, which inactivate p53 and ATM, respectively. This mechanism likely allows the cell to periodically assess its DNA damage status. If substantial DNA damage persists unrepaired over time, p53 will initiate apoptosis via transcriptional up-regulation of targets BAX, NOXA, and PUMA (Ciccia and Elledge 2010).

1.3) Recruitment of ATM and DNA-PKcs to DNA DSBs.

ATM is present in the cell in inactive dimeric form. It is rapidly recruited to DNA DSBs by the MRE11-NBS1-RAD50 (MRN) complex through a direct interaction with NBS1 . Once present at the break site, it is converted from inactive dimers to active monomers through an autophosphorylation step (Bakkenist and Kastan 2003, Lee and Paull 2004, Lee and Paull 2005). A mutant form of ATM that cannot be phosphorylated at this site still activates a subset of downstream DDR targets, however, suggesting that there are alternative ways to activate ATM.

Indeed, ATM can be activated by hydrogen peroxide via direct oxidation in a DSB- and MRN-independent manner (Guo, Kozlov et al. 2010).

DNA-PKcs is recruited to DNA DSBs by the ring-shaped Ku70:Ku80 heterodimer. The positioning of the broken DNA ends in this toroidal channel suggests that Ku may play a role in proper alignment of the ends prior to joining (Ciccia and Elledge 2010, Helmink and Sleckman 2012). After binding the DNA ends, Ku loads and activates the catalytic subunit of DNA-PK (DNA-PKcs). Binding of DNA-PKcs to Ku causes a shift in Ku80, allowing DNA-PKcs to contact the broken DNA directly. Activation of DNA-PKcs's catalytic activity depends on its interaction with both broken DNA and Ku. Once activated, it phosphorylates a number of protein targets involved in the repair of the DNA DSB. DNA-PKcs also associates with both broken DNA and various repair factors in a kinase- independent fashion, suggesting that it may serve as a "bridge" linking a DNA DSB to the factors that will process and repair it (DeFazio, Stansel et al. 2002, Merkle, Douglas et al. 2002). Among these repair factors in DNA ligase IV, which is required for NHEJ-mediated joining of broken DNA ends. Ligase IV associates with XRCC4, which promotes its stability. In addition, XRCC4 facilitates the adenylation of lysine residues within the catalytic core of ligase IV—this is essential in the formation of the phosphodiester bond that will allow the broken DNA ends to be re-ligated (Gellert 2002, Helmink and Sleckman 2012).

1.4) DNA damage influences cell-type-specific processes

After DNA damage-induced activation, ATM phosphorylates a broad range of targets that are important in shaping an appropriate cellular response. Importantly, this response is not limited to the canonical response to DNA damage, as it also leads to the induction of cell-type-specific genetic programs (Bredemeyer, Helmink et al. 2008, Sherman, Kuraishy et al. 2010, Bednarski, Nickless et al. 2012, Innes, Hesse et al. 2013). Once activated by DNA DSBs, ATM regulates gene expression by modulating the activity of a variety of transcription factors. As previously mentioned, ATM and DNA-PKcs phosphorylate p53, which in turn promotes the expression of factors that will promote cell death if the damage persists unrepaired. However, previous studies have shown that in response to genotoxic DSBs, ATM also activates pro-survival pathways via activation of NF-κB (Wu, Shi et al. 2006). RAG-induced DSBs in developing lymphocytes also activate pro-survival pathways that integrate with p53-dependent pro-apoptotic pathways in determining cell fate. In particular, RAG DSBs activate the classical (p50/RelA) NF-κB pathway, which is known to activate a cohort of genes that promote cell survival, including *Pim2* (Bredemeyer, Helmink et al. 2008, Bednarski, Nickless et al. 2012). In response to DNA damage, ATM phosphorylates NEMO in the nucleus. After translocating to the cytoplasm, it is able to activate NF-κB (Bredemeyer, Helmink et al. 2008). Notably, the transcriptional program that was activated in response to RAG-induced DSBs is not limited to pro-survival and pro-apoptotic pathways. RAG breaks activated over 200 genes in developing pre-B cells, many of which were downstream of transcription factors other than NF-κB and p53. Approximately half of these genes were dependent on ATM, which suggests that other kinases play a role in regulating DSB- dependent transcriptional programs (Bredemeyer, Helmink et al. 2008, Helmink and Sleckman 2012). Predictably, many of the identified genes had established

roles in the canonical response to DNA damage, such as caspase 9 and Bcl3. Surprisingly, however, a large cohort of the genes had no known function in the DDR and instead participated in diverse processes such as lymphocye homing and migration, such as CD62L, CD69, and SWAP70 (Bredemeyer, Helmink et al. 2008). A subset of these genes were also activated by genotoxic breaks, suggesting that DNA damage—irrespective of the source—can induce some aspects of this genetic program in developing lymphocytes (Bredemeyer, Helmink et al. 2008, Innes, Hesse et al. 2013). Similarly, AID-induced DSBs in activated B cells activate an ATM-dependent genetic program that is critical for plasma cell differentiation (Sherman, Kuraishy et al. 2010, Sherman, Bassing et al. 2011). Taken together, these findings suggest that context-dependent DNA DSBs introduced either by physiologic processes or genotoxic agents may influence cell-type-specific processes. Notably, some cell types, such as phagocytes, produce genotoxic agents as part of their normal physiologic response to pathogenic stimuli. Thus, we are interested in assessing whether genotoxin-induced DNA damage impacts the function of activated macrophages in the innate immune response to infection.

1.5) Classically activated macrophages in the immune response.

Macrophages are a heterogenous population of cells that play a key role in the generation, maintenance, and regulation of the immune response. The macrophages generated during cell-mediated immune responses have traditionally been designated classically activated macrophages (Mosser and Edwards 2008). Originally, a combination of interferon-γ (IFN-γ) and tumor-necrosis factor (TNF) were found to generate a macrophage population that had enhanced microbicidal capacity and secreted high levels of pro-inflammatory cytokines (O'Shea and

Murray 2008). Though sustained IFN-γ production is provided by T helper 1 (T$_H$1) lymphocytes during an adaptive immune response, NK cells are an important innate source of this cytokine early in innate immune responses (Mosser and Edwards 2008). IFN-γ can prime macrophages to secrete pro-inflammatory cytokines and produce reactive oxygen and nitrogen radicals to enhance their killing ability (Edelson and Unanue 2000, Dale, Boxer et al. 2008). TNF, which synergizes with IFN-γ to generate a classically activated macrophage, is transcribed downstream of Toll-like receptor (TLR) engagement. However, some TLR ligands can also induce IFN-β production in a signaling cascade that depends on the adaptor TRIF and the interferon regulatory factor IRF3 (Yamamoto, Sato et al. 2003). It was discovered that IFN-β can replace the IFN-γ that is produced by NK and T cells, synergizing with TNF to generate a fully activated macrophage. Thus, certain TLR agonists can activate macrophages through the production of both TNF and IFN-β. Both IFN-γ and IFN-β signal through the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway, leading to the transcription of interferon-stimulated genes (ISGs) (Ivashkiv and Donlin 2014). Thus, TNF and interferon (IFN) signaling pathways synergize to dramatically influence the transcriptional profile of the activated macrophage. In addition to producing pro-inflammatory cytokines and reactive oxygen and nitrogen intermediates, classically activated macrophages up-regulate co-stimulatory and MHC class II molecules (Dale, Boxer et al. 2008). Thus, in addition to their role in the direct killing of microorganisms, they are able to shape the adaptive immune response that is generated subsequently.

1.6) Production of reactive oxygen and nitrogen intermediates.

Classically activated macrophages produce a diverse array of reactive oxygen and nitrogen intermediates after sensing microbes. Though ROS can be generated in several ways, the main source of ROS in response to bacterial infection is the phagocyte respiratory burst oxidase (NADPH oxidase), which is a multi-subunit phagosome- and plasma membrane-associated enzyme that is assembled by phagocytes upon activation by inflammatory stimuli (Nunes, Demaurex et al. 2013). The catalytic core of NADPH oxidase is composed of two integral membrane proteins, $p22^{phox}$ and $gp91^{phox}$ (NOX2). $p22^{phox}$ is responsible for the recruitment of the otherwise-cytoplasmic regulatory *phox* subunits $p40^{phox}$, $p67^{phox}$, and $p47^{phox}$, whereas the $gp91^{phox}$ subunit mediates the transfer of electrons across plasma or phagosomal membranes (Nunes, Demaurex et al. 2013). The active enzyme produces superoxide radicals from molecular oxygen, which are subsequently converted to hydrogen peroxide spontaneously or through enzymatic dismutation (Fang 2011, Nunes, Demaurex et al. 2013). One of the main targets of ROS within the pathogen is DNA (Imlay and Linn 1988). Base oxidation can be mutagenic and DNA strand breaks are lethal to the pathogen unless effectively repaired. Additionally, iron sulfur-cluster-containing proteins are also modified by ROS, which may restrict bacterial metabolic pathways even if the damage is not fatal (Imlay 2006, Fang 2011). Both phagocytes and many types of bacteria express antioxidant enzymes such as catalases, peroxiredoxins, and superoxide dismutases which function to scavenge hydrogen peroxide and superoxide (Fang 2011, Nathan and Cunningham-Bussel 2013). In particular, the intracellular bacterium *Salmonella enterica* expresses three distinct catalases, three peroxiredoxins, and four superoxide dismutases as well as repair enzymes that can reverse oxidative DNA lesions within the pathogen genome (Imlay 2008, Fang 2011).

In macrophages, nitric oxide is produced by the inducible nitric oxide synthase (iNOS), which is encoded by the *Nos2* gene. Previous studies established that iNOS is synthesized downstream of PRR engagement; indeed, the *Nos2* promoter contains a binding site for NF-κB (Kleinert, Schwarz et al. 2003). In LPS-treated or pathogen-infected murine cells, full transcriptional induction of *Nos2* depends on the production of type I interferons and signaling through the Janus-kinase (JAK)-STAT pathway (Gao, Filla et al. 1998, Bogdan 2001). However, type II interferon (IFN-γ) also augments LPS-induced *Nos2* transcription in a manner that requires signaling through the interferon-γ receptor (IFNGR) and active STAT1 (Meraz, White et al. 1996). From these data, it is clear that PRR-elicited NF-κB cooperates with IFN-receptor activated STATs in the full transcriptional induction of *Nos2* and the production of NO. NOS2 is homodimeric enzyme that converts L-arginine and oxygen into L-citrulline and NO, a labile radical that has many reaction partners that mediate its antimicrobial and regulatory properties (Bogdan 2015). NO groups can be covalently added to the thiol groups of cysteine residues within proteins or can react with superoxide, giving rise to the reactive peroxynitrite, which can also modify proteins and DNA (Anand and Stamler 2012, Radi 2013). Like ROS, NO can modify iron-sulfur clusters, which can regulate the function of many transcription factors or enzymes. Indeed, direct antimicrobial effects of NO include (but are not limited to) modification of nucleic acids, replication machinery, and virulence-associated molecules (Bogdan 2015). Though reactive oxygen and nitrogen intermediates are required for indirect and direct antimicrobial activity, their toxic effects are not restricted to the pathogen and thus pose a significant threat to the host cell genome.

Given that reactive oxygen and nitrogen intermediates can cause significant damage to macrophage DNA, we hypothesize that these intermediates will initiate a DDR in activated macrophages. As was observed previously in other immune cell types (Bredemeyer, Helmink et al. 2008, Sherman, Kuraishy et al. 2010), we postulate that this DDR will impact the function of the macrophages in the immune response. Here, we have developed an experimental system to assess DDR activation in macrophages that have been exposed to LPS and IFN-γ or the intracellular bacterium *Listeria monocytogenes*. In the next few sections, we will review the signaling cascades and cellular responses that are triggered by these agents.

1.7) *Listeria monocytogenes:* **an established bacterial infection model.**

L. monocytogenes is a Gram-positive, facultative intracellular bacterium that can survive in the environment. In humans, ingestion of contaminated food causes listeriosis. Clinical symptoms range from gastroenteritis to more severe forms of infection, such as meningo-encephalitis or sepsis. After ingestion, the bacteria cross the intestinal epithelium and disseminate to deeper tissues via the bloodstream and lymph (Stavru, Archambaud et al. 2011). In murine models of infection, *L. monocytogenes* is typically administered intraperitoneally or intravenously, resulting in the rapid infection of the spleen and liver.

Bacterial entry into non-phagocytic cells is mediated by bacterial surface proteins called internalins. Internalin A (InlA) binds the adherens junction protein E-cadherin on an intestinal epithelial cell while InlB is a ligand for the receptor tyrosine kinase Met (Mengaud, Ohayon et al. 1996, Shen, Naujokas et al. 2000). In addition to mediating pathogen uptake, interactions

between internalins and their cognate receptors leads to the activation of P13K, type II PI4-kinases, and MAPK signaling pathways (Shen, Naujokas et al. 2000). In contrast, phagocytes engulf circulating bacteria, which are taken up into phagosomal compartments. After uptake, *L. monocytogenes* escapes into the cytoplasm via secretion of the hemolysin listeriolysin O (LLO), which destroys phagosomal membranes (Portnoy, Jacks et al. 1988). Invasion of the cytosol triggers innate inflammatory responses and is important for the induction of lasting protective immunity (Pamer 2004). Bacterial mobility within the cytosol is conferred by actin-assembly-inducing protein (ActA), which enables the formation of actin polymers that propel the bacteria through the cytoplasm and into neighboring cells without inducing cell lysis (Stavru, Archambaud et al. 2011). Cell-to-cell spread is critical for the survival of *L. monocytogenes*, as it allows the bacteria to evade extracellular immune recognition.

Innate immune responses are rapidly triggered following *L. monocytogenes* infection and are required for host survival. The effectiveness of the innate immune system in responding to and controlling *L. monocytogenes* infection was established in severe combined immunodeficiency (*Scid*) and athymic NUDE mice, which lack T cell-mediated and humoral immunity (Nickol and Bonventre 1977, Bancroft, Schreiber et al. 1991). These mice were surprisingly resistant to infection in the short-term but were ultimately unable to clear the infection. It is established that lymphocytes are in fact detrimental during the early stages of *L. monocytogenes* infection, as wild type mice displayed enhanced bacterial titers in both spleen and liver relative to *Scid* mice early after infection (Carrero, Calderon et al. 2006). This is due in part to macrophage secretion of anti-inflammatory mediators such as prostaglandin E2 and IL-10 after sensing massive lymphocyte apoptosis. Thus, *L. monocytogenes* establishes an infective

niche via the induction of host cell death (Ren, Stuart et al. 2001, Carrero, Calderon et al. 2006, Carrero and Unanue 2006).

A variety of innate immune cell types participate in the early eradication of infection. Polymorphonuclear neutrophils (PMNs) are among the first responders to arrive at the inflammatory site; their migration is directed by chemokine-secreting hepatocyes. The inflammatory cytokine IL-1, produced by resident macrophages at the site of infection, also plays an important role in *L. monocytogenes*-dependent neutrophil migration and activation (Rogers, Tripp et al. 1994). Once neutrophils have arrived at the site of infection, they phagocytose bacteria and generate large quantities of antimicrobial reactive oxygen and nitrogen species (ROS and NOS). Given that neutrophils also have a role in amplifying the inflammatory response via the secretion of cytokines and chemokines, it is not surprising that neutrophil-deficient mice are more susceptible to infection and display an increased bacterial burden in both liver and spleen (Rogers and Unanue 1993).

Resident macrophages, particularly liver Kupffer cells, play a key and well- established role in *L. monocytogenes* infection. Upon infection, macrophages secrete TNF and IL-12, which triggers IFN-γ production by NK cells. NK cells begin producing IFN-γ within 24 hours after infection and are the predominant source of this cytokine until an adaptive immune response is generated several days later (Bancroft, Schreiber et al. 1991, Tripp, Wolf et al. 1993). In addition, it is known that optimal NK cell activation also depends on the inflammatory cytokine IL-18, which is processed and released by multimeric complexes known as inflammasomes

(Akira 2000, Lamkanfi and Dixit 2014). IFN-γ is critical in the control of the bacteria, as it activates macrophages to produce reactive oxygen and nitrogen species (ROS and NOS), which inhibit bacterial escape from vacuoles (Myers, Tsang et al. 2003). Indeed, several reports suggest that IFN-γ also functions in part to accelerate the fusion of phagosomes with lysosomes, resulting in the eradication of the bacteria before it is able to access the cytosol (Portnoy, Schreiber et al. 1989, Alvarez-Dominguez and Stahl 1998, Via, Fratti et al. 1998). Both the oxidative burst and the production of nitric oxide (NO) are required for pathogen clearance *in vivo,* as iNOS-deficient mice or mice deficient an essential subunit of the NADPH oxidase (NOX2) and are more susceptible to infection and mice deficient in both succumb rapidly to virulent *L. monocytogenes* infection (MacMicking, Nathan et al. 1995, North, Dunn et al. 1997, Shiloh, MacMicking et al. 1999). Macrophages also produce chemokines that regulate trafficking of other cell types that will be important in combating the infection. Upon internalization of *L. monocytogenes*, resident macrophages up-regulate CC-chemokine ligand 2 (CCL2), which initiates the recruitment of circulating monocytes that express CCR2. Microbial products released by these macrophages activate the monocytes via TLRs; these monocytes then differentiate into TNF- and iNOS-producing dendritic cells (TipDCs), which are highly bacteridical (Pamer 2004). Though these DCs play a key role in controlling *L. monocytogenes* infection, they are not required for T-cell priming *in vivo* (Tam and Wick 2004). Other types of DCs, however, are critical for bridging the gap between innate and adaptive immunity via antigen presentation to naïve lymphocytes. They also are important sources of IL-12 and IL-18, which activate both NK and T cells to produce IFN-γ (Hsieh, Macatonia et al. 1993, Okamura, Tsutsi et al. 1995, Akira 2000). Unlike macrophages, DCs are able to prime naïve CD8^{+} cytotoxic T cells, enabling them to kill infected cells (Stavru, Archambaud et al. 2011).

Histological analysis of spleens from *L. monocytogenes*-infected mice demonstrated that cells containing live bacteria migrate to the T cell-containing zones of the splenic white pulp within 24 hours after infection. This migration is followed by significant lymphocyte apoptosis in the T-cell compartment, though the majority of the T cell casualties are non-pathogen specific (Merrick, Edelson et al. 1997). It has been demonstrated that type I interferon sensitizes lymphocytes to LLO-dependent apoptosis, and drives macrophage IL-10 secretion, which dampens the inflammatory response (Carrero, Calderon et al. 2006). Thus, both type I and type II interferon play a significant role in shaping the innate and adaptive immune responses to *L. monocytogenes* infection.

Murine infection with *L. monocytogenes* is a widely used model for studying cell-mediated immunity. Though both CD8$^+$ and CD4$^+$ T cells are involved in the response, a specific cytotoxic CD8$^+$ T cell response is required for complete clearance of *L. monocytogenes* (Stavru, Archambaud et al. 2011). Intracellular bacteria secrete proteins that are loaded onto MHC class I molecules for presentation to CD8+ T cells after proteasomal degradation and transport through the ER. Among the most antigenic proteins in activating T cell responses is LLO (Villanueva, Sijts et al. 1995). CD8$^+$ T cells produce perforin and granzymes that lyse *L. monocytogenes-* infected cells, resulting in the release of bacteria for phagocytosis (and subsequent destruction) by macrophages and neutrophils. Both CD8$^+$ and CD4$^+$ T cells secrete IFN-γ, which will activate macrophages to full listericidal capacity (Stavru, Archambaud et al. 2011). Thus, the interplay between the innate and adaptive immune responses is critical in the clearance of *L. monocytogenes* and the generation of sterilizing immunity.

1.8) The role of Toll-like receptor signaling in macrophage activation.

Macrophages express myriad receptors that allow them to recognize pathogen components and endogenous danger signals released from necrotic cells. Among these are germline-encoded pattern recognition receptors (PRR) known as Toll-like receptors (TLRs). TLRs are type I transmembrane receptors that are present on the surface of host cells or are localized to a variety of intracellular compartments (Akira, Uematsu et al. 2006, Kawai and Akira 2010). To date, 12 TLRs have been identified in mice; these receptors recognize a wide variety of pathogen-associated molecular patterns (PAMPs) such as lipoproteins, lipids, nucleic acids, and proteins that are derived from bacteria, fungi, and viruses. TLR1, TLR2, TLR4, TLR5, and TLR6 are expressed on the cell surface and primarily recognize components of microbial membranes such as lipoproteins and flagellin (Kawai and Akira 2011). TLR3, TLR7, TLR8, and TLR9 are localized within intracellular compartments such as endosomes and lysosomes and bind to pathogen-derived DNA and RNA (Blasius and Beutler 2010). After recognizing a specific PAMP, TLRs activate signaling pathways that will elicit specific immune responses appropriate for combating the pathogen expressing that PAMP. The nature of the signaling cascade that is triggered by TLR engagement depends on the adaptor molecule with which the receptor interacts.

TLR4 forms a complex with MD2 on the cell surface and, in conjunction with CD14, binds lipopolysaccharide (LPS), a cell wall component of Gram-negative bacteria that can potently induce septic shock (Kawai and Akira 2010). It signals through two pathways with distinct kinetics, one involving the adaptor MyD88 and the other involving an adaptor protein

known as TRIF. MyD88, the first adaptor to be identified, interacts with all TLRs except TLR3 and is also utilized by members of the IL-1 receptor family (Kawai and Akira 2011). It induces inflammatory cytokine production through the activation of both NF-κB and mitogen-activated protein kinases (MAPK) (Akira, Uematsu et al. 2006). The other adaptor, TRIF, is utilized by both TLR4 and TLR3 and induces type I interferon and inflammatory cytokine production. Thus, TLR4 regulates the production of inflammatory mediators both spatially and temporally. In the initial phase, the plasma membrane-localized adaptor TIRAP acts as a bridge that connects TLR to MyD88 upon engagement with LPS (Barton and Kagan 2009). MyD88 subsequently recruits IRAKs, TRAF6, and the TAK1 complex, which promote activation of NF-κB and MAPK (Kawai and Akira 2011). In the later phase, TLR4 it is trafficked to intracellular compartments where it complexes with adaptors TRAM and TRIF. TRAF3 is subsequently recruited along with the protein kinases TBK1 and IKKi, which initiate IRF3 activation and the production of type I interferon (Barton and Kagan 2009). The TRAM-TRIF complex also promotes a "late-phase" activation of NF-κB and MAPK through recruitment of TRAF6 and TAK1 (Kawai and Akira 2011). Thus, detection of LPS leads to the production of inflammatory cytokines and ultimately, type I interferon through distinct MyD88-dependent and-independent signaling cascades.

TLR2 complexed in a heterodimer with TLR6 is responsible for the recognition of another cell wall component, peptidoglycan (PGN), from Gram-positive bacteria such as *L. monocytogenes* (Takeuchi, Hoshino et al. 1999, Dziarski and Gupta 2010). TLR2 can also be stimulated by other potential *L. monocytogenes*-derived ligands such as lipoteichoic acid (LTA) and lipoproteins (Travassos, Girardin et al. 2004, Kawai and Akira 2010). However, TLR2-

deficient mice exhibit little to no increased susceptibility as compared with wild type mice upon

L. monocytogenes infection, suggesting that other TLRs may be important in the response to the

bacterium (Edelson and Unanue 2002, Torres, Barrier et al. 2004, Janot, Secher et al. 2008).

Indeed, MyD88^{-/-} mice have a 3-4-log greater bacterial burden in the spleen and liver post-*L.*

monocytogenes infection relative to wild type mice and succumb rapidly to the bacterial

infection (Edelson and Unanue 2000). Thus, it is evident that MyD88-dependent TLR signaling

distinct from TLR2 is critical in the innate response to *L. monocytogenes*. TLR4 is unlikely to

contribute to the recognition of *L. monocytogenes*-derived ligands, as it primarily recognizes

LPS, which is not present in Gram-positive bacteria (Witte, Archer et al. 2012). Though *L.*

monocytogenes-derived flagellin is recognized by TLR5, this recognition is irrelevant in studies

with murine macrophages, which do not express TLR5 (Uematsu, Jang et al. 2006). MyD88-

dependent TLR signaling from vacuolar compartments requires a transmembrane protein known

as Unc93b, which localizes to TLR-containing endosomal compartments from the ER (Tabeta,

Hoebe et al. 2006, Barbalat, Ewald et al. 2011). After exposure to LLO-deficient *L.*

monocytogenes, macrophages derived from Unc93b-deficient mice exhibit reduced expression of

IL-12 and TNF, suggesting that *L. monocytogenes*-derived nucleic acids contribute to the

MyD88-dependent response. Additionally, a lysozyme-sensitive *L. monocytogenes* mutant is

rapidly degraded within phagosomes and elicits enhanced MyD88-dependent cytokine

production as compared to wild type bacteria (Boneca, Dussurget et al. 2007, Rae, Geissler et al.

2011). Taken together, these data suggest that TLRs localized to both the cell surface and

vacuolar compartments recognize multiple *L. monocytogenes*-derived ligands and contribute to

the resulting MyD88-dependent production of inflammatory cytokines and chemokines.

1.9) Intracellular bacteria activate a cytosolic surveillance pathway.

Unmethylated CpG DNA motifs that are derived from many different pathogens potently stimulate immune responses via endosomal TLR signaling. However, pathogen-derived B-form DNA as well as single-stranded DNA with distinct structural characteristics can stimulate robust immune responses after sensing in the host cytosol (Hemmi, Takeuchi et al. 2000, Ishii, Coban et al. 2006, Stetson and Medzhitov 2006). In a variety of bacterial infections, the critical signaling molecule in the innate immune response to cytosolic nucleic acid ligands is STING ("stimulator of interferon genes"; also known as TMEM173, MPYS, MITA, and ERIS) (Ishikawa and Barber 2008, Ishikawa, Ma et al. 2009, de Almeida, Carvalho et al. 2011, Parker, Martin et al. 2011, Manzanillo, Shiloh et al. 2012). STING is anchored in the endoplasmic reticulum by four transmembrane domains and is activated by upstream DNA sensing events in a manner that is as of yet incompletely defined (Paludan and Bowie 2013). However, it is established that in response to cytosolic dsDNA, STING relocalizes to a perinuclear region of the cytosol where it forms complexes with TANK-binding kinase 1 (TBK-1) (Ishikawa, Ma et al. 2009, Burdette and Vance 2013). The carboxy-terminal domain (CTD) of STING acts as a scaffold to bring TBK-1 in close proximity to IRF-3, thereby facilitating IRF-3 phsophorylation (Tanaka and Chen 2012). Phosphorylated IRF-3 promotes the expression of IFN-β and co-regulated genes (Leber, Crimmins et al. 2008).

Wild type *L. monocytogenes* activates the STING-IRF3 cytosolic surveillance pathway upon gaining entry to the host cytosol but until recently, the bacterial ligands responsible for this activation were unknown. A forward genetic screen designed to identify bacterial mutants that

altered IFN-β expression revealed that multidrug resistance (MDR) transporters are critical for

IFN-β expression in *L. monocytogenes*-infected cells. This finding suggested that MDRs are

perhaps responsible for the transport of bacterial ligands, likely small nucleic acids, into the host

cytosol (Witte, Archer et al. 2012). Indeed, subsequent analyses revealed that cyclic-di-AMP

and cyclic-di-GMP activate the STING-IRF3 pathway and that induction of IFN-β by either

dinucleotide depends on STING (McWhirter, Barbalat et al. 2009, Woodward, Iavarone et al.

2010, Jin, Hill et al. 2011, Sauer, Sotelo-Troha et al. 2011). Given that STING does not directly

bind to dsDNA, it remains an open question as to which proteins act upstream of STING to

detect *L. monocytogenes*-derived DNA. However, it is clear that *L. monoctyogenes* DNA and/or

cyclic dinucleotides induce the STING-dependent production of type I interferon, which in turn

regulates the host response to the infection.

1.10) Intracellular bacteria promote inflammasome activation.

Inflammasomes make up another cytosolic surveillance pathway, as they sense and

respond to microbe-derived PAMPs as well as a variety of danger-associated molecular patterns

(DAMPs) such as ATP, uric acid crystals, or heat-shock proteins that are primarily derived from

dying or damaged cells (Martinon, Mayor et al. 2009, Lamkanfi and Dixit 2014). Binding of a

subset of NOD-like receptors (NLRs) such as NLRP3 and NLRC4 or the PYHIN family member

absent in melanoma 2 (AIM2) to cognate ligands leads to the recruitment of the inactive cysteine

protease pro-caspase 1, which associates with the NLR or AIM2 through the adaptor ASC.

Inflammasome activation results in the proteolytic cleavage of pro-caspase 1 to active caspase 1,

which subsequently cleaves pro-IL-1β and pro-IL-18 to yield bioactive IL-1β and IL-18,

respectively (Lamkanfi 2011). The involvement of various inflammasomes in the activation of

caspase 1 during *L. monocytogenes* infection has proved controversial. The NLRP3

inflammasome, which recognizes a diverse array of stimuli including ATP, particulate matter,

and pore-forming toxins such as LLO, was initially reported to be required for inflammasome

activation during *L. monocytogenes* infection (Mariathasan, Weiss et al. 2006). However,

subsequent reports suggested that other inflammasomes are involved as well (Franchi,

Kanneganti et al. 2007, Kim, Bauernfeind et al. 2010). Indeed, AIM2-deficient macrophages

exhibit a significant defect in caspase 1 activation and IL-1β production after infection with *L.*

monocytogenes (Rathinam, Jiang et al. 2010). AIM2 is known to be required for caspase 1

activation in response to dsDNA specifically, as AIM2-deficient macrophages produce wild type

levels of IL-1β upon treatment with NLRP3 and NLRC4 inflammasome agonists but exhibit a

near-complete defect in IL-1β production after exposure to B-form dsDNA (Fernandes-Alnemri,

Yu et al. 2010, Rathinam, Jiang et al. 2010). Taken together, these data suggest that AIM2

contributes to inflammasome activation by *L. monocytogenes* DNA. Though *L. monocytogenes*

was reported to activate caspase 1 through the NLRC4 (Ipaf) inflammasome as well, its

activation is known to depend on flagellin, which is not expressed by all strains of *L.*

monocytogenes at physiologic temperatures (Way, Thompson et al. 2004). Indeed, variations in

the expression of various *L. monocytogenes* factors may influence the mechanism by which

caspase 1 is activated to induce the processing of pro-inflammatory cytokines IL-1β and IL-18.

1.11) Hypothesis

We hypothesize that genotoxic reactive oxygen and nitrogen intermediates produced by activated macrophages may induce DNA damage in the macrophage genome. As in developing and activated B cells, this damage and the resulting DDR may act as potent signaling intermediates that impact macrophage function in the innate immune response (Figure 1). Here, we have developed an experimental system to assess whether DDR is activated in macrophages that have been exposed to LPS and IFN-γ or the intracellular bacterium *Listeria monocytogenes* and to address the impact of the DDR on macrophage function.
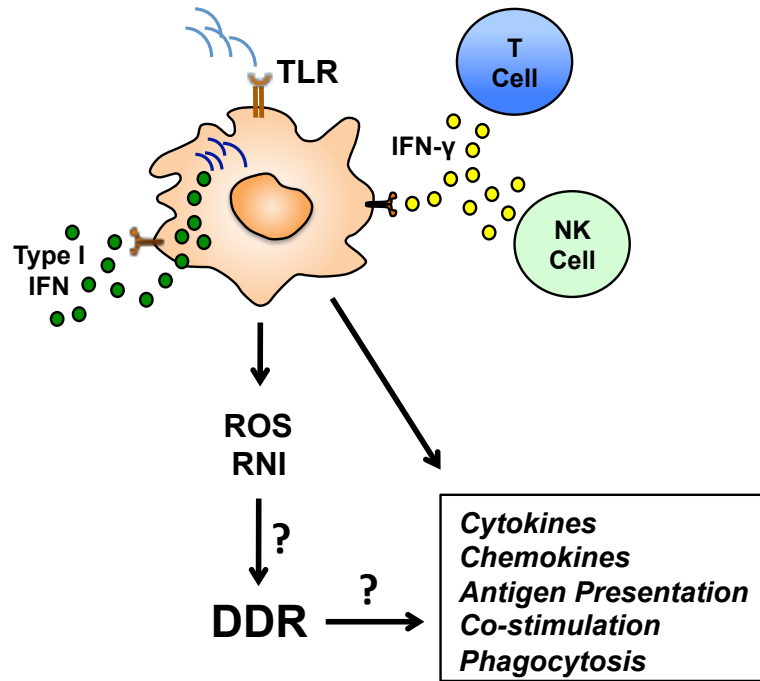
Figure 1: Genotoxic intermediates produced by classically activated macrophages may initiate a DDR.

Chapter 2: Materials and Methods

Mice. All mice were bred and maintained under specific pathogen-free conditions at the Washington University School of Medicine and were handled in accordance with the guidelines set forth by the Division of Comparative Medicine of Washington University. *Atm$^{c/c}$* mice (Zha, Sekiguchi et al. 2008) were extensively backcrossed to the C57BL/6 background and were monitored by the analysis of microsatellite markers at the Rheumatic Disease Core Center, Washington University School of Medicine (St. Louis, MO). They were then crossed to *LysM$^{cre/+}$* mice (Clausen, Burkhardt et al. 1999) and bred onto a *Scid* (*Prkdc^{scid}*) background. *Atm$^{-/-}$* mice were generated through germline Cre-mediated deletion of the aforementioned conditionally targeted ATM allele. *Lysm$^{cre/+}$, Ifnar1$^{-/-}$, Sting$^{-/-}$, Myd88$^{-/-}$, Atm$^{-/-}$, Scid, Nos2$^{-/-}$* and *gp91$^{phox-/-}$* mice were all maintained on a C57BL/6 background. In a subset of experiments, *Atm$^{-/-}$* mice on a mixed genetic background were used with *Atm$^{+/+}$* littermates as controls. *Mre11$^{ATLD1/ATLD1}$* have been described previously (Theunissen, Kaplan et al. 2003). All mice were analyzed between 4 and 8 wks. of age.

Bacteria. *Listeria monocytogenes* strains used in this study were the wild-type strain EGD and the listeriolysin O (LLO) deletion mutant EJL1. *L. monocytogenes* was stored as glycerol stocks at -80°C. For all *ex vivo* experiments with bone marrow-derived macrophages (BMDMs), cultures of *L. monocytogenes* were grown for 12-15 hr. in Brain Heart Infusion (BHI) liquid media (Becton Dickinson) at 37°C without agitation. *L. monocytogenes* concentration was estimated from a standard curve at OD560. *L. monocytogenes* was diluted into DMEM supplemented with 10% heat-inactivated FBS (HyClone, Thermo Scientific) before infection of BMDMs. Heat-killed *L. monocytogenes* (hk *L. m.* strain EGD) was prepared by incubation of mid-log bacteria at 70°C for 3 hr. followed by three washes with sterile 1X PBS.

Primary Cell Culture. Bone marrow was harvested and cultured for 6 days in complete

DMEM containing 10% heat- inactivated FBS, 5% heat- inactivated horse serum (Sigma), and

20% culture supernatant from L929 fibroblasts as a source of macrophage colony-stimulating

factor (M-CSF). On day 6, BMDMs were removed from tissue culture dishes and re-plated in 6-

well plates at a density of 2.5 x 10^6/well in the media detailed above. 16 hr. later, BMDMs were

treated with 100 ng/mL LPS (*Escherichia coli* serotype 055:B5) (Sigma), 100 U/mL murine

IFN-γ (PBL Interferon Source), or both. In a subset of experiments, BMDMs were co-treated

with a specific chemical inhibitor of the inducible nitric oxide synthase (iNOS), aminoguanidine

hemisulfate (1 mM) (Sigma) or with the cell-permeable superoxide scavenger manganese (III)

tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride (MnTMPyP) (100 uM) (Alexis). In

experiments in which DNA-PKcs activity was blocked chemically, the specific ATP-competitive

inhibitor NU7026 was used (20 uM) (Sigma).

In *ex vivo L. monocytogenes* experiments, BMDMs were re-plated in antibiotic-free complete

media (containing 20% culture supernatant from L929 fibroblasts) and infected 16 hr. later with

L. monocytogenes at a multiplicity of infection (MOI) of 5. In experiments where live *L.

monocytogenes* was used, Gentamicin (5 ug/mL) (Gibco) was administered 30 minutes post-

infection to kill extracellular *L. monocytogenes*. All *ex vivo L. monocytogenes* experiments were

done in the presence or absence of 100 ng/mL murine IFN-γ (R&D Systems).

Primary peritoneal macrophages were generated by harvesting resident peritoneal exudate cells

from C57BL/6 mice via peritoneal lavage. Cells were plated in 24-well plates at a density of 1-2

x10^6 cells/well and were incubated at 37°C for 4 hr. in complete DMEM. At this time, media was removed and the remaining adherent macrophages were incubated for 24 hr. in complete DMEM containing LPS (100 ng/mL), IFN-γ (100 U/mL), or both.

Bleocin Damage Assay. Wild type BMDMs and mouse embryonic fibroblasts (MEFs) were treated with bleomycin (1 ug/mL) (Bleocin^{TM}) (Millipore) for 6 hr. without or with IFN-β (100 U/mL) (PBL Interferon Source) pre-treatment for 3 hr.

Southern Blot Analysis. Southern blot analyses were performed on genomic DNA digested with restriction enzyme KpnI using the 3' ATM conditional probe as previously described (Zha, Sekiguchi et al. 2008). The 3' probe was generated by PCR amplification using the following oligonucleotides:

5'-GGCATCTGCTTGA CTGCAGTAAATCAGGCGG-3′ and

5′-GGGGTACTGCAGCATAGGGCTGGAAGAGG-3′.

Western Blot Analysis. BMDMs and MEFs were isolated in RIPA buffer and whole cell lysates were generated with LDS sample buffer (Invitrogen) supplemented with dithiothreitol (DTT). For immunoblot analysis of proteins present in culture supernatants, protein was precipitated with 7.2% w/v trichloroacetic acid (TCA) (Sigma) followed by two acetone wash steps. Standard immunoblotting techniques were used as previously described (Helmink, Tubbs et al. 2011). Primary antibodies used were anti-γ-H2AX (clone JBW301) (Millipore), anti-

H2AX (Millipore), anti-phospho-KAP-1 (Bethyl Laboratories), anti-KAP-1 (GeneTex), anti-

caspase 1 (p20, Casper-1) (AdipoGen), anti-AIM2 (Cell Signaling Technology), anti-ATM

(clone 5C2) (GeneTex), anti-DNA-PK (Ab-4, Cocktail) (NeoMarkers), anti-vinculin (Cell

Signaling Technology), and anti- glyceraldehyde-3-phosphate dehydrogenase (GAPDH)

(Sigma). Secondary reagents were horseradish peroxidase–conjugated anti–mouse IgG

(Promega) or horseradish peroxidase–conjugated anti–rabbit IgG (Cell Signaling Technology).

Immunoflurescence. BMDMs were plated on 12 mm glass coverslips (2.5 x 10^5 cells/coverslip)

in 24-well plates and infected with *L. monocytogenes* as described above. 9 hr. post-infection, the

cells were fixed with 4% formaldehyde in 1X PBS for 10 min. at room temperature,

permeabilized in 0.5% Triton X-100 in PBS for 5 min., and then washed with 1X PBS.

Coimmunostaining with primary and secondary antibodies was performed with a blocking

solution of 3% bovine serum albumin (BSA) in 1X PBS at 37°C for 30 min., and cells were

mounted with ProLong Gold Antifade reagent containing 4′,6-diamidino-2-phenylindole (DAPI)

(Invitrogen). Antibodies used for staining were anti-γ-H2AX (clone JBW301) (1:2,000 dilution)

(Millipore) and Difco Listeria O Antiserum Poly Serotypes 1, 4 (1:200 dilution) (BD).

Antibodies used for secondary staining were Alexa Fluor 488–goat anti-rabbit IgG (1:2,000)

(Invitrogen), and Alexa Fluor 594–goat anti-mouse IgG (1:2,000) (Invitrogen). Imaging was

performed with a microscope (BX-53; Olympus), using an ApoN 60×/1.49-numerical-aperture

(NA) oil immersion lens and cellSens Dimension software.

Gene Arrays. Wild type BMDMs and mouse embryonic fibroblasts (MEFs) were treated with bleomycin (1 ug/mL) (Bleocin™) (Millipore) for 8 hr. without or with IFN-β (100 U/mL) (PBL Interferon Source) pre-treatment for 3 hr. RNA was isolated from two independent BMDM and MEF cultures using the RNeasy Mini Kit (Qiagen). Gene expression profiling was performed using Ilumina MouseRef-8 expression microarrays by the Washington University Genome Technology Access Center, Washington University School of Medicine (St. Louis, MO) according to the manufacturer's protocols. Signal intensities were quantile normalized and all negative intensities were set to 1.

RNA was isolated as described above from two independent BMDM cultures for each genotype (*LysM$^{cre/+}$* and *Scid:Atm$^{c/c}$:LysM$^{cre/+}$*) after no infection or infection with *L. monocytogenes* for 24 hrs. Gene expression profiling was performed using Illumina MouseRef-8 expression microarrays by the Washington University Genome Technology Access Center, Washington University School of Medicine (St. Louis, MO) according to the manufacturer's protocols. As with the other gene array, signal intensities were quantile normalized and all negative intensities were set to 1. Fold changes were calculated based on the average of two biological replicates for each genotype. Genes with a ≥ 5-fold change in *L. m.*-infected versus uninfected *LysM$^{cre/+}$* BMDMs were selected. Of these, a secondary filter identified genes with decreased expression (fold change of ≥ 1.5) in *Scid:Atm$^{c/c}$:LysM$^{cre/+}$* relative to *LysM$^{cre/+}$* BMDMs. Only these genes were considered for further analysis.

Quantitative RT-PCR Analysis. RNA was isolated using the RNeasy Mini Kit (Qiagen) and reversed transcribed using a poly-dT primer and SuperScriptII reverse transcriptase (Invitrogen) according to the manufacturer's protocol. RT-PCR was performed using Brilliant II SYBR Green (Agilent Technologies) and acquired on an Mx3000P (Agilent Technologies). Primer sequences are detailed in Table 1.

Cytokine and Nitrite Determinations. Supernatant IL-1β and IL-18 levels were measured using the OptEIA ELISA Set (BD Biosciences) and Mouse IL-18 ELISA Set (MBL), respectively. Both sets were used in accordance with the manufacturer's instructions. Nitric oxide levels were determined in thawed supernatants by measuring the amount of nitrite, a stable metabolic product of nitric oxide. The assay mixture contained supernatant and Griess reagent in a 1:1 ratio in a flat-bottom 96-well tissue culture plate, and absorption was measured at 540 nm using a microplate reader. The amount of nitrite was determined by comparison of unknowns to a $NaNO_2$ standard curve.

NK-BMDM Co-Culture Assay. BMDMs were cultured in 12-well plates at a density of 1×10^6 cells/well and infected with *L. monocytogenes* as described above. Splenic NK cells were magnetically sorted from whole splenocytes obtained from C57BL/6 mice using CD49b (DX5) MicroBeads and MS columns (Miltenyi). 12 hr. post-*L. monocytogenes* infection of BMDM, 0.5 $\times 10^6$ purified splenic NK cells were added to each well in the presence of IL-2 (50 U/mL) (PeproTech) to foster NK cell survival with or without murine IL-18 (10 ng/mL) (MBL). 10 hr. after adding the purified NK cells to the BMDMs, protein transport was inhibited with GolgiStop

(BD Biosciences). 5 hr. later, cells were harvested and nonspecific binding was blocked with 5 ug/mL of anti-CD16/32 (2.4G2; BD Pharmingen) before cell surfaces were stained with anti-NK1.1 (PK136; eBioscience), anti- CD11b (M1/70; BD Pharmingen), and anti-F4/80 (BM8; BioLegend). Cells were fixed and permeabilized according to standard protocol and intracellular staining for IFN-γ (XMG1.2, eBioscience) was performed. Data were acquired on a FACSCanto II (BD Biosciences) and were analyzed with FlowJo software version 9.6.4 (TreeStar).

Coverslip Assay. BMDM were plated on 12 mm glass coverslips (2.5×10^5 cells/coverslip) in 24-well plates and infected with *L. monocytogenes* as described previously. At several timepoints post-infection, coverslips were washed in warm 1X PBS and then placed into 10 ml cold sterile deionized water and vortexed for 30 s. to lyse the BMDMs. Each condition was assayed in triplicate. Serial dilutions of lysates were plated on BHI-agar plates. Bacterial colonies were counted after overnight incubation at 37°C.

LDH Release Assay. Percent lactate dehydrogenase (LDH) release in culture supernatants was determined with the LDH Cytotoxicity Detection Kit (Clontech) in accordance with the manufacturer's recommendations. Data for *Scid:Atm^{c/c}:LysM^{cre/+}* and *LysM^{cre/+}* are given as a percentage of the LDH released by equivalent cell numbers of each genotype lysed with 1% Triton X-100.

Statistical analysis. All P-values were generated via Student's two-tailed t test using Prism

Version 5. P-values below 0.05 were considered statistically significant.

	Forward Primer	**Reverse Primer**
Gapdh	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA
Cxcl1	CTGGGATTCACCTCAAGAACATC	CAGGGTCAAGGCAAGCCTC
Cxcl10	CCAAGTGCTGCCGTCATTTTC	GGCTCGCAGGGATGATTTCAA
Ccl2	TTAAAAACCTGGATCGGAACCAA	GCATTAGCTTCAGATTTACGGGT
Ccl3	TTCTCTGTACCATGACACTCTGC	CGTGGAATCTTCCGGCTGTAG
Ccl4	TTCCTGCTGTTTCTCCTCTTACACCT	CTGTCTGCCTCTTTTGGTCAG
Ccl5	GCTGCTTTGCCTACCTCTCC	TCGAGTGACAAACACGACTGC
Marco	GCACAGAAGACAGAGCCGATTT	GCCACAGCACATCTCTAGCATCT
Cd69	TGGTGAACTGGAACATTGGA	CAGTGGAAGTTTGCCTCACA
Il1b	AGCTTCCTTGTGCAAGTGTCT	GACAGCCCAGGTCAAAGGTT
Il18	TCAAAGTGCCAGTGAACCCC	GGTCACAGCCAGTCCTCTTAC
Aim2	CGGGAAATGCTGTTGTTGAC	TGCTCCTGGCAATCTGAAA
Nlrp3	TCCTGCAGAGCCTACAGTTG	ACGCCTACCAGGAAATCTCG
Il12p40	ACCTGTGACACGCCTGAAGAAGAT	TCTTGTGGAGCAGCAGATGTGAGT

Table 1. Oligonucleotide sequences used in quantitative RT-PCR analysis.

Chapter 3: Signaling requirements for the DDR in activated macrophages

In response to Toll-like receptor (TLR) engagement coupled with type I or type II interferon receptor signaling, macrophages become classically activated. Activated macrophages up-regulate MHC class II and co-stimulatory molecules, have enhanced phagocytic capacity, and express a wide variety of inflammatory cytokines and chemokines. They also produce genotoxic reactive oxygen and nitrogen intermediates which function to compromise or eliminate invading pathogens (Mosser and Edwards 2008, Nish and Medzhitov 2011). The primary source of reactive oxygen species (ROS) in classically activated macrophages is the NADPH oxidase, which is rapidly assembled at plasma and phagosomal membranes upon exposure to inflammatory stimuli (Nunes, Demaurex et al. 2013). Nitric oxide (NO), by contrast, is produced by the inducible nitric oxide synthase (iNOS) when signals downstream of pattern recognition receptors (PRR) cooperate with type I or II interferon signaling in the full transcriptional induction of the *Nos2* gene. ROS such as free radicals or one-electron oxidants can react with DNA to form a variety of different lesions, including modified bases, inter- and intra-strand crosslinks, and strand breaks (Jena, Anand et al. 2012). Similarly, reactive nitrogen species (RNS) generated by a reaction between NO and oxygen radicals can cause significant DNA damage, including base modifications and DNA breaks (Sawa and Ohshima 2006, Bogdan 2015). Additionally, both ROS and RNS can alter protein function through the covalent modification of key residues within the protein (Finkel 2011, Bogdan 2015). Given that reactive oxygen and nitrogen species are highly genotoxic, we hypothesize that these intermediates may damage the macrophage host genome, leading to the initiation of a DNA damage response (DDR) in these cells. In G1-phase cells, this response is initiated through the activation of PI3-like kinase family members ataxia telangiectasia mutated (ATM) and the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) (Shiloh 2003). Thus, we asked three major

questions: (1) Is the DDR initiated in classically activated macrophages? (2) If so, what signaling pathways are required for the response? (3) Are PI3-like kinases ATM and/or DNA-PKcs responsible for initiating a DDR in activated macrophages?

3.1) Experimental system to assess DDR in activated macrophages.

BMDMs were generated from whole bone marrow isolated from the femurs of 4-8-week-old mice in complete media containing supernatant from L929 fibroblasts as a source of macrophage-colony stimulating factor (M-CSF) (Chapter 2) (Figure 2A). After 6 days in culture, adherent cells were removed from culture dishes and re-plated in the same media. Flow cytometric analysis revealed that the resulting cell cultures were overwhelmingly composed of murine bone marrow-derived macrophages, as >95% expressed the common myeloid marker CD11b and the macrophage marker F4/80 (Figure 2B) (Ho and Springer 1982, McGarry and Stewart 1991). 16-20 hours later, the BMDMs were classically activated either with the TLR4 agonist lipopolysaccharide (LPS) in the presence of murine IFN-γ, or with the intracellular bacterium *Listeria monocytogenes*, which activates a variety of TLRs.

To assess whether the DDR was initiated in activated macrophages, we asked whether known DDR substrates were phosphorylated in macrophages after exposure to LPS and IFN-γ or *L. monocytogenes*. During the canonical response to DNA damage, hundreds of downstream target proteins are phosphorylated by PI3-like kinases ATM and DNA-PKcs (Matsuoka, Ballif et al. 2007, Helmink and Sleckman 2012). Specifically, we examined the phosphorylation of the chromatin H2A variant H2AX at serine 139 (known as γ-H2AX) or phosphorylation of KAP-1, a

soluble protein that is phosphorylated at serine 824 after the induction of DNA damage. Though these proteins do not have any known function in activated macrophages, detection of their phosphorylated forms using specific antibodies is a reliable indicator that a DDR has been initiated.

3.2) DDR is initiated in classically activated macrophages.

We found that treatment of BMDMs with LPS in the presence of IFN-γ activated them to produce genotoxins, as nitrite was detectable in the cell supernatants 9 hours post-treatment when assayed with the Griess reagent (Figure 3A) (Mosser and Zhang 2008). Treatment of BMDMs with LPS and IFN-γ (but with neither agent alone) resulted in the initiation of a robust DDR, as evidenced by γ-H2AX formation and KAP-1 phosphorylation (Figure 3B). To address whether DDR is similarly activated in primary macrophages, we isolated exudate from the peritoneal cavity of C57BL/6 mice. After several hours in culture, we removed non-adherent cells and treated the remaining adherent macrophages with LPS and IFN-γ. As with BMDMs, we observed γ-H2AX formation and KAP-1 phosphorylation in these cells, indicative of DDR activation (Figure 3C). We conclude that a robust DDR is initiated in peritoneal and bone marrow-derived macrophages after exposure to LPS in the presence of type II interferon (IFN-γ).

We next sought to test whether BMDMs activated by a pathogen would likewise induce a DDR. To address this question, we designed an experimental system to assay the DDR in BMDMs infected with the intracellular bacterium *L. monocytogenes*, which activates both TLR and cytosolic signaling pathways. We first asked whether *in vitro L. monocytogenes* infection

activates DDR. Strikingly, we observed robust γ-H2AX formation and KAP-1 phosphorylation in *L. monocytogenes*-infected macrophages without the addition of IFN-γ (Figure 4A). In contrast to LPS, *L. monocytogenes* is taken up into phagosomal vesicles upon infection and subsequently escapes into the host cytosol in a process that depends on the pore-forming hemolysin listeriolysin O (LLO), which destroys phagosomal membranes (Portnoy, Jacks et al. 1988, Stavru, Archambaud et al. 2011). Cytosolic invasion of *L. monocytogenes* occurs rapidly after infection and triggers myriad signaling pathways within infected cells (Stavru, Archambaud et al. 2011). Among them is the production of type I interferon downstream of the ER-localized sensor STING, which is activated by cyclic dinucleotides produced by *L. monocytogenes* itself (Woodward, Iavarone et al. 2010, Witte, Archer et al. 2012). We therefore asked whether cytosolic entry is required for a *L. monocytogenes*-elicited DDR. To address this, wild type BMDMs were either treated with heat-killed *L. monocytogenes* or infected with a LLO-deficient strain of live *L. monocytogenes* (EJL1). Both activate TLR signaling; however neither can initiate signaling pathways in the host cytoplasm (Edelson and Unanue 2002, Stavru, Archambaud et al. 2011). Neither treatment with heat-killed *L. monocytogenes* nor infection with LLO-deficient *L. monocytogenes* activated the DDR. However, co-treatment of the *L. monocytogenes*-infected cells with exogenous IFN-γ led to robust γ-H2AX formation in both cases (Figure 4B and 4C). Taken together, we conclude that cytosolic entry is required for the initiation of a DDR in *L. monocytogenes*-infected cells in the absence of type II interferon.

3.3) DDR depends on TLR and interferon signaling.

Classical activation of macrophages depends on the sensing of various microbial components by TLRs. Thus, we asked whether the initiation of the DDR in *L. monocytogenes*-infected macrophages similarly requires TLR signaling. To address this, we infected BMDMs deficient in the TLR adaptor protein MyD88 (*Myd88$^{-/-}$*) with *L. monocytogenes*. We found that there was a significant reduction in γ-H2AX formation in infected *Myd88$^{-/-}$* BMDMs relative to WT BMDMs (Figure 5A). Thus, initiation of the DDR depends, at least in part, on TLR signaling mediated by the adaptor MyD88. The slight DDR that is detected may be mediated by MyD88-independent TLR or NLR signaling pathways. For example, the cytosolic NOD family members Nod1 and Nod2 activate NF-κB in *L. monocytogenes*-infected macrophages through the adaptor RIP2 (Park, Kim et al. 2007).

Given that cytosolic entry of *L. monocytogenes* is required for the initiation of DDR, we hypothesized that this may be due to a requirement for type I interferon production downstream of the cytosolic sensor STING (Figure 4B and 4C) (Paludan and Bowie 2013). Indeed, infection of STING-deficient (*Sting$^{-/-}$*) BMDMs with *L. monocytogenes* did not lead to DDR activation (Figure 5B). The DDR also depends on signaling through the type I interferon receptor (IFNAR), as we observed a near-complete block in the DDR in *L. monocytogenes*-infected type I interferon receptor-deficient (*Ifnar1$^{-/-}$*) BMDMs (Figure 5C). Taken together, we conclude that the activation of macrophages through TLR and interferon receptor signaling leads to the initiation of a robust DDR.

3.4) ATM and DNA-PKcs kinases initiate DDR in activated macrophages.

In G1-phase cells, the DDR is initiated through the activation of ATM and DNA-PKcs, both members of the PI3-like family of serine-threonine kinases (Shiloh 2003). Once activated, these kinases phosphorylate many downstream targets that mediate diverse responses to DNA damage (Matsuoka, Ballif et al. 2007). In most cell types analyzed to date, ATM is the primary kinase responsible for activating DNA damage responses in G1-phase cells, with DNA-PKcs able to partially compensate by phosphorylating several known DDR substrates in ATM-deficient cells (Rouse and Jackson 2002, Callen, Jankovic et al. 2009). Thus, we wished to interrogate the requirements for ATM and DNA-PKcs in the initiation of the DDR in activated BMDMs. To address this experimentally, we generated BMDMs from wild type, ATM-deficient (*Atm*$^{-/-}$), and *Scid* mice, which have a point mutation in the DNA-PKcs gene that results in low-level expression of a truncated, non-functional DNA-PKcs protein (Blunt, Gell et al. 1996). Phosphorylation of the known ATM substrate KAP-1 was almost completely abrogated in ATM-deficient (*Atm*$^{-/-}$) BMDMs after infection with *L. monocytogenes* (Figure 6A). However, we observed robust KAP-1 phosphorylation in *L. monocytogenes*-infected *Scid* BMDMs, indicating that DNA-PKcs is not required for KAP-1 phosphorylation in *L. monocytogenes*-infected BMDMs (Figure 6A). Intriguingly, we observed robust γ-H2AX formation in wild type, *Atm*$^{-/-}$, and *Scid* BMDMs after infection with *L. monocytogenes* (Figure 6B). Taken together, these data indicate that though KAP-1 phosphorylation depends on ATM, robust γ-H2AX formation is initiated in *L. monocytogenes*-infected in the absence of either ATM or DNA-PKcs. To determine whether this is due to the activation of both ATM and DNA-PKcs in these cells, we wished to generate macrophages deficient in both ATM and DNA-PKcs. However, mice with germline loss of ATM and DNA-PKcs exhibit early embryonic lethality, precluding the

generation of BMDMs (Sekiguchi, Ferguson et al. 2001). Thus, we generated mice that were

homozygous for both the conditionally targeted ATM allele (Atm^c) and for a Cre knock-in at the

lysozyme M (*Lyz2)* locus ($Atm^{c/c}:LysM^{cre/cre}$) (Clausen, Burkhardt et al. 1999, Zha, Sekiguchi et

al. 2008). Lysozyme M is expressed in subsets of monocytic lineage cells and thus, the Cre

knock-in should delete ATM in macrophages and a subset of other myeloid cell types (Clausen,

Burkhardt et al. 1999). Indeed, BMDMs from $Atm^{c/c}:LysM^{cre/cre}$ mice had no detectable ATM

protein, whereas thymocytes from the same mice did not exhibit any appreciable reduction in

ATM protein levels (Figure 7A). We then crossed the $Atm^{c/c}:LysM^{cre/cre}$ mice to the *Scid*

background to eliminate both kinases in BMDMs generated from these mice. The resulting

Scid:$Atm^{c/c}:LysM^{cre/+}$ mice were viable and macrophages derived from these mice exhibited

efficient deletion of the Atm^c allele, generating the Atm^- allele (Figure 7B). Thus, we

successfully generated a mouse model in which myeloid lineage cells, including macrophages,

are deficient in both ATM and DNA-PKcs.

After infection with *L. monocytogenes,* we observed a near-complete block in KAP-1

phosphorylation in ATM-deficient ($Atm^{-/-}$) BMDMs, but not in wild type and DNA-PKcs (*Scid*)

BMDMs (Figure 6A). In contrast, robust γ-H2AX formation was observed in all three genotypes

after exposure to *L. monocytogenes* (Figure 6B). To determine whether this is due to the

activation of both ATM and DNA-PKcs during *L. monocytogenes* infection, we examined γ-

H2AX formation and KAP-1 phosphorylation in *L. monocytogenes*-infected

Scid:$Atm^{c/c}:LysM^{cre/+}$ BMDMs, which are deficient in both ATM and DNA-PKcs. As was

observed with wild type BMDMs, $LysM^{cre/+}$ BMDMs exhibited a robust DDR post-*L.*

monocytogenes infection (Figure 8A). In contrast, ATM- and DNA-PKcs-deficient

(*Scid*:*Atm^{c/c}*:*LysM^{cre/+}*) BMDMs exhibited a near-complete abrogation of both γ-H2AX

formation and KAP-1 phosphorylation upon infection with *L. monocytogenes* (Figure 8A). This

is not due to a rapid clearance of *L. monocytogenes* infection in ATM- and DNA-PKcs-deficient

(*Scid*:*Atm^{c/c}*:*LysM^{cre/+}*) BMDMs as compared to *LysM^{cre/+}* controls, as the two genotypes

harbored similar levels of *L. monocytogenes* throughout the course of infection (Figure 8B).

Additionally, the near-complete block in the DDR in *Scid*:*Atm^{c/c}*:*LysM^{cre/+}* BMDMs was not due

to a general activation defect in these cells, as equivalent levels of nitrite were detected in the

supernatants of *LysM^{cre/+}* and *Scid*:*Atm^{c/c}*:*LysM^{cre/+}* *LysM^{cre/+}* BMDMs after *L. monocytogenes*

infection (Figure 8C). The kinase requirements for DDR activation in LPS- and IFN-γ-activated

BMDMs were similar to those observed in *L. monocytogenes*-infected BMDMs. We observed γ-

H2AX formation in ATM-deficient (*Atm^{-/-}*) BMDMs and wild type BMDMs that were treated

with a specific chemical inhibitor of DNA-PKcs, NU7026 (Figure 9A). KAP-1 phosphorylation,

however, depended entirely on ATM, as we observed robust KAP-1 phosphorylation in LPS-

and IFN-γ-treated BMDMs that were co-treated with NU7026, which blocks DNA-PKcs kinase

activity (Figure 9B). As was observed in *L. monocytogenes*-infected BMDMs, LPS- and- IFN-γ-

induced DDR was nearly completely abrogated in ATM-deficient (*Atm^{-/-}*) BMDMs when DNA-

PKcs kinase activity was also blocked (Figure 9A and 9B). Taken together, we conclude that

ATM and DNA-PKcs are both activated and participate in the DDR that is initiated in activated

macrophages.

3.5) Summary and Discussion

Here we demonstrate that a robust DDR is initiated in macrophages that are activated with LPS and IFN-γ or the intracellular pathogen *L. monocytogenes*. In *L. monocytogenes*-infected macrophages, this response depends on signals downstream of TLRs in combination with type I or type II interferon signaling. γ-H2AX formation is observed in wild type, ATM-deficient, and DNA-PKcs-deficient macrophages after exposure to *L. monocytogenes*, but is nearly abrogated in macrophages deficient in both kinases, indicating that both ATM and DNA-PKcs are activated and participate in the DDR that is elicited by *L. monocytogenes* infection. Similarly, γ-H2AX formation is observed after LPS- and IFN-γ-treatment in both ATM-deficient macrophages and in macrophages in which DNA-PKcs kinase activity has been blocked. In contrast, KAP-1 phosphorylation depends on ATM in activated macrophages. Taken together, these data suggest that ATM and DNA-PKcs have differential abilities in the phosphorylation of H2AX and KAP-1 and thus, have both unique and overlapping functions in the initiation of the DDR in activated macrophages. The differential requirements for ATM and DNA-PKcs in the activation of these two substrates may be related to the localization of the substrates within the nucleus. KAP-1 is a diffusible soluble protein, whereas the histone H2A variant H2AX is a key component of chromatin and is therefore closely associated with DNA DSB sites. Indeed, γ-H2AX spans hundreds of kilobases from the DNA break site, forming a platform for DNA repair factors. As both ATM and DNA-PKcs are specifically recruited to DNA DSBs by the MRN and Ku sensors, respectively, it is likely that both kinases will be in close proximity with chromatin that is associated with the break. Macrophages are frequently exposed to a variety of genotoxic intermediates that are necessary byproducts of their function in the innate immune response to infection. Thus, these cells may have evolved a complex response to DNA damage that involves

the activity of both ATM and DNA-PKcs downstream of the repair platform that is assembled after the propagation of γ-H2AX. It is possible that once activated at the break site, these kinases phosphorylate both common and distinct targets to enable the macrophage to cope with extensive genotoxic stress. The requirement for ATM but not DNA-PKcs in the phosphorylation of soluble KAP-1 may support this idea. Unfortunately, a lack of specific antibodies for many of the putative downstream targets of ATM and DNA-PKcs precludes a deeper understanding of the requirements for each kinase in the DDR that is initiated in activated macrophages. Thus, it is difficult to assess the roles of each kinase in promoting different aspects of the DDR. However, we observe that ATM and DNA-PKcs have both distinct and overlapping roles in the regulation of the genetic program and inflammasome activation in *L. monocytogenes*-infected macrophages (Chapter 5). These findings support the notion that both kinases are critical for a fully functional DDR in activated macrophages.

A

6d in M-CSF

LPS + IFN-γ
or
Listeria monocytogenes

B

F4/80

CD11b

CD11b
Iso

CD11b

F4/80
Iso

F4/80
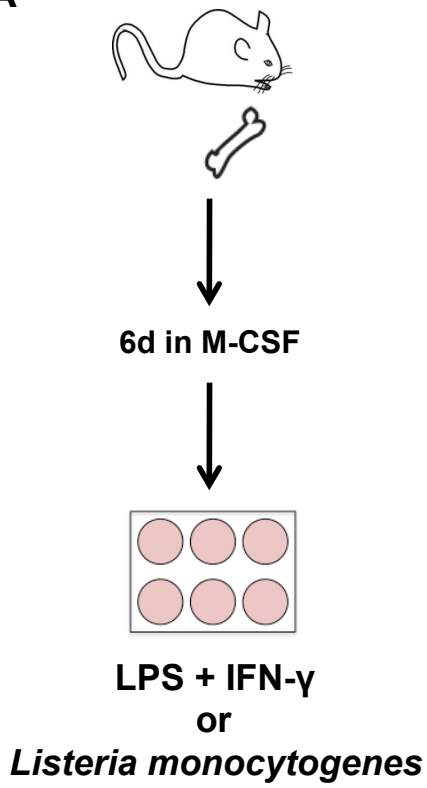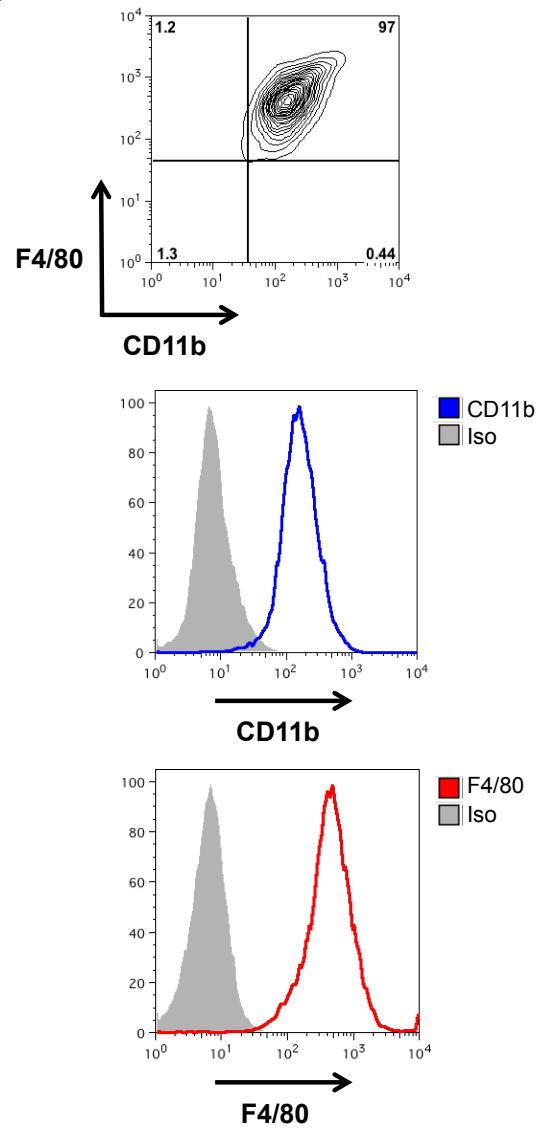
Figure 2. Generation and activation of BMDMs.

Figure 2 Legend. Generation and activation of BMDMs. (A) Schematic outlining the isolation of whole bone marrow from the femurs of mice and the generation of BMDMs in M-CSF-containing media. BMDMs are subsequently activated with LPS and IFN-γ or *L. monocytogenes*. **(B)** Flow cytometric analysis of CD11b and F4/80 surface expression on wild type BMDMs generated from whole bone marrow cultured in M-CSF-containing media for 6 days.   Grey = Isotype control, Blue = CD11b, Red = F4/80.
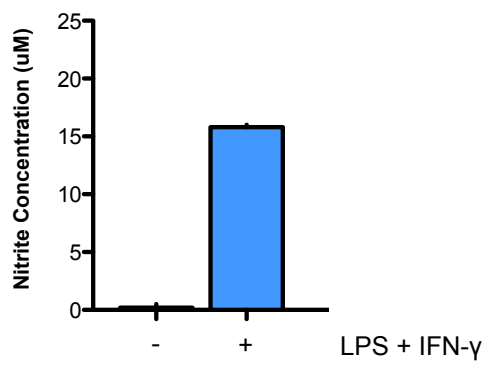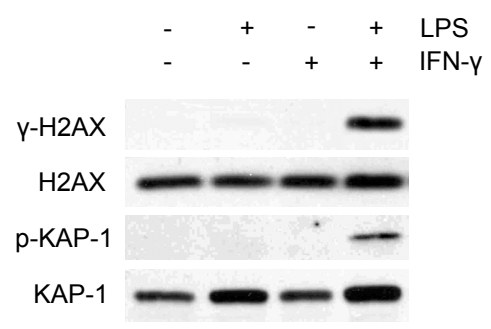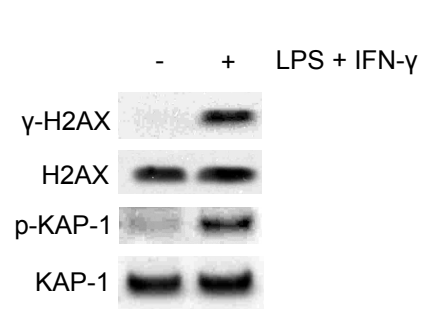
Figure 3. Classically activated macrophages initiate DDR.

Figure 3 Legend. Classically activated macrophages initiate DDR. (A) Nitrite concentration

in culture supernatants collected from wild type BMDMs after no treatment (-) or treatment (+)

with LPS and IFN-γ for 9 hrs. Data depict the mean and standard deviation of three independent

experiments. **(B-C)** Western blotting for γ-H2AX, H2AX, phosphorylated KAP-1 (p-KAP-1)

and KAP-1 in whole cell lysates from **(B)** WT BMDMs after no treatment (-) or treatment (+)

with LPS, IFN-γ, or both for 9 hrs. and **(C)** WT peritoneal macrophages after no treatment (-) or

treatment (+) with LPS + IFN-γ for 24 hrs. Data are representative of three or more experiments.
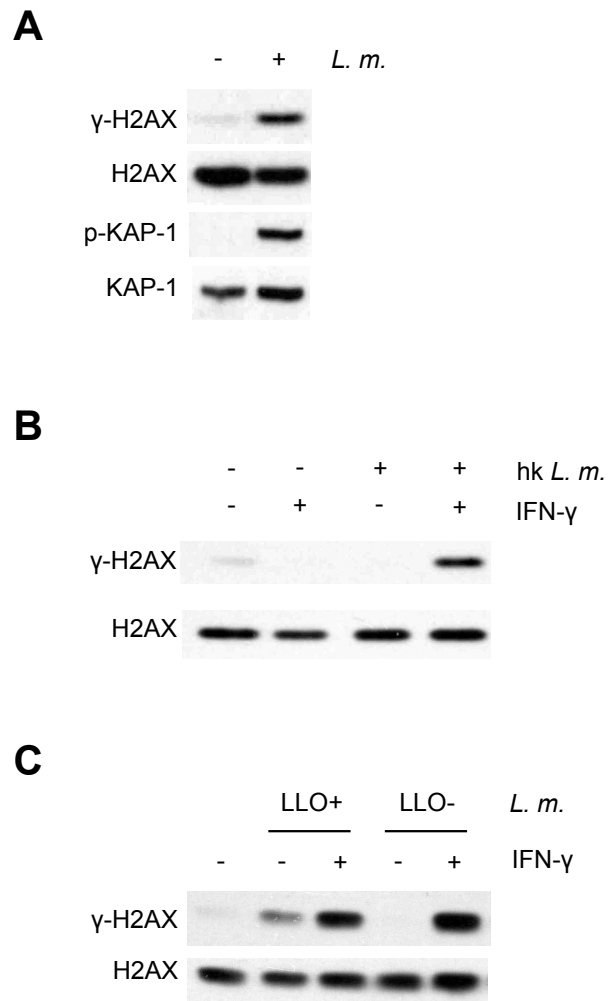
Figure 4. *L. monocytogenes* **induces a DDR that depends on entry into the macrophage cytosol.**

Figure 4 Legend. *L. monocytogenes* **induces a DDR that depends on entry into the**

macrophage cytosol. **(A)** Western blotting for γ-H2AX, H2AX, p-KAP-1, and KAP-1 in whole

cell lysates from WT BMDMs after no infection (-) or infection (+) with *L. monocytogenes* (*L.*

m.) for 24 hrs. **(B-C)** Western blotting for **(B)** γ-H2AX and H2AX in whole cell lysates from

WT BMDMs after no treatment (-) or treatment (+) with IFN- γ, heat-killed *L. monocytogenes*

(hk *L. m.*), or both for 9 hrs. or **(C)** WT BMDMs after no infection (-) or infection (+) with WT

(LLO+) or LLO-deficient (LLO-) *L. m.* with (+) or without (-) IFN-γ for 9 hrs. Data are

representative of two or more experiments.

A

WT   *Myd88⁻ᐟ⁻*

-   +   -   +   *L. m.*

γ-H2AX

H2AX

B

WT   *Sting⁻ᐟ⁻*

-   +   -   +   *L. m.*

γ-H2AX

H2AX

p-KAP-1

KAP-1

C

WT   *Ifnar1⁻ᐟ⁻*

-   +   -   +   *L. m.*

γ-H2AX

H2AX

p-KAP-1

KAP-1
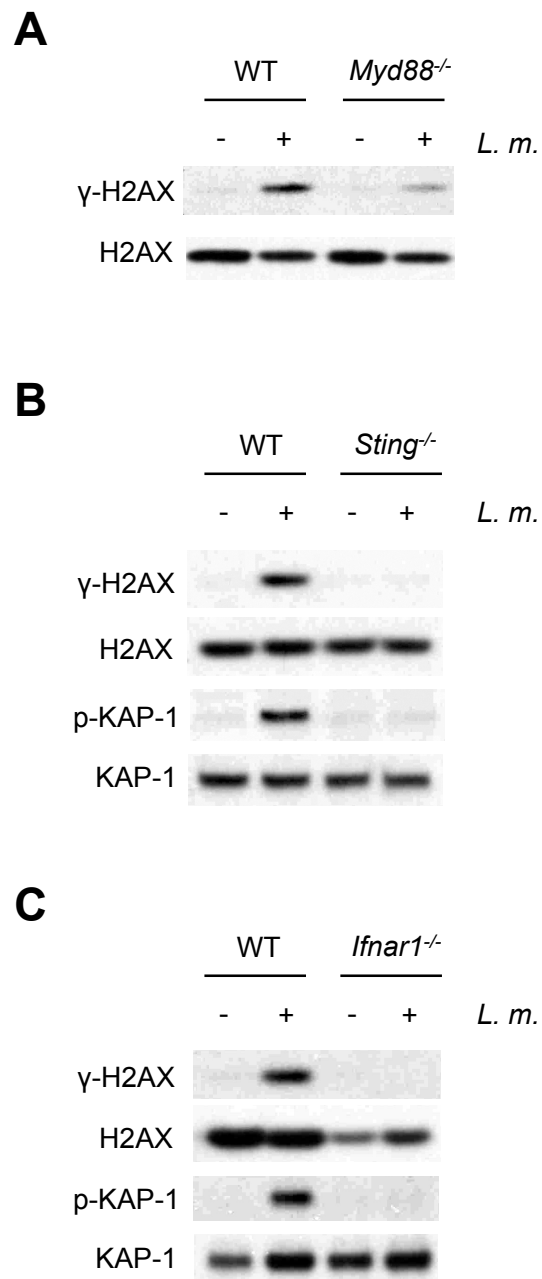
Figure 5. The DDR in *L. monocytogenes*-infected macrophages depends on TLR and interferon signaling.

55

Figure 5 Legend. The DDR in *L. monocytogenes*-infected macrophages depends on TLR and interferon signaling. (A) Western blotting for γ-H2AX and H2AX in whole cell lysates from WT and *Myd88*^{-/-} BMDMs after no infection (-) or infection (+) with *L. m.* for 24 hrs. **(B-C)** Western blotting for γ-H2AX, H2AX, p-KAP-1, and KAP-1 in whole cell lysates from WT and *Sting*^{-/-} BMDMs **(B)** or WT and *Ifnar1*^{-/-} BMDMs **(C)** after no infection (-) or infection (+) with *L. m.* for 24 hrs. Data are representative of two or more experiments.

A

WT   *Atm*^{-/-}   *Scid*

- +   - +   - +   *L. m.*

p-KAP-1

KAP-1

B

WT   *Atm*^{-/-}   *Scid*

- +   - +   - +   *L. m.*

γ-H2AX
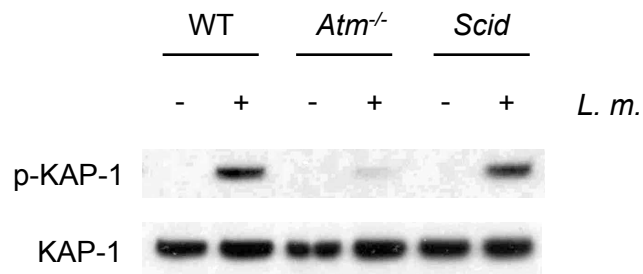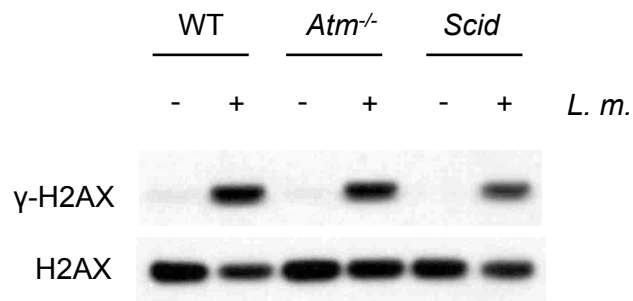
H2AX

Figure 6. KAP-1 phosphorylation depends on ATM in *L. monocytogenes*-infected macrophages.

Figure 6 Legend. KAP-1 phosphorylation depends on ATM in *L. monocytogenes*-infected macrophages. **(A-B)** Western blot analysis of p-KAP-1 and KAP-1 **(A)** and γ-H2AX and H2AX **(B)** in whole cell lysates from WT, *Atm*^{-/-}, and *Scid* BMDMs after no infection (-) or infection (+) with *L. m.* for 24 hrs. Data are representative of two or more independent experiments.

A

Thymocytes

BMDMs

WT $Atm^{-/-}$ $Atm^{c/c}\cdot LysM^{cre/cre}$ WT $Atm^{-/-}$ $Atm^{c/c}\cdot LysM^{cre/cre}$

ATM

DNA-PKcs

Vinculin

B

$Atm^{c/c}$ $Atm^{-/-}$ $Scid\cdot Atm^{c/c}\cdot LysM^{Cre/+}$

12 ——
10 ——

◄ (-)
◄ C

Figure 7. Generation of ATM- and DNA-PKcs-deficient macrophages.

Figure 7 Legend. Generation of ATM- and DNA-PKcs-deficient macrophages. (A) Western blot analysis of ATM, DNA-PKcs, and vinculin in whole cell lysates from WT, *Atm*$^{-/-}$, and *Atm*$^{c/c}$*:LysM*$^{cre/cre}$ thymocytes and BMDMs. **(B)** Southern blot analysis of genomic DNA from BMDMs generated from *Atm*$^{c/c}$, *Atm*$^{-/-}$, and *Scid:Atm*$^{c/c}$*:LysM*$^{cre/+}$ mice. DNA was digested with the restriction enzyme KpnI and hybridized to the 3' ATM conditional probe. Arrows indicate the conditional (c) and deleted (-) alleles. Molecular weight (kb) markers are indicated.
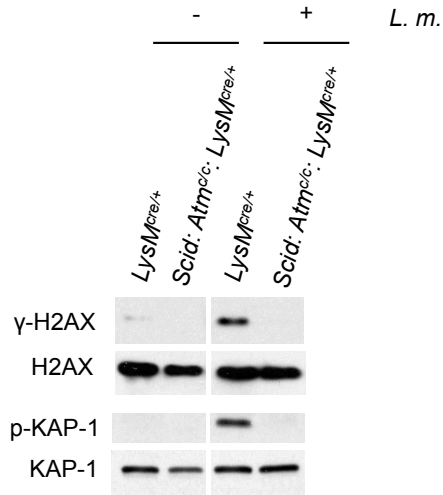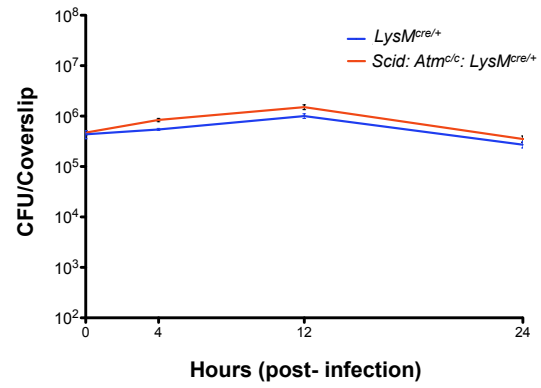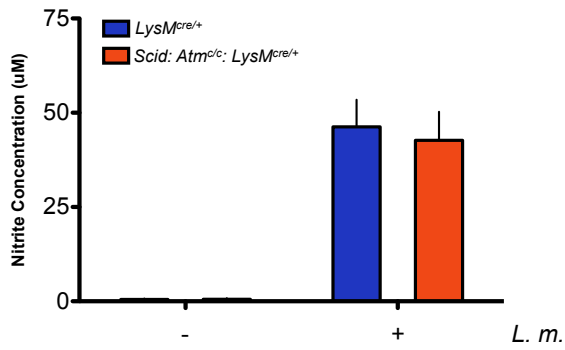
Figure 8. ATM and DNA-PKcs activate the DDR in *L. monocytogenes*-infected macrophages.

Figure 8 Legend. ATM and DNA-PKcs activate the DDR in *L. monocytogenes*-infected macrophages. (A) Western blotting for γ-H2AX, H2AX, p-KAP-1, and KAP-1 in whole cell lysates from *LysM^{cre/+}* and *Scid:Atm^{c/c}:LysM^{cre/+}* BMDMs after no infection (-) or infection (+) with *L. m.* for 24 hrs. Data are representative of three independent experiments. **(B)** *LysM^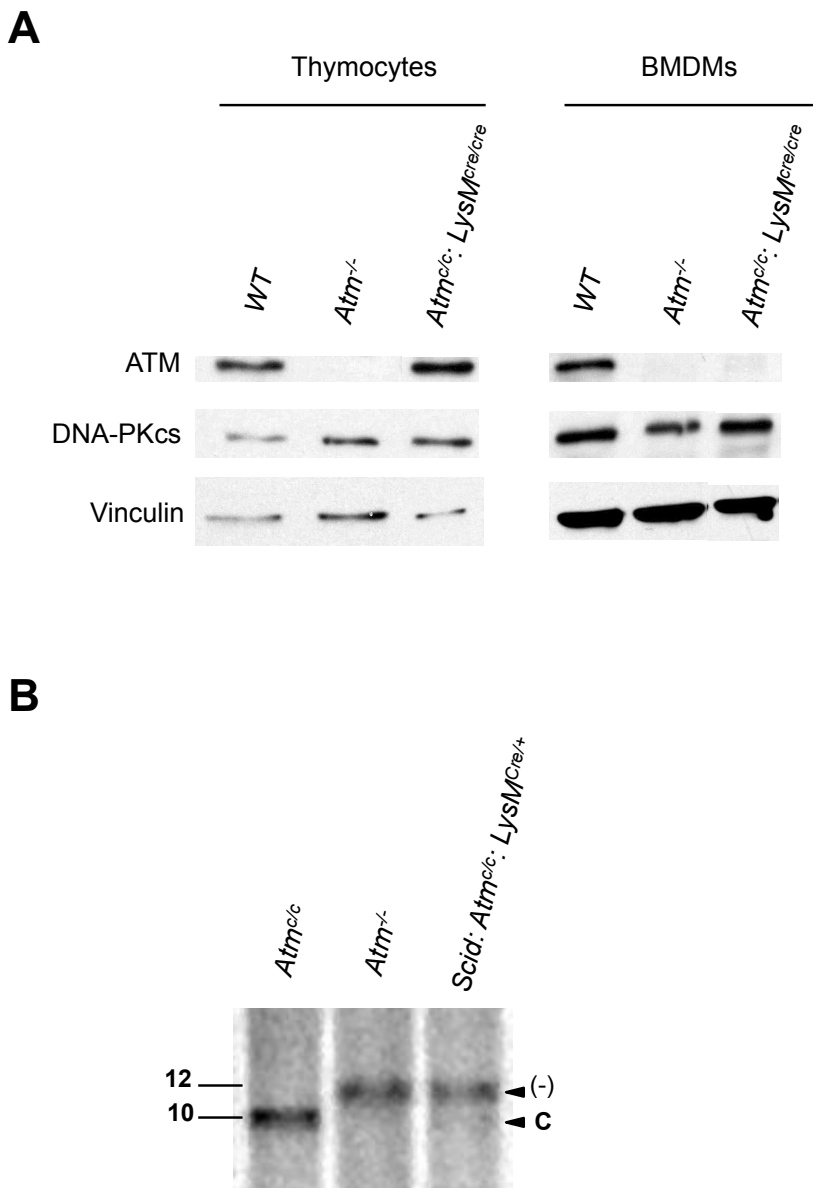{cre/+}* and *Scid:Atm^{c/c}:LysM^{cre/+}* BMDMs were infected with *L. m.* and colony-forming units (CFU) per coverslip were determined at the indicated timepoints post-infection. Shown are the mean and standard deviation of three technical replicates per timepoint. Data are representative of two independent experiments. **(C)** Nitrite concentration in culture supernatants collected from *LysM^{cre/+}* and *Scid:Atm^{c/c}:LysM^{cre/+}* BMDMs after no infection (-) or infection (+) with *L. m.* for 24 hrs. Data are a compilation of three independent experiments and represent mean and standard deviation. Blank space in **(A)** indicates that blot has been cropped.

A

WT   *Atm⁻ᐟ⁻*

-		+		-		+		NU7026

| - | + | - | + | - | + | - | + | LPS + IFN-γ |

γ-H2AX

H2AX

B

WT   *Atm⁻ᐟ⁻*

-		+		-		+		NU7026

| - | + | - | + | - | + | - | + | LPS + IFN-γ |
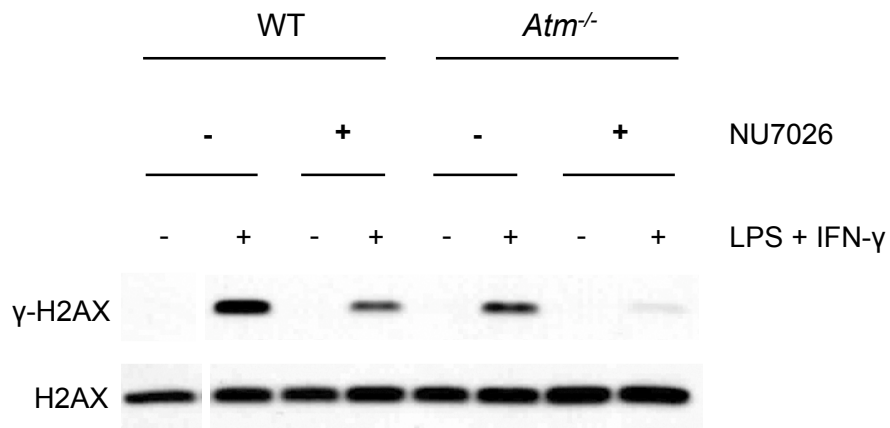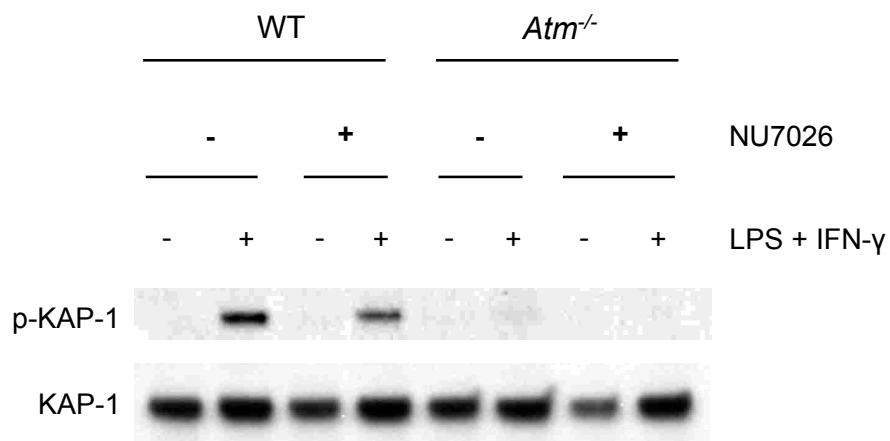
p-KAP-1

KAP-1

Figure 9. ATM and DNA-PKcs activate the DDR in classically activated macrophages.

Figure 9 Legend. ATM and DNA-PKcs activate the DDR in classically activated macrophages. (A-B) Western blot analysis of γ-H2AX and H2AX **(A)** and p-KAP-1 and KAP-1 **(B)** in whole cell lysates from WT and *Atm^-/-^* BMDMs after no treatment (-) or treatment (+) with LPS + IFN-γ for 9 hrs. in the presence (+) or absence (-) of NU7026. Blank space in **(A)** indicates that blot has been cropped. Data are representative of two or more experiments.

Chapter 4: Mechanisms of DDR initiation in activated macrophages

Classically activated macrophages produce reactive oxygen and nitrogen intermediates that enable the cell to damage or eliminate invading pathogens. Indeed, mice deficient in both the inducible nitric oxide synthase (iNOS) ($Nos2^{-/-}$) and a critical subunit of the NADPH oxidase, $gp91^{phox-/-}$, harbor massive abscesses containing enteric bacteria and are unable to kill virulent *L. monocytogenes* (Shiloh, MacMicking et al. 1999). Though important for the control of both commensal and exogenous bacteria, nitric oxide (NO) and reactive oxygen species (ROS) are highly genotoxic and have DNA damaging properties (Sawa and Ohshima 2006, Jena, Anand et al. 2012, Bogdan 2015). In addition, several reports suggest that metabolites of these agents may impair repair factors by chemically modifying key residues within these proteins (Finkel 2011, Bogdan 2015). Exposure to ROS can induce DNA DSBs, which activate ATM indirectly in a process that depends on the recruitment of ATM to the DSB site by the MRE11-RAD50-NBS1 (MRN) sensor (Lee and Paull 2004, Driessens, Versteyhe et al. 2009). However, ATM can also be directly activated in an MRN-independent fashion by the ROS-mediated modification of a specific cysteine residue, enabling ATM to form disulfide-cross-linked dimers (Guo, Kozlov et al. 2010). Thus, we hypothesize that reactive oxygen and nitrogen intermediates may initiate the DDR in activated macrophages. This DDR could be activated by direct oxidation of the DDR kinases (ATM and/or DNA-PKcs), or may be activated indirectly through the generation of DNA DSBs in macrophage genomic DNA.

4.1) A DDR is initiated by nitric oxide in activated macrophages.

To address whether reactive oxygen or nitrogen intermediates induce a DDR in activated macrophages, we activated BMDMs with LPS and IFN-γ in the presence of a cell-permeable

superoxide scavenger (MnTMPyP) or a specific chemical inhibitor of the inducible nitric oxide synthase (iNOS), aminoguanidine hemisulfate (AGHS). Treatment with AGHS, but not MnTMPyP, blocked γ-H2AX in activated BMDMs, suggesting that NO but not ROS activates the DDR in these cells (Figure 10A). In agreement with this, we found that LPS- and IFN-γ-treatment of iNOS-deficient BMDMs results in a near-complete abrogation of γ-H2AX formation as compared with wild type BMDMs (Figure 10B). In contrast, we observed a robust DDR in activated NADPH oxidase-deficient ($gp91^{phox-/-}$) BMDMs (Figure 10B). Taken together, we conclude that the induction of the DDR in LPS- and IFN-γ-activated macrophages depends on NO production.

Given that the DDR depends on NO in LPS- and IFN-γ-activated macrophages, we asked whether the induction of DDR in BMDMs exposed to *L. monocytogenes* requires the same genotoxic agent. We assayed BMDMs treated with heat-killed *L. monocytogenes* in the presence of both IFN-γ and AGHS, the specific chemical inhibitor of iNOS. As with LPS- and IFN-γ-treated BMDMs, we found that γ-H2AX formation in BMDMs activated with heat-killed *L. monocytogenes* and IFN-γ depends on NO production (Figure 11A). BMDMs infected with a LLO-deficient strain of *L. monocytogenes* (EJL1) in the presence of IFN-γ also required NO for the initiation of a robust DDR, as γ-H2AX formation was markedly reduced in activated iNOS-deficient ($Nos2^{-/-}$) BMDMs relative to wild type BMDMs (Figure 11B). Strikingly, γ-H2AX formation was not significantly reduced in $Nos2^{-/-}$ BMDMs infected with live *L. monocytogenes* unless the infected BMDMs were co-treated with IFN-γ (Figure 11C and B). Similar results were observed in AGHS-treated wild type BMDMs infected with wild type or LLO-deficient *L. monocytogenes* in the presence and absence of IFN-γ (data not shown). Notably, neither heat-

killed nor LLO-deficient *L. monocytogenes* are able to activate cytosolic signaling pathways (Stavru, Archambaud et al. 2011). Similarly, a number of reports suggest that IFN-γ promotes enhanced phagosome-lysosome fusion, thereby limiting the cytosolic escape of live *L. monocytogenes* (Portnoy, Schreiber et al. 1989, Alvarez-Dominguez and Stahl 1998, Via, Fratti et al. 1998). Taken together, these data suggest that though NO is able to activate a robust DDR in *L. monocytogenes*-infected macrophages, live *L. monocytogenes* can activate the DDR through an additional pathway or pathways. This activation may occur after the bacteria gains access to the host cytosol. Another non-mutually exclusive possibility is that *L. monocytogenes* activates the DDR through the production of various virulence factors.

4.2) The DDR is activated through DNA DSB intermediates.

In addition to ATM's role in the response to DNA DBSs, it is known that ROS can activate ATM directly, raising the possibility that NO may also modify key residues within ATM that lead to its DSB-independent activation (Guo, Kozlov et al. 2010). However, several lines of evidence indicate that the DDR in activated BMDMs occurs, at least in part, through the generation of DSBs in macrophage genomic DNA. γ-H2AX is generated in chromatin flanking DSBs and extends for kilobases, forming discrete nuclear foci that can be detected by immunostaining, whereas direct, DSB-independent activation of ATM will lead to diffuse, pan-nuclear γ-H2AX staining (Rogakou, Boon et al. 1999). We found that wild type BMDMs *monocytogenes* exhibited discrete γ-H2AX foci after infection with *L. monocytogenes*, suggesting that the DDR has been activated by DNA DSBs (Figure 12A). Notably, a higher percentage of *L. monocytogenes*-infected BMDMs harbored γ-H2AX foci as compared with

uninfected BMDMs (Figure 12B). In support of these findings, a previous study established that the direct activation of ATM by hydrogen peroxide leads to the activation of ATM targets p53 and CHK2 but does not lead to γ-H2AX formation (Guo, Kozlov et al. 2010).

We then hypothesized that a block in the repair of DNA DSBs should result in an amplified DDR if the response is in fact initiated by DSBs. A deficiency in DNA Ligase IV, which is required for DSB repair by non-homologous end joining (NHEJ), leads to late embryonic lethality. However, mice homozygous for a conditionally targeted DNA Ligase IV allele and heterozygous for the $LysM^{cre}$ allele ($LigIV:LysM^{cre/+}$) are viable and have BMDMs deficient in DNA Ligase IV. Thus, if the DDR is initiated by DSBs in activated BMDMs, we should observe amplified DDR signaling in $LigIV:LysM^{cre/+}$ BMDMs, as these cells are unable to repair DSBs by NHEJ (Figure 13A). Indeed, we find that *L. monocytogenes* infection of $LigIV:LysM^{cre/+}$ BMDMs leads to increased γ-H2AX formation relative to infected $LysM^{cre/+}$ controls (Figure 13B). Similarly, LPS- and IFN-γ-activated $LigIV:LysM^{cre/cre}$ BMDMs exhibit increased phosphorylation of KAP-1 (Figure 13C).

Finally, we reasoned that if the DDR in activated macrophages is initiated through DNA DSBs, it should depend on DSB sensing machinery. The activation of ATM by DSBs depends on the recruitment of ATM to the DSB by the MRE11, RAD50 and NBS1 (MRN) complex once it binds to the broken DNA end (Lee and Paull 2005). In contrast, the DSB-independent activation of ATM by ROS does not require the MRN complex (Guo, Kozlov et al. 2010). Indeed, BMDMs that express a hypomorphic Mre11 allele ($Mre11^{ATLD1/ATLD1}$) with severely

compromised MRN activity exhibit a near-complete block in γ-H2AX formation when DNA-PKcs kinase activity is inhibited with NU7026 (Figure 14A) (Theunissen, Kaplan et al. 2003). Additionally, KAP-1 phosphorylation, which depends on ATM in activated BMDMs, is significantly reduced in LPS- and IFN-γ- activated $Mre11^{ATLD1/ATLD1}$ macrophages (Figures 9B and 14B). Taken together, we conclude that DNA DSBs are generated in the genome of activated macrophages and that these DSBs initiate a DDR.

4.3) Sustained DDR requires type I interferon signaling.

In Chapter 3, we established that the activation of DDR in *L. monocytogenes*-infected BMDMs depends on type I interferon ($Ifnar1^{-/-}$) signaling (Figure 5C). This signaling is required for NO production, as $Ifnar1^{-/-}$ BMDMs do not produce NO after infection with *L. monocytogenes* (Figure 15A) (Farlik, Reutterer et al. 2010). Addition of IFN-γ to *L. monocytogenes*-infected $Ifnar1^{-/-}$ BMDMs restored NO production in these cells (Figure 15A). This was not unexpected, given that IFN response region of the murine *Nos2* promoter contains binding sites for STAT1 dimer (GAS) and interferon regulatory factors (IRF), both of which are assembled downstream of IFN-γ signaling (Xie, Whisnant et al. 1993, Kamijo, Harada et al. 1994, Meraz, White et al. 1996, Spink and Evans 1997). Though robust NO production was detected in IFN-γ-treated $Ifnar1^{-/-}$ BMDMs 24 hours after *L. monocytogenes* infection, the DDR was nearly undetectable, despite the fact that a DDR was observed in IFN-γ-treated $Ifnar1^{-/-}$ BMDMs 12 hours post-infection (Figure 15A and 15B and data not shown). Taken together, these data suggest that type I interferon signaling is required to maintain the DDR in *L. monocytogenes*-infected macrophages, and that this requirement extends beyond the role of type

I interferon signaling in promoting NO production. We conclude that in BMDMs, sustained

activation of the DDR depends on type I interferon signaling.

4.4) Type I interferon is required for optimal DDR activation in macrophages.

We find that an optimal DDR in *L. monocytogenes*-infected BMDMs depends on type I

interferon signaling even in the presence of the DNA damaging agent NO. Thus, we asked

whether the DDR is generally augmented by type I interferon signaling in macrophages. To

address this, we treated BMDMs and mouse embryonic fibroblasts (MEFs) with the

chemotherapeutic agent bleomycin, which induces DNA DSBs, in the presence or absence of

IFN-β. We found that treatment of MEFs with bleomycin elicited robust phosphorylation of

KAP-1 that was not further augmented by the addition of IFN-β (Figure 16). In contrast,

bleomycin treatment of BMDMs led to KAP-1 phosphorylation that was barely detectable unless

IFN-β was also administered (Figure 16). Given that type I interferon receptor signaling results

in the activation of several distinct transcription factors (Ivashkiv and Donlin 2014), we reasoned

that type I interferon may promote DDR in macrophages by altering the genetic program of the

cell. To address whether type I interferon signaling regulates the transcriptional profile of

macrophages that have been exposed to DNA damaging agents, we carried out gene expression

profiling in BMDMs and MEFs after bleomycin treatment in the presence or absence of IFN-β.

Notably, treatment of BMDMs with bleomycin did not alter the transcriptional profile of the cell,

whereas 69 genes were up-regulated by bleomycin in MEFs (Table 2). Addition of type I

interferon (IFN-β) to bleomycin-treated BMDMs induced the expression of more than 1000

genes; however IFN-β treatment alone also dramatically altered the transcriptional profile of

BMDMs (Table 2). Thus, we asked how many genes in BMDMs were induced after treatment with bleomycin and IFN-β but not IFN-β alone. Of the genes that were not regulated by IFN-β, we found that 148 were up-regulated ($\geq$ 2-fold) after treatment with both bleomycin and IFN-β. These findings suggest that bleomycin-induced DDR signaling synergizes with type I interferon signaling to activate a transcriptional program in BMDMs.

Previously, we found that MEFs exhibit IFN-independent DDR signaling in response to bleomycin, whereas the bleomycin-induced DDR in BMDMs depends on type I interferon (Figure 16). Thus, we hypothesized that a subset of the genes that are regulated by IFN-β in BMDMs but not in MEFs may have a role in promoting DDR in macrophages. Indeed, we found that a variety of the genes that are differentially up-regulated by IFN-β in BMDMs have a putative role in the DDR (Table 3). Notably, type I interferon regulates the gene expression of several factors that are involved in regulating cell cycle progression, such as the CDK inhibitor p21 and the myeloid differentiation factor Ifi204 (Table 3). ATM itself is also transcriptionally regulated by IFN-β in BMDMs, as is the nuclease Artemis (*Dclre1c*) and the H2AX deubiquitinase *Brcc3* (Table 3). Though IFN-β induces a transcriptional program in BMDMs that includes genes with established functions in the DDR, it is also possible that type I interferon signaling regulates DDR factors post-translationally. Taken together, we conclude that optimal induction of the DDR in macrophages depends on type I interferon signaling.

4.5) Summary and Discussion

Here we show that the genotoxic intermediate NO can initiate a robust DDR in activated macrophages, and that this DDR is initiated, at least in part, through the generation of DNA DSBs. Intriguingly, we find that the DDR is activated in macrophages that are deficient in NO production after infection with wild type *L. monocytogenes*, suggesting that live *Listeria* is capable of activating the DDR through an alternate pathway or pathways. Notably, the DDR depends on NO in IFN-γ–treated macrophages that are infected with phagosomally-confined (LLO-deficient) bacteria, suggesting that *L. monocytogenes* may activate the DDR after gaining access to the host cytosol. In support of this idea, the DDR also primarily depends on NO in *L. monocytogenes*-infected macrophages that have been treated with IFN-γ, which functions to limit the cytosolic escape of *L. monocytogenes* by promoting enhanced phagosome-lysosome fusion (Portnoy, Schreiber et al. 1989, Alvarez-Dominguez and Stahl 1998, Via, Fratti et al. 1998). Thus, it is tempting to speculate that IFN-γ inhibits the *L. monocytogenes*-mediated activation of DDR by preventing the bacteria from activating cytosolic signaling pathways. Another non-mutually exclusive possibility is that IFN-γ treatment may inhibit a *L. monocytogenes*-induced agent that can, in the absence of IFN-γ, activate the DDR.

We established that in the late phase of *L. moncytogenes* infection, a sustained DDR depends specifically on type I interferon receptor signaling. This is not due to a requirement for type I interferon to promote NO production, as addition of type II interferon results in robust NO production in *Ifnar1*$^{-/-}$ macrophages but not a DDR. Signaling through both the type I and type II interferon receptors results in the activation of the latent cytosolic transcription factor signal

73

transducer and activator of transcription 1 (STAT1). STAT1 homodimers translocate to the

nucleus, where they bind a consensus site known as a gamma-activated sequence (GAS)

(Ivashkiv and Donlin 2014). Given that both signaling pathways activate the transcription factor

STAT1, it is intriguing that type I interferon signaling is specifically required to sustain the DDR

in *L. monocytogenes*-infected macrophages. This could be due to the fact that signaling

downstream of the type I interferon also activates the transcription factor STAT2. STAT1-

STAT2 dimers translocate to the nucleus, where they assemble with interferon-regulatory factor

9 (IRF9) to form a complex known as IFN-stimulated gene factor 3 (ISGF3). ISGF3 specifically

binds to a consensus sequence known as interferon-stimulated response element (ISRE)

(Ivashkiv and Donlin 2014). Though some reports indicate that type II interferon signaling can

lead to a low-level of ISGF3 complex formation, ISGF3 is predominantly activated downstream

of type I interferon signaling (Schroder, Hertzog et al. 2004). Thus, it is possible that type I

interferon signaling regulates the expression of factors that are critical for an optimal DDR

through ISGF3-mediated transcription of IRSE-driven interferon-stimulated genes (ISGs).

Indeed we find that in macrophages, type I interferon induces the expression of several genes

with a putative role in the DDR. Whether there is a role for type I interferon signaling in the

post-translational modification of various DDR factors remains an open question worthy of

investigation.

A

B
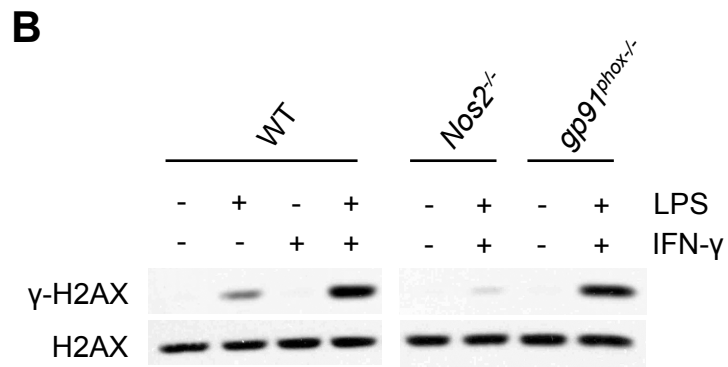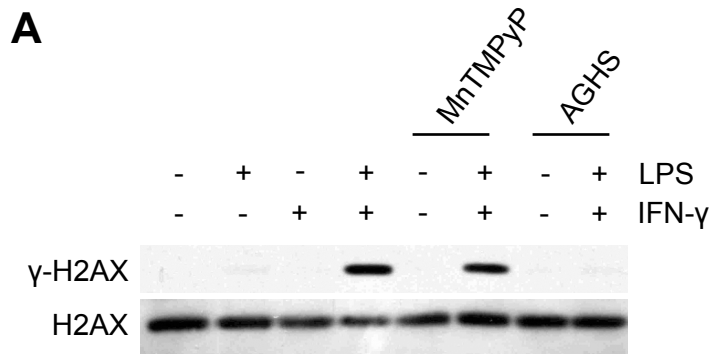
Figure 10. Nitric oxide activates the DDR in activated macrophages.

Figure 10 Legend. Nitric oxide activates the DDR in activated macrophages. Western blot

analysis of γ-H2AX and H2AX in whole cell lysates from **(A)** WT BMDMs after no stimulation

(-) or stimulation (+) with LPS, IFN-γ, or LPS + IFN-γ for 9 hrs. in the presence or absence of

MnTMPyP or AGHS. **(B)** WT, iNOS-deficient (*Nos2$^{-/-}$*), or NADPH oxidase-deficient (*gp91^{phox-}*

$^{/-}$) BMDMs after no stimulation (-) or stimulation (+) with LPS, IFN-γ, or LPS + IFN-γ for 9 hrs.

Blank space in **(B)** indicates that blot has been cropped. Data are representative of two

independent experiments.

A

B

C
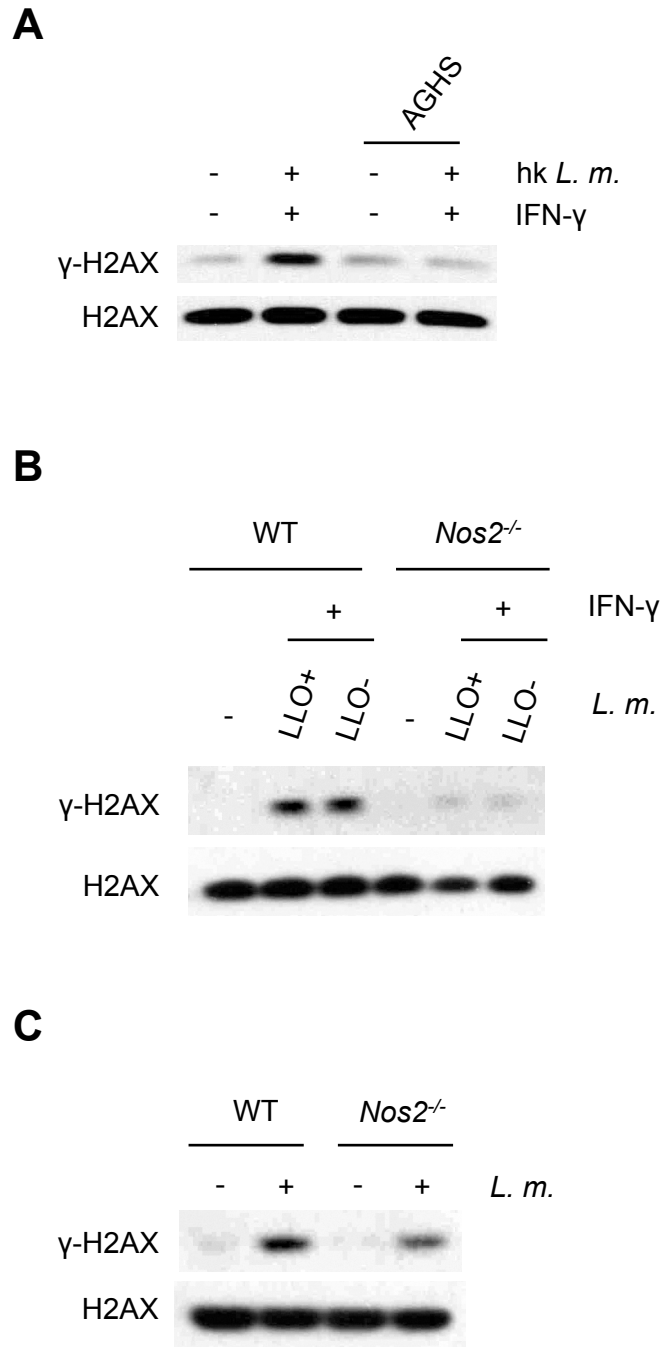
Figure 11. Nitric oxide can activate DDR in *L. monocytogenes*-infected macrophages.

Figure 11 Legend. Nitric oxide can activate DDR in *L. monocytogenes*-infected macrophages. Western blot analysis of γ-H2AX and H2AX in whole cell lysates from **(A)** WT BMDMs after no stimulation (-) or stimulation (+) with heat-killed *L. monocytogenes* (hk *L. m.*) + IFN-γ for 9 hrs. in the presence or absence of AGHS. **(B)** WT and *Nos2*$^{-/-}$ BMDMs after no infection (-) or infection (+) with *L. m.* for 9 hrs. **(C)** WT and *Nos2*$^{-/-}$ BMDMs after no infection (-) or infection (+) with WT (LLO+) or LLO-deficient (LLO-) *L. m.* in the presence (+) or absence (-) of IFN-γ for 9 hrs. Results are representative of two or more independent experiments.

A

γ–H2AX
LM
DAPI

B
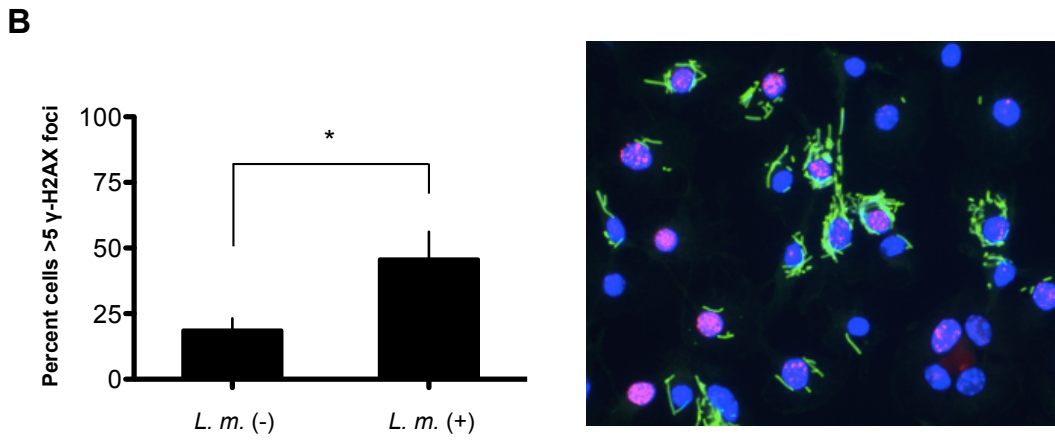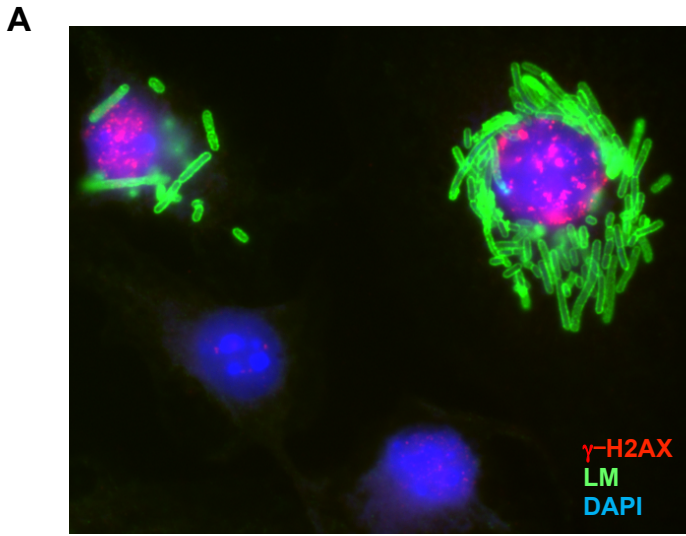
Figure 12. *L. monocytogenes* infection induces discrete γ-H2AX foci in macrophages.

Figure 12 Legend. *L. monocytogenes* infection induces discrete γ-H2AX foci in macrophages. (A) Immunofluorescence for *L. m.* (green) and γ-H2AX (red) in WT BMDMs 9 hrs. post-infection. Nuclei are revealed by DAPI (blue). **(B)** Quantitation of γ-H2AX foci in *L. m.*-positive (+) BMDMs and *L. m.*-negative (-) BMDMs 9 hrs. after infection (left). A representative cross-section of the immunofluorescence used for quantitation (right). Data are a compilation of 3 independent biological experiments and represent mean and standard deviation. *, $P = 0.0150$.

Anthony Tubbs contributed to this work.

A

B

$L.\ m.$

	-		+
	$LysM^{cre/+}$	$LigIV^{c/c};LysM^{cre/+}$	$LysM^{cre/+}$	$LigIV^{c/c};LysM^{cre/+}$

γ-H2AX

H2AX

C

	$LysM^{cre/cre}$		$LigIV^{c/c};LysM^{cre/cre}$	
	-	+	-	+	LPS + IFN-γ

p-KAP-1

KAP-1
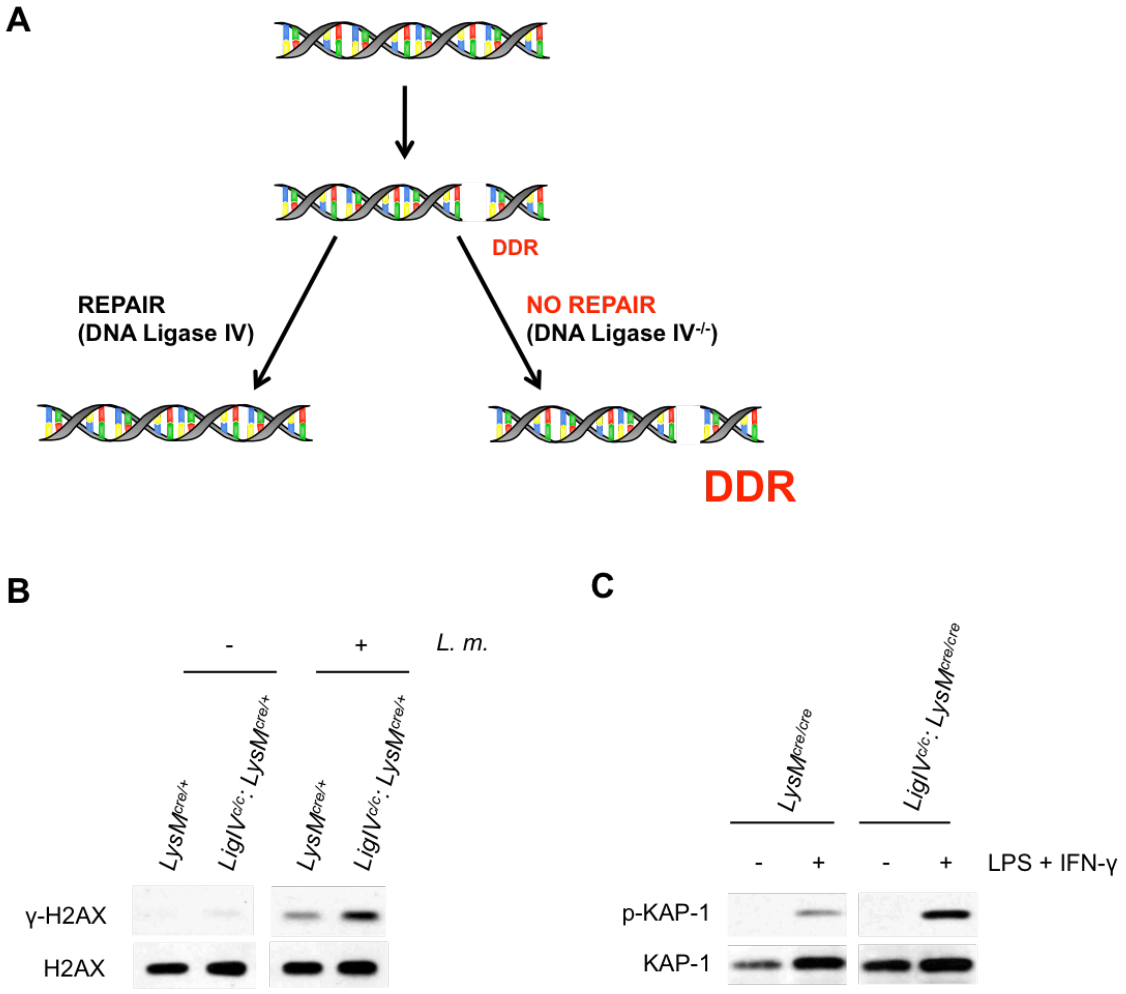
Figure 13. The DDR is amplified in DNA Ligase IV-deficient activated macrophages.

81

Figure 13 Legend. The DDR is amplified in DNA Ligase IV-deficient activated macrophages. **(A)** Schematic outlining the persistence of DDR signaling in the absence of DNA Ligase IV. **(B)** Western blot analysis of γ-H2AX and H2AX in whole cell lysates from *LysM^{cre/+}* and *LigIV^{c/c}:LysM^{cre/+}* BMDMs after no infection (-) or infection (+) with *L. m.* for 24 hrs. **(C)** Western blotting for p-KAP-1 and KAP-1 in *LysM^{cre/cre}* and *LigIV^{c/c}:LysM^{cre/cre}* BMDMs after no treatment (-) or treatment (+) with LPS + IFN-γ for 9 hrs. Blank spaces in **(B)** and **(C)** indicate that blots have been cropped. Data are representative of two or more independent experiments.
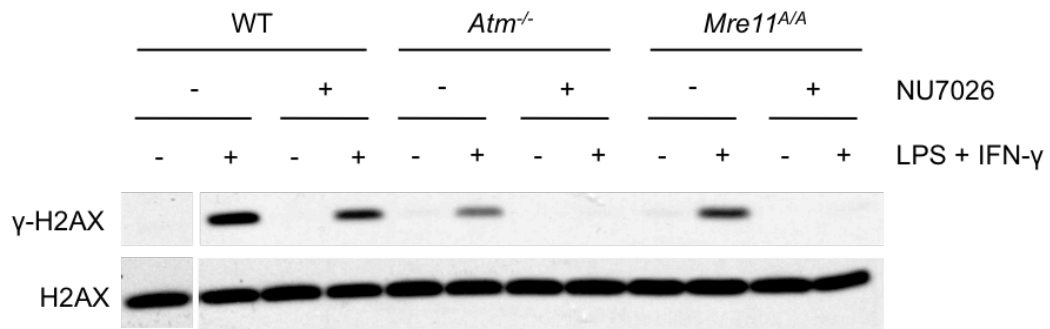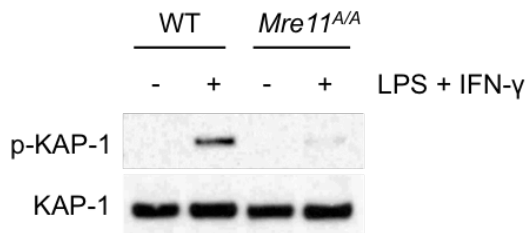
Figure 14. DDR initiation in activated macrophages depends on the MRN sensor.

Figure 14 Legend. DDR initiation in activated macrophages depends on the MRN sensor.

(A) Western blot analysis for γ-H2AX and H2AX in whole cell lysates from WT, $Atm^{-/-}$, and

$Mre11^{A/A}$ BMDMs after no treatment (-) or treatment (+) with LPS + IFN-γ in the presence (+) or

absence (-) of NU7026 for 9 hrs. **(B)** Western blotting for p-KAP-1 and KAP-1 in WT and

$Mre11^{A/A}$ BMDMs after no treatment (-) or treatment (+) with LPS + IFN-γ for 9 hrs. Blank

space in **(A)** indicates that blot has been cropped. Data are representative of two independent

experiments.
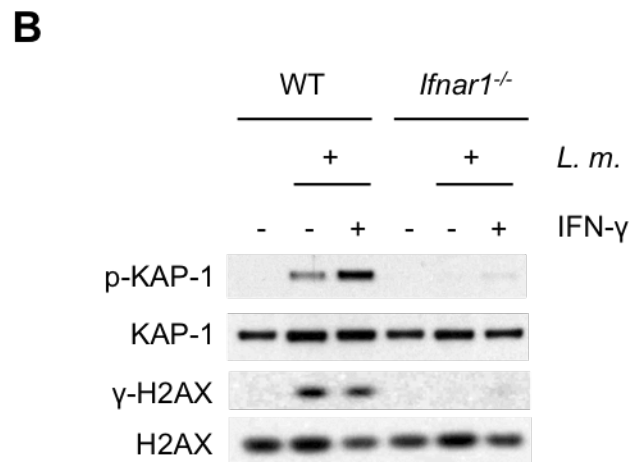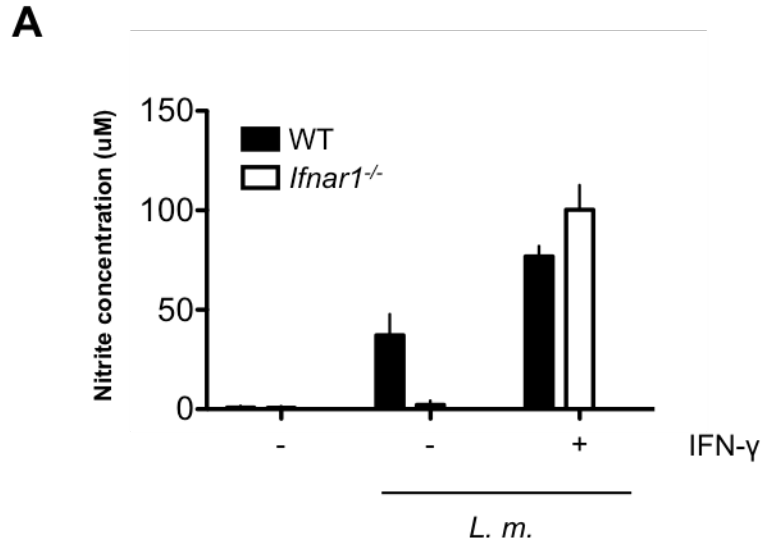
Figure 15. A sustained DDR depends on type I interferon signaling in *L. monocytogenes*-infected macrophages.

Figure 15 Legend. A sustained DDR depends on type I interferon signaling in *L. monocytogenes*-infected macrophages. (A) Nitrite concentration in culture supernatants collected from WT and *Ifnar1*$^{-/-}$ BMDMs after no infection (-) or infection (+) with *L. m.* for 24 hrs. Data are a compilation of three independent experiments and depict mean and standard deviation. **(B)** Western blot analysis of p-KAP-1, KAP-1, γ-H2AX, and H2AX in whole cell lysates from WT and *Ifnar1*$^{-/-}$ BMDMs after no infection (-) or infection (+) with *L. m.* for 24 hrs. Data are representative of three independent experiments.
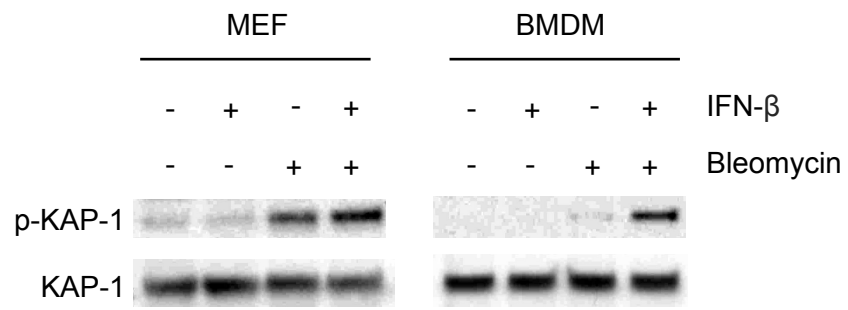
Figure 16. Optimal DDR depends on type I interferon in macrophages.

Figure 16 Legend. Optimal DDR depends on type I interferon in macrophages.   Western

blot analysis of WT mouse embryonic fibroblasts (MEFs) or BMDMs after no stimulation (-) or

stimulation (+) with bleomycin, IFN-β, or bleomycin + IFN-β for 6 hrs. Data are representative

of three independent experiments.

Cell type Treatment	BMDM	MEF
Bleomycin	0	69
IFN-β	1110	257
Bleomycin + IFN-β	1080	292

Table 2. Treatment with bleomycin and type I interferon regulates gene expression in macrophages and fibroblasts.

Table 2 Legend. Treatment with bleomycin and type I interferon regulates gene expression in macrophages and fibroblasts. Number of genes up-regulated ($\geq$ 2-fold) after treatment with bleomycin, IFN-β, or bleomycin + IFN-β for 8 hrs in BMDMs and MEFs.

Putzer Hung contributed to this work.

Gene Symbol	Function	BMDM		MEF	
		Exp 1	Exp 2	Exp 1	Exp 2
Brcc3	Deubiquitinase that recognizes K63-polyubiquitinated H2AX at DSBs, forms part of the BRCA1/2 complex	3.7	4.5	1.07	1.33
Dclre1c (Artemis)	Opens hairpin-sealed ends at RAG breaks, required for DSB repair in heterochromatin	3.9	4.4	ns	ns
Eya3	Dephosphorylates Y142 on H2AX, promoting recruitment of MDC1 to DSBs	2.8	2.6	0.90	1.45
Atm	PI3-like kinase	2.34	2.92	ns	ns
Ccnd1	Cyclin D1, binds to RAD51 and recruited to DSBs in a BRCA2-dependent fashion to promote HR	7.4	6.4	1.25	1.33
Ifi204	Inhibits cell cycle progression in response to DNA damage, implicated in myeloid differentiation	25	31	ns	ns
Cdkn1a (p21)	CDK inhibitor, G1/S checkpoint	3.5	3.4	0.97	0.94
Pttg1	Securin, proto-oncogene that inhibits NHEJ (possibly through its interaction with Ku70), down-regulated by p53 in response to DNA damaging agents	7.1	11	ns	ns
Nupr1	Transcription factor that promotes a "resistance program" in response to environmental stress (e.g. DNA damage), binds to MORF4L1 (HDAC)	4.1	4.6	1.10	1.25
Nono	May promote NHEJ by stabilizing free DNA ends	2.9	2.1	0.86	0.96

Table 3. Genes that are regulated by type I interferon in macrophages.

Table 3 Legend. Genes that are regulated by type I interferon in macrophages. A subset of genes with a putative role in the DDR that are regulated by IFN-β in BMDMs but not in MEFs. Fold-change over untreated is given for two biological replicates for each cell type. ns = not significant (probe did not meet intensity cut-off).

Putzer Hung contributed to this work.

Chapter 5: DDR activation impacts macrophage function in immune responses

In addition to canonical responses, the DDR can also regulate cellular processes unrelated to the repair of damaged DNA. Several studies have established that DNA DSBs and the DDR they elicit are important signaling intermediates that can impact cell-type-specific processes. DSBs introduced by the RAG endonuclease in pre-B cells rearranging their antigen receptor loci activate a broad transcriptional program in these cells, a significant part of which depends on ATM. Notably, a cohort of the ATM-regulated genetic program involves genes involved in pre-B cell development, survival, and migration (Bredemeyer, Helmink et al. 2008, Bednarski, Nickless et al. 2012). RAG activity in lymphocyte and NK progenitors can also influence mature T and NK cell function, including their survival following virus-driven proliferative bursts. These capabilities are presumably endowed through the generation of DSBs in the progenitors themselves (Karo, Schatz et al. 2014). Finally, AID-induced DNA DSBs in mature B cells activate an ATM-dependent transcriptional program that impacts plasma cell differentiation in germinal centers (Sherman, Kuraishy et al. 2010). Notably, many of the ATM-dependent gene expression changes elicited by RAG breaks in developing pre-B cells also occur in lymphocytes treated with genotoxic DNA damaging agents such as ionizing radiation (Bredemeyer, Helmink et al. 2008, Innes, Hesse et al. 2013). As we have established that genotoxins can induce an ATM- and DNA-PKcs-dependent DDR in macrophages, we asked whether this DDR impacts the transcriptional profile of *L. monocytogenes*-infected macrophages. We also assessed the effect of DDR-deficiency on macrophage innate immune responses that are not generally regulated at the level of transcription, such as the processing and release of pro-inflammatory cytokines IL-1β and IL-18 by the inflammasome. Taken together, we establish *L. monocytogenes*-elicited DSBs and the resulting DDR as important signaling intermediates that impact macrophage function in the innate immune response to bacterial infection.

5.1) DDR regulates the genetic program of activated macrophages.

To address whether the DDR regulates the genetic program that is activated in BMDMs after exposure to *L. monocytogenes,* we compared the gene expression profiles of ATM- and DNA-PKcs-deficient (*Scid:Atm^{c/c}:LysM^{cre/+}*) and wild type (*LysM^{cre/+}*) BMDMs with and without *L. monocytogenes* infection (Figure 17). There were 435 genes that exhibited a $\geq$ 5-fold induction in expression in wild type (*LysM^{cre/+}*) BMDMs after infection with *L. monocytogenes* (Figure 17). Of these, 138 exhibited a $\geq$ 1.5-fold reduction in expression in ATM- and DNA-PKcs-deficient (*Scid:Atm^{c/c}:LysM^{cre/+}*) as compared with wild type (*LysM^{cre/+}*) BMDMs (Figure 17). Thus, approximately one-third of the genes that are up-regulated by 5-fold or more during *L. monocytogenes* infection are positively regulated by ATM and/or DNA-PKcs, as the expression of these genes is diminished in the absence of one or both kinases. These genes include many with an established role in the immune response to infection, including a variety of chemokines, cytokines, and cell surface molecules (Figure 17).

We validated a number of the gene expression changes with known relevance to macrophage immune function. Expression of the gene encoding the class A scavenger receptor MARCO, which has a role in the phagocytosis of bacteria, exhibits a strong dependence on DDR signaling (Figure 18A) (Kraal, van der Laan et al. 2000). Optimal gene expression of CD69, which functions in cell localization and migration, also depends on ATM and DNA-PKcs (Figure 18A) (Schwab and Cyster 2007). Notably, the DDR regulates the gene expression of a variety of chemokines that are key mediators of leukocyte migration during acute inflammation (Kuziel, Morgan et al. 1997, Griffith, Sokol et al. 2014). The gene expression of CXCL1, which

regulates neutrophil migration, is reduced in *Scid:Atm^{c/c}:LysM^{cre/+}* relative to *LysM^{cre/+}* controls (Figure 18B) (Griffith, Sokol et al. 2014). CXCL10 is DDR-regulated at the level of gene expression, as are CCL2, CCL3 (MIP-1α), and CCL4 (MIP-1β), all of which are secreted after exposure to bacterial endotoxins and function to recruit immune cells such as monocytes, macrophages, and NK cells to the site of infection (Figure 18B) (Griffith, Sokol et al. 2014). Expression of *Ccl5*, which encodes the chemokine RANTES, is DDR-independent, suggesting that the transcriptional regulation imposed by ATM and/or DNA-PKcs is limited to a subset of chemokine genes (Figure 18B). Thus, DDR-deficient BMDMs do not exhibit a global defect in chemokine gene expression after *L. monocytogenes* infection. Taken together, we conclude that the DDR influences the transcriptional profile of macrophages infected with *L. monocytogenes*.

Previous analyses revealed that ATM and DNA-PKcs have distinct functions in regulating DNA damage responses in activated macrophages (Chapter 3). Thus, we asked whether the two kinases also have distinct roles in regulating the genetic program that is induced upon infection with *L. monocytogenes*. The analysis of BMDMs deficient in ATM (*Atm^{-/-}*) and DNA-PKcs (*Scid*) revealed that ATM and DNA-PKcs compensate for one another in the regulation of some genes, such as *Ccl2* (Figure 19A). However, the expression of *Marco* and *Cxcl10* depends on both ATM and DNA-PKcs, as expression of both genes is reduced in *L. monocytogenes*-infected *Atm^{-/-}* and *Scid* BMDMs relative to wild type controls (Figure 19B). Thus, after the induction of a DDR by DNA DSBs, ATM and DNA-PKcs both have a role in the regulation of the *L. monocytogenes*-induced genetic program in macrophages.

5.2) DDR regulates IL-1β production in activated macrophages.

During infection with viral and bacterial pathogens, macrophages produce a variety of inflammatory cytokines and chemokines, the majority of which are induced transcriptionally upon macrophage activation. However, two key inflammatory cytokines, IL-1β and IL-18, are regulated post-translationally, as they are converted to their biologically active forms by a multi-protein complex known as the inflammasome. This process is initiated when cytosolic sensors of bacterial products complex with the adaptor ASC, which binds to the inactive protease pro-caspase 1(Lamkanfi 2011, von Moltke, Ayres et al. 2013, Lamkanfi and Dixit 2014). Inflammasome activation results in the cleavage of pro-caspase 1 to active caspase 1, which then cleaves and activates pro-IL-1β and pro-IL-18 (Lamkanfi 2011, von Moltke, Ayres et al. 2013, Lamkanfi and Dixit 2014).

As previously discussed, *L. monocytogenes* activates a variety of signaling pathways when it escapes phagosomal vesicles and enters the host cytoplasm. Bacteriolysis in the cytosol is thought to activate absent-in melanoma 2 (AIM2), a cytosolic receptor that mediates activation of the inflammasome (Sauer, Witte et al. 2010, Warren, Armstrong et al. 2010, Witte, Archer et al. 2012). A number of reports have also implicated the cytosolic sensor NLRP3, which recognizes a wide range of endogenous and bacterial ligands, in *L. monocytogenes*-elicited inflammasome activation (Mariathasan, Weiss et al. 2006, Kim, Bauernfeind et al. 2010, Wu, Fernandes-Alnemri et al. 2010). Strikingly, we find that ATM- and DNA-PKcs-deficient (*Scid:Atm^{c/c}:LysM^{cre/+}*) BMDMs produce significantly less IL-1β than wild type (*LysM^{cre/+}*) BMDMs after infection with *L. monocytogenes* (Figure 20A). This is not due to an inability of

ATM- and DNA-PKcs- deficient (*Scid:Atm^{c/c}:LysM^{cre/+}*) BMDMs to promote pro-IL-1β gene

expression, as *Il1b* transcripts are equivalent in wild type (*LysM^{cre/+}*) and ATM- and DNA-PKcs-

deficient (*Scid:Atm^{c/c}:LysM^{cre/+}*) BMDMs (Figure 20B). Indeed, ATM and DNA-PKcs are also

not required to promote the gene expression of inflammasome components pro-caspase 1, ASC,

NLRP3, or AIM2 (Figure 21A-D). Active caspase 1 can promote cell death by pyroptosis, an

event thought to be mediated by AIM2 upon infrequent bacteriolysis in the host cytosol

(Bergsbaken, Fink et al. 2009, Miao, Leaf et al. 2010, Sauer, Witte et al. 2010). However, the

observed differences in IL-1β production between the two genotypes are not due to differences in

cell viability, as ATM- and DNA-PKcs-deficient (*Scid:Atm^{c/c}:LysM^{cre/+}*) and wild type

(*LysM^{cre/+}*) BMDMs exhibit similar LDH release after *L. monocytogenes* infection (Figure 22).

Rather, after infection with *L. monocytogenes*, ATM- and DNA-PKcs-deficient

(*Scid:Atm^{c/c}:LysM^{cre/+}*) BMDMs produce nearly undetectable levels of active caspase 1 as

compared with wild type (*LysM^{cre/+}*) BMDMs, whereas expression levels of pro-caspase 1 and

AIM2 are equivalent between the two genotypes (Figure 23A and B). These findings indicate

that in *L. monocytogenes*-infected BMDMs, the DDR is required for efficient conversion of pro-

caspase 1 to its active form. Notably, conversion of pro-caspase 1 to active caspase 1 is not

impaired in ATM- and DNA-PKcs-deficient (*Scid:Atm^{c/c}:LysM^{cre/+}*) BMDMs in response to all

inflammasome-activating stimuli. When LPS-primed BMDMs are treated with ATP, robust

inflammasome activation and IL-1β release occurs. We find that ATM- and DNA-PKcs-

deficient (*Scid:Atm^{c/c}:LysM^{cre/+}*) and wild type (*LysM^{cre/+}*) BMDMs produce equivalent amounts

of IL-1β after LPS- and ATP-treatment (Figure 24A). Consistent with this finding, we observe

no defect in the conversion of pro-caspase 1 to its active form in ATM- and DNA-PKcs-deficient

(*Scid:Atm^{c/c}:LysM^{cre/+}*) BMDMs after treatment with LPS- and ATP (Figure 24B). Taken

together, we conclude that ATM- and DNA-PKcs-deficient macrophages have a defect in IL-1β production after exposure to *L. monocytogenes*, and this defect is due to an inability of these cells to efficiently convert pro-caspase 1 to active caspase 1.

5.3) ATM and DNA-PKcs have distinct roles in cytokine production.

Given that both ATM and DNA-PKcs are activated in *L. monocytogenes*-infected BMDMs and both kinases have a role in regulating the expression of a variety of genes, we asked whether both ATM and DNA-PKcs were similarly required to promote IL-1β production in *L. monocytogenes*-infected BMDMs. Indeed, we find that ATM-deficient (*Atm*$^{-/-}$) and DNA-PKcs-deficient (*Scid)* BMDMs each have a defect in IL-1β production after infection with *L. monocytogenes* (Figure 25A). Inflammasome activation also leads to the processing and release of related cytokine IL-18 (von Moltke, Ayres et al. 2013, Lamkanfi and Dixit 2014). Strikingly, IL-18 production in *L. monocytogenes*-infected BMDMs requires DNA-PKcs but not ATM, as *L. monocytogenes*-infected *Atm*$^{-/-}$ BMDMs produce IL-18 at a slightly elevated level relative to wild type BMDMs (Figure 25B). This is not due to a requirement for DNA-PKcs to promote pro-IL-18 gene expression, as *Il18* transcripts are equivalent in wild type and *Scid* BMDMs post-infection with *L. monocytogenes* (Figure 25C). Taken together, we conclude that while both ATM and DNA-PKcs regulate IL-1β production in *L. monocytogenes*-infected BMDMs, IL-18 production is regulated by DNA-PKcs.

5.4) DNA-PKcs promotes IFN-γ production by NK cells.

IL-12 and IL-18 are key cytokines that stimulate both T cells and NK cells to produce IFN-γ early in immune responses (Hsieh, Macatonia et al. 1993, Okamura, Tsutsi et al. 1995, Akira 2000). IFN-γ production by NK cells is known to be critical for the activation of macrophages during the response to *L. monocytogenes* infection in mice lacking B and T lymphocytes (Bancroft, Schreiber et al. 1991). Given that DNA-PKcs-deficient (*Scid*) BMDMs have a defect in IL-18 production after infection with *L. monocytogenes*, we asked whether these cells are able to effectively activate naïve NK cells to produce IFN-γ. DNA-PKcs-deficient (*Scid*) and wild type BMDMs, either uninfected or infected with *L. monocytogenes*, were cultured with purified splenic NK cells, and the percentage of NK cells producing IFN-γ was assessed by flow cytometry (Figure 26A). Wild type and DNA-PKcs-deficient (*Scid*) BMDMs exhibited equivalent induction of IL-12 p40 gene expression upon infection with *L. monocytogenes* (Figure 26B). Moreover, the addition of exogenous IL-18 to wild type and *Scid* BMDM-wild type NK co-cultures led to equivalent percentages of IFN-γ-producing NK cells (Figure 27A). Taken together, we conclude that in *L. monocytogenes*-infected macrophages, DNA-PKcs signaling is required for optimal IFN-γ production in NK cells, due to the role of DNA-PKcs in promoting IL-18 production by macrophages.

5.5) Summary and Discussion

Here we show that the DDR regulates the genetic program induced in macrophages after infection with *L. monocytogenes*, including the expression of a number of chemokines. NF-κB is a critical transcription factor that regulates chemokine gene expression downstream of pattern

recognition receptors as well as TNF and IL-1 receptors (Amiri and Richmond 2003). DNA

damage can also lead to the ATM-dependent activation of NF-κB (Huang, Wuerzberger-Davis et

al. 2003, Wu, Shi et al. 2006). Thus, it is possible that the DDR in activated macrophages

regulates chemokine gene expression by augmenting NF-κB activation. Many chemokines are

transcriptionally regulated by NF-κB in combination with other factors. For example, *Cxcl1*

expression is regulated by NF-κB and poly(ADP-ribose) polymerase (PARP)-1, which is

activated by DNA damage and has a broad range of functions in the DDR (Nirodi, NagDas et al.

2001, Amiri and Richmond 2003, Rouleau, Patel et al. 2010). Thus, in activated macrophages,

the initiation of a DDR may regulate gene expression through multiple pathways. Intriguingly,

ATM and DNA-PKcs are not able to compensate for one another in the regulation of a number

of genes, suggesting that they may activate distinct signaling pathways leading to the

transcription of these genes. Another non-mutually exclusive possibility is that in some cases,

they are both required for optimal activation of the same pathway.

RAG-induced DSBs regulate the expression of a number of genes that have established

functions in lymphocyte homing and migration, such as CD62L, SWAP70, and CD69

(Bredemeyer, Helmink et al. 2008). Notably, CD69 expression is also regulated by the DDR in

L. monocytogenes-infected macrophages, suggesting that in both lymphoid and myeloid cells,

context-specific DSBs activate a DDR that modulates the trafficking of immune cells. CD69

down-regulates the expression of the receptor for sphingosine 1 phosphate (S1P), limiting the

egress of immune cells from a particular niche (Schwab and Cyster 2007). Thus, it is tempting

to speculate that in macrophages, the DDR may promote the retention of macrophages in

infectious foci by maintaining the expression of CD69.

The mechanism by which the DDR regulates inflammasome function in *L. monocytogenes*-infected macrophages remains unclear. We established that DDR signaling is required for efficient conversion of pro-caspase 1 to active caspase 1 during infection with *L. monocytogenes* but not in response to LPS and ATP, suggesting that there is not a cell-intrinsic requirement of the DDR in the activation of caspase 1. Thus, is it possible that the DDR regulates the sensing of bacterial ligands upstream of caspase 1 activation. Though we have ruled out a role for the DDR in the transcriptional regulation of key inflammasome components (AIM2, NLRP3, ASC, and Caspase 1), it is possible that the DDR kinases (ATM and/or DNA-PKcs) regulate one of the inflammasome components through phosphorylation at SQ/TQ motifs. Indeed, ATM in particular is known to phosphorylate hundreds of proteins in response to DNA damage at these motifs specifically (Matsuoka, Ballif et al. 2007).

It is not yet clear whether both ATM and DNA-PKcs are required for optimal processing of caspase 1 in *L. monocytogenes* infection. The requirement for DNA-PKcs and not ATM in the production of IL-18 suggests that ATM is dispensable for the activation and processing of pro-caspase-1, as IL-18 production is unaffected by a deficiency in ATM. The kinase requirements for this conversion and the mechanism by which it occurs will be areas of further exploration.
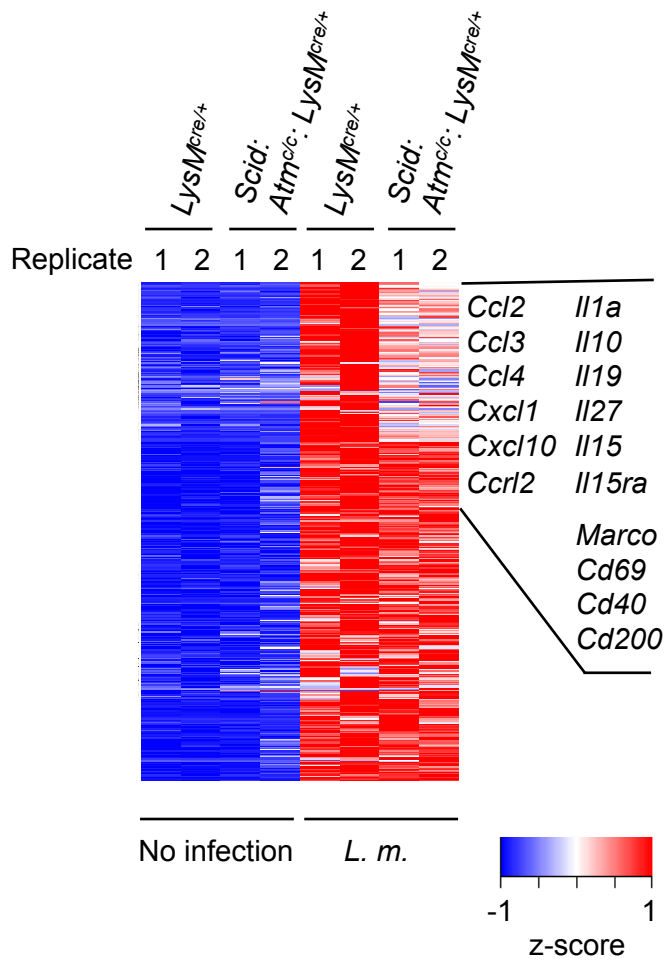
Figure 17. **DDR influences the transcriptional profile of *L. monocytogenes*-infected macrophages.**

Figure 17 Legend. DDR influences the transcriptional profile of *L. monocytogenes*-infected macrophages. Heat map showing genes that are up-regulated ≥ 5-fold in *L. m.*-infected *LysM^{cre/+}* BMDMs relative to no infection. Expression of the same set of genes in *L. m.*-infected *Scid:Atm^{c/c}:LysM^{cre/+}* BMDMs is indicated. Red indicates relatively higher expression; blue indicates relatively lower expression. Genes with decreased expression (≥ 1.5-fold) in *L. m.*-infected *Scid:Atm^{c/c}:LysM^{cre/+}* relative to *L. m.*-infected *LysM^{cre/+}* controls are clustered in the upper right corner of the heat map. Some of the genes in this group are indicated. Shown are two biological replicates (1 and 2) for each condition and genotype.
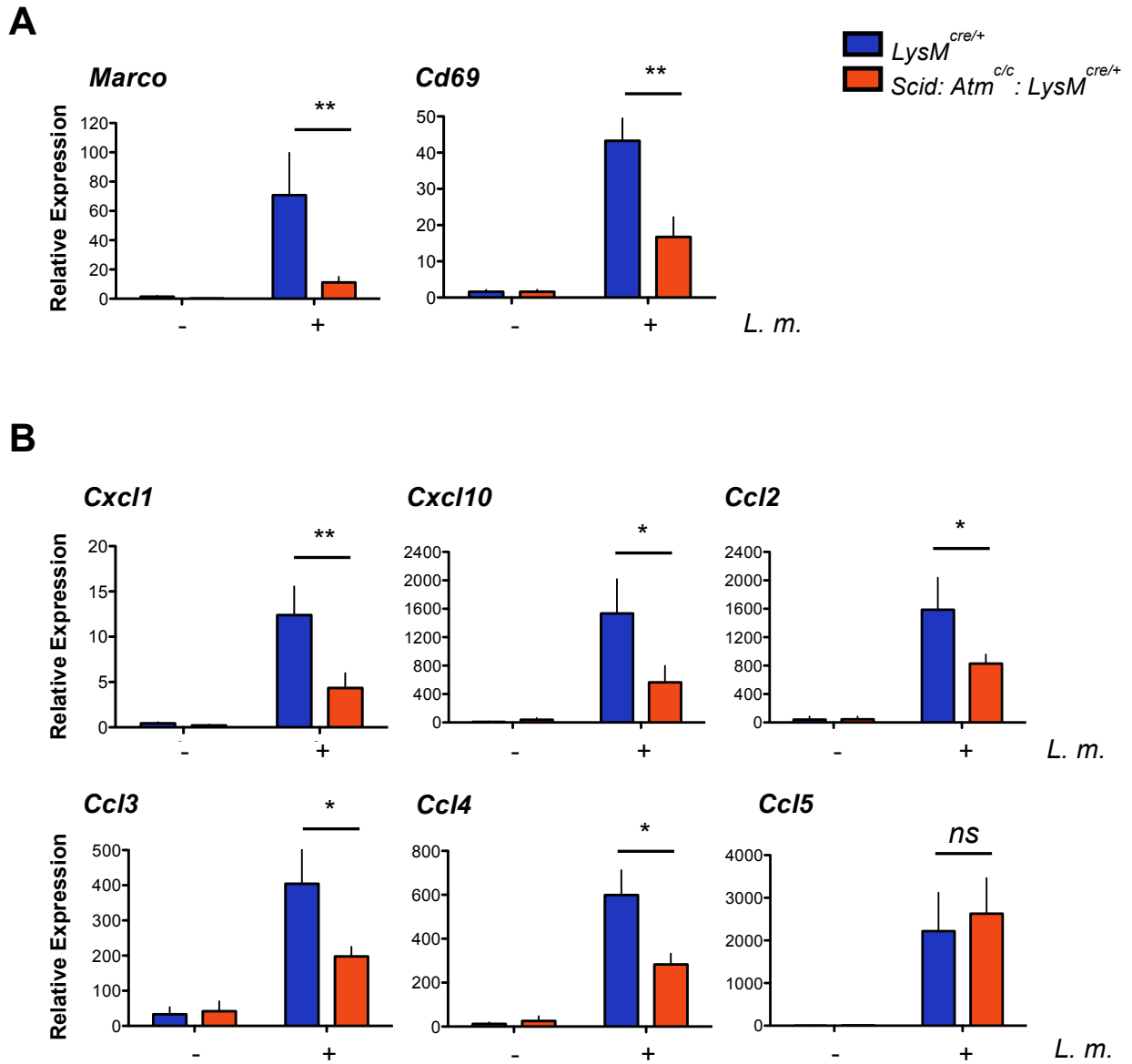
Figure 18. The DDR regulates the expression of immune-related genes.

Figure 18 Legend. The DDR regulates the expression of immune-related genes. (A,B)

Quantitative real-time PCR (RT-PCR) analysis of gene expression in *Scid:Atm^{c/c}:LysM^{cre/+}* and

LysM^{cre/+} BMDMs after no infection (-) or infection (+) with *L. m.* for 24 hrs. Data are a

compilation of three or more independent experiments and depict the mean and standard

deviation. *, $P < 0.05$, **, $P < 0.01$, *ns* = not significant.
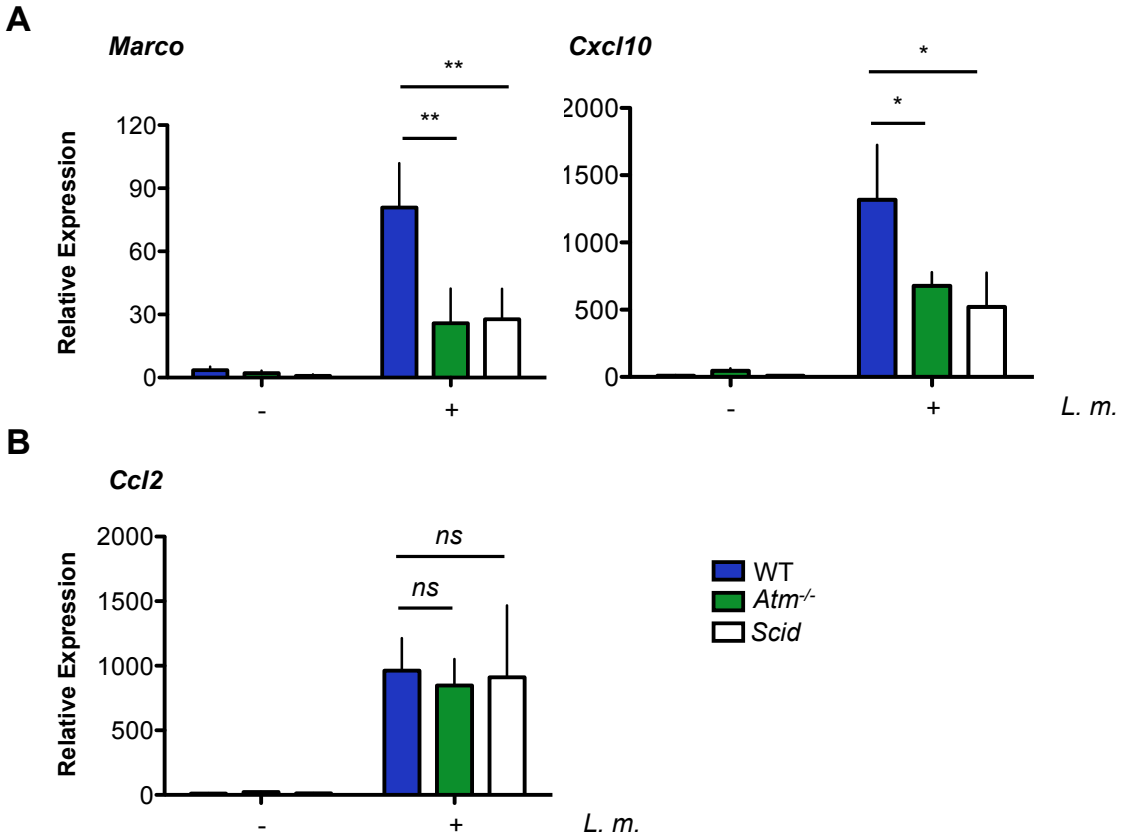
Figure 19. ATM and DNA-PKcs have distinct roles in the regulation of a subset of *L. monocytogenes*-induced genes.

Figure 19 Legend. ATM and DNA-PKcs have distinct roles in the regulation of a subset of

***L. monocytogenes*-induced genes. (A,B)** Quantitative real-time PCR (RT-PCR) of gene

expression in WT, *Atm*$^{-/-}$, and *Scid* BMDMs after no infection (-) or infection (+) with *L. m.* for

24 hrs. Data are a compilation of three or more independent experiments and represent the mean

and standard deviation. *, *P* < 0.05, **, *P* < 0.01, *ns* = not significant.
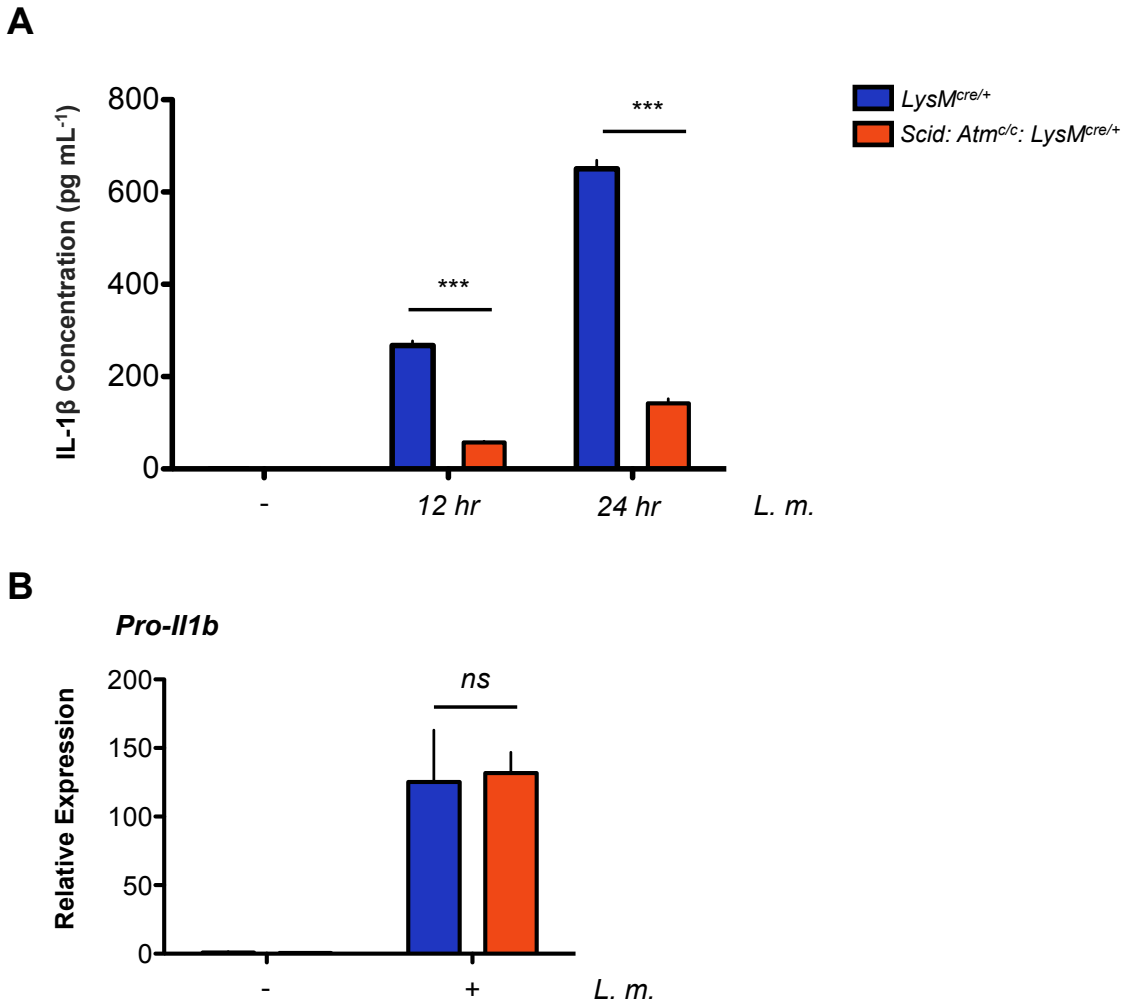
Figure 20. The DDR regulates IL-1β production in *L. monocytogenes*-infected macrophages.

Figure 20 Legend. The DDR regulates IL-1β production in *L. monocytogenes*-infected

macrophages. (A) IL-1β protein concentration as determined by ELISA in supernatants from

uninfected (-) and *L. m.*-infected *Scid:Atm^{c/c}:LysM^{cre/+}* and *LysM^{cre/+}* BMDMs 12 and 24 hrs.

post-infection. **(B)** Quantitative RT-PCR analysis of *Il1b* expression in *Scid:Atm^{c/c}:LysM^{cre/+}* and

LysM^{cre/+} BMDMs after no infection (-) or infection (+) with *L. m.* Data are representative of

four independent experiments **(A)** or a compilation of three independent experiments **(B)** and

depict mean and standard deviation. ***, *P* < 0.0001, *ns* = not significant.
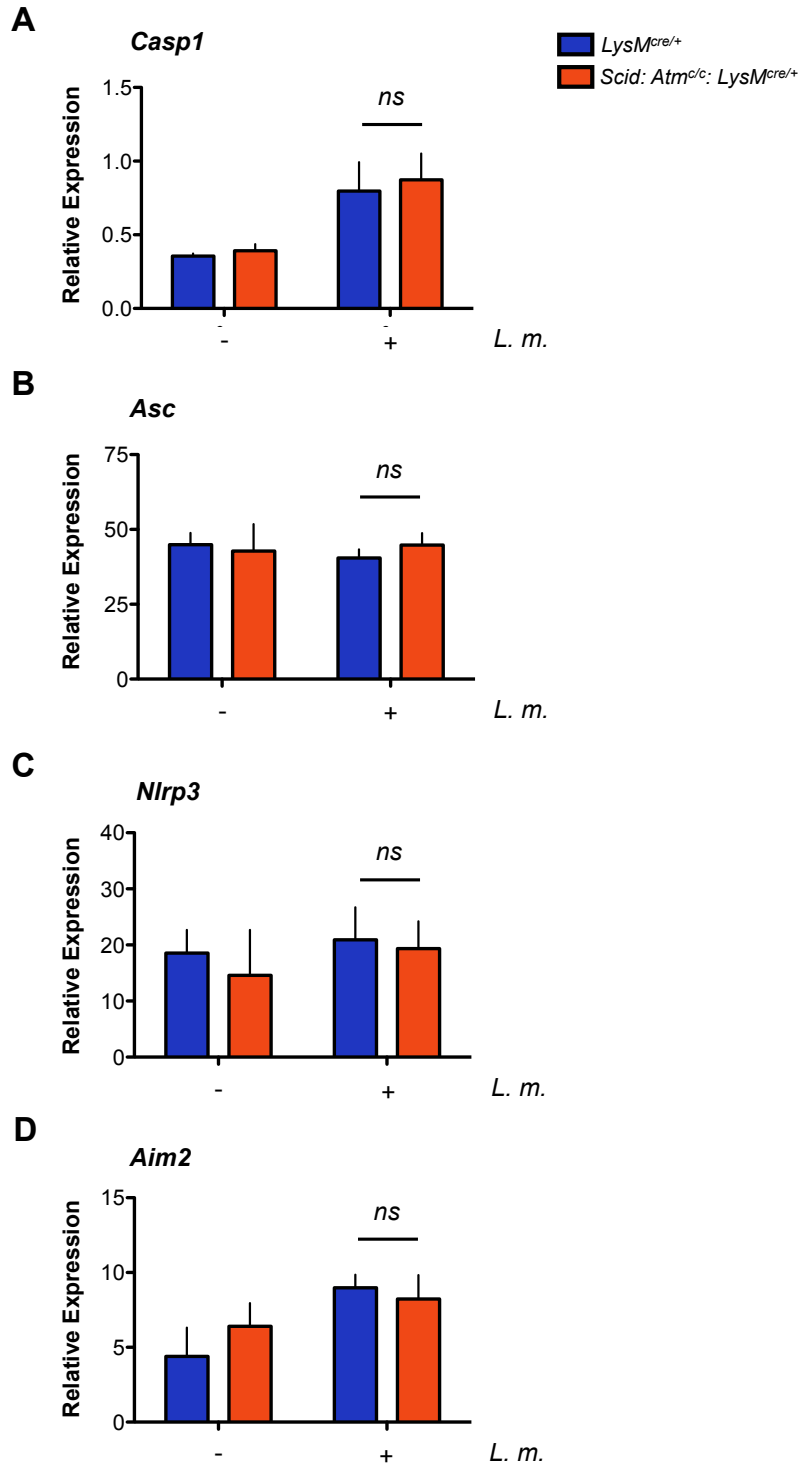
Figure 21. The DDR does not regulate the gene expression of inflammasome components.

Figure 21 Legend. The DDR does not regulate the gene expression of inflammasome components. (A-C) Quantitative RT-PCR analysis of *Casp1*, *Asc*, *Nlrp3*, and *Aim2* gene in *Scid:Atm^{c/c}:LysM^{cre/+}* and *LysM^{cre/+}* BMDMs after no infection (-) or infection (+) with *L. m.* Data are a compilation of three independent experiments and depict mean and standard deviation. *ns* = not significant.
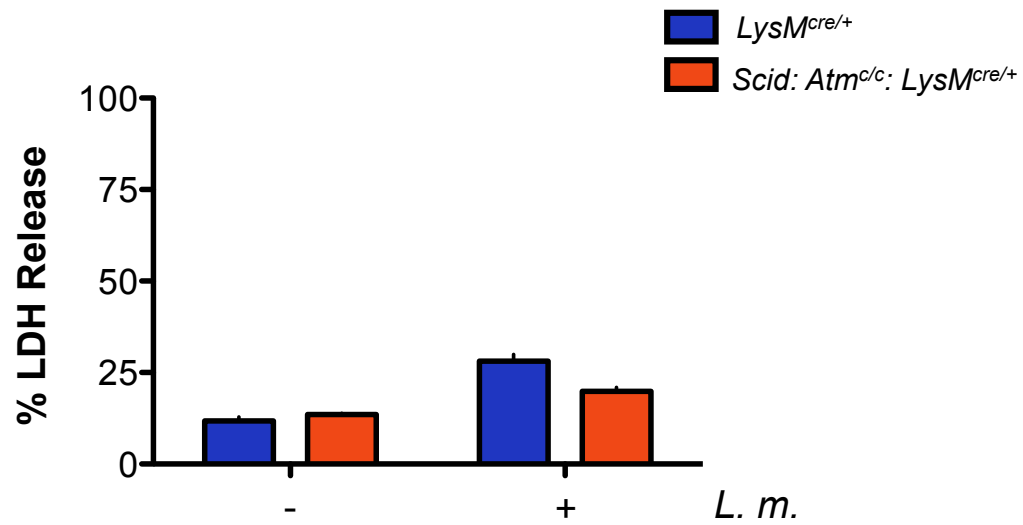
Figure 22. Deficiency in DDR does not affect cell viability in *L. monocytogenes*-infected macrophages.

Figure 22 Legend. Deficiency in DDR does not affect cell viability in *L. monocytogenes*-infected macrophages.   Lactate dehydrogenase (LDH) activity of cell-free supernatants collected from BMDMs 24 hrs. post-infection with *L. m.* Data for *Scid:Atm^{c/c}:LysM^{cre/+}* and *LysM^{cre/+}* are given as a percentage of the LDH released by equivalent cell numbers of each genotype lysed with 1% Triton X-100. Data are representative of three independent experiments and depict the mean and standard deviation.

A

B
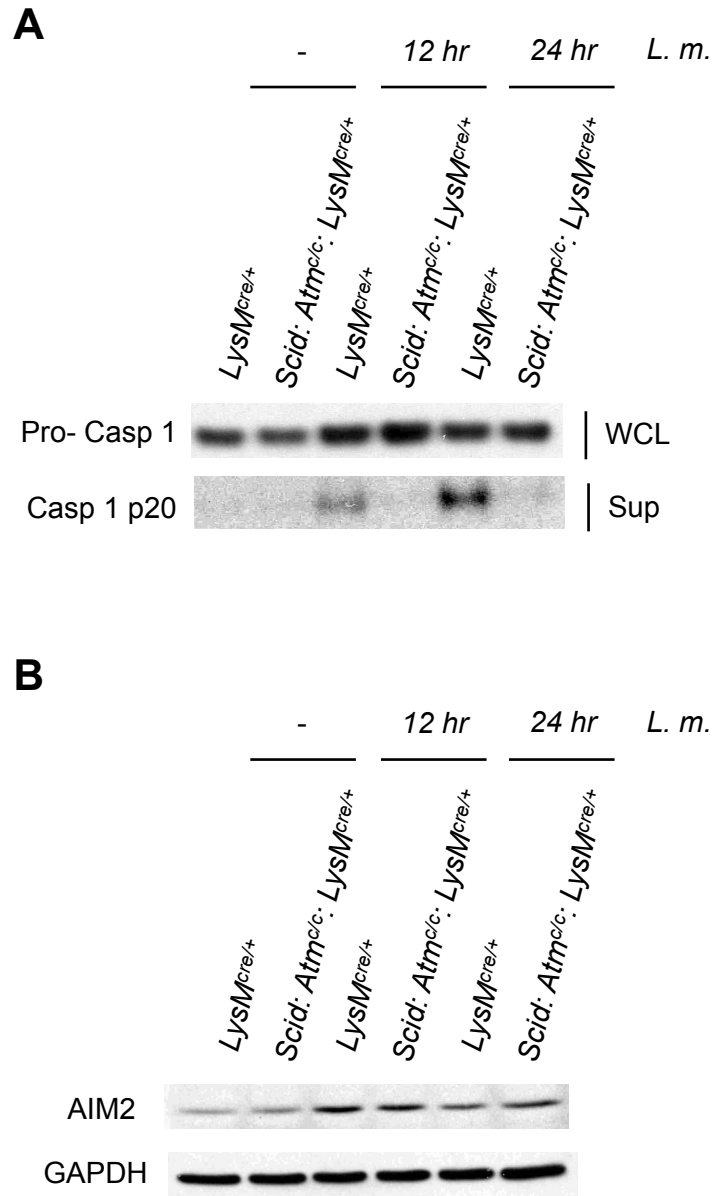
Figure 23. Conversion of pro-caspase 1 to active caspase-1 is impaired in DDR-deficient _L. monocytogenes_-infected macrophages.

Figure 23 Legend. Conversion of pro-caspase 1 to active caspase-1 is impaired in DDR-deficient *L. monocytogenes*-infected macrophages. Western blotting analysis of **(A)** pro-caspase 1 in whole cell lysates (WCL) and active caspase 1 (p20) in supernatants (Sup) and **(B)** AIM2 in whole cell lysates from *LysM$^{cre/+}$* and *Scid:Atm$^{c/c}$:LysM$^{cre/+}$* BMDMs 12 and 24 hrs. post-*L. m.* infection. GAPDH in **(B)** serves as a protein loading control. Data are representative of two independent experiments.
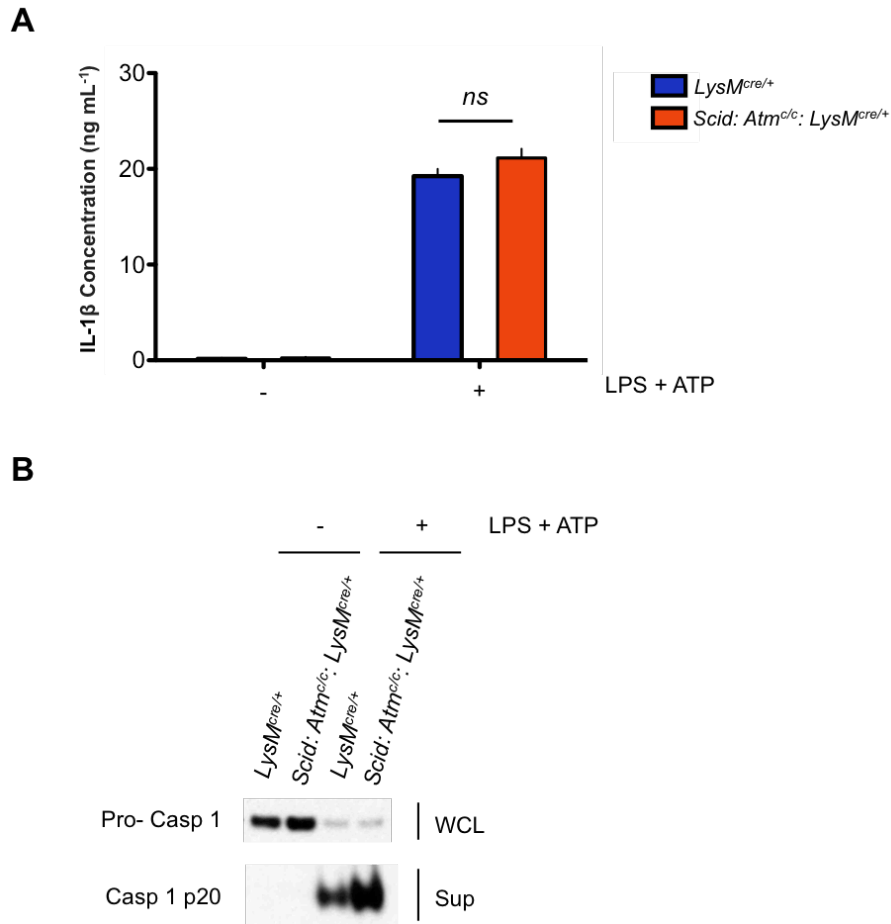
Figure 24. ATM- and DNAPKcs-deficiency does not impact inflammasome function in LPS- and ATP-treated macrophages.

Figure 24 Legend. ATM- and DNAPKcs-deficiency does not impact inflammasome function in LPS- and ATP-treated macrophages.   **(A)** IL-1β protein concentration as determined by ELISA in supernatants from *Scid:Atm^{c/c}:LysM^{cre/+}* and *LysM^{cre/+}* BMDMs after no treatment (-) or treatment with LPS for 4 hrs. and co-treated with ATP for the last 30 min. Data are representative of three independent experiments and depict the mean and standard deviation. *ns* = not significant. **(B)** Western blotting analysis of pro-caspase 1 in whole cell lysates (WCL) and active caspase 1 (p20) in supernatants (Sup) from *Scid:Atm^{c/c}:LysM^{cre/+}* and *LysM^{cre/+}* BMDMs after no treatment (-) or treatment with LPS for 4 hrs. and co-treatment with ATP for the last 30 min.
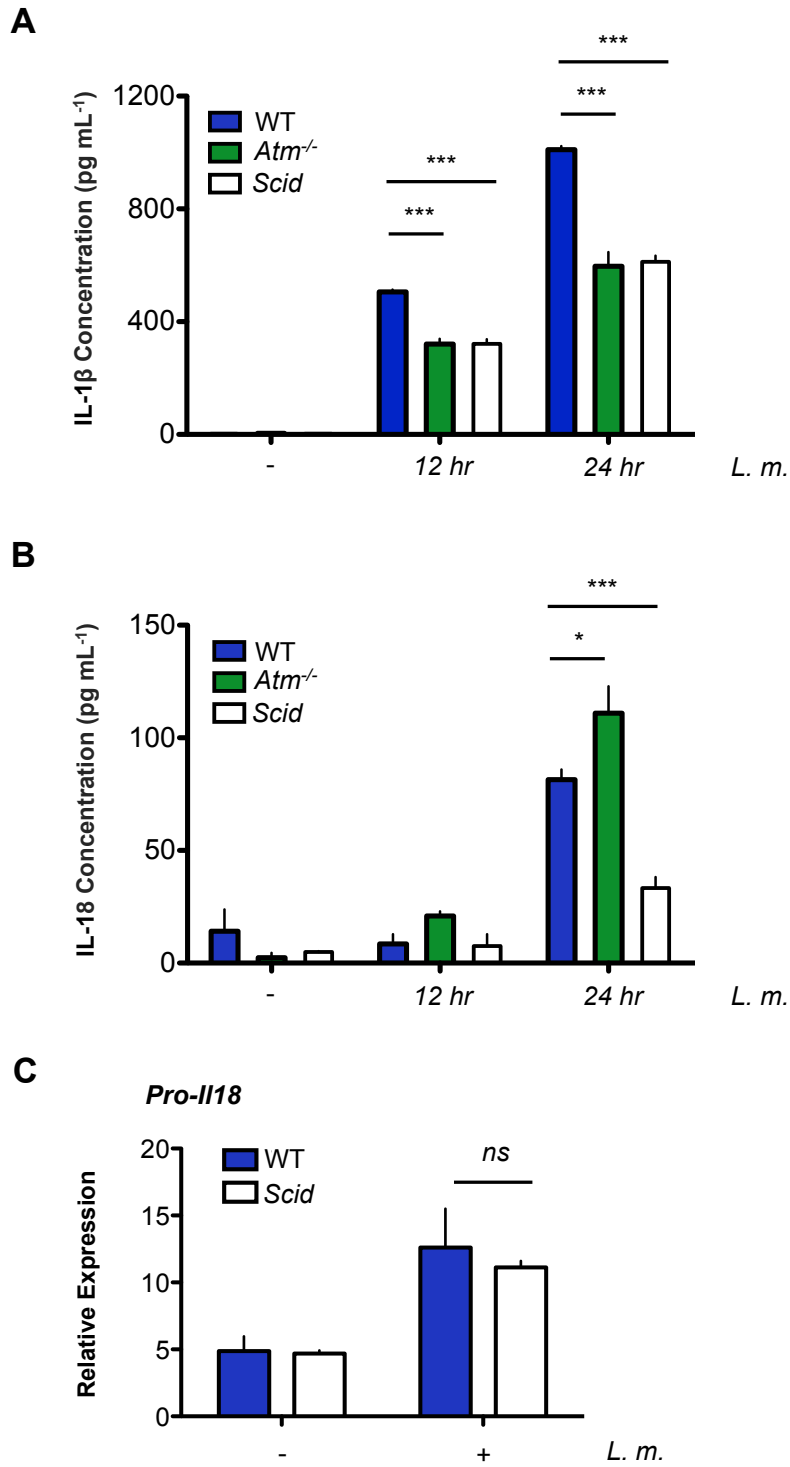
Figure 25. ATM and DNA-PKcs have distinct roles in the production of IL-1β and IL-18.

Figure 25 Legend. ATM and DNA-PKcs have distinct roles in the production of IL-1β and IL-18. **(A)** IL-1β protein concentration and **(B)** Il-18 protein concentration as determined by ELISA in supernatants from uninfected (-) and *L. m.*-infected WT, *Atm*$^{-/-}$, and *Scid* BMDMs 12 and 24 hrs. post-infection. **(C)** RT PCR analysis of *Il18* expression in WT and *Scid* BMDMs after no infection (-) or infection (+) with *L. m*. Data are representative of three or more experiments **(A,B)** or a compilation of three independent experiments **(C)** and depict mean and standard deviation. *, $P = 0.0160$, **, $P \leq 0.0002$, *ns* = not significant.

A

B

Il12 p40
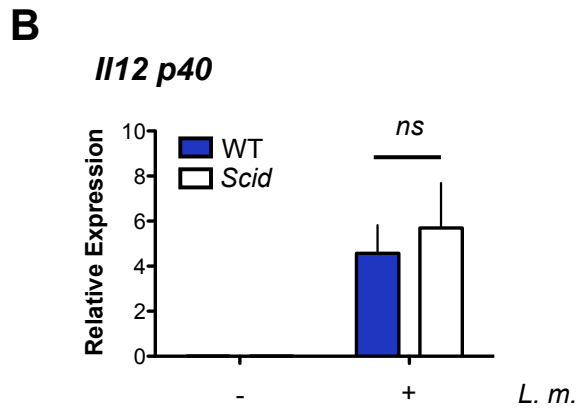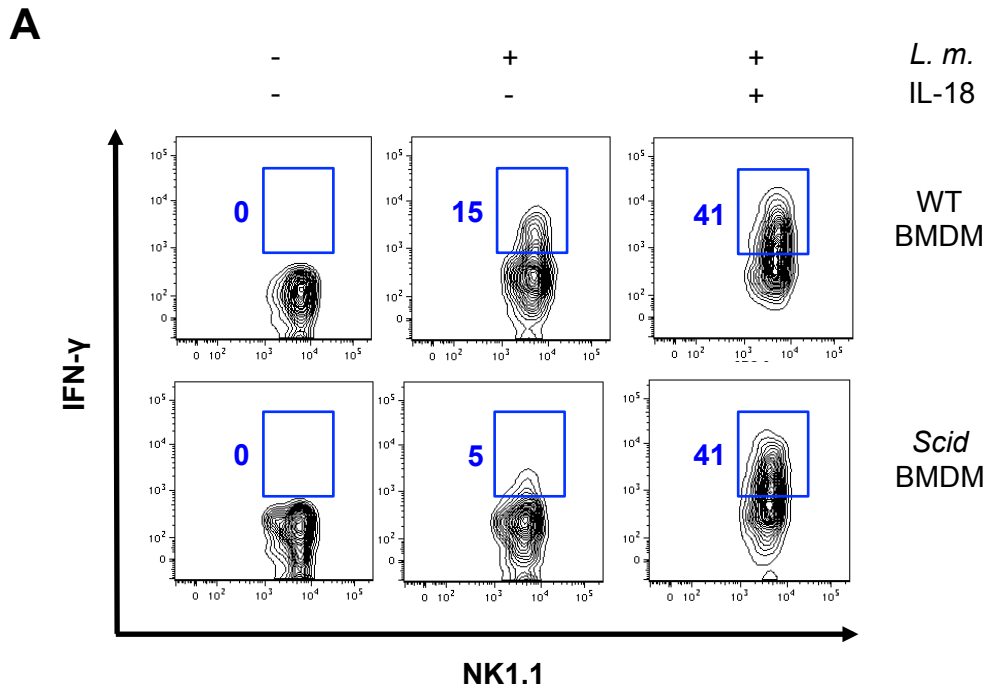
Figure 26. DNA-PKcs-deficient *L. monocytogenes*-infected macrophages cannot optimally stimulate NK cells to produce IFN-γ.

Figure 26 Legend. DNA-PKcs-deficient *L. monocytogenes*-infected macrophages cannot optimally stimulate NK cells to produce IFN-γ. (A) Flow cytometric analysis of intracellular IFN-γ production by WT splenic NK cells co-cultured with uninfected (-) or *L. m.*-infected (+) WT or *Scid* BMDMs with (+) or without (-) the addition of IL-18 for 15 hrs. **(B)** Quantitative RT-PCR analysis of *Il12 p40* gene expression in WT and *Scid* BMDMs uninfected (-) or infected (+) with *L. m.* Data are representative of four independent experiments **(A)** or a compilation of three independent experiments and depict mean and standard deviation **(B).** *ns* = not significant.

Chapter 6: Conclusions and Discussion

Upon exposure to infectious agents, phagocytes produce reactive oxygen and nitrogen intermediates that are highly microbicidal. Though critical for damaging or eradicating microbes, these agents pose a significant threat to the host genome. In this study, we show that activated macrophages produce genotoxic bactericidal agents that damage host cell genomic DNA (Figure 27). This DNA damage, primarily DNA DSBs, activates a robust DDR that depends on ATM and DNA-PKcs (Figure 27). In addition to promoting canonical DDR, ATM and DNA-PKcs regulate inflammasome activation and the genetic program of activated macrophages (Figure 27). These findings establish the generation of DNA DSBs and the initiation of DDR as important signaling events in innate immune responses mediated by macrophages.

6.1) Cell-type-specific DDRs regulate diverse cellular processes.

Developing lymphocytes activate the RAG endonuclease, which generates DSBs at precise genomic locations during antigen receptor gene assembly. Similarly, AID is induced in activated B cells and generates DNA DSBs at immunoglobulin loci to initiate class switch recombination. Through the activation of DDR, RAG- and AID-induced DSBs influence lymphocyte development and plasma cell differentiation, respectively. In macrophages, we find that a genotoxic agent produced upon macrophage activation, NO, can be responsible for the generation of DSBs and the initiation of the DDR. Unlike the site-specific DSBs generated by RAG and AID, these DSBs are likely generated at random locations throughout the genome (Fugmann, Lee et al. 2000, Chaudhuri and Alt 2004, Helmink and Sleckman 2012). Nevertheless, these DSBs and the DDR that they initiate are important signaling intermediates

that regulate macrophage function in the innate immune response to infection. Thus, in all three very different contexts, site-specific or random DNA DSBs and the DDR they elicit act as critical signaling intermediates that impact tissue-specific functions.

Notably, infection with a variety of different pathogens can activate the DDR (Nougayrede, Homburg et al. 2006, Cuevas-Ramos, Petit et al. 2010, Toller, Neelsen et al. 2011, Bergounioux, Elisee et al. 2012, Vielfort, Soderholm et al. 2012, Chumduri, Gurumurthy et al. 2013, Elsen, Collin-Faure et al. 2013, Leitao, Costa et al. 2014, Samba-Louaka, Pereira et al. 2014). However, the pathways affected and the impact on cellular function varies with both the pathogen and the cell type. *L. monocytogenes* infection of HeLa cells leads to the inhibition of ATM-mediated DDR(Samba-Louaka, Pereira et al. 2014). In contrast, we find that ATM is activated in murine macrophages after *L. monocytogenes* infection and that it mediates key functions in the macrophage immune response. Thus, cell-type-specific pathways may have evolved to allow the DDR to regulate a variety of functions in immune cells specifically.

6.2) DDRs in activated macrophages require several signaling pathways.

Although we examined DDR in macrophages activated by either LPS and IFN-γ or infection with *L. monocytogenes*, all bacteria activate NF-κB downstream of recognition by TLRs and NLRs which, in the presence of type I or II IFN receptor signaling, will lead to NO production (Farlik, Reutterer et al. 2010). Thus, we speculate that DDR will be activated in macrophages after infection with a broad variety of bacterial pathogens. It is clear that during *L. monocytogenes* infection, agents other than NO are capable of activating the DDR in its absence.

Why might there be more than one mechanism by which *L. monocytogenes* activates the DDR? It is conceivable that the way in which the DDR is activated in *L. monocytogenes*-infected cells depends on the location of the bacteria within the macrophage. Upon infection, *L. monocytogenes* activates TLR signaling pathways at the cell surface and possibly within phagosomal vesicles after uptake (Kagan and Iwasaki 2012, Witte, Archer et al. 2012). *L. monocytogenes* subsequently escapes from the mildly acidic phagosome to the host cytosol, where it adapts its metabolism to the cytosolic environment to promote effective replication (Stavru, Archambaud et al. 2011). Our data support the notion that *L. monocytogenes* activates the DDR through NO-dependent mechanism while confined to phagosomal vesicles, as both heat-killed and LLO-deficient *L. monocytogenes* fail to elicit a response in macrophages that are deficient in NO production. In contrast, live *L. monocytogenes* that is capable of cytosolic escape activates a robust DDR in the absence of NO signaling. Thus, it is tempting to speculate that *L. monocytogenes* elicits the production of DNA damaging agents once it gains access to the host cytosol. These agents could potentially be generated by the activated macrophage, or may be produced by the bacterium itself. It is possible, for example, that virulence factors produced by *L. monocytogenes* could activate the DDR either directly or indirectly. Indeed, we observe robust DDR after infection with wild type *L. monocytogenes* but not upon infection with bacteria that is deficient in the virulence factor LLO, suggesting that LLO may be capable of activating a DDR in infected macrophages. Though we find that the genotoxic agent ROS does not activate the DDR in LPS- and IFN-γ-treated macrophages, we cannot rule out a role for ROS in *L. monocytogenes* infection. Perhaps *L. monocytogenes* stimulates production of genotoxic ROS upon entering the host cytoplasm. It is known that *L. monoctoygenes* secretes a superoxide dismutase that is post-translationally controlled within macrophages (Archambaud, Nahori et al.

2006). Notably, it is down-regulated by phosphorylation at serine and threonine residues after the bacterium enters the cytoplasm, suggesting that perhaps ROS may potentially be present at sufficient concentrations to induce a DDR at this time. Thus, it is possible that the host is able to control the *L. monocytgenes*-elicited production of genotoxic ROS through the post-translational modification of bacterial virulence factors.

The relative importance of ROS in the initiation of DDR may also be cell-type-specific. A recent study established that the infection of epithelial cells with *Chlamydia trachomatis* leads to the generation of DNA DSBs through the production of ROS (Chumduri, Gurumurthy et al. 2013). Whether *Chlamydia*-elicited ROS similarly induces DNA damage in macrophages is not known; however, it is established that macrophages express high levels of proteins that rapidly inactivate ROS, such as superoxide dismutase and catalase (Nathan and Cunningham-Bussel 2013). Perhaps, then, these enzymes prevent DDR activation by ROS in macrophages specifically. As already alluded to, another source of ROS-inactivating enzymes can be the pathogen itself, as *L. monocytogenes* expresses the ROS-inactivating enzyme superoxide dismutase. This is not unique to *L. monocytogenes*, as the Gram-negative bacterium *Salmonella enterica* expresses three distinct catalases, three peroxiredoxins, and four superoxide dismutases (Fang 2011). The expression of ROS-inactivating enzymes by some bacteria clearly confers a survival advantage upon the pathogen, as these agents are highly mutagenic and microbicidal. It is tempting to speculate, however, that pathogens expressing enzymes that inactivate genotoxic species such as ROS and NO may additionally benefit from eliminating a key signaling intermediate, genotoxin-induced host DNA DSBs, that will modulate the host cell's handling of the infection.

6.3) DDR activation is temporally regulated.

The generation of DSBs by NO in activated macrophages has important implications for the temporal activation of the DDR. NO is produced only after TLR signaling synergizes with IFN receptor signaling, leading to full transcriptional induction of the *Nos2* gene and iNOS expression. Thus, the production of NO, the resulting DDR, and its effect on macrophage physiologic processes will be delayed for several hours after infection. This delay in DDR activation may provide an important checkpoint for the macrophage, ensuring that a DDR is not activated in macrophages that are only transiently exposed to microbial stimuli. Instead, multiple pathways downstream of pathogen sensing, including interferon production, must be activated in order for the DDR to be initiated. Indeed, we find that a sustained DDR in *L. monocytogenes*-infected macrophages specifically depends on type I interferon receptor signaling. This suggests that once signaling through the type I interferon receptor is terminated, perhaps by resolution of the infection, the DDR will also cease. Thus, type I interferon signaling regulates the DDR in *L. monocytogenes*-infected cells in at least two distinct ways. First, by promoting NO production, which can damage host DNA and initiate a DDR. And second, by sustaining the DDR while an inflammatory response is ongoing.

Why would a sustained DDR in activated macrophages be important? We find that the DDR regulates the expression of a variety of genes that have an established role in both the response to *L. monocytogenes* infection and in macrophage innate immune responses generally. Likely, some genes are transcriptionally up-regulated early after infection, while others are induced later on. Thus, the DDR may be involved in regulating different aspects of the *L.*

monocytogenes-elicited genetic program at at various times during infection. DDR activation

also regulates activation of the inflammasome, which is critical for the processing and release of

pro-inflammatory cytokines IL-1β and IL-18 (von Moltke, Ayres et al. 2013). Thus, a sustained

DDR in macrophages may be important for fine-tuning macrophage function at various times

after exposure to *L. monocytogenes.*

6.4) Cell-type-specific requirements for optimal DDR.

DNA damage has been linked to the production of type I IFNs, which can occur after

exposure to genotoxic agents or through the activation of STING by genomic DNA fragments

generated as a byproduct of defects in DSB repair (Brzostek-Racine, Gordon et al. 2011,

Hartlova, Erttmann et al. 2015). In some settings, type I interferon has been implicated in

augmenting the DDR, resulting in cellular senescence (Yu, Katlinskaya et al. 2015). In addition

to a requirement for type I interferon signaling to sustain *L. monocytogenes*-induced DDR, we

find that type I interferon is also required for optimal DDR activation in macrophages that have

been treated with the DSB-inducing chemotherapeutic agent bleomycin. This finding suggests

that the requirement for type I interferon receptor signaling is not specific to a pathogen-induced

DDR. Notably, optimal DDR does not depend on type I interferon in all cell types, as mouse

embryonic fibroblasts (MEFs) exhibit a robust bleomycin-induced DDR that is not further

augmented by type I interferon. Indeed, gene expression profiling revealed that though

bleomycin treatment induces the expression of many genes in MEFs, treating macrophages with

bleomycin does not alter their gene expression profile unless the cells are simultaneously treated

with type I interferon. Though many of these genes are also induced by type I interferon alone,

we were able to identify a subset of genes that were not type I interferon-regulated but were specifically induced by bleomycin in conjunction with type I interferon signaling. Additionally, we identified a cohort of genes that were specifically up-regulated by type I interferon in macrophages but not in fibroblasts, a subset of which have established functions in the DDR. The PI3-like kinase ATM itself was transcriptionally up-regulated by type I interferon in macrophages but not fibroblasts, as was the nuclease Artemis, which has an established role in the processing of DNA ends. Notably, Artemis activity is required for the repair of a subset of genotoxic DSBs and therefore may be required to process broken DNA ends with complex structures that would otherwise prevent repair by non-homologous end joining (NHEJ) (Riballo, Kuhne et al. 2004, Helmink and Sleckman 2012). Intriguingly, we find that the phosphatase EYA3 is also up-regulated transcriptionally by type I interferon in macrophages. In addition to the DNA damage-induced phosphorylation of H2AX at serine 139 (S139), H2AX is constitutively phosphorylated at tyrosine 142 (Y142) by the tyrosine kinase Williams syndrome transcription factor (WSTF) (Xiao, Li et al. 2009). A combination of S139 and Y142 phosphorylation promotes the recruitment of the pro-apoptotic kinase JUN amino-terminal kinase 1 (JNK1) and prevents binding of MDC1. MDC1 binding to H2AX S139 (known as γ-H2AX) leads to the recruitment of DNA repair factors in a focus that surrounds the break. The phosphatases EY1 and EY3 promote the dephosphorylation of Y142, allowing binding of MDC1 and thus guiding DSB signaling pathways toward repair instead of apoptosis (Panier and Durocher 2013). Thus in macrophages but not in fibroblasts, type I interferon promotes the gene expression of several factors that are known to regulate the processing of broken DNA ends or the repair of those ends. Further work will focus on the importance of these factors in the DDR initiated by both genotoxic and pathogen-induced DSBs in macrophages specifically.

Additionally, it is possible that type I interferon signaling regulates the DDR in macrophages by promoting the post-translational modification of DDR factors.

6.5) ATM and DNA-PKcs have distinct and overlapping roles in the DDR.

In most cell types analyzed to date, ATM is the predominant kinase that activates a DDR in G1-phase cells, though DNA-PKcs is able to phosphorylate a subset of the same downstream targets (Rouse and Jackson 2002, Callen, Jankovic et al. 2009). In activated macrophages, ATM and DNA-PKcs have both distinct and overlapping functions in the activation of a DDR. We find that both ATM and DNA-PKcs are able to robustly phosphorylate the histone H2A variant H2AX (forming γ-H2AX), whereas phosphorylation of the transcriptional repressor KAP-1 depends on ATM. It is unclear why both kinases function in γ-H2AX formation but only ATM is required for KAP-1 phosphorylation. The differential requirements for the two kinases could be due to differences in the way each kinase responds upon activation by DNA DSBs. ATM exists as an inactive homodimer that is recruited to the site of DNA DSBs by the MRE11-RAD50-NBS1 (MRN) complex through a specific interaction with NBS1. Once bound to MRN, ATM is activated by an autophosphorylation step that converts ATM to active monomers (Helmink and Sleckman 2012). Once activated, ATM can diffuse throughout the nucleus and phosphorylate hundreds of downstream target proteins with diverse functions in the DDR (Matsuoka, Ballif et al. 2007). Some of these targets, such chromatin-containing histone H2AX, are directly associated with the DNA break, whereas other targets, such as the transcription factor p53, are not associated with regions of DNA damage. ATM can also be activated by ROS in a DSB- and MRN-independent manner that results in the phosphorylation of targets

throughout the nucleus (Guo, Kozlov et al. 2010). Like ATM, DNA-PKcs is recruited to DNA

DSB sites, though its recruitment is mediated by the Ku70:Ku80 heterodimer. DNA-PKcs

kinase activity depends on its association with both Ku and the broken DNA end. Once

activated, DNA-PKcs can phosphorylate a number of targets associated with the DNA DSB,

such as H2AX. However, there is no evidence that it phosphorylates downstream target proteins

that are not associated with the DNA break. This could explain why DNA-PKcs can

phosphorylate H2AX but not KAP-1, while ATM phosphorylates both targets.

6.6) DDRs regulate the genetic program of activated macrophages.

In addition to their role in DDR activation, both ATM and DNA-PKcs are involved in

regulating gene expression in *L. monocytogenes*-infected macrophages. ATM and DNA-PKcs

have unique functions required for the expression of some genes and overlapping roles in the

regulation of others. Both kinases are required for optimal expression of *Marco* and *Cxcl10*, as

expression of both genes is markedly reduced in macrophages deficient in either kinase. In

contrast, ATM and DNA-PKcs can compensate for one another in the regulation of *Ccl2*. How

do the DDR kinases regulate chemokine gene expression? NF-κB is known to regulate

chemokine gene expression downstream of PRRs and notably, DNA damage also leads to the

ATM-dependent activation of NF-κB (Amiri and Richmond 2003, Huang, Wuerzberger-Davis et

al. 2003, Wu, Shi et al. 2006). Thus, it is possible that the *L. monocytogenes*-induced DRR

regulates chemokine gene expression in macrophages by enhancing NF-κB activation. Some

chemokines are transcriptionally regulated by several pathways, such as *Cxcl1*, which is co-

regulated by NF-κB and the known DDR factor poly (ADP-ribose) polymerase 1 (PARP-1)

(Nirodi, NagDas et al. 2001, Amiri and Richmond 2003, Rouleau, Patel et al. 2010). Thus, in activated macrophages, DDR initiation may regulate gene expression through multiple pathways. Whether ATM and DNA-PKcs have distinct or overlapping targets in regulating the *L. monocytogenes*-induced genetic program remains to be determined.

Intriguingly, the genetic programs induced in both developing pre-B cells and activated macrophages include a variety of factors that are important in the migration of immune cells during lymphocyte development and inflammation, respectively. The DDR regulates expression of SWAP70 and L selectin (CD62L) in developing pre-B cells and the expression of a variety of chemokine genes in activated macrophages. Notably, *Cd69* expression is regulated by the DDR in both developing pre-B cells and activated macrophages. CD69 down-regulates the expression of the receptor for sphingosine 1 phosphate (S1P), limiting the egress of immune cells from a particular niche (Schwab and Cyster 2007). Thus, it is tempting to speculate that in activated macrophages, the DDR is required for optimal expression of CD69, which will in turn promote the retention of macrophages in lymphoid organs by rendering them unresponsive to S1P. The *L. monocytogenes*-induced DDR also regulates the expression of a large cohort of chemokines that are involved in the trafficking of many different immune cell types during an inflammatory response, including *Ccl2, Ccl3, Ccl4, Cxcl1,* and *Cxcl10* (Griffith, Sokol et al. 2014). CXCL1 has a role in the trafficking of neutrophils during bacterial infection (Griffith, Sokol et al. 2014). Neutrophils are key in the immune response to *L. monocytogenes* infection, as mice in which neutrophils have been depleted display increased susceptibility to *L. monocytogenes* and have an increased bacterial burden in both the spleen and the liver (Rogers and Unanue 1993, Czuprynski, Brown et al. 1994). CCL2, CCL3, CCL4, and CXCL10 regulate the migration of

other immune cell types, such as macrophages and NK cells (Griffith, Sokol et al. 2014). Notably, resident macrophages up-regulate CCL2 during *L. monocytogenes* infection, which allows for the recruitment of CCR2-expressing monocytes. These monocytes then become activated by microbial ligands and differentiate into highly bactericidal TNF- and iNOS-producing TipDCs (Pamer 2004). Thus, the *L. monocytogenes*-induced DDR may promote the activity of highly bactericidal monocytes by regulating the gene expression of CCL2. As was previously established in developing lymphocytes, it is clear that in activated macrophages, the DDR regulates the gene expression of critical mediators of immune cell trafficking.

6.7) The DDR regulates inflammasome function.

L. monocytogenes infection results in the triggering of several signaling networks that do not require *de novo* transcription and translation. Among these networks is the multi-protein complex known as the inflammasome. Inflammasome activation results in the processing and release of the pro-inflammatory cytokines IL-1β and IL-18. We find that in *L. monocytogenes*-infected macrophages, IL-1β production depends on both ATM and DNA-PKcs, as IL-1β levels are significantly reduced in the absence of both kinases or in the absence of either kinase individually. Notably, this is not due to a role for the kinases in promoting pro-IL-β gene or protein expression. Our findings suggest that the DDR is involved in the processing of the enzyme caspase-1 to its proteolytically active form, as active caspase 1 is significantly reduced in DDR-deficient macrophages during *L. monocytogenes* infection. *L. monocytogenes* is reported to activate caspase 1 through the AIM2, NLRP3, and NLRC4 inflammasomes (Mariathasan, Weiss et al. 2006, Franchi, Kanneganti et al. 2007, Franchi and Nunez 2010, Kim,

Bauernfeind et al. 2010, Warren, Armstrong et al. 2010, Wu, Fernandes-Alnemri et al. 2010).

NLRP3 recognizes a wide variety of bacterial-derived ligands and endogenous danger signals,

known as DAMPs, while AIM2 recognizes bacterial dsDNA (Eitel, Suttorp et al. 2010). NLCR4

specifically recognizes flagellin; however, the strain of *L. monocytogenes* used in this study does

not express flagellin at physiologic temperatures, rendering the NLRC4 inflammasome an

unlikely contributor to caspase 1 activation in our experimental system (Way, Thompson et al.

2004, Eitel, Suttorp et al. 2010). Given that ATP-mediated inflammasome activation requires

NLRP3, it is also unlikely that the DDR regulates this inflammasome, as we see no defect in

caspase 1 proccessing or IL-β production in DDR-deficient macrophages after ATP stimulation.

Thus, it is tempting to speculate that the DDR is specifically involved in regulating the AIM2

inflammasome, though we have established that this regulation is not at the level of AIM2 gene

or protein expression. In the response to DNA DSBs, the DDR kinases activate hundreds of

substrates through phosphorylation of SQ/TQ motifs. Thus, it is possible that ATM and/or

DNA-PKcs phosphorylate one or more inflammasome components, thus promoting their activity

through post-translational modification.

Intriguingly, production of the cytokine IL-18, which also produced downstream of

inflammasome-dependent caspase 1 activation, depends on DNA-PKcs but not ATM. This

finding suggests that ATM and DNA-PKcs may regulate the inflammasome in distinct ways,

though the mechanism of this remains elusive and will be an area of further investigation.

Notably, the defect in IL-18 production in *L. monocytogenes*-infected DNA-PKcs-deficient

macrophages renders the cells unable to optimally stimulate purified splenic NK cells to produce

IFN-γ. NK cells are an important source of IFN-γ early in the innate immune response to

infection (Bancroft, Schreiber et al. 1991, Unanue 1997, Edelson and Unanue 2000). Previous work in lymphocyte-deficient mice established that during *L. monocytogenes* infection, macrophages produce IL-12 and TNF, which activate NK cells to produce IFN-γ (Tripp, Wolf et al. 1993). The NK-derived IFN-γ then acts in synergy with TNF to initiate full macrophage activation, including the up-regulation of MHC class II molecules and production of microbicidal intermediates (Edelson and Unanue 2000). Thus, DNA-PKcs may contribute to the full activation of macrophages by positively regulating IL-18 production, thus promoting optimal IFN-γ production by NK cells.

6.8) Conclusions and Future Directions

Here we show that activated macrophages initiate a robust DDR that depends on the kinases ATM and DNA-PKcs. The genotoxic agent NO can activate this response and does so primarily through DNA DSB intermediates. Notably, an optimal DDR in macrophages depends on type I interferon signaling in response to both pathogen-induced DSBs and DSBs elicited by the chemotherapeutic agent bleomycin. Finally, ATM and DNA-PKcs have both distinct and overlapping functions in regulating the genetic program and inflammasome activation in *L. monocytogenes*-infected macrophages, suggesting that the DDR is important for fine-tuning the innate immune response of macrophages during *L. monocytogenes* infection. In the future, we plan to investigate the role of type I interferon signaling in promoting DDR in macrophages specifically. We also plan to interrogate the mechanism by which the DDR regulates inflammasome activation in macrophages that have been infected with bacterial pathogens.
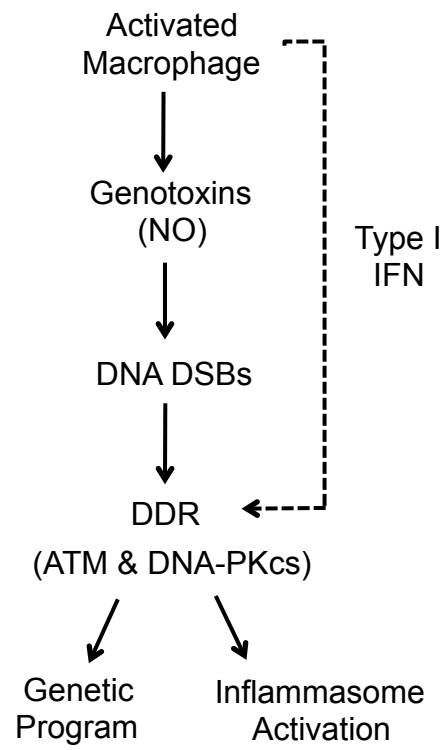
Figure 27: Regulation of macrophage functions by DDR.

References

Akira, S. (2000). "The role of IL-18 in innate immunity." <u>Curr Opin Immunol</u> **12**(1): 59-63.

Akira, S., S. Uematsu and O. Takeuchi (2006). "Pathogen recognition and innate immunity." <u>Cell</u> **124**(4): 783-801.

Alvarez-Dominguez, C. and P. D. Stahl (1998). "Interferon-gamma selectively induces Rab5a synthesis and processing in mononuclear cells." <u>J Biol Chem</u> **273**(51): 33901-33904.

Amiri, K. I. and A. Richmond (2003). "Fine tuning the transcriptional regulation of the CXCL1 chemokine." <u>Prog Nucleic Acid Res Mol Biol</u> **74**: 1-36.

Anand, P. and J. S. Stamler (2012). "Enzymatic mechanisms regulating protein S-nitrosylation: implications in health and disease." <u>J Mol Med (Berl)</u> **90**(3): 233-244.

Archambaud, C., M. A. Nahori, J. Pizarro-Cerda, P. Cossart and O. Dussurget (2006). "Control of Listeria superoxide dismutase by phosphorylation." <u>J Biol Chem</u> **281**(42): 31812-31822.

Bakkenist, C. J. and M. B. Kastan (2003). "DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation." <u>Nature</u> **421**(6922): 499-506.

Bancroft, G. J., R. D. Schreiber and E. R. Unanue (1991). "Natural immunity: a T-cell-independent pathway of macrophage activation, defined in the scid mouse." <u>Immunol Rev</u> **124**: 5-24.

Barbalat, R., S. E. Ewald, M. L. Mouchess and G. M. Barton (2011). "Nucleic acid recognition by the innate immune system." <u>Annu Rev Immunol</u> **29**: 185-214.

Barton, G. M. and J. C. Kagan (2009). "A cell biological view of Toll-like receptor function: regulation through compartmentalization." <u>Nat Rev Immunol</u> **9**(8): 535-542.

Bednarski, J. J., A. Nickless, D. Bhattacharya, R. H. Amin, M. S. Schlissel and B. P. Sleckman (2012). "RAG-induced DNA double-strand breaks signal through Pim2 to promote pre-B cell survival and limit proliferation." <u>J Exp Med</u> **209**(1): 11-17.

Bergounioux, J., R. Elisee, A. L. Prunier, F. Donnadieu, B. Sperandio, P. Sansonetti and L. Arbibe (2012). "Calpain activation by the Shigella flexneri effector VirA regulates key steps in the formation and life of the bacterium's epithelial niche." <u>Cell Host Microbe</u> **11**(3): 240-252.

Bergsbaken, T., S. L. Fink and B. T. Cookson (2009). "Pyroptosis: host cell death and inflammation." <u>Nat Rev Microbiol</u> **7**(2): 99-109.

Blasius, A. L. and B. Beutler (2010). "Intracellular toll-like receptors." <u>Immunity</u> **32**(3): 305-315.

Blunt, T., D. Gell, M. Fox, G. E. Taccioli, A. R. Lehmann, S. P. Jackson and P. A. Jeggo (1996). "Identification of a nonsense mutation in the carboxyl-terminal region of DNA-dependent protein kinase catalytic subunit in the scid mouse." Proc Natl Acad Sci U S A **93**(19): 10285-10290.

Bogdan, C. (2001). "Nitric oxide and the immune response." Nat Immunol **2**(10): 907-916.

Bogdan, C. (2015). "Nitric oxide synthase in innate and adaptive immunity: an update." Trends Immunol **36**(3): 161-178.

Boneca, I. G., O. Dussurget, D. Cabanes, M. A. Nahori, S. Sousa, M. Lecuit, E. Psylinakis, V. Bouriotis, J. P. Hugot, M. Giovannini, A. Coyle, J. Bertin, A. Namane, J. C. Rousselle, N. Cayet, M. C. Prevost, V. Balloy, M. Chignard, D. J. Philpott, P. Cossart and S. E. Girardin (2007). "A critical role for peptidoglycan N-deacetylation in Listeria evasion from the host innate immune system." Proc Natl Acad Sci U S A **104**(3): 997-1002.

Bredemeyer, A. L., B. A. Helmink, C. L. Innes, B. Calderon, L. M. McGinnis, G. K. Mahowald, E. J. Gapud, L. M. Walker, J. B. Collins, B. K. Weaver, L. Mandik-Nayak, R. D. Schreiber, P. M. Allen, M. J. May, R. S. Paules, C. H. Bassing and B. P. Sleckman (2008). "DNA double-strand breaks activate a multi-functional genetic program in developing lymphocytes." Nature **456**(7223): 819-823.

Brzostek-Racine, S., C. Gordon, S. Van Scoy and N. C. Reich (2011). "The DNA damage response induces IFN." J Immunol **187**(10): 5336-5345.

Burdette, D. L. and R. E. Vance (2013). "STING and the innate immune response to nucleic acids in the cytosol." Nat Immunol **14**(1): 19-26.

Caldecott, K. W. (2008). "Single-strand break repair and genetic disease." Nat Rev Genet **9**(8): 619-631.

Callen, E., M. Jankovic, N. Wong, S. Zha, H. T. Chen, S. Difilippantonio, M. Di Virgilio, G. Heidkamp, F. W. Alt, A. Nussenzweig and M. Nussenzweig (2009). "Essential role for DNA-PKcs in DNA double-strand break repair and apoptosis in ATM-deficient lymphocytes." Mol Cell **34**(3): 285-297.

Carrero, J. A., B. Calderon and E. R. Unanue (2006). "Lymphocytes are detrimental during the early innate immune response against Listeria monocytogenes." J Exp Med **203**(4): 933-940.

Carrero, J. A. and E. R. Unanue (2006). "Lymphocyte apoptosis as an immune subversion strategy of microbial pathogens." Trends Immunol **27**(11): 497-503.

Chapman, J. R., M. R. Taylor and S. J. Boulton (2012). "Playing the end game: DNA double-strand break repair pathway choice." Mol Cell **47**(4): 497-510.

Chaudhuri, J. and F. W. Alt (2004). "Class-switch recombination: interplay of transcription, DNA deamination and DNA repair." Nat Rev Immunol **4**(7): 541-552.

Chumduri, C., R. K. Gurumurthy, P. K. Zadora, Y. Mi and T. F. Meyer (2013). "Chlamydia infection promotes host DNA damage and proliferation but impairs the DNA damage response." Cell Host Microbe **13**(6): 746-758.

Ciccia, A. and S. J. Elledge (2010). "The DNA damage response: making it safe to play with knives." Mol Cell **40**(2): 179-204.

Clausen, B. E., C. Burkhardt, W. Reith, R. Renkawitz and I. Forster (1999). "Conditional gene targeting in macrophages and granulocytes using LysMcre mice." Transgenic Res **8**(4): 265-277.

Cuevas-Ramos, G., C. R. Petit, I. Marcq, M. Boury, E. Oswald and J. P. Nougayrede (2010). "Escherichia coli induces DNA damage in vivo and triggers genomic instability in mammalian cells." Proc Natl Acad Sci U S A **107**(25): 11537-11542.

Czuprynski, C. J., J. F. Brown, N. Maroushek, R. D. Wagner and H. Steinberg (1994). "Administration of anti-granulocyte mAb RB6-8C5 impairs the resistance of mice to Listeria monocytogenes infection." J Immunol **152**(4): 1836-1846.

Dale, D. C., L. Boxer and W. C. Liles (2008). "The phagocytes: neutrophils and monocytes." Blood **112**(4): 935-945.

de Almeida, L. A., N. B. Carvalho, F. S. Oliveira, T. L. Lacerda, A. C. Vasconcelos, L. Nogueira, A. Bafica, A. M. Silva and S. C. Oliveira (2011). "MyD88 and STING signaling pathways are required for IRF3-mediated IFN-beta induction in response to Brucella abortus infection." PLoS One **6**(8): e23135.

DeFazio, L. G., R. M. Stansel, J. D. Griffith and G. Chu (2002). "Synapsis of DNA ends by DNA-dependent protein kinase." EMBO J **21**(12): 3192-3200.

Driessens, N., S. Versteyhe, C. Ghaddhab, A. Burniat, X. De Deken, J. Van Sande, J. E. Dumont, F. Miot and B. Corvilain (2009). "Hydrogen peroxide induces DNA single- and double-strand breaks in thyroid cells and is therefore a potential mutagen for this organ." Endocr Relat Cancer **16**(3): 845-856.

Dziarski, R. and D. Gupta (2010). "Review: Mammalian peptidoglycan recognition proteins (PGRPs) in innate immunity." Innate Immun **16**(3): 168-174.

Edelson, B. T. and E. R. Unanue (2000). "Immunity to Listeria infection." Curr Opin Immunol **12**(4): 425-431.

Edelson, B. T. and E. R. Unanue (2002). "MyD88-dependent but Toll-like receptor 2-independent innate immunity to Listeria: no role for either in macrophage listericidal activity." J Immunol **169**(7): 3869-3875.

Eitel, J., N. Suttorp and B. Opitz (2010). "Innate immune recognition and inflammasome activation in listeria monocytogenes infection." Front Microbiol **1**: 149.

Elsen, S., V. Collin-Faure, X. Gidrol and C. Lemercier (2013). "The opportunistic pathogen Pseudomonas aeruginosa activates the DNA double-strand break signaling and repair pathway in infected cells." Cell Mol Life Sci **70**(22): 4385-4397.

Fang, F. C. (2011). "Antimicrobial actions of reactive oxygen species." MBio **2**(5).

Farlik, M., B. Reutterer, C. Schindler, F. Greten, C. Vogl, M. Muller and T. Decker (2010). "Nonconventional initiation complex assembly by STAT and NF-kappaB transcription factors regulates nitric oxide synthase expression." Immunity **33**(1): 25-34.

Fernandes-Alnemri, T., J. W. Yu, C. Juliana, L. Solorzano, S. Kang, J. Wu, P. Datta, M. McCormick, L. Huang, E. McDermott, L. Eisenlohr, C. P. Landel and E. S. Alnemri (2010). "The AIM2 inflammasome is critical for innate immunity to Francisella tularensis." Nat Immunol **11**(5): 385-393.

Finkel, T. (2011). "Signal transduction by reactive oxygen species." J Cell Biol **194**(1): 7-15.

Franchi, L., T. D. Kanneganti, G. R. Dubyak and G. Nunez (2007). "Differential requirement of P2X7 receptor and intracellular K+ for caspase-1 activation induced by intracellular and extracellular bacteria." J Biol Chem **282**(26): 18810-18818.

Franchi, L. and G. Nunez (2010). "AIM2 joins the gang of microbial sensors." Cell Host Microbe **7**(5): 340-341.

Fugmann, S. D., A. I. Lee, P. E. Shockett, I. J. Villey and D. G. Schatz (2000). "The RAG proteins and V(D)J recombination: complexes, ends, and transposition." Annu Rev Immunol **18**: 495-527.
Gao, J. J., M. B. Filla, M. J. Fultz, S. N. Vogel, S. W. Russell and W. J. Murphy (1998). "Autocrine/paracrine IFN-alphabeta mediates the lipopolysaccharide-induced activation of transcription factor Stat1alpha in mouse macrophages: pivotal role of Stat1alpha in induction of the inducible nitric oxide synthase gene." J Immunol **161**(9): 4803-4810.

Gellert, M. (2002). "V(D)J recombination: RAG proteins, repair factors, and regulation." Annu Rev Biochem **71**: 101-132.

Griffith, J. W., C. L. Sokol and A. D. Luster (2014). "Chemokines and chemokine receptors: positioning cells for host defense and immunity." Annu Rev Immunol **32**: 659-702.

Guo, Z., S. Kozlov, M. F. Lavin, M. D. Person and T. T. Paull (2010). "ATM activation by oxidative stress." <u>Science</u> **330**(6003): 517-521.

Hartlova, A., S. F. Erttmann, F. A. Raffi, A. M. Schmalz, U. Resch, S. Anugula, S. Lienenklaus, L. M. Nilsson, A. Kroger, J. A. Nilsson, T. Ek, S. Weiss and N. O. Gekara (2015). "DNA damage primes the type I interferon system via the cytosolic DNA sensor STING to promote anti-microbial innate immunity." <u>Immunity</u> **42**(2): 332-343.

Helmink, B. A. and B. P. Sleckman (2012). "The response to and repair of RAG-mediated DNA double-strand breaks." <u>Annu Rev Immunol</u> **30**: 175-202.

Helmink, B. A., A. T. Tubbs, Y. Dorsett, J. J. Bednarski, L. M. Walker, Z. Feng, G. G. Sharma, P. J. McKinnon, J. Zhang, C. H. Bassing and B. P. Sleckman (2011). "H2AX prevents CtIP-mediated DNA end resection and aberrant repair in G1-phase lymphocytes." <u>Nature</u> **469**(7329): 245-249.

Hemmi, H., O. Takeuchi, T. Kawai, T. Kaisho, S. Sato, H. Sanjo, M. Matsumoto, K. Hoshino, H. Wagner, K. Takeda and S. Akira (2000). "A Toll-like receptor recognizes bacterial DNA." <u>Nature</u> **408**(6813): 740-745.

Ho, M. K. and T. A. Springer (1982). "Mac-1 antigen: quantitative expression in macrophage populations and tissues, and immunofluorescent localization in spleen." <u>J Immunol</u> **128**(5): 2281-2286.

Hsieh, C. S., S. E. Macatonia, C. S. Tripp, S. F. Wolf, A. O'Garra and K. M. Murphy (1993). "Development of TH1 CD4+ T cells through IL-12 produced by Listeria-induced macrophages." <u>Science</u> **260**(5107): 547-549.

Huang, T. T., S. M. Wuerzberger-Davis, Z. H. Wu and S. Miyamoto (2003). "Sequential modification of NEMO/IKKgamma by SUMO-1 and ubiquitin mediates NF-kappaB activation by genotoxic stress." <u>Cell</u> **115**(5): 565-576.

Imlay, J. A. (2006). "Iron-sulphur clusters and the problem with oxygen." <u>Mol Microbiol</u> **59**(4): 1073-1082.

Imlay, J. A. (2008). "Cellular defenses against superoxide and hydrogen peroxide." <u>Annu Rev Biochem</u> **77**: 755-776.

Imlay, J. A. and S. Linn (1988). "DNA damage and oxygen radical toxicity." <u>Science</u> **240**(4857): 1302-1309.

Innes, C. L., J. E. Hesse, S. S. Palii, B. A. Helmink, A. J. Holub, B. P. Sleckman and R. S. Paules (2013). "DNA damage activates a complex transcriptional response in murine lymphocytes that includes both physiological and cancer-predisposition programs." <u>BMC Genomics</u> **14**: 163.

Ishii, K. J., C. Coban, H. Kato, K. Takahashi, Y. Torii, F. Takeshita, H. Ludwig, G. Sutter, K. Suzuki, H. Hemmi, S. Sato, M. Yamamoto, S. Uematsu, T. Kawai, O. Takeuchi and S. Akira (2006). "A Toll-like receptor-independent antiviral response induced by double-stranded B-form DNA." Nat Immunol **7**(1): 40-48.

Ishikawa, H. and G. N. Barber (2008). "STING is an endoplasmic reticulum adaptor that facilitates innate immune signalling." Nature **455**(7213): 674-678.

Ishikawa, H., Z. Ma and G. N. Barber (2009). "STING regulates intracellular DNA-mediated, type I interferon-dependent innate immunity." Nature **461**(7265): 788-792.

Ivashkiv, L. B. and L. T. Donlin (2014). "Regulation of type I interferon responses." Nat Rev Immunol **14**(1): 36-49.

Jackson, S. P. and J. Bartek (2009). "The DNA-damage response in human biology and disease." Nature **461**(7267): 1071-1078.

Janot, L., T. Secher, D. Torres, I. Maillet, J. Pfeilschifter, V. F. Quesniaux, R. Landmann, B. Ryffel and F. Erard (2008). "CD14 works with toll-like receptor 2 to contribute to recognition and control of Listeria monocytogenes infection." J Infect Dis **198**(1): 115-124.

Jena, S., C. Anand, G. B. Chainy and J. Dandapat (2012). "Induction of oxidative stress and inhibition of superoxide dismutase expression in rat cerebral cortex and cerebellum by PTU-induced hypothyroidism and its reversal by curcumin." Neurol Sci **33**(4): 869-873.

Jin, L., K. K. Hill, H. Filak, J. Mogan, H. Knowles, B. Zhang, A. L. Perraud, J. C. Cambier and L. L. Lenz (2011). "MPYS is required for IFN response factor 3 activation and type I IFN production in the response of cultured phagocytes to bacterial second messengers cyclic-di-AMP and cyclic-di-GMP." J Immunol **187**(5): 2595-2601.

Kagan, J. C. and A. Iwasaki (2012). "Phagosome as the organelle linking innate and adaptive immunity." Traffic **13**(8): 1053-1061.

Kamijo, R., H. Harada, T. Matsuyama, M. Bosland, J. Gerecitano, D. Shapiro, J. Le, S. I. Koh, T. Kimura, S. J. Green and et al. (1994). "Requirement for transcription factor IRF-1 in NO synthase induction in macrophages." Science **263**(5153): 1612-1615.

Karo, J. M., D. G. Schatz and J. C. Sun (2014). "The RAG recombinase dictates functional heterogeneity and cellular fitness in natural killer cells." Cell **159**(1): 94-107.

Kawai, T. and S. Akira (2010). "The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors." Nat Immunol **11**(5): 373-384.

Kawai, T. and S. Akira (2011). "Toll-like receptors and their crosstalk with other innate receptors in infection and immunity." Immunity **34**(5): 637-650.

Kawanishi, S., Y. Hiraku, S. Pinlaor and N. Ma (2006). "Oxidative and nitrative DNA damage in animals and patients with inflammatory diseases in relation to inflammation-related carcinogenesis." Biol Chem **387**(4): 365-372.

Kim, S., F. Bauernfeind, A. Ablasser, G. Hartmann, K. A. Fitzgerald, E. Latz and V. Hornung (2010). "Listeria monocytogenes is sensed by the NLRP3 and AIM2 inflammasome." Eur J Immunol **40**(6): 1545-1551.

Kleinert, H., P. M. Schwarz and U. Forstermann (2003). "Regulation of the expression of inducible nitric oxide synthase." Biol Chem **384**(10-11): 1343-1364.
Kraal, G., L. J. van der Laan, O. Elomaa and K. Tryggvason (2000). "The macrophage receptor MARCO." Microbes Infect **2**(3): 313-316.

Kuziel, W. A., S. J. Morgan, T. C. Dawson, S. Griffin, O. Smithies, K. Ley and N. Maeda (1997). "Severe reduction in leukocyte adhesion and monocyte extravasation in mice deficient in CC chemokine receptor 2." Proc Natl Acad Sci U S A **94**(22): 12053-12058.

Lamkanfi, M. (2011). "Emerging inflammasome effector mechanisms." Nat Rev Immunol **11**(3): 213-220.

Lamkanfi, M. and V. M. Dixit (2014). "Mechanisms and functions of inflammasomes." Cell **157**(5): 1013-1022.

Leber, J. H., G. T. Crimmins, S. Raghavan, N. P. Meyer-Morse, J. S. Cox and D. A. Portnoy (2008). "Distinct TLR- and NLR-mediated transcriptional responses to an intracellular pathogen." PLoS Pathog **4**(1): e6.

Lee, J. H. and T. T. Paull (2004). "Direct activation of the ATM protein kinase by the Mre11/Rad50/Nbs1 complex." Science **304**(5667): 93-96.

Lee, J. H. and T. T. Paull (2005). "ATM activation by DNA double-strand breaks through the Mre11-Rad50-Nbs1 complex." Science **308**(5721): 551-554.

Leitao, E., A. C. Costa, C. Brito, L. Costa, R. Pombinho, D. Cabanes and S. Sousa (2014). "Listeria monocytogenes induces host DNA damage and delays the host cell cycle to promote infection." Cell Cycle **13**(6): 928-940.

Lieber, M. R. (2010). "The mechanism of double-strand DNA break repair by the nonhomologous DNA end-joining pathway." Annu Rev Biochem **79**: 181-211.

Lindahl, T. and D. E. Barnes (2000). "Repair of endogenous DNA damage." Cold Spring Harb Symp Quant Biol **65**: 127-133.

MacMicking, J. D., C. Nathan, G. Hom, N. Chartrain, D. S. Fletcher, M. Trumbauer, K. Stevens, Q. W. Xie, K. Sokol, N. Hutchinson and et al. (1995). "Altered responses to bacterial infection and endotoxic shock in mice lacking inducible nitric oxide synthase." Cell **81**(4): 641-650.

Manzanillo, P. S., M. U. Shiloh, D. A. Portnoy and J. S. Cox (2012). "Mycobacterium tuberculosis activates the DNA-dependent cytosolic surveillance pathway within macrophages." Cell Host Microbe **11**(5): 469-480.

Mariathasan, S., D. S. Weiss, K. Newton, J. McBride, K. O'Rourke, M. Roose-Girma, W. P. Lee, Y. Weinrauch, D. M. Monack and V. M. Dixit (2006). "Cryopyrin activates the inflammasome in response to toxins and ATP." Nature **440**(7081): 228-232.

Martinon, F., A. Mayor and J. Tschopp (2009). "The inflammasomes: guardians of the body." Annu Rev Immunol **27**: 229-265.

Matsuoka, S., B. A. Ballif, A. Smogorzewska, E. R. McDonald, 3rd, K. E. Hurov, J. Luo, C. E. Bakalarski, Z. Zhao, N. Solimini, Y. Lerenthal, Y. Shiloh, S. P. Gygi and S. J. Elledge (2007). "ATM and ATR substrate analysis reveals extensive protein networks responsive to DNA damage." Science **316**(5828): 1160-1166.

McGarry, M. P. and C. C. Stewart (1991). "Murine eosinophil granulocytes bind the murine macrophage-monocyte specific monoclonal antibody F4/80." J Leukoc Biol **50**(5): 471-478.

McWhirter, S. M., R. Barbalat, K. M. Monroe, M. F. Fontana, M. Hyodo, N. T. Joncker, K. J. Ishii, S. Akira, M. Colonna, Z. J. Chen, K. A. Fitzgerald, Y. Hayakawa and R. E. Vance (2009). "A host type I interferon response is induced by cytosolic sensing of the bacterial second messenger cyclic-di-GMP." J Exp Med **206**(9): 1899-1911.

Mengaud, J., H. Ohayon, P. Gounon, R. M. Mege and P. Cossart (1996). "E-cadherin is the receptor for internalin, a surface protein required for entry of L. monocytogenes into epithelial cells." Cell **84**(6): 923-932.

Meraz, M. A., J. M. White, K. C. Sheehan, E. A. Bach, S. J. Rodig, A. S. Dighe, D. H. Kaplan, J. K. Riley, A. C. Greenlund, D. Campbell, K. Carver-Moore, R. N. DuBois, R. Clark, M. Aguet and R. D. Schreiber (1996). "Targeted disruption of the Stat1 gene in mice reveals unexpected physiologic specificity in the JAK-STAT signaling pathway." Cell **84**(3): 431-442.

Merkle, D., P. Douglas, G. B. Moorhead, Z. Leonenko, Y. Yu, D. Cramb, D. P. Bazett-Jones and S. P. Lees-Miller (2002). "The DNA-dependent protein kinase interacts with DNA to form a protein-DNA complex that is disrupted by phosphorylation." Biochemistry **41**(42): 12706-12714.

Merrick, J. C., B. T. Edelson, V. Bhardwaj, P. E. Swanson and E. R. Unanue (1997). "Lymphocyte apoptosis during early phase of Listeria infection in mice." Am J Pathol **151**(3): 785-792.

Miao, E. A., I. A. Leaf, P. M. Treuting, D. P. Mao, M. Dors, A. Sarkar, S. E. Warren, M. D. Wewers and A. Aderem (2010). "Caspase-1-induced pyroptosis is an innate immune effector mechanism against intracellular bacteria." Nat Immunol **11**(12): 1136-1142.

Mosser, D. M. and J. P. Edwards (2008). "Exploring the full spectrum of macrophage activation." Nat Rev Immunol **8**(12): 958-969.

Mosser, D. M. and X. Zhang (2008). "Activation of murine macrophages." Curr Protoc Immunol **Chapter 14**: Unit 14 12.

Myers, J. S. and D. Cortez (2006). "Rapid activation of ATR by ionizing radiation requires ATM and Mre11." J Biol Chem **281**(14): 9346-9350.

Myers, J. T., A. W. Tsang and J. A. Swanson (2003). "Localized reactive oxygen and nitrogen intermediates inhibit escape of Listeria monocytogenes from vacuoles in activated macrophages." J Immunol **171**(10): 5447-5453.

Nathan, C. and A. Cunningham-Bussel (2013). "Beyond oxidative stress: an immunologist's guide to reactive oxygen species." Nat Rev Immunol **13**(5): 349-361.

Nickol, A. D. and P. F. Bonventre (1977). "Anomalous high native resistance to athymic mice to bacterial pathogens." Infect Immun **18**(3): 636-645.

Nirodi, C., S. NagDas, S. P. Gygi, G. Olson, R. Aebersold and A. Richmond (2001). "A role for poly(ADP-ribose) polymerase in the transcriptional regulation of the melanoma growth stimulatory activity (CXCL1) gene expression." J Biol Chem **276**(12): 9366-9374.

Nish, S. and R. Medzhitov (2011). "Host defense pathways: role of redundancy and compensation in infectious disease phenotypes." Immunity **34**(5): 629-636.

North, R. J., P. L. Dunn and J. W. Conlan (1997). "Murine listeriosis as a model of antimicrobial defense." Immunol Rev **158**: 27-36.

Nougayrede, J. P., S. Homburg, F. Taieb, M. Boury, E. Brzuszkiewicz, G. Gottschalk, C. Buchrieser, J. Hacker, U. Dobrindt and E. Oswald (2006). "Escherichia coli induces DNA double-strand breaks in eukaryotic cells." Science **313**(5788): 848-851.

Nunes, P., N. Demaurex and M. C. Dinauer (2013). "Regulation of the NADPH oxidase and associated ion fluxes during phagocytosis." Traffic **14**(11): 1118-1131.

O'Shea, J. J. and P. J. Murray (2008). "Cytokine signaling modules in inflammatory responses." Immunity **28**(4): 477-487.

Okamura, H., H. Tsutsi, T. Komatsu, M. Yutsudo, A. Hakura, T. Tanimoto, K. Torigoe, T. Okura, Y. Nukada, K. Hattori and et al. (1995). "Cloning of a new cytokine that induces IFN-gamma production by T cells." Nature **378**(6552): 88-91.

Paludan, S. R. and A. G. Bowie (2013). "Immune sensing of DNA." Immunity **38**(5): 870-880.

Pamer, E. G. (2004). "Immune responses to Listeria monocytogenes." <u>Nat Rev Immunol</u> **4**(10): 812-823.

Panier, S. and D. Durocher (2013). "Push back to respond better: regulatory inhibition of the DNA double-strand break response." <u>Nat Rev Mol Cell Biol</u> **14**(10): 661-672.

Park, J. H., Y. G. Kim, C. McDonald, T. D. Kanneganti, M. Hasegawa, M. Body-Malapel, N. Inohara and G. Nunez (2007). "RICK/RIP2 mediates innate immune responses induced through Nod1 and Nod2 but not TLRs." <u>J Immunol</u> **178**(4): 2380-2386.

Parker, D., F. J. Martin, G. Soong, B. S. Harfenist, J. L. Aguilar, A. J. Ratner, K. A. Fitzgerald, C. Schindler and A. Prince (2011). "Streptococcus pneumoniae DNA initiates type I interferon signaling in the respiratory tract." <u>MBio</u> **2**(3): e00016-00011.

Paull, T. T., E. P. Rogakou, V. Yamazaki, C. U. Kirchgessner, M. Gellert and W. M. Bonner (2000). "A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage." <u>Curr Biol</u> **10**(15): 886-895.

Portnoy, D. A., P. S. Jacks and D. J. Hinrichs (1988). "Role of hemolysin for the intracellular growth of Listeria monocytogenes." <u>J Exp Med</u> **167**(4): 1459-1471.

Portnoy, D. A., R. D. Schreiber, P. Connelly and L. G. Tilney (1989). "Gamma interferon limits access of Listeria monocytogenes to the macrophage cytoplasm." <u>J Exp Med</u> **170**(6): 2141-2146.

Radi, R. (2013). "Protein tyrosine nitration: biochemical mechanisms and structural basis of functional effects." <u>Acc Chem Res</u> **46**(2): 550-559.

Rae, C. S., A. Geissler, P. C. Adamson and D. A. Portnoy (2011). "Mutations of the Listeria monocytogenes peptidoglycan N-deacetylase and O-acetylase result in enhanced lysozyme sensitivity, bacteriolysis, and hyperinduction of innate immune pathways." <u>Infect Immun</u> **79**(9): 3596-3606.

Rathinam, V. A., Z. Jiang, S. N. Waggoner, S. Sharma, L. E. Cole, L. Waggoner, S. K. Vanaja, B. G. Monks, S. Ganesan, E. Latz, V. Hornung, S. N. Vogel, E. Szomolanyi-Tsuda and K. A. Fitzgerald (2010). "The AIM2 inflammasome is essential for host defense against cytosolic bacteria and DNA viruses." <u>Nat Immunol</u> **11**(5): 395-402.

Ren, Y., L. Stuart, F. P. Lindberg, A. R. Rosenkranz, Y. Chen, T. N. Mayadas and J. Savill (2001). "Nonphlogistic clearance of late apoptotic neutrophils by macrophages: efficient phagocytosis independent of beta 2 integrins." <u>J Immunol</u> **166**(7): 4743-4750.

Riballo, E., M. Kuhne, N. Rief, A. Doherty, G. C. Smith, M. J. Recio, C. Reis, K. Dahm, A. Fricke, A. Krempler, A. R. Parker, S. P. Jackson, A. Gennery, P. A. Jeggo and M. Lobrich (2004). "A pathway of double-strand break rejoining dependent upon ATM, Artemis, and proteins locating to gamma-H2AX foci." <u>Mol Cell</u> **16**(5): 715-724.

Rogakou, E. P., C. Boon, C. Redon and W. M. Bonner (1999). "Megabase chromatin domains involved in DNA double-strand breaks in vivo." J Cell Biol **146**(5): 905-916.

Rogers, H. W., C. S. Tripp, R. D. Schreiber and E. R. Unanue (1994). "Endogenous IL-1 is required for neutrophil recruitment and macrophage activation during murine listeriosis." J Immunol **153**(5): 2093-2101.

Rogers, H. W. and E. R. Unanue (1993). "Neutrophils are involved in acute, nonspecific resistance to Listeria monocytogenes in mice." Infect Immun **61**(12): 5090-5096.

Rooney, S., J. Chaudhuri and F. W. Alt (2004). "The role of the non-homologous end-joining pathway in lymphocyte development." Immunol Rev **200**: 115-131.

Rouleau, M., A. Patel, M. J. Hendzel, S. H. Kaufmann and G. G. Poirier (2010). "PARP inhibition: PARP1 and beyond." Nat Rev Cancer **10**(4): 293-301.

Rouse, J. and S. P. Jackson (2002). "Interfaces between the detection, signaling, and repair of DNA damage." Science **297**(5581): 547-551.

Samba-Louaka, A., J. M. Pereira, M. A. Nahori, V. Villiers, L. Deriano, M. A. Hamon and P. Cossart (2014). "Listeria monocytogenes dampens the DNA damage response." PLoS Pathog **10**(10): e1004470.

Sauer, J. D., K. Sotelo-Troha, J. von Moltke, K. M. Monroe, C. S. Rae, S. W. Brubaker, M. Hyodo, Y. Hayakawa, J. J. Woodward, D. A. Portnoy and R. E. Vance (2011). "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): 688-694.

Sauer, J. D., C. E. Witte, J. Zemansky, B. Hanson, P. Lauer and D. A. Portnoy (2010). "Listeria monocytogenes triggers AIM2-mediated pyroptosis upon infrequent bacteriolysis in the macrophage cytosol." Cell Host Microbe **7**(5): 412-419.

Savic, V., B. Yin, N. L. Maas, A. L. Bredemeyer, A. C. Carpenter, B. A. Helmink, K. S. Yang-Iott, B. P. Sleckman and C. H. Bassing (2009). "Formation of dynamic gamma-H2AX domains along broken DNA strands is distinctly regulated by ATM and MDC1 and dependent upon H2AX densities in chromatin." Mol Cell **34**(3): 298-310.

Sawa, T. and H. Ohshima (2006). "Nitrative DNA damage in inflammation and its possible role in carcinogenesis." Nitric Oxide **14**(2): 91-100.

Schroder, K., P. J. Hertzog, T. Ravasi and D. A. Hume (2004). "Interferon-gamma: an overview of signals, mechanisms and functions." J Leukoc Biol **75**(2): 163-189.

Schwab, S. R. and J. G. Cyster (2007). "Finding a way out: lymphocyte egress from lymphoid organs." Nat Immunol **8**(12): 1295-1301.

Sekiguchi, J., D. O. Ferguson, H. T. Chen, E. M. Yang, J. Earle, K. Frank, S. Whitlow, Y. Gu, Y. Xu, A. Nussenzweig and F. W. Alt (2001). "Genetic interactions between ATM and the nonhomologous end-joining factors in genomic stability and development." Proc Natl Acad Sci U S A **98**(6): 3243-3248.

Shen, Y., M. Naujokas, M. Park and K. Ireton (2000). "InIB-dependent internalization of Listeria is mediated by the Met receptor tyrosine kinase." Cell **103**(3): 501-510.

Sherman, M. H., C. H. Bassing and M. A. Teitell (2011). "Regulation of cell differentiation by the DNA damage response." Trends Cell Biol **21**(5): 312-319.

Sherman, M. H., A. I. Kuraishy, C. Deshpande, J. S. Hong, N. A. Cacalano, R. A. Gatti, J. P. Manis, M. A. Damore, M. Pellegrini and M. A. Teitell (2010). "AID-induced genotoxic stress promotes B cell differentiation in the germinal center via ATM and LKB1 signaling." Mol Cell **39**(6): 873-885.

Shiloh, M. U., J. D. MacMicking, S. Nicholson, J. E. Brause, S. Potter, M. Marino, F. Fang, M. Dinauer and C. Nathan (1999). "Phenotype of mice and macrophages deficient in both phagocyte oxidase and inducible nitric oxide synthase." Immunity **10**(1): 29-38.

Shiloh, Y. (2003). "ATM and related protein kinases: safeguarding genome integrity." Nat Rev Cancer **3**(3): 155-168.

Spink, J. and T. Evans (1997). "Binding of the transcription factor interferon regulatory factor-1 to the inducible nitric-oxide synthase promoter." J Biol Chem **272**(39): 24417-24425.

Stavru, F., C. Archambaud and P. Cossart (2011). "Cell biology and immunology of Listeria monocytogenes infections: novel insights." Immunol Rev **240**(1): 160-184.

Stetson, D. B. and R. Medzhitov (2006). "Type I interferons in host defense." Immunity **25**(3): 373-381.

Stucki, M., J. A. Clapperton, D. Mohammad, M. B. Yaffe, S. J. Smerdon and S. P. Jackson (2005). "MDC1 directly binds phosphorylated histone H2AX to regulate cellular responses to DNA double-strand breaks." Cell **123**(7): 1213-1226.

Tabeta, K., K. Hoebe, E. M. Janssen, X. Du, P. Georgel, K. Crozat, S. Mudd, N. Mann, S. Sovath, J. Goode, L. Shamel, A. A. Herskovits, D. A. Portnoy, M. Cooke, L. M. Tarantino, T. Wiltshire, B. E. Steinberg, S. Grinstein and B. Beutler (2006). "The Unc93b1 mutation 3d disrupts exogenous antigen presentation and signaling via Toll-like receptors 3, 7 and 9." Nat Immunol **7**(2): 156-164.

Takeuchi, O., K. Hoshino, T. Kawai, H. Sanjo, H. Takada, T. Ogawa, K. Takeda and S. Akira (1999). "Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components." Immunity **11**(4): 443-451.

Tam, M. A. and M. J. Wick (2004). "Dendritic cells and immunity to Listeria: TipDCs are a new recruit." Trends Immunol **25**(7): 335-339.

Tanaka, Y. and Z. J. Chen (2012). "STING specifies IRF3 phosphorylation by TBK1 in the cytosolic DNA signaling pathway." Sci Signal **5**(214): ra20.

Theunissen, J. W., M. I. Kaplan, P. A. Hunt, B. R. Williams, D. O. Ferguson, F. W. Alt and J. H. Petrini (2003). "Checkpoint failure and chromosomal instability without lymphomagenesis in Mre11(ATLD1/ATLD1) mice." Mol Cell **12**(6): 1511-1523.

Toller, I. M., K. J. Neelsen, M. Steger, M. L. Hartung, M. O. Hottiger, M. Stucki, B. Kalali, M. Gerhard, A. A. Sartori, M. Lopes and A. Muller (2011). "Carcinogenic bacterial pathogen Helicobacter pylori triggers DNA double-strand breaks and a DNA damage response in its host cells." Proc Natl Acad Sci U S A **108**(36): 14944-14949.

Torres, D., M. Barrier, F. Bihl, V. J. Quesniaux, I. Maillet, S. Akira, B. Ryffel and F. Erard (2004). "Toll-like receptor 2 is required for optimal control of Listeria monocytogenes infection." Infect Immun **72**(4): 2131-2139.

Travassos, L. H., S. E. Girardin, D. J. Philpott, D. Blanot, M. A. Nahori, C. Werts and I. G. Boneca (2004). "Toll-like receptor 2-dependent bacterial sensing does not occur via peptidoglycan recognition." EMBO Rep **5**(10): 1000-1006.

Tripp, C. S., S. F. Wolf and E. R. Unanue (1993). "Interleukin 12 and tumor necrosis factor alpha are costimulators of interferon gamma production by natural killer cells in severe combined immunodeficiency mice with listeriosis, and interleukin 10 is a physiologic antagonist." Proc Natl Acad Sci U S A **90**(8): 3725-3729.

Uematsu, S., M. H. Jang, N. Chevrier, Z. Guo, Y. Kumagai, M. Yamamoto, H. Kato, N. Sougawa, H. Matsui, H. Kuwata, H. Hemmi, C. Coban, T. Kawai, K. J. Ishii, O. Takeuchi, M. Miyasaka, K. Takeda and S. Akira (2006). "Detection of pathogenic intestinal bacteria by Toll-like receptor 5 on intestinal CD11c+ lamina propria cells." Nat Immunol **7**(8): 868-874.

Unanue, E. R. (1997). "Inter-relationship among macrophages, natural killer cells and neutrophils in early stages of Listeria resistance." Curr Opin Immunol **9**(1): 35-43.

Via, L. E., R. A. Fratti, M. McFalone, E. Pagan-Ramos, D. Deretic and V. Deretic (1998). "Effects of cytokines on mycobacterial phagosome maturation." J Cell Sci **111 (Pt 7)**: 897-905.

Vielfort, K., N. Soderholm, L. Weyler, D. Vare, S. Lofmark and H. Aro (2012). "Neisseria gonorrhoeae infection causes DNA damage and affects the expression of p21, p27 and p53 in non-tumor epithelial cells." J Cell Sci **126**(Pt 1): 339-347.

Villanueva, M. S., A. J. Sijts and E. G. Pamer (1995). "Listeriolysin is processed efficiently into an MHC class I-associated epitope in Listeria monocytogenes-infected cells." J Immunol **155**(11): 5227-5233.

von Moltke, J., J. S. Ayres, E. M. Kofoed, J. Chavarria-Smith and R. E. Vance (2013). "Recognition of bacteria by inflammasomes." Annu Rev Immunol **31**: 73-106.

Warren, S. E., A. Armstrong, M. K. Hamilton, D. P. Mao, I. A. Leaf, E. A. Miao and A. Aderem (2010). "Cutting edge: Cytosolic bacterial DNA activates the inflammasome via Aim2." J Immunol **185**(2): 818-821.

Way, S. S., L. J. Thompson, J. E. Lopes, A. M. Hajjar, T. R. Kollmann, N. E. Freitag and C. B. Wilson (2004). "Characterization of flagellin expression and its role in Listeria monocytogenes infection and immunity." Cell Microbiol **6**(3): 235-242.

Witte, C. E., K. A. Archer, C. S. Rae, J. D. Sauer, J. J. Woodward and D. A. Portnoy (2012). "Innate immune pathways triggered by Listeria monocytogenes and their role in the induction of cell-mediated immunity." Adv Immunol **113**: 135-156.

Woodward, J. J., A. T. Iavarone and D. A. Portnoy (2010). "c-di-AMP secreted by intracellular Listeria monocytogenes activates a host type I interferon response." Science **328**(5986): 1703-1705.

Wu, J., T. Fernandes-Alnemri and E. S. Alnemri (2010). "Involvement of the AIM2, NLRC4, and NLRP3 inflammasomes in caspase-1 activation by Listeria monocytogenes." J Clin Immunol **30**(5): 693-702.

Wu, Z. H., Y. Shi, R. S. Tibbetts and S. Miyamoto (2006). "Molecular linkage between the kinase ATM and NF-kappaB signaling in response to genotoxic stimuli." Science **311**(5764): 1141-1146.

Xiao, A., H. Li, D. Shechter, S. H. Ahn, L. A. Fabrizio, H. Erdjument-Bromage, S. Ishibe-Murakami, B. Wang, P. Tempst, K. Hofmann, D. J. Patel, S. J. Elledge and C. D. Allis (2009). "WSTF regulates the H2A.X DNA damage response via a novel tyrosine kinase activity." Nature **457**(7225): 57-62.

Xie, Q. W., R. Whisnant and C. Nathan (1993). "Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon gamma and bacterial lipopolysaccharide." J Exp Med **177**(6): 1779-1784.

Yamamoto, M., S. Sato, H. Hemmi, K. Hoshino, T. Kaisho, H. Sanjo, O. Takeuchi, M. Sugiyama, M. Okabe, K. Takeda and S. Akira (2003). "Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway." <u>Science</u> **301**(5633): 640-643.

Yu, Q., Y. V. Katlinskaya, C. J. Carbone, B. Zhao, K. V. Katlinski, H. Zheng, M. Guha, N. Li, Q. Chen, T. Yang, C. J. Lengner, R. A. Greenberg, F. B. Johnson and S. Y. Fuchs (2015). "DNA-damage-induced type I interferon promotes senescence and inhibits stem cell function." <u>Cell Rep</u> **11**(5): 785-797.

Zha, S., J. Sekiguchi, J. W. Brush, C. H. Bassing and F. W. Alt (2008). "Complementary functions of ATM and H2AX in development and suppression of genomic instability." <u>Proc Natl Acad Sci U S A</u> **105**(27): 9302-9306.

Zhou, B. B. and S. J. Elledge (2000). "The DNA damage response: putting checkpoints in perspective." <u>Nature</u> **408**(6811): 433-439.

Ziv, Y., D. Bielopolski, Y. Galanty, C. Lukas, Y. Taya, D. C. Schultz, J. Lukas, S. Bekker-Jensen, J. Bartek and Y. Shiloh (2006). "Chromatin relaxation in response to DNA double-strand breaks is modulated by a novel ATM- and KAP-1 dependent pathway." <u>Nat Cell Biol</u> **8**(8): 870-876.