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The Regulation of TLR7 and TLR9 in the Prevention of Autoimmunity

By

Zachary Robert Newman

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

Committee in charge:

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Abstract

The Regulation of TLR7 and TLR9 in the Prevention of Autoimmunity

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Doctor of Philosophy in Molecular and Cell Biology

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Recognition of nucleic acids is an evolutionarily conserved trait of the immune system that enables detection of a diverse array of microbial species with relatively few receptors. However, the inappropriate recognition of self-derived nucleic acids is well documented for its role in autoimmune diseases. Two such receptors that recognize nucleic acids, Toll-like receptor (TLR) 7 and TLR9, are highly regulated in order to maintain the proper discrimination between self and non-self. In this dissertation I discuss the background of what is known about TLRs, with an emphasis on nucleic acid sensing TLRs and evidence for their involvement in autoimmune disease. I also provided an introduction to the phenomenon of codon usage bias and discuss the debate surrounding its functional significance. I then present our findings that codon bias may be playing a significant role in the balance of protein levels between TLR7 and TLR9. In an endogenous context, we observe a markedly different measured translational efficiency for TLR7 compared to TLR9, correlating with their degree of codon bias. Codon optimization of TLR7's coding sequence dramatically increases the translation efficiency and subsequent protein levels in an exogenous setting. Furthermore, by scaling the level of optimization we reveal how the different mechanisms of transcription, mRNA stability, and translation, all of which contribute to overall protein levels, are affected by codon bias. In contrast to the exogenous setting, codon optimization of TLR7 at the endogenous locus did not result in an increase in TLR7 function suggesting additional levels of regulation may be involved. In this dissertation I also introduce a novel method to study the functional significance of misregulated localization of nucleic acid sensing TLRs in an endogenous context. Our studies introduced a conditional mutant allele of TLR9 that is capable of signaling from the cell surface. The mutant is well expressed in a controlled, cell-specific manner with preliminary results suggesting potential autoimmune consequences. Combined, our studies significantly add to the literature by illuminating the importance of two distinct mechanisms regulating nucleic acid sensing TLRs in the prevention of autoimmunity.

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Chapter 1: Introduction to the Role of Nucleic Acid Sensing Toll-Like Receptors in the Immune System and Disease

Toll-Like Receptors and Their Role in the Immune System

The immune system is a coordinated group of cells and biological processes whose role it is to effectively prevent harm to the host by microorganisms. To protect the host, often the microorganisms, such as bacterial or viral pathogens, are killed by the toxic compounds or antibodies produced by the immune system to mediate clearance of the infection. Critically, the immune system has to balance its protective role with its own potential to do harm, as many of these means to kill microorganisms are also toxic to the host. Examples of inappropriate responses can be unnecessary inflammation due to benign elements, excessive or unchecked inflammation, or autoimmunity where the host attacks itself. Therefore, the immune system requires the ability to discriminate between microbes it encounters from benign elements or self-derived ligands.

To aid in discerning what is a bona fide microbe, the innate immune system recognizes conserved features of microorganisms, termed pathogen associated molecular patterns (PAMPs), via germline encoded pattern recognition receptors (PRRs). Recognition of a PAMP by its cognate PRR can lead to a variety of subsequent outcomes depending on the PAMP/PRR engaged and the cell type involved. One such outcome is the activation of the innate immune system to help control an infection directly through the production of inflammatory cytokines, such as TNFα or IL-1, that lead to recruitment of additional innate immune cells. Once recruited, killing of microbes is mediated by direct engulfment or release of toxic agents. However, many of the mechanisms of killing employed by the innate immune system, such as reactive oxygen species, can be indiscriminate in their effects and potentially lead to host tissue damage. Thus, by requiring PRR engagement by a PAMP, this inflammatory response by the innate immune system should only be triggered during an appropriate response to an infection.

Recognition of a PAMP by a PRR is also critical in mediating the appropriate activation of the adaptive immune response. Charles Janeway in his seminal paper proposed that PAMPs were necessary to provide context to the adaptive immune system when it encountered antigen (1). The context of a specific PAMP can establish two things: the type of microorganism the antigen is derived from and therefore the most appropriate way to clear the infection, and that the antigen is not self-derived thereby avoiding the immune system targeting the host itself (2). The activation of T-cells is the classic example of the necessity for this contextual signal. Antigen presenting cells (APCs), such as dendritic cells (DCs), possess a variety of PRRs. When APCs encounter antigen associated with microbial PAMPs, PRR/PAMP engagement signals to the cell to upregulate the co-stimulatory molecules CD80 and CD86 (B7.1 and B7.2) on their cell-surface. Simultaneously, APCs process and present the captured antigen also on the cell-surface. T-cells that encounter their cognate antigen on an APC that is co-expressing these co-stimulatory molecules become activated and trigger the

appropriate adaptive immune response. However, the mechanisms that govern antigen presentation are not selective and thus APCs may also present self-derived antigens. Importantly, because they are self-derived, in principal these antigens are not associated with PAMPs and therefore APCs presenting them do not upregulate co-stimulatory molecules. A T-cell encountering an APC presenting its cognate antigen in the absence of co-stimulation is no longer activated, but instead rendered anergic as part of a regulatory mechanism termed peripheral tolerance (3). Thus, it is the PRR/PAMP interaction that establishes the context that an antigen is derived from a microbial source, and this context is critical in determining the functional outcome of the adaptive immune response.

Despite an accumulation of evidence that strongly suggested that there were PRRs necessary for proper immune activation, the identity of these receptors remained elusive for a long period (1). Toll-like receptors (TLRs) were the first class of PRRs identified with the discovery of TLR4. Interestingly, the story of how TLR4 was discovered highlights both the need and potential harm discussed previously. In the first approach, TLR4 was discovered by looking for receptors that contained a motif known to act through the NFκB pathway and shown to signal the upregulation of co-stimulatory molecules (4). Importantly, this finding clearly demonstrated that the immune system has defined signaling pathways whose specific role it is to determine the origin of the antigen it is encountering and instruct the adaptive immune system accordingly. The second approach aimed to determine the molecular basis for a subset of mice who do not respond to lipopolysaccharide (LPS) (5). The justification for looking for the LPS receptor was to better understand the mechanisms involved in septic shock, as wild-type strains of mice would die upon administration of a given amount of LPS. Septic shock is a “proper” but extreme response to a microbial infection that leads to a systemic cytokine response highlighted by a large drop in blood pressure that frequently results in organ failure and death (6). Therefore, even though the immune system is acting “properly” by responding to a microbial infection and producing cytokines normally thought beneficial, left unregulated the response itself can result in death. Eventually this response to LPS was mapped to TLR4. Together, the reports and subsequent research unequivocally established that the immune system contains receptors which recognize specific microbially derived products, and that this recognition is critical for providing context so that only appropriate immune responses are triggered.

The Regulation of Nucleic Acid Sensing Toll-Like Receptors

Toll-like receptors, as discussed, are key to the immune system’s proper activation by providing the context of an antigen when encountered by an immune cell. The TLR4 example above exemplifies one set of TLRs that are found on the cell-surface and are capable of encountering their respective PAMPs extracellularly. Together with TLRs 1/2/6, 5, and 11/12 which recognize bacterial lipoproteins, flagellin, and profilin-like proteins respectively (7, 8), TLRs found at the cell-surface detect PAMPs that are attractive ligands for the innate immune system because they are uniquely foreign, and their importance in microbial viability restricts the ability to mutate

these PAMPs in order to avoid immune detection. These PAMPs are also broadly associated with bacteria and parasites allowing a limited number of fixed receptors to respond to the vast complexity of microorganisms. Importantly, encountering these components provides immediate context to the immune system that there is an infection and that any antigens associated with these PAMPs are bacterially derived.

Some microbes, however, can lack conserved foreign molecular structures. For example, viruses frequently evade immune detection by mutating their antigens, especially those on the surface (9). The viral life cycle is also almost completely dependent on the host's cellular machinery and therefore does not possess distinct biochemical processes that generate uniquely foreign products (10). Thus, a highly conserved structural target that can be recognized by a germline encoded receptor is less likely in the case of viruses. The immune system, though, still requires the ability to discriminate virally derived antigens it encounters from self. To address the need for a viral PAMP, the innate immune system instead utilizes another ligand that defines viruses; nucleic acid (10). TLRs 3, 7 (also 8 in humans), 9, and 13 recognize double stranded RNA (dsRNA) (11), single stranded RNA (ssRNA) and small imidazoquinoline derived compounds (12–15), unmethylated CpG motifs in DNA (16), and ribosomal RNA respectively (17). It should be noted that other immune receptors besides TLRs recognize nucleic acid, such as RIG-I like receptors (RLRs) for RNA species (18, 19), and proteins such as DAI (20), AIM2 (21–24), STING (25), and the recently discovered cGAS in DNA detection (26). This need for a viral PAMP, however, comes at the cost of the nucleic acid ligand not being uniquely foreign and the potential risk of improperly responding to self-derived ligands.

To provide additional context for microbially derived nucleic acid, the innate immune system has evolved to discriminate the source of ligand through two different but non-mutually exclusive mechanisms. The first is similar to that of the cell-surface localized TLRs, where the mechanism of discrimination is at the level of the ligand itself through recognition of features generally only found in virally or bacterially derived nucleic acid. This discrimination is demonstrated by the increased capability of DNA with unmethylated CpG motifs to stimulate TLR9, which differentiates DNA ligands found in bacteria and viruses from the generally hypermethylated DNA found in mammals (27). However, this mechanism for discrimination is not perfect as illustrated by the ability of antibody bound to self-derived immune complexes to stimulate TLR9 after uptake by autoreactive B-cells (28). Thus, while mammalian derived DNA may be less stimulatory, it nevertheless has the potential to be recognized by the innate immune system. Likewise, recognition of ssRNA species by TLR7 and TLR8 appears to be mostly mediated by a preference for uridine-rich sequences (29, 30). Therefore, TLR7 and TLR9 cannot rely on "specific" features of nucleic acids to discriminate self from non-self.

The second mechanism to provide context is the spatial restriction of nucleic acid sensing TLRs to intracellular compartments (31, 32). Due to evidence that recognition of DNA requiring proper endosomal maturation, it had been proposed soon after its

discovery that TLR9 may localize to endosomal compartments (2). Since then it has been appreciated that all nucleic-acid sensing TLRs require trafficking to endosomal compartments for proper function. The strongest evidence for this necessity is demonstrated by a mutation (H412R) in the trafficking chaperone UNC93B1 that disrupts proper nucleic-acid TLR trafficking and subsequent signaling capability (33). On one level this endosomal localization may facilitate TLR interaction with microbially derived nucleic acid, as it is argued these ligands may only be exposed after internalization and degradation (34), or after transport of viral replication intermediates to these compartments (35). However, an important distinction is that nucleic-acid sensing TLRs are not merely located to endosomal compartments, but their functional signaling capability is restricted solely to these compartments. It has been argued that limiting recognition in this manner restricts the ability of TLRs to respond to self-nucleic acid in the serum because these nucleic acids are degraded before reaching a mature endosome (31). Importantly, a mechanism that can explain this spatial restriction was demonstrated by studies identifying a need for cleavage of the ectodomains of the nucleic-acid sensing TLR family members by pH dependent endosomal proteases (36). However, despite the restriction to endosomal compartments, several examples exist of self-nucleic acid reaching functional endosomal TLRs, generally through uptake mediated by interactions with other proteins such as antibodies (28), the antimicrobial peptide LL37 (37, 38), or the DNA-binding protein HMGB1 (39, 40). Therefore, all current evidence suggests it is an important regulatory feature of the innate immune system to limit signaling capacity of nucleic acid sensing TLRs to endosomal compartments, although examples exist of this restriction being subverted.

Beyond the broader implications of avoiding improper activation by restricting nucleic acid sensing by TLRs to endosomes, the ability to regulate localization has also been implicated in the differential regulation of these receptors that may control more specialized signaling outcomes. An important role for nucleic acid sensing TLRs is the production of type I interferon (IFN), particularly by TLR7 and TLR9 in plasmacytoid dendritic cells (pDCs), a cell type considered specialized for the production of type I IFN. The functional importance of IFN will be discussed in the next chapter. Evidence suggests there may be a specialized endosome required for type I IFN production. Separate groups have identified the trafficking adaptor complex AP-3 as being required for TLR7 and TLR9 mediated IFN production, although they conflict as to whether it is a specific defect for interferon or if production of other cytokines is also affected (41, 42). Additional evidence for specialized compartments comes from differential responses to artificial TLR9 ligands, where depending on the exact sequence of a CpG oligo and the delivery method of the ligand, signaling can be biased towards production of either pro-inflammatory cytokines or type I IFN (43). Therefore, proper signaling through nucleic acid TLRs may not only depend on reaching endosomal compartments, but may in fact require regulated trafficking to specialized endosomes for proper signaling.

Further levels of trafficking regulation have also been implicated at the level of individual nucleic acid sensing TLRs. Despite their shared signaling pathways and general regulation, a more detailed analysis of TLR7 and TLR9 has revealed that these

receptors differ significantly in their trafficking (44). Both receptors traffic from the ER to the Golgi mediated by UNC93B1 and COP-II vesicles. However, their routes diverge following exit from the Golgi. TLR9 transiently traffics to the surface where it is then loaded into endosomes via AP-2 mediated endocytosis. TLR7, on the other hand, traffics directly to endosomes via AP-4 mediated mechanisms. Interestingly, the chaperone UNC93B1 was demonstrated to play a significant role in this trafficking regulation, as point mutations in its C-terminal region disrupt AP-2 mediated TLR9 trafficking. Furthermore, a point mutation (D34A) in UNC93B1 has been shown to bias its interaction with TLR7 and TLR9 (45). The exact functional significance of this differential trafficking remains unclear, however one possible outcome is that these TLRs reach two separate endosomal populations. Additionally, as will be discussed in Chapter 3, this divergence may also reflect a difference in selective pressure to avoid self-recognition.

In vivo Consequences of Improper TLR7 and TLR9 Signaling

Despite the previously discussed checkpoints put in place by the innate immune system, discrimination between self and non-self nucleic acids can break down and may contribute to diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) (46). A defining symptom of SLE is the production of auto-antibodies targeting nucleic acids or other nuclear proteins. A model auto-reactive B-cell, which possesses a low affinity B-cell receptor (BCR) for the IgG2a antibody isotype isolated from a mouse model of SLE, proliferates in response to IgG2a anti-chromatin immune complexes (28). Importantly, in this model direct sensing of nucleic acid contained in these complexes was demonstrated to be necessary for proliferation both *in vitro* and *in vivo*. Furthermore, when TLR7 and 9, which are expressed in both human and murine B-cells, are inhibited or deficient, proliferation of these auto-reactive B-cells is impaired (47). The normal exclusion of self-nucleic acid from the TLR7/9 signaling compartment is circumvented in this case by BCR mediated uptake and delivery, illustrating the importance of proper spatial regulation.

Nucleic acid signaling is also implicated in SLE because of its importance in type I IFN production mentioned earlier. Type I IFN is presumed to play a role in SLE because studies demonstrate that patients and mouse models have a transcriptional phenotype described as the IFN signature (48, 49), patients frequently have increased IFNα in their serum (50), and IFNα treatments increase risk of developing SLE in humans and mice (51, 52). IFN is known to be involved in a wide array of processes involved in producing an “anti-viral state.” Of particular interest, IFN can lead to plasma-cell differentiation, class-switching to pathogenic antibody classes, and increased sensitivity to apoptotic signals. Plasmacytoid dendritic cells (pDCs) are considered to be the specialized producers of IFN (27), and importantly TLR7 and 9 are critical for IFN production in this cell type (53). Of note, in humans the only cell types believed to express TLR7 and TLR9 are pDCs and B-cells, and not conventional dendritic cells

(cDCs) or macrophages (54). This restricted expression in humans is in contrast to mice, in which express TLR7 and TLR9 in each of these cell types (15, 16). Although the importance of this distinct expression is not understood, it is interesting to speculate that expression of these receptors is under evolutionary pressure due to their potential role is auto-immunity. Further establishing an interconnected role for pDCs, type I IFN, and B-cells, studies using the same auto-reactive B-cells described earlier found that treating the B-cells with IFNα (a type I IFN) increased their responsiveness to sub-optimal TLR9 ligands contained in the immune complexes (55). Importantly, additional studies tie this IFN loop to the importance of spatial restriction in humans by demonstrating the necessity for FcγRIIa mediated uptake of SLE patient derived IgG complexes to produce IFN (56). Together, these studies illustrate both the interconnectedness of B-cells, pDCs, and IFN production, as well as the possible deleterious effects in human disease of bypassing the spatial regulation of nucleic acid TLR sensing.

SLE is a cyclical disease as it potentially involves multiple feedforward loops that result in disease progression (57). The molecular causes for progression to disease appear to be multifaceted, as multiple mutations have been described that may confer susceptibility to SLE by genome wide association studies, although importantly type I IFN, B-cell, nucleic acid clearance, and TLR signaling pathways have all been implicated (58). However, the molecular basis for the initial triggers that ultimately lead to disease progression is still unclear. Several animal models have demonstrated the ability for specific genes and mutations to confer disease susceptibility. Examples include deficiency in the Src family kinases Lyn and Fyn involved in regulating B-cell signaling (59), as well as deficiency in the complement protein C1q and DNAses involved in clearance of nucleic acid (60–63). A direct role for TLRs to predispose mice towards a lupus like disease was demonstrated by determining that the Y-linked autoimmune accelerator (*Yaa*) locus, which predisposes mice to a lupus like disease, was primarily a duplication of the X-linked *Tlr7* gene onto the Y-chromosome (64). This finding was subsequently confirmed by introducing multiple copies of *Tlr7* via bacterial artificial chromosome (BAC) transgenesis, coupled with the interesting finding that disease type and severity correlated with the number of copies introduced (65). These studies indicate that mutations which affect TLR7 protein levels, not function per se, are potentially responsible for triggering lupus like disease. Additionally, the UNC93B1 mutation discussed earlier, which biases signaling towards TLR7 at the expense of TLR9, is sufficient to predispose mice to a lupus like disease suggesting alterations in the levels of functional TLR7 receptor, but not necessarily the total TLR7 pool itself, can also trigger disease (66). This balance between TLR7 and TLR9 is also illustrated by TLR9 deficiency exacerbating disease in a TLR7 dependent fashion (67, 68). Other mouse models have also demonstrated that improper TLR9 signaling can confer disease following mutations that alter localization and the requirement for receptor processing (69). Together, these studies demonstrate that while SLE disease may be multifactorial, the proper regulation of nucleic acid sensing TLRs is paramount in

preventing autoimmune disease and deserves further study to fully elucidate the mechanisms involved.

Chapter 2: Introduction to Codon Bias and its Biological Role

Historical Background

The central dogma of biology is a cell's ability to read genetic information encoded by DNA and convert it into protein. Following that monumental insight, it was soon established that genetic material is decoded into protein through tRNA recognition of a codon that consists of three nucleotides. As DNA consists of 4 nucleotides, there are 64 possible codons in nature, yet there are only 20 naturally occurring amino acids. Thus, multiple codons encode for the same amino acid, and it was eventually determined that almost every amino acid is encoded for by at least 2 codons. Importantly, most variation between codons encoding for a given amino acid occurs at the third position, and more specifically usually a difference in whether the final nucleotide is a G/C or an A/T, which will be discussed further. Interestingly, while the assignment of each codon for a particular amino acid has remained almost universally fixed, it was recognized early on that usage of particular codons is uneven across organisms (70). The frequency with which one codon is used within a genome, and the argued evolutionary pressure for this apparent preference, is termed codon bias.

Evolution of Codon Bias

Ever since it was recognized that codon usage is skewed between genes and between genomes, the mechanisms for the evolutionary causes and functional significance of this bias have been examined and debated. It has been appreciated for quite some time that nucleotide composition of genomes varies between unicellular organisms, as well as within vertebrate genomes, and that the variation in GC content correlates with the GC composition of codons used (71–73). Furthermore, this variation has been argued to be the driving force behind codon bias (74). In mammals, it is believed GC variation between genes is mainly reflective of the mosaic nature of the mammalian genome, which contains large stretches of homogeneous GC content termed isochores (75, 76). The molecular mechanisms that maintain the nucleotide composition of isochores are largely unknown, but it is speculated to be reflective of the recombination rates of an isochore's respective genomic location (77). Therefore, in this neutral theory of codon bias, variation is due to passive differences resulting from mutational mechanisms. However, this isochore effect cannot explain the whole story as "silent" mutation rates at the third position of codons within a coding region have a higher GC content than accounted for by the mutation rate of flanking introns or intergenic regions (78–80). This phenomenon suggests that evolutionary selection for particular codons does exist (81), although conclusions about the strength of this selective process and the reasons for it can vary depending on methodology (75).

The Debated Functional Significance of Codon Bias

While selective pressures appear to influence codon usage, debate surrounds the exact functional significance of this bias, particularly as it relates to translation efficiency. Early evidence for a presumed functional role in optimizing translational efficiency came from studies of unicellular organisms which saw a strong correlation between using frequent codons and genes that are considered the most abundantly expressed in both yeast and *E coli* (82, 83). These studies and others also noted a positive correlation between codon usage and cognate tRNA abundance, although, it should be noted, that there are conflicting reports as to whether there is a correlation between tRNAs, expression, and codon bias in humans. The evolutionary basis of this correlation has also been debated, with one model suggesting highly expressed genes use codons that match the most abundant tRNAs so that the available tRNA pool is not depleted, but the rate of translation itself would remains unchanged under normal conditions. A second model proposes that matching codons to abundant tRNAs would increase the rate and accuracy of translation (74). Differentiating between these two models is difficult, however, as it is hard to address if codon usage during evolution was influenced tRNA abundance, vice versa, or likely a co-evolution of the two resulting in a correlated skewing of both (75).

While adapting codons to tRNA abundance in order to maximize translational efficiency by improving ribosomal translocation provides a simple model, it is debated how practical this selective process would be, as a single codon change would need to confer enough of a fitness advantage for natural selection to act on. In comparison, a single nucleotide change in a promoter region could have a potentially dramatic effect on mRNA expression, enabling a selectable fitness advantage (74). Furthermore, it is argued that initiation is the limiting factor in protein production, and therefore altering translational efficiency would have a minimal impact on protein levels. This is in addition to other mechanisms, such as inherent mRNA differences in transcription, stability, secondary structure, regulatory elements, etc. that have been implicated (75). However, experimental evidence formally demonstrating the mechanisms for a selecting bias is conflicting. New technology has allowed closer examination of these potential mechanisms in endogenous genes on a genome wide scale. In particular, the new technique of ribosome profiling in yeast and mammalian cells revealed no significant correlation between codon bias and the elongation rate of ribosomes (84, 85), although the exact conclusions of the data have been challenged and may involve codon bias affecting mRNA folding energy (86–88). Of note, these reports all noted the importance of the 5’ end of the coding sequence thus strengthening the idea that translational initiation is likely a critical element. These studies, though, had to make key assumptions to infer resulting protein levels, although current technology in the field will likely remove that barrier.

An example of the future of the field for studying endogenous genes will likely resemble the recent report which directly monitored mRNA, translational, and protein kinetics (89). This report combined the power of RNAseq to assess mRNA kinetics, ribosome profiling to assess translational kinetics, and SILAC-labeling based mass-spectrometry to directly assess overall protein kinetics. By combining these approaches, key assumptions are largely removed from analysis. As an example, the previous assumption that protein stability remained unaffected is no longer required and instead changes in stability can be quantitated. In addressing open question in the field, the study concluded that following a stimulus the majority of the protein changes are reflective of their relative change in mRNA levels. However, a subset of proteins, particularly abundant proteins involved in cellular maintenance, were seen to have altered protein levels as a result of differential translation efficiency or changes in protein stability following stimulation. Thus, while certain mechanisms for altering protein levels may dominate, alternative mechanisms may nevertheless contribute and their relative importance depend on context. Yet, it should be noted that this study did not address the role of codon bias directly, leaving open the intriguing possibility that many of the unresolved questions surrounding the functional significance of codon usage are still to be explored.

Studies have also looked to answer questions about codon bias in direct experimental models by manipulating codon usage in a given gene, typically by matching codons to those considered optimal within an organism. While it is not debated that altering codons can have a profound influence on subsequent protein levels, such as with GFP, in a heterologous system, the mechanisms altered by codon differences are strongly debated. In one defining study, 154 synonymous variants of GFP were analyzed for resulting protein expression in *E. coli* and no correlation with codon adaption was observed, but instead with mRNA folding energy (90). In other studies involving mammalian systems, protein expression was argued to correlate with GC content altering the rate of transcription (91, 92). Contradicting these findings, further studies in mammalian systems that altered codon bias were argued to affect translational efficiency directly as assessed by *in vitro* translation assays and ribosomal density (93, 94). In addition to these conflicting results, a major caveat in inferring general mechanisms from these studies is the use of heterologous expression systems, which due to the common use of strong promoters and multiple gene copy number, may bias results because of possible massive overexpression of the gene of interest and subsequent predominance in the total mRNA pool (74). Importantly, one study attempted to address a physiological role for codon bias by altering the endogenous locus of their protein of interest and detected a modest difference in the rate of translation (94). Furthermore, with the advent of CRISPR-Cas9 technology that allows for more efficient target of endogenous genes, it should now be feasible for future studies to address the functional role of codon bias under physiological conditions.

In conclusion, codon bias is a phenomenon long debated in the field and still debated today. While selection for specific codons in coding sequences appears to be generally accepted, the mechanisms that account for this bias and the subsequent

functional significance in expression remain unclear despite decades of investigation. However, there is renewed excitement in the field due to the rapid advances in technology that may finally allow researchers to fully address the roles for codon bias in biology.

Chapter 3: The Role of Codon Bias in the Differential Regulation of TLR7 and TLR9

Background

Toll-like receptors (TLRs) recognize broadly conserved microbial features and initiate downstream signaling events critical for proper responses to infection, including production of key cytokines and initiation of the secondary immune response. A subset of these TLRs recognize nucleic-acid ligands (TLR3, TLR7, TLR8, TLR9, TLR13). While these nucleic acid-sensing TLRs recognize features generally associated with viruses and bacteria, the potential exists for recognition of self-derived nucleic-acid ligands. This self-recognition is proposed to play an important role in the breaks in tolerance which promote auto-immune diseases such as systemic lupus erythematosus (SLE) and Rheumatoid Arthritis (RA), particularly in driving the production of auto-antibodies against nucleic-acid and nucleic-acid containing complexes (46). Therefore a balance exists between the ability to properly respond to infection and the avoidance of improper activation against self.

Several lines of evidence point to the importance of proper regulation of the nucleic-acid receptors TLR7 and TLR9, whose ligands are single-stranded RNA and DNA with unmethylated CpG motifs respectively, in limiting inappropriate responses to self-derived nucleic acid that may result in autoimmunity (32). At the transcriptional level, TLR7 and TLR9 are both restricted in their expression to distinct immune cells types in mice. Interestingly, in humans their expression is even further restricted beyond that of other TLRs, suggesting an evolutionary need to limit expression (54). TLR7 and TLR9 also share the regulatory feature of limiting ligand recognition to intracellular endolysosomal compartments through restricted cellular localization and requirement for pH dependent receptor processing (36). Therefore current evidence supports the notion that evolution has imparted a regulatory need to limit nucleic-acid TLR signaling.

Despite the similarities in TLR7 and TLR9 regulation, a more detailed analysis reveals that differences exist in the regulation of the two receptors. One area of active investigation is the mechanisms of cellular trafficking to endolysosomes where recent evidence suggests differences exist in both the nature of the endosomes reached and the trafficking routes used for TLR7 and TLR9 (41, 44). Furthermore, in an *in vitro* setting it has been shown that TLR7 and TLR9 can compete with each other for factors required for signaling (95). Recent studies also suggest this interplay is cell-type specific and may involve other TLRs depending on cell-type (96). Additionally, a point mutation in the trafficking chaperone Unc93b1 can bias signaling towards TLR7 at the expense of TLR9 (66). Type-I interferons, cytokines strongly linked to auto-immune diseases such as SLE (97), have been shown to differentially regulate the expression of TLR7 and TLR9 in B-cells (98). Altogether, while the detrimental effects of mis-

regulated nucleic-acid TLR sensing have been appreciated for some time, there is accumulating evidence that distinct regulatory mechanisms must be controlling TLR7 and TLR9 function.

Evidence for the critical need *in vivo* to regulate nucleic-acid sensing TLRs, particularly in limiting TLR7 function, is demonstrated by mouse models of SLE. Both TLR7 and TLR9 have been demonstrated to promote autoantibody production in these models (28, 99, 100). Furthermore, TLR7 is shown to be important in these models as mice lacking TLR7 have ameliorated disease. However, TLR9 deficiency paradoxically exacerbates disease in a TLR7 dependent fashion demonstrating a differential need for regulation of respective receptor levels (67, 68). Importantly, TLR7 itself has been shown to have a direct role in autoimmunity in mice by triggering disease through introduction of multiple copies of TLR7 (65). Interestingly, this study also found that varying levels of TLR7 gene dosage lead to different phenotypic disease outcomes ranging from SLE like disease to acute inflammation. This implies different regulatory barriers are overcome as the amount of TLR7 protein increases. Of importance, copy number variations of TLR7 have also been shown in patients with SLE (101), as well as a point mutation in the 3' untranslated region of TLR7 in SLE patients that correlates with increased TLR7 mRNA levels (102). Additionally, the point mutation discovered in the chaperone Unc93b which biases signaling towards TLR7 and way from TLR9 promotes autoimmunity in mice (66). Altogether, evidence suggests regulating the level of TLR7 protein is critical in the balance between proper and improper responses, while protein levels of TLR9 may play a lesser role or even be deleterious if limited further. Therefore, further elucidation of the mechanisms involved in regulating protein levels of TLR7 and TLR9 protein levels is critical for fully understanding their various roles in autoimmunity.

The mechanism by which codon bias, the unequal use of synonymous codons encoding for a given amino acid, impacts translation efficiency remains a hotly debated subject (74, 75). Recent advances in technology and experimental design, in particular ribosome profiling (84), have allowed researchers to explore mechanisms related to codon bias and protein expression on a global scale. However, disagreement in the field persists, and conclusions about the role of codon bias seem heavily influenced by the method of analysis (85, 86, 88). On a smaller scale, optimizing codons of individual proteins can lead to increased protein expression. However, the mechanism(s) underlying the increased protein remain(s) controversial. Some groups report that the protein increase is a result of an increase in the rate of translation (93, 94), while others observe an increase in the rate of transcription (91, 92). Furthermore, a recent study concluded that while transcriptional control is the broadest means of controlling protein expression, a fraction of proteins are differentially regulated at the translational level (89). The field, however, has tended to focus on extreme examples, either by comparisons to fully optimized sequences or limiting global analysis to genes that meet certain thresholds. Absent from the field are concrete, biologically relevant examples where the various potential mechanisms of codon optimization are thoroughly distilled down into their component contributions by varying the level of optimization. Doing so

allows one to investigate the more nuanced, but nevertheless potentially critical, effects that may play important regulatory roles.

Here we investigate the role codon bias plays in the biologically important regulation of TLR7 and TLR9 protein expression. We observe that these proteins display remarkably different translation efficiencies, correlating with their degree of codon optimization. Additionally, by scaling the extent of codon optimization we are able to elucidate, and importantly separate, the multiple mechanistic contributions of codon bias that converge to limit TLR7 but not TLR9 protein expression. Importantly, elucidating these mechanisms widens the regulatory pathways to be explored, not only as it relates to therapies for SLE which focus on TLR7 and TLR9 pathways as drug targets, but also for other biologically important systems which demonstrate a codon bias.

Results

TLR7 and TLR9 mRNAs are in distinct translation states

A previous study suggested that codon bias may be important for the expression of TLRs, noting that TLR7 uses a high percentage of infrequent codons while TLR9 uses more common codons (103). We have also reported that codon optimization of TLR7 dramatically increases protein levels in an *in vitro* setting whereas TLR9 required no such optimization (44). However, these studies did not elucidate the mechanisms responsible for the increase in protein, nor was the biological significance of potential differences in translation addressed. Because of the clear link between increased TLR7 levels and autoimmune disease, we sought to investigate whether inefficient translation limits TLR7 responses as well as the mechanisms underlying such potential regulation.

As a first step, we aimed to establish the translation state of endogenous TLR7 and TLR9 mRNAs. Ribosome profiling provides the ability to look at both positional differences of ribosome density as well as kinetics of movement along transcripts. We took advantage of a data set previously generated from bone-marrow derived dendritic cells (BMDCs) to perform our analysis for TLR7 and TLR9 translation state. Using ribosomal density on mRNAs as a read-out for translational efficiency (TE) of an mRNA, we calculated the TE values of TLR7 and TLR9 (85). TLR9's translational efficiency was calculated to be three times greater than TLR7's, suggesting a more optimal translational state for TLR9 (Figure 3.1A).

Dendritic cells were also treated with harringtonine in order to monitor translation kinetics. Harringtonine blocks translation initiation yet allows ribosomes already on transcripts to translocate normally. By comparing positional changes of ribosomes between treated and untreated samples, inferences can be made about the rate of translation. In the absence of harringtonine, the distribution of ribosomes along TLR7 and TLR9 is largely similar (Figure 3.1A-C "CHX"). Following harringtonine treatment, ribosome density shifts to the 3' end for both TLR7 and TLR9 as expected, but with a

greater apparent shift for TLR7 (Figure 3.1A-C “Harr”). However, the greater shift in TLR7 normalized to itself cannot distinguish between increased run-off from the 5’ end and increased retention on the 3’ end. Normalizing harringtonine treatment to TLR9 revealed that a greater fraction of ribosomes remained bound to the 3’ end of TLR7 relative to TLR9 (Figure 3.1C). This difference suggests that the rate of ribosome translocation on TLR9 is fast enough that most ribosomes complete translation during the harringtonine treatment. In contrast, ribosomes on TLR7 appear to translocate more slowly and therefore accumulate on the 3’ end of TLR7 mRNA. No distinct pause sites along TLR7 were noted in the untreated samples, but minor stalling sites did appear around base pair 1800 and 2700 following harringtonine treatment. Overall these ribosome profiling data suggest that the general rate of translation for TLR7 is slower than that of TLR9, although we cannot completely rule out that stalling at a few distinct stretches may also contribute.

As a next step to confirm this differential translation state of TLR7 and TLR9 in our hands, we performed sucrose gradient ultracentrifugation of RAW264.7, a macrophage like cell line that expresses TLR7 and TLR9, and collected the polysome containing fractions. Analysis by quantitative RT-PCR (qPCR) revealed that mRNAs for TLR7 and TLR9 were contained in different fractions, with proportionally more TLR7 present in fractions with few ribosomes (Figure 3.1D-F, black arrow) while TLR9 was present predominantly in fractions with many ribosomes (Figure 3.1D-F, grey arrow). Importantly, the coding sequence length for TLR7 and TLR9 are comparable (3152bp and 3098bp respectively), so differences in ribosome occupancy most likely reflect differences in density. Therefore, these results establish a distinct difference between TLR7 and TLR9 in terms of their ribosome density, suggesting potential regulation mediated by codon bias at the level of translational efficiency. When considered together with the ribosome profiling results described above, these results provide strong evidence that the translational states for TLR7 and TLR9 transcripts are quite distinct.

TLR7 and TLR9 Display Distinct Codon Biases

Having demonstrated that the translation states of TLR7 and TLR9 differ quite dramatically, we turned to the investigation of mechanisms that may underlie these differences. As mentioned earlier, several lines of evidence suggested that differential codon usage may influence expression of TLRs, so we performed an analysis of codon usage of the TLR7 and TLR9 genes in humans and mice. Using the codon adaptation index (CAI) as a measure of bias, we compared TLR7 and TLR9’s transcripts to the rest of the coding sequences (CDS) for both mouse and human genomes (Figure 3.2A). We chose to use the CAI as our measure because it is designed to be predictive of protein expression (104), whereas other measures such as GC content at the third codon position (GC3) and the Effective Codon Number (Nc) are designed to measure bias as deviation from a null selection hypothesis of equal codon usage (73, 105). Relative to

all mouse and human genes, TLR7 scores in the bottom 5th and 17th percentile, respectively, while TLR9 scores in the 81st and 88th percentile (Figure 3.2C). Therefore, TLR7 and TLR9 fall on opposite ends of the distribution of genes scored for usage of optimal codons.

More frequently used codons in mice and humans correlate with a higher GC content, and genes reflect the GC content of their chromosomal context. This correlation has led some scientists to argue that codon bias is a passive process and not actively selected for by evolution (74). Accordingly, we sought to address whether the codon bias we observe for TLR7 and TLR9 could be entirely explained by the genomic context of these genes, or, on the other hand, if we could detect positive selection for codon bias (poor or favored) by comparison to genes in similar chromosomal contexts. We performed further analyses of CAI taking into account the local GC content of all intron-containing CDS (Figure 3.2B); the local GC content was calculated from intronic sequences, with the assumption that any bias in the GC content of these sequences primarily reflects non-selective genetic drift. Comparison to genes within a 2.5% of the GC content window revealed that even when accounting for chromosomal context TLR7 still reflects a codon bias towards infrequent codons (7th mouse, 15th human), while TLR9 displays less of a bias (54th mouse, 76th human) (Figure 3.2C). This suggests the coding region of TLR7 may reflect evolutionary pressure to maintain suboptimal codons even below that of its chromosomal context, while the optimal codons used by TLR9 may simply reflect the GC content of its chromosomal context.

Based on the different CAI scores for TLR7 and TLR9, we looked differences in the usage of specific codons representative of differences in codon bias between TLR7 and TLR9. We reasoned that leucine codon usage may be significant due to the relative abundance of leucine in TLR genes due to the leucine rich repeats (LRRs) within TLR ectodomains. Strikingly, TLR7 and TLR9 varied in their usage of almost all leucine encoding codons (Figure 3.2D). Most dramatic was in their use of the frequently used CTG and infrequently used TTA. TLR7 only used CTG 18.6% of the time (31/167), less than half of the 39.6% genome wide average, while TLR9 used the codon 56.0% of the time (107/191). TLR7 also used TTA almost twice as frequently as average (14.4% vs 7.5%, 24/167), while TLR9 only used TTA once (0.5%). Therefore, although TLR9 may be mainly reflecting its chromosomal context, TLR7 has an inherent bias towards infrequent codons within its coding region, particularly in its usage of the codons encoding leucine, suggesting an evolutionary pressure and possible regulatory role in protein expression.

Codon bias in TLR7 affects protein production and ribosome density in a heterologous system

We next sought to address the functional consequences of the differential CDS codon bias between TLR7 and TLR9 protein by directly comparing protein expression in

a heterologous system. For our studies we used the Flp-In T-REx 293 system, which integrates a single copy of the sequence of interest into a fixed genomic location containing a Dox-inducible CMV promoter. This system enables comparisons of expression that are less prone to experimental variations such as transfection efficiency or differences in genomic context. Directly comparing wild-type (WT) coding sequences for TLR7 and TLR9 revealed a greater than 40-fold difference in protein levels (Figure 3.3A). While in agreement with the endogenous polysome and ribosome profile data suggesting greater translation of TLR9, we could not formally differentiate between codon bias affecting expression of protein and other mechanisms, such as differences in protein stability. To formally show a role for codon bias we fully optimized TLR7's coding sequence using Invitrogen's GeneArt algorithm (CO). Placed in the T-REx system, optimization increased TLR7's protein levels close to that of TLR9 (34.4 fold) (Figure 3.3A). Importantly, this increase in protein has functional consequences as signaling capacity, as measured by IL-8 production, also increased following codon optimization (Figure 3.3B). The increased level of functional protein suggests TLR7 still undergoes proper folding, as well as that regulation at the level of protein can have functional consequences in downstream signaling consistent with previous studies investigating TLR7 overexpression. We therefore established with this system that differential protein expression of TLR7 and TLR9 protein is a direct reflection of codon bias, with functional implications for regulating signaling.

Having improved protein expression of TLR7 to levels similar to that of TLR9, we wanted to know if the translation state had been altered as assessed by polysome profiling. Optimizing the coding sequence of TLR7 dramatically shifted the transcript to ribosome-dense fractions, consistent with the hypothesis that the phenotypic difference between endogenous TLR7 and TLR9 is due to codons (Figure 3.3C-F). Importantly, other transcripts, such as GAPDH, were not affected indicating the general state of translation was the same between clones. Taken together, these results suggests codon bias plays an important role in regulating ribosome density with potential consequences for protein production.

While fully optimizing coding sequences certainly increases protein expression in mammalian systems, it is unknown whether more modest coding changes can impact expression in a meaningful way. Put another way, is codon optimization scalable or bimodal? To investigate the consequences of more modest changes to TLR7's sequence, we focused on codons encoding for leucine because their usage is so different between TLR7 and TLR9 (Figure 3.2D). Replacing codons TTA, TTG, CTT, and CTA all with the codon CTG increased protein by more than 20-fold (LO, Figure 3.3A). A more modest change of replacing only the codon TTA with CTG increased protein close to 10-fold (TTA, Figure 3.3A). Importantly, signaling also displayed a similar trend to the increase in protein levels (Figure 3.3B). To rule out a role for untranslated regions (UTRs), we generated T-REx lines with only the 5' or both 5' and 3' UTRs added and saw no additional change in protein levels (data not shown). Therefore, codon bias, even to a minor degree, is a scalable effect that may play an

important role in the regulation of protein levels and subsequent signaling capacity of cells.

Codon bias affects mRNA levels by altering transcription in a heterologous system

We next examined the effect of codon optimization on RNA levels in the T-REx system as other systems which optimized mammalian proteins noted a correlation between codon bias and RNA levels. However, stark disagreement in the field exists as to whether the increase in RNA is playing a minor or major role in protein levels, as well as the mechanisms responsible for the increase. RNA levels in our T-REx system quantified by Northern blot revealed a large difference (50-fold) in mRNA levels between the WT sequences of TLR7 and TLR9 (Figure 3.4A), reflective of the difference seen at the protein level. Additionally, the fully optimized version of TLR7 led to a significant increase in RNA levels (42.5-fold), again closely resembling the levels seen at the protein level. Importantly, we see that more modest codon changes to TLR7 correlate with more modest increases in RNA levels. This is in agreement with reports that codon optimization, due to an increase in GC content, has a dramatic effect on RNA levels and can account for most of the overall increase in protein levels. Additionally, we have shown here that this effect is also related to the degree to which the coding sequence is changed.

Previous reports also conflict as to whether the mechanism of RNA increase was an increase in transcription or an increase in transcript stability. To elucidate the mechanism of this RNA difference in our T-REx system, we used 4-thiouracil (4sU) labeling to track rates of transcription. Following a short pulse with 4sU, the rate of transcription was indeed greater comparing WT TLR7 to TLR9 in the T-REx clones (Figure 3.4B). Likewise, fully optimizing codons in TLR7 lead to a significant increase in the rate of transcription. Importantly, this increase in transcription rate again scaled with the level of optimization. Therefore, in our system codon bias plays an important role in the rate of transcription.

Previous reports have also suggested RNA differences can be attributed to differences in transcript stability due to translational stalling and no-go decay (NGD). To test this possibility we assessed RNA half-lives by inhibiting transcription with ActinomycinD and monitoring subsequent RNA levels. In our T-REx system, TLR9 displayed a modest but reproducible increase in stability, however not to a degree which adequately explains the overall difference in RNA and subsequent protein levels (Figure 3.4C). Interestingly, fully optimizing the coding sequence of TLR7 lead to a reproducible 2-fold increase in stability, although again not adequately explaining the more than 40-fold increase in total TLR7 RNA. However, we cannot differentiate in this assay if the stability increase is due to decreasing stalling and subsequent NGD, or to GeneArt's optimization algorithm being designed to eliminate RNA instability motifs. Furthermore, in TLR7 constructs featuring more modest changes, RNA stability is

indistinguishable from the WT sequence, noticeably in contrast to our previous assays which all displayed a scalable effect. Taken together, codon optimization has a profound role in total RNA levels through its effect on the rate of transcription, and not on mRNA stability, in a manner directly proportional to the overall increase in protein levels.

In vitro systems reveal modest contribution of rate of translation

While the predominant role of codon optimization may be at the level of transcription in the T-REx system, our results did not rule out an improvement in the rate of translation. To tease apart the contribution of codon bias in translation, we looked at protein production in the absence of any contribution of transcription that could potentially mask more modest differences. Using a T7 *in vitro* transcription system, we produced capped, poly-adenylated transcripts and transfected equivalent amounts of RNA transcript into HEK293 cells (Figure 3.5A). Quantification revealed a reproducible increase in the level of protein when comparing the WT TLR7 sequence to both TLR9 and the fully optimized construct (Figure 3.5B). While the increase was still modest compared to the effect of codon bias on transcription, it nevertheless suggests a potential role for codon bias in the rate of translation, although we cannot rule out the contribution of the modest increase in stability. However, in comparison to the construct which replaced the codons TTA, TTG, CTT, and CTA all with the codon CTG, a modest but reproducible 2-fold increase in protein was observed even though RNA stability was equivalent. Taken together, codon bias may play a role in overall translation rate, but in a more modest manner than its effect on transcription.

Modest alterations of codon bias in endogenous TLR7 have no affect

Having explored the role of codon bias in a heterologous system, we next wanted to address if altering codon bias in an endogenous context would alter protein production and subsequent signaling capacity. To do this we took advantage of the fact that TLR7 is located on the X-Chromosome, and therefore the RAW264.7 cell-line derived from Balb/C males only contains one copy of TLR7. The general scheme of the locus is depicted in Figure 3.6A. We also took advantage of the fact that the entire coding sequence of TLR7 except for the ATG-Start codon is encoded by exon 3. The general targeting scheme is illustrated in Figure 3.6B. Briefly, using Crispr/Cas9 technology to facilitate homologous recombination, we first replaced exon 3 with the fluorophore tdTomato then sorted targeted clones via FACS and confirmed proper integration via Southern and loss of responsiveness to the TLR7 ligand R848 (Figure 3.6C and data not shown). We then retargeted the locus to replace tdTomato with various versions of TLR7, sorted by FACS and confirmed proper integration by Southern and sequencing (Figure 3.6C and 3.7).

We compared WT TLR7 to the more modest leucine optimizations using a functional readout of TNFα production to assay for increases in protein. No significant differences in TNFα were detected between clones (Figure 3.6D and E). We also confirmed general signaling capacity was still intact for all lines as CpG stimulation revealed no significant difference in between lines. Importantly, in contrast to the heterologous system, no significant difference in RNA levels was detected (Figure 3.6F). This is potentially explained by our finding that transcription in the heterologous system is the dominant mechanism for differential RNA levels, but in the endogenous context a large (21.5kb) low GC intron is now present and may act as a transcriptional buffer (Figure 3.2B and 3.6A). In other words, the modest changes that were sufficient to alter the rate of transcription of the CDS in T-REx cells may not be sufficient to alter overall rate of transcription when introns are present in the endogenous locus. It is also possible that additional regulatory mechanisms that we are unaware of are involved in limiting RNA levels and subsequent TLR7 signaling capacity. Unfortunately, due to the still relative inefficient process of homologous recombination mediated targeting, we were unable to generate a line containing the fully optimized TLR7 to see if more extreme alterations could alter signaling. Altogether, we have demonstrated that multiple mechanisms related to codon bias have the potential to converge on limiting TLR7 protein and function, but that in the endogenous context the cell can withstand modest changes in codon bias with the biological significant outcome of limiting TLR7 signaling capacity.

Discussion

The regulation of TLR7 at the protein level is generally accepted to be important for the prevention of autoimmune diseases, and has been convincingly demonstrated in mouse models (65). We therefore were interested in exploring in greater mechanistic detail the cell biology involved in maintaining proper levels of TLR7 protein. Here we report that codon bias can play an important role in limiting TLR7 protein expression. To begin we demonstrated that the endogenous TLR7 transcript has a low observed translational efficiency, particularly when compared to the closely related TLR9. We attribute this translational difference to a dramatic skewing in codon bias. To directly test this apparent role for codons we explored the consequences of altering the codon bias of TLR7. Increased protein levels resulted from fully optimizing TLR7's coding sequence in an exogenous context coupled with a measured improvement in translational efficiency, demonstrating convincingly that codon bias can play a role in protein expression. Interestingly, we also detected a marked increase in RNA levels leading us to ask what exact mechanisms were affected by altering codon bias. To tease apart the various potential mechanisms responsible for these phenotypes, we took the novel approach of scaling the level of optimization for TLR7's coding sequence. Careful analysis revealed the major cellular mechanism affected was transcriptional enhancement resulting in increased RNA levels that scaled with the degree of optimization, although more minor roles for mRNA stability and translational

enhancement were also evident. We then explored the consequences of altering TLR7's endogenous locus. Curiously, moderate changes to codon bias to TLR7's endogenous locus did not result in increased TLR7 function suggesting either further limitations imposed by TLR7's genomic context or additional regulation that requires further exploration.

Our study focuses on the role for codon bias in the biologically relevant system of nucleic-acid sensing TLRs TLR7 and TLR9. Our interest in the regulation of TLR7 and TLR9 stems from several lines of evidence that point not only to the importance of proper regulation of these two proteins in limiting inappropriate responses to self-derived nucleic, but also their seemingly paradoxical role in mouse models of autoimmunity. Of particular importance appears to be the relative protein levels of these receptors in mouse models of lupus (65, 67, 68). Our findings that TLR7 and TLR9 have dramatically divergent translational efficiencies in an endogenous context (Figure 3.1), and protein expression of TLR7 can be markedly increased if codon bias is manipulated (Figure 3.3) strongly supports the hypothesis that there is a consistent bias towards limiting TLR7 function relative to TLR9. While it should be noted that modest changes to TLR7's endogenous locus do not result in a functional increase (Figure 3.6), this finding may actually support the importance to the cell to limit TLR7 function, as there may be additional regulatory mechanisms which buffer sequence changes in TLR7. It is also exciting to think that the observed difference in codon bias between TLR7 and TLR9 adds to the mechanisms that can be exploited for therapeutic purposes.

Our findings also add to the decades old debate of the functional significance of codon bias in gene regulation (74–76). The recent advance of ribsome profiling has reinvigorated this debate since direct analysis of translation on a per transcript basis can now be explored. However, despite the advance in technology, the functional significance of codon bias still remains unclear. While codon bias has been shown to potentially play a role in translation efficiency (87, 88), the regions of mRNA involved and the global extent of correlation are still controversial (85, 87). Our results suggest there does exist the very real potential for codon bias to play an important part in the regulation of genes, particularly in limiting protein expression. Importantly, we formally demonstrate that manipulation of codons can directly affect the observed translational efficiency (Figure 3.3).

It also interesting to speculate that translation efficiency may be able to be regulated. Related to this hypothesis, a recent study investigating global mechanisms of protein level regulation in an immune context found that while most changes in protein levels are due to transcriptional changes, the increase in a subset of proteins is more reflective of changes in their respective translational kinetics (89). Particularly noteworthy, TLR2 was identified as one of the 233 proteins in the study to be translated faster after lipopolysaccharide treatment suggesting TLRs may be subject to translational regulation (Table S7 Jovanovic et al., 2015). Translational kinetics of TLR7 was not seen to be affected in the study, but it would be interesting to explore its

behavior in additional cell types or following alternative treatments such as type-I interferon. Additional mechanisms of regulation to explore involve the more recent evidence for plasticity of tRNAs (106). Interestingly, studies in this area have demonstrated that mammalian cells may actually guard against tRNA manipulation by viruses that could alter the codon bias of a cell, particularly by preventing an increase in tRNAs for AT-rich codons (107). Altogether, there is growing evidence that the rate of translation may be more plastic than previously thought and may be subject to levels of regulation not yet appreciated. Therefore, additional studies exploring the role of translational regulation of TLRs may prove fruitful, particularly in how they may be manipulated by pathogens or in a disease context.

Our study also attempts to resolve controversies in the literature concerning the mechanisms affected by codon bias in mammalian systems. Several groups have attributed increase in protein following codon optimization predominantly to an increase in translation (93, 94). It should be noted that these studies also observed increased mRNA levels, although the authors argued that the differences were not sufficient to explain the increase in protein levels. Instead the authors attributed the increased RNA levels to reduction in No-Go Decay (NGD) mechanisms that affect mRNA stability. In direct contradiction, other studies see that the predominant effect of altering codon bias is in altering transcription due to modulating GC content (91, 92). Our results agree that transcription is the most affected mechanism (Figure 3.4). This conclusion is particularly supported by the TLR7 mutant in which replacement of TTA codons with CTG lead to an increase in transcription and subsequent protein levels, but had no detectable effect on mRNA stability or translation of *in vitro* transcribed RNA. This conclusion is also supported by a recent study that attributed a direct role for GC content in the translocation of the RNA polymerase II during transcription (108). However, our study importantly reveals other mechanisms involved by scaling the level of codon optimization. While the fully optimized version of TLR7 displayed a dramatic increase in RNA and transcription rate, a more modest contribution of directly improving translation rate is evident by increased protein from *in vitro* transcribed RNA as well as a modest increase in mRNA stability. We cannot directly address from our results if this stability is inherent to the RNA or due to altering NGD, although replacing the leucines encoded by TTA, TTG, CTT, and CTA saw a modest increase in translation rate but no detectable difference in stability suggesting the two mechanisms may be separate. Importantly, the increase in translation rate and mRNA stability of fully optimized TLR7 can explain the increased ribosome density detected. Intriguingly, the phenotypes of fully optimized TLR7 are consistent with all the phenotypes of TLR9 in the same conditions, strongly suggesting all the mechanisms potentially involved in limiting TLR7's expression are also involved in increasing TLR9's expression. Altogether our results suggest that transcription of TLR7 and TLR9 may be affected by codon bias, although there is evidence of additional roles for stability and translation rate but further exploration is needed to tease apart the exact contributions in an endogenous setting.

Regulating TLR7 and TLR9 function is clearly important due to the number of mechanisms implicated in restricting signaling. Our study here adds to the mechanisms

potentially involved, as well as furthering the hypothesis that these two closely related receptors are differentially regulated. Additional exploration into *in vivo* consequences of altering this codon bias is still required, as well as exploration into other TLRs. Nevertheless, it is exciting to think about the potential ability to exploit the mechanism of codon bias in regulating TLR function.

Methods and Materials

Antibodies and Reagents

The following antibodies were used for immunoblots, flow cytometry, or ELISAs: anti-HA (3F10; Roche, Indianapolis, IN), anti-tubulin (DM1A; EMD Millipore, Billerica, MA), Alexa Fluor 680 goat anti-rat IgG H+L (Life Technologies, Grand Island, NY), IRDye 800CW goat anti-mouse (Li-Cor, Licoln, NE), anti-TNFα-APC (MP6-XT22; eBiosciences, San Diego, CA), Human IL-8 ELISA kit (BioLegend, San Diego, CA), anti-CD16/32 (2.4G2, UC San Francisco, CA). The following ligands were used for stimulation: CpG ODN 1668, R848 (InvivoGen, San Diego, CA), ssRNA40/LyoVec (InvivoGen),

qPCR Primers: 18s (CATTCGAACGTCTGCCCTAT/CCTGCTGCCTTCCTTGGA), Human GAPDH (AGAAGGCTGGGGCTCATTTG/AGGGGCCATCCACAGTCTTC), Mouse GAPDH (GAAGGTCGGTGTGAACGGA/GTTAGTGGGGTCTCGCTCCT), T-REx common (GTCAGATCGCCTGGAGACGCC/GGATCCGAGCTCGGTACCAAGC), Luciferase (GCGCGGTCGGTAAAGTTGTTCC/GACTTTCCGCCCTTCTTGGCC)

Guide RNA target sequences (PAM in bold): 5' TLR7-AGGTGTTTTCGATGTGGACA**CGG**, 3' TLR7- ACAAAAGCAGCTACTGGTAC**AGG**, tdTomato- ACGGAAGAGACAAGTCGACA**TGG**

TLR7 Constructs

Optimized versions of TLR7 were synthesized by Invitrogen's GeneArt services and subsequently subcloned into additional vectors. The fully optimized TLR7 sequence was generated by Invitrogen's GeneArt algorithm for optimized expression in mice. Other derivatives were generated in MacVector and submitted for synthesis.

Cell Culture Lines and Reagents

HEK293 and RAW264.7 cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA). The Flp-In T-REx 293 system was purchased from Life Technologies. All cell culture lines were cultured in DMEM supplemented with 10% (vol/vol) FCS, L-glutamine, penicillin-streptomycin, sodium pyruvate, and HEPES (pH 7.2) (Life Technologies). Cell culture reagents used: cycloheximide cell culture tested

(Sigma-Aldrich, St. Louis, MO), Actinomycin D (Sigma), Lipofectamine LTX (Life Technologies), Lipofectamine 2000 (Life Technologies).

Flp-In T-REx 293 Lines: C-terminally HA-tagged constructs were subcloned into pcDNA5/FRT/TO (Life Technologies) then co-transfected with pOG44 using Lipofectamine LTX. Following manufacturer's protocol for Hygromycin-B selection (Life Technologies), individual colonies were isolated and tested for protein expression following addition of doxycycline (Sigma).

RAW Cas9 Lines: *tdTomato* and *Tlr7* sequences lacking the ATG translation start codon were cloned into pUC19 (Life Technologies) flanked by homology arms derived from the genomic sequence adjacent to Exon 3 of *Tlr7* (741bp upstream, 845bp downstream). Guide RNAs were designed and synthesized as gBlocks as previously described (Mali et al Science 2013) then subcloned into pUC19. Humanized Cas9(D10A)-2xNLS-GFP was a gift from the Doudna lab. RAW264.7 cells were transfected using Lipofectamine LTX with equal amounts the guide RNA plasmid, Cas9 plasmid, and donor template plasmid. For the initial round looking for replacement of *Tlr7* with *tdTomato*, cells were sorted first for Cas9 expression by GFP fluorescence then for tdTomato fluorescence by FACS (Influx sorter, BD Biosciences, San Jose, CA). Clones were verified for proper integration by Southern blot analysis and loss of response to the TLR7 ligand R848. Following proper integration of *tdTomato*, the process was repeated with various *Tlr7* donor templates, with the exception that cells were sorted by FACS for loss of tdTomato fluorescence and gain of response to R848.

Polysome Profiling

Cell lines were lysed in buffer (20mM Tris, 150mM NaCl, 5mM $MgCl_2$, 1mM DTT, 100µg/ml cycloheximide, 1% Triton X-100, 25 U Turbo DNAse, 20 U SUPERase-In, pH7.4) and loaded onto 8-48% sucrose gradients composed of lysis buffer lacking Triton and DNAse and generated by mixing on a Gradient Station IP (BioComp Instruments, Fredericton, Canada). Samples were centrifuged at 36,000 RPM in an SW-41 rotor using an ultracentrifuge (L8-80M; Beckmann Coulter, Brea, CA). Fractions were collected every 12 seconds in 2.0ml Eppendorf tubes on a Gradient Station while monitoring UV absorbance (Econo UV Monitor; Bio-Rad, Hercules, CA), then spiked with 1ng Luciferase Control mRNA (Promega, Madison, WI). Total RNA was harvested using TRI Reagent LS and 1-Bromo-3-Chloropropane (Sigma), with the modification that following isopropanol addition samples were then passed over RNA Clean and Concentrator 5 columns (Zymo, Irvine, CA). For qPCR experiments, cDNA was generated using iScript Reverse Transcription Supermix (Bio-Rad) then analyzed using SsoAdvanced SYBR Green reagents (Bio-Rad) on a StepOnePlus Real-Time PCR system (Life Technologies), and quantitated using the $\Delta\Delta$Ct method of quantitation.

Cell Stimulation Assays

293 T-REx cells were treated with doxycycline overnight then stimulated with various ligands and supernatant collected after 18 hours then analyzed by ELISA for human IL-8 production.

RAW264.7 cells were stimulated with various ligands and analyzed for TNF production by intracellular cytokine staining. Briefly cells were stimulated, then after 30 minutes GolgiPlug was added and incubated for an additional 5 hours. Cells were harvested and stained using Cytofix/Cytoperm (BD), then analyzed on a LSR Fortessa X20 (BD) with further analysis performed on FlowJo (FlowJo, Ashland, OR).

Western Blot Analysis

Cell lysates were prepared with RIPA buffer (25 mM Tris [pH 8.0], 150 mM NaCl, 1% Triton X-100, 1% NaDeoxycholate, 0.1% SDS, 4 mM EDTA and supplemented with EDTA-free complete protease inhibitor cocktail; Roche). Cellular debris was pelleted by centrifugation at 4-degrees. 6X-SDS loading buffer was added to lysates and separated by SDS-PAGE. Protein was transferred to an Immobilon-FL membrane (Millipore), probed using the indicated antibodies, and fluorescence detected using an Odyssey CLx Infrared Imaging System (Li-Cor). All images were processed and quantitated using ImageJ software (NIH, Bethesda, MD).

RNA Analysis

For Northern analysis of T-REx lines, cell were treated overnight with doxycycline and the next day harvested for total RNA using RNAzol RT (Molecular Research Center, Cincinnati, OH). Total RNA was run on a NorthernMax denaturing gel (Life Technologies) and transferred with 20X SSC to a Hybond N+ membrane (GE Healthcare, Pittsburg, PA). A common T-REx probe was generated by PCR using the common T-REx qPCR primers to the 5' UTR then radiolabeled with using P^{32}-dCTP (Perkin Elmer, Waltham, MA) and the RadPrime DNA Labeling System (Life Technologies). The membrane was hybridized, washed, then exposed to a Phosphor Screen and detected with a Typhoon Imager (GE Healthcare). Images were processed and quantitated using ImageJ.

For 4sU labeling, T-REx lines were treated overnight with doxycycline, then media replaced with fresh media containing 0.5mM 4-thiouracil (Sigma) and doxycycline. After 30 minutes total RNA was harvested with RNAzol, labeled with EZ-Link biotin-HPDP (Thermo Scientific Pierce, Waltham, MA), captured with streptavidin magnetic beads (Thermo Scientific Pierce), and eluted with 100mM DTT. cDNA was generated using iScript Reverse Transcription Supermix (Bio-Rad) then analyzed using SsoAdvanced SYBR Green reagents (Bio-Rad) on a StepOnePlus Real-Time PCR system (Life Technologies), and quantitated using the $\Delta\Delta$Ct method of quantitation.

For Actinomycin-D RNA stability assays, T-REx lines were treated overnight with doxycycline, then 5µg/ml Actinomycin-D (Sigma) was added for the indicated time periods. Total RNA was harvested with RNAzol. cDNA was generated using iScript Reverse Transcription Supermix (Bio-Rad) then analyzed using SsoAdvanced SYBR Green reagents (Bio-Rad) on a StepOnePlus Real-Time PCR system (Life Technologies), and quantitated using the ΔΔCt method of quantitation.

Bioinformatics

Genome wide analysis of CAI was calculated using the CAI algorithm from the EMBOSS suite available at bioweb2.pasteur.fr/docs/EMBOSS/cai.html. The appropriate codon usage table for murine or human expression were selected.

Ribosome profile data was analyzed from the previously generated data set as previously described (85, 89).

***In vitro* Translation**

TLR constructs were cloned into pcDNA3 vectors containing a T7 promoter element. To generate RNA, constructs were first linearized and extracted with phenol-chloroform, then used in the mMessage mMachine T7 Ultra *in vitro* RNA tranascription kit (Life Technologies) following the manufacturer's protocol for generating polyadenylated transcripts. Synthesized RNA was then transfected into HEK293 cells using Lipofectamine 2000 then harvested the next day in RIPA buffer and analyzed by Western blot using the indicated antibodies. Images were processed and quantitated using ImageJ.

Southern Blot Analysis

Genomic DNA was harvested following Proteinase K digestion (Thermo Fisher) by isopropanol precipitation. DNA was digested overnight then run on a 1% agarose gel. Gels were pre-incubated in denaturation solution (1.5M NaCl, 0.5M NaOH) for 1 hour, then transferred to a Zeta-Probe GT membrane (Bio-Rad, Hercules, CA) overnight via capillary action. Blots were washed briefly with neutralization buffer (0.5M Tris-HCl, 1.5M NaCl), UV crosslinked with the auto setting on a UV Stralinker 2400 (Stratagene/Agilent, Santa Clara, CA), then placed in pre-hybridization solution (50% formamide, 5X SSCPE, 5X Denharts, 500µg/ml sheared salmon sperm DNA, 1% SDS) with rotation overnight at 42 degrees. DNA probes were generated by PCR then purified by gel extraction. Probes were radiolabeled with using P^{32}-dCTP (Perkin Elmer, Waltham, MA) and the RadPrime DNA Labeling System (Life Technologies) then added to the membranes in hybridization solution (50% formamide, 5X SSCPE, 1X Denharts, 100µg/ml sheared salmon sperm DNA, 20% dextran sulfate, 1% SDS). The membrane

was washed in 2X SSC three times, then exposed to a Phosphor Screen and detected with a Typhoon Imager (GE Healthcare). Images were processed and quantitated using ImageJ.

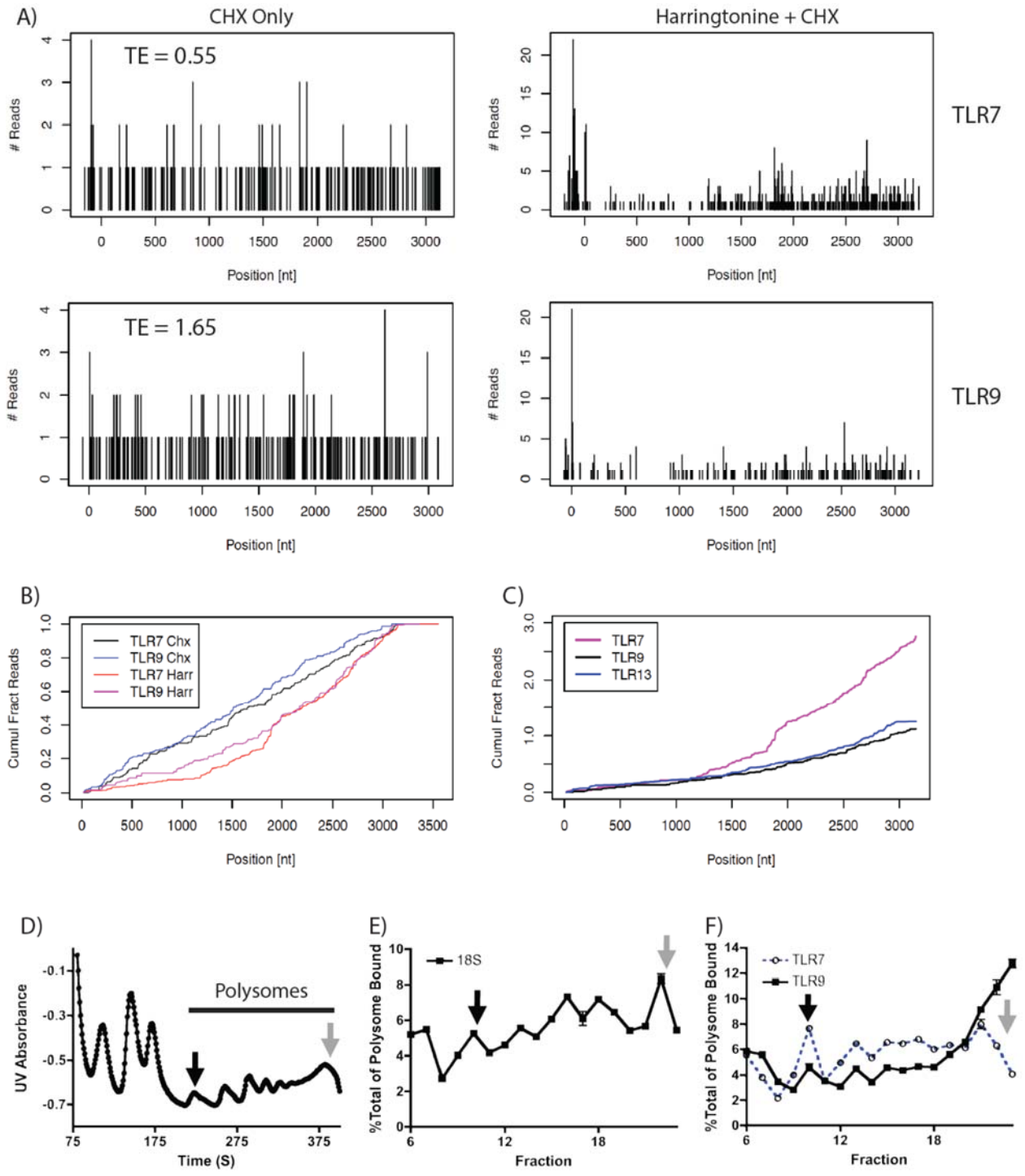
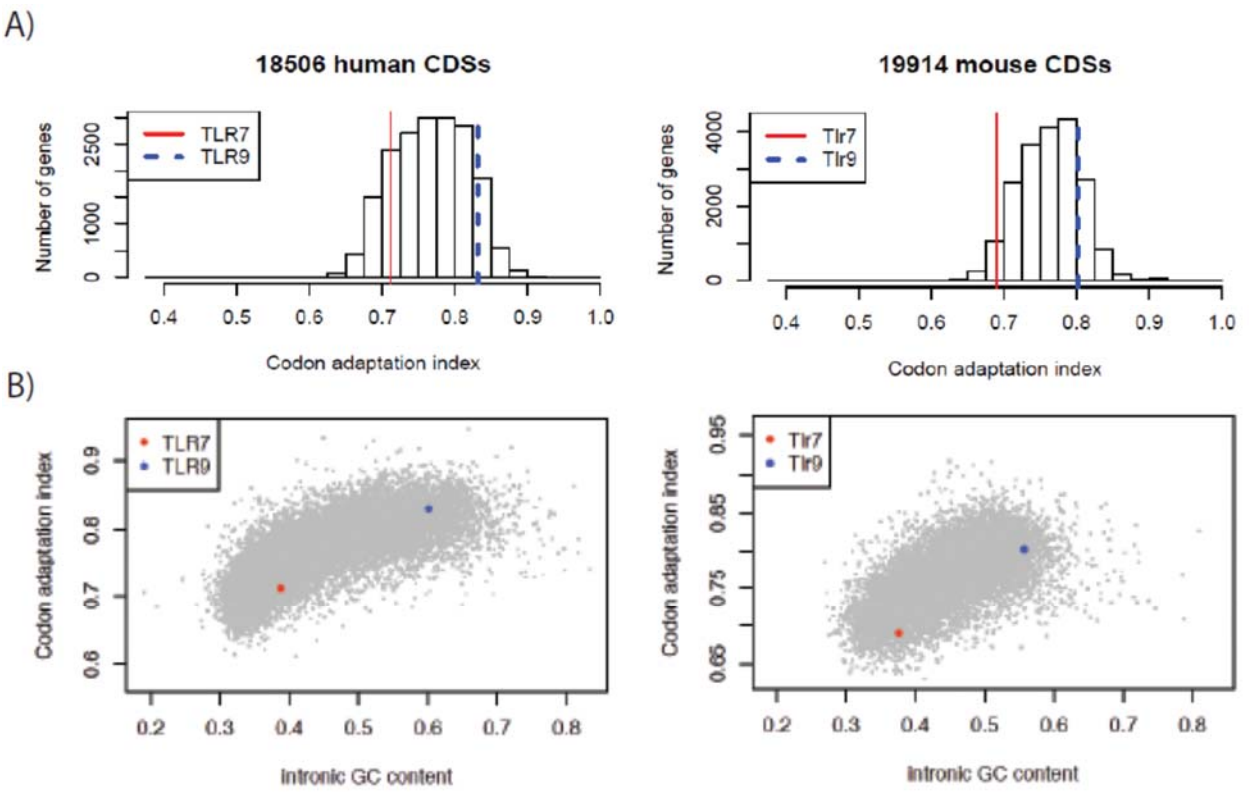

A)
CHX Only
Harringtonine + CHX
TE = 0.55
TE = 1.65
# Reads
Position [nt]
TLR7
TLR9
B)
Cumul Fract Reads
TLR7 Chx
TLR9 Chx
TLR7 Harr
TLR9 Harr
C)
TLR7
TLR9
TLR13
D)
UV Absorbance
Polysomes
Time (S)
E)
18S
%Total of Polysome Bound
Fraction
F)
TLR7
TLR9

Figure 3.1- TLR7 and TLR9 mRNAs are in distinct translational states

A) TLR7 and TLR9 have different translation efficiencies and behave differently after harringtonine treatment. Positional reads of ribosomes from BMDCs bound to either TLR7 (top) or TLR9 (bottom) transcripts, in cells either treated with cycloheximide (CHX) only to inhibit ribosome translocation (left) or pre-treated with harringtonine to inhibit ribosome initiation before CHX addition (right). TE values for the respective transcripts are noted. B) Quantitation of ribosome profiles in A represented as the cumulative fraction of reads summed in the 5' to 3' direction along the transcript normalized to each genes total ribosome counts. C) As in B, with the exception of normalizing TLR7 reads to TLR9. D) Polysome profile of RAW264.7 lysate. Following ultracentrifugation of sucrose gradients, polysomes were fractionated while monitoring UV absorbance. mRNAs containing few ribosomes are in the earlier fractions (black arrow), and mRNAs containing numerous ribosomes are in heavier fractions (grey arrow). Fractions were spiked with 1ng luciferase RNA for normalization, then harvested for total RNA and used for subsequent cDNA synthesis. E) qPCR of fractions accurately represents polysome profile. qPCR of ribosomal RNA (18S) in polysome containing fractions (6 to 23) normalized to luciferase and plotted as percent of total polysome 18S RNA. F) TLR7 and TLR9 have different profiles. qPCR of TLR7 and TLR9 in polysome containing fractions normalized to luciferase and plotted as percent of total respective mRNA bound by polysomes. Figures D-F are representative of two independent experiments.



C)

Percentile of CAI Relative to All Genes

	Mouse	Human
Tlr7	5th	17th
Tlr9	81st	88th

Percentile of CAI Relative to Genes Within +/-2.5% Intronic GC Content

	Mouse	Human
Tlr7	7th	15th
Tlr9	54th	76th

D)

Frequency of Leu Codons (%)

	TLR7	TLR9	Genome Avg
CTG	18.6%	56.0%	39.6%
TTA	14.4%	0.5%	7.5%
TTG	19.8%	7.3%	12.5%
CTT	18.6%	5.8%	13.0%
CTA	10.2%	8.4%	7.2%
CTC	18.6%	22.0%	20.0%

Total Leu Codons

	TLR7	TLR9
CTG	31	107
TTA	24	1
TTG	33	14
CTT	31	11
CTA	17	16
CTC	31	42
Total	167	191

Figure 3.2- TLR7 and TLR9 display distinct codon biases

A) TLR7 and TLR9 differ in their use of codons considered optimal. Histograms displaying the calculated codon adaptation index (CAI) for all coding sequences (CDS) in humans and mice, with TLR7 (red) and TLR9 (blue) highlighted. B) Codon optimization correlates with GC content. CAI is plotted against intronic GC content for all human and mouse coding sequences. C) TLR7 CAI is low even accounting for GC content. Overall percentile ranks of CAI for TLR7 and TLR9 in humans and mice (top), and percentile ranks within a 5% intronic GC content window (bottom). D) Frequency of codons encoding for the amino acid leucine differ between TLR7 and TLR9. Frequency (left) and absolute number (right) of leucine encoding codons for murine TLR7 and TLR9. The optimal codon CTG (green), the suboptimal codon TTA (red), and other leucine codons displaying a bias (grey) are highlighted.

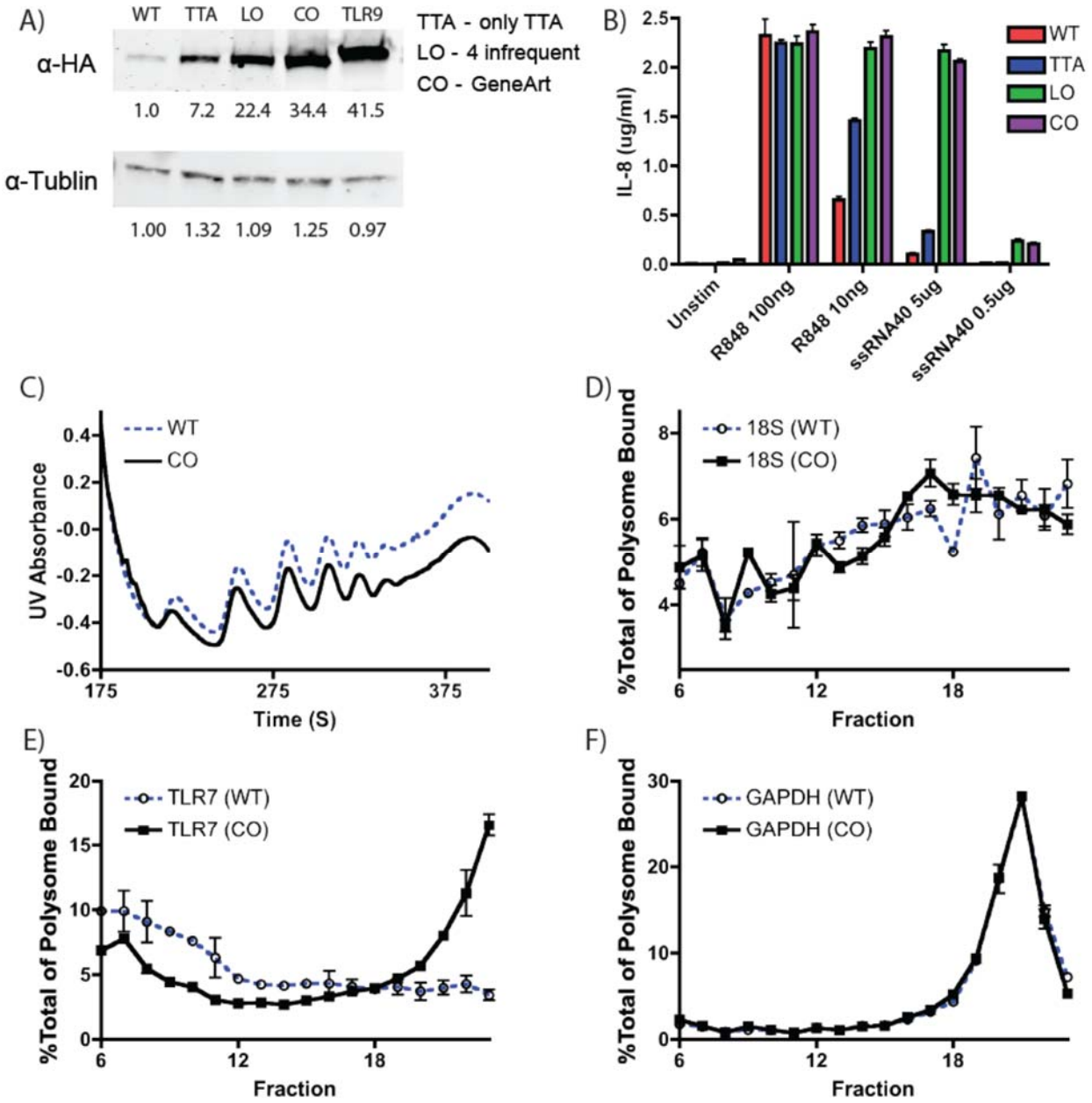


Figure 3.3- Codon bias alters protein levels and translation efficiency in a heterologous system

A) Increased protein expression after codon optimization. TLR7 coding sequences were synthesized replacing either all leucine TTA codons with CTG (TTA), all leucine TTA/TTG/CTT/CTA codons with CTG (LO), or optimized with Invitrogen's GeneArt algorithm (CO) were cloned into pcDNA5-FRT-TO and stably integrated into HEK293 Flp-In T-REx cells. Following 18 hours of doxycycline (Dox) treatment, cells were lysed and analyzed by SDS-PAGE Western blot with the indicated antibodies. B) Signaling in increased in optimized cells lines. T-REx lines were treated with Dox overnight then stimulated with the indicated ligands for 18hr and supernatants analyzed for human IL-8 production by ELISA. (C-F) Polysome profiles reveal increased ribosome density upon optimization. Lysates of T-REx lines were analyzed by sucrose gradient ultracentrifugation. (C) UV trace of polysomes for WT (blue-dashed) or CO (black-solid). RNA levels were assessed by qPCR for 18S rRNA (D), common 5' T-REx/TLR7 UTR (E), or GAPDH (F). Figure A is representative of at least 3 independent experiments, figure B is Mean+SEM of N=3, and figure C-F is Mean+SEM of N=2.

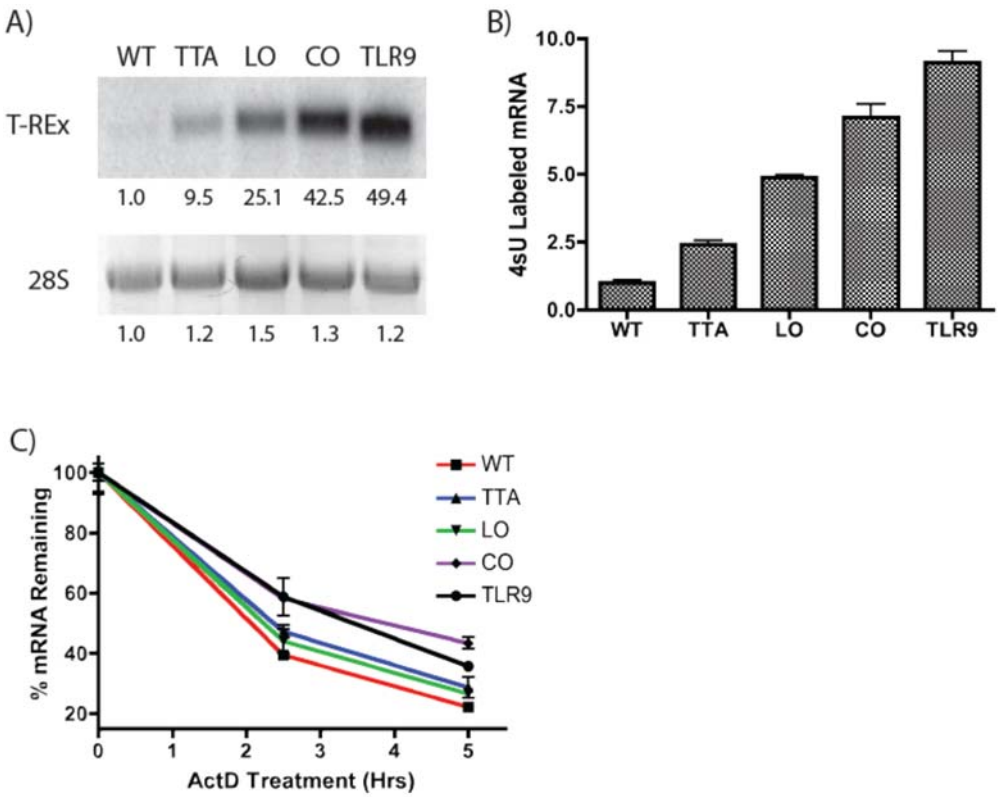


Figure 3.4- Codon bias affects mRNA levels by altering transcription in a heterologous system

A) mRNA levels differ in T-REx lines. Northern blot analysis of mRNA levels of T-REx lines treated with Dox overnight and probed with P^{32} labeled probe common to the 5' UTR of all mRNAs (top). 28S rRNA band after EtBr staining on a denaturing agarose gel (bottom). B) Rate of transcription differs in T-REx lines. Lines were treated with Dox overnight then pulsed with 4-thiouracil for 30 min, total RNA harvested, labeled with biotin, pulled down with streptavidin magnetic beads, and eluted RNA used for cDNA synthesis. Labeled mRNA levels were assessed by qPCR of common 5' T-REx UTR normalized to GAPDH. C) Minor stability differences in T-REx lines. T-REx lines were treated with Dox overnight and total RNA harvested after Actinomycin-D treatment for the indicated times (0, 2.5, 5 hours). mRNA levels were assessed by qPCR and normalized to 18S rRNA. Figure A is representative of at least 3 independent experiments, figure B and C are Mean+SEM N=2.

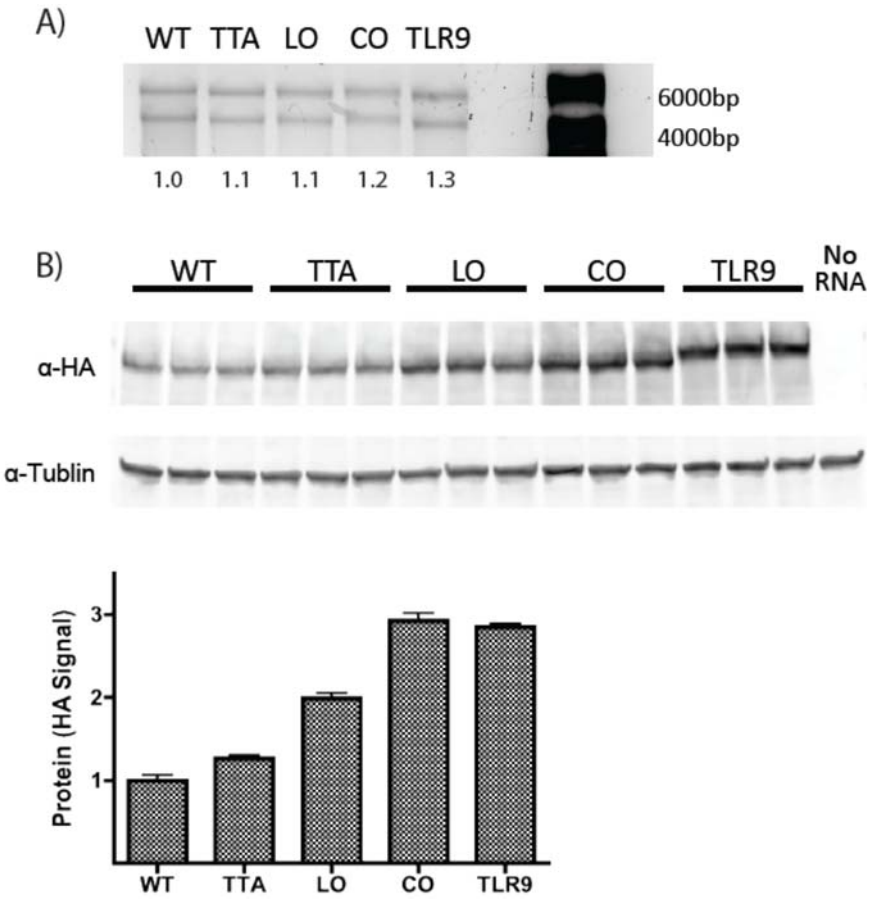


Figure 3.5- *In vitro* systems reveal modest contribution of rate of translation

A) Capped, polyadenylated mRNA was synthesized *in vitro* with T7 polymerase and products run on a denaturing agarose gel and stained with EtBr. B) Codon bias modestly alters translation. HEK293 cells were transfected with the indicated mRNA constructs and 18hrs later lysed and analyzed by SDS-PAGE Western blot with the indicated antibodies (above) and quantified (below). Figure B is Mean+SEM N=3 and representative of 3 independent experiments.

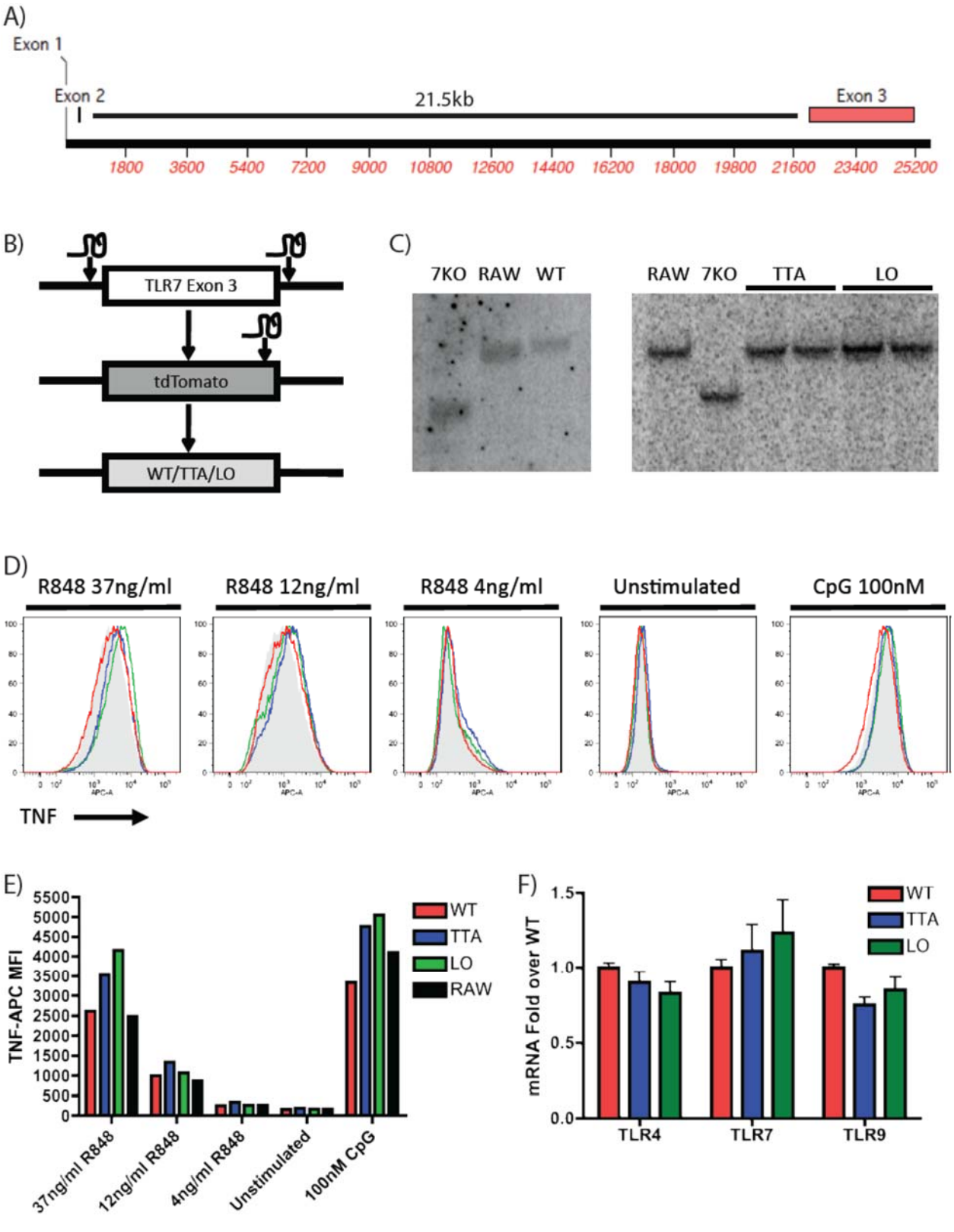

A)
Exon 1
Exon 2
21.5kb
Exon 3
1800 3600 5400 7200 9000 10800 12600 14400 16200 18000 19800 21600 23400 25200
B)
TLR7 Exon 3
tdTomato
WT/TTA/LO
C)
7KO RAW WT
RAW 7KO TTA LO
D)
R848 37ng/ml
R848 12ng/ml
R848 4ng/ml
Unstimulated
CpG 100nM
TNF
E)
TNF-APC MFI
5500 5000 4500 4000 3500 3000 2500 2000 1500 1000 500 0
WT
TTA
LO
RAW
37ng/ml R848
12ng/ml R848
4ng/ml R848
Unstimulated
100nM CpG
F)
mRNA Fold over WT
1.5 1.0 0.5 0.0
WT
TTA
LO
TLR4
TLR7
TLR9

Figure 3.6- Modest alterations of codon bias in endogenous TLR7 have no affect

A) To scale representation of the genomic locus of TLR7. Exons and the length of the intron between exons 2 and 3 are annotated. B) General scheme used to alter the endogenous locus of TLR7. Homologous recombination was facilitated by CRISPR-Cas9 targeting of regions flanking exon 3 of TLR7 with the D10A nickase variant of Cas9, and a donor template containing tdTomato was co-transfected for the first round of targeting. For the second round, tdTomato was targeted while co-transfecting donor templates containing the TLR7 variants. C) Proper targeting of the genomic locus was confirmed by Southern blot analysis. Genomic DNA was digested with NcoI and probed for a 5' region outside of the homology arms. The endogenous fragment is 6.2kb, while proper integration of tdTomato generates a 4.8kb band. Confirmation of the wild-type allele is in the left panel, while introduction of altered alleles is confirmed in the right panel. D) Modest TLR7 alterations do not increase TNFα production. RAW264.7 cell lines with altered codon biases were stimulated with the indicated concentrations of the TLR7 ligand R848 then assayed for TNFα production by intracellular cytokine staining and flow cytometry and displayed as histograms overlays of TNF-APC. E) Quantitation mean fluorescent intensity (MFI) in D. F) TLR7 mRNA expression levels are not significantly altered. Quantitation was performed using qPCR with normalization to GAPDH. Figure D and E are representative of 3 independent experiments. Figure F is Mean+SEM N=4

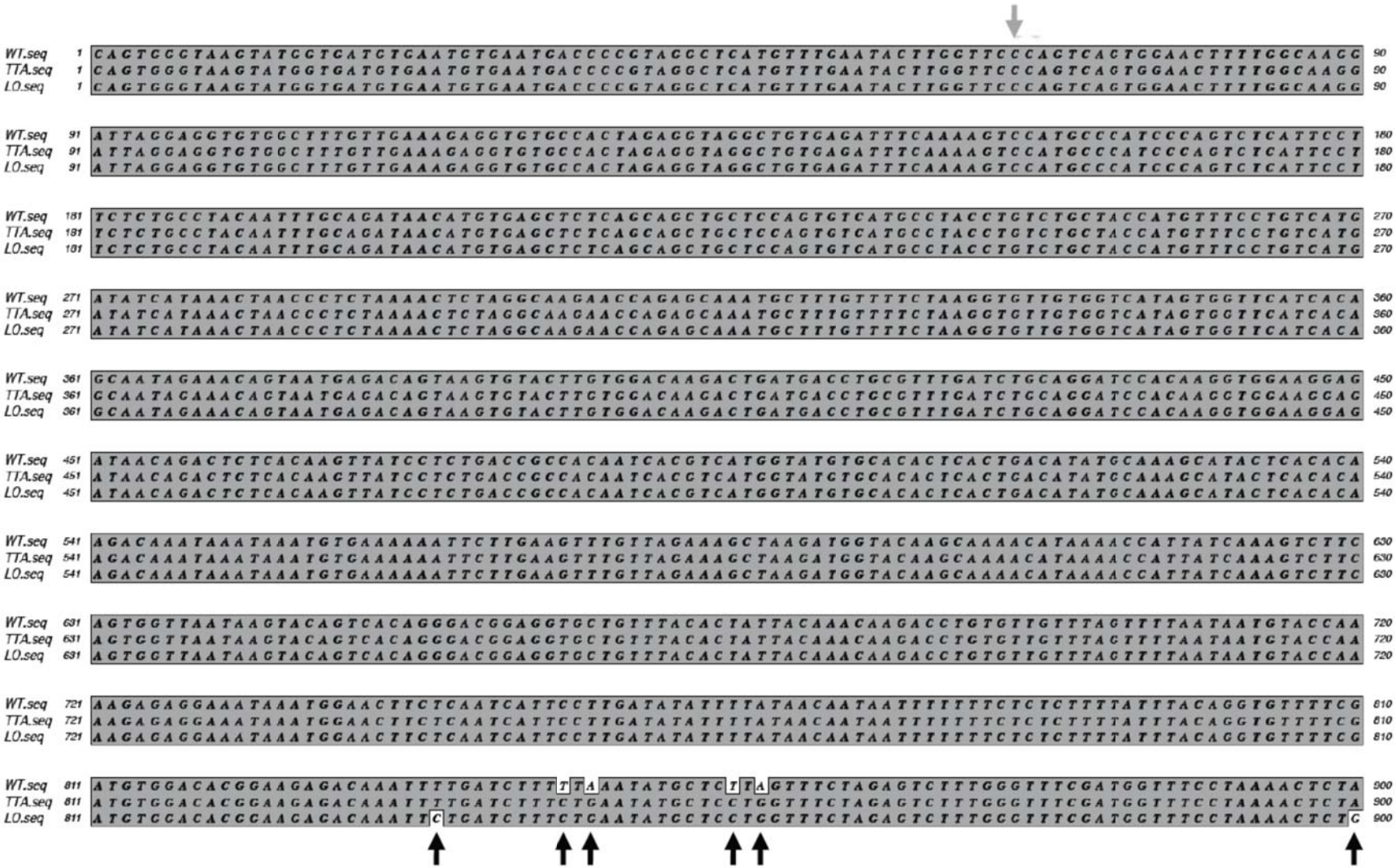

WT.seq 1 CAGTCCCTAACTATCCTCATCTCAATCTCAATCACCCCCTACCCTCATCTTTCAATACTTCCTTCCCACTCACTCGAACTTTTCCCAAGG 90
TTA.seq 1 CAGTGGGTAAGTATGGTGATGTGAATGTGAATGACCCCGTAGGCTCATGTTTGAATACTTGGTTCCCAGTCAGTGGAACTTTTGGCAAGG 90
LO.seq 1 CAGTGGGTAAGTATGGTGATGTGAATGTGAATGACCCCGTAGGCTCATGTTTGAATACTTGGTTCCCAGTCAGTGGAACTTTTGGCAAGG 90
WT.seq 91 ATTAGCAGGTCTGCCTTTCTTGAAAGAGGTCTGCCACTACAGGTAGGCTGTGAGATTTCAAAAGTCCATGCCCATCCCAGTCTCATTCCT 180
TTA.seq 91 ATTAGGAGGTGTGGCTTTGTTGAAAGAGGTGTGCCACTAGAGGTAGGCTGTGAGATTTCAAAAGTCCATGCCCATCCCAGTCTCATTCCT 180
LO.seq 91 ATTAGGAGGTGTGGCTTTGTTGAAAGAGGTGTGCCACTAGAGGTAGGCTGTGAGATTTCAAAAGTCCATGCCCATCCCAGTCTCATTCCT 180
WT.seq 181 TCTCTGCCTACAATTTGCAGATAACATGTGAGCTCTCAGCAGCTGCTCCAGTGTCATGCCTACCTGTCTGCTACCATGTTTCCTGTCATG 270
TTA.seq 181 TCTCTGCCTACAATTTGCAGATAACATGTGAGCTCTCAGCAGCTGCTCCAGTGTCATGCCTACCTGTCTGCTACCATGTTTCCTGTCATG 270
LO.seq 181 TCTCTGCCTACAATTTGCAGATAACATGTGAGCTCTCAGCAGCTGCTCCAGTGTCATGCCTACCTGTCTGCTACCATGTTTCCTGTCATG 270
WT.seq 271 ATATCATAAACTAACCCTCTAAAACTCTAGGCAAGAACCAGAGCAAATGCTTTGTTTTCTAAGGTGTTGTGGTCATAGTGGTTCATCACA 360
TTA.seq 271 ATATCATAAACTAACCCTCTAAAACTCTAGGCAAGAACCAGAGCAAATGCTTTGTTTTCTAAGGTGTTGTGGTCATAGTGGTTCATCACA 360
LO.seq 271 ATATCATAAACTAACCCTCTAAAACTCTAGGCAAGAACCAGAGCAAATGCTTTGTTTTCTAAGGTGTTGTGGTCATAGTGGTTCATCACA 360
WT.seq 361 GCAATAGAAACAGTAATGAGACAGTAAGTGTACTTGTGGACAAGACTGATGACCTGCGTTTGATCTGCAGGATCCACAAGGTGGAAGGAG 450
TTA.seq 361 GCAATACAAACACTAATCACACACTAACTGTACTTCTGCACAAGACTCATGACCTCCCTTTCATCTGCACGCATCCACAACCTCCAACCAG 450
LO.seq 361 GCAATAGAAACAGTAATGAGACAGTAAGTGTACTTGTGGACAAGACTGATGACCTGCGTTTGATCTGCAGGATCCACAAGGTGGAAGGAG 450
WT.seq 451 ATAACAGACTCTCACAAGTTATCCTCTGACCGCCACAATCACGTCATGGTATGTGCACACTCACTGACATATGCAAAGCATACTCACACA 540
TTA.seq 451 ATAACAGACTCTCACAAGTTATCCTCTGACCGCCACAATCACGTCATGGTATGTGCACACTCACTGACATATGCAAAGCATACTCACACA 540
LO.seq 451 ATAACAGACTCTCACAAGTTATCCTCTGACCGCCACAATCACGTCATGGTATGTGCACACTCACTGACATATGCAAAGCATACTCACACA 540
WT.seq 541 AGACAAATAAATAAATGTGAAAAAATTCTTGAAGTTTGTTAGAAAGCTAAGATGGTACAAGCAAAACATAAAACCATTATCAAAGTCTTC 630
TTA.seq 541 AGACAAATAAATAAATGTGAAAAAATTCTTGAAGTTTGTTAGAAAGCTAAGATGGTACAAGCAAAACATAAAACCATTATCAAAGTCTTC 630
LO.seq 541 AGACAAATAAATAAATGTGAAAAAATTCTTGAAGTTTGTTAGAAAGCTAAGATGGTACAAGCAAAACATAAAACCATTATCAAAGTCTTC 630
WT.seq 631 AGTGGTTAATAAGTACAGTCACAGGGACGGAGGTGCTGTTTACACTATTACAAACAAGACCTGTGTTGTTTAGTTTTAATAATGTACCAA 720
TTA.seq 631 AGTGGTTAATAAGTACAGTCACAGGGACGGAGGTGCTGTTTACACTATTACAAACAAGACCTGTGTTGTTTAGTTTTAATAATGTACCAA 720
LO.seq 631 AGTGGTTAATAAGTACAGTCACAGGGACGGAGGTGCTGTTTACACTATTACAAACAAGACCTGTGTTGTTTAGTTTTAATAATGTACCAA 720
WT.seq 721 AAGAGAGGAAATAAATGGAACTTCTCAATCATTCCTTGATATATTTTATAACAATAATTTTTTTCTCTCTTTTATTTACAGGTGTTTTCG 810
TTA.seq 721 AAGAGAGGAAATAAATGGAACTTCTCAATCATTCCTTGATATATTTTATAACAATAATTTTTTTCTCTCTTTTATTTACAGGTGTTTTCG 810
LO.seq 721 AAGAGAGGAAATAAATGGAACTTCTCAATCATTCCTTGATATATTTTATAACAATAATTTTTTTCTCTCTTTTATTTACAGGTGTTTTCG 810
WT.seq 811 ATGTGGACACGGAAGAGACAAATTTTGATCTTTTTAAATATGCTCTTAGTTTCTAGAGTCTTTGGGTTTCGATGGTTTCCTAAAACTCTA 900
TTA.seq 811 ATGTGGACACGGAAGAGACAAATTTTGATCTTTCTGAATATGCTCCTGGTTTCTAGAGTCTTTGGGTTTCGATGGTTTCCTAAAACTCTA 900
LO.seq 811 ATGTGCACACCGAAGAGACAAATTCTGATCTTTCTGAATATGCTCCTGGTTTCTAGAGTCTTTGCGTTTCGATGGTTTCCTAAAACTCTG 900

Figure 3.7- Sequencing confirmation of genomic locus for Cas9 targeted clones

Genomic DNA from the Cas9 targeted clones was isolated and the 5' region of TLR7's exon 3 was PCR amplified and sequenced by conventional methods. The genomic locus was still intact since no disruption of the genome beyond the start of the 5' targeting arm (grey arrow) was detected. Proper integration of the respective donor templates was also confirmed by detection of the expected polymorphisms (black arrows).

Chapter 4: The *In Vivo* Consequences of Improper TLR9 Signaling

Background

Toll-like receptors (TLRs) are a family of pattern recognition receptors (PRRs) that recognize features generally associated with pathogens. This recognition provides context to the immune system when it encounters specific antigens and directs the appropriate immune response needed to control the particular pathogen encountered. TLR9 is important in the recognition of viral and bacterial DNA. However, TLR9 is also capable of recognizing self-derived DNA, potentially leading to an inappropriate immune response and subsequent autoimmunity.

Signaling by TLR9 is normally restricted to endolysosomal compartments. This spatial restriction is proposed to maintain separation between TLR9 and potential self-ligands, preventing responses to self. Previous studies have circumvented this regulation by delivering self-derived nucleic acids to the TLR9 signaling compartment, generally through uptake mediated by interactions with other proteins such as antibodies, the antimicrobial peptide LL37, or the DNA-binding protein HMGB1 (32). In contrast, our lab has explored the consequences of directly disrupting the endolysosomal localization of TLR9. Retroviral bone-marrow chimeras with a TLR9 mutant capable of signaling from the surface develop fatal autoimmune disease (69). The induced disease was largely inflammatory due to the production of TNFα and presence of anemia. Additionally, depletion of CD11c+ conventional dendritic cells was able to rescue disease implicating their role as drivers of inflammation. However, the conditions present in the bone-marrow chimeras may not accurately represent the disease that would develop under more homeostatic conditions. For example, TLR signaling may be sensitive to the overall levels of receptor present, thus expression from a strong retroviral promoter may play an additional role in disease progression. To illustrate this point, in another autoimmune model where multiple copies of TLR7 are introduced, disease type and severity is affected by overall copy number (65). Additionally, the process of making bone-marrow chimeras involves whole body lethal irradiation of the mice, a process likely to promote cell death and release of self-DNA into the serum. Therefore, a model that investigates the consequences of misregulated localization of TLR9 at endogenous levels under homeostatic conditions may reveal dramatically differential phenotypes then those of a bone-marrow chimera model.

Systemic lupus erythematosus (SLE) is a multifactorial disease that involves multiple feed forward loops and thought to require self-nucleic acid sensing at multiple points within the loop (57). TLR9 is expressed in B-cells, plasmacytoid dentritic cells (pDCs), conventional dendritic cells (cDCs), macrophages, and potentially neutrophils in

mice (32, 109). Thus, an outstanding question in the field is the role of individual cell types. Of particular interest is what cell type(s) are responsible for the initial break in tolerance. By controlling the cell specific expression of the TLR9 mutant capable of signaling from the surface we can bias the cell type where the initial triggering event is likely to occur and monitor the outcome associated.

Results

Generating a Conditional TLR9-TM-Mut Mouse

In order to address the *in vivo* consequences and cell specific roles of misregulated TLR9 signaling, we generated a conditional TLR9 mutant mouse using a "Floxed-Stop" strategy. The mutation, which consists of swapping a portion of the transmembrane domain with that of TLR3, results in misregulated TLR9 signaling from the cell surface as previously described (69) (Figure 4.1A, 9TM-Mut). The "Stop Cassette" consists of a retroviral splice acceptor site, which prevents skipping of the stop cassette by alternative splicing, and two polyadenylation sites to terminate transcription (Figure 4.1B). Upon expression of Cre recombinase, the Stop-Cassette is removed allowing transcriptional read-through. To monitor that expression is properly regulated, a GFP translated from an internal ribosome entry site (IRES) was inserted into the C-terminal portion of TLR9's genomic locus.

Embryonic stem (ES) cells were targeted by conventional methods and screened for proper homologous recombination by Southern blot analysis (Figure 4.1C and D). An important consideration when screening ES cells for proper integration was that two foreign elements, the 5' Stop-Cassette and a 3' IRES-GFP, were simultaneously being introduced flanking the TLR9 exon. Therefore, there was the possibility that the TLR9 exon would act as a homology arm instead of the intended 5' and 3' homology arms and subsequently fail to introduce one of the two elements. This in fact was the case as the majority of the ES cells in the Southern analysis exhibited a banding pattern consistent with the 5' homology arm and the TLR9 exon mediating recombination (10.6kb vs expected 6kb 3' probe Figure 4.1D). Yet, despite this technical limitations, several properly targeted ES clones were generated (black arrows Figure 4.1D). A properly targeted founder was generated from the ES cells and subsequent progeny were shown to have inherited the allele (Figure 4.1E).

TLR9 is Properly Expressed upon Expression of Cre-Recombinase

To confirm that the expression of the conditional TLR9 mutant was properly regulated, we analyzed peripheral blood cells for expression of GFP. No fluorescent signal was detected in the original founder line in the absence of Cre recombinase, suggesting the Stop-Cassette was properly disrupting transcription of the locus (Figure 4.2A Parent). Importantly, when crossed to a ubiquitous Cre driver (E2A), GFP signal

was detected (Figure 4.2A E2A Cre). We also generated TLR9 mutant mice crossed to the tamoxifen inducible ERT2 Cre line. Expression of the mutant was confirmed to be inducible as GFP fluorescence was only seen following tamoxifen injection (Figure 4.2A comparing ERT2+Tamox to ERT2–Tamox). Further characterization also revealed that GFP expression was restricted to cell types previously characterized to express TLR9, such as B-cells (Fig 4.2B B220+), but not in immune cell not believed to express TLR9, such as T-cells (Figure 4.2B CD8+). Thus, we have successfully generated a conditional mouse line expressing the signaling mutant of TLR9.

TLR9 Mutant May Mediate Inflammation and Embryonic Lethality

We next looked to explore the functional consequences of expression of the mutated TLR9 construct. We first focused on requirement for proper regulation of TLR9 under homeostatic conditions, in other words in the absence of conditions known to induce release of endogenous ligands. Our initial results suggest that restriction of TLR9 signaling may be crucial early during development. Mice heterozygous for the mutant TLR9 allele were bred to ERT2 Cre mice or E2A Cre mice. Progeny of ERT2 mice inherited the mutant allele at Mendelian ratios (16/29 mice, Figure 4.2C). However, progeny of the heterozygous mutant crossed to the ubiquitous E2A Cre driver possessed the mutant allele in less than 5% of pups (2/42 mice), dramatically lower than the expected ratio of 50% (Figure 4.2C). Pups possessing the mutant allele expressed GFP. However, the frequency of GFP expression was lower than expected in cell types considered to universally express TLR9, such as B-cells. This may reflect not yet appreciated differences in expression within these populations, or possibly reflect the known mosaic effect of Cre mediated recombination in the F1 progeny of E2A Cre litters (110). Furthermore, due to the lower than expected GFP expression levels, these results suggest there may be a threshold level of mutant TLR9 expression to cause embryonic lethality. Nevertheless, proper regulation of TLR9 signaling appears to play a critical role during development.

Additional evidence for the importance of proper regulation of TLR9 under homeostatic conditions came from studies of mutant mice crossed to the inducible ERT2 Cre line. In this system, Cre mediated removal of the stop cassette was induced by administration of tamoxifen, allowing temporal control of mutant TLR9 expression. Importantly, this system allowed us to circumvent the apparent embryonic lethality and study the role of misregulated TLR9 in older mice. Littermates with and without the mutant TLR9 allele were injected with tamoxifen to induce Cre mediated recombination. Approximately a week after the final injection of tamoxifen, peripheral blood cells, bone marrow, and splenic cells were harvested and stained for various markers. Notably, B220+ B-cells from the blood and the spleen of the mouse containing the mutant allele displayed higher levels of the co-stimulatory molecule CD86, a marker of activation (Figure 4.2D). This surface phenotype suggests differential activation of B-cells occurs upon expression of misregulated TLR9. Interestingly, both GFP negative and GFP

positive B-cells appeared to upregulate activation markers, suggesting upregulation may not be due to a cell intrinsic effect of expressing the mutant TLR9, but instead may be due to a broad activation state promoted by expression of the mutant. It should be noted, though, that GFP positive cells appeared to express higher levels of CD86, suggesting there may be an additional cell intrinsic role for the mutant receptor in B-cells. Additionally, neutrophils in the bone marrow, despite no evidence of GFP or TLR9 intrinsic expression, appeared to upregulate MHC-II on their surface (Figure 4.2E). Although the functional significance of this upregulation is not known, this phenotype in the bone marrow suggests as possible systemic state of inflammation occurring. Together, our preliminary studies suggests a critical importance for regulating TLR9 function under homeostatic conditions, both during development and afterward. However, additional studies are needed to confirm this preliminary outcome of expressing a misregulated TLR9 receptor, to dissect the cell types responsible, and to further elucidate the nature of the disease.

Discussion

We have described here the generation of a mouse model to explore the functional significance of proper spatial regulation of TLR9 signaling. Using a mutant version of TLR9 previously establish to improperly localize to and signal from the cell surface, we can begin to answer outstanding questions in the literature concerning the role for improper activation of nucleic acid sensing TLRs. Our mouse model also allows controlled expression of the mutant TLR9 by using the conditional “Floxed-Stop” system. We have demonstrated that our system is well regulated, as GFP expression is only observed after Cre mediated excision of the stop cassette, as well as GFP expression limited to cell types known to express TLR9 (Figure 4.2). Our preliminary results also suggest that disrupting the spatial regulation of TLR9 may have functional consequences even under homeostatic conditions, particularly during development (Figure 4.2).

Misregulation of TLR9 appears to have a dramatic role during development, as mice derived from a cross to a ubiquitous Cre driver inherit the mutant allele of TLR9 at a 10-fold lower frequency than expected. It is interesting to speculate that this may be due to an abundance of free nucleic acid during the developmental process, although it raises the question of why TLR9 is expressed during development. Importantly, our system will allow us to dissect the role of specific cell lineages mediating this apparent lethality by crossing TLR9 mutant mice to lineage specific Cre drivers. It will also be interesting to elucidate the timing of this lethality during development, as this may better implicate the mechanism causing lethality, as well as shed light on the timing and role of TLR9 expression during development.

Our results, while preliminary, also suggests that misregulated TLR9 may lead to a broad activation state in older mice, even under homeostatic conditions. Compared to wild-type B-cells from a mouse injected with tamoxifen, B-cells from a mouse with

tamoxifen-induced expression of mutant TLR9 displayed upregulation of CD86. Intriguingly, both GFP positive and negative B-cells displayed upregulated markers. This suggests the potential for systemic activation of cells being mediated by expression of the mutant. Additionally, the upregulation of MHC-II by neutrophils in the bone marrow also provides evidence for systemic inflammation. It will be important in future studies to better characterize the nature of this activation state, particularly in determining if there are classic inflammatory cytokines present, and/or if there is type I IFN signature. These results, while demonstrating activation of B-cells, do not necessarily implicate B-cells themselves as the drivers of this activation. Instead, improper activation of macrophages of cDCs may lead to subsequent stimulation of B-cells. A more careful analysis will need to be performed using lineage specific Cre drivers to dissect the cell types responsible. Additionally, the functional outcomes may differ following crosses to separate lineage specific Cre lines. Thus, our system can also address open questions in the field concerning the importance of improper nucleic acid TLR signaling in specific cell types.

While our results suggest that the spatial regulation of TLR9 is important under homeostatic conditions, it will also be interesting to investigate the outcomes of misregulated TLR9 under non-homeostatic circumstances. Our results currently differ from the previous study investigating this mutant of TLR9 as mice expressing the mutant in our system do not rapidly succumb to disease, unlike the bone-marrow chimera model (69). Importantly, the bone-marrow chimera model implicated an inflammatory response dependent on cDCs in disease progression. Investigating various mechanisms believed to induce release of DNA ligands, such as radiation, infection (both viral and bacterial), and other forms of tissue damage, may elucidate whether or not the type and severity of disease is circumstance and cell type dependent. Thus, our system may help address circumstances that lead to disease progression, particularly towards a lupus like phenotype.

While still early on, our preliminary results suggest a critical importance for the spatial regulation of TLR9. Importantly, our results also demonstrate the generation of a model with the potential to address many still unanswered questions in the field of autoimmunity.

Materials and Methods

Flow Cytometry

The following antibodies were used for flow cytometry analysis and purchased from eBiosciences (San Diego, CA): anti-CD8-PerCP-Cy5.5 (53-6.7), anti-B220-AlexaFluor 700 (RA3-6B2), anti-CD86-PE (GL1), anti-MHC-II-APC-780 (M5/114.15.2), anti-CD11b-AlexaFluor 700 (M1/70), anti-Ly6C-PE (HK1.4), anti-Ly6G-PE-Cy7 (1A8). Anti-CD16/32 (2.4G2, UC San Francisco, CA) was used to block Fc receptor mediated binding.

Blood was collected into tubes containing heparan sulfate (24 U/ml; Sigma-Aldrich, St. Louis, MO) and lymphocytes were isolated using Lymphocyte Separation Medium (MP Biomedicals, Santa Ana, CA). Bone marrow was harvested by flushing femurs with a syringe followed by ACK lysing. Splenic cells were isolated by Collagenase 8 digestion and lysing red blood cells with ACK buffer (Life Technologies, Grand Island, NY). Cells were stained in FACS buffer (PBS, 3% FCS, 0.1% Sodium Azide, 1mM EDTA) then analyzed on a LSR Fortessa X20 (BD) with further analysis performed on FlowJo (FlowJo, Ashland, OR).

Southern Blot Analysis

Genomic DNA was harvested following Proteinase K digestion (Thermo Fisher) by isopropanol precipitation. DNA was digested overnight then run on a 1% agarose gel. Gels were pre-incubated in denaturation solution (1.5M NaCl, 0.5M NaOH) for 1 hour, then transferred to a Zeta-Probe GT membrane (Bio-Rad, Hercules, CA) overnight via capillary action. Blots were washed briefly with neutralization buffer (0.5M Tris-HCl, 1.5M NaCl), UV crosslinked with the auto setting on a UV Stralinker 2400 (Stratagene/Agilent, Santa Clara, CA), then placed in pre-hybridization solution (50% formamide, 5X SSCPE, 5X Denharts, 500µg/ml sheared salmon sperm DNA, 1% SDS) with rotation overnight at 42 degrees. DNA probes were generated by PCR then purified by gel extraction. Probes were radiolabeled with using P^{32}-dCTP (Perkin Elmer, Waltham, MA) and the RadPrime DNA Labeling System (Life Technologies) then added to the membranes in hybridization solution (50% formamide, 5X SSCPE, 1X Denharts, 100µg/ml sheared salmon sperm DNA, 20% dextran sulfate, 1% SDS). The membrane was washed in 2X SSC three times, then exposed to a Phosphor Screen and detected with a Typhoon Imager (GE Healthcare). Images were processed and quantitated using ImageJ.

Generation of TLR9-TM-Mut Mice

Targeting of mice was performed by the UC Davis Mouse Biology Program. Briefly JM8.N4 ES cells were electroporated with the donor construct containing the Floxed-Stop mutant described, an internal FRT flanked neomycin resistance cassette, flanking homology arms (5' 3.7kb, 3' 1.5kb), and a DTAA cassette external to the homology arms. ES clones were grown under selection with G418 and genomic DNA screened by Southern blot analysis for proper targeting then expanded for microinjection into Balb/C donors. Pups chimeric for coat color were set up for breeding and progeny were screened for germline transmission by PCR and confirmed by Southern blot analysis.

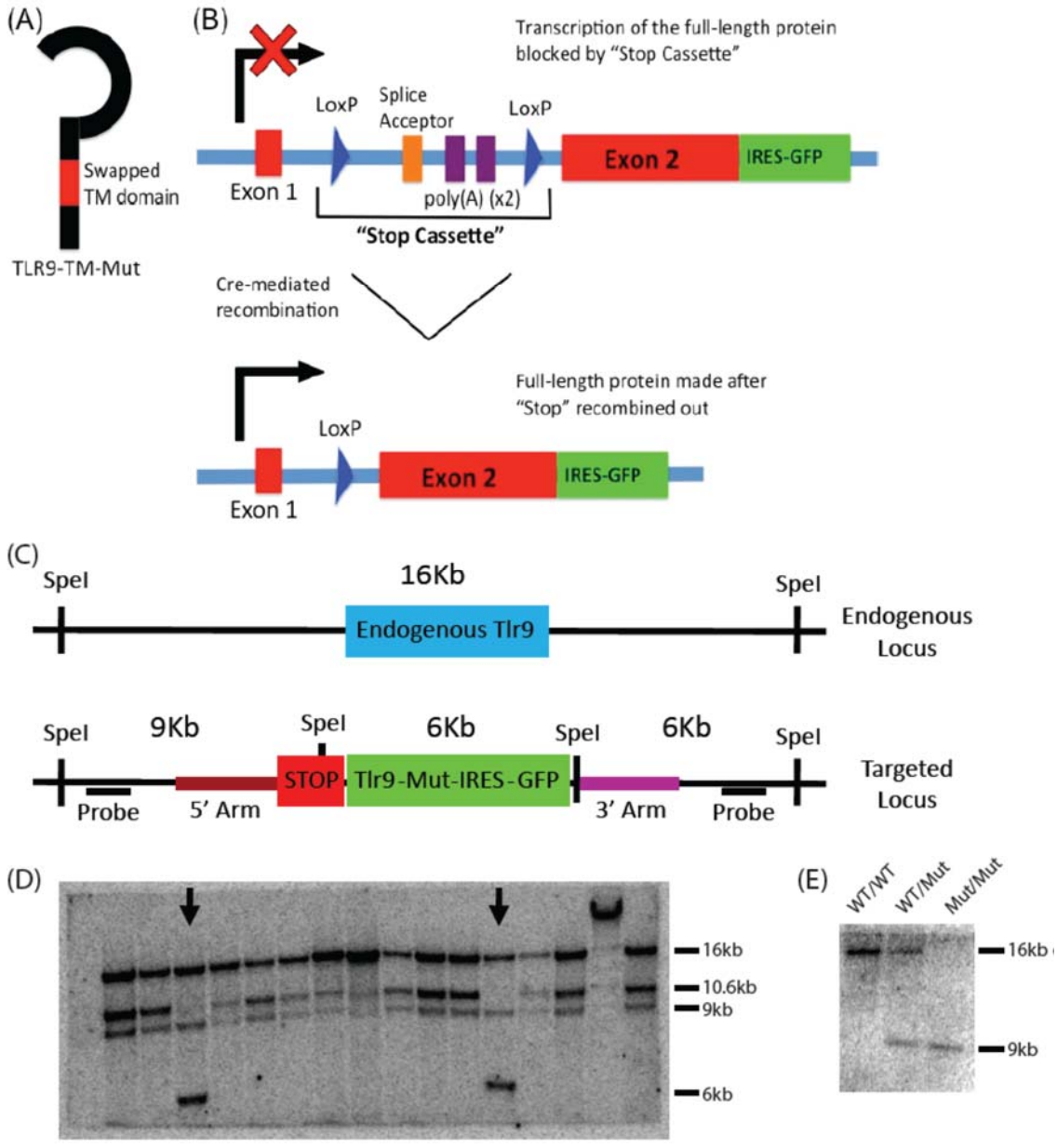
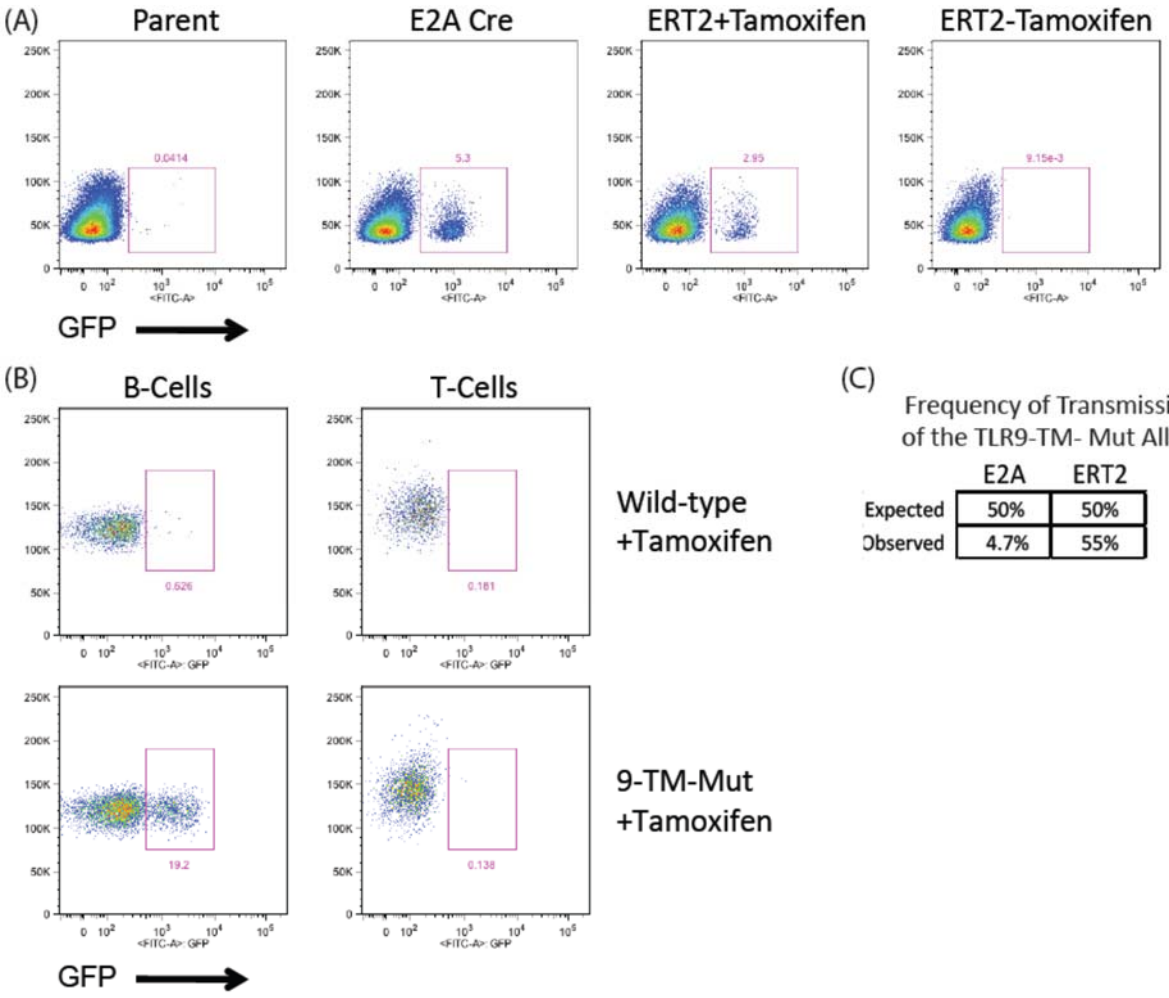

(A)
Swapped
TM domain
TLR9-TM-Mut
(B)
Transcription of the full-length protein
blocked by "Stop Cassette"
LoxP
Splice
Acceptor
LoxP
Exon 1
poly(A) (x2)
Exon 2
IRES-GFP
"Stop Cassette"
Cre-mediated
recombination
Full-length protein made after
"Stop" recombined out
LoxP
Exon 1
Exon 2
IRES-GFP
(C)
SpeI
16Kb
SpeI
Endogenous Tlr9
Endogenous
Locus
SpeI
9Kb
SpeI
6Kb
SpeI
6Kb
SpeI
STOP
Tlr9-Mut-IRES-GFP
Probe
5' Arm
3' Arm
Probe
Targeted
Locus
(D)
16kb
10.6kb
9kb
6kb
(E)
WT/WT
WT/Mut
Mut/Mut
16kb
9kb

Figure 4.1- General schematic and strategy of the TLR9-TM-Mut conditional system

A) Depiction of the TLR9 mutant (TLR9-TM-Mut). Briefly, a region within the transmembrane domain of TLR9 was replaced with that of TLR3's resulting in mislocalization and a gain in function signaling at the cell surface. B) Schematic of the "Floxed-Stop" model for controlling expression of the TLR9 mutant. Labeled are the exons of TLR9, the "Stop-Cassette" that contains are retroviral splice acceptor and two poly-adenylation signals to terminate transcription, LoxP recombination sites which mediated excision of the stop cassette following expression of Cre recombinase, and an IRES-GFP to monitor proper regulation of the system. C) Targeting strategy of the endogenous TLR9 locus. D) ES were properly targeted. Genomic DNA was harvested from ES cell clones and proper integration was confirmed by Southern blot analysis. DNA was digested with SpeI and probed for regions outside of both the 5' and 3' homology arms. The endogenous locus generates a 16kb fragment, while proper integration of the targeting construct generates a 9kb and 6kb band (black arrows). E) Confirmation of germline transmission of the mutant allele by Southern blot analysis. Genomic DNA was isolated from ear punches of mice negative, heterozygous, or homozygous for the mutant construct and analyzed as in D using the probe outside the 5' arm.



(C)

Frequency of Transmission of the TLR9-TM- Mut Allele

	E2A	ERT2
Expected	50%	50%
Observed	4.7%	55%

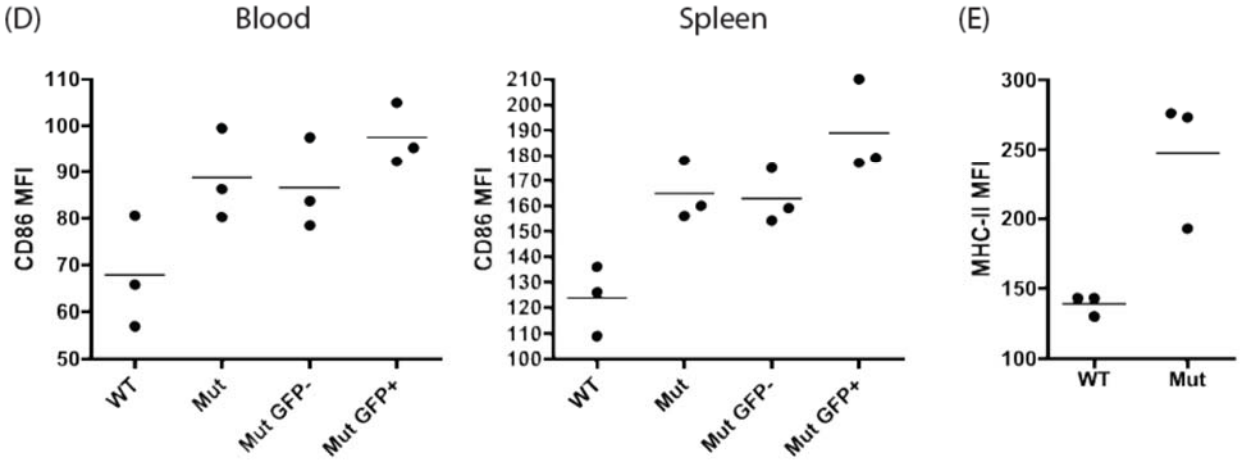


Figure 4.2- Expression of the TLR9 mutant is well regulated and may have functional consequences upon expression

A) Expression of the TLR9 mutant allele is only present following Cre mediated excision of the stop cassette. Mice heterozygous for the TLR9-TM-Mut allele were crossed to both the ubiquitous E2A and tamoxifen inducible ERT2 Cre lines. Splenocytes were harvested and analyzed for GFP signal by flow cytometry for the parental line (ie no Cre present), following a cross to the E2A Cre line, and following a cross to the ERT2 Cre line and either administered with tamoxifen or not. Percentage of GFP expressing cells in the gated population is displayed. B) TLR9 expression is limited to proper cell types. Littermates either containing the mutant allele or only the wild-type allele were injected with tamoxifen and their splenocytes analyzed for GFP signal. Only B-cells (gated on B220+) containing TLR9-TM-Mut had a detectable GFP signal, and not T-cells (gated on CD8+). C) Frequency of progeny containing the mutant allele of TLR9 following a cross between a heterozygous founder and either E2A or ERT2 Cre lines. D) B-cells from a mouse expressing the TLR9 mutant display signs of activation. Cells from peripheral blood (left panel) and spleens (right panel) were harvested from ERT2 Cre crossed littermates approximately a week after the final tamoxifen injection. Staining was performed and analysis gated of B220+ cells. Displayed are geometric means of fluorescent intensity of CD86 from wild-type, total mutant, mutant GFP- and mutant GFP+ cells. E) Neutrophils display signs of systemic activation. Bone marrow was collected from wild-type and mutant mice. Displayed is the geometric mean of MHC-II gated on CD11b+/Ly6C+/Ly6G+ neutrophils. N=42 for E2A and 29 for ERT2 in figure C, and results in D and E are N=3.

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