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Abstract
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Doctor of Philosophy in Molecular and Cell Biology

University of California, Berkley

Professor Gregory M. Barton, Chair

Most of our understanding about the cellular response to microbes by innate immune cells is shaped by our work with macrophages in culture. Because there exist such a large number of specialized innate immune cells, it stands to reason that the response to pathogen by different cell types will be specialized, even if the same innate immune receptors are used – my work documents two such incidents. Here we report that TLR2 activation by multiple viruses leads to production of type I interferon (IFN) only in $Ly6C^{high}$ inflammatory monocytes. Importantly, TLR2-dependent induction of type I IFN only occurs in response to viruses, not bacterial TLR2 ligands, indicating that TLR2 is capable of discriminating between these pathogen classes. Separately, we demonstrate that bone marrow neutrophils need to be "armed" by GM-CSF before they respond to TLR ligands, unlike other innate immune cells. We then go on to show that certain pro-inflammatory signals *in vivo* are sufficient to "arm" neutrophils so they could more readily respond to pathogen associated microbial patterns.

This manuscript is dedicated to Saul Katsap.

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Chapter 1: Introduction to Innate Immunity

As the first line defense against microbes, the innate immune system is essential for the clearance of environmental microbes and for restricting the replication of pathogenic microbes until the adaptive immune system attempts to clear the infection.

Since the discovery of toll-like receptors, the innate immune system has been viewed as a singular entity – this view has reduced the analysis of the innate immune system to the study of a macrophage in the petri dish and then hoping that the observation applies *in vivo*. Recently, it has become appreciated that the innate immune system consists of a collection of parts that must work together within a diverse environment. It has become clear that "splenic/macrophage immunology" ignores many interesting aspects of how the immune system addresses persistent symbiotic microbial interactions throughout the gastrointestinal tract and transient interaction with microbes within other mucosal tissues, such as the lungs. Many immunoregulatory pathways that are absent from the cultured macrophages have been shown to be important in maintaining homeostasis in the gastrointestinal tract. In all, these recent efforts have enabled us to appreciate the spatial specialization that the innate immune uses to deal with microbes in tissues that are not normally sterile.

Importantly, this multi-organ view has reinforced the idea that the innate immune system is not monolithic, but has multiple levels of specialization. Yet even this multi-organ view of the innate immune system does not incorporate the temporal nature of the immune response; upon infection there are waves of specialized innate immune cells that encounter the pathogen – whether these cells respond to pathogens in a specialized manner is not well understood. Again, most of our understanding about the response to microbes by innate immune cells is shaped by our work with macrophages in culture. *Because there exist such a large number of specialized innate immune cells, it stands to reason that the response to pathogen by different cell types will be specialized, even if the same innate immune receptors are used – my work documents two such incidents.*

Inception

The recent resurgence of innate immunity can most directly be attributed to Charles Janeway Jr. who in 1989 gave a lecture in which he argued that the innate immune system had been largely overlooked (Janeway, 1989). He pointed out that, that the need for complete Freund's

adjuvant in order to elicit a strong immune response against a foreign antigen – something he termed the "immunologist's dirty little secret" – suggested that microbial ligands are needed to provide "signal two", which was known to be necessary to activate lymphocytes. He went on to argue that a series of pattern recognition receptors (PRRs) must exist that recognizes microbial molecules, which are the product of "complex and critical enzymology in the microorganism" and these pathogen associated microbial patterns (PAMPs) must be unique to microorganisms. Eight years later, Janeway's hypothesis was proven correct by the cloning of the first PRR (Medzhitov et al., 1997), which was later recognized to be Toll-like receptor 4 (TLR4). Quickly thereafter, TLR4 was shown to recognize lipopolysaccharide (Poltorak et al., 1998).

Membrane-bound Receptors

Since the initial cloning and identification of TLR4 and its ligand, the cloning of additional PPRs and the identification of their ligands has been accomplished at an outstanding rate. TLR4 was recognized to be a member of a family – the toll-like receptors (TLRs). TLRs are all transmembrane proteins with ectodomains comprised of a series of leucine-rich repeats which recognize PAMPS and a Toll/interleukin-1 receptor-like domain in the cytosol that transduces signal and ultimately leads to Nf-κB activation. Additional families of transmembrane PRRs have been identified – most notably, Dectin-1 which is a C-type lectin. Interestingly, Dectin-1 uses entirely different domains to bind ligand and transduce signal (Takeuchi and Akira, 2010).

The ligands for these receptors have provided several insights into both host and microbe biology. TLR2, TLR4 and Dectin-1 all recognize components of bacteria or fungal cell wall – which as Janeway described are the product of "complex and critical enzymology in the microorganism". Yet other TLRs bind much more enigmatic ligands - TLRs 3,7&9 all recognize nucleic acids – which are obviously not uniquely foreign, as RNA & DNA play a indispensible role in the body. How the host discriminates between foreign and self DNA is still not entirely understood but is an area of intense research (Ewald and Barton, 2011). Lastly, TLR5 has been shown to recognize bacterial flagellin. Flagellin is an intriguing target because it is a bacterial protein; as proteins can be easily mutated, it is interesting to consider that most bacteria are unable to mutate flagellin to avoid recognition – suggesting that with sufficient structural limitations placed upon proteins, the host can recognize some as being foreign.

Cytosolic Receptors

While the first family of PRRs discovered were the membrane-bound TLR family of receptors, several other families of receptors have been identified that reside within the cytosol of cells and recognize a wide diversity of ligands.

Like the TLRs, the Nod-like receptors (NLRs) activate an Nf-κB cascade upon activation. The NLRs importance was first recognized when Nod2 was found to be associated with susceptibility to Crohn's disease. These observations with the knowledge that the NLRs contained a LRR domain, made the NLRs candidate PRRs. Ultimately, it was shown that Nod1 and Nod2 recognize bacterial cell wall – just like TLR2. Specifically, they recognize bacterial peptidoglycans, Nod1 recognizes g-D-glutamyl-meso-diaminopimelic acid and Nod2 recognizes muramyl dipeptide(Takeuchi and Akira, 2010).

Another family of receptors that are found in the cytosol recognizes flagellin – the same protein that TLR5 recognizes on the cell surface. This again suggests that flagellin is a unique protein, in that it is one of the few proteins that are sufficiently restrained in its ability to mutate, and therefore the innate immune system has targeted it as a PAMP. Interestingly this family of receptors – IPAF and Naip5 – does not activate the Nf-κB pathway unlike most other PRRs – this family of receptors activates a pro-inflammatory cell death – termed "inflammasome activation" (which is reviewed in chapter two). Why the activation of these receptors leads to inflammasome activation and cell death instead of Nf-κB like is still not understood (Lightfield et al., 2008).

Additionally, there exist multiple receptors that recognize nucleic acids in the cytosol of cells. Chapter two provides a complete summary of them, their regulation, and their ligands.

Chapter 2: Cytosolic sensors of nucleic acids

Nucleic acid sensing TLRs sample the lumenal contents of endolysosomal compartments and respond to ligands released from degraded microbes. Accordingly, TLRs enable the detection of viruses prior to productive infection of a cell. A different family of receptors is required to detect nucleic acids derived from microbes that enter the cytosol. In the last few years, a number of proteins involved in cytosolic nucleic acid sensing have been described. These pathways are expressed in many more cell types than TLRs, which presumably enables any cell to sense a cytosolic pathogen in a cell-autonomous fashion. In the following section, we will review our knowledge of the nucleic acid cytosolic sensors and their known ligand specificity.

Recognition of RNA by cytosolic receptors

After the identification of the nucleic acid–sensing TLRs and their cognate ligands, investigators soon realized that another class of receptors must exist. Although the response to Poly(I:C) was reduced in TLR3-deficient cells, it was not completely abrogated(Alexopoulou et al., 2001). Subsequent studies from Reis e Sousa's group demonstrated that delivery of Poly(I:C) into the cytosol of cells induced type I IFN in a TLR3-independent manner (Diebold et al., 2003). This observation led to the identification of an entire family of receptors that are required for the sensing of viral RNA products in the cytosol of infected cells.

RIG-I like receptors

In a cDNA screen to identify genes that enhance activation of an IFN-β promoter in response to cytosolic Poly(I:C), the Fujita lab cloned a gene, retinoic acid inducible gene-I (RIG-I), which became the founding member of the RLR family of receptors (Yoneyama et al., 2004). Interestingly, this initial RIG-I clone encoded a protein truncated after the two N-terminal caspase recruitment domains (CARDs) and was able to activate IRF3 independently of ligand. Full-length RIG-I also contains a central DEAD box helicase/ATPase domain whose ATPase function is necessary for IRF3 activation, and a C-terminal regulatory domain that prevents constitutive activation of the protein.

The two other members of the RLR family are Mda5 and Lgp2. Mda5 has a domain structure similar to RIG-I: N-terminal CARD domains, a central DEAD box helicase/ATPase domain, and a C-terminal regulatory

domain (Yoneyama et al., 2005). Similar to RIG-I, Mda5 has been shown to activate the IRF3 pathway in response to Poly (I:C). In contrast, Lgp2 lacks the N-terminal CARD domains necessary for IRF3 activation and consists only of the central DEAD box helicase/ATPase domain and the C-terminal domain (Yoneyama et al., 2005). Lack of a CARD domain suggests that Lgp2 cannot induce downstream signaling, and an initial report supported the conclusion that Lgp2 functions as a negative regulator of the RIG-I/Mda5 pathways (Yoneyama et al., 2005). However, more recent work using Lgp2-deficient mice suggests that Lgp2 acts a co-receptor for some Rig-I and Mda5 ligands (Satoh et al., 2010). These conflicting results are likely due to the use of different ligands, different cell types, and the difficulty with interpreting protein over-expression data.

A recent report has implicated Nod2 as another cytosolic sensor of ssRNA (Sabbah et al., 2009), which is quite surprising because Nod2 is also a sensor of muramyldipeptide (MDP) derived from peptidoglycan (Girardin et al., 2003; Inohara et al., 2003). Whereas Nod2 induces a classic pro-inflammatory signature in response to MDP, the receptor induces type I IFN in response to ssRNA. Nod2 has an N-terminal CARD domain, a central nucleotide-binding domain (NBD), and a C-terminal LRR domain. The double life of Nod2 raises several questions relevant to a number of other innate immune sensors. How can one receptor recognize molecules with such distinct molecular structures (such as MDP and ssRNA)? Moreover, as discussed in the previous section with regard to TLR7 and TLR9, how does recognition of distinct ligands by the same receptor lead to such different signaling pathways?

Protein kinase R (PKR) has also been implicated in dsRNA recognition (Williams, 1999). The identification of RIG-I and Mda5, however, has raised questions regarding the role played by PKR in dsRNA sensing. Little work has been done to understand how PKR fits within the framework of these newly discovered RLRs, but a recent paper suggests that PKR is necessary for stabilizing IFN-α/β transcripts downstream of Mda5 but not RIG-I signaling (Schulz et al., 2010). While this observation might explain why PKR-deficient cells and animals respond poorly to virus, it is still not understood how PKR is activated. While PKR contains a dsRNA binding domain, it is still not clear whether it functions as a PRR independently of RIG-I and Mda5.

Little is known about how any of these receptors RIG-I, Mda5, or Nod2 recognizes RNA. While none of the proteins contain classic RNA binding domains, a substantial amount of work has defined which domains of RIG-I are important for binding RNA. Two reports have demonstrated

that the C-terminal regulatory domain of RIG-I, which is necessary to prevent constitutive activation (see discussion above), also binds RNA (Cui et al., 2008; Takahasi et al., 2008). While the mechanism of RLR activation remains incomplete, a model has been proposed where the regulatory domain maintains RIG-I in an inactive form until RNA is bound (Yoneyama and Fujita, 2008). Upon binding RNA, a conformational change occurs which frees the N-terminal CARD domains and allow recruitment of downstream adapters.

Ligand specificity of RLR proteins

The discovery of RIG-I and Mda5 raised the central question of how these receptors discriminate between self and non-self RNA. Unlike endolysosomes or phagosomes, the cytosol contains many self-RNAs, which RIG-I and Mda5 must somehow ignore while retaining the ability to respond to pathogen-derived molecules. Most of the studies in the field have used synthetic ligands to define the ligand specificity of RIG-I and Mda5. While the use of such ligands does not lead to better understanding of host-pathogen interactions, it can address how RIG-I and Mda5 are able to discriminate between self and non-self RNAs.

When first characterized, both RIG-I and Mda5 were implicated in recognition of Poly(I:C). With the generation of gene-targeted mice, though, the roles of these two receptors have been dissected with more precision. Initial reports suggested that Mda5, but not RIG-I, recognizes cytosolic Poly(I:C) (Gitlin et al., 2006; Kato et al., 2006), while a more recent report has implicated both family members in Poly(I:C) sensing (Kato et al., 2008). These conflicting results may be explained by the observation that long (>2kb) polymers of Poly(I:C) are preferentially recognized by Mda5 while smaller polymers (as short as 70bp) are recognized by RIG-I (Kato et al., 2008). Thus, the preferential recognition of different sizes of RNA may be an important functional difference between RIG-I and Mda5, although the relevance of these distinct specificities for pathogen recognition remains unclear.

Another synthetic ligand recognized by RIG-I is short, single-stranded, uncapped 5'-triphosphate RNA (Hornung et al., 2006; Pichlmair et al., 2006). Whereas host mRNAs are capped with a 7-methyl-guanosine group, many viral RNAs remain uncapped, providing a potential mechanism for self/non-self discrimination by RIG-I. Recent work has suggested that single-stranded uncapped 5'-triphosphate RNAs are unable to activate RIG-I without small regions of basepairing (Schlee et al., 2009; Schmidt et al., 2009). To explain these discrepancies, the more recent reports suggest that

T7 transcribed RNAs (which were used in the earlier studies) contain a small amount of double-stranded RNAs. An additional complication to understanding the requirements for RIG-I activation has been reports that short double-stranded RNAs without 5'-triphosphate can also activate RIG-I (Kato et al., 2008; Takahasi et al., 2008).

In parallel with efforts to understand the molecular mechanisms underlying RNA detection by RIG-I and MDA5, the role these receptors play in immunity to specific viral pathogens has received a great deal of attention. Viruses can generally be divided into three categories: those only recognized by RIG-I (flavivirus, orthomyxovirus), those only recognized by Mda5 (picornavirus), and those recognized by both RIG-I and Mda5 (paramyxovirus, reovirus, flavivirus) (Fredericksen et al., 2008; Gitlin et al., 2006; Kato et al., 2006; Wilkins and Gale, 2010). For many of the viruses listed above, *in vivo* work has confirmed the importance of these receptors in host defense (Gitlin et al., 2006; Gitlin et al., 2010; Kato et al., 2006; Koyama et al., 2007; Suthar et al., 2010). While functional interactions between different families of viruses and different receptors have been described – very little work has been done to understand why some viruses activate certain receptors and not others. The simplest explanation is that certain viruses either lack the appropriate ligand for RIG-I or Mda5 or have evolved evasion strategies for specific RLR family members. Ultimately, more comparative analysis will need to be performed between different viruses and different receptors to understand the nature of these specificities.

RLR accessory proteins

The initial experiments that elucidated the function of the RLRs used downstream events such as type I IFN production or IRF3 activation as a proxy for RLR activation. While there is substantial overlap between the multi-protein complexes that TLRs and RLRs used to activate IRF3 (e.g., TBK1, IKKi, and TRAF3) (Fitzgerald et al., 2003; Häcker et al., 2006; Oganesyan et al., 2006), as reviewed below, many of the proximal adapters used by RLRs to activate IRF3 are unique to the RLR family of receptors.

MAVS

MAVS (also known as IPS-1, CARDIF, and VISA) is an essential adapter that connects RIG-I/Mda5/Nod2 activation with IRF3 phosphorylation. Identified by four different groups simultaneously using a combination of candidate gene approaches and cDNA screening (Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005; Xu et al., 2005), MAVS contains an N-terminal CARD domain that mediates its association with

RLRs and is essential for activation of the IRF3 axis. In addition to a CARD domain, MAVS contains a C-terminal transmembrane domain that targets MAVS to the mitochondrial outer membrane (Seth et al., 2005). This mitochondrial localization is essential for function as cytosolic MAVS is unable to activate IRF3 (Seth et al., 2005). However, the underlying mechanism for this localization requirement remains unclear. To further complicate matters, Dixit et al. have recently demonstrated that MAVS is also localized to peroxisomes (Dixit et al.). Moreover, the subcellular localization of MAVS appears to have functional consequences: MAVS signaling from the peroxisomes induces antiviral genes without type I IFN, while MAVS signaling from the mitochondria leads to production of type I IFN (Dixit et al.). The mitochondrial response is delayed compared to signaling from the peroxisome, suggesting that MAVS signaling from distinct compartments may occur with distinct kinetics. The mechanisms responsible for this transcriptional specificity remain unclear.

Despite these recent breakthroughs, many questions remain about the cell biology of RLR signaling. Why does MAVS require membrane association for signaling? RIG-I and Mda5 are cytosolic, so why must the downstream signaling events take place on very specific cellular organelles? One possibility is that MAVS associates with other signaling components that only localize to specific organelles. However, it is difficult to reconcile this model with the observation that MAVS can signal from the cytosol after being artificially dimerized (Tang and Wang, 2009). It will be important to examine whether additional intracellular receptors require association with specific organelles to organize signaling complexes. As of now only the nucleic acid sensing intracellular receptors seem to require this level of organization (see below for DNA).

TRIM25

TRIM25 is an E3 ligase that directly ubiquitinates RIG-I (Gack et al., 2007). TRIM25 dependent ubiquitination of RIG-I is necessary for recruitment of MAVS and subsequent signal transduction. While these data could suggest that ubiquitination of RIG-I is sufficient to activate the IRF3 pathway, it has been shown that RIG-I is ubiquitinated independently of activation, indicating that additional steps are required to initiate signaling. Thus, TRIM25 dependent ubiquitination might serve to prime RIG-I prior to binding viral RNA.

STING

The transmembrane protein STING (also known as MITA and ERIS) has also been implicated in the RLR signaling pathway, although its precise role is somewhat confusing (Ishikawa and Barber, 2008; Sun et al., 2009; Zhong et al., 2008). STING is required for maximal RIG-I signaling but it is not necessary for Mda5 signaling (Ishikawa and Barber, 2008). These data are difficult to reconcile with the observations that overexpression of MAVS leads to STING-dependent IRF3 activation, yet Mda5 activation of IRF3 is MAVS-dependent but STING-independent (Ishikawa and Barber, 2008). Another confusing aspect of STING biology is its localization; while controversial, several groups have demonstrated that STING is ER resident (Sun et al., 2009). However, STING also has been shown to interact with MAVS (Zhong et al., 2008), which is primarily found on the mitochondrial membrane (see above). Why activation of the IRF3 axis requires signaling across so many different organelles is not understood, but understanding these requirements may provide insight into antiviral immunity.

Recognition of DNA by cytosolic receptors

In 2006 the Medzhitov and Akira groups demonstrated the existence of an additional pathway of DNA recognition (Ishii et al., 2006; Stetson and Medzhitov, 2006a). While no receptor for this pathway was identified in these initial studies, the two reports demonstrated that a "DNA sensor(s)" in the cytoplasm of cells could lead to the activation of the IRF3 pathway. While both groups demonstrated that multiple sources of DNA could activate the DNA sensor, they each focused on different DNA molecules. While the Akira group used a long polymer of a poly(dA-dT)•poly(dT-dA) DNA, the Medzhitov lab used a much smaller (45mer) dsDNA oligo (called immunostimulatory DNA, or ISD) that lacked CpG motifs. Both ligands activate similar pathways in cells, but it is unclear if the same receptors recognize both ligands.

To date, very little is known about the specificity of putative cytosolic DNA sensors for substrate DNA molecules. Mice that lack DNaseII die from an autoimmunity before birth likely due to the presence of self-DNA in the cytosol. This observation suggests that DNA sensor(s) cannot discriminate between self and non-self DNA that gains access to the cytosol (Yoshida et al., 2005). This lack of specificity is probably tolerated because the cytosolic compartment normally does not contain DNA.

Finally, it has been demonstrated that transfected poly(dA-dT) can be transcribed by RNA polymerase III (PolIII) to generate RNA lacking a 5' cap. This transcribed RNA can activate a RIG-I dependent cytosolic

response (Ablasser et al., 2009; Chiu et al., 2009). While the role for Pol III in host defense is not well understood, this pathway has certainly made the analysis of the "DNA sensor(s)" more complicated.

DNA sensor(s) and accessory proteins

While many of the primary receptors and adapters for the sensing of cytosolic RNA were discovered relatively quickly, the receptors for the sensing of cytosolic DNA have remained controversial and elusive.

Using a candidate gene approach, the Taniguchi group identified DAI (also known as DLM-1 and ZBP1) as the putative DNA sensor. In L929 cells, knocking down DAI led to a decrease in type I IFN in response to transfected dsDNA (Takaoka et al., 2007). Additionally, DAI was shown to bind DNA and interact with the key transcription factor IRF3. While these data certainly suggested that DAI is the DNA sensor, subsequent analyses of mice lacking DAI found no defect in the response to transfected DNA in mouse embryonic fibroblast (MEFS) or DCs (Ishii et al., 2008). The lack of a phenotype suggests either that DAI is not a DNA sensor or that there is functional redundancy in certain cell types. More recent work by the Taniguchi group has argued the latter point, showing that many cell types possess multiple DNA sensors (Wang et al., 2008).

The only gene product that has been shown to be essential for the activation of the IRF3 pathway in response to cytoplasmic DNA is STING (Ishikawa et al., 2009). While STING is necessary for maximal RIG-I signaling, there is stronger evidence for its role in sensing DNA (Ishikawa et al., 2009). STING is absolutely essential for type I IFN production in response to cytosolic DNA. How STING mediates signal transduction is completely unknown. STING has multiple transmembrane domains that are necessary for function, suggesting that it may function as an adapter or scaffold protein (Ishikawa and Barber, 2008). Interestingly, several groups have noted that STING translocates from the ER to punctate perinuclear structures upon stimulation, yet the significance of this translocation is not understood (Ishikawa and Barber, 2008; Saitoh et al., 2009).

Cyclic dinucleotides

Bacterial-derived cyclic dinucleotides that enter the host cytosol have recently been shown to activate cytosolic innate receptors. These small molecules are used by bacterial cells as secondary messengers (Schirmer and Jenal, 2009). Initially, c-di-GMP was shown to be immunostimulatory when injected into mice (Karaolis et al., 2007). This immunostimmulatory activity was shown to be dependent on the delivery of c-di-GMP to the cytosol of

cells but appears to be independent of all known cytosolic sensing pathways (McWhirter et al., 2009). The relevance of cyclic dinucleotides as ligands for innate sensors was unclear until quite recently, when it was shown that secreted c-di-AMP from *Listeria monocytogenes* activates a cystolic immune surveillance program in infected cells (Woodward et al., 2010). Interestingly, many bacteria have been reported to activate a type I IFN response upon accessing the cytosol of eukaryotic cells (Monroe et al., 2010). It will be interesting to see if other bacteria are sensed via the presence of various bacterial cyclic dinucleotides in the cytosol of host cells.

Inflammasome activation by cytosolic DNA

All of the cytosolic RNA and DNA sensing pathways discussed thusfar induce type I IFN production, yet another nucleic acid sensing receptor was recently reported that leads to a very different cellular response – activation of the inflammasome. The inflammasome cleaves pro-IL-1β to its active form and induces a form of rapid cell death - termed pyroptosis (Mariathasan and Monack, 2007). For several years, activation of the inflammasome was known to be induced either by the presence of cytosolic flagella that activates the IPAF/NAIP5 inflammasome or by several forms of extracellar particles that activate the NALP3 inflammasome (Mariathasan and Monack, 2007). In 2008, the Tschopp group demonstrated the existence of an additional inflammasome responsible for sensing cytosolic DNA (Muruve et al., 2008). Using a variety of ligands, they have demonstrated that the DNA sensing inflammasome responds to dsDNA over 250bp in length (Muruve et al., 2008). While the inflammasome DNA sensor responds to a long polymer of poly(dA-dT)•poly(dT-dA)), it does not recognize the synthetic ISD ligand (used previously by the Medzhitov group), suggesting that the inflammasome DNA sensor has an overlapping but unique specificity relative to the DNA sensor(s). It remains unclear how the decision to induce type I IFN versus activate the inflammasome is made.

Using a candidate gene approach, several groups have identified AIM2 as the sensor that activates the inflammasome upon the introduction of cytosolic DNA (Burckstummer et al., 2009; Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Roberts et al., 2009). The two domains that are important for AIM2 function (and that were used to identify AIM2) are the HIN2000 DNA binding domain and the pyrin domain that interacts with inflammasome adapter protein ASC. While the role of AIM2 was first demonstrated by siRNA knockdown, AIM2-deficient mice have recently been generated and are unable to activate the inflammasome in response to DNA (Fernandes-Alnemri et al., 2010; Jones et al., 2010; Rathinam et al.,

2010). Very little is known about the specificity of AIM2. It seems that AIM2 is able to recognize DNA in a sequence-independent manner and that both bacterial and viral DNA are able to activate AIM2 (Rathinam et al., 2010; Sauer et al., 2010). This apparent lack of specificity is not surprising because host DNA is normally not found in the cytoplasm of eukaryotic cells.

Chapter 3: Toll-like receptor 2 on inflammatory monocytes induces type I interferon in response to viral but not bacterial ligands.

Introduction

Receptors of the innate immune system have evolved to recognize conserved microbial features that represent broad pathogen classes (Medzhitov, 2007). This strategy ensures that diverse pathogen species can be quickly recognized by the host, as long as these microbial features are sufficiently constrained that they remain invariant. Examples of such features are the bacterial cell wall components lipopolysaccharide (LPS) and peptidoglycan. Members of the Toll-like receptor (TLR) family recognize these and other microbial ligands and induce signals important for initiation of both innate and adaptive immunity (Medzhitov, 2007). Accordingly, mice lacking TLR function show increased susceptibility to infection.

Viral recognition by the innate immune system is more challenging than recognition of other pathogen classes because of the relative paucity of conserved features (Kawai and Akira, 2006). Viruses replicate within host cells, and they do not generate any of the unique biochemical products present in bacterial and fungal cell walls. It has been argued that this lack of conserved viral features has forced the innate immune system to use nucleic acid as a means of detecting viral infection. Indeed, several members of the TLR family recognize nucleic acids: TLR3 recognizes dsRNA, TLR7 and TLR8 recognize ssRNA, and TLR9 recognizes CpG motifs in DNA (Kawai and Akira, 2006). In addition, a family of cytosolic receptors, including RIG-I, MDA-5, DAI, and AIM2, recognizes various nucleic acid species in the cytosol (Burckstummer et al., 2009; Fernandes-Alnemri et al., 2009; Hornung et al., 2009; Kawai and Akira, 2006; Roberts et al., 2009). Targeting nucleic acids allows for the recognition of highly diverse viral species with only a few innate receptors.

One of the key components of antiviral immunity is induction of the type I interferon (IFN) family of cytokines, hereafter referred to as type I IFN (Stetson and Medzhitov, 2006b). Type I IFN induces hundreds of genes that promote an antiviral state in cells. The importance of this signaling network is illustrated by the extreme susceptibility of mice lacking the type I IFN receptor (van den Broek et al., 1995). All of the nucleic acid sensing TLRs induce type I IFN, underscoring the importance of the cytokine family in antiviral immunity. For TLR7 and TLR9, though, induction of type I IFN only occurs in plasmacytoid dendritic cells (pDCs) via the common signaling adaptor MyD88. In other cell types, activation of TLR7 and TLR9 does not lead to type I IFN production (Gilliet et al., 2008). Similarly, most

TLRs involved in bacterial or fungal recognition (TLR2 and TLR5) are not expressed in pDCs (Kadowaki et al., 2001) and do not induce type I IFN in other cell types. The notable exception is TLR4, which can induce type I IFN in macrophages and conventional DCs via the signaling adaptor TRIF (Yamamoto et al., 2003). Nevertheless, type I IFN clearly plays a less critical role for antibacterial immunity than for antiviral immunity (Auerbuch et al., 2004; O'Connell et al., 2004).

By multiple criteria, viral proteins would seem poor choices as targets for innate receptors relative to nucleic acids. First, any given viral protein is unlikely to be shared among diverse viruses. Second, innate recognition of a viral protein would likely select for mutants that escape recognition yet retain function, if at all possible. Nevertheless, several viruses do encode proteins that are capable of stimulating TLR2, a receptor known to recognize multiple bacterial and fungal cell wall components. The best-characterized example is stimulation of TLR2 by glycoprotein B from human cytomegalovirus (HCMV) (Boehme et al., 2006; Compton et al., 2003), but mouse cytomegalovirus (MCMV) (Szomolanyi-Tsuda et al., 2006), Herpes simplex virus 1 and 2 (HSV-1 and HSV-2) (Kurt-Jones et al., 2004; Sato et al., 2006), Hepatitis C virus (Chang et al., 2007), Lymphocytic choriomeningitis virus (Zhou et al., 2005), measles virus (Bieback et al., 2002), and vaccinia virus (Zhu et al., 2007) are also capable of stimulating TLR2. In some of these cases, it seems that viruses benefit in some way from the stimulation of TLRs. For instance, measles virus may have evolved the ability to activate TLR2 as a means of upregulating the viral entry receptor, CD150 (Bieback et al., 2002)). In other examples, however, there is evidence that TLR2 activation contributes to protection. Most notably, mice lacking TLR2 are impaired in their ability to mount an innate or adaptive immune response against vaccinia virus (Zhu et al., 2007). One problematic aspect of any general role for TLR2 in antiviral immunity, however, is the apparent inability of this receptor to induce type I IFN (Doyle et al., 2002; Kawai et al., 2001; Toshchakov et al., 2002).

Here we describe a specialized role for TLR2 in innate recognition of several viruses. In contrast to the well-documented transcriptional response induced by bacterial ligands, we show that TLR2 induces type I IFN when activated by viruses. This signaling pathway is unique to inflammatory monocytes. The functional specialization of these cells is conceptually analogous to the role played by pDCs in TLR7 and TLR9 signaling and likely represents a general strategy to achieve specificity within innate immune signaling.

Results

TLR2-mediated recognition of DNA viruses

TLR2 has been implicated in the recognition of several DNA viruses, including vaccinia virus, HCMV, MCMV, HSV-1, and HSV-2. These viruses contain ligands that can activate additional TLRs (e.g., TLR9), so we first sought to determine the relative contribution made by TLR2 for viral recognition. To minimize potential viral interference with innate immune signaling, we UV-inactivated each virus prior to stimulating cells. Both vaccinia virus and MCMV induced NF-kB activation in HEK293 cells stably expressing murine TLR2 but not in control cells (**Fig. 3.1a**), supporting a role for TLR2 in recognition of these viruses. We directly tested the relative contributions of TLR2 and TLR9 by stimulating bone marrow derived dendritic cells (DCs) from TLR2-deficient, TLR9-deficient, Myd88-deficient, and control mice. Surprisingly, TNF production by DCs was entirely TLR2-dependent (**Fig. 3.1b**). TLR9 appeared to play little role in these cells. Similar results were obtained with bone marrow derived macrophages (**Fig. 3.1c**). These data suggest that TLR2 may play a primary role in the recognition of certain viruses.

TLR2 induces type I IFN

The lack of TLR9 activation in DCs and macrophages treated with vaccinia virus or MCMV is at odds with the well-documented role played by TLR9 in recognition of DNA viruses (Krug et al., 2004). One potential explanation for this discrepancy is that TLR9-mediated virus recognition is best observed in pDCs. To address this possibility, we used bone marrow cells as a source of *ex vivo* pDCs and measured the production of type I IFN in response to MCMV and vaccinia virus. As previously described, MCMV induced potent production of type I IFN and this response was reduced in TLR9-deficient cells (**Fig. 3.2a**). Surprisingly, type I IFN production in response to MCMV was also partially TLR2-dependent. Moreover, the production of type I IFN in response to vaccinia virus was entirely TLR2-dependent (**Fig. 3.2a**). A similar dependence on TLR2 was observed in splenocytes (data not shown).

There is no known pathway by which TLR2 can induce type I IFN, so a requirement for TLR2 in IFN production by MCMV and vaccinia virus was quite unexpected. There are two known mechanisms of type I IFN induction by TLR family members. TLR3 and TLR4 induce IRF3 activation via the signaling adaptor TRIF (Yamamoto et al., 2003). TLR7 and TLR9 can induce IRF7 activation downstream of MyD88, but this interaction only occurs in pDCs, which do not express TLR2 (Kadowaki et al., 2001). To

define the signaling pathway responsible for TLR2-dependent type I IFN production, we examined the requirement for a number of known TLR signaling components. Type I IFN production in response to vaccinia virus or MCMV was markedly reduced in MyD88-deficient and MyD88, TRIF double-deficient cells but not in TRIF-deficient cells (Fig. 2a). Both IRF3 and IRF7 appeared to contribute to type I IFN production by TLR2 because IRF3, IRF7 double-deficient cells were completely impaired in type I IFN production (**Fig. 3.2b**). In contrast, the response to vaccinia virus was unaffected in IRF1-deficient cells (**Fig. 3.3a**). As expected, production of type I IFN was also impaired in cells lacking the type I IFN receptor (**Fig. 3.3b**). To rule out any contribution of the nucleic acid sensing TLRs we tested the response to vaccinia virus in "*3d*" mice containing a non-functional allele of the *Unc93b1* gene. These mice respond normally to TLR2 and TLR4 ligands but are unable to respond to TLR3, TLR7, and TLR9 ligands (Tabeta et al., 2006). Importantly, the response to vaccinia virus in cells from 3d mice was equivalent to wild-type controls, ruling out any role for nucleic acid recognition in the type I IFN production (**Fig. 3.2c**). Finally, to address whether the recently described RNA polymerase III-dependent activation of RIG-I or MDA-5 was involved in the induction of type I IFN (Ablasser et al., 2009; Chiu et al., 2009), we examined the response of cells lacking the common signaling adapter MAVS. Type I IFN production in response to vaccinia virus was equivalent between MAVS-deficient and control cells, ruling out a role for this pathway (**Fig. 3.3c**). Collectively, these data indicate that in response to virus a MyD88-dependent pathway downstream of TLR2 leads to activation of IRF3, IRF7 and transcription of type I IFN genes.

We next measured which specific type I IFN genes were induced by TLR2 in response to viruses. We focused on the vaccinia virus response because unlike MCMV, its recognition was entirely TLR2-dependent. Vaccinia virus induced IFN-b and IFN-a4 production by bone marrow cells in a TLR2-dependent manner (**Fig. 3.2d**). The induction of each cytokine peaked at 12 h while the response to CpG oligonucleotides peaked at 4 h. PDCs are responsible for the rapid type I IFN induction in response to CpG ODN (Asselin-Paturel et al., 2001). The delayed response to VV suggests that a cell type other than pDCs may be responsible for the TLR2 response.

Differential TLR2 response to viral and bacterial ligands

The data presented thus far contradict a large body of work demonstrating that TLR2 does not induce type I IFN (Doyle et al., 2002; Kawai et al., 2001; Toshchakov et al., 2002). One major difference between

our work and previous studies is the use of viral as opposed to bacterial TLR2 ligands. To address whether the nature of the microbial ligands could account for differential induction of type I IFN, we compared induction of IFNb and IFNa4 in response to vaccinia virus and TLR2 ligands. In contrast to vaccinia virus, the triacylated lipid Pam_3SK_4 (a TLR2/1 agonist (Jin et al., 2007)) did not induce IFN-b or IFN-a4 in bone marrow cultures (**Fig 3.4a**).

As an alternative way to measure IFN-b induction, we used IFN-b reporter mice in which cDNA encoding yellow fluorescent protein has been knocked in downstream of the IFN-b locus (hereafter referred to as MOB mice) (Scheu et al., 2008). YFP was clearly detectable in bone marrow and splenocytes from these mice after stimulation with vaccinia virus. In contrast, there was no YFP signal when the same cells were stimulated with Pam_3SK_4 or Fsl-1 (a TLR2/6 agonist (Okusawa et al., 2004)) (**Fig. 3.4b**). This lack of type I IFN production was not due to poor stimulation because TNF was induced by all TLR2 ligands (**Fig. 3.5**). Thus, a population of cells in the bone marrow and spleen is able to discriminate between viral and bacterial TLR2 stimuli and selectively induce type I IFN in response to virus.

Both bone marrow-derived DCs and macrophages express TLR2 and respond to vaccinia virus. If the ability to discriminate between bacterial and viral ligands is a general property of TLR2 when expressed on all cells, then we would expect to observe TLR2-dependent type I IFN in response to viral ligands. However, type I IFN production by bone marrow-derived DCs and HEK-293Ts was TLR2-independent and likely due to the activation of the cytosolic DNA sensor(s) (**Fig. 3.6**). Therefore the ability to produce type I IFN in a TLR2-dependent manner is likely restricted to a specialized cell type present in spleen and bone marrow.

Inflammatory monocytes produce TLR2-dependent type I IFN

We next sought to identify the population of cells in the bone marrow and spleen responsible for TLR2-dependent type I IFN induction. As an initial approach we used magnetic bead cell sorting to separate bone marrow cells based on expression of the common myeloid marker CD11b or the common DC marker CD11c. Strikingly, CD11b positively sorted cells were enriched for TLR2-dependent type I IFN production in response to vaccinia virus, while the CD11b negative cells no longer responded (**Fig. 3.7a**). In contrast, sorting based on CD11c did not enrich for cells producing IFN **Fig. 3.7b**). We obtained similar results with cells from transgenic mice expressing the diptheria toxin receptor (DTR) driven by the CD11b promoter (CD11b-DTR mice) (Cailhier et al., 2005). Splenocytes harvested

from CD11b-DTR mice injected with diptheria toxin no longer responded to vaccinia virus (**Fig. 7c**). These results suggest that a $CD11b^{+}CD11c^{-}$ population of cells was responsible for TLR2-dependent type I IFN production.

Although the lack of CD11c expression suggested that pDCs were not responsible for the TLR2-dependent type I IFN production, we addressed this possibility directly using MOB mice. We compared the surface markers expressed by responding cells (YFP^{+}) in bone marrow and spleen stimulated with vaccinia virus or CpG oligos. CpG oligos induce TLR9-dependent type I IFN production by pDCs, and the YFP^{+} cells in these cultures were $B220^{+}CD11c^{+}$, a surface phenotype consistent with that of pDCs (**Fig. 3.8a**). In contrast, the cells responding to vaccinia virus were $B220^{-}$ and expressed lower amounts of CD11c (**Fig. 3.8a**). These distinct surface phenotypes clearly demonstrate that different cell types are responding to vaccinia virus and CpG oligos.

To characterize more completely the $CD11b^{+}$ cells producing IFN-b in response to vaccinia virus we stained bone marrow cells and splenocytes with a panel of antibodies against common hematopoetic surface markers. Based on the absence of certain lineage markers, we were able to exclude B cells, T cells, NK cells and neutrophils as the source of type I IFN (data not shown). The expression of CD11b suggested that the cells responding to vaccinia virus may represent a subset of monocytes. Monocytic subsets have been classified based on differential expression of the surface markers Ly6C and Ly6G (Auffray et al., 2009; Geissmann et al., 2003; Serbina et al., 2008). Cells expressing YFP in response to vaccinia virus were $Ly6C^{hi}Ly6G^{-}$ (**Fig. 3.8b**), suggesting that they represent the subset of monocytes often referred to as "inflammatory" monocytes (Fitzgerald et al.). While the expression of CD11c on IMs is inconsistent with published reports describing these cells, one possible explanation for this discrepancy is that activation of these cells leads to CD11c upregulation (Auffray et al., 2009; Serbina et al., 2008). Taken together with the fact that sorting based on CD11c staining did not alter the response to vaccinia (Fig. 4b), we conclude that the cells that make TLR2-dependent type I IFN are not initially $CD11c^{+}$, but upregulate CD11c expression upon stimulation with virus. Staining with a TLR2-specific antibody confirmed that IMs in the spleen and bone marrow express TLR2 (**Fig. 3.8c**). Moreover, treatment of MOB bone marrow with TLR2 blocking antibodies prior to stimulation with vaccinia virus greatly reduced the number of YFP^{+} cells, demonstrating that the type I IFN production by these cells requires TLR2 (**Fig. 3.8d**).

To demonstrate formally that IMs are solely responsible for the TLR2-dependent type I IFN, we sorted these cells based on Ly6C, CD11b, CD11c, and B220 (**Fig. 3.8e**). $Ly6C^{hi}CD11b^{+}CD11c^{-}B220^{-}$ cells sorted from the bone marrow produced type I IFN when stimulated with vaccinia virus in a TLR2-dependent manner. Moreover, the "negative" population (i.e., all other cells falling outside the $Ly6C^{hi}CD11b^{+}CD11c^{-}B220^{-}$ gate) did not produce type I IFN in response to virus, indicating that IMs were solely responsible for the response (**Fig. 3.8e**). Importantly, these "negative" cells did produce type I IFN when stimulated with CpG oligos, while the IMs did not, demonstrating that our sorting parameters had effectively separated pDCs from IMs. Finally, quantitative PCR analysis of cDNA generated from the sorted IMs confirmed that they expressed elevated levels of CCR2 as previously reported for these cells (Geissmann et al., 2003), and we observed slightly elevated levels of IRF7 transcripts in IMs, although not as high as the expression in pDCs (**Fig. 3.9**). These data suggest that $Ly6C^{hi}$ IMs have the unique ability to link viral recognition by TLR2 to type I IFN production.

Role of inflammatory monocytes during viral infection

Our *in vitro* analyses of cells from bone marrow and spleen implicate IMs in the recognition of vaccinia virus and suggest that TLR2 activation in these cells leads to production of type I IFN. To address the relevance of these cells during vaccinia virus infection *in vivo*, we utilized the CD11b-DTR mice described earlier. Although the DTR transgene is driven by the CD11b promoter, previous analyses of these mice have demonstrated that only a limited population of CD11b positive cells are deleted upon injection of diptheria toxin (DT) (Cailhier et al., 2005). While monocytes and some tissue resident macrophages are removed, other CD11b positive cells (such as neutrophils and activated lymphocytes) remain largely unaffected. Indeed, we observed similar numbers of $Ly6G^{+}CD11b^{+}$ neutrophils in mice injected with DT or PBS, and the overall profile of CD11b-expressing cells in the spleen remained mostly unchanged (**Fig. 3.0a**). In contrast, IMs were deleted quite efficiently, providing an efficient system with which to probe the functional relevance of these cells *in vivo*. Remarkably, when DTR-injected mice were subsequently challenged with vaccinia virus, serum concentrations of type I IFN were reduced to amounts comparable to uninfected mice (**Fig. 3.10b**). Injection of DT into non-transgenic mice followed by vaccinia virus infection had no effect on type I IFN production, as expected (**Fig. 3.11**). To assess the relevance of these cells for viral clearance, we determined viral titers in CD11b-DTR mice depleted of IMs

prior to infection. Mice lacking IMs displayed higher titers of vaccinia virus in the liver and ovaries (**Fig. 3.10c** and **data not shown**). Collectively, these data indicate that IMs are a key early source of type I IFN during viral infection and are necessary for early restriction of viral replication.

Induction of IFN by TLR2 requires receptor internalization

Finally, we sought to address the selective production of type I IFN in response to viruses by IMs. Because IMs produce TNF in response to both viral and bacterial ligands, the differential type I IFN response is unlikely to be due to lack of recognition. Recent studies of TLR4 signal transduction have revealed that induction of type I IFN requires receptor internalization while signals leading to TNF and IL-6 production can occur at the plasma membrane (Kagan et al., 2008). To determine whether similar cell biological regulation controls type I IFN production downstream of TLR2, we blocked endocytosis with the actin depolymerizing drug cytochalasin D or blocked endosomal maturation with chloroquine. Treatment of bone marrow cultures with either inhibitor prior to stimulation with vaccinia virus completely abrogated type I IFN production (**Fig. 3.12a**). In contrast, the production of TNF was unaffected, indicating that these agents do not prevent overall recognition of vaccinia virus or signaling by TLR2 (**Fig. 3.12b**). Instead, receptor internalization appears necessary only for TLR2-dependent production of type I IFN.

Discussion

Here we report the identification of a TLR2-dependent antiviral signaling pathway that leads to type I IFN production. Prior to this work, TLR2-dependent type I IFN production had not been reported, and, indeed, in most cell types TLR2 does not induce this antiviral response. We demonstrate that inflammatory monocytes are uniquely capable of responding to viral TLR2 ligands by producing type I IFN, and our work suggests that these cells represent another specialized antiviral cell type with functional and conceptual parallels to pDCs. In addition, these data solidify the interpretation that viral recognition by TLR2 is a host strategy, as opposed to manipulation by viruses, and argue that certain viral proteins are sufficiently constrained to serve as targets for innate immune receptors. Overall, this work has important implications for our understanding of how the innate immune system recognizes viruses.

Until recently, the specific and differing roles played by monocytic subpopulations during immune responses have not been well appreciated. In the last few years, however, several studies have identified IMs as a largely bone marrow resident cell type that is rapidly recruited to sites of infection in a CCR2-dependent manner (Auffray et al., 2009; Geissmann et al., 2003; Serbina et al., 2008). These cells have been named "inflammatory monocytes" to distinguish them from Ly6C^{-} monocytes, which are thought to play a more important role in maintaining tissue homeostasis (Auffray et al., 2009). IMs can differentiate into a number of different DC subsets at sites of inflammation, including TNF- and inducible nitric oxide synthase (iNOS)-producing DCs (TipDCs) as well as inflammatory DCs (Auffray et al., 2009; Geissmann et al., 2003; Serbina et al., 2008). These cells are implicated in bacterial, parasitic, and viral immunity (Serbina et al., 2008). An additional role for Ly6C^{hi} monocytes has been observed in a mouse model of induced lupus. In this model, Ly6C^{hi} monocytes accumulate in the peritoneal cavity of mice after injection of 2,6,10,14-tetramethylpentadecane (Lee et al., 2008). Surprisingly, the Ly6C^{hi} monocytes express type I IFN in this model. Although the activation signal for these cells has not been defined in this context, the observation that IMs can produce type I IFN during disease supports our contention that they may function as specialized IFN-producing cells. The ability of IMs cells to secrete pro-inflammatory cytokines and type I IFN suggests that these cells may play a key role early during viral infection. In addition, differentiation of IMs into DCs upon activation by viruses may further enhance their contribution to the antiviral immune response through induction of adaptive responses.

A surprising aspect of TLR2 function on IMs is the ability to distinguish between viral and bacterial ligands; type I IFN is only induced in response to viral ligands while TNF production occurs in response to both classes of stimuli. This differential response may be partially explained by the observation that activation of the signal transduction pathway leading to type I IFN production requires receptor internalization, as both chloroquine and cytochalasin D disrupt IFN but not TNF production. This dichotomy is reminiscent of TLR4 signaling, in which TRIF activation occurs at endosomal membranes while MyD88 activation occurs at the plasma membrane (Kagan et al., 2008). Our data suggest that TLR2 signaling is also regulated through localization, yet, in contrast to TLR4, all TLR2 signaling is MyD88-dependent. In this sense, differential signaling by TLR2 may be more conceptually similar to TLR9 signaling in that different ligands produce distinct responses through the same signaling adaptor (MyD88). For example, in pDCs class B CpG oligos lead to the production of TNF and IL-12 but not to type I IFN, while class C CpG oligos lead to the production of TNF, IL-12 and type I IFN (Gilliet et al., 2008; Krieg, 2006). IMs have a similar ability to use one TLR and produce two unique responses. How such specificity can be generated downstream of a particular TLR is not understood in pDCs or in our system. It is possible that viruses are more efficiently internalized than bacterial ligands or specifically traffic to a specialized compartment from which type I IFN signaling can be initiated. Still, such a mechanism cannot explain why cDCs or macrophages are unable to make type I IFN in a TLR2-dependent manner when stimulated with virus. Thus, it seems possible that a specialized co-receptor might be required to generate the specificity that we have discovered.

The fact that innate immune recognition of vaccinia virus is so heavily TLR2-dependent is somewhat unexpected based on the additional innate receptors capable of viral recognition. For example, TLR9 clearly plays a role in innate recognition of many DNA viruses, yet our data as well as the work of others (Zhu et al., 2007) suggest that TLR9 does not play a major role in the recognition of vaccinia virus. The ability of some viruses to evade certain innate receptors has undoubtedly required the host to evolve additional strategies of viral recognition. Cytosolic DNA sensors play a major role in detection of viral nucleic acid in the host cytosol (Ishii et al., 2006; Stetson and Medzhitov, 2006a; Takaoka et al., 2007). In DCs and macrophages stimulated with vaccinia virus, we observe a TLR-independent type I IFN response, which is presumably due to activation of cytosolic DNA sensors. In IMs as well as during *in vivo* infection, though, induction of type I IFN is largely TLR2-dependent. This dependence suggests that

vaccinia virus can evade innate receptors, such as TLR9 and cytosolic DNA sensors, yet remains detectable by TLR2.

While nucleic acid recognition appears to be the dominant strategy employed by the innate immune system to detect viral infection, there is accumulating evidence that the host can recognize certain viruses independently of nucleic acid. The work presented here supports this view. Our data and the work of others suggest that the innate immune system must have evolved specificity for certain viral proteins that are unable to mutate and escape recognition. Such a scenario is not unprecedented. Bacterial flagellin is recognized by several innate receptors (TLR5 (Hayashi et al., 2001), Ipaf (Franchi et al., 2006; Miao et al., 2006), and Naip5 (Lightfield et al., 2008)), yet most bacteria appear unable to mutate flagellin to avoid recognition. Importantly, mutations that abrogate flagellin recognition result in nonmotile bacteria (Lightfield et al., 2008; Smith et al., 2003). Thus, it would appear that certain pathogen encoded proteins are sufficiently constrained that they can serve as targets for innate immune receptors.

Following this reasoning, it is possible that the innate immune system has evolved to recognize viral proteins that are under similar functional constraints as flagellin. The viral fusion apparatus is a particularly attractive target in this regard. All viruses possess a fusion apparatus that is absolutely essential for propagation, making it an ideal target of the innate immune system. While fusion proteins from unrelated viruses share no homology at the amino acid level, structural studies have demonstrated that, in some cases, these proteins share surprising structural homology (Steven and Spear, 2006). It is possible that TLR2 has evolved to recognize a conserved structural feature associated with certain viral fusion proteins. In support of this hypothesis, HCMV glycoprotein B, which is required for viral fusion, has been reported to activate TLR2 (Boehme et al., 2006). Additionally, a number of reports suggest that TLR4 can recognize viral structural proteins (Georgel et al., 2007; Jiang et al., 2005; Jude et al., 2003; Kurt-Jones et al., 2000; Triantafilou and Triantafilou, 2004). Identification of ligands from other viruses recognized by TLR2 and TLR4 will be necessary to address whether a common structural feature is targeted.

Methods

Mice and Viruses

C57BL/6, FVB/N, FVB/N-CD11b-DTR and *Irf1*$^{-/-}$ mice were purchased from Jackson Laboratories. UNC93b1 mutant mice were purchased from the Mutant Mouse Regional Resource Centers. *Tlr2*$^{-/-}$, *Tlr9*$^{-/-}$, *Myd88*$^{-/-}$, and *Myd88 Trif*$^{/-}$ mice (kindly provided by S. Akira) were backcrossed at least 7 generations onto the C57BL/6 background. *Irf3*$^{-/-}$ and *Ifnαβr*$^{-/-}$ mice were provided by D. Portnoy (UC Berkeley). *Irf7*$^{-/-}$ and *Irf3 Irf7*$^{-/-}$ mice were provided by K. Fitzgerald (U. Mass). *Mavs*$^{-/-}$ mice were provided by Z. Chen (UT Southwestern). MOB mice have been previously described(Scheu et al., 2008). All mice were housed within the animal facilities at the University of California, Berkeley according to IACUC guidelines.

Vaccinia virus (Western Reserve strain) was a gift from D. Raulet (UC Berkeley). MCMV (Smith strain) was a gift from L. Coscoy (UC Berkeley). VV was propagated and plaqued on BHK21 cells. MCMV was propagated and plaqued on NIH 3T3 cells.

Flow cytometry and FACS

Antibodies were from eBioscience or BD Biosciences: anti-CD11b-APC-Cy7 and -PE-Cy5 (clone M1/70), anti-CD11c-FITC and -PE-Cy7 (clone N418), anti-B220-PE-Cy5 and -FITC (clone RA3-6B2), anti-Ly6C-PerCP-Cy5.5 (clone HK1.4), anti-Ly6G-PE and -FITC (clone 1A8), anti-TNFα-PE and-PacificBlue (clone MP6-XT22), and anti-TLR2-PE (clone T2.5). Before staining for surface markers, cells were incubated with an anti-CD16/CD32 antibody (clone 2.4G2, UCSF Monoclonal Antibody Core). Intracellular cytokine staining was performed with Fixation & Permeabilization Kit (eBioscience) according to manufacturer's instructions. Brefeldin was added 30 min after stimulation and cell were harvested after an additional 5 hours. Data were collected on a LSR II (Becton Dickinson) or FC-500 (Beckman Coulter). The data were analyzed with FloJo software (Tree Star).

Cell sorting was performed on a BD INFLUX. Isolation of IMs is described above. Sorted pDCs (Supplementary Fig. 4) were purified from bone marrow by sorting $B220^{+}Cd11c^{+}$ cells.

Cell lines and tissue culture

HEK293, NIH3T3, and BHK21 cells were obtained from ATCC, and HEK293-TLR2 cells have been previously described(Sato et al., 2006).

ISRE-L929 cells were obtained from D. Portnoy (UC Berkeley)(Crimmins et al., 2008) and have been previously described. Briefly, these cells have been stably transfected with an interferon-sensitive responsive element upstream of the luciferase gene.

Cell lines were cultured in DMEM supplemented with 10% FCS, L-glutamine, penicillin/streptomycin, sodium pyruvate and HEPES (Invitrogen). Primary cells were cultured in RPMI 1640 supplemented with 10% FCS, L-glutamine, penicillin/streptomycin, sodium pyruvate and HEPES.

Bone-marrow-derived conventional DCs and macrophages were differentiated in RPMI supplemented with GM-CSF or M-CSF containing supernatant, respectively, as previously described(Ewald et al., 2008). After 5 days, cells were replated in RPMI media, and stimulated as indicated.

Luciferase Assays

ISRE-L929 cells were used to measure the amount of type I IFN present in supernatants or sera. In parallel, ISRE-L929 cells were treated with serial dilutions of recombinant IFNb (R&D) to generate a standard curve in each experiment. In some cases supernatants or sera were diluted to achieve signal within the linear range of the standard curve.

All cells were lysed using Passive Lysis Buffer (Promega) and luciferase was measured on a LMaxII-384 luminometer (Molecular Devices).

Bone marrow and spleen cell harvesting/stimulation

Spleens were dispersed into single cell suspensions by passage through nylon mesh (BD Falcon). Bones were washed with ethanol and RPMI media, and bone marrow cells were released by gentle grinding with a mortar and pestle. Cells were passed through a filter to remove debris, red blood cells were removed by hypotonic lysis, and the remaining cells were washed three times with RPMI media, counted, and plated.

All viral stimulations were performed at an MOI of 0.2 using UV inactivated particles, unless otherwise noted. CpG oligonucleotide (TCCATGACGTTCCTGACGTT with phosphorothioate linkages from IDT or Invitrogen) was used at 1μM. CpG A oligonucleotide (G*GTGCATCGATGCAG*G*G*G*G*G with phosphorothioate linkages indicated with an asterisk were from IDT) was used at 1μM. Cultures were stimulated with FSL-1, Pam_3SK_4 and R848 (Invivogen) at 0.1 μg/ml, 1 μg/ml, and 1 μg/ml respectively. TLR2 blocking antibody (clone T2.5 - eBioscience) was added to cells 30 minutes before stimulation at 50 μg/ml.

Quantitative real-time RT-PCR

For IFNb/IFna4 RT-PCR, at indicated times post-stimulation, cells were harvested and resuspended in TRIzol. For CCR2/IRF7 RT-PCR, cells were sorted on a BD INFLUX and were resuspended in TRIzol.

For all samples, the Trizol Plus RNA Purification System (Invitrogen) was used to isolate RNA. Quantative PCR was performed on an Applied Biosystems 7300 (Applied Biosystems) using SYBR green-based quantification. Gene-specific transcript levels were normalized to the amount of RPS17 mRNA. The following primers were used: RPS17: 5'-cgccattatccccagcaag-3', 5'-tgtcgggatccacctcaatg-3'; IFNb: 5'-ataagcagctccagctccaa-3', 5'-ctgtctgctggtggagttca-3'; IFNa4: 5'-gcaatgacctccatcagcagc-3', 5'-cactccttctcctcactcagtcttg-3'; IRF7: 5'-ctacaccatctacctgggttttgg-3', 5'- agacaagcacaagccgagactg-3'; CCR2: 5'-tccttgggaatgagtaactgtgt-3', 5'- tggagagataccttcggaactt-3'.

Magnetic Cell Sorting

$CD11b^+$ and $CD11c^+$ bone marrow cells were positively sorted by magnetic cell sorting. Cells were stained with anti-CD11b or anti-CD11c biotinylated antibodies and then incubated with anti-biotin microbeads (Miltenyi Biotec). Cells were sorted on an AutoMACS cell sorter (Miltenyi Biotec). The positive and negative (flow-through) populations were collected. The positive populations were enriched for the target cells but also contained $CD11b^-$ or $CD11c^-$ cells.

CD11b-DTR mice

CD11b-DTR mice were injected intravenously with 500ng diptheria toxin (Sigma). For in vitro experiments, splenocytes were harvested 24h later and stimulated with vaccinia virus (0.2 MOI) or CpG oligos (1μM). Type I IFN was measured in supernatants after 24h by bioassay.

For *in vivo* experiments, mice were infected with live vaccinia virus ($1x10^6$ pfu) 24h after diptheria toxin injection. 24 h post-infection serum was collected and type I IFN was measured by bioassay. 48 h post-infection organs where collected and PFU counts where performed.

Inhibition of receptor internalization

To measure IFN production, bone marrow from MOB mice was treated with chloroquine (15μM) or cytochalasin D (1μg/ml) for 30 minutes before the addition of ligands. 20h post stimulation, cells where harvested and analyzed.

To measure TNF production, bone marrow from B6 mice was pretreated with chloroquine (15μM) or cytochalasin D (1μM) for 14h before the addition of ligands. 30 minutes after the addition of ligands, Brefeldin was added. Cells were harvested after an additional 5 hours. In all, the cells spent the same amount of time in drug to control for potential toxicity.

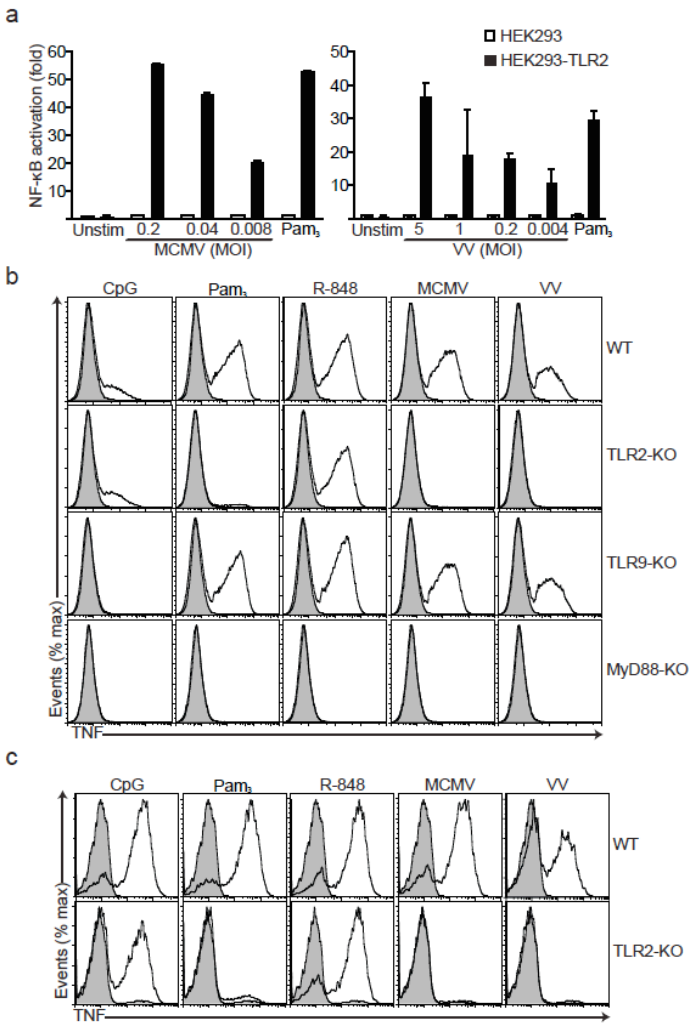

Figure 3.1 - TLR2-mediated recognition of vaccinia virus and MCMV. **(a)** Luciferase activity in HEK293 cells stably transfected with a NF-κB luciferase reporter (HEK293) or the reporter together with plasmids encoding human TLR2 and human CD14 (HEK293-TLR2), then left unstimulated (Unstim) or stimulated with ultraviolet irradiation–inactivated MCMV or vaccinia virus (VV; multiplicity of infection (MOI), horizontal axes) and assessed 10 h after activation. Pam_3SK_4 (Pam_3) serves as a positive control. Results are presented relative to activation in unstimulated cells. (**b**,**c**) Flow cytometry of intracellular TNF in bone marrow–derived DCs (BMDC; **b**) or macrophages (MΦ; **c**) stimulated with TLR ligands or viruses (above plots). Plots of stimulated cells (black lines) are overlaid on plots of unstimulated cells (shaded). DC plots **(b)** are gated on $CD11c^+$ cells. Genotype (right margin): WT, wild-type; KO, knockout. Data are representative of at least three experiments (error bars **(a)**, s.d.).

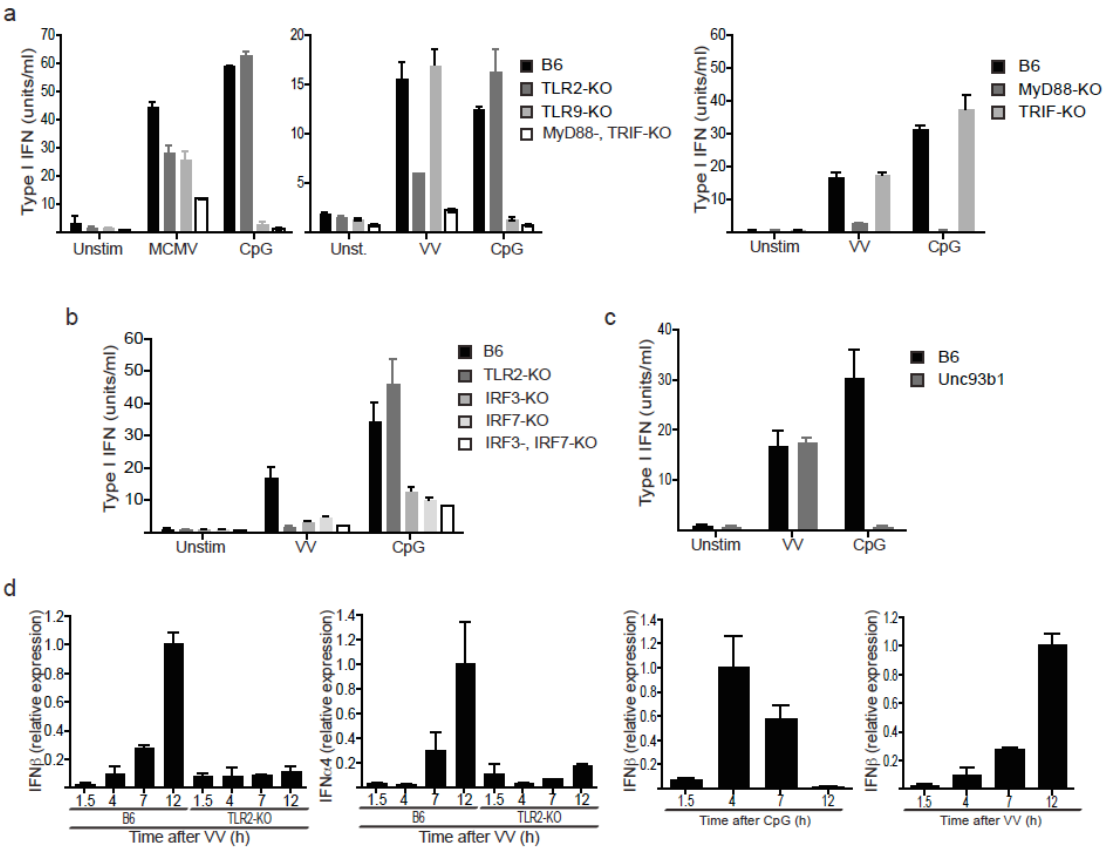

Figure 3.2 - TLR2 induces the production of type I interferon in response to virus. (**a**–**c**) Bioassay of type I interferon in supernatants of bone marrow cells (genotype, key) 24 h after stimulation (horizontal axis). B6, C57BL/6; Unc93b1, 3d. (**d**) Real-time PCR analysis of transcripts for IFN-β and IFN-α4 in bone marrow cells stimulated with vaccinia virus or CpG oligonucleotides, presented relative to expression of mRNA for the ribosomal protein S17 (RPS17). Data are representative of at least two experiments (error bars, s.d.).

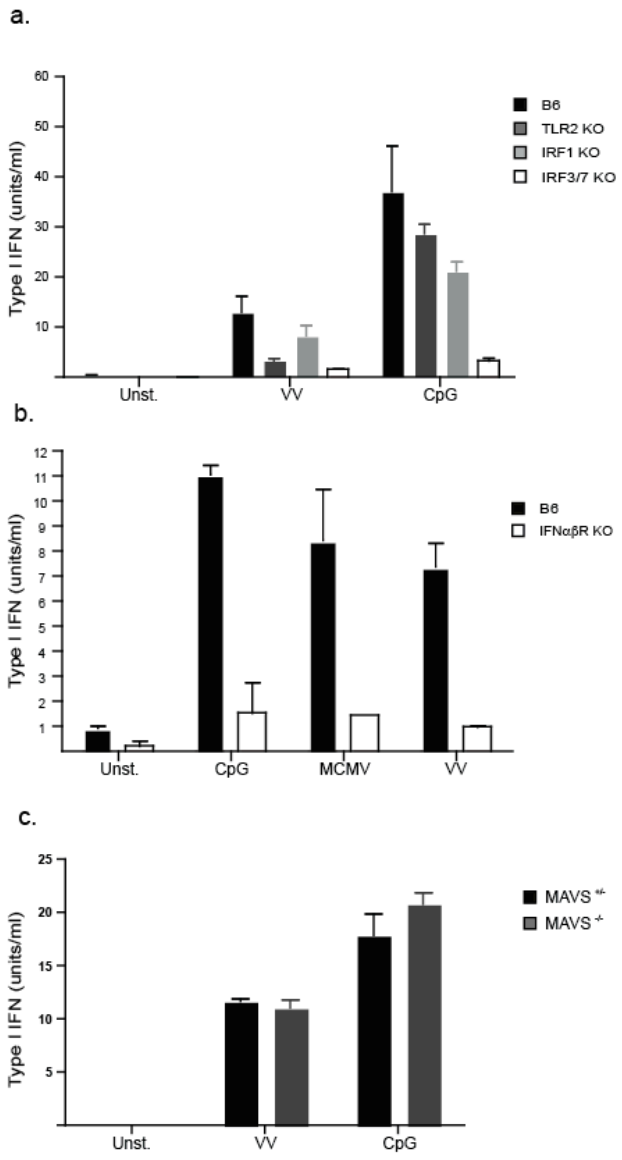

Figure 3.3 - IFNαβ Receptor is necessary for optimal type I IFN production, while IRF1 and MAVS are dispensable. (a, b, c) Bone marrow cells from the indicated mice were stimulated as described. Type I IFN was measured by bioassay in the supernatants 24 h after stimulation.

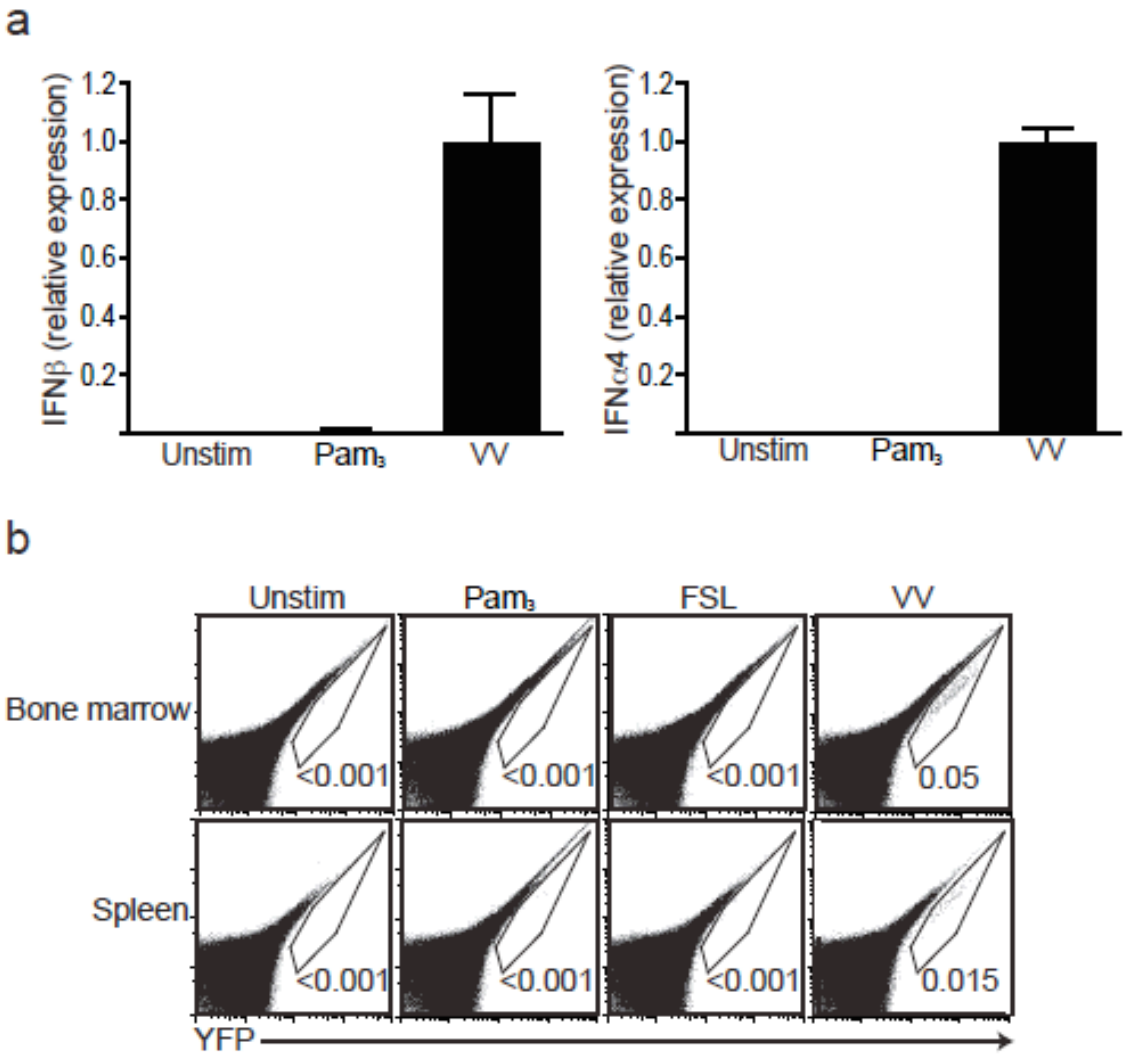

Figure 3.4 - Differences in the induction of type I interferon by TLR2 in response to viral and bacterial ligands. (**a**) IFNβ transcripts in bone marrow cells left unstimulated or treated for 12 h with Pam_3SK_4 or vaccinia virus, presented relative to the expression of RPS17 mRNA. (**b**) Flow cytometry of YFP production by bone marrow cells (BM) or splenocytes (Spleen) obtained from MOB mice and left unstimulated or stimulated for 20 h with Pam_3SK_4, FSL-1 or vaccinia virus. Numbers in bottom right corners indicate percent YFP^+ cells. Data are representative of at least three experiments (error bars (**a**), s.d.).

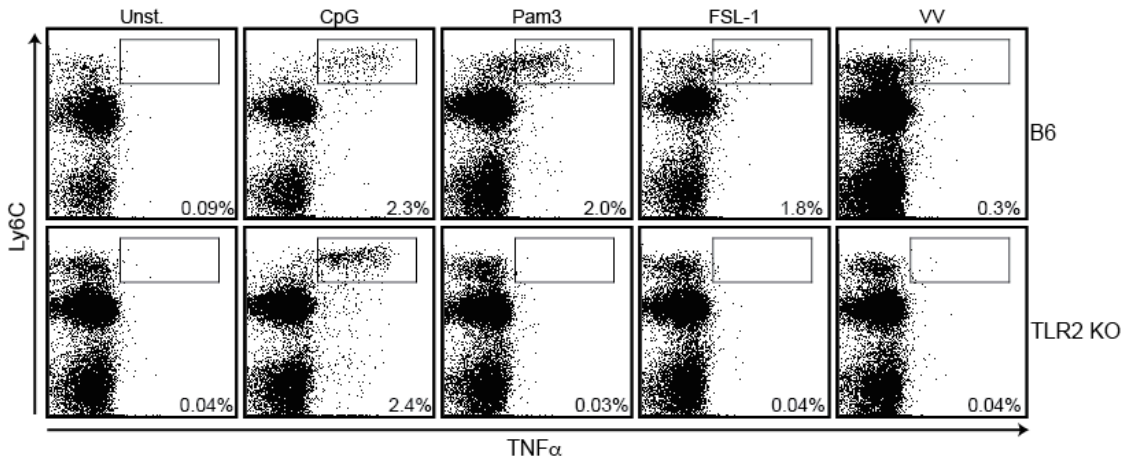

Figure 3.5 - All TLR2 ligands lead to the production of TNF in Ly6C^{hi} cells. Bone marrow cells were stimulated with the indicated TLR ligands or vaccinia virus(VV) and intracellular TNF was measured by flow cytometry in Ly6C^{hi} cells.

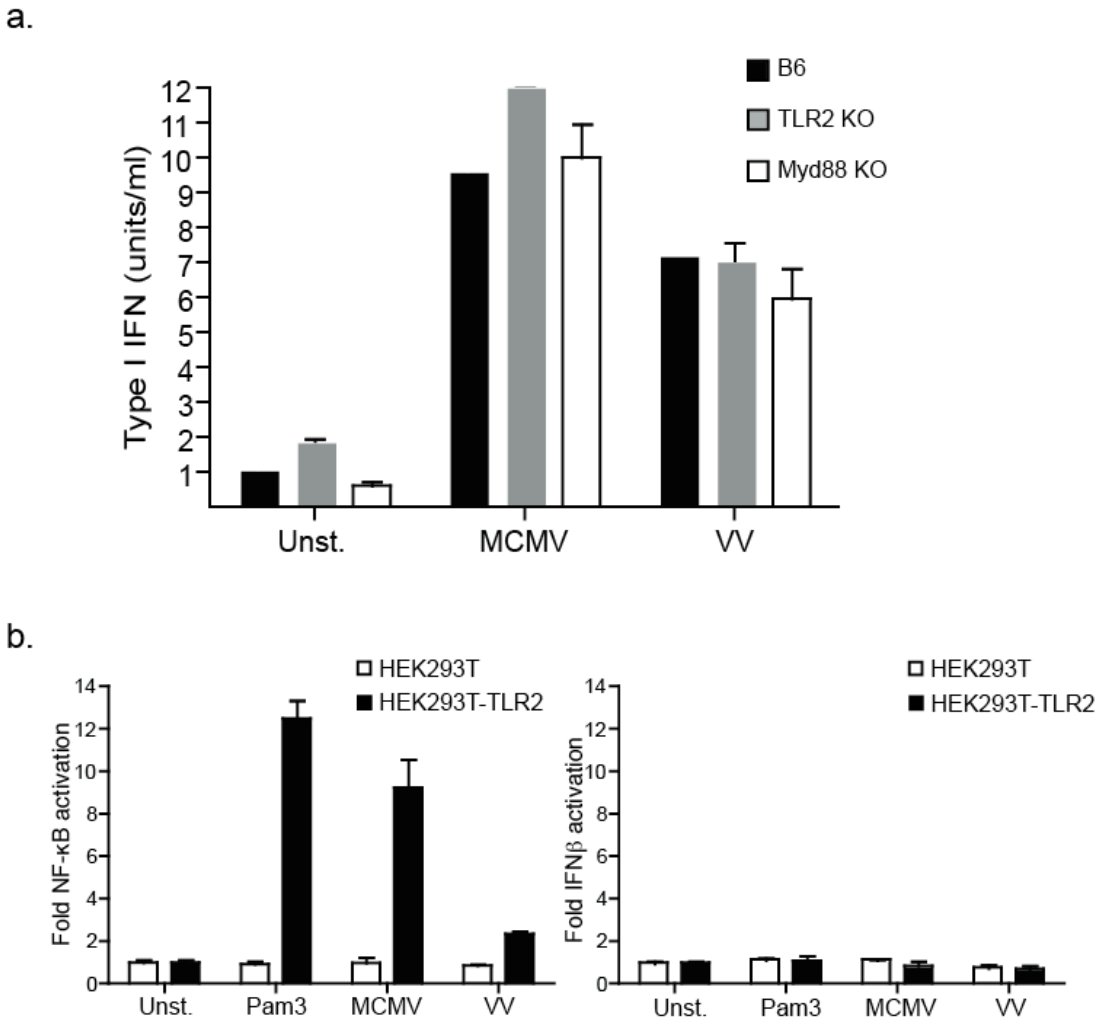

Figure 3.6 - TLR2 is not required for virus induced type I IFN production in cDCs or HEK-293Ts. (a) Bone marrow DCs were derived from the indicated mouse strains, stimulated with CpG, MCMV or vaccinia virus (VV), and type I IFN production was measured after 24 hours by bioassay. **(b)** HEK293T cells were transiently transfected with a NF-ϰB or IFN-β luciferase reporter and either empty plasmid or plasmid encoding human TLR2. 24 h post-transfection, cells were stimulated with UV-inactivated MCMV, VV, or Pam3. Luciferase activity was measured 10 h after stimulation. Fold activation was calculated relative to unstimulated cells.

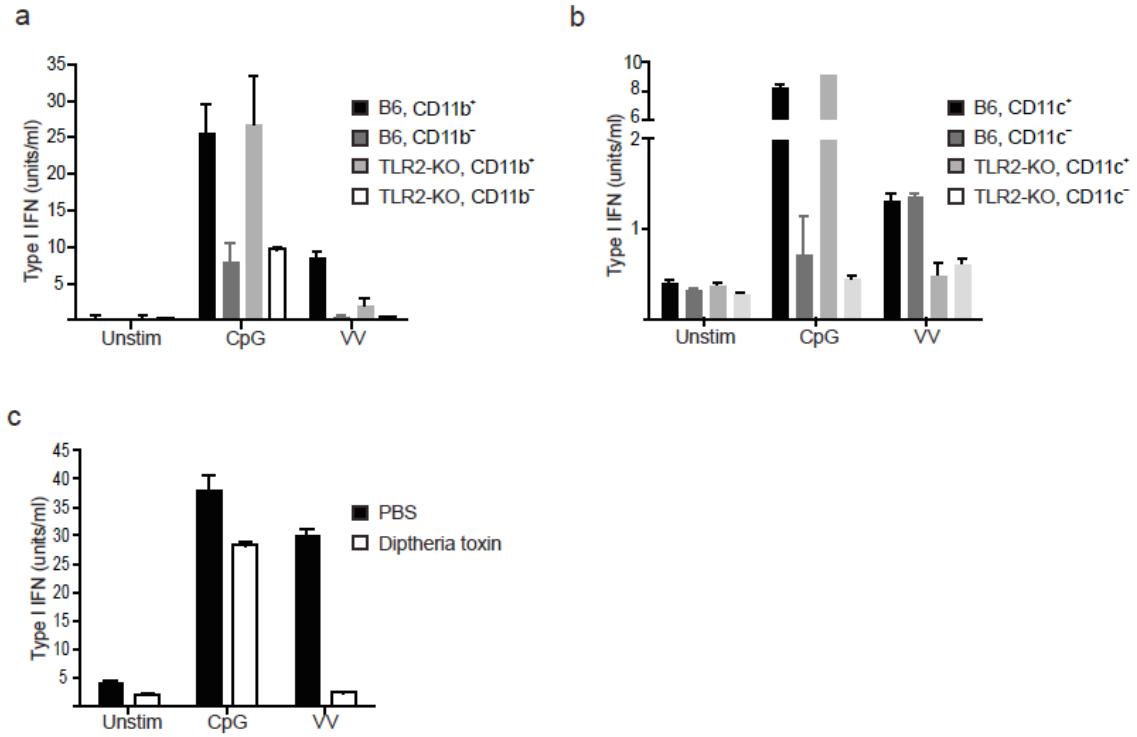

Figure 3.7 - A population of CD11b+CD11c- cells is responsible for TLR2-dependent production of type I interferon. (**a**,**b**) Bioassay of type I interferon in supernatants of bone marrow cells collected from B6 or TLR2-deficient mice and sorted into $CD11b^+$ and $CD11b^-$ populations (**a**) or $CD11c^+$ and $CD11c^-$ populations (**b**), then left unstimulated or stimulated for 24 h with CpG or vaccinia virus. (**c**) Bioassay of type I interferon in supernatants of splenocytes collected from CD11b-DTR–transgenic mice 24 h after injection with saline (PBS) or diphtheria toxin (DT), then left unstimulated or stimulated for 24 h with CpG or vaccinia virus. Data are representative of at least three experiments (error bars, s.d.).

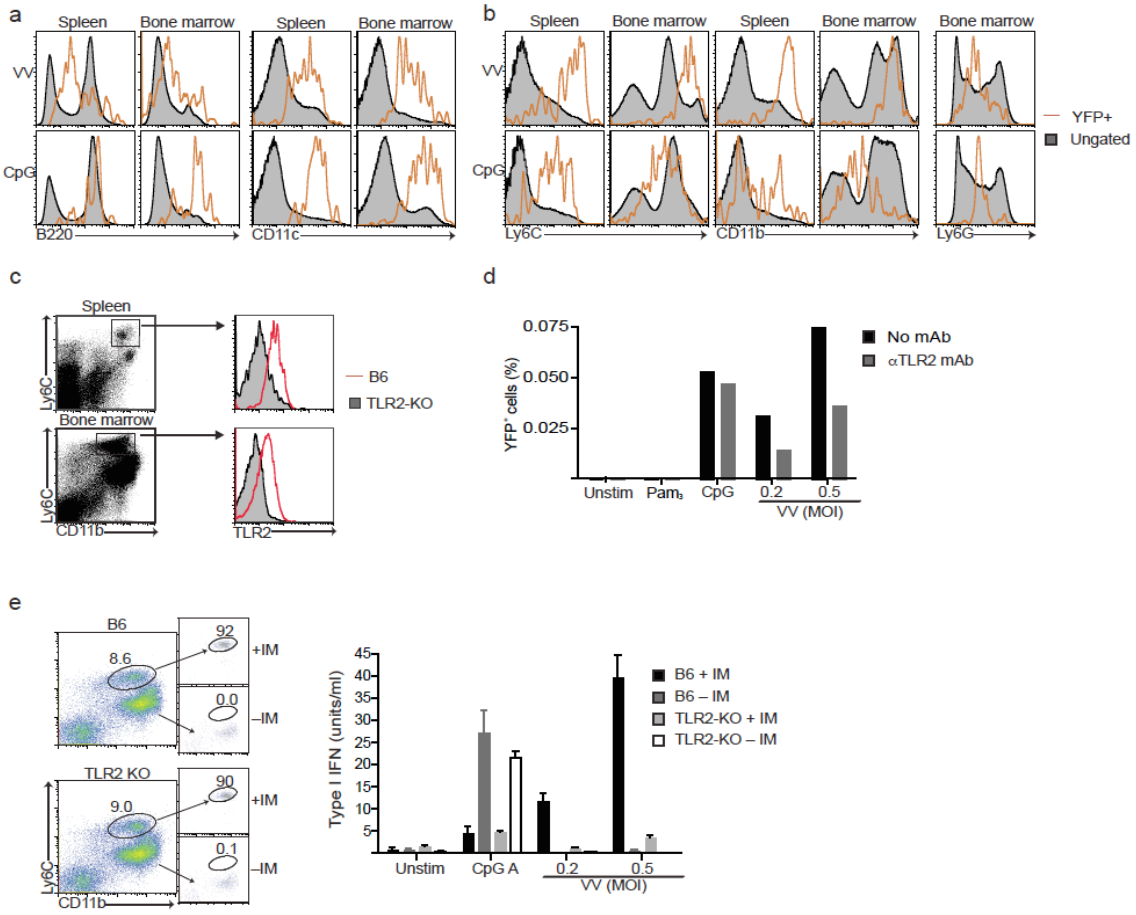

Figure 3.8 - Ly6C^{hi} IMs produce IFNβ in response to vaccinia virus. (**a**,**b**) Flow cytometry of splenocytes or bone marrow cells collected from MOB mice, stimulated for 20 h with CpG or vaccinia virus and stained with antibody to B220 (anti-B220) or anti-CD11c (**a**) or with anti-Ly6C, anti-CD11b or anti-Ly6G (**b**). Orange lines, YFP-gated cells; shaded histograms, total ungated cells. (**c**) Flow cytometry of bone marrow cells and splenocytes stained with anti-CD11b, anti-Ly6C and anti-TLR2. (**d**) Frequency of YFP^{+} cells among bone marrow cells obtained from MOB mice and cultured in the presence (mAb to TLR2) or absence (No mAb) of a TLR2-blocking monoclonal antibody, then left unstimulated or stimulated with Pam_3SK_4, CpG or vaccinia virus. (**e**) Flow cytometry of bone marrow cells sorted on the basis of expression of CD11b and Ly6C (left) and postsort analysis (middle) of populations within (positive (+IM)) or outside (negative (-IM)) the Ly6C^{hi}CD11b^{+}CD11c^{-}B220^{-} gate. B220^{+} and CD11c^{+} cells were excluded by gating; numbers above outlined areas indicate percent IMs. Right, bioassay of the production of type I interferon by positive (+IM) and negative (-IM) populations left unstimulated or stimulated for 24 h with CpG A or vaccinia virus. Data are representative of at least three experiments (error bars (**e**), s.d.).

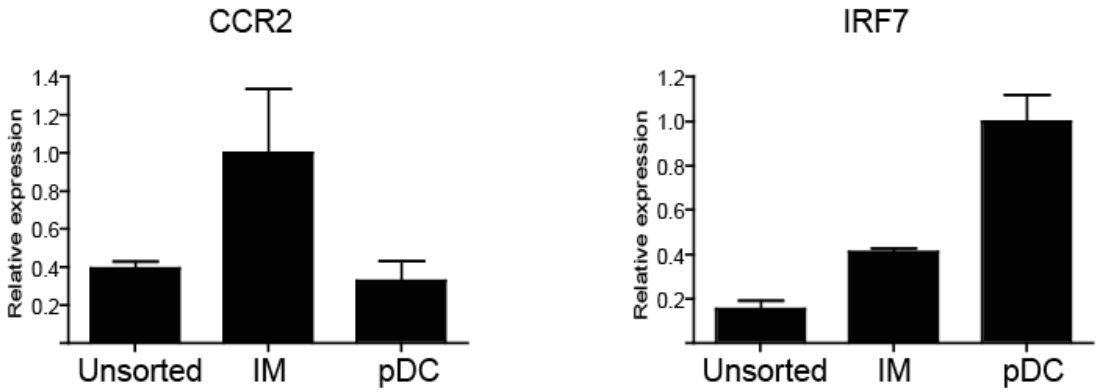

Figure 3.9 - Expression analysis of sorted inflammatory monocytes. Transcripts for CCR2 and IRF7 were measured by real-time PCR in bone marrow cells, sorted Ly6Chi inflammatory monocytes (Fitzgerald et al.), and sorted pDCs.

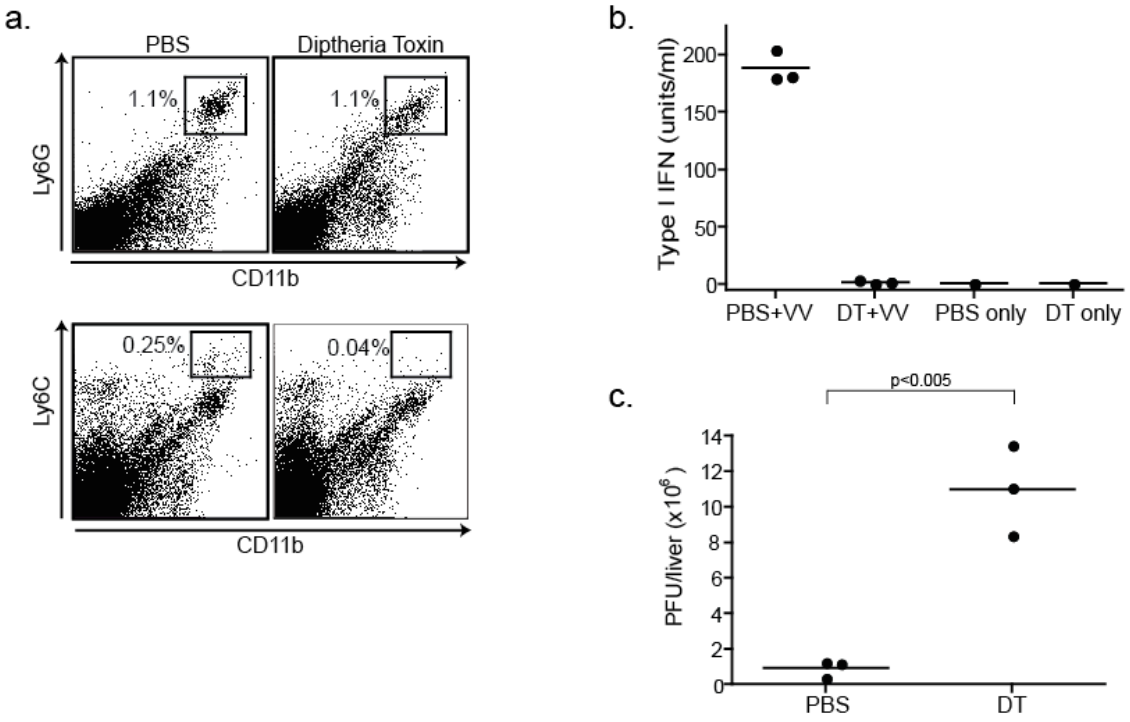

Figure 3.10 - IMs are required for early production of type I interferon and efficient viral clearance in vivo. (**a**) Flow cytometry of splenocytes collected from CD11b-DTR–transgenic mice 24 h after intravenous injection of PBS or diphtheria toxin, then stained with anti-Ly6C, anti-CD11b or anti-Ly6G. Numbers adjacent to outlined areas indicate $Ly6G^+CD11b^+$ cells (top row) or $Ly6C^+CD11b^+$ cells (bottom row).(**b**) Bioassay of type I interferon in serum from CD11b-DTR–transgenic mice injected with PBS or diphtheria toxin 24 h before infection with vaccinia virus ($1x10^6$ plaque-forming units), assessed 24 h after infection. (**c**) Viral titers in the liver of CD11b-DTR–transgenic mice treated as described in **b**, assessed 48 h after infection. Each symbol represents an individual mouse; small horizontal lines indicate the mean. *P* value, unpaired *t*-test. Data are representative of at least two experiments.

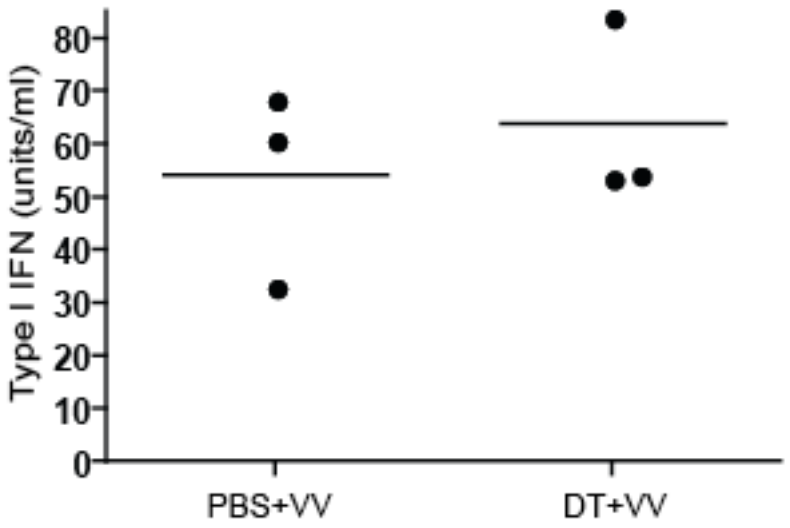

Figure 3.11 - The CD11b-DTR transgene is necessary for diptheria toxin to effect type I IFN production *in vivo*. FVB/N mice where injected with diptheria toxin or PBS 24 h before infection with 1x106 PFU of vaccinia virus. Serum was collected 24 h post-infection and type I IFN were quantified by bioassay.

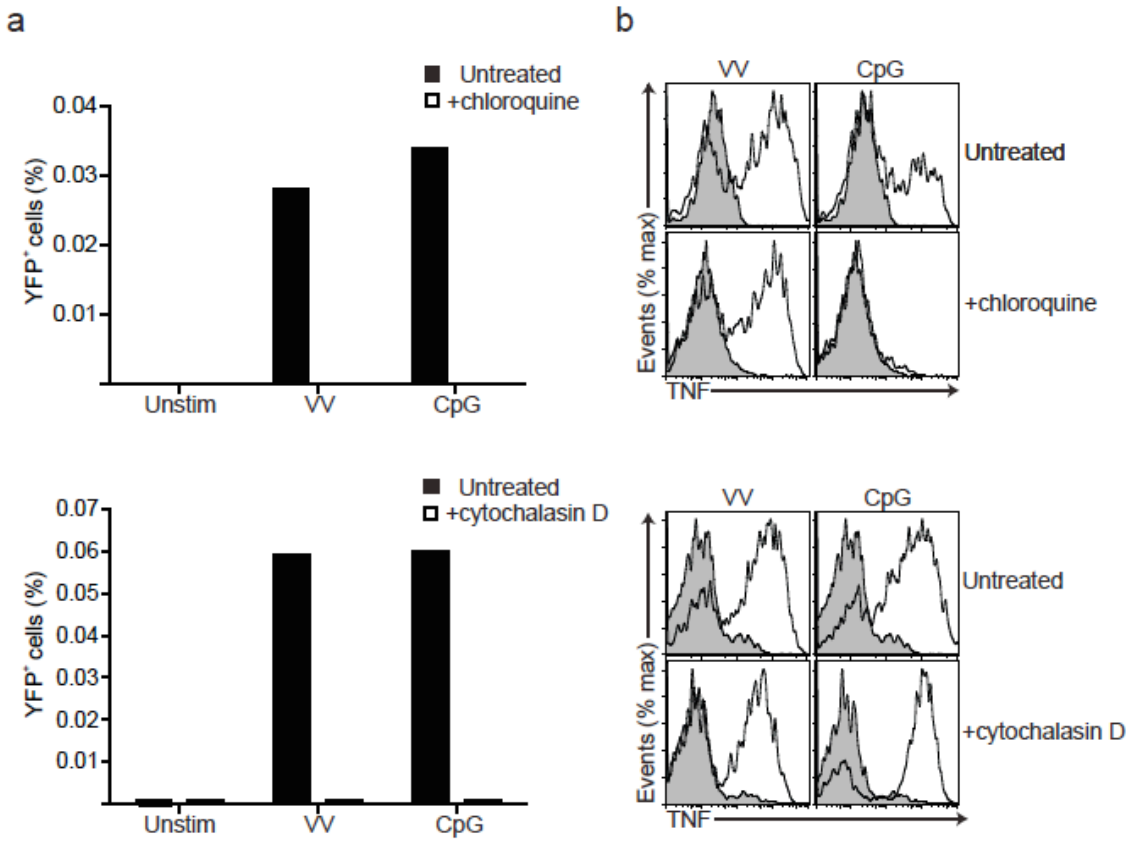

Figure 3.12 - TLR2-dependent production of type I interferon requires receptor internalization. (**a**) Flow cytometry of YFP$^+$ cells among bone marrow obtained from MOB mice and then incubated with 15 μM chloroquine, 1 μM cytochalasin D or left untreated before stimulation for 20 h with vaccinia virus or CpG. (**b**) Flow cytometry of intracellular TNF in IMs treated as described in **a**, measured by intracellular cytokine staining. Data are representative of at least two experiments.

Chapter 4: Neutrophils and TLRs – a complicated relationship

Introduction

It has long been appreciated that neutrophils are one of the first cell types recruited to fight off infection. Their overall importance to human health is also well appreciated (Borregaard, 2010; Nathan, 2006). They are important for protection against bacterial(Rogers and Unanue, 1993), viral(Tumpey et al., 1996), and parasitic (Bliss et al., 2001)infections. While the recent resurgence in innate immunity research has provided us with great insight about how pattern recognition receptors (PRRs) activate macrophages and dendritic cells, the PRRs that neutrophils use to activate themselves are not well understood.

While neutrophils are viewed as a very blunt tool of the innate immune system, their responsibility within the body is immense. Neutrophils must be able to enter a new tissue and quickly understand their environment. Do they need to start producing pro-inflammatory cytokines and release granules that can efficiently kill bacteria, while potentially causing collateral tissue damage? Or are have they been summoned because the host has just experienced a severe physical shock, which has lead to sterile inflammation – in that case, should neutrophils now promote a programmed tissue repair process? All the other cells that are recruited to the site of inflammation afterwards pick up on these queues and potentially reinforce the early decisions that are made by neutrophils.

Prior to the discovery of Toll-like receptors, there was no molecular paradigm to understand how neutrophils could "sense" their environments. It was known that an infection or cellular damage could initiate a series of events that would lead to neutrophil influx from the blood. But it was not well understood how neutrophils themselves could be activated in an intrinsic manner upon their extravasation. After the discovery of TLRs and other PRRs, it was mostly likely assumed that neutrophils would be a key cell type that would use these innate immune receptors to modulate their activity. While for other innate immune cells, like macrophages and dendritic cells, it has been demonstrated that PRRs are very important for their activation, there is little (or very conflicting data) to indicate how neutrophils use PRRs to govern their own activation. It is counterintuitive to think that the first cell that is elicited from the blood in order to respond to infection – the neutrophil – would not use PRRs to regulate its activation step, considering it is the cell that is most likely to sense pathogen-associated molecular patterns (PAMPs). To demonstrate the confusing state of neutrophil activation by PAMPs, see the review by Cassatella (Cassatella,

1999) which reviews dozens of papers which either report that neutrophils do or do not see a respond to LPS. Why the field is so divided on this singular, but very important question is unknown. I have carried out a series of experiments with the hope of providing some clarity on the relationship between neutrophils and TLRs.

While some of the cell intrinsic aspects of neutrophil activation are unanswered, extrinsic signals that are necessary (but likely insufficient) to activate neutrophils have been clearly established. Most importantly, it has been shown that integrin ligation is an important step in the activation of neutrophils (Abram and Lowell, 2009). On the molecular level, it has been shown that certain kinases, like Syk, are activated by integrin ligation and these kinases are absolutely essential for neutrophil activation (Mocsai et al., 2002). This close relationship is intellectually very appealing because it tightly links extravasation with neutrophil activation. Neutrophils will only leave the blood if they are summoned to a disruption in normal cellular processes in host tissue, and it is in these tissues that neutrophils will first encounter microbes. Integrin ligation will be the first but not the only step that is necessary to activate neutrophils because the act of extravasation will not provide the neutrophils with the contextual cues for them to understand what kind of environment they are entering i.e. microbially infiltrated tissue or sterile inflammation.

Here we provide several important insights into neutrophil biology: 1. Naïve neutrophils do not respond to purified TLR ligands (unlike macrophages and other key innate immune cells). 2. Pro-inflammatory signals can "license" neutrophils to respond to purified TLR ligand. 3. While naïve neutrophils do not respond to purified TLR ligands, they can respond to whole bacteria suggesting that neutrophils use multiple PRR families in a complementary manner to decide whether or not to respond to PAMPs when they are in a naïve state.

Results

Neutrophils make TNFα in response to bacteria but not purified PAMPs.

While previously focusing our studies on inflammatory monocytes (see chapter three), we became interested in the ways in which neutrophils responded to PAMPs because their behavior was rather unexpected. While inflammatory monocytes ($Ly6C^{hi}$) made large amounts of TNFα in response to the well established PAMPs CpG (a TLR9 ligand) and Pam_3 (a TLR2 ligand), neutrophils ($Ly6C^{int}$ – additionally, we verified that these cells where $Ly6g^{+}F4/80^{-}CD115^{-}$) did not made any detectible TNFα in response to either TLR ligand (**Fig. 4.1**).

This observation was confusing because it has been demonstrated that neutrophils make TNFα when they encounter bacteria (Van Ziffle and Lowell, 2009). In agreement with the published data, when we challenged bone marrow cells with *Listeria monocytogenes* ΔLLO – we detected a significant amount of TNFα production (**Fig. 4.1**). We presumed that this response was cell-autonomous because brefeldin was added to the media, which prevents cell-to-cell communication.

There are two distinct possibilities that could explain why *Listeria monocytogenes* ΔLLO is able to elicit a TNFα response in neutrophils, while TLR ligands cannot: either neutrophils can sense a pathogenic property of the bacteria that purified PAMPs lack or *Listeria* contains additional PAMPs that are necessary to fully activate neutrophils (or the PAMPs are presented in a unique context see Goodrige *et al* (Goodridge et al., 2011)).

We decided to first understand if there was additional virulence pathogenic properties that purified PAMPs lacked. Because *Listeria monocytogenes* ΔLLO is effectively avirulent (due to its inability to escape the phagasome), we speculated that the response to the bacterium is likely not due to the neutrophils ability to sense an additional pathogenic property of the bacteria. To insure that this observation was not isolated to *Listeria*, but was a more generalized response to bacteria, we challenged neutrophils with classical lab strains of *Escherichia coli*. Neutrophils made ample amounts of TNFα in response to *E. coli* suggesting that the neutrophil response was not specific to *Listeria* and was not due to *LLO* independent virulence factors, as lab strains of *E. coli* should be completely avirulent (**Fig. 4.2**).

Lastly, we observed that neutrophils could respond to heat killed bacteria, suggesting that neutrophils were not simply responding to "living"

microorganisms (**Fig. 4.2**). Because simply challenging neutrophils with bacterial extracts was sufficient to activate them, we conclude that *Listeria* contains PAMPs that are necessary to fully activate neutrophils and these PAMPs are not simply TLR ligands.

TLR signaling is necessary but not sufficient to activate neutrophils.

We next wanted to determine if TLRs played a role in sensing bacteria in neutrophils. In bone marrow neutrophils that were deficient for Myd88 and Trif, two key signaling adapter molecules in TLR signaling(Akira and Takeda, 2004), we could not detect TNFα in response to *Listeria* (**Fig 4.3)**. Suggesting that TLRs played a role in detecting *Listeria* in neutrophils

One possible explanation for this result is that neutrophils only express a subset of TLRs and they are simply ignorant to the purified TLR ligands we choose to use. Therefore, bone marrow neutrophils were stimulated with ligands for TLRs 2,4,5,7 and 9. None of these stimulations lead to TNFα production in neutrophils. Therefore we conclude that no singular TLR ligand was sufficient to lead to TNFα production in bon marrow resident neutrophils (data not shown).

In a reciprocal set of experiments, we exposed bone marrow cells that were doubly deficient for TLRs 2 and 4 or bone marrow cells that lacked signaling by all nucleic acid sensing cells (Unc93b1 mice) with *Listeria* (data not shown). In both groups, we saw a reduced, but still detectible level of TNFα in neutrophils, suggesting that multiple TLRs can sense *Listeria* in neutrophils.

Additionally, we tested whether multiple TLRs needed to be stimulated in neutrophils at the same time for them to produce TNFα. As of yet, we did not find any combination of TLR ligands that could lead to TNFα production in neutrophils (data not shown)..

Overall, these results suggested that TLR signaling was necessary but not sufficient to lead to TNFα production in neutrophils. Because most bacteria are sensed by multiple TLRs (Arpaia et al., 2011), it is difficult to determine which TLRs can lead to TNFα production in neutrophils.

We were very interested in identify the second signal that emanates from bacteria that is necessary for TNFα production in bone marrow neutrophils. Of note, we have tested if N-formylated peptide - which is known to strongly activate neutrophils via the G-protein coupled formyl peptide receptor (FPR) (Le et al., 2002) – is the source of the second signal that emanates from bacteria. Currently, we have not observed any synergy between TLR and FPR signaling when analyzing TNFα production in bone

marrow neutrophils (data not shown).. Fortunately, there are some hints about additional signaling pathways that might synergize with TLR signaling in bone marrow neutrophils (see below), but so far none of our data provide us with the identification of "signal two" or its receptor.

Thioglycolate elicited neutrophils do not require "signal two"

All of our previous experiments were performed with neutrophils that were isolated from the bone marrow. While the use of neutrophils isolated from the bone marrow is a readily accepted source for the cells, we were concerned that analyzing bone marrow neutrophils might provide a biased view of neutrophil function.

Our worries stemmed from the realization that neutrophils do not do their jobs in the bone marrow. Upon inflammation, neutrophils are recruited from the blood into potentially infected tissues. This complex process exposes neutrophils to several factors that they do not encounter when they are still in the bone marrow. Integrin signaling (which is necessary for neutrophil extravasation) and chemokine receptor signaling (which is necessary for neutrophil homing) can both influence the behavior of neutrophils once they actually encounter bacteria (Borregaard, 2010).

To address these concerns, we decided to isolate neutrophils that were elicited to a site of inflammation. Thioglycolate is a well-known irritant that is commonly used to elicit peritoneal macrophages. We took advantage of the fact that before a massive macrophage influx occurs in response to thioglycolate, a substantial portion of the cells that first infiltrate into the peritoneum is neutrophils.

To our surprise, thioglycolate elicited neutrophils ($Ly6G^{hi}$) made copious amounts of TNFα in response to TLR ligands (**Fig 4.4**). This stands in marked difference to bone marrow neutrophils where we saw no TNFα production in response to TLR ligands (**Fig. 4.1**). Just like bone marrow neutrophils, thioglycolate elicited neutrophils were still able to make TNFα in response to *Listeria*. It is also important to note that thioglycolate is insufficient to lead to the production of these pro-inflammatory cytokines in neutrophils – suggesting that thioglycolate itself is not the trigger.

This response is not specific to thioglycolate elicitation because other irritants like incomplete freund's adjuvant can also lead to the elicitation of neutrophils that do not require "signal two" from the bacteria (**Fig. 4.5**).

Overall, these observations lead us to conclude that "signal two", which is necessary for the production of TNFα in bone marrow neutrophils, is not required for TNFα production in elicited neutrophils.

These data lead us to propose a model in which neutrophils can be "armed." When neutrophils are in the bone marrow, they are naïve cells and there is a high threshold set for them before they can initiate a release of pro-inflammatory cytokines. In our case, they need multiple, unique, signaling pathways to be activated before they can secrete TNFα. But when neutrophils are recruited in response to the presence of inflammatory agents, the threshold within the neutrophils is lowered – afterwards, these neutrophils can more readily alert the body to the presence of microorganisms. In our case, they need a single signaling pathway to be activated before they can secrete TNFα.

Neutrophils elicited by sterile cell death still require "signal two"

While neutrophils clearly play an important role in host defense, they also home to sites of sterile inflammation. The role of neutrophils in sterile inflammation is not well understood, but it is reasonable to assume that the role of neutrophils during sterile inflammation and microbial infection might be quite different.

One well-established system that is used to study the recruitment of neutrophils in response to sterile inflammation was developed by the Rock group (Chen et al., 2006). In this model, necrotic cells are injected into the peritoneum of mice and neutrophil influx occurs within the following 16 hours. Because the kinetics of influx are similar between the thioglycolate elicited neutrophils and the necrotic cells elicited neutrophils, we decided to compare the two elicitation factors.

Surprisingly, we observed that neutrophils elicited by necrotic cells act like bone marrow neutrophils and not thioglycolate elicited neutrophils. In response to *Listeria*, both sets of neutrophils were able to make TNFα (**Fig. 4.6**). But when the cells are treated with a purified TLR ligand, the necrotic cell elicited neutrophils failed to make TNFα (**Fig. 4.6**) – phenocopying bone marrow neutrophils (**Fig. 4.1**).

The inability to "arm" neutrophils while they are being elicited by necrotic cells suggests that extravasation is not the step that leads to "arming". This was a distinct possibility because it is known that integrin signaling occurs during extravasation and that integrin signaling within neutrophils is essential for certain functions (Abram and Lowell, 2009), like TNFα production.

On a more fundamental level, these data suggest that the local environment can modulate the activation state of neutrophils. During some inflammatory contexts, neutrophils are "armed", which enables them to

release pro-inflammatory cytokines with a lower threshold of activation in the future. During sterile conditions, where a "clean-up" response is more appropriate, neutrophils are not "armed" and presumably promote the resolution of inflammation (or at least do not contribute to additional inflammation unless multiple, unique signaling pathways are activated).

Priming bone marrow cells with GM-CSF alleviates the requirement for "signal two" in bone marrow neutrophils

So far, we have demonstrated that bone marrow neutrophils can adopt one of two fates when they are elicited with thioglycolate or necrotic cells. They presumably receive multiple signals between exiting the bone marrow and being harvested during a peritoneal lavage. To identify the signal(s) that leads to the "arming" of the bone marrow neutrophils, we took a reductionist approach.

We harvested bone marrow from mice and pretreated the cells with a series of stimuli that are know to act on neutrophils, including M-CSF, GM-CSF, IL-1β, TNFα and F-MLP. Out of these agents, only GM-CSF recapitulated the "armed" phenotype: after treating bone marrow with GM-CSF overnight, we could detect bone marrow neutrophils making TNFα in response to purified TLR ligands, while untreated bone marrow neutrophils could not (**Fig. 4.7**).

It should be pointed out that adding GM-CSF in combination with TLR ligands did not lead to the same response, the GM-CSF must be added prior to the addition of the TLR ligand. This suggests that it is not simply GM-CSF signaling which leads to "arming" the neutrophils, but it is likely the activity of a GM-CSF target gene that leads to the "arming" of bone marrow neutrophils.

Additionally, we have sorted bone marrow neutrophils to purity and then pretreated them with GM-CSF before the addition of TLR ligands. With this experimental setup, we again observed that GM-CSF can "arm" bone marrow neutrophils (**Fig 4.8)**. By sorting the cells prior to the addition of GM-CSF, we excluded the possibility that an additional cell type, which is found in bone marrow, is necessary for "arming".

Syk activation is not necessary for licensed neutrophils to make TNFα

As discussed above, it has been long appreciated that intergrin signaling can activate neutrophils. Furthermore, it has been demonstrated that intergrin signaling in neutrophils relies on the kinase Syk (Mocsai et al., 2002). More recently, it has been shown that Syk is phosphorylated in

response to bacteria and this is necessary in bone marrow neutrophils for TNFα production (Van Ziffle and Lowell, 2009). Therefore, we wanted to examine the phosphorylation state of Syk to determine if TNFα production correlated with the phosphorylation of Syk in both bone marrow and thioglycolate elicited neutrophils.

As expected, we observed the phosphorylation of Syk in bone marrow and thioglycolate elicited cells when stimulated with bacteria – two conditions under which we have observed TNFα production (**Fig 4.9**).

When we stimulated bone marrow cells with TLR ligands we did not observe phosphorylated Syk – which correlates with this condition being unable to lead to TNFα production. Surprisingly, thioglycolate elicited neutrophils which were stimulated with TLR ligands did not have detectible levels of phosphorylated Syk either (**Fig 4.9**). This result was unexpected because thioglycolate elicited neutrophils under these conditions produce TNFα.

These data suggest that the TNFα produced by "naïve" bone marrow neutrophils may be dependent on Syk activation as suggested by Van Ziffle et al. But more interestingly, these data suggest that "armed" neutrophils might be using a novel pathway to lead to TNFα production in response to TLR ligands – a pathway that is independent of Syk kinase activation.

Discussion

In this study, we examined how different types of neutrophils respond to TLR ligands and whole bacteria.

We report the unexpected observation that bone marrow neutrophils fail to make TNFα in response to purified TLR ligands, while thioglycolate elicited neutrophils can. We suggest that this bifurcation is not due to the lack of maturity of bone marrow resident neutrophils, as bone marrow neutrophils can make TNFα in response to bacteria. We argue that this bifurcation is likely due to a difference in the activation state of neutrophils. Our data suggests that certain inflammatory conditions can cause the "arming" of neutrophils. These "armed" neutrophils can then go on to make pro-inflammatory cytokines upon TLR activation, while naïve neutrophils require multiple receptors to be activated for them to make TNFα.

The observation that naïve neutrophils require signals in addition to TLRs to make TNFα is also unexpected. The well-studied cells of the innate immune system, macrophage and dendritic cells, can make TNFα in response to TLR ligands without the need for additional pathways to be activated. Why neutrophils require supplementary signals to be activated is not clear. As neutrophils make up the first line of defense against pathogens, it might be expected that they would readily make pro-inflammatory cytokines to initiate immune responses.

Our results with neutrophils elicited by sterile cell death compared to neutrophils elicited by inflammatory signals suggest that the function of neutrophils can be rather plastic, it is possible to elicit neutrophils without "arming" them. Based on the type of elicitation, neutrophils seem to be able to adopt different levels of pro-inflammatory behavior. Plasticity within the innate immune system is not unheard of, as it is known that macrophages can be polarized to be M1 macrophages or M2 macrophages (Rauh et al., 2005), but so far there has been little evidence to suggest any kind of polarization for neutrophils (Fridlender et al., 2009).

It is tempting to speculate that due to the destructive capacity of neutrophils, additional regulatory hurdles have been placed upon them. It would likely be very harmful if simply low levels of TLR ligands that are found in the blood, but which emanated from distant organs (Shi et al., 2011) could lead to neutrophil activation, as this would lead to a great amount of unnecessary tissue damage.

Before a naïve neutrophil acts, it must know that it is truly encountering a foreign microbe; to be sure of this, naïve neutrophils require multiple PRRs to be activated before they make TNFα. While a neutrophil

that has been called into an inflammatory environment, is licensed and can TNFα in response to purified TLR ligands. We have evidence that GM-CSF can provide neutrophils with such a license. We currently do not know if GM-CSF is unique in its ability to license neutrophils or if there are many such factors. In the future it will be interesting to determine the molecular difference between naïve and licensed neutrophils.

Methods

Mice and Viruses

C57BL/6 mice were purchased from Jackson Laboratories. UNC93b1 mutant mice were purchased from the Mutant Mouse Regional Resource Centers. *Myd88 Trif*$^{-/-}$ mice were kindly provided by S. Akira and were backcrossed at least 7 generations onto the C57BL/6 background. All mice were housed within the animal facilities at the University of California, Berkeley according to IACUC guidelines.

Flow cytometry and FACS

Antibodies were from eBioscience, BD Biosciences or Cell Signaling Technology: anti-Ly6C-PerCP-Cy5.5 (clone HK1.4), anti-Ly6G-PE (clone 1A8), anti-TNFα-PE and-FITC (clone MP6-XT22), and phospho-specific Syk (Tyr525/526).

Before staining for surface markers, cells were incubated with an anti-CD16/CD32 antibody (clone 2.4G2, UCSF Monoclonal Antibody Core). Intracellular cytokine staining was performed with Fixation & Permeabilization Kit (eBioscience) according to manufacturer's instructions. Brefeldin was added 30 min after stimulation and cell were harvested after an additional 5 hours. Phoso-Syk staining was performed as described by Underhill etl al. Data were collected on a LSR II (Becton Dickinson) or FC-500 (Beckman Coulter). The data were analyzed with FloJo software (Tree Star).

TNF in supernatants was measured by BD Cytometric Bead Array according to manufacturer's instructions.

Cell sorting was performed on a BD INFLUX. Neutrophils were isolated by sorting for Ly6^{hi} cells from bone marrow.

Tissue culture

Cells were cultured in RPMI 1640 supplemented with 10% FCS, L-glutamine, penicillin/streptomycin, sodium pyruvate, HEPES and Beta-mercaptoethanol.

Bone marrow and peritoneal cell harvesting.

Bones were washed with ethanol and RPMI media, and bone marrow cells were released by gentle grinding with a mortar and pestle. Cells were passed through a filter to remove debris, red blood cells were removed by hypotonic lysis, and the remaining cells were washed three times with RPMI media, counted, and plated.

For isolation of thioglycolate neutrophils, mice were injected with 2 ml of aged 4% thioglycolate medium (BD Diagnostics Systems, Sparks, MD). Sterile inflammation by necrotic cells was performed as described by Chen et al. For all elicited neutrophils: 16 hours later, the mice were euthanized, injected intraperitoneally with 8 ml of PBS supplemented with 3% FCS, L-glutamine, penicillin/streptomycin, and lavaged. Red blood cells were removed by hypotonic lysis, and the remaining cells were washed two times with RPMI media, counted, and plated.

Bone marrow and peritoneal cell stimulation

After plating cells were stimulated with TLR ligands: CpG oligonucleotide (TCCATGACGTTCCTGACGTT with phosphorothioate linkages from IDT or Invitrogen) at 1μM or Pam_3SK (Invivogen) at 1 μg/ml. Bacteria were grown at 37ºC, washed twice with PBS, and spin-infected. 20 minutes after spinfection, gentamicin was added. Brefeldin was added 30 min later and cell were harvested after an additional 5 hours.

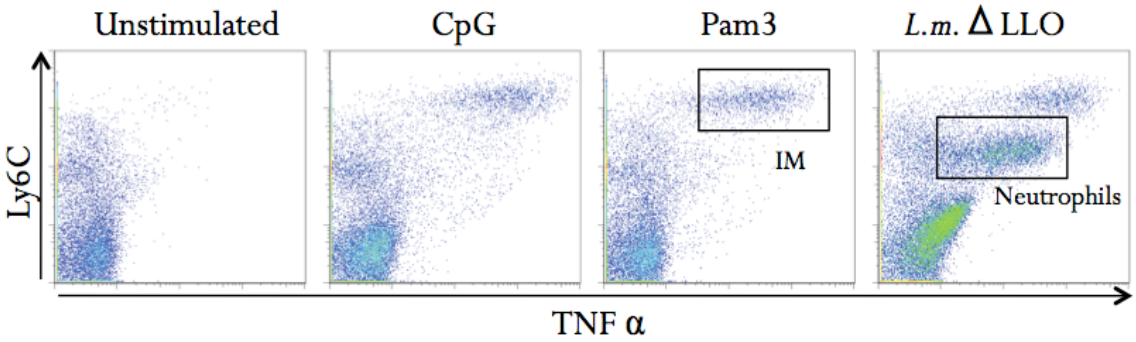

Figure 4.1 - Neutrophils make TNFα in response to bacteria but not purified PAMPs. Bone marrow cells were isolated and either left unstimulated or stimulated for 6 hours with CpG, Pam_3 or Listeria. Intracellular TNF was measured by flow cytometry in inflammatory monocytes ($Ly6C^{hi}$ cells) and neutrophils ($Ly6C^{int}$ cells).

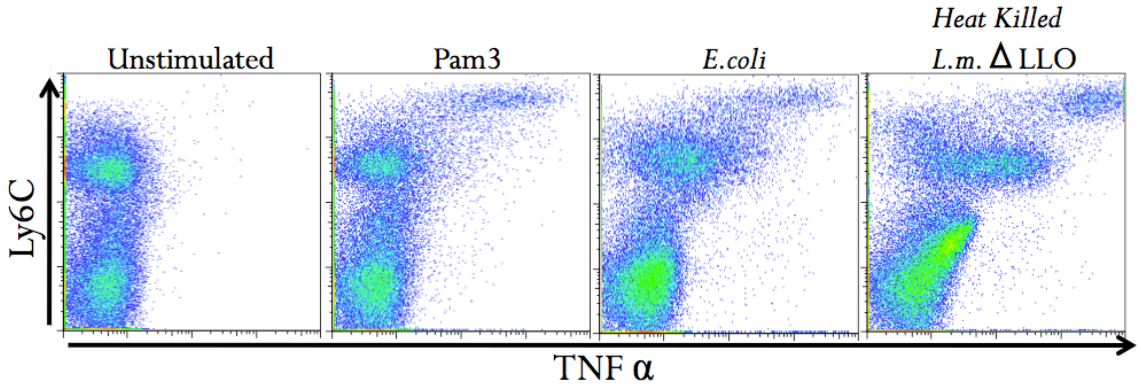

Figure 4.2 - Neutrophils make TNFα in response to non-pathogenic and dead bacteria. Bone marrow cells were isolated and either left unstimulated or stimulated for 6 hours with Pam_3, *E. coli, or* Listeria that was incubated at 100ºC for 10 minutes. Intracellular TNF was measured by flow cytometry in inflammatory monocytes ($Ly6C^{hi}$ cells) and neutrophils ($Ly6C^{int}$ cells).

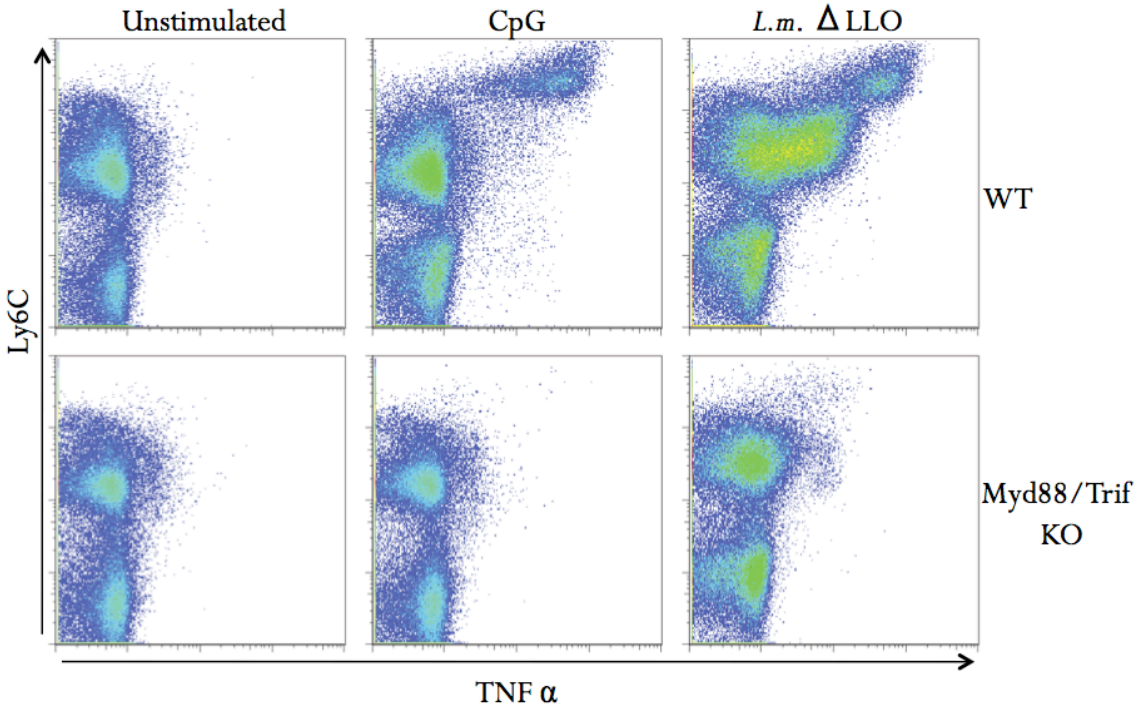

Figure 4.3 - TLR signaling is to activate neutrophils. Bone marrow cells from the indicated genotype were isolated and either left unstimulated or stimulated for 6 hours with CpG, or Listeria. Intracellular TNF was measured by flow cytometry in inflammatory monocytes ($Ly6C^{hi}$ cells) and neutrophils ($Ly6C^{int}$ cells).

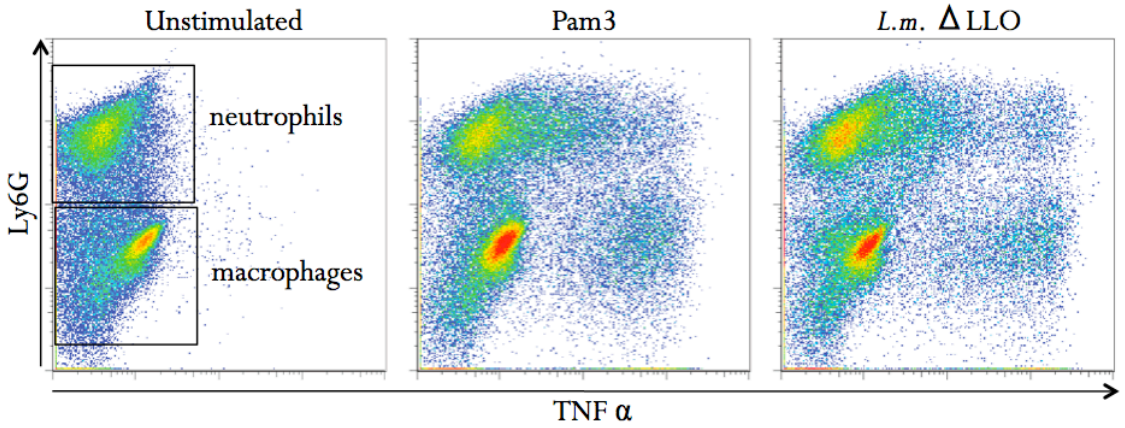

Figure 4.4 - Thioglycolate elicited neutrophils can make TNFα in response to TLR ligands. 16 hours after the injection of thioglycolate, peritoneal cells were isolated. Cells either left unstimulated or stimulated for 6 hours with Pam_3, or Listeria. Intracellular TNF was measured by flow cytometry in neutrophils (Ly6G+ cells) and macrophages (Ly6G- cells).

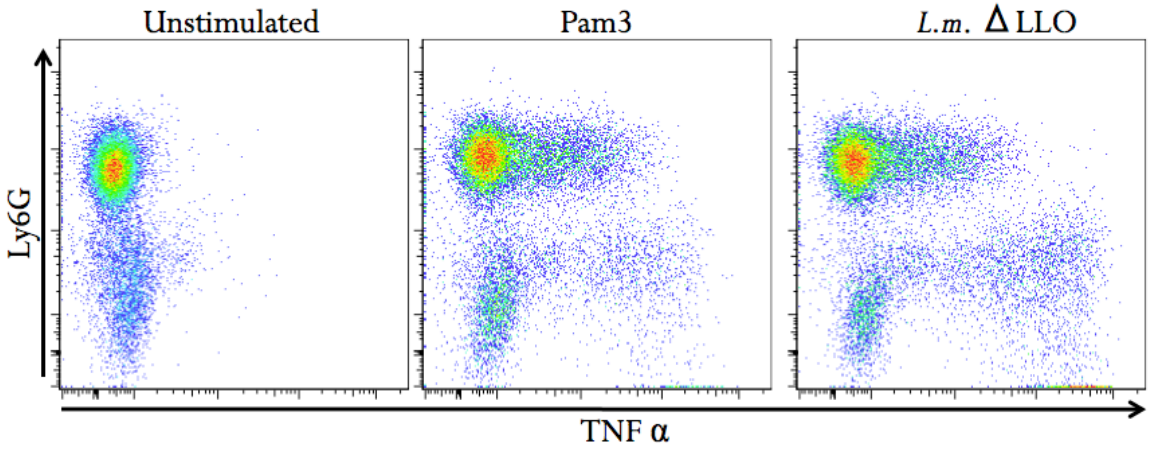

Figure 4.5 - Incomplete freund's adjuvant elicited neutrophils can make TNFα in response to TLR ligands. 16 hours after the injection of incomplete freund's adjuvant, peritoneal cells were isolated. Cells either left unstimulated or stimulated for 6 hours with Pam_3, or Listeria. Intracellular TNF was measured by flow cytometry in neutrophils (Ly6G+ cells) and macrophages (Ly6G- cells).

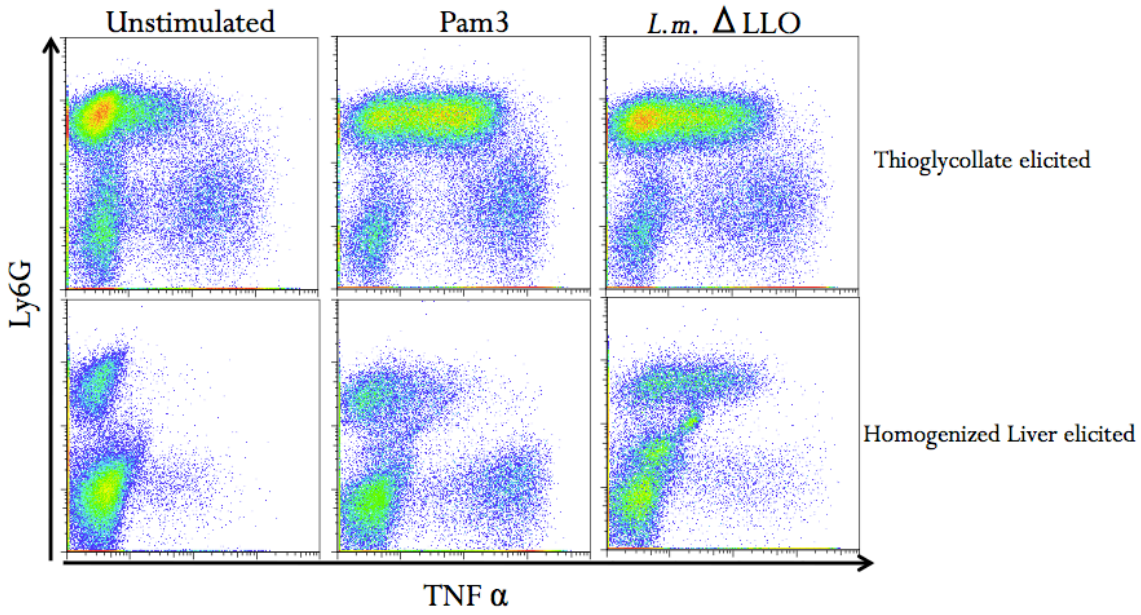

Figure 4.6 - Neutrophils elicited by sterile cell death cannot respond to TLR ligands. 16 hours after the injection of thioglycolate or necrotic homogenized liver extracts, peritoneal cells were isolated. Cells either left unstimulated or stimulated for 6 hours with Pam_3 or Listeria. Intracellular TNF was measured by flow cytometry in neutrophils (Ly6G+ cells) and macrophages (Ly6G- cells).

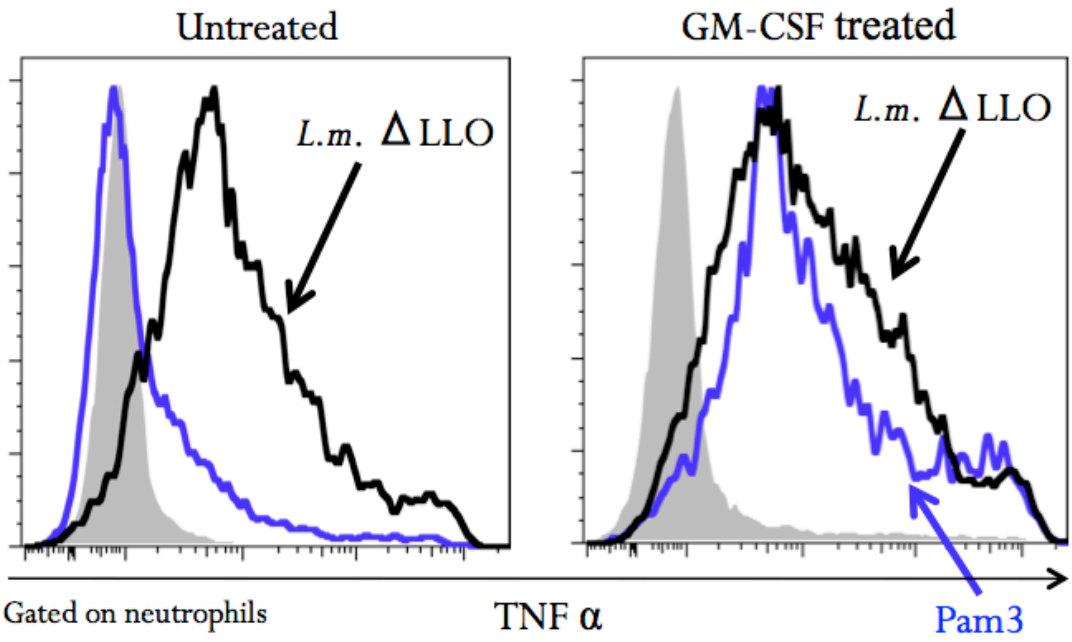

Figure 4.7 - GM-CSF treated neutrophils can make TNFα in response to TLR ligands. Bone marrow cells were isolated and either left untreated or treated with GM-CSF for 12 hours. Cells were then either left unstimulated(gray shading) or stimulated for 6 hours with Pam_3 or Listeria. Intracellular TNF was measured by flow cytometry in neutrophils by gating on Ly6C+ cells.

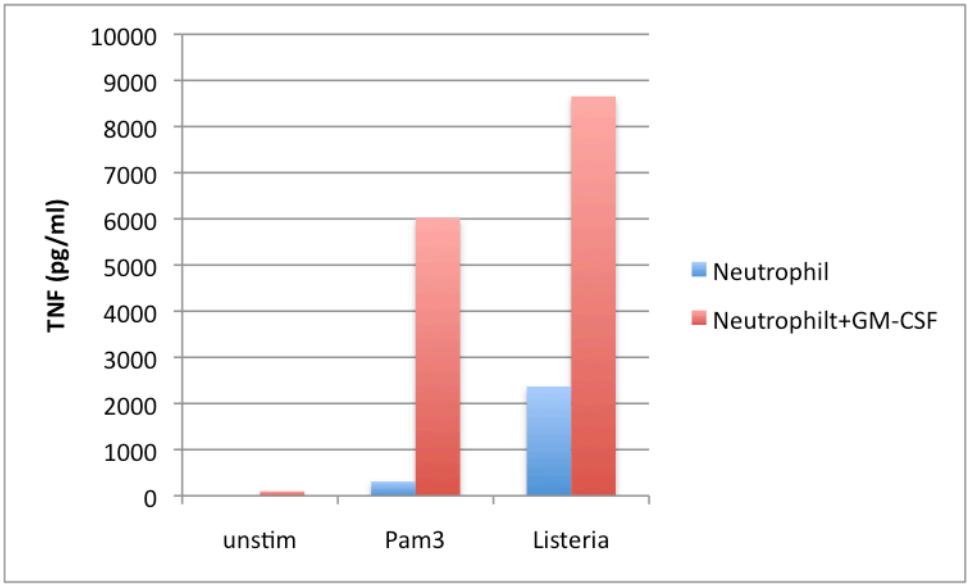

Figure 4.8 – Purified bone marrow neutrophils treated with GM-CSF can make TNFα in response to TLR ligands. Ly6G positive cells were sorted from bone marrow and either left untreated or treated with GM-CSF for 12 hours. Cells were then either left unstimulated or stimulated overnight with Pam_3 or Listeria. TNF in supernatants was measured by CBA (cytokine bead array).

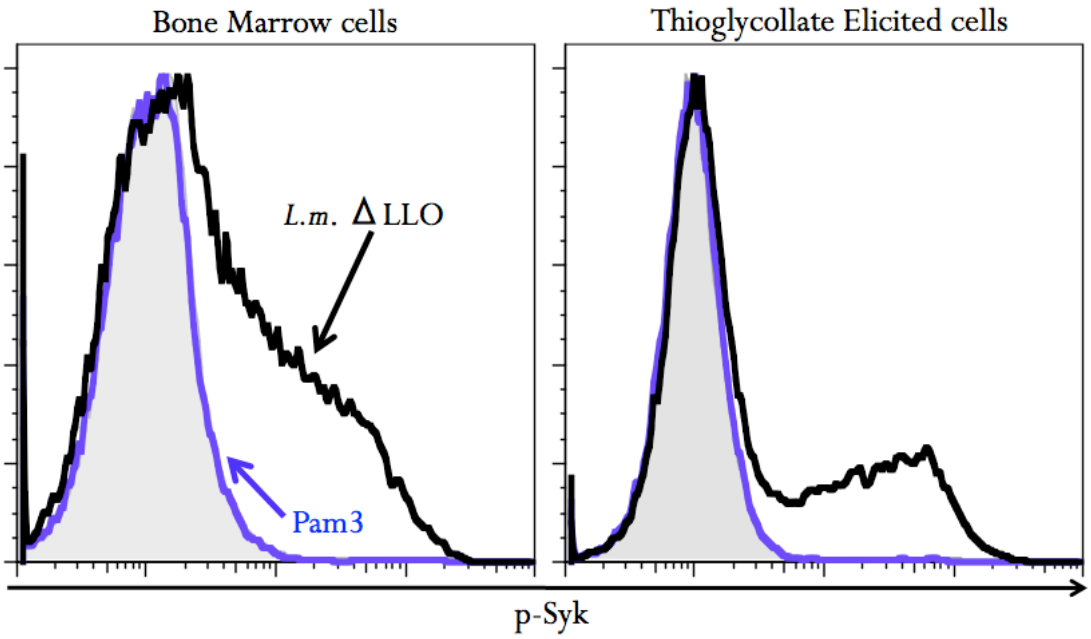

Figure 4.9 - Syk activation does not correlate with TNFα production. Bone marrow cells or Thioglycollate elicited cells were isolated and either left unstimulated or stimulated for 30 minutes with Pam or Listeria in the presence of 1 mM sodium orthovanadate. Phospho-Syk levels were measured by using a phospho-specific Syk antibody.

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