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Publication Date
2011-01-01

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Self/Non-self Discrimination by Toll-like Receptor 9

by

Maria Luz Mouchess

A dissertation submitted in partial satisfaction of the requirements for the

degree of

Doctor of Philosophy

in

Molecular and Cell Biology

in the

Graduate Division

of the University of California, Berkeley

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Spring 2011
Abstract

Self/Non-self Discrimination by Toll-like Receptor 9

By

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Doctor of Philosophy in Molecular and Cell Biology

University of California, Berkeley

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Recognition of nucleic acids as a signature of infection by Toll-like receptors (TLRs) 7 and 9 exposes the host to potential self-recognition and autoimmunity. It has been proposed that intracellular compartmentalization is largely responsible for reliable self/non-self discrimination by these receptors. Our lab has previously shown that TLR9 and TLR7 require processing prior to activation, which may further restrict TLR9 and TLR7 compartmentalization and reinforce self/non-self discrimination; although, this possibility has remained untested. We have identified residues within the TLR9 transmembrane (TM) region that confer the requirement for ectodomain proteolysis. TLR9 TM mutants responded to extracellular DNA and mice expressing such receptors died from systemic inflammation and anemia. This inflammatory disease did not require lymphocytes and appears to require recognition of self-DNA by dendritic cells. These results provide the first demonstration that TLR-intrinsic mutations can lead to a break in tolerance and support the hypothesis that ectodomain processing has evolved to reinforce self/non-self discrimination by nucleic acid-sensing TLRs.
I would like to dedicate this work to my parents,
Maria Luz Jaramillo Mouchess
and
Luis Alberto Mouchess
Table of Contents

Abstract 1

Chapter 1: An introduction to nucleic-acid sensing TLRs and mechanisms that prevent responses to self nucleic acid
Nucleic-acid sensing Toll-like receptors 1
Mechanisms of self/non-self discrimination by nucleic acid receptors 2
The nature of self-ligands 3
Mechanisms of self-ligand clearance 4
Compartmentalization of innate receptors 6
Regulation of cytosolic sensors 7

Chapter 2: Transmembrane mutations in TLR9 bypass the requirement for ectodomain proteolysis
Background 11
Materials and Methods 12
Results 15
Discussion 25

Chapter 3: TLR9 proteolysis is a regulatory mechanism that prevents fatal autoinflammation
Background 27
Materials and Methods 29
Results 31
Discussion 46

Chapter 4: Contribution of the TLR9 cytosolic domain to TLR9 trafficking
Background 48
Materials and Methods 51
Results 52
Discussion 56

Chapter 5: Future Directions and Closing Remarks
Future Directions 57
Closing Remarks 59
References 60
List of Figures

Figure 1.1: Overview of TLR9 trafficking ................................................. 9
Figure 1.2: Multiple mechanisms reinforce self/nonself discrimination by innate receptors that recognize nucleic acids. ...................... 10
Figure 2.1: Alternate approaches do not allow testing of regulatory role of TLR9 processing and TLR9^TM-MUT does not induce altered downstream signaling pathways. 18
Figure 2.2: TLR9^TM-MUT does not require processing for activation and responds to extracellular ligands. 20
Figure 2.3: TLR9^TM-MUT localizes to the cell surface and bypasses the endolysosome. 22
Figure 2.4: TLR9 TM mutants identify residues critical in dictating requirement for proteolysis. 23
Figure 2.5: A model for TLR9^TM-MUT signaling. 24
Figure 3.1: Schematic of TLR9^TM-MUT trafficking in comparison to wild-type TLR9. 34
Figure 3.2: Expression of TLR9^TM-MUT in vivo leads to fatal inflammation. 35
Figure 3.3: Analysis of transduction efficiencies, cellularity and serum cytokines in bone marrow chimeras. 36
Figure 3.4: TLR9^TM-MUT mediated inflammatory disease is driven by CD11c+ dendritic cells. 38
Figure 3.5: Depletion of CD11c+ cells in TLR9^TM-MUT radiation chimera using CD11c-DTR HSCs. 40
Figure 3.6: Lack of TNF and lymphotoxin αβ and not type I interferon receptor (IFNAR) partially rescues inflammatory disease in TLR9^TM-MUT expressing bone marrow chimeras. 41
Figure 3.7: Lack of Type I interferon signaling does not rescue TLR9^TM-MUT mediated inflammatory disease. 43
Figure 3.8: TLR9 TM mutants identify residues critical in preventing self-reactivity. 45
Figure 4.1: TLR9 truncation mutants of the cytosolic region suggest the presence of Golgi to lysosome trafficking motif/s in TLR9. 54
Figure 4.2: Identification of amino acids in TLR9 important for proper trafficking to the lysosome. 55
Acknowledgements

First, I want to thank Greg for being a great example of how to be successful in academia. I would like to thank Nicholas Arpaia, Roman Barbalat and Laura Lau for giving me so many great memories and for all good times. I would also like to thank Victor Aguilera, Michelle I. Aguilar, Patty Garcia and Maria Tokuyama for the priceless support during difficult times and for all the love and laughs. Lastly, I want to thank my family and the rest of my comunidad for being my motivation, for teaching me perserverance and for all their sacrifices to make my education a priority.

Si se puede!
Chapter 1: An introduction to nucleic acid sensing TLRs and mechanisms that prevent responses to self nucleic acid

Nucleic acid sensing Toll-like receptors

Toll-like Receptors (TLRs) are receptors of the innate immune system that have evolved to recognize conserved features of microbes. There are 12 TLR family members in mouse and 10 in human (Kawai and Akira, 2005). These receptors are critical for eliciting both innate and adaptive immune responses (Akira et al., 2001). In contrast to adaptive immune responses, which are based on the randomly generated specificity of its antigen receptors, TLR specificity is germline encoded and shaped by natural selection through pressures applied by infectious microbes (Janeway, 1989). All TLRs have highly conserved protein domains: an extracellular ligand binding domain containing leucine rich repeats (LRRs), a single-pass transmembrane region, a short linker region and a cytosolic Toll/IL-1R (TIR) domain responsible for interaction with the signaling adaptor MyD88. A subset of TLRs (TLR3, TLR7, TLR8, and TLR9) link the recognition of nucleic acids to induction of innate and adaptive immune responses (Medzhitov, 2007; Takeda et al., 2003). Most similar in terms of expression profile and signaling between nucleic acid sensing TLRs are TLR7 and TLR9, which recognize CpG DNA motifs and single-stranded DNA, respectively. TLR3 has more restricted expression and signals only through the adapter TRIF. Although this recognition strategy enables detection of viral nucleic acid within the degradative environment of intracellular compartments, the cost of this strategy is the potential recognition of self DNA and RNA, which has been implicated in autoimmune diseases such as systemic lupus erythematosus and psoriasis (Lande et al., 2007a; Marshak-Rothstein, 2006). Accordingly, regulatory mechanisms must exist that prevent responses to self-derived nucleic acids and will be discussed in the latter part of this introduction.

Our long-standing hypothesis has been that the intracellular localization of TLR9 regulates self/non-self discrimination by this receptor. Strikingly, all the TLRs (TLR3, 7 and 9) that recognize nucleic acid are localized within intracellular compartments (Ahmad-Nejad et al., 2002; Funami et al., 2004; Leifer et al., 2004; Matsumoto et al., 2003). The importance of this compartmentalization is made evident in that, if self-nucleic acid reaches these intracellular compartments through internalization by, for example Fc receptors, it can become stimulatory (Means et al., 2005). The localization motif for TLR7 and TLR9 has been proposed to be within the transmembrane domain whereas for TLR3, it is within the linker region. We and others have shown that intracellular localization is important for establishing the balance between avoidance of self-recognition and detection of foreign nucleic acid in endolysosomes (Barton et al., 2006; Diebold et al., 2004; Latz et al., 2004; Leifer et al., 2004; Lund et al., 2003). However, during the beginning of our studies, little was known about the trafficking of nucleic acid sensing TLRs. In order to better understand how mislocalization can lead to loss of tolerance, more information on the cell biology of these receptors was necessary.

Our lab has made considerable progress in this area within the last few years. We have discovered that TLR9 does, in fact, traffic through the Golgi, contrary to previous reports (Ewald et al., 2008). More surprisingly, the majority of the TLR9 protein that is present in the endolysosome where the receptor encounters ligand is actually a cleaved product of the full-length protein (Ewald et al., 2011b; Park et al., 2008a) (Figure 1.1).
This suggests that the cleaved protein present in the phagosome is the functional form of the protein that binds ligand and leads to downstream signaling of TLR9. The processing for TLR9 also seems to proceed in a stepwise fashion, where multiple cathepsins and Asparagine endopeptidase (AEP) are necessary to generate the functional form (Ewald et al., 2011b). Acidification of these compartments is also necessary as blocking the vacuolar H+ ATPase with Bafilomycin A1 blocks TLR9 processing and signaling. This processing seems to be conserved between all nucleic acid TLRs since inhibitors that block the function of cathepsins and AEP also inhibit signaling from TLRs 3, 7 and 9 although much of what is known in terms of cell biology for nucleic acid sensing TLRs has been deduced from studies using TLR9 (Ewald et al., 2011b). Whether there are cell type differences in terms of the proteases necessary to cleave TLR9 remains controversial (Sepulveda et al., 2009). Another recent finding that has suggested that trafficking of nucleic acid sensing TLRs is unique and may require additional regulation is the recent finding of the relationship between Unc93B and intracellular TLRs. Unc93B is a 12-pass transmembrane identified by Bruce Beutler’s group in an ENU mutagenesis screen where mice containing a mutated form of Unc93b have defective TLR3, 7 and 9 signaling (Tabeta et al., 2006). Other chaperones such as PRAT4 or gp96 have been proposed to be general TLR chaperones but Unc93B seems to have a function specific to only nucleic sensing TLRs (Takahashi et al., 2007; Yang et al., 2007). Although many other questions have become uncovered due to these unexpected findings, this information allows us to postulate that perhaps processing serves as a mechanism to limit the functional form of the receptor to endolysosomal compartments. As discussed in chapter 2, we have evidence that this is indeed the case. Altogether, it is clear that nucleic acid sensing TLRs require strict regulation in order to prevent responses to self and maintenance of tolerance is dependant on these mechanisms.

Mechanisms of self/non-self discrimination by nucleic acid receptors

While innate immune recognition of nucleic acids plays an important role in the effective detection of pathogens, this strategy introduces the potential for self-recognition leading to autoimmune or autoinflammatory disorders. Multiple regulatory mechanisms have evolved to ensure that self/non-self discrimination occurs reliably. Much of our understanding of this regulation has been gained from the study of gene-targeted mice or from the study of mouse models of autoimmune diseases, especially systemic lupus erythematosus (SLE). In particular, the MRL strain of mice as well as an MRL strain lacking functional Fas (MRL/lpr) develop SLE-like disease (Marshak-Rothstein, 2006). A number of additional mouse models have been developed, some of which will be discussed in the following sections, but the majority of our knowledge regarding self/non-self discrimination by nucleic acid receptors has been gained from the approaches listed above. In our discussion of self/non-self discrimination by nucleic acid specific receptors, we will emphasize regulation of TLR activity, although similar regulatory mechanisms may apply to cytosolic sensors and are likely to emerge as our understanding of these pathways increases.

One of the signatures of SLE that indicates a break in tolerance to nucleic acids is the presence of antibodies with specificity for nucleic acids or nucleic acid associated proteins, often referred to as anti-nuclear antibodies (ANA) based on their specificity for nuclear structures. These antibodies are commonly used as an indicator of SLE-like
autoimmunity in mouse models, and each of the mouse models described above do develop ANA. TLR9 and TLR7 have been implicated in the development of ANA against DNA and RNA, respectively (Christensen et al., 2005; Christensen et al., 2006; Lau et al., 2005; Leadbetter et al., 2002; Viglianti et al., 2003). Despite their similarity in function and specificity, though, TLR7-deficiency is protective on an MRL/lpr background, while TLR9-deficient mice suffer accelerated disease (Christensen et al., 2006; Nickerson et al., 2010). The explanation for this unexpected difference in the roles for TLR7 and TLR9 remains unclear, although recent work has demonstrated that the increased pathology in TLR9-deficient mice still requires TLR7 function (Nickerson et al., 2010). Future work will surely focus on understanding the mechanism(s) behind TLR9’s protective effects. Further evidence for the primary role of TLR7 in SLE pathology comes from the discovery that a duplication of the tlr7 gene is responsible for the Y chromosome autoimmune accelerator (Yaa) locus phenotype in various strains of mice (Fairhurst et al., 2008; Pisitkun et al., 2006; Subramanian et al., 2006), and overexpression of TLR7 through transgenesis is sufficient to induce SLE-like disease (Deane et al., 2007).

As discussed in greater detail below, the self nucleic acids that activate TLRs (or other innate receptors) during autoimmune disease are typically released from dead or dying cells. TLR-mediated pathology is primarily driven by activation of antigen presenting cells such as DCs, macrophages and B cells; these cells express TLRs and have the ability to acquire nucleic acid ligands, either by receptor-mediated uptake or endocytosis/phagocytosis. Recently, a type I IFN signature has been described in SLE patients as well as mice with SLE-like disease (Baechler et al., 2003; Blanco et al., 2001). Activation of pDCs via TLR7 and/or TLR9 contributes significantly to this cytokine profile. The production of type I IFN enhances many aspects of the immune response, including B cell activation and the production of autoantibodies (Green et al., 2009). Thus, linking viral nucleic acid recognition by innate receptors to induction of type I IFN can potentially amplify the risk of autoimmunity associated with self nucleic acid recognition.

Any innate immune response to self nucleic acids occurs despite multiple checkpoints that collectively function to establish a threshold for activation that ensures self/non-self discrimination. First, the specificity of innate receptors may favor recognition of foreign nucleic acids. Second, mechanisms function to reduce the levels of self-ligands. Finally, the accessibility of receptors is regulated by subcellular compartmentalization. In the following sections we will discuss examples of each of these checkpoints with particular emphasis on their relevance to TLR activation.

The nature of self nucleic acid ligands

In principle, the simplest mechanism to avoid self-recognition would be to target sequence motifs or chemical modifications unique to microbes. For TLR9 there is evidence that such mechanisms may reduce the likelihood of self-DNA recognition. Unmethylated CpG DNA is a much more potent activator of TLR9, and CpG motifs are largely methylated in mammalian genomes (Krieg, 2002). Such suppression of stimulatory motifs in the genome decreases the frequency of TLR9 stimulatory ligands, yet it is quite clear that the remaining motifs are sufficient to activate TLR9 when additional regulatory mechanisms fail. As discussed earlier in the article, it has been
more difficult to define motif preferences for TLR7 and TLR8. While certain sequences may be more likely to activate TLR7, many self-RNAs appear capable of stimulating the receptor. Thus, self/non-self discrimination by TLR7 and TLR9 cannot be assured simply based on the sequence of ligands.

Despite the stimulatory potential inherent to host DNA and RNA, these ligands are typically not accessible to TLRs. The topology of TLRs requires ligands to be extracellular or within vesicles whose lumen is topologically equivalent to the extracellular space (i.e., endosomes and lysosomes). Thus, for any TLR to respond to self nucleic acid, DNA or RNA must be released from a cell. Typically, release of self nucleic acid only occurs during cell death, although there are some interesting exceptions, such as release of DNA “nets” by neutrophils, which will not be discussed here but has been elsewhere (Brinkmann et al., 2004; Fuchs et al., 2007). Conceptually, necrosis is the most likely form of cell death to result in release of stimulatory self-DNA and RNA. In addition, pyroptosis, a form of cell death that results from inflammasome activation, can also lead to nuclear content release. In contrast, apoptosis leads to laddering of the genome and packaging of cellular contents into apoptotic bodies, which are typically non-inflammatory. However, recent evidence suggests that DNA from apoptotic cells retains the ability to stimulate TLR9. The average length of the DNA found in the DNA:anti-DNA antibody immune complexes (IC) in the blood from lupus patients is similar to the size of DNA generated from cleaved chromatin during apoptosis (Means et al., 2005). Moreover, repeated injection of apoptotic cells can lead to autoantibody production, suggesting that apoptotic cells can stimulate autoreactive B cells with specificities for nuclear antigens (Mevorach et al., 1998). Perhaps the strongest evidence that apoptotic nucleic acids remain potential self-ligands comes from the observation that failure to clear apoptotic cells in vivo is associated with a number of autoimmune disorders. As discussed in greater detail below, apoptotic cells that are not efficiently cleared can undergo secondary necrosis, which also leads to release of self nucleic acids.

Mechanisms of self-ligand clearance

Most, if not all, of the self nucleic acid ligands associated with innate immune activation during autoimmunity originate from dying cells. Accordingly, a number of regulatory mechanisms function to remove these stimulatory ligands before they engage innate receptors.

Clearance of apoptotic cells is an essential aspect of homeostasis in all multicellular organisms. Macrophages recognize features on apoptotic cells and engulf them via phagocytosis. The most well characterized and evolutionarily conserved “eat me” signal is phosphatidylserine (PS), a component of the inner leaflet of the plasma membrane that becomes exposed in apoptotic cells. The known secreted molecules that bind to PS and facilitate apoptotic cell uptake are MFG-E8, Gas6 and Protein S (Fadok et al., 2000). MFG-E8 binds a,b3 integrins on phagocytic cells whereas Gas 6 and Protein S bind the receptor tyrosine kinase family members Tyro3, Axl and Mer (TAM receptors). MFG-E8-deficient female mice develop a SLE-like autoimmune disease with ANA and glomerular nephritis (Hanayama et al., 2004). Mice deficient in all 3 TAM receptors develop splenomegaly and hyperresponsive APCs (Rothlin et al., 2007). TAM receptors have been implicated in negative regulation of TLR signaling, so the phenotype of TAM receptor-deficient mice cannot be solely attributed to defective apoptotic cell clearance.
Nevertheless, this process likely leads to release of self nucleic acid ligands and contributes to the disease in these mice. In addition to the TAM receptors, members of the T-cell immunoglobulin and mucin domain (TIM) family have been shown to bind PS and facilitate apoptotic cell uptake. In fact, a number of putative PS receptors have been identified, although the importance of individual receptors for apoptotic cell clearance and whether receptors bind PS directly has remained controversial (Böse et al., 2004; Fadok et al., 2000; Park et al., 2007; Park et al., 2008b).

An important issue that remains poorly understood is why macrophages are not activated by nucleic acid ligands within engulfed apoptotic cells. One potential explanation is that these ligands are degraded by lysosomal hydrolases such as DNase II, thereby preventing TLR recognition. DNase II is a ubiquitously expressed endonuclease that localizes in lysosomal compartments and degrades chromosomal DNA from apoptotic cells and expelled nuclei from erythroid precursors (Kawane et al., 2001; Yoshida et al., 2005). DNase II-deficient mice die from severe anemia due to an inability to breakdown expelled erythrocyte nuclei in the fetal liver. Although the severe anemia and embryonic lethality was rescued by type I IFN receptor (IFNAR) deficiency (Yoshida et al., 2005), the mice later developed arthritis and produced several other inflammatory cytokines such as TNF, IL-1b and IL-6 (Kawane et al., 2006). The sensor responsible for the type I IFN production has not been identified, yet the fact that additional deficiency in MyD88 and TRIF did not rescue the embryonic lethality suggests that a cytosolic sensor is involved (Yoshida et al., 2005). Whether TLRs are responsible for the later production of inflammatory cytokines observed in DNase II x IFNAR-deficient mice is not yet clear.

In addition to clearance of apoptotic cells, mechanisms have evolved to facilitate removal of necrotic cells. One proposed mechanism by which necrotic cells may be cleared is through the serum complement protein, C1q. C1q can clear apoptotic and necrotic cells in an IgM-dependant manner (Ogden et al., 2005). Another protein involved in avoiding self-ligand recognition is serum amyloid P (SAP). SAP is believed to bind chromatin and prevent DNA:protein complexes from becoming immunogenic (Bickerstaff et al., 1999). Interestingly, C1q and SAP deficient mice develop ANA. Moreover, most humans deficient in C1q develop severe SLE (Botto et al., 1998). More recently, receptors for necrotic cell clearance, such as CLEC9A and Mincle, have been identified (Sancho et al., 2009; Yamasaki et al., 2008). Whether deficiency in these receptors increases the likelihood of responses to self nucleic acids has not been examined.

An additional mechanism that reduces the potential for recognition of self nucleic acids is the degradation of extracellular nucleic acids prior to recognition by innate receptors. Dnase I is the major endonuclease found in serum and urine where it functions to degrade extracellular dsDNA into tri- or tetra- oligonucleotides (Martínez Valle et al., 2008). DNase I-deficient mice develop features of SLE, including ANA, glomerulonephritis and eventual death (Napirei et al., 2000). In humans, mutations in dnase I are associated with SLE, and low DNase I activity correlates with glomerulonephritis in patients (Yasutomo et al., 2001). These studies point to DNase I as having a critical role in degrading free DNA and resulting in destruction of an otherwise stimulatory self-ligand.
Compartmentalization of innate receptors

Failure of the clearance mechanisms discussed in the previous section does not a priori result in activation of nucleic acid TLRs. As discussed in earlier sections of this review, TLR3, TLR7, TLR8, and TLR9 are localized intracellularly within endolysosomal compartments. For reasons that are not well understood, free self nucleic acids are not efficiently delivered to these compartments. Instead, multiple mechanisms have been described that facilitate uptake of nucleic acids ligands and bypass the compartmentalization of TLRs. There are four main examples that illustrate how delivery of self-DNA to intracellular compartments can lead to immune cell activation by self-DNA: Fc receptors (FcRs), B cell receptor (BCR) crosslinking, LL37 and HMGB1.

Binding to FcRs or BCRs can lead to internalization of self-ligands followed by trafficking to compartments containing TLRs. The important role of B cells in the pathogenesis of SLE is clear from the break in tolerance evident through the presence of ANA. How the initial break in tolerance occurs for autoreactive B cells is believed to involve activation of B cells by immune complexes containing nucleic acid. B cells primarily utilize the BCR as a method to internalize these immune complexes. Elegant studies involving autoreactive B cells have shown that the BCR can facilitate internalization of ICs, which leads to synergistic activation through the BCR and TLR9 or TLR7 (Leadbetter et al., 2002; Viglianti et al., 2003). Interestingly, there is recent evidence that BCR signals are sufficient to causes relocalization of TLR9 to autophagosomes where it colocalizes with the BCR after internalization of ICs (Chaturvedi et al., 2008). Although this strategy may allow TLRs to sample the contents of antigens internalized by the BCR, this relocalization of TLR9 has been proposed to be responsible for the increased responsiveness of autoreactive B cells to mammalian DNA ligands after BCR stimulation (Avalos et al., 2009). These studies illustrate that the uptake of ICs by the BCR can lead to nucleic acid sensor and autoreactive B cell activation, which can tilt potentially the balance towards autoimmunity. Another method of internalization of self-ligands is through FcRs. FcRs are expressed on a wide range of immune cell types where they bind Fc regions of antibodies and provide inhibitory or activating signals. In addition to the contribution of FcRs to activating or inhibitory signals, further studies using serum from SLE patients have shown that CD32 participates in the delivery of DNA containing immune complexes into TLR9 containing compartments in human pDCs (Means et al., 2005). Thus, BCRs and FcRs allow internalization of immune complexes that potentially carry self-ligands.

Another protein that has been found to facilitate entry of self nucleic acids into immune cells is HMGB1. HMGB1 is a ubiquitous, highly conserved DNA binding protein that recognizes the minor groove of DNA with low sequence specificity to enable bending during transcription initiation. During apoptosis, chromatin deacetylation enhances HMGB1-DNA association resulting in nuclear retention of the protein; however, during necrotic cell death the deacetylation program is not engaged and HMGB1 is passively released from dying cells (Scaffidi et al., 2002). Interestingly, monocytes and DCs have also been shown to secrete HMGB1 in response to pro-inflammatory cytokines or TLR ligands (Wang et al., 1999). These studies along with the observation that administering HMGB1-blocking antibodies during sepsis is sufficient to delay lethality in mice has led to the suggestion that HMGB1 is a mediator...
of inflammation (Wang et al., 1999). Since these initial reports, it has been shown that extracellular HMGB1 associates with DNA released from necrotic cells or DNA-containing immune complexes thereby inducing TLR9 responses to self-DNA rather than directly stimulating immune cells itself (Ivanov et al., 2007; Tian et al., 2007a). Some reports have indicated that HMGB1 association with Receptor for advanced glycosylation end products (RAGE) is required to engage TLR9 responses. In this model, HMGB1 facilitates uptake of self nucleic acids by engaging RAGE at the cell surface and mediating internalization. Recently, HMGB1 and the related family members HMGB2 and HMGB3 have all been shown to bind model DNA and RNA ligands as well as mediate viral recognition (Yanai et al., 2009). Simultaneous siRNA knockdown of all three family members blocked sensing by TLRs as well as the cytosolic nucleic acid sensors suggesting that these proteins play a universal role in nucleic acid sensing (Yanai et al., 2009). These results indicate that the presence of HMGB proteins during infection may increase the probability of detecting rare viral ligands; however, this enhanced sensitivity may come at the potential cost of recognizing self nucleic acids that may be present in the local environment as well. Understanding whether HMGB family members are absolutely required for nucleic acid sensing as well as the role these proteins may play in self/non-self discrimination are important areas of future investigation.

Recently a role for the anti-microbial peptide LL37 was also defined in mediating TLR9 and TLR7 responses to self-nucleic acids during psoriasis. When extracts derived from psoriatic lesions were compared with healthy skin in their ability to elicit type I IFN responses from pDCs, LL37 was identified as the immunologically active component in psoriatic samples (Lande et al., 2007a). This cationic, amphipathic anti-microbial peptide is highly induced in psoriatic lesions and bears structural similarity to α-helical peptides used to transfect DNA (Dufourcq et al., 1998). LL37 was shown to bind and protect DNA and RNA from nucleases and greatly enhance uptake into early endosomes (Ganguly et al., 2009; Lande et al., 2007a). Interestingly, LL37 complexed with class B CpG ODN induced increased type I IFN production, suggesting that the cationic peptide can alter uptake and/or trafficking (Lande et al., 2007b).

Thus, the four mechanisms discussed in this section (uptake through the BCR and FcRs or binding to HMGB1 and LL37) share the property of facilitating delivery of self nucleic acid ligands to intracellular, TLR-containing compartments. In this way, the sequestration of the receptors that appears central to self/non-self discrimination is short-circuited. In addition, these complexes appear to protect nucleic acids from nucleases. Whether LL37 and HMGB simply enhance general uptake of nucleic acids to endosomal compartments or actively influence the delivery of these complexes to compartments specialized for interferon production is yet to be shown. Finally, it is also possible that these proteins may play a role in directly interacting with the TLR itself, as has been suggested for HMGB1 (Ivanov et al., 2007).

**Regulation of cytosolic sensors**

Although extensive studies have been performed to address the role of TLRs in autoimmune disease, much less is known about the role of cytosolic nucleic acid sensors in the pathogenesis of autoimmunity. For most cytosolic RNA and DNA sensors, the mechanisms of self/non-self discrimination remain incompletely defined. It may be the
case that the specificity of certain cytosolic sensors provides the basis for self/non-self discrimination. For example, RIG-I’s specificity for uncapped 5’-triphosphate RNAs favors recognition of foreign RNAs (Hornung et al., 2006). However, not all host RNAs are capped, suggesting that additional regulatory mechanisms may aid self/non-self discrimination. Determination of the origin of DNA may be largely based on localization, as DNA outside of the nucleus is clearly an anomaly; although, as discussed below, mechanisms clearly regulate cytosolic DNA recognition as well.

One of the first studies to highlight the contribution of other DNA sensors to autoimmune pathology was the analysis of DNase II-deficient mice. As mentioned earlier, DNase II degrades DNA from apoptotic cells and expelled nuclei from erythroid cells. DNaseII knockout mice are embryonic lethal due to severe anemia but DNaseII x IFNAR-deficient mice that are rescued from the anemia phenotype later develop polyarthritis dependent on TNFa (Kawane et al., 2006). It is not yet clear which innate sensors are activated in these animals but IFNβ appears to be responsible for the anemia phenotype, as IFNAR deficiency rescues the embryonic lethality (Yoshida et al., 2005). The type I IFN production correlates with an increase in macrophage number in the fetal liver and thymus. Moreover, these macrophages contain undigested DNA, suggesting that macrophages, not pDCs, are responsible for the type I IFN made. In macrophages type I IFN is typically induced by cytosolic sensors whereas TLR signaling induces inflammatory cytokines, suggesting that ligands produced by DNaseII knockouts may engage cytosolic sensors rather than TLRs.

More recently, Trex1 (also known as DNASEIII) was identified as a negative regulator of cytosolic DNA sensors. Trex1 is the most abundant 3’ to 5’ exonuclease in the cell. It localizes to the ER and cytosol and acts on both ssDNA and dsDNA (Stetson et al., 2008). Furthermore, Trex1 is the only 3’ to 5’ exonuclease known to have type I IFN inducible expression. Trex1-deficient mice succumb to autoimmune inflammatory myocarditis that is rescued by additional deficiency in IRF3, IFNAR or RAG2. However, Trex1xRAG2-deficient mice still induce IFNb implying that disease is initiated by innate immune activation. Remarkably, Trex1 deficiency led to an increase in cytosolic DNA derived from endogenous retroelements, indicating that one of the functions of Trex1 is to degrade cytosolic DNA derived from reverse transcribed retroelements. This work suggests that cytosolic DNA sensors require regulation to prevent autoimmunity in a cell intrinsic manner, as opposed to TLRs that work through a non-cell autonomous manner. Moreover, these data provide mechanistic insight for earlier findings that Trex1 is a susceptibility locus for patients with the neurological inflammatory disease, Aicardi-Goutieres Syndrome (AGS). It is also known that heterozygous mutations in Trex1 cause familial chilbain lupus, which is considered a SLE-subtype and has been also linked to SLE (Rice et al., 2007). Both AGS and chilbain lupus can show evidence of ANA.
Figure 1.1. Overview of TLR9 trafficking. TLR9 is found mostly in the endoplasmic reticulum as well as endolysosomal compartments. From the ER, the receptor then traffics through the Golgi where it is then routed to the endolysosome. It is not yet clear whether TLR9 accesses the cell surface in route to endosomes. Once it reaches endosomes, it requires acidicification of these compartments to generate the functional form of the receptor. TLR9 is cleaved in a stepwise fashion, first by both cathepsins and asparagine endopeptidase and then further trimmed by multiple cathepsins. Unc93B is a 12-pass transmembrane protein that has been proposed to serve as a chaperone for nucleic acid sensing TLRs to exit the ER and for delivery to the endolysosome. We hypothesize that the requirement for processing in the endolysosome to generate the functional form of the receptor prevents any TLR9 that may reach the surface in its full-length form from becoming activated by potentially accessible self-nucleic acid.
Figure 1.2. Multiple mechanisms reinforce self/nonself discrimination by innate receptors that recognize nucleic acids. Self nucleic acid ligands for innate immune receptors are released from necrotic cells or from apoptotic cells that undergo secondary necrosis. Receptors that bind and clear apoptotic or necrotic cells reduce the likelihood that self-ligands will encounter innate receptors. DNaseI can also destroy extracellular DNA ligands prior to internalization; DNaseII plays a similar role within lysosomes after ligand internalization. Self nucleic acids that bypass these clearance or degradation mechanisms may associate with proteins that facilitate uptake and delivery to intracellular compartments. Antibodies (either on the B cell surface or as immune complexes), antimicrobial peptides (LL37), or HMGB1 can all mediate nucleic-acid uptake. DNA that escapes the phagosome or otherwise gains access to the cytosol (e.g., reverse transcribed retroelements) may activate cytosolic sensors. Trex1 (DNaseIII) can degrade these cytosolic ligands. Whether self-RNA can in some instances activate RLRs is not yet known.
Chapter 2: Transmembrane mutations in TLR9 bypass the requirement for ectodomain proteolysis

Background

We hypothesize that aspects of TLR9 cell biology regulate its response and allow for discrimination between foreign and self nucleic acid. One such regulatory mechanism appears to be the intracellular localization of TLR9 and TLR7, which sequesters these receptors from extracellular self nucleic acid released from necrotic cells or apoptotic cells that undergo secondary necrosis (Barton and Kagan, 2009; Marshak-Rothstein, 2006). Ligand recognition occurs in endocytic compartments, although the mechanistic details of receptor trafficking and localization remain somewhat enigmatic and, in some cases, controversial (Brinkmann et al., 2007a; Ewald et al., 2008a; Kim et al., 2008; Latz et al., 2004; Leifer et al., 2006; Leifer et al., 2004).

Mechanisms that facilitate delivery of self nucleic acid to these compartments, such as internalization via surface receptors or association with cationic peptides, can trigger TLR activation (Lande et al., 2007a; Leadbetter et al., 2002; Marshak-Rothstein, 2006). It is worth noting that all examples of TLR-mediated recognition of self nucleic acids, especially in vivo, involve aberrant ligand delivery as opposed to receptor intrinsic mutations. One exception comes from our previous work in which we showed that a chimeric TLR9/TLR4 receptor, which traffics to the cell surface, gains the ability to respond to extracellular vertebrate DNA in vitro (Barton et al., 2006). However, the TLR4-based signaling of this receptor prevented us from performing definitive experiments regarding the effect of altered TLR9 localization (Barton et al., 2006). Unlike TLR9 that signals only through the common TLR adaptor MyD88, TLR4 utilizes both MyD88 and TRIF, so the in vitro gain of function we observe with this chimera cannot be unequivocally attributed to altered localization. Moreover, this receptor does not result in autoimmune disease when expressed in vivo (Fig. 2.1A). This lack of disease may be due to the inability of this receptor to signal in pDCs and B cells but could also imply that additional regulatory mechanisms exist that prevent responses to self.

In addition to physical sequestration in intracellular compartments, we and others have recently described another regulatory step that may limit TLR activation to endolysosomes; namely, the ectodomain of TLR9 is cleaved prior to receptor activation (Ewald et al., 2008; Park et al., 2008a). We have recently demonstrated that TLR7 and TLR3 are similarly regulated, suggesting that ectodomain proteolysis may be a general mechanism to restrict nucleic acid sensing TLR activation to intracellular compartments (Ewald et al., 2011a). Receptor processing consists of at least two distinct proteolytic steps mediated by aspartagine endopeptidase and cathepsins (Ewald et al., 2011a; Ewald et al., 2008; Park et al., 2008a; Sepulveda et al., 2009), and treatment of cells with inhibitors of these proteases prevents TLR9, TLR7, and TLR3 activation (Ewald et al., 2011a). An attractive possibility is that this requirement for proteolysis by lysosomal proteases prevents mislocalized receptors (e.g., receptors that access the cell surface en route to the endolysosome) from responding to self nucleic acid because they remain unprocessed and thus non-functional (Barton and Kagan, 2009; Ewald et al., 2008). In this way, the requirement for proteolysis may obviate the need for strict receptor
compartmentalization; however, the importance of receptor processing, and the consequential localized receptor activation, for maintaining tolerance to self nucleic acid remains unexplored. We provide the first evidence that receptor proteolysis has evolved to regulate self/non-self discrimination by nucleic acid sensing TLRs.

**Materials and Methods**

**Reagents.**

All chemicals and reagents, unless noted otherwise, were purchased from Fisher Scientific. CpG ODN (TCCATGACGTTCTACGTT, all phosphorothioate linkages) and 5'-biotinylated CpG ODN of the same sequence were from Integrated DNA Technologies. LPS, salmon sperm DNA (endotoxin free), and R848 were purchased from Invivogen. PolyI:C was purchased from Sigma. GM-CSF was purchased from Peprotech. The following antibodies were from eBioscience unless otherwise stated: anti-HA (clone 3F10, Roche), anti-FLAG (M2 and M5, Sigma), Alexa-647 goat anti-mouse IgG (Invitrogen). TNF was quantified in cell culture supernatants with the CBA mouse inflammation kit (BD Biosciences).

**DNA cloning.**

TLR9™-MUT was constructed by 'PCR sewing' of cDNA corresponding to amino acids 1–810 and 834–1032 of mouse TLR9 and amino acids 698–720 of mouse TLR3. cDNA encoding precleaved TLR9 (corresponding to amino acids 471–1032) was kindly provided by H. Ploegh (MIT). All TLR-encoding cDNAs were cloned into the mouse stem cell virus–based retroviral vector MSCV2.2, provided by M. Schlissel (UC Berkeley). TLR9-HA and TLR9™-MUT-HA were constructed by the addition of a hemagglutinin epitope (YPYDVPDYA) to the C-terminal end of each receptor in MSCV2.2. Unc93b1 and the non-functional H412R mutant were cloned into MSCV2.2. Flag TLR9 and Flag TLR9™-MUT were constructed in pCMV-Flag-8 (Sigma) and were then transferred into MigR2 (a MSCV2.2-based vector in which GFP has been replaced by human CD2).

**Cell lines, plasmids and tissue culture.** HEK293 and HEK 293T cells were from American Type Culture Collection. Cell lines were cultured in DMEM supplemented with 10% (vol/vol) FCS, L-glutamine, penicillin-streptomycin, sodium pyruvate and HEPES, pH 7.2 (Invitrogen). Macrophages and DCs were cultured in M-CSF and GM-CSF, respectively, containing RPMI-1640 medium supplemented with 10% (vol/vol) FCS, L-glutamine, penicillin-streptomycin, sodium pyruvate and HEPES, pH 7.2 as previously described (Barbalat et al., 2009). Unless otherwise noted, stable lines were generated by transducing cells with MSCV2.2 retroviruses encoding the target cDNA. Immortalized macrophages were generated as previously described (Blasi et al., 1985). Briefly, bone marrow-derived macrophages were cultured in supernatant containing M-CSF as well as virus encoding both v-raf and v-myc. After 8 days, macrophages were removed from M-CSF containing media and cultured in RPMI-1640 media with added supplements as described above.

**Mice.** Tlr9™ (provided by S. Akira) were backcrossed at least seven generations onto the C57BL/6 background. Unc93b1™™ mice (generated by B. Beutler) were obtained from
the MMRRC at UC Davis. All mice were housed in the animal facilities at the University of California, Berkeley according to guidelines of the Institutional Animal Care and Use Committee.

**Retroviral transduction.** Transduction of HSCs was performed by first enriching for HSCs by isolating bone marrow from donor mice that had been injected intraperitoneally 4d earlier with 5mg of 5-fluorouracil. Cells were cultured in stem cell media (DMEM supplemented with 15% FCS, 10mM sodium pyruvate, 2mM L-glutamine, 100U/ml of penicillin, 100g/ml of streptomycin, 100ng/ml of stem cell factor, 10ng/ml of IL-6 and 10ng/ml of IL-3, cytokines from R&D systems). 48h later, these cells were transduced with retroviral supernatant (supplemented with stem cell factor, IL-6, IL-3 and polybrene) on two successive days. Virus was produced with the ØNX-E packaging line (provided by G. Nolan, Stanford University). For retroviral transduction of immortalized macrophages, VSV-G–psuedotyped retroviral supernatant was made with GP2-293 packaging cells as previously described (Ewald et al., 2008).

**Immunoprecipitation and western blot analyses.** Cells were lysed as previously described (Ewald et al., 2008). Briefly, cells were lysed in TNT buffer (20 mM Tris pH 8.0, 200 mM NaCl, 1% Triton X-100) supplemented with Complete protease inhibitor cocktail (Roche). Lysates were cleared of insoluble material by centrifugation. For immunoprecipitations, lysates were incubated with anti-HA matrix (Roche) and precipitated proteins were boiled in SDS buffer, separated by SDS–PAGE, and probed by anti-HA, or anti-FLAG immunoblot.

**Luciferase assays.** Cells were stimulated with ligands and lysed with Passive Lysis buffer (Promega) after 16h. Luciferase activity was measured on a LMaxII-384 luminometer (Molecular Devices). For plate-bound CpG assays, strepavidin coated plates were blocked overnight with 0.1% BSA in PBS followed by incubation with biotin-CpG ODN (2h at room temperature). After extensive washing, plates were seeded with HEK293 cells stably expressing a NF-kB luciferase reporter and the TLR of interest. In parallel, cells were seeded onto TC plates for stimulation with soluble CpG ODN. Cells were lysed after 6h and luciferase activity was measured as described above. All assays were performed in triplicate.

**Pulse–chase analysis.** Cells were starved for 1 h in cysteine/methionine-free media, then pulsed with 0.25 mCi 35S-cysteine/methionine (Perkin-Elmer). After a 45-min pulse, cells were washed and cultured in 5 ml chase media with 10,000-fold molar excess of L-cysteine, L-methionine or harvested as the zero time point. Time points were harvested as follows: cells were washed twice in 2 ml PBS and lysed in 1 ml RIPA plus protease inhibitor cocktail. Labeled HA-tagged proteins were immunoprecipitated using anti-HA-matrix and visualized using SDS–PAGE.

**Flow cytometry.** For surface marker analysis, cells were pre-incubated with anti-CD16-CD32 (2.4G2; Monoclonal Antibody Core, University of California, San Francisco) followed by staining with the indicated antibodies. To measure TNF production, brefeldinA was added 30 min after stimulation, cells were collected after an additional
4h, and cells were stained for intracellular cytokines with a Fixation & Permeabilization kit according to manufacturer's instructions (eBioscience). For FLAG-TLR surface expression, HEK293T cells stably expressing FLAG-TLR9, FLAG-TLR9\textsuperscript{TM-MUT}, or FLAG-TLR2 were stained with anti-FLAG (M5) antibody followed by Alexa-647 goat anti-mouse IgG secondary antibody. All data were collected on LSR II (Becton Dickinson) or FC-500 (Beckman Coulter) flow cytometers and were analyzed with FloJo software (TreeStar).

**Statistical analysis.** Student’s paired t-test was used for statistical comparison. P-values represent comparison of TLR9\textsuperscript{TM-MUT} with wild type TLR9. Prism software was used for all analyses (Graphpad Software).
Results

*Mutations in the TLR9 TM domain enable signaling in the absence of ectodomain proteolysis.*

A direct way to test the role of receptor processing in self/non-self discrimination would be to express TLR9 truncation mutants already lacking the portion of the ectodomain that is normally removed by cleavage. We constructed a number of different “pre-cleaved” receptors, including a truncated TLR9 mutant recently reported to complement TLR9-deficiency (Park et al., 2008a); however, when expressed in HEK293T cells or TLR9-KO macrophages, these receptors responded very poorly or not at all to CpG oligonucleotides (ODN) (Fig. 2.1B). Analysis of N-linked sugars indicated that truncated TLR9 mutants were unable to exit the ER, presumably due to misfolding of the ectodomains (Fig. 2.1C). Thus, we concluded that expression of pre-truncated receptors would not allow us to test the physiological relevance of receptor cleavage for maintaining tolerance.

As an alternative approach, we sought to construct mutant TLR9 receptors that bypass the requirement for ectodomain processing prior to activation. Ligand binding studies indicate that proteolysis does not regulate the receptor’s ability to bind ligands; instead, processing of the ectodomain may be required to allow ligand-bound receptors to undergo a conformational change that initiates MyD88 recruitment and signal transduction. Indeed, the initiation of signal transduction by TLR9 homodimers appears to require a membrane proximal shift in the cytosolic domains. These data as well as the presence of atypical amino acids for a membrane spanning region led us to focus on the transmembrane (TM) domain of TLR9 as potentially involved in mediating the requirement for ectodomain processing. Our strategy was to create a chimeric receptor (hereafter called TLR9\(^{TM-MUT}\)) in which the TLR9 TM region was replaced with a more conventional, largely hydrophobic membrane spanning sequence (Fig. 2.2A).

To determine the impact of TM substitutions on TLR9 signaling, we tested the ability of cells expressing TLR9\(^{TM-MUT}\) to respond to TLR9 ligands. HEK293 cells transduced with TLR9\(^{TM-MUT}\) responded to CpG ODN (with the nuclease-resistant phosphorothioate backbone), although the response was weak relative to cells expressing wildtype TLR9 (Fig. 2.2B). Similar results were observed in TLR9-deficient macrophages and dendritic cells reconstituted with each receptor (Fig. 2.2C and Fig. 2.1D). As expected, TLR9\(^{TM-MUT}\) signaling still required the ER-resident protein, Unc93b1 (Fig. 2.1F). Additionally, expression of TLR9\(^{TM-MUT}\) did not perturb the balance between Unc93b1 and other nucleic acid sensing TLRs, as TLR7 and TLR3 responses were largely unaltered in TLR9\(^{TM-MUT}\) expressing macrophages and DCs (Fig. 2.1D, E). Remarkably, despite the reduced signaling of TLR9\(^{TM-MUT}\) to CpG DNA ligands mentioned above, activation of TLR9\(^{TM-MUT}\) was no longer blocked by bafilomycinA1 or z-FA-FMK (Fig. 2.2D, E), compounds that potently block receptor processing (Ewald et al., 2011a; Ewald et al., 2008; Park et al., 2008a). BafilomycinA1, in particular, leads to accumulation of a post-ER full-length form of TLR9, the immediate precursor of the cleaved receptor (Ewald et al., 2008). This unprocessed form of TLR9\(^{TM-MUT}\) also accumulated with z-FA-FMK treatment (Fig. 2.2F, see asterisk). However, in contrast to TLR9, this accumulation of the full-length form of TLR9\(^{TM-MUT}\) correlated with increased responsiveness to ligands, further supporting the conclusion that mutations in the TM
enable TLR9\textsuperscript{TM-MUT} to signal independently of processing. Thus, altering the TM region of TLR9 can create a receptor (TLR9\textsuperscript{TM-MUT}) that no longer requires proteolytic processing prior to activation.

To determine the impact of this altered regulation on self/non-self discrimination by TLR9, we first compared the responses of TLR9 and TLR9\textsuperscript{TM-MUT} to phosphodiester CpG ODN and salmon sperm genomic DNA, ligands to which wildtype TLR9 responds poorly due to degradation of ligands before reaching the TLR9-containing endolysosome or exclusion from endolysosomes, respectively (Fig. 2.2C, G). TLR9\textsuperscript{TM-MUT} responded robustly to both ligands while TLR9 did not, suggesting that the unprocessed TLR9\textsuperscript{TM-MUT} receptor had gained the ability to respond to ligands at the cell surface. To test this possibility directly, we compared the abilities of cells expressing TLR9 and TLR9\textsuperscript{TM-MUT} to respond to biotin-CpG ODN immobilized by conjugation to streptavidin-coated plates. While cells expressing wildtype TLR9 were not activated, TLR9\textsuperscript{TM-MUT} expressing cells responded robustly (Fig. 2.2H). These data suggested that bypassing the cleavage requirement enabled TLR9\textsuperscript{TM-MUT} to access and respond to ligands at the cell surface.

Whether TLR9 trafficking includes a plasma membrane step is not well understood. Most studies have not detected the receptor at the cell surface, although it remains possible that the receptor localizes to the plasma membrane in certain cell types or during an intermediate trafficking step (Latz et al., 2004; Lee et al., 2006). To examine whether surface localization could explain the robust response of TLR9\textsuperscript{TM-MUT} to extracellular ligands, we measured surface expression of N-terminal FLAG-tagged receptors by flow cytometry. Use of an N-terminal tag allows us to assess how much, if any, of the unprocessed receptors are at the cell surface. TLR9\textsuperscript{TM-MUT} was clearly detectable on the cell surface at levels higher than TLR9 albeit at lower levels than TLR2 (Fig. 2.3A). This difference in localization was not due to differences in overall expression, as anti-FLAG immunoblot revealed similar levels of each protein, and the cells expressed similar levels of a bicistronic human CD2 reporter (Fig. 2.3A, inset, and figure not shown). Consistent with the slightly elevated levels of TLR9\textsuperscript{TM-MUT} at the cell surface, the cleaved form of TLR9\textsuperscript{TM-MUT} was reduced relative to wild-type TLR9, suggesting that TLR9\textsuperscript{TM-MUT} does not efficiently traffic to the endolysosome (Fig. 2.3B, filled arrowheads). This reduction in processing was not due to inefficient ER exit, as the shifted form of the full-length receptor (representing the pool of TLR9 that has exited the ER and trafficked through the Golgi) was detectable in cells expressing TLR9\textsuperscript{TM-MUT} (see asterisk in Fig. 2.3B). As expected, this shifted form of the protein was resistant to endoglycosidase H (endoH) treatment (Fig. 2.3C and (Ewald et al., 2008). Pulse/chase analysis confirmed that the post-ER full-length forms of TLR9 and TLR9\textsuperscript{TM-MUT} appeared with similar kinetics (asterisk in Fig. 2.3D). However, the shifted form of TLR9\textsuperscript{TM-MUT} was more stable than the shifted form of TLR9 (Fig. 2.3D, 3h versus 4h), which agrees with the higher levels of TLR9\textsuperscript{TM-MUT} at the cell surface. Thus, receptor trafficking of TLR9\textsuperscript{TM-MUT} was largely similar to wildtype except for detectable cell surface expression and reduced cleavage of the receptor. However, TM mutations enable TLR9\textsuperscript{TM-MUT} to respond in its full-length form to extracellular ligands while at the cell surface.

The phenotype associated with expression of the TLR9\textsuperscript{TM-MUT} receptor suggests that elements of the TLR9 TM impose the requirement for ectodomain proteolysis. To define more precisely which residues are required to maintain TLR9 tolerance, we made
additional mutants (TLR9\textsuperscript{TM-MUT2}, TLR9\textsuperscript{TM-MUT3}, and TLR9\textsuperscript{TM-MUT4}) in which fewer residues within the TLR9 TM domain were altered (Fig. 2.4A). Analysis of the processing of each receptor indicated that only one of the mutants, TLR9\textsuperscript{TM-MUT4}, recapitulated the altered trafficking and proteolysis we had observed with TLR9\textsuperscript{TM-MUT} (Fig. 2.4B). Moreover, TLR9\textsuperscript{TM-MUT4} responded, albeit more weakly than TLR9\textsuperscript{TM-MUT}, to plate-bound CpG ODN and was insensitive to protease inhibitors, while TLR9\textsuperscript{TM-MUT2} and TLR9\textsuperscript{TM-MUT3} behaved like wildtype TLR9 in these assays (Fig. 2.4C, D). Consistent with these functional data, the levels of TLR9\textsuperscript{TM-MUT4} on the cell surface were below the level of detection (data not shown). Altogether these data suggest that TLR9\textsuperscript{TM-MUT4} has a weaker gain-of-function phenotype than TLR9\textsuperscript{TM-MUT}.
Figure 2.1. Alternate approaches do not allow testing of regulatory role of TLR9 processing and TLR9\textsuperscript{TM-MUT} does not induce altered downstream signaling pathways. TLR9N4C does not induce inflammatory disease in vivo. Hematopoietic stem cells (HSC) from TLR9-deficient mice were transduced with retroviruses encoding TLR9, TLR9\textsuperscript{TM-MUT}, TLR9N4C, or vector control followed by transfer into lethally irradiated C57BL/6 recipient mice (as described in Fig. 3.1A). A, Analysis of RBC and
hemoglobin levels in blood (top) collected from radiation chimeras expressing the indicated receptors and representative analysis of percentages of CD19+ B cells (bottom) in radiation chimeras expressing the indicated receptors.

**B and C.** Pre-truncated TLR9 does not exit the ER and cannot respond to ligands. **B**, Macrophages (top) and HEK293T cells (bottom) expressing precleaved TLR9 receptors (amino acids 471-1032) do not respond to CpG ODN. Top, intracellular cytokine staining for TNF after stimulation with LPS or CpG. Bottom, NF-kB luciferase activity measured in HEK293T cells transiently transfected with the indicated constructs. Cells were stimulated with 3µM CpG ODN 24h post-transfection. **C**, Representative immunoblot showing that precleaved TLR9 receptors are entirely Endoglycosidase H sensitive. Data are representative of at least three experiments.

**D-F.** TLR9\(^{TM-MUT}\) does not induce altered downstream signaling pathways. **D and E**, TLR9\(^{TM-MUT}\) expressing cells respond normally to other nucleic acid TLR ligands. TLR9-deficient DCs (D) and macrophages (E) transduced with retroviruses encoding TLR9, TLR9\(^{TM-MUT}\), or empty vector were stimulated as indicated and the percentage of GFP-positive cells producing TNF was measured by intracellular staining (D and E, left) or Cytometric Bead Array (E, right). Data shown are representative of two experiments. **F**, TLR9\(^{TM-MUT}\) cannot respond to CpG DNA ligands in the absence of Unc93b1. Unc93b 3d/3d mutant (non-functional) macrophages transduced with retroviruses encoding TLR9 or TLR9\(^{TM-MUT}\) and reconstituted with either wildtype or mutant (H412R) Unc93b1 were stimulated as indicated and the percentage of GFP-positive cells producing TNF was measured by intracellular staining and flow cytometry. Data shown are representative of two experiments.
Figure 2.2. TLR9<sup>TM-MUT</sup> does not require processing for activation and responds to extracellular ligands. A, Schematic of TLR9 and TLR9<sup>TM-MUT</sup>. TLR9<sup>TM-MUT</sup> consists of the ectodomain and cytosolic domain of TLR9 (blue) and the transmembrane domain of TLR3 (yellow). B, TLR9<sup>TM-MUT</sup> can respond to CpG DNA ligands. HEK293 cells stably expressing a NF-κB luciferase reporter and TLR9 or TLR9<sup>TM-MUT</sup> were stimulated with CpG ODN (phosphorothioate backbone) as indicated and luciferase activity was measured 16h later. C, Macrophages expressing TLR9<sup>TM-MUT</sup> respond to CpG ODN and extracellular genomic DNA. TLR9-deficient macrophages transduced with retroviruses encoding TLR9, TLR9<sup>TM-MUT</sup> or empty vector (control) were stimulated as indicated and the percentage of GFP-positive cells producing TNF was measured by intracellular staining and flow cytometry. Data shown are representative of two experiments. D and E, Signaling by TLR9<sup>TM-MUT</sup> does not require proteolysis. TLR9-deficient macrophages
expressing TLR9 or TLR9<sup>TM-MUT</sup> were pretreated with bafilomycinA1 for 2 hours (D) or z-FA-FMK overnight (E), stimulated with CpG ODN, and stained to measure TNF production, as described in (C). F, Stabilization of shifted form of TLR9<sup>TM-MUT</sup> in the presence of z-FA-FMK. Anti-HA immunoblot of TLR9 and TLR9<sup>TM-MUT</sup> from lysates of macrophage treated with DMSO (vehicle) or z-FA-FMK. Open triangle indicates full-length TLR9. Asterisk denotes the shifted form of the full-length receptor. Immunoblot shown is representative of two experiments. Unless stated otherwise, experiments in this figure were performed at least three times with similar results. G, TLR9<sup>TM-MUT</sup> is more responsive to extracellular DNA ligands. Luciferase assays were performed as described in (B) except stimulations were with either phosphodiester (PD) backbone CpG ODN or salmon sperm DNA (V. DNA). Data shown are representative of two experiments. H, TLR9<sup>TM-MUT</sup> can respond to immobilized CpG ODN at the cell surface. Cells described in (B) were plated on streptavidin-coated plates to which biotinylated-CpG ODN had been previously conjugated. Luciferase activity was measured in lysates after 6h.
Figure 2.3 TLR9<sup>TM-MUT</sup> localizes to the cell surface and bypasses the endolysosome.
A, Full-length TLR9<sup>TM-MUT</sup> is expressed at the cell surface. Flow cytometry histograms of anti-FLAG staining of HEK293T cells stably expressing N-terminal FLAG-TLR9 (grey), FLAG-TLR9<sup>TM-MUT</sup> (black), FLAG-TLR2 (blue), or empty vector control (shaded). Anti-FLAG immunoblot of whole cell lysates (WCL) of the indicated cells (inset). B, The ectodomain of TLR9<sup>TM-MUT</sup> is processed inefficiently. Anti-HA immunoblot of lysates from TLR9-deficient macrophages expressing C-terminally HA-tagged TLR9 or TLR9<sup>TM-MUT</sup>. Open triangle indicates full-length TLR9. Closed triangle indicates processed TLR9. Asterisk denotes the shifted form of the full-length receptor. C, TLR9<sup>TM-MUT</sup> traffics through the Golgi apparatus. Immunoprecipitated TLR9 or TLR9<sup>TM-MUT</sup> from macrophage lysates were treated with Endoglycosidase H (E), PNGase F (P) or left untreated (U). D, TLR9 and TLR9<sup>TM-MUT</sup> exit the ER with similar efficiency and kinetics. Pulse-chase analysis of macrophages expressing TLR9 or TLR9<sup>TM-MUT</sup>. TLR9 or TLR9<sup>TM-MUT</sup> were harvested at the indicated chase times, immunoprecipitated and visualized by SDS-PAGE. Data presented are representative of at least three independent experiments.
Figure 2.4. TLR9 TM mutants identify residues critical in dictating requirement for proteolysis.  

A, Schematic showing an alignment of TM regions of TLR9 mutants. Underline indicates residues from TLR9. Arrowhead indicates the last conserved cysteine of the ectodomain. B, TLR9TM-MUT4 has altered trafficking to the endolysosome. Anti-HA immunoblots of lysates from TLR9-deficient macrophages expressing the indicated TLR9 TM mutants, as described in (A). C, TLR9TM-MUT4 can respond to ligands at the cell surface. HEK293 cells stably expressing TLR9 or the indicated TLR9 mutants were plated on streptavidin-coated plates to which biotinylated CpG ODN had been previously conjugated. Luciferase activity was measured in lysates after 6h. D, TLR9TM-MUT4 activation does not require proteolysis. TLR9-deficient macrophages expressing the indicated TLR9 TM mutants were stimulated with CpG ODN after treatment with DMSO (vehicle) or z-FA-FMK. TNF production was measured by flow cytometry. The data are normalized for each TLR9 TM mutant by dividing by the percentage of responding cells in vehicle treated control samples. Results shown for B-D are representative of at least three experiments.
Figure 2.5. A model for TLR9<sup>TM-MUT</sup> signaling. Receptors assemble as homodimers prior to ligand binding. (Top) In the endolysosome, cleaved TLR9 binds ligand, which induces a conformational change (arrows) and receptor activation. (Middle) Full-length TLR9 can bind ligand but does not undergo this conformation change. (Bottom) Changes in the TM sequence allow TLR9<sup>TM-MUT</sup> to adopt the ligand-induced conformational change in the absence of ectodomain proteolysis.
Discussion

One of the most surprising aspects of our study is that the TM region of TLR9 confers both the regulatory requirement for ectodomain proteolysis as well as localization of the receptor. Precisely how these amino acids mediate this regulation remains unclear. Experiments using split GFP molecules fused to the TLR9 ectodomain and TM indicate that receptor activation leads to increased proximity of TM domains within the TLR9 dimer (Latz et al., 2007). Previous work from our group has shown that both cleaved and uncleaved TLR9 can bind CpG ODN, yet adapter recruitment and receptor activation is only a property of cleaved receptors (Ewald et al., 2008). Thus, amino acids within the TLR9 TM domain may prevent the formation of this active conformation prior to processing of the ectodomain. Therefore, we postulate that TLR9TM-MUT has gained the ability to undergo ligand-induced conformational changes in the absence of cleavage due to alteration of these residues in the TM (Fig. 2.5F). Notably, the disrupted TM region did not result in constitutive activation of TLR9TM-MUT since signaling does not occur in the absence of ligand (Fig. 2.2B, C). Structural studies of TLR9 may provide clarity on whether the ectodomain conformation after ligand binding is indeed altered upon cleavage of receptor.

Although the requirement for processing is common to all nucleic acid sensing TLRs, it is becoming increasingly clear that these TLRs are each regulated through distinct mechanisms. For example, TLR7 overexpression is sufficient to break tolerance in vivo. However, the lack of disease in wild-type TLR9 expressing radiation chimeras (see Figure 3.2) suggests that the disease in mice expressing TLR9TM-MUT is due to a different form of dysregulation and not simply overexpression. Additionally, in the MRL/lpr model of lupus, TLR9-deficient animals have exacerbated disease while TLR7-deficient mice are partially protected. This surprising result suggests that, despite similar expression profiles and signaling by both receptors, there are important differences in the regulation of TLR7 and TLR9 that influence responses to self-ligands that remain poorly understood. Whether the proteolysis of TLR7 and TLR3 is regulated through residues within the TM of these receptors is not yet clear, and it is possible that there are other regions of these proteins that regulate processing for each receptor. Due to the similarities between TLR9 and TLR7, we attempted to make a comparable “TLR7TM-MUT”, but we were unable to generate a functional receptor. There is also little similarity between the TM regions of these receptors which also supports the idea that regulation of proteolysis may be different for each of the nucleic acid sensing TLRs. Further studies are needed to better understand how each receptor is differentially regulated as well as how signaling from one can influence another within this subfamily of TLRs. Nonetheless, the requirement of receptor processing for activation is a unique property of nucleic acid sensing TLRs and it is tempting to speculate that self/non-self discrimination by TLR7 and TLR3 is similarly regulated by proteolysis as has been shown here for TLR9.

It is also possible that the TLR9 TM mediates interaction with an as yet unidentified regulatory protein that influences signaling and trafficking of the receptor. It is notable that this region of TLR9 contains an aspartic acid (D818), which is quite unusual in TM domains. Charged residues within TM domains can mediate association with other proteins, or in this case may govern the interactions between the TM domains within the TLR9 dimer (Latz et al., 2007). Our strategy did not directly address the
importance of D818; however, a TLR9 mutant with only D818 changed to alanine was inhibited by protease inhibitors similarly to wildtype TLR9, indicating that this amino acid does not, by itself, dictate the requirement for ectodomain proteolysis (data not shown). Additional TM mutants containing a proline instead of the leucine at position 815 or changing amino acids 817-820 to ELL as is present in TLR9 TM-MUT4 is also not sufficient to make these receptors gain the ability to signal without proteolysis in vitro (data not shown). Therefore, it seems likely that multiple residues cooperate to confer this regulation or that the phenotypes of TLR9^{TM-MUT} and TLR9^{TM-MUT4} are dependent on the precise amino acids that we substituted into these receptors.
Chapter 3: TLR9 proteolysis is a regulatory mechanism that prevents fatal autoinflammation

Background

TLRs that recognize nucleic acids are potentially dangerous. As discussed in Chapter 1, these receptors may gain access to self nucleic acid released from necrotic cells. Thus, mechanisms that prevent self-reactivity by TLRs must have evolved within mammalian immune systems. Such mechanisms include suppression of unmethylated CpG DNA motifs in mammalian genomes and the secretion of DNAses important for the degradation of extracellular self-DNA (Figure 1.2) (Napirei et al., 2000; Okabe et al., 2005; Yasutomo et al., 2001). However, self-nucleic still has the inherent ability to stimulate TLR9 under certain pathological circumstances (Ivanov et al., 2007; Park et al., 2006; Tian et al., 2007b).

One shared feature of TLRs that recognize nucleic acids is that they are all localized in intracellular compartments. Recent studies suggest that this intracellular localization contributes to avoidance of self-nucleic acid recognition. Restricting TLR9 ligand recognition to endolysosomes limits the likelihood that self nucleic acid is encountered. While this mechanism is appealing, the signals responsible for establishing TLR9’s intracellular localization remain undefined. Multiple motifs within TLR9 have been reported as necessary for maintaining proper localization. Indeed, both the transmembrane (TM) region and the cytoplasmic regions (TIR domain) of TLR9 have been proposed to be sufficient for localization (Kajita et al., 2006; Leifer et al., 2006; Nishiya et al., 2005).

Not only are the necessary motifs within TLR9 poorly defined, but until very recently, the route by which TLR9 reaches the endolysosomes remained unclear. As illustrated in Figure 1.1, we now know that TLR9 traffics through the secretory pathway en route to the endolysosome rather than a non-canonical pathway from the ER to the lysosome. TLR9 is then cleaved by cathepsins and AEP within acidic compartments to generate the fully functional receptor. These findings that the cleaved and not the full-length receptor is functional led us to hypothesize whether receptor processing is a regulatory mechanism that prevents uncleaved receptors from being able to respond to self ligands that may be accessible at the cell surface. In support of this hypothesis is previous work from our lab that showed that wild-type TLR9, when forced to the cell surface by fusing it to the yeast localization motif Ist-2, cannot signal in response to CpG and is not functional. We sought to make receptors that were competent for signaling at the cell surface.

As mentioned in Chapter 2, we had previously identified a chimeric receptor (T9N4C) that mislocalized to the cell surface and had gained responsiveness to extracellular vertebrate DNA. However, for reasons we do not understand, this receptor was not functional in plasmacytoid dendritic cells or B cells which limited our ability to use this as a receptor to test the relevance of TLR9 for tolerance to self nucleic acid in vivo. Additionally, the TLR4-based signaling of this receptor prevented us from performing definitive experiments regarding the effect of altered TLR9 localization in vivo (Barton et al., 2006). Unlike TLR9 that signals only through the common TLR adaptor MyD88, TLR4 utilizes both MyD88 and TRIF, so the in vitro gain of function we observe with this chimera can not be unequivocally attributed to altered localization,
especially if one seeks to test the importance of this regulation in vivo. Therefore, whether altered localization can disrupt self/non-self discrimination has remained an open question. Now with this new information on the cell biology of TLR9 and the requirement for processing, we can now test whether proteolysis serves as a mechanism to prevent responses to self-ligands. Whether processing has implications for self/non-self discrimination in vivo remains unexplored.

Supporting the idea that avoidance of self-recognition by TLRs is important to prevent autoimmunity, it has been observed that TLRs have a role in the development of SLE (Christensen et al., 2005; Lau et al., 2005; Leadbetter et al., 2002; Viglianti et al., 2003). SLE is a chronic, potentially fatal autoimmune disease characterized by the production of autoantibodies to nuclear antigens (Shlomchik et al., 2001). Marshak-Rothstein’s group has shown that self-DNA associated with antinuclear antibodies can activate TLR9 in autoreactive B cells (Leadbetter et al., 2002). Although the causes of SLE are unknown, TLR9 and TLR7 have both been implicated in the exacerbation of disease in mouse models of SLE (Berland et al., 2006). The contribution of these receptors to disease progression seems complex but nonetheless the production of autoantibodies to nucleic acid is a signature of SLE and TLR9 and TLR7 contribute to this break in tolerance (Christensen et al., 2006). The role for TLRs is further supported by the fact that a TLR7 gene duplication in Yaa mice results in autoreactive B cell responses to RNA-related antigens (Pisitkun et al., 2006). In related work, simple overexpression of TLR7 through transgenesis in vivo is sufficient to cause autoimmunity (Deane et al., 2007). Thus, activation of TLR9 and/or TLR7 by self nucleic acid appears to be a critical step in the development of SLE.

Using the TLR9 TM mutants described in Chapter 2, we can express these receptors in vivo using radiation chimeras and test whether our in vitro findings translate into a loss of self tolerance in vivo. What would be the in vivo consequences of dysregulated TLR9 and how would the disease, if any, manifest itself? To test our hypothesis, we used TLR9\textsuperscript{TM-MUT}, which has the strongest phenotype in vitro i.e., it could signal robustly in the absence of proteolysis and can be detected at the cell surface. What we observed was that expression of dysregulated TLR9 in mice results in lethal autoinflammatory disease. Unexpectedly, we find that this disease is independent of B and T cells yet requires dendritic cells. Thus, dysregulated activation of TLR9 can lead to autoinflammatory disorders in certain contexts. Overall, this work provides the first in vivo demonstration that mutations in TLR9 can break tolerance to self-nucleic acid.
Materials and methods

Reagents.
All chemicals and reagents, unless noted otherwise, were purchased from Fisher Scientific. The following antibodies were from eBioscience unless otherwise stated: PE/Cy7–conjugated anti-CD11c (N418), PE/Cy5–conjugated anti-B220 (RA3-6B2), PE–conjugated anti-TNF (MP6-XT22), PE/Cy7–conjugated CD19 (1D3), PE/Cy5–conjugated anti-CD2 (RPA-2.10, DBBiosciences), APC/Alexa750–conjugated anti-CD11b (M1/70), APC-conjugated anti-MHC II (M5), Alexa647–conjugated anti-SiglecH (ebio440c).

DNA cloning.
TLR9\textsuperscript{TM-MUT} was constructed by 'PCR sewing' of cDNA corresponding to amino acids 1–810 and 834–1032 of mouse TLR9 and amino acids 698–720 of mouse TLR3. cDNA encoding precleaved TLR9 (corresponding to amino acids 471–1032) was kindly provided by H. Ploegh (MIT). All TLR-encoding cDNAs were cloned into the mouse stem cell virus–based retroviral vector MSCV2.2, provided by M. Schlissel (UC Berkeley).

Cell lines, plasmids and tissue culture.
Cell lines were cultured in DMEM supplemented with 10% (vol/vol) FCS, L-glutamine, penicillin-streptomycin, sodium pyruvate and HEPES, pH 7.2 (Invitrogen).

Mice. C57BL/6, Rag1\textsuperscript{−/−}, and Tnf\textsuperscript{−/−} mice were purchased from Jackson Laboratories. Tlr9\textsuperscript{−/−}, Trif\textsuperscript{−/−}, and Myd88\textsuperscript{−/−} mice (provided by S. Akira) were backcrossed at least seven generations onto the C57BL/6 background. Ifnar1\textsuperscript{−/−} mice on the C57BL/6 background were provided by D. Portnoy (UC Berkeley). CD11c-DTR transgenic mice (Jung et al., 2002) on the C57BL/6 background were kindly provided by the Cancer Research Lab at UC Berkeley. Unc93b1\textsuperscript{3d/3d} mice (generated by B. Beutler) were obtained from the MMRRC at UC Davis. All mice were housed in the animal facilities at the University of California, Berkeley according to guidelines of the Institutional Animal Care and Use Committee.

Retroviral transduction. Transduction of HSCs was performed by first enriching for HSCs by isolating bone marrow from donor mice that had been injected intraperitoneally 4d earlier with 5mg of 5-fluorouracil. Cells were cultured in stem cell media (DMEM supplemented with 15% FCS, 10mM sodium pyruvate, 2mM L-glutamine, 100U/ml of penicillin, 100g/ml of streptomycin, 100ng/ml of stem cell factor, 10ng/ml of IL-6 and 10ng/ml of IL-3, cytokines from R&D systems). 48h later, these cells were transduced with retroviral supernatant (supplemented with stem cell factor, IL-6, IL-3 and polybrene) on two successive days. Virus was produced with the ØNX-E packaging line (provided by G. Nolan, Stanford University). After the second transduction, cells were washed three times with PBS before injection into recipient mice. For retroviral transduction of immortalized macrophages, VSV-G–psuedotyped retroviral supernatant was made with GP2-293 packaging cells as previously described (Ewald et al., 2008).

Bone Marrow chimera mice. At least 1 wk before irradiation and BM transfer, mice were placed on trimethoprim-sulfamethoxazole (via drinking water).Recipient mice
received lethal total body irradiation (1000 rad) from a $^{137}$Cs source. Twenty-four hours later they were reconstituted with syngeneic HSCs that had been cultured and transduced as specified in the retroviral transduction section. After reconstitution, animals were monitored until they displayed severe morbidity and/or loss of 10% of their starting body weight. For DC depletion experiments, HSCs derived from CD11c-DTR mice (Jung et al., 2002) were used to generate chimeras as described above followed by intravenous injection with 200ng of diphtheria toxin (Sigma) or saline as indicated in the schematic in Figure 3.4D. At the time of sacrifice, bone marrow and spleens were harvested, processed into single-cell suspensions for counting, and analyzed as previously described (Barbalat et al., 2009). Experimental groups typically consisted of three to five mice. Mice were analyzed individually.

**Blood collection and analysis.** Blood was collected by retroorbital bleeding or cardiac puncture. After collection, blood was divided into aliquots for preparation of sera and for haematological analysis. Serum was prepared using serum separator tubes (BD Biosciences) according to manufacturer’s instructions. Erythrocyte, leukocyte, and hematocrit counts were assayed by the UCSF Comprehensive Cancer Center Mouse Pathology Core facility, using a HEMAVET Multispecies Hematology Analyzer. Cytokines in sera samples were analyzed using SearchLight custom multiplex protein array (Pierce Biotechnology, Inc.).

**Flow cytometry.** For surface marker analysis, cells were pre-incubated with anti-CD16-CD32 (2.4G2; Monoclonal Antibody Core, University of California, San Francisco) followed by staining with the indicated antibodies. To measure TNF production, brefeldinA was added 30 min after stimulation, cells were collected after an additional 4h, and cells were stained for intracellular cytokines with a Fixation & Permeabilization kit according to manufacturer’s instructions (eBioscience). All data were collected on LSR II (Becton Dickinson) or FC-500 (Beckman Coulter) flow cytometers and were analyzed with FloJo software (TreeStar).

**Statistical analysis.** Student’s paired t-test was used for statistical comparison. P-values represent comparison of TLR9$^{TM\text{-MUT}}$ with wild type TLR9. Prism software was used for all analyses (Graphpad Software).
Results

Expression of TLR9\textsuperscript{TM-MUT} in vivo causes lethal autoinflammatory disease

Because the TM mutations enable TLR9\textsuperscript{TM-MUT} to signal independently of ectodomain proteolysis, we used TLR9\textsuperscript{TM-MUT} to test the relevance of this regulatory mechanism in vivo. We transduced TLR9-deficient hematopoietic stem cells (HSCs) with retroviruses encoding TLR9, TLR9\textsuperscript{TM-MUT}, or empty vector (control) and transferred these HSCs into lethally irradiated C57BL/6 recipients (Fig. 3.2A). HSCs were transduced with similar efficiencies and expressed similar levels of GFP driven from a bicistronic vector (Fig. 3.3A). Strikingly, all mice receiving TLR9\textsuperscript{TM-MUT} HSCs died within 4 weeks of transfer while mice receiving control or TLR9 HSCs remained healthy and viable (Fig. 3.2B).

TLR9\textsuperscript{TM-MUT} expressing mice were profoundly anemic, as measured by erythrocyte counts, hemoglobin levels, and hematocrit (Fig. 3.2C). The overall cellularity of bone marrow in TLR9\textsuperscript{TM-MUT} expressing mice was reduced relative to TLR9-expressing mice, whereas the cellularity of the spleen was similar between all groups (Fig. 3.3C, D). Analysis of the cellular composition of bone marrow and spleens from TLR9\textsuperscript{TM-MUT} expressing mice revealed an expansion of CD11c\textsuperscript{+} cells relative to mice receiving TLR9 and control transduced HSCs (Fig. 3.2D and Fig. 3.3B, E). Moreover, TLR9\textsuperscript{TM-MUT} expressing mice had elevated levels of a number of inflammatory cytokines, including TNF, Rantes, and IL-18, consistent with TLR9\textsuperscript{TM-MUT} activation in DCs (Fig. 3.2E and Fig. 3.3H). Surprisingly, the bone marrow and spleens of TLR9\textsuperscript{TM-MUT} mice had drastically reduced numbers of CD19\textsuperscript{+} B cells, indicating a block in B cell development at an early stage (Fig. 3.2F). B cell development was blocked for both transduced and non-transduced cells, supporting the conclusion that this phenotype was not due to cell autonomous defects in HSCs (Fig. 3.2G). No abnormalities in the blood by complete blood count (CBC) or in the cellular composition of the spleen and bone marrow were observed in mice receiving control or TLR9 expressing HSCs (Fig. 3.2C-F).

TLR9\textsuperscript{TM-MUT} induced autoinflammatory disease is independent of B and T cells but dependent on CD11c\textsuperscript{+} dendritic cells.

The expansion of DCs and lack of B cells suggested that disease in TLR9\textsuperscript{TM-MUT} mice was driven by DC activation and did not require autoantibody production. To test this possibility directly, we generated radiation chimeras using retrovirally transduced HSCs from Rag1-deficient mice. Mice receiving wildtype or Rag1-deficient HSCs expressing TLR9\textsuperscript{TM-MUT} developed similarly severe disease, indicating that lymphocytes were not required for TLR9\textsuperscript{TM-MUT} induced disease (Fig. 3.4A, B). Therefore, we considered the possibility that expression of TLR9\textsuperscript{TM-MUT} on DCs enabled these cells to recognize DNA released from apoptotic cells undergoing secondary necrosis, as might be expected in irradiated mice. Expansion of DCs was only observed in GFP-positive cells, indicating that this aspect of the phenotype was cell autonomous and required TLR9\textsuperscript{TM-MUT} (Fig. 3.3E, F). These DCs expressed high levels of MHC class II and CD11b but were negative for SiglecH, which suggests they are conventional DCs (Fig. 3.4C).

To test more directly the role of DCs in TLR9\textsuperscript{TM-MUT}-mediated disease we transduced HSCs from mice expressing the diphtheria toxin receptor driven by the CD11c promoter (CD11c-DTR mice) and transferred these HSCs into irradiated recipients. Injecting these radiation chimeras with diphtheria toxin (DT) depleted...
CD11c-positive cells and prevented anemia and B cell depletion, while mice receiving saline still developed disease typical of TLR9\textsuperscript{TM-MUT} expressing mice (Fig. 3.4D, E, Fig. 3.5A). As expected, the rescue mediated by DT was only apparent when HSCs expressing CD11c-DTR were used, ruling out any non-specific effects of DT treatment (Fig. 3.5B). Collectively, these data indicate that DCs in TLR9\textsuperscript{TM-MUT} expressing mice are necessary for the anemia and B cell depletion; although, we cannot formally rule out that another CD11c-positive population of cells is depleted by DT treatment and contributes to disease. Importantly, these results further support that the observed inflammatory disease is not simply due to cell autonomous defects in the HSCs associated with ectopic TLR9\textsuperscript{TM-MUT} expression since depletion of a specific cell type rescues these mice from disease.

\textit{Autoinflammatory disease in TLR9\textsuperscript{TM-MUT} is partially rescued in the absence of TNF but remains intact in IFNAR deficient mice.}

Systemic inflammation may account for the anemia and B cell deficiency observed in TLR9\textsuperscript{TM-MUT} mice, as inflammatory mediators such as TNF and type I interferon (IFN) have been shown to negatively impact B cell development and erythropoiesis (Lin et al., 1998; Ueda et al., 2005; Ueda et al., 2004; Yamada et al., 1991; Zoubos et al., 1985). Indeed, when we generated radiation chimeras using TNF x lymphotoxinαβ triple knockout HSCs, the mice expressing TLR9\textsuperscript{TM-MUT} showed prolonged survival, reduced anemia, and less severe B cell depletion (Fig. 3.6A-C and data not shown). Using mice lacking the type I IFN receptor (IFNAR) or IFNAR-deficient HSCs, however, did not ameliorate the disease (Fig 3.6D-F, Fig. 3.7A-D), which is consistent with our observation that the expanded DCs do not express the plasmacytoid DC marker SiglecH (Fig. 3.5C) and we could not detect elevated levels of type I interferon in the serum of these mice (data not shown).

While these data suggest that bypassing the requirement for ectodomain proteolysis leads to a break in tolerance, an alternative interpretation is that TLR9\textsuperscript{TM-MUT} has altered signaling properties or engages pathways not normally accessible to wildtype TLR9. We tested this possibility by determining the role of known TLR9 signaling components in TLR9\textsuperscript{TM-MUT}-based disease. As expected for a receptor with unaltered downstream signaling, the disease was entirely MyD88-dependent and did not require Trif (Fig. 3.7E-G). These data, along with our TLR9\textsuperscript{TM-MUT} in vitro results argue that the TLR9\textsuperscript{TM-MUT} signaling pathways are unaltered relative to wildtype TLR9, other than the ability to access extracellular ligands at the cell surface and signal independently of cleavage.

\textit{Disruption of 5 amino acids within the TLR9 transmembrane region is sufficient to cause autoinflammatory disease.}

Previous data discussed in chapter 2 suggested that TLR9\textsuperscript{TM-MUT4} has a weaker gain-of-function phenotype than TLR9\textsuperscript{TM-MUT}. Nevertheless, mice receiving HSCs expressing TLR9\textsuperscript{TM-MUT4} succumbed to inflammatory disease characterized by severe anemia much like TLR9\textsuperscript{TM-MUT} expressing mice, while TLR9\textsuperscript{TM-MUT2} and TLR9\textsuperscript{TM-MUT3} expressing mice were unaffected (Fig. 3.8E). These results indicate that replacement of only five amino acids (Leu, Ser, Trp, Asp, and Cys) within the TLR9 TM is sufficient to render the receptor self reactive, although we cannot formally rule out that these residues
are positioned just outside the TM region rather than within the membrane. Regardless, this region of the protein clearly plays a key regulatory role.
Figure 3.1. Schematic of TLR9<sup>TM-MUT</sup> trafficking in comparison to wild-type TLR9. Similar to wild-type TLR9, TLR9<sup>TM-MUT</sup> is found in the ER, golgi and traffics to the endolysosome. As shown in chapter 2, it has increased expression at the cell surface compared to wild-type and is present in reduced amounts in the endolysosome. By altering the amino acid sequence of the TM of TLR9, this receptor was stabilized at the cell surface and signals in its full-length form in the absence of proteolysis.
Figure 3.2. Expression of TLR9<sup>TM-MUT</sup> in vivo leads to fatal inflammation.  

A, Schematic of the approach used to express TLR9 or TLR9<sup>TM-MUT</sup> in mice. Hematopoietic stem cells (HSC) from TLR9-deficient mice were transduced with retroviruses encoding TLR9, TLR9<sup>TM-MUT</sup>, or vector control followed by transfer into lethally irradiated C57BL/6 recipient mice.  

B, Mice expressing TLR9<sup>TM-MUT</sup> die from inflammatory disease. Survival plot of radiation chimeric mice receiving HSCs expressing either TLR9, TLR9<sup>TM-MUT</sup>, or empty vector (control). C, TLR9<sup>TM-MUT</sup> expressing mice develop anemia. Mean values of hematocrit, red blood cells (RBC), and hemoglobin (Hg) in the blood of mice at day 19 post-HSC transfer.  

D, Expression of TLR9<sup>TM-MUT</sup> in mice causes an expansion of DCs. Graph showing the total number of GFP-positive, CD11<sup>c</sup> cells in the bone marrow at day 19 after injection of HSCs.  

E, TLR9<sup>TM-MUT</sup> expression leads to inflammatory cytokine induction. TNF was measured in sera from the indicated mice at day 19-29 post-HSC transfer. Each point represents an individual mouse. Horizontal line represents the mean. F, Expression of TLR9<sup>TM-MUT</sup> in mice leads to block in B cell development. The total number of CD19<sup>+</sup> cells in the bone marrow and spleens of the indicated mice, as measured by flow cytometry, is shown. Unless stated otherwise, experiments in this figure were performed at least three times with similar results. See also Figure 3.3.
Figure 3.3. Analysis of transduction efficiencies, cellularity and serum cytokines in bone marrow chimeras. A, Percentage of total GFP-expressing cells from bone marrow and spleen of the indicated radiation chimeras (as described in Fig. 3.2) was assessed by flow cytometry. B, Graph showing the total number of GFP-positive gated, CD11c+ cells in the spleen at day 19 after injection of HSCs. C and D, Cellularity of bone marrow (C) and spleen (D) in radiation chimeras. Data are representative of at least three experiments and represent mean values from 5 animals per group. Representative flow
cytometry plots from TLR9, TLR9\textsuperscript{TM-MUT} and control mice. \textbf{E and F}, Dendritic cell expansion is specific to GFP-positive cells. Representative plots of bone marrow cells and splenocytes from the indicated mice gated on transduced (GFP-positive gate) or untransduced (GFP-negative gate) cells. The data presented are representative of groups of 5 mice. \textbf{G}, Deficit in CD19\textsuperscript{+} B cells in the bone marrow and spleen of TLR9, TLR9\textsuperscript{TM-MUT} or empty vector expressing radiation chimeras (d19 post-transfer). TLR9\textsuperscript{TM-MUT} bone marrow chimeras have increased levels of inflammatory cytokines in serum. \textbf{H}, Levels of RANTES (top) and IL-18 (bottom) in sera of TLR9 and TLR9\textsuperscript{TM-MUT}-expressing mice (day 19-29 post-transfer) were measured by multiplex protein array. Horizontal line indicates mean. Each point represents an individual mouse.
Figure 3.4. TLR9\textsuperscript{TM-MUT} mediated inflammatory disease is driven by CD11c+ dendritic cells. \textbf{A} and \textbf{B}, Disease caused by TLR9\textsuperscript{TM-MUT} does not require B or T cells. Experiments were performed as in (3.2), except Rag1-deficient HSCs were used to generate chimeric mice. \textbf{A}, Survival plot of chimeric mice receiving transduced HSCs of the indicated genotypes. \textbf{B}, Blood analyses at day 19 post-HSC transfer are shown. The data shown are representative of two independent experiments. \textbf{C}, Surface phenotype of CD11c+ cells in TLR9\textsuperscript{TM-MUT} radiation chimeras. Bone marrow cells from radiation chimeras expressing the indicated receptors were analyzed for cell surface marker expression by flow cytometry (d20 post-transfer). The histograms show expression of the CD11b, MHC class II, and SiglecH on GFP-gated CD11c+ cells. Representative histograms are shown for individual mice in two separate experiments containing four mice. \textbf{D} and \textbf{E}, CD11c+ cells are required for inflammatory disease in TLR9\textsuperscript{TM-MUT} mice. \textbf{D}, Schematic of experimental setup for depletion of CD11c+ cells in radiation chimeras expressing TLR9\textsuperscript{TM-MUT}. Mice were given either PBS and Diphtheria Toxin (DT) every
five days after introduction of TLR9$^{\text{TM-MUT}}$ transduced HSCs and analyzed twenty days after HSC injection. E, Block in B cell development and anemia seen in TLR9$^{\text{TM-MUT}}$ radiation chimeras is rescued by depletion of CD11c+ cells. The total number of CD19+ cells in the spleens of the indicated mice as well as mean hematocrit are shown for PBS or Diphtheria toxin (DT)-treated mice. Data are representative of two experiments with four mice per group. See also Figure 3.5.
Figure 3.5. Depletion of CD11c+ cells in TLR9^{TM-MUT} radiation chimera using CD11c-DTR HSCs. A, Diphtheria toxin (DT) depletes CD11c+ cells in TLR9^{TM-MUT} expressing CD11c-DTR radiation chimeras. DT or saline was injected intravenously at timepoints shown in Figure 3.4D and GFP-gated CD11c+ cells in the spleen were measured by flow cytometry (d20 post-transfer). The mean values are shown from one of two representative experiments. B, The effect of DT injection is specific. TLR9^{TM-MUT} radiation chimeras reconstituted with C57BL/6 cells without the CD11c-DTR transgene were treated as described in (A). Mean values for red blood cells (RBC), hemoglobin (Hg) and hematocrit for each group of mice are shown.
Figure 3.6. Lack of TNF and lymphotoxin ab and not IFNAR partially rescues inflammatory disease in TLR9\textsuperscript{TM-MUT} expressing bone marrow chimeras. A-C, Autoinflammatory disease mediated by TLR9\textsuperscript{TM-MUT} is partially ameliorated in the absence of tnf/lymphotoxinαβ. A, Schematic of bone marrow chimera experiment to address the role of TNF in TLR9\textsuperscript{TM-MUT} mediated disease. B, Partial recovery of B cell development in TLR9\textsuperscript{TM-MUT} expressing radiation chimeras generated with TNF x LTαβ-deficient HSCs. The number of CD19\textsuperscript{+} B cells in the bone marrow and spleen of TLR9 and TLR9\textsuperscript{TM-MUT} expressing radiation chimeras in C57BL/6 or TNFxLTαβ-deficient cells (d20 post-transfer) is shown. C, TNFxLTαβ-deficient TLR9\textsuperscript{TM-MUT} expressing mice do not develop anemia at early time points. Mean values of hematocrit in the blood of mice at day 20 post-HSC transfer. Data shown are representative of two experiments. D-F, Loss of Ifnar does not rescue TLR9\textsuperscript{TM-MUT} mediated disease in vivo. D, Schematic of bone marrow chimera experiment to address role of type I interferon in TLR9\textsuperscript{TM-MUT} mediated disease. Initial experiment utilized IFNAR-deficient or C57BL/6 donors. E, Expression of TLR9\textsuperscript{TM-MUT} using IFNAR-deficient HSCs in mice causes an expansion of dendritic cells similar to wild type. Graph showing the average number of GFP-gated CD11c\textsuperscript{+} cells in the bone marrow and spleen at day 25 after injection of HSCs. F, TLR9\textsuperscript{TM-MUT}
expression in IFNAR-deficient HSCs expressing mice develop severe anemia. Mean values of hematocrit, red blood cells (RBC), and hemoglobin (Hg) in the blood at day 25 post-HSC transfer. See also Figure 3.7
Figure 3.7. Lack of Type I interferon signaling does not rescue TLR9<sup>TM-MUT</sup> mediated inflammatory disease. **A**, Schematic of bone marrow chimera experiment to further address role of type I interferon in TLR9<sup>TM-MUT</sup> mediated disease. Experiments were performed as in Figure 3.6D-F, except IFNAR-deficient mice were used as recipients. IFNAR-deficient (IFNAR) HSCs were used as donor cells. Radiation chimeras expressing TLR9<sup>TM-MUT</sup> still display **B**, an expansion of dendritic cells, **C**, anemia and **D**, block in B cell development regardless of recipient genotype. **E-G** The inflammatory disease induced by TLR9<sup>TM-MUT</sup> does not induce altered downstream TLR9 signaling pathways. **E**, The inflammatory disease induced by TLR9<sup>TM-MUT</sup> requires MyD88 but not Trif. Expression of TLR9<sup>TM-MUT</sup> using Trif-deficient but not
MyD88xTrif-KO HSCs in mice causes an expansion of dendritic cells. Graph showing the average number of GFP-gated CD11c+ cells in the bone marrow and spleen at day 27 after injection of HSCs. F, Trif-deficient cells retain block in B cell development. G, TLR9$^{TM\text{MUT}}$ expressing mice using Trif-deficient HSCs develop severe anemia. Mean values of hematocrit (left), red blood cells (RBC), and hemoglobin (Hg) (right) in the blood of mice at day 27 post-HSC transfer.
Figure 3.8. TLR9 TM mutants identify residues critical in preventing self-reactivity. **A**, Schematic of bone marrow chimera experiment to assess disease in mice expressing TLR9 TM mutants. **B**, TLR9$^{TM-MUT}$ induces inflammatory disease in vivo. Mean values of hematocrit, red blood cells (RBC), and hemoglobin (Hg) in the blood of mice at day 19 post-HSC transfer. Data are representative of two independent experiments.
Discussion

The results presented here provide the first demonstration that mutations within a TLR can result in a break of tolerance. There are many examples of autoimmune or autoinflammatory diseases resulting from perturbations in cell-extrinsic pathways that ultimately lead to excessive generation or altered delivery of self-ligands for innate immune receptors (Nagata et al., 2010; Napirei et al., 2000; Yasutomo et al., 2001; Yoshida et al., 2005). One key piece of evidence indicating that TLR9 can contribute to the break in tolerance in autoimmune disease has been work demonstrating TLR9’s ability to synergize with autoreactive B cell antigen receptors bound to antibody-chromatin immune complexes and lead to potent activation of these cells. This work has suggested that TLR9 signaling in response to self-DNA can lead to the production of potentially pathological self-reactive antibodies, likely due to delivery of ligands to intracellular compartments. What distinguishes our work from these previous studies is the finding that receptor-intrinsic mutations can alter TLR activation and break tolerance to self. The biophysical changes associated with signal transduction remain poorly described for TLR family members, but our work suggests that residues in the TM domain play a key regulatory role for TLR9 and impose the requirement for ectodomain processing that is necessary to avoid self recognition.

Use of radiation chimeras in our studies demonstrated that bypassing the requirement for TLR9 ectodomain processing enables cells to respond to self-DNA released from dying cells. Activation of these cells results in production of inflammatory cytokines that feed back on hematopoiesis. The features of inflammatory disease that we describe here are similar to those observed in other models of dysregulated innate immune activation (Deane et al., 2007; O’Connell et al., 2008; Yoshida et al., 2004). Most relevant to our work is the observation that transgenic mice overexpressing TLR7 develop a lupus-like disease characterized by DC activation and anemia within two to ten months (Deane et al., 2007). Importantly, though, the disease in TLR9\textsuperscript{TM-MUT} expressing animals is much more acute and cannot simply be attributed to overexpression, as TLR9 expressing mice in our system are healthy in the time frame analyzed. Anemia is also observed in DNAse II-deficient mice, which produce type I IFN due to undigested DNA expelled during erythropoiesis in the fetal embryo (Yoshida et al., 2004). The disease induced by TLR9\textsuperscript{TM-MUT} was not rescued in IFNAR-deficient mice but was partially ameliorated when HSCs lacking the genes encoding TNF, lymphotoxin a, and lymphotoxin b were used, suggesting that a combination of inflammatory cytokines may be responsible for the hematopoietic defects in these mice. The resulting anemia we observe in TLR9\textsuperscript{TM-MUT} expressing mice is accompanied by reduced white blood cells, red blood cells and platelet counts which is similar to aplastic anemia in humans, a form of anemia which is largely idiopathic and correlates with increases in serum TNF.

One unexpected aspect of the disease is the independence of disease progression on B and T cells. This independence may be due to the severity of the phenotype resulting from the abundance of self-nucleic acid that is present after irradiation and the presence of a dysregulated mutant of TLR9 that can respond to those ligands. Another unexpected result was that TLR9\textsuperscript{TM-MUT} expressing mice were not rescued by the loss of type I interferon signaling. SLE and other autoimmune dieases has been associated with having an “interferon signature,” due to analyzing the expression profile of peripheral blood from SLE patients and observing increased expression of genes induced by type I
interferon (Baechler et al., 2003; Blanco et al., 2001). Whether this will be the case when less self-ligands are present remains to be determined. Although the observed disease is not lupus-like, it clearly affirms the gain of function to self nucleic acid we initially observed in vitro; the resulting loss of self-tolerance in vivo demonstrates the importance of the necessity for linking receptor function with proteolysis within intracellular compartments.

Regarding the likelihood that mutations in the TM region of TLR9 could predispose individuals to autoimmune or autoinflammatory disorders, the severe phenotype observed in TLR9\textsuperscript{TM-MUT} expressing mice suggests that similar alleles in humans may be fatal as congenital mutations. For this reason, genome-wide association studies may be unlikely to identify such mutations, unless certain substitutions result in less severe, non-fatal alleles. In our system, we were unable to identify any substitutions that resulted in such alleles, although this possibility certainly remains possible. It would seem more likely, though, that TM mutations could play a role in somatic cells, resulting in rare cells with self-reactive TLRs. These cells may facilitate the initial break in tolerance responsible for certain autoimmune disorders.
Chapter 4: Contribution of the TLR9 cytosolic domain to TLR9 trafficking

Background

Toll-like receptors sense invading microbes through recognition of conserved microbial products, such as the bacterial cell wall components lipopolysaccharide and peptidoglycan. To detect viruses, TLRs employ a more general strategy of targeting nucleic acid (Janeway; Medzhitov and Janeway). For example, TLR9 recognizes unmethylated CpG motifs which are enriched in viral and bacterial genomes. Although the frequency of unmethylated CpG motifs in mammalian genomes is suppressed, mammalian genomic DNA is still capable of stimulating TLR9 and inappropriately activating the immune system. This breakdown in tolerance can lead to autoimmune disorders such as systemic lupus erythematosus (Christensen et al., 2005; Leadbetter et al., 2002). Defining the mechanisms that prevent TLR recognition of self nucleic acid is critical for our understanding of these autoimmune diseases and will have profound implications for human health.

In spite of the similarity of their cytosolic domains, nucleic acid sensing TLRs (TLR3, 7 and 9) are retained intracellularly while others are localized to the plasma membrane. We speculate that the intracellular localization is a strategy that has evolved to limit immune responses to foreign pathogens and promote self-tolerance. Recent studies suggest that this intracellular localization contributes to avoidance of self-nucleic acid recognition. Restricting TLR9 ligand recognition to endolysosomes limits the likelihood that self nucleic acid is encountered. While this mechanism is appealing, the signals responsible for establishing TLR9’s intracellular localization remain undefined. Multiple motifs within TLR9 have been reported as necessary for maintaining proper localization. Indeed, both the transmembrane (TM) region and the cytoplasmic regions (TIR domain) of TLR9 have been proposed to be sufficient for localization (Kajita et al., 2006; Leifer et al., 2006; Nishiya et al., 2005).

Not only are the necessary motifs within TLR9 poorly defined, but the route by which TLR9 reaches the endolysosomes, until recently, remained unclear. Previous work had shown that TLR9 is retained in the endoplasmic reticulum and gains access to DNA in endolysosomal compartments by a poorly defined trafficking mechanism (Ahmad-Nejad et al., 2002; Leifer et al., 2004). This route of trafficking from the ER to the endolysosome differed from known routes of trafficking employed by lysosomal proteins such as LAMP and cathepsins. The ER retention signal for TLR9 and other intracellular TLRs has remained elusive as no KDEL sequence or other known ER retention mechanisms have been found within the TLR9 protein. Therefore, it has been proposed that TLR9 traffics directly from the ER to the endolysosome, bypassing the Golgi completely. Such a trafficking pathway has never been described in mammalian cells, so there is considerable interest in elucidating the mechanistic details controlling TLR9 trafficking.

Data from our lab has recently shown that, contrary to current dogma, TLR9 traffics through the Golgi. This was unexpected since several groups have shown that TLR9 is primarily ER resident and is recruited to the endolysosome after stimulation with CpG DNA. Moreover, our lab has shown that, in addition to trafficking through the Golgi, the form that is present in the endolysosome is proteolytically cleaved at the N-terminus (Ewald et al., 2008). Although we are still examining the mechanism and
regulation of this cleavage event, we have clearly demonstrated that the functional form of TLR9 that encounters ligand in the endolysosome is the cleaved TLR9 product (CP). Our lab has also found that TLR9 protein that passes through the Golgi is modified and migrates at a higher molecular weight than the “full-length” protein that is in the ER (see asterisk band in Figure 2.3B). Pulse-chase analysis indicates that the precursor of the cleaved TLR9 product is the high molecular weight (HM) full-length TLR9. Therefore, our current model of TLR9 trafficking is that a significant portion of the protein leaves the ER and continues through the canonical secretory pathway through the Golgi where it receives additional carbohydrate modifications. From the golgi, TLR9 is routed to the endolysosome where it is proteolytically cleaved (Figure 1.1). What remains unclear is how TLR9 is directed to the endolysosome. Understanding how this routing to the endolysosome is regulated is critical because access to self nucleic acid at the cell surface can potentially cause inappropriate activation and autoimmunity. Although our new hypothesis based on TLR9^{TM-MUT} (discussed in Chapter 2 and 3) is that proteolysis is a regulatory mechanism that prevents self-recognition, whether increased cell surface TLR9 can ultimately lead to self-reactivity is not known. Therefore, we are working to understand how TLR9 is sorted to the endolysosome and how this process is regulated.

These discoveries related to the cell biology of TLR9 provide a unique opportunity to apply this newfound knowledge toward the identification of the mechanisms controlling TLR9 trafficking and localization. We would like to determine the TLR9 motif/s that are responsible for its appropriate intracellular compartmentalization and identify associated factors that may be mediating TLR9 localization to the endolysosome. With these new data from our lab demonstrating proteolytic processing of TLR9, we can now use TLR9 processing as a readout to evaluate the contribution of putative motifs within the cytosolic domain toward proper localization.

Our lab has shown that a chimeric receptor (T9N4C) with the TLR9 extracellular domain and TLR4 transmembrane region and cytosolic domain trafficks through the golgi and is localized to the cell surface (Barton et al., 2006). From the altered localization of this receptor, we can conclude that the transmembrane region and/or cytosolic region contain a sorting motif to the lysosome. Although it is not clear what role the transmembrane region plays in trafficking, we are currently investigating whether the cytosolic region of TLR9 contains a sorting motif. The cytoplasmic tails of other membrane proteins contain sorting motifs to various intracellular destinations and this may also be the case for TLR9 (Bonifacino and Traub, 2003). Sorting of proteins destined for the lysosome from the golgi is well understood and with the new information regarding TLR9 trafficking through the golgi, we can now dissect the sorting mechanism of TLR9. It is likely that this information will translate to the other intracellular TLRs.

**Known endosomal sorting motifs and clathrin-associated proteins.**

The two main classes of endosomal sorting signals are tyrosine- (YXXφ) or leucine- (LL) based motifs, where Y is tyrosine residue, φ is hydrophobic, and X is any amino acid. Both classes have been found to interact with the heterotetrameric adaptor complexes AP1, AP2 and AP3 for clathrin-dependant sorting to intracellular compartments. Clathrin coated vesicles that associate with the trans Golgi and endosome contain AP-1 and the monomeric adaptor proteins GGA1, GGA2 and GGA3. Tyrosine
motifs are generally important for internalization from the surface but also have been shown to be important for targeting to lysosomes (e.g., in the cases of LAMP and HLA-DM). These proteins have been shown to be sorted via the adaptor protein complexes AP-1 and AP-2. A putative tyrosine-based motif has been identified in TLR9 but it has been reported to not be phosphorylated as is common for these YXXφ motifs (Leifer et al., 2006). These motifs are also frequently located proximal to the transmembrane region, which is not the case for the putative motif in TLR9. Additionally, this motif is not conserved between TLR7 and TLR9, which suggests it may not serve as a sorting signal, since we would expect the sorting of this subfamily of TLRs to be similar.

It is also possible that TLR9 contains di-leucine motifs in the cytosolic domain. Truncation mutants may be the simplest way to identify these important regions. The general arrangement of the motifs is either DXXXLL or DXXLL where the acidic residue (D or E) and leucine residues are critical to the interaction with AP complexes and proper sorting. Dileucine motifs have been found in various lysosomal proteins such as invariant chain (Ii) and LIMPII (Bakke and Nordeng, 1999; Honing et al., 1998). In addition, TLR9 may contain more than one sorting motif, as has been described for invariant chain (Ii). DXXL are a different class of dileucine motifs, which have been implicated in trafficking of proteins known to cycle between the TG and endosome (Bakke and Nordeng, 1999; Bonifacino and Traub, 2003).

Ubiquitin labeling as a lysosomal sorting signal has also been reported in other proteins. Another possibility is that there is a receptor that specifically recognizes TLR9 or a modification of TLR9. For example, mannose-6 phosphate (M6P) moieties that are added on the N-linked carbohydrates of some proteins in the cis-Golgi, and recognition of these sugars by M6P receptors can target protein to endolysosomes. Recognition of M6P has been shown to be important for delivery of acidic hydrolases to the lysosome.

Finally, another route to the lysosome could be used is the autophagy pathway. Suffice it to say, trafficking from the Golgi to endolysosomes has been studied extensively, and this literature will aid us in the identification and analysis of proteins involved in TLR9 trafficking. Ultimately, we wish to understand how appropriate TLR9 trafficking contributes to self/non-self discrimination. Our findings suggest that mutations within the TLR9 cytosolic domain can disrupt proper trafficking to the endolysosome.
Materials and Methods

Reagents.
All chemicals and reagents, unless noted otherwise, were purchased from Fisher Scientific. Antibodies were from the following: anti-HA (clone 3F10, Roche), anti-Giantin (ab24586, Abcam), anti-rat Alexa 555 (Molecular Probes) and donkey anti-rabbit Cy5 (Jackson Immunoresearch).

DNA cloning.
TLR9<sub>890EE, 890E</sub> was constructed by 'PCR sewing' of cDNA corresponding to amino acids 896EE and 890 on mouse TLR9. TLR9 truncations were made by PCR adding a stop codon at the desired location. All TLR-encoding cDNAs were cloned into the mouse stem cell virus–based retroviral vector MSCV2.2, provided by M. Schlissel (UC Berkeley). TLR9-HA and TLR9 mutants with HA were constructed by the addition of a hemagglutinin epitope (YPYDVPDYA) to the C-terminal end of each receptor in MSCV2.2.

Cell lines, plasmids and tissue culture. RAW cells were from American Type Culture Collection. Cell lines were cultured in RPMI-1640 medium supplemented with 10% (vol/vol) FCS, L-glutamine, penicillin-streptomycin, sodium pyruvate and HEPES, pH 7.2 as previously described (Barbalat et al., 2009). Unless otherwise noted, stable lines were generated by transducing cells with MSCV2.2 retroviruses encoding the target cDNA.

Retroviral transduction. For retroviral transduction of RAW macrophages, VSV-G–psuedotyped retroviral supernatant was made with GP2-293 packaging cells as previously described (Ewald et al., 2008).

Immunoprecipitation and western blot analyses. Cells were lysed as previously described (Ewald et al., 2008). Briefly, cells were lysed in TNT buffer (20 mM Tris pH 8.0, 200 mM NaCl, 1% Triton X-100) supplemented with Complete protease inhibitor cocktail (Roche). Lysates were cleared of insoluble material by centrifugation. For immunoprecipitations, lysates were incubated with anti-HA matrix (Roche) and precipitated proteins were boiled in SDS buffer, separated by SDS–PAGE, and probed by anti-HA immunoblot.

Immunofluorescence microscopy. Cells were plated on coverslips and allowed to attach overnight. The cells were then fixed with 4% paraformaldehyde for 15 minutes. After washing and permeabilizing with 1% Triton X, cells were blocked using normal goat serum in 0.5% Triton X for 30 minutes. Following the blocking step, cells were stained with anti-HA and anti-giantin for 1 hour, washed and stained with anti-rat Cy3 and anti-rabbit Cy5 antibodies for 30 minutes. After washing, the coverslips were mounted on slides and sealed for fluorescence microscopy. Cells were visualized using a Nikon E800 fluorescent microscope.
Results

**TLR9 contains a golgi to lysosome sorting motif in the cytosolic region.**

Our initial approach was to create mutants that are truncated to exclude various domains of TLR9 within the cytosol. Although mutants truncated immediately after the putative transmembrane region were not stably expressed, a TLR9 mutant excluding the tail after the TIR domain was competent for signaling and was proteolytically cleaved, similar to wild-type TLR9 (data not shown). We also made a truncation mutant that excluded the TIR domain (LNK-STP). This receptor exited the ER and had reduced amount of the cleaved form of TLR9 (data not shown). Using this information we made truncations within the more N-terminal region of the TLR9 TIR, shortly after the linker region (Figure 4.1A). Our preliminary data show that there may be a sorting motif contained in the cytosolic domain downstream of amino acid 888 of the TLR9 protein. In order to facilitate biochemical experiments, truncation mutants were made to include a hemaglutinin (HA) epitope tag at the C-terminus. The new constructs were then cloned into a retroviral vector (MSCV2.2) to transduce RAW macrophages. To assess the localization of truncated TLR9 receptors, we used the enzyme EndoglycosidaseH (EndoH). As shown in Figure 2.3C, if a protein is sensitive to digestion by this enzyme, the protein has not passed through the medial golgi and is considered to be in the ER. Once proteins have passed through the medial golgi, the additional sugar modifications sterically hinder EndoH from cleaving off the N-linked carbohydrates from the protein. This results in the protein gaining EndoH resistance and remaining unchanged in molecular weight, as visualized by SDS-PAGE. As a positive control, the enzyme PNGaseF cleaves off all sugars from the protein. Using this assay, the TLR9 mutant receptor truncated immediately before amino acid 888 (888 STP) domain showed an increased amount of the high molecular weight TLR9 (Figure 4.1B). This protein is EndoH resistant (unchanged by EndoH treatment), which indicates that the protein has trafficked through the medial golgi as has been shown for TLR9. Interestingly, TLR9-888-STA also does not have detectable amounts of the TLR9 cleavage product. This indicates that this mutant receptor is trafficking through the Golgi but may not reach the endolysosome where cleavage normally occurs. Another truncation mutant, TLR9-897-STA has a similar phenotype (Figure 4.1B). We did attempt to make a mutant truncated at residue 902 but expression of this protein was not stable (data not shown). These results suggest that the cytosolic region of TLR9 contains a motif or motifs that allow for sorting to the endolysosome.

Previous work from Leifer et al. has suggested that there is a motif in the cytosolic domain that allows TLR9 to traffic from the ER to the lysosome (Leifer et al., 2006). They identified a region from amino acids 888 to 902 that they concluded was important for TLR9 intracellular localization. To examine this region further, we made four full-length mutants in this region, changing the amino acids conserved between TLR9 and TLR7. The mutants were again analyzed using EndoH digestion. One of the mutants, TLR9-896EE, had a similar phenotype to the truncation mutants described above (Figure 4.2A). By EndoH analysis, TLR9-896EE accumulates the high molecular weight full-length form of the protein but does not show any cleaved product. The full-length mutant, TLR9-890E, has an intermediate phenotype, where the amount of cleavage product is reduced relative to wildtype yet still detectable. Both
receptors were not competent for signaling, which may be due to their inability to traffic to the endolysosome and be cleaved.

Another approach we have used to analyze the cellular location of these mutants is immunofluorescence microscopy (IF). We have visualized TLR9 and TLR9 mutants together with various sub-cellular compartments in RAW macrophage cell lines. By merging the individual confocal images of the compartment-specific staining and the TLR9 staining, we can assess whether there is increased co-localization relative to wild-type TLR9. As seen biochemically, TLR9-896EE shows increased co-localization with the Golgi marker giantin than both the wild-type TLR9 and TLR9-890E (Figure 4.2B). This increased colocalization may indicate that TLR9-896EE is retained in Golgi. Alternatively, this mutant may localize to the cell surface. Further immunofluorescence or flow cytometry experiments will be necessary to discriminate between these possibilities.
Figure 4.1. TLR9 truncation mutants of the cytosolic region suggest the presence of Golgi to lysosome trafficking motif/s in TLR9. **A,** Schematic of TLR9 domains including extracellular ligand binding domain (ECD), transmembrane(TM) region and Toll/IL-1 receptor signaling domain (TIR domain). Region within the TIR outlined includes amino acid sequence of region of interest, amino acids 888 to 902 of TLR9. Underline indicates location of truncations, where TLR9 888 STP is before line and TLR9 897 STP is at the end of the line. Point mutations were made in the Glutamic Acid (E) in position 890 and two Glutamic Acids in positions 896 and 897. **B,** Endoglycosidase H digestion of immunoprecipitated TLR9 (wild-type, 888 or 897 STP truncation mutants). Untreated(-) and PNGase(P) controls included. PNGase removes all glycosylation. Asterix indicates high migrating protein in truncation mutants.
Figure 4.2. Identification of amino acids in TLR9 important for proper trafficking to the lysosome. **A**, Endoglycosidase H digestion of immunoprecipitated HA tagged TLR9 (wild-type, 890E or 896EE mutants) in stably expressing RAW macrophages. Single asterix marks high-migrating protein. Double asterix indicates position of cleaved TLR9 product. **B**, Immunofluorescence in RAW cell lines showing increased golgi colocalization with 896EE using markers for the golgi (anti-giantin in red) and TLR (anti-HA in green). Shown are representative images of wild-type TLR9, 890E and 896EE.
Discussion

These results suggest that the glutamic acid residues at position 896 and 897 seem to be important for localization to the endolysosome. However, the preliminary analysis of TLR9 truncation mutants (see TLR9-897-STP in Figure 4.1B), suggests that these residues are not sufficient for sorting to the endolysosome. Thus, we have yet to determine the minimal motif within TLR9 that is sufficient for proper endolysosomal trafficking. However, these mutants demonstrate that TLR9 contains information in its cytosolic domain for sorting from the Golgi to the endolysosome. Given our data with a TLR9\(^{TM\text{-MUT}}\) discussed in chapter 2, the TM and cytosolic region may both be involved in proper trafficking to the endolysosome. Careful examination of post-golgi trafficking for TLR9 is necessary to determine to what extent wild-type TLR9 accesses the cell surface. Whether TLR9 at the cell surface is “leaking” en route to the endolysosome and most is directly trafficked to intracellular compartments is not known. It is possible that the cytosolic region ensures golgi exit but may also be involved in internalization after accessing the cell surface.

Interestingly, we have not observed other TLR9 mutants accumulate in the same manner as we have observed with TLR9 888-STP. The fact that the high molecular weight form accumulates suggests that we have not simply made a misfolded protein since it is able to exit the ER. Although we know that TLR9\(^{TM\text{-MUT}}\) can reach the cell surface and is inefficiently trafficked to the endolysosome, we do not observe an accumulation of the higher molecular weight form of full-length TLR9. Why this is the case is not yet clear and may imply that post golgi trafficking is regulated at multiple levels, including at the level of protein stability.

Given our results with the full-length TLR9 mutants versus the truncations, it would be important to know whether the full-length mutants are located in the same place within the cell as the truncations. These studies may be best performed in cells where there is no endogenous TLR9 in order to ensure that the phenotypes seen are not skewed by association with wild-type TLR9. Nonetheless, it is interesting that the disruption of one or two amino acids within the TLR cytosolic domain was sufficient to prevent signaling in response to CpG DNA. We attempted to immunoprecipitate full-length mutant 896EE with MyD88 to determine if the cytosolic domain was properly folded but the results were inconclusive. The inability of the receptors to signal may be due to the retention we observe in the golgi but may also be due to the much reduced amounts of the cleaved form of the protein. Using immunofluorescence, we were able to detect colocalization of TLR9 896EE with the golgi. Although we know that wild-type TLR9 traffics through the golgi, it may not be within the limit of detection and only if it is retained, can it be detected by immunofluorescence. Future studies may allow better resolution of the differences between various TLR9 mutants.
Chapter 5: Future Directions and Closing Remarks

Future Directions

In the introduction, we have discussed how TLRs utilize nucleic acids as a signature of microbial infection. These receptors have evolved to sample the lumenal contents of endolysosomal compartments. This strategy enables recognition of diverse microbes with a limited set of receptors. A trade-off associated with innate immune recognition of nucleic acids is the potential for self-recognition. As discussed above, inappropriate recognition of self nucleic acids has been implicated in several autoimmune disorders. The risk stems from the inherent similarity between self versus foreign nucleic acids. Accordingly, various mechanisms have evolved to ensure reliable self/nonself discrimination. Although some of these mechanisms have been defined, additional mechanisms likely remain undiscovered. The fact that failure of these mechanisms can result in quite distinct diseases (e.g., SLE versus inflammatory myocarditis) underscores the nonoverlapping roles of other innate immune pathways involved in nucleic acid sensing.

Expression of a TLR9 TM mutant that could signal in the absence of proteolysis in vivo resulted in a severe autoinflammatory phenotype. This finding is the first demonstration that mutations in TLR9 are sufficient to break tolerance in vivo to self-nucleic acid. Although TLR9 has been linked to the pathogenesis of SLE as discussed in chapter 2, the phenotype we observe is more autoinflammatory i.e. no B or T cell dependence and partially rescued by TNF deficiency. This may be due to the availability of self-ligand in our radiation chimeras and the resulting severity of the disease in mice expressing this dysregulated receptor. One outstanding question is whether under steady-state conditions, would disease develop if TLR9\textsuperscript{TM-MUT} were present. If disease does develop, will this disease manifest itself with SLE-like pathology such as autoantibody production and glomerulonephritis or as a milder inflammatory phenotype? These questions have important implications for how TLR9 can contribute to the break in tolerance seen in autoimmune diseases. Future studies to be performed in our lab using a conditional knockin-TLR9\textsuperscript{TM-MUT} should help answer these questions.

Another area that remains unanswered from our studies is the cell biology of TLR9 post-golgi before reaching the acidic compartments. It is not yet clear how TLR9 traffics once it leaves the golgi. Our hypothesis is that TLR9 may access the surface but due to the requirement for cleavage, it will remain inactive. We have seen some evidence that a small amount of wild-type TLR9 is able to reach the cell surface (Figure 2.3A). Although our hypothesis is that altering the residues in the transmembrane region resulted in a version of TLR9 that can signal in its full-length form, the details of where the full-length form is found, are not yet clear. It is possible that even though TLR9\textsuperscript{TM-MUT} is present in its full-length form at the cell surface, it may also be in recycling endosomes in its unprocessed form. Our data using inhibitors of proteolysis such as Bafilomycin A1 and ZFA-FMK show that TLR9\textsuperscript{TM-MUT} can signal in the absence of proteolysis but where that signaling is precisely occurring is a challenging question to answer. However in order to better to understand how TLR9 is routing to the endolysosome, better tools to determine its localization post-golgi are necessary.

Our preliminary results defined a region in the TLR9 cytosolic domain that is necessary for trafficking to the endolysosome. In the future, we can further define this
region using full-length mutants to determine the amino acid residues necessary for proper TLR9 localization and to define the contribution of the cytosolic domain of TLR9 in trafficking and localization. These studies as well as other mutants made in the lab further emphasize the necessity to gain a better understanding of the events that occur once TLR9 leaves the golgi. Elucidating the role of Unc93B in ER export for nucleic acid sensing TLRs as well as any role it may have post-golgi are also of interest. There have also been recent studies that suggest that mutations in the N-terminal region of TLR9 or TLR7 that is cleaved off during processing can affect function of the receptor (Iavarone et al., 2011; Peter et al., 2009). Whether these mutations are affecting an undescribed role for the TLR9 N-terminus remains to be determined. Ensuring these mutations are not causing misfolding and retention in the ER is critical in interpreting these results. What has become clear from this work is that it is likely that there are several layers of regulation that remain to be discovered. These mechanisms are ultimately critical in restricting nucleic acid sensing TLRs from self-reactivity.
Closing Remarks

Although TLRs have evolved a strategy to detect nucleic acid from viruses, stringent regulatory mechanisms to prevent self-reactivity are necessary. It is known that in certain autoimmune diseases, these mechanisms have been bypassed and activation of nucleic acid sensing TLRs by self-ligands can contribute to the pathogenesis of these diseases. In the last few years, cell biological studies from our lab and others have allowed us to propose new hypotheses regarding the regulation of TLR9 trafficking to maintain proper self/non-self discrimination. The implications of better understanding these regulatory mechanisms are that we can gain insight into how tolerance to self-ligands is maintained. Given the importance of innate immune activation for robust adaptive immune responses to pathogens, it is likely that TLRs can also greatly exacerbate immune responses seen in autoimmune conditions. Additional studies dissecting the contribution of nucleic acid sensing TLRs to the breakdown in self-tolerance will be exciting. However, what will be most fascinating will be the mechanistic details of TLR9 trafficking before reaching its signaling compartment within the cell. Future studies will unravel the intricacies of the cell biology for this receptor that are likely to be necessary for strict self/non-self discrimination.
REFERENCES


