Cell-intrinsic TLR7 signaling drives Treg-mediated tissue repair

By

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Abstract

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The Toll-like receptors (TLRs) are a family of pattern recognition receptors with a well-established role promoting activation and cytokine production by innate immune cells. Expression of TLRs on T cells is controversial, with conflicting reports of various functional outcomes of T-cell intrinsic TLR signaling. Using a series of novel TLR reporter mice I have identified TLR7, a sensor of single-stranded RNA, as the only TLR expressed at detectable levels on T cells.

This dissertation seeks to characterize the role of TLR7 signaling on T cells. Stimulation of $CD8^+$ T cells with TLR7 ligand boosts TCR activation-induced proliferation and effector cytokine production. This result suggests an alternative means of co-stimulation that could be beneficial during immune responses to viral infections. While this finding provides needed clarity to the field, it matches previously proposed theories for the role of TLR signaling on T cells, prompting me to focus instead on the role of TLR7 on $Foxp3^+$ regulatory T cells (Tregs).

The pattern of TLR7 expression on Tregs suggested a critical functional role. Returning to the TLR7 reporter mice, I demonstrated that TLR7 is expressed by nearly all Tregs upon exit from the thymus. Tregs in the lymphoid tissues are bimodal for TLR7 expression, while those in non-lymphoid tissues tend to downregulate TLR7. Intriguingly, expression of TLR7 on Tregs is anticorrelated with antigen experience, implying that this receptor might be most critical on naïve Tregs.

The functional impact of TLR7 signaling on Tregs was novel and unexpected. Tregs in non-lymphoid tissues are capable of promoting repair following injury or infection, a phenotype characterized by production of the EGF family member amphiregulin. The stimuli that cause Tregs to upregulate amphiregulin and accumulate in damaged tissue

are not well understood. Using a series of *in vitro* experiments, I established that Treg-intrinsic TLR7 signaling has no impact on suppressive capacity, but instead results in proliferation and elevated production of amphiregulin. These results suggest that TLR7 signaling primes Tregs for tissue repair.

To test the role of TLR7 on Tregs *in vivo*, I generated mice with a Treg-specific deletion of TLR7. When challenged with influenza, an ssRNA virus capable of stimulating TLR7, these mice exhibit severe disease and impaired lung function. Single-cell RNA sequencing of Tregs from influenza experiments indicate that TLR7 is important for maintenance of a population of lung-resident Tregs with that express elevated levels of amphiregulin. This data demonstrates that TLR7 signaling can directly enhance the tissue repair capacity of Tregs, a critical aspect of the host response to respiratory infections. Additionally, the ability of TLR7 to recognize self-RNA in certain contexts led me to test its role on Tregs in non-viral lung damage models. I again observed impaired lung function in mice with the Treg-specific deletion of TLR7. This result suggests a mechanism that may enable Tregs to sense and repair tissue damage more generally.

My exciting findings about the role of TLR7 on Tregs are applicable to humans as well. The final chapter of this dissertation shows that TLR7/8 stimulation of human Tregs drives proliferation and amphiregulin production, the first known method for driving human Tregs to adopt a phenotype consistent with tissue repair. This result opens the door to a new type of cell-based Treg therapy, which may be used to treat tissue damage induced by, for example, severe viral infection.

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Acknowledgments

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Mom, Dad, and Stephanie, thank you for the love that was present as early as I can remember and continues to this day. Mom and Dad, the examples you set for me, both personal and professional, have been a guiding light during every challenge that I have ever faced. My goal as a parent is to do as good a job with the boys as you did for me.

To Kristin and the boys, Philip, Lucas, and Patrick, it is difficult to put into words the gratitude I feel for having you as part of my life. Failed experiments are so much easier to take when you come home to pure joy and unconditional love. Kristin, my return to graduate school and this dissertation literally do not happen without your support. I will forever be grateful to you for encouraging me to take this dramatic change of course, despite my doubts about the financial impact, because you knew it would make me happier. To me, that is the definition of love.

Chapter 1: Introduction[1]

The Toll-like receptors (TLRs) are a family of innate immune receptors whose activation is critical for the induction of innate and adaptive immunity. TLRs recognize conserved microbial features shared by broad pathogen classes, which enables a limited set of receptors to recognize the tremendous diversity of microbes potentially encountered by the host. The TLRs can be grouped into two categories: 1) those that localize to the cell surface and recognize structures associated with bacteria, and 2) those that localize to the endosome and respond to nucleic acids. The first category includes TLRs 2, 4 and 5 which are activated by bacterial lipoproteins, lipopolysaccharide, and flagellin respectively. The second category of TLRs each recognize different nucleic acid sequences: TLR3 recognizes double-stranded RNA; TLR7, TLR8, and TLR13 recognize fragments of single-stranded RNA with distinct sequence preferences; and TLR9 recognizes single-stranded DNA containing unmethylated CpG motifs. These nucleic acid-sensing TLRs are particularly relevant for detection of viruses because viruses generally lack other common, invariant features suitable for innate immune recognition.

Targeting nucleic acids (hereinafter abbreviated as "NAs") greatly expands the breadth of microbes that can be recognized but comes with the tradeoff of potentially sensing self-NAs. Indeed, improper activation of NA-sensing TLRs by self-NAs leads to autoinflammation and/or autoimmunity.[1-5] One possible strategy for limiting such adverse outcomes is recognition of specific features that distinguish self-NAs from foreign NAs. However, while ligand preferences based on sequence or chemical modifications can somewhat limit responses to self-NAs, discrimination between foreign and self-NAs is largely independent of these differences. Instead, NA-sensing TLRs depend on mechanisms that 1) reduce the likelihood that they will encounter self-NAs and/or 2) dampen the response when self-NAs are nevertheless detected. These mechanisms collectively set a precisely tuned threshold for receptor activation: too low a threshold will result in sensing of self-NAs and autoimmunity, while too high a threshold will hinder defense against the very pathogens that the NA-sensing TLRs aim to detect.

The mechanisms which regulate NA-sensing TLRs can be classified into four categories: compartmentalization, ligand availability, receptor expression, and signal transduction. Detailed discussion of each of these categories is beyond the scope of this dissertation and are covered in a review recently submitted for publication. Here, the focus will be on the activation and regulation of TLR7 which, as will be

[1] Pages 1 and 2 of this Introduction have been partially adapted from "Regulation of nucleic acid-sensing Toll-like receptors," a review article that I co-authored with Victoria Rael, Dr. Kathleen Pestal, Dr. Bo Liu, and Professor Greg Barton. At the time of writing, this article has been submitted but not yet published. Thank you to my co-authors for granting permission to use this material in my dissertation.

demonstrated below, is the only TLR expressed on T cells. TLR7 and its closely related neighbor on the X chromosome, TLR8, are at first glance strange pattern recognition receptors because they are capable of sensing extremely small RNA fragments that have no “pathogenic” qualities whatsoever.[6, 7] For TLR7, a single guanosine and a trimer of uracil have proven sufficient to trigger dimerization and signaling.[7]

The minimalist sequence requirement for TLR7 signaling greatly raises the chances of autoimmunity because such small ssRNA fragments are not unique to viruses, and are certainly present in the host in the absence of infection. There are several examples of breakdowns in TLR7 regulation leading directly to autoimmunity. In mice and humans, increasing gene dosage of TLR7 can lead to immune pathology.[2-4, 8-13] This finding stands in stark contrast to studies of overexpression of TLR9 in mice, where there is no major pathology observed even with two additional Tlr9 transgene copies expressed in every cell.[14] There also seems to be a requirement for excess TLR7 ligands to be continuously pumped out of the endosomal lumen and into the cytosol. One clear example of this principle comes from recent analysis of mice lacking SLC29a3, a member of the solute carrier family that functions to maintain nucleoside homeostasis. These mice accumulate endosomal nucleosides and develop disease with many of the hallmarks observed in other models of TLR7 dysregulation.[15] Disruptions in TLR7 signaling is also sufficient to drive autoimmunity, as demonstrated by previous studies from our laboratory on the NA-sensing TLR chaperone protein Unc93b1. Upon TLR7 stimulation, Unc93b1 recruits Syntenin-1 to the Unc93b1-TLR7 complex, leading to the sorting of the complex into multivesicular bodies, and ultimately terminating signaling.[16] Mutations in Unc93b1 that prevent binding of Syntenin-1 induce TLR7 hyperresponsiveness and severe TLR7-dependent autoimmune disease in mice.

Why has TLR7 been maintained over the course of evolution if it has the inherent potential to cause significant harm to the host? One possibility is straightforward, that TLR7 facilitates beneficial immunity to pathogens and is therefore a net positive for the host. While this hypothesis may prove true, studies assessing the response of $TLR7^{KO}$ mice to ssRNA viruses have only revealed mild to moderate phenotypes.[17, 18] Despite clear TLR7-mediated production of inflammatory cytokines by innate immune cells in the presence of viral ssRNA, $TLR7^{KO}$ mice are capable of combating ssRNA viruses. Another, arguably more intriguing, possibility is that the promiscuous nature of TLR7 pattern recognition is actually beneficial in some circumstances. In other words, there may be physiological scenarios where particular immune cells need to respond in the presence of ssRNA ligands, whether derived from pathogens or the host. Tissue damage and inflammation, for example, might result in elevated levels of nucleic acids released by dying cells. These nucleic acids could be used as a “damage signal” for regulatory immune cells that are necessary to control inflammation and initiate repair. Such a theory was previously relegated to the realm of pure speculation, but the

findings contained in this dissertation suggest that regulatory T cells may use TLR7 in precisely this manner.

A) TLRs and T cells

The TLRs have traditionally been viewed as pattern recognition receptors for innate immune cells, facilitating activation in the presence of microbial threats and indirectly leading to the initiation of an adaptive immune response. Multiple studies have demonstrated the validity of this principle, and it is now well-accepted that TLR signaling on innate immune cells can drive T cell responses through at least two mechanisms. First, cell-intrinsic TLR signaling on antigen-presenting cells can cause maturation characterized by enhanced antigen presentation and elevated expression of costimulatory molecules. Mice deficient in MyD88, the signaling adaptor downstream of most TLRs, exhibit reduced dendritic cell activation and antigen-specific $CD4^+$ T cell priming following immunization.[19] The contribution of TLRs to $CD8^+$ T cell activation is more nuanced and depends on which TLR is stimulated. All TLRs can induce dendritic cell maturation and subsequent $CD8^+$ T cell activation *in vitro*, but the NA-sensing TLRs 3 and 9 appear better equipped than TLRs 2 and 4 to support $CD8^+$ T cell responses *in vivo*.[20, 21] Second, TLR stimulation of innate immune cells can drive T cell responses indirectly by reducing the suppressive capacity of regulatory T cells. Dendritic cells that receive signaling via either TLR4 or TLR9 release IL-6, a cytokine that can block the suppressive effect of Tregs.[22] This cytokine-mediated reduction in Treg function is necessary to properly prime antigen-specific naive T cells.[23] These findings have recently been applied to cancer immunotherapy, and it has been shown that TLR1/2 stimulation enhances intratumoral Treg depletion when combined with CTLA-4 blockade.[23]

The existence and outcome of T cell-intrinsic TLR signaling is far more controversial. Direct detection of microbial patterns by T cells is an appealing concept, perhaps providing an activating signal that could synergize with T cell receptor signaling. On the other hand, direct activation of T cells via TLRs would seemingly obviate the need for costimulation by antigen presenting cells, elevating the risk of T cell responses against self-peptides. There is also a question as to whether it would even be useful for naive T cells to express TLRs given that they generally reside in lymphoid tissues, whereas microbes tend to be confined to barrier tissues. Tissue-resident T cells might therefore stand to benefit the most from expression of TLRs, allowing them to quickly respond in the event of a breach. These and other ruminations on the propriety of TLR expression by T cells has led to a significant number of publications on the subject over the past twenty years.

Publications on T cell-intrinsic TLR signaling, unfortunately, have not provided conclusive answers. Some studies have claimed that T cells express all of the TLRs,

while some assert that only specific TLRs are expressed. The lack of reliable antibodies to individual TLRs has likely contributed to these discrepancies, forcing researchers to rely on quantitative PCR of purified T cell populations, wherein even slight contamination by innate immune cells that express high levels of TLRs can lead to confounding results. A massive range of functional outcomes for T cell-intrinsic TLR signaling have also been proposed, from predictable claims of costimulatory effect to counterintuitive findings that TLR signaling can actually restrain immune activation. Some of these results are likely explained by overreliance on poorly controlled *in vitro* systems, where contaminating non-T cells respond to TLR stimulation by releasing inflammatory cytokines that skew T cell responses. Other findings on the topic may be valid, but failed to gain general acceptance due to the sheer amount of contradictory data in the literature. To provide context for the conclusions contained in this dissertation, the initial goal of which was to conclusively establish which TLRs are expressed by T cells, it is important to carefully consider these previous studies.

1) T cell-intrinsic TLR signaling promotes activation

The function most commonly attributed to T cell-intrinsic TLR signaling is enhanced activation, usually described as a costimulatory or adjuvant-like effect. An early study of TLR function on activated $CD4^+$ T cells concluded that TLRs 3 and 9 are expressed and promote T cell survival but not proliferation *in vitro*.[24] Expression of the TLRs was assessed by RT-PCR and the authors attempted to control for APC contamination by also assaying for MHC-II. This control is helpful, but does not rule out contamination by non-T cells that do not express MHC II. It is worth noting that the authors also detected TLR5 on both naive and activated $CD4^+$ T cells, and TLR4 on naive but not activated $CD4^+$ T cells. Stimulation of the T cells with TLR2 or TLR4 ligands did not yield a discernible effect. From these results, the authors concluded that TLRs 3 and 9 can promote survival of activated $CD4^+$ T cells. A later study also came to the conclusion that TLR9 stimulation on $CD4^+$ T cells could act as a costimulatory signal, but it also suffered from a lack of controls to rule out contributions by other immune cells.[25]

Two studies of human $CD4^+$ T cells described a similar outcome of T cell-intrinsic TLR signaling, but differed on which TLRs are capable of producing that effect. The first found expression of TLRs 2 and 4 on $CD4^+$ T cells by RT-PCR, with TLR2 increasing following stimulation with anti-CD3 and IFN-α.[26] There was no innate cell control provided for the RT-PCR, and flow cytometry showed an extremely small population of positive cells using anti-TLR2 and anti-TLR compared to an isotype control. TLR4 stimulation had no effect, but TLR2 increased IFN-ɣ production by $CD4^+$ T cells when used in combination with anti-CD3. The second study, also examining human $CD4^+$ T cells, used RT-PCR to demonstrate expression of TLRs 1-5, 7 and 9.[27] The levels of

TLR mRNA varied between different donors, but receptors expressed were consistent. Ligands for TLRs 2, 5 and 7 increased proliferation and levels of IFN-ɣ produced by the $CD4^+$ T cells *in vitro*, while TLR4 ligand had no discernible effect.

TLR2 has also been described in multiple publications to have a stimulatory effect for mouse T cells. One study found that TLRs 2, 7 and 9 mRNA are expressed by $CD8^+$ T cells, albeit at levels much lower than B cells or macrophages.[28] Only TLR2 was thoroughly tested, and the authors found that it boosted proliferation and survival of $CD8^+$ T cells when combined with anti-CD3 *in vitro.* A major caveat of this study is that the primary comparison was between T cells isolated from a WT mouse with T cells isolated from a $TLR2^{KO}$ mouse in separate cultures. The results could therefore be confounded by contaminating innate immune cells, which would only be able to receive the TLR2 stimulus in the WT condition. A different publication identified TLR2 as a gene specifically upregulated in Th17 T cells when compared to either Th1 or Th2.[29] From this study, the most convincing experiment suggesting a functional role for TLR2 was a transfer of either WT or $TLR2^{KO}$ $CD4^+$ T cells into mice that had been subjected to experimental autoimmune encephalitis (EAE). Mice receiving the WT $CD4^+$ T cells developed much more severe EAE than those receiving $TLR2^{KO}$ $CD4^+$ T cells, providing evidence that cell-intrinsic TLR2 amplifies the function of Th17 cells.

2) <u>TLR signaling prevents activation or differentiation</u>

Costimulation may be the most common function associated with T cell-intrinsic TLR signaling, but there are examples of publications claiming a near-opposite effect. TLR4 stimulation has been described to reduce IFN-ɣ production by $CD4^+$ T cells, and to lessen the severity of colitis induced by the transfer of $CD4^+$ T cells into Rag^{KO} mice.[30] This result is difficult to reconcile with the above-described studies, some of which do not observe expression of TLR4 on T cells at all, and others that observe no impact of TLR4 signaling on T cells. A somewhat similar conclusion was reached in a publication which asserted a role for direct TLR7 signaling in preventing the conversion of both human and mouse $CD4^+$ T cells into Th1 or Th17 lineages.[31] The authors found that TLR7 could cause already-established Th17 cells to produce less IL-17, and used imiquimod treatment of mice in an EAE model to conclude that this pathway leads to reduction in Th17 cells *in vivo*. This latter finding does not provide evidence for a T cell-intrinsic role of TLR7, and also directly conflicts with a finding from another group that TLR7 stimulus exacerbates EAE.[32]

An additional proposed role for TLR7 on human $CD4^+$ T cells is to induce anergy during chronic HIV infection.[33] *In vitro* stimulation of purified human $CD4^+$ T cells with CD3 and CD28, along with a variety of TLR7 ligands, revealed a reduction in proliferation in the presence of certain TLR7 ligands. Intriguingly, shRNA-mediated knockdown of TLR7 in $CD4^+$ T cells reduced infection by HIV, which led the authors to

conclude that T cell anergy induced via TLR7 is actually beneficial for spread of the virus. This conclusion is problematic from an evolutionary perspective, because it suggests that T cells have maintained expression of a pattern recognition receptor that renders them more vulnerable to viral infection. The authors speculate that their proposed mechanism may have evolved to prevent reactivity to endogenous retroviruses.

3) Treg-intrinsic TLR signaling

The logic behind the function of Treg-intrinsic TLR signaling is somewhat more complicated than on other T cell subsets. For $CD8^+$ and $CD4^+Foxp3^-$ T cells, the presence of microbial patterns would seemingly favor activation and cytokine production to facilitate clearance of pathogens. Both positive and negative modulation of Treg function, however, may be useful during an immune response to microbes. Reducing the suppressive capacity of Tregs may facilitate quicker and more robust activation of immune cells needed to clear a potentially threatening pathogen. Alternatively, increasing the suppressive capacity of Tregs might prevent an immune response to a given microbe from spiraling out of control, or reduce the likelihood of an undesirable response to commensal microbes.

4) Suppressive function of Tregs is enhanced by TLR signaling

Even within the subset of publications that agree Treg-intrinsic TLR signaling increases suppressive capacity, there are significant differences regarding which TLRs are expressed and mediate this effect. An early study stated that RT-PCR analysis of Tregs revealed expression of TLRs 4-8, and found that LPS stimulation of TLR4 caused a “10-fold” enhancement of *in vitro* suppressive capacity.[34] *In vivo* models of Treg suppression did not reveal increased suppression, but rather confirmed that LPS-treated Tregs are still functional in preventing colitis. A different study made the bold claim that human Tregs express TLR5 at the same level as monocytes and dendritic cells.[35] This determination was made using a combination of RT-PCR, western blots, and flow cytometry using traditional antibodies. Stimulation via TLR5 increased suppressive capacity and Foxp3 expression, whereas TLR4 ligand had no effect. Finally, TLR7 signaling in Tregs has also been described to enhance suppressive capacity by boosting Foxp3 and CD25 expression.[36] However, the data provided does not show that the effect observed is based on Treg-intrinsic TLR7 signaling, and potentially conflicts with another publication showing an increase in Tregs in $TLR7^{KO}$ mice subjected to a model of EAE.[32]

5) Suppressive function of Tregs is reduced by TLR signaling

Mice with a global TLR2-deficiency have a reported reduction in $CD4^+CD25^+$ T cells, and multiple studies have shown that Treg-intrinsic TLR2 signaling reduces suppressive capacity. The first found that TLR2 transiently decreases suppression by Tregs *in vitro*, and provided a reasonably well-designed experiment to determine the existence of an *in vivo* effect.[37] WT Tregs were first transferred into a $TLR2^{KO}$ mouse, followed by infection with *Candida albicans* and provision of either exogenous TLR2 ligand or a vehicle control. Mice that received the TLR2 ligand exhibited greater bacterial burden and reduced IFN-ɣ production by splenocytes. Since the transferred Tregs were the only cells capable of receiving the TLR2 stimulus, it was reasonable for the authors to conclude that Treg-intrinsic TLR2 signaling modulated the function of those Tregs. A separate study the same year agreed with this general principle, concluding that TLR2 triggered proliferation of Tregs and transient loss of suppressive capacity.[38] These findings are also in line with a 2015 study finding that Tregs in multiple sclerosis (MS) patients express high levels of TLR2, and that stimulation with TLR2 ligand can cause Tregs from MS patients to adopt a Th17 phenotype *in vitro*.[39] The field is not in total agreement on the function of TLR2 on Tregs, however, as one study determined that intranasal treatment of mice with TLR2 ligand expands the number of lung Tregs and provides protection in a model of allergic asthma.[40]

Several NA-sensing TLRs have also been described to directly reduce the suppressive capacity of Tregs. The earliest and highest profile example is a 2005 study that found TLR8 on human Tregs can “reverse” their suppressive function.[41] Using novel Treg cell lines, the authors initially determined that CpG-A reduced suppressive capacity. Although CpG-A is usually considered a TLR9 ligand, the study identified a poly-G region that was mediating the observed effect. A similar outcome was seen after treatment with multiple RNA sequences and imiquimod, but not loxoribine. Coupled with RT-PCR data, the authors concluded that TLR8 was responsible for modulation of Treg function. The finding was then expanded to a tumor model, where Tregs co-transferred with Poly-G were less suppressive, allowing for improved tumor clearance. The first author has since started his own group, and recently published an additional study attributing the TLR8-mediated reduction in suppressive capacity to an inhibition of glucose uptake and glycolysis.[42]

6) State of the Field

The inconsistent or outright conflicting publications described above highlight the unsettled nature of our collective understanding of the existence and function of T cell-intrinsic TLR signaling. There is currently no consensus as to whether T cells express TLRs at all, which individual TLRs might be expressed, or what the function of signaling

through these receptors might be. The only common thread is that most investigations of TLR signaling on effector T cells find a costimulatory effect, but even that general conclusion is muddied by a handful of conflicting studies. This dissertation seeks to provide resolution to these open questions by demonstrating conclusively that T cells express TLR7, and that signaling through TLR7 1) drives Tregs to proliferate and adopt a tissue repair phenotype, and 2) boosts the expansion and effector capacity of effector T cells.

Chapter 2: T cells express TLR7

Multiple barriers have prevented a conclusive determination of which TLRs are expressed by T cells. Flow cytometry would be an ideal method to address this issue, but antibodies designed to determine expression of individual TLRs have proven unreliable. Western blots also require the use of good antibodies and come with the added complication of needing to purify a pure population of T cells to assess TLR expression. RT-PCR, the most common used technique in the above-summarized publications, sidesteps the antibody problem but amplifies the potential for contamination. If the T cell population to be tested is not exceptionally pure, TLR expression on even a small number of contaminating innate immune or B cells will yield a band that also makes the T cells appear positive.

To circumvent the problems with assessment of TLR expression by traditional immunological techniques, previous members of the laboratory generated a series of TLR reporter mice to measure expression of TLRs 2, 4, 5, 7, and 9. Each of the reporter mouse lines was designed according to the same general strategy: an internal ribosome entry site (IRES), coupled with coding sequence for a fluorescent protein, was inserted immediately after the endogenous locus for the TLR to be measured.[14] As a result, every time the TLR transcript is made in a cell, a fluorescent protein transcript is made as well. Flow cytometry can then be used to evaluate TLR expression on specific cell types. These reporter mice have allowed us to conclusively determine which TLRs are expressed by the different subsets of T cells.

A) Characterization of TLR expression on T cells

TLR expression on T cells was determined by harvesting spleens from each of the five lines of TLR reporter mice. T cells were identified by expression of TCRb divided by expression of CD4 or CD8. The $CD4^+$ T cells were further subdivided into $CD25^-$ or $CD25^+$, since the latter population is composed largely by regulatory T cells. Expression of the reporter fluorescent protein was compared to a C57/BL6 spleen as a negative control, as well as $CD11b^+$ splenocytes from the same mouse as a positive control. The $CD11b^+$ population contains many innate immune cells while the CD19 antibody marks B cells, and both of these populations are known to express substantial levels of TLRs.

The differences in expression of the individual TLRs on T cells was striking. **(Fig. 1).** TLRs 2, 5, and 9 were not expressed at all in any T cell subset, with reporter fluorescence completely overlapping with the C57/BL6 control. Signal for each of these reporters was strong on the $CD11b^+$ and $CD19^+$ splenocytes, indicating that the mouse lines were functioning as expected. All T cells were largely negative for TLR4, although we note the appearance of a small shoulder that extends beyond the negative control.

If this shoulder represents genuinely TLR4$^+$ T cells, it is a very small population with extremely low expression when compared to innate immune and B cells.

TLR7 is the clear outlier on T cells, with strong expression on all subsets of T cells. CD8$^+$ T cells generally express TLR, while CD4$^+$CD25$^-$ T cells appear to span a range of expression from low to high. Strikingly, CD4$^+$CD25$^+$ T cells have bimodal expression of TLR7 in the spleen, with more than half expressing TLR7 at levels comparable to innate immune cells and the remainder appearing to lack TLR7 altogether. **(Fig. 1).** This bimodal expression hints that TLR7 on Tregs has an important function, necessitating differential regulation in undetermined subpopulations.

To better understand the underlying principles of TLR expression on T cells, we expanded our analysis of the TLR reporter mice to the thymus and Peyer's Patches. The reasoning behind this expansion is that previous publications have suggested that activation of T cells can induce TLR expression.[26, 29] The thymus should contain very few activated T cells, while the Peyer's Patches contain a high percentage compared to the spleen. For TLRs 2, 4, 5, and 9, examining the thymus and Peyer's Patches did not change the result, with no detectable expression on any T cells present in these anatomical locations. (**Fig. 2a-b).** Expression of TLR7 on CD4$^+$CD25$^+$ T cells, however, did change significantly. In the thymus, an even higher percentage of CD4$^+$ single-positive CD4$^+$CD25$^+$ T cells express TLR7 than in the spleen, suggesting that the near-universal default state for developing Tregs is to express TLR7. In contrast, relatively few CD4$^+$CD25$^+$ T cells in the Peyer's Patches express TLR7. This evidence suggested that activation status of Tregs may be inversely correlated to TLR7 expression, a hypothesis that will be investigated below.

B) <u>TLR7 expression on Tregs varies by anatomical location</u>

The CD4$^+$CD25$^+$ T cell subset is known to be composed largely of Tregs, highlighted by the fact that these markers were used as the primary defining parameters for Tregs prior to the discovery of Foxp3.[43, 44] However, activated CD4$^+$ T cells also upregulate CD25 expression, meaning that the CD4$^+$CD25$^+$ population in the TLR7 reporter mice technically include a mix of true CD4$^+$Foxp3$^+$ Tregs and CD4$^+$Foxp3 effector T cells.

To obtain an accurate representation of TLR7 expression on Tregs, we crossed the TLR7 reporter mouse to a Foxp3^{GFP} reporter mouse developed by the Rudensky laboratory.[45] The resulting Foxp3^{GFP}TLR7^{KI} mice were then analyzed to confirm that the pattern of TLR7 expression on Tregs matched that of the CD4$^+$CD25$^+$ T cells in the original TLR7 reporter mice. As expected, the vast majority of Foxp3$^+$ Tregs in the thymus express TLR7, while the spleen showed majority expression and the Peyer's Patches greatly reduced expression. **(Fig. 3a).** Inguinal and mesenteric lymph node

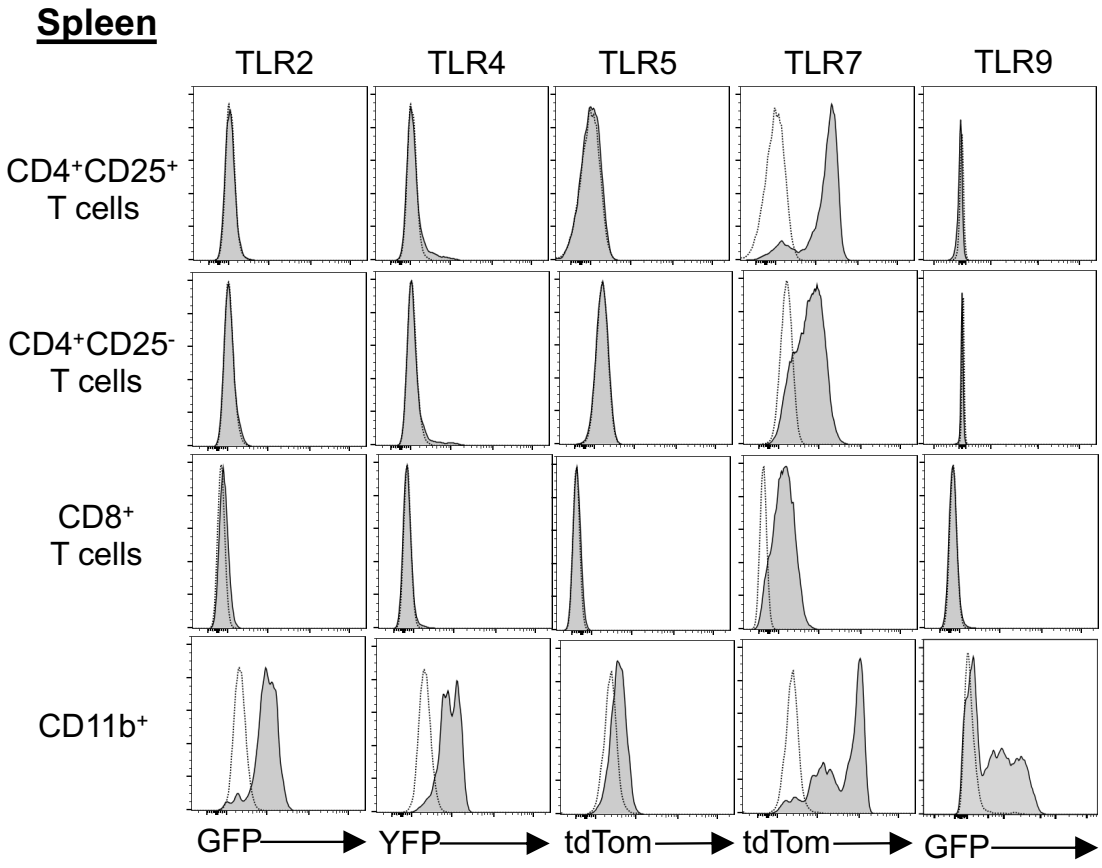


Figure 1. T cells express TLR7. TLR expression of splenic T cells and CD11b$^+$ cells from indicated reporter mice was assayed by flow cytometry (shaded histograms). Dotted unfilled histograms represent fluorescence from the matching cell population in a C57/BL6 mouse using identical cytometer settings in the same experiment.

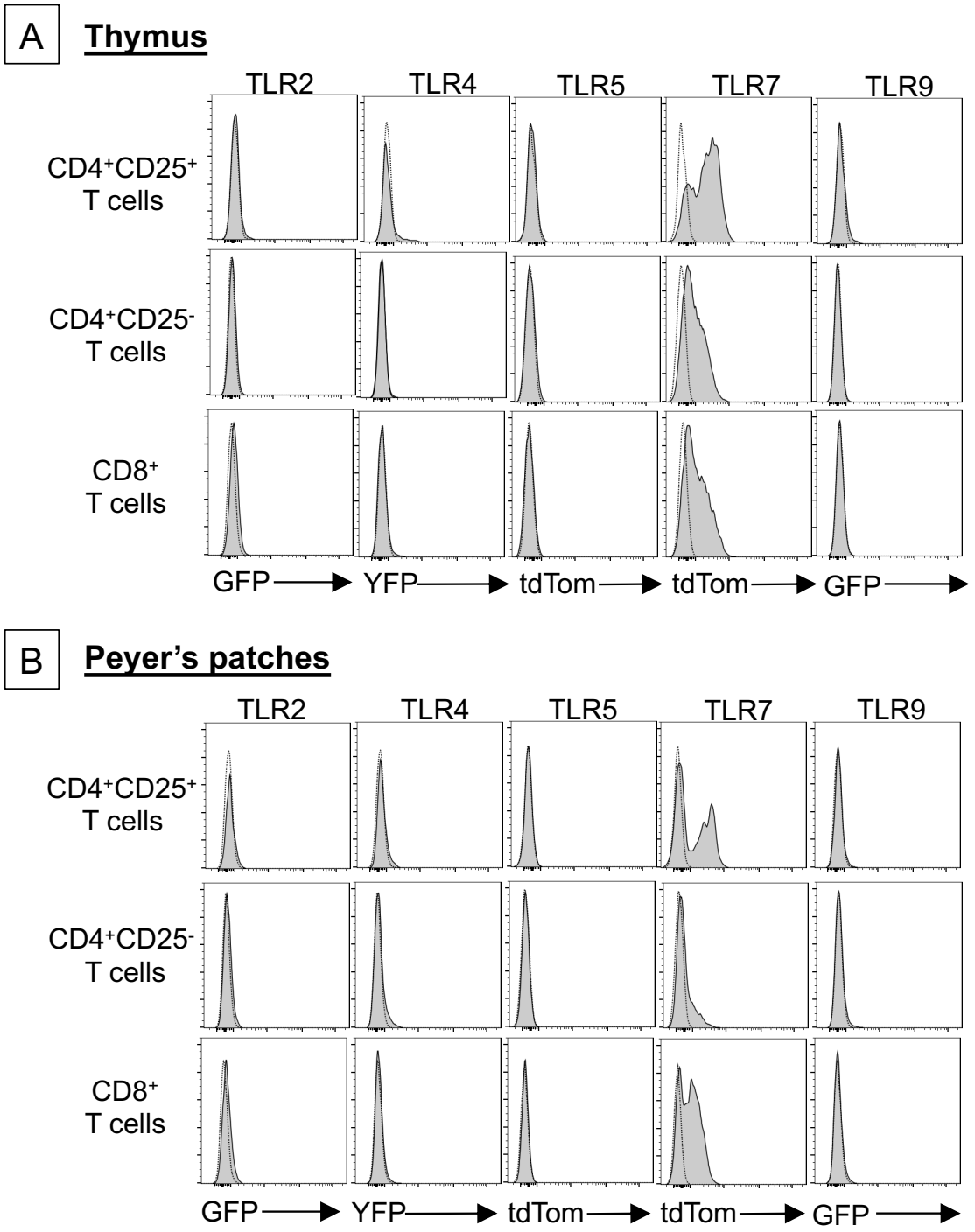


Figure 2. TLR expression on T cells in (a) thymus and (b) Peyer's patches. TLR expression of T cells isolated from thymus and Peyer's patches of indicated reporter mice was assayed by flow cytometry (shaded histograms). Dotted unfilled histograms represent fluorescence from the matching cell population in a C57/BL6 mouse using identical cytometer settings in the same experiment.

Tregs were added to the analysis and also exhibited high frequency of TLR7 expression, again suggesting an inverse correlation with activation status.

Reduced TLR7 expression on Tregs in the Peyer's Patches led us to investigate non-lymphoid tissues known to contain significant numbers of regulatory T cells.[46-48 49] In the visceral adipose tissue and intestinal lamina propria, an extremely low percentage of Tregs express in TLR7. **(Fig. 3b).** The lungs and gastrocnemius muscle (calf), home to a stable but small population of Tregs at steady-state that swells following injury, had a higher percentage of $TLR7^+$ Tregs than the fat or intestine but still relatively few compared to the lymphoid tissues. These results suggest that while TLR7 is expressed on the vast majority of Tregs upon exit from the thymus, expression is downregulated on many Tregs when they enter and establish residency in non-lymphoid tissues.

C) Activation status of T cells correlates with TLR7 expression

The high levels of TLR7 expression in anatomical locations generally populated by naive T cells, and low expression in tissues with high numbers of antigen-experienced T cells, led us to investigate the potential connection between TLR7 expression and activation status. To discriminate between naive and activated T cells, we added antibodies specific for CD44 and CD62L to our analysis of the $Foxp3^{GFP}TLR7^{KI}$ mice. For splenic Tregs, activation status correlated almost perfectly with TLR7 expression; nearly all $CD44^{low}CD62L^{hi}$ "naive" Tregs express TLR7, while the $CD44^{hi}CD62L^{low}$ population was divided into substantial populations of both $TLR7^+$ and $TLR7^-$. **(Fig. 4a).** This correlation carried over into all anatomical locations, lymphoid and non-lymphoid, with $CD44^{low}CD62L^{hi}$ Tregs generally expressing TLR7. **(Fig. 4b).** The driving factors behind overall TLR7 expression on Tregs in a given tissue therefore is a combination of 1) the proportion of the Treg population that is activated, and 2) within that activated population, how many of the Tregs downregulate TLR7. This latter factor appears to be driven by undetermined environmental cues, since the proportion of $TLR7^+$ Tregs within the $CD44^{hi}CD62L^{low}$ compartment varies by anatomical location. Although we have not dedicated significant time to identifying these environmental signals to date, we observe that activated Tregs in the adipose tissue and intestine appear to strongly favor downregulation of TLR7.

D) TCR stimulation causes downregulation of TLR7 on T cells

The strong correlation between activation status of T cells and TLR7 expression led us to determine whether T cell receptor stimulation can directly cause TLR7 downregulation. This question was addressed by isolating splenocytes from a $Foxp3^{GFP}TLR7^{KI}$ mouse and placing them in culture with either anti-CD3 or the TLR7 agonist R848. As early as 1 day later, T cells in the anti-CD3 condition had greatly

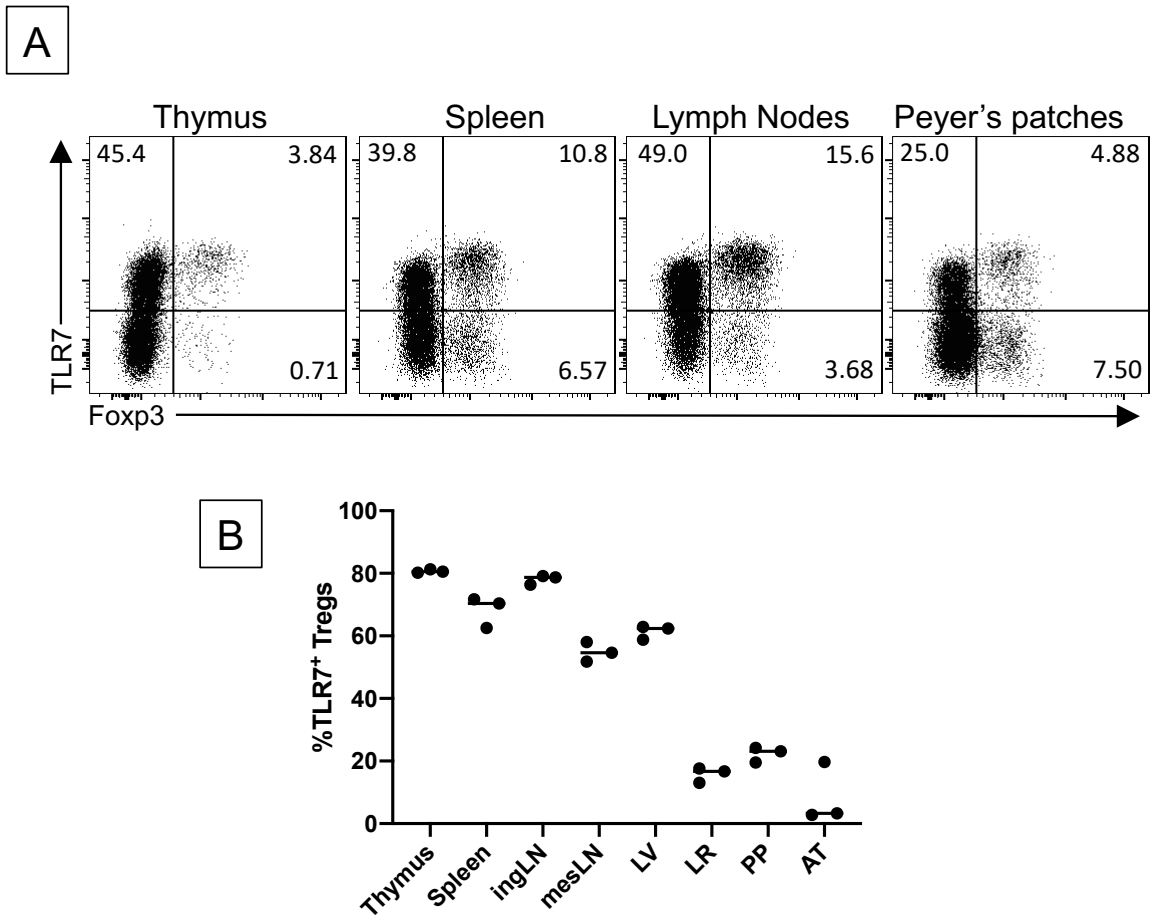


Figure 3. TLR7 expression on Tregs varies by anatomical location. (a) Representative FACS plot of Live, TCRb^{+}CD4^{+} T cells isolated from indicated anatomic location of Foxp3^{GFP}TLR7^{KI} mice. (b) Summary graph of TLR7-positive Tregs in each anatomical location, isolated from three male littermates. (ingLN=inguinal lymph node, mesLN=mesenteric lymph node, LV=lung vasculature, LR=lung-resident, PP=Peyer's patches, AT=abdominal adipose tissue).

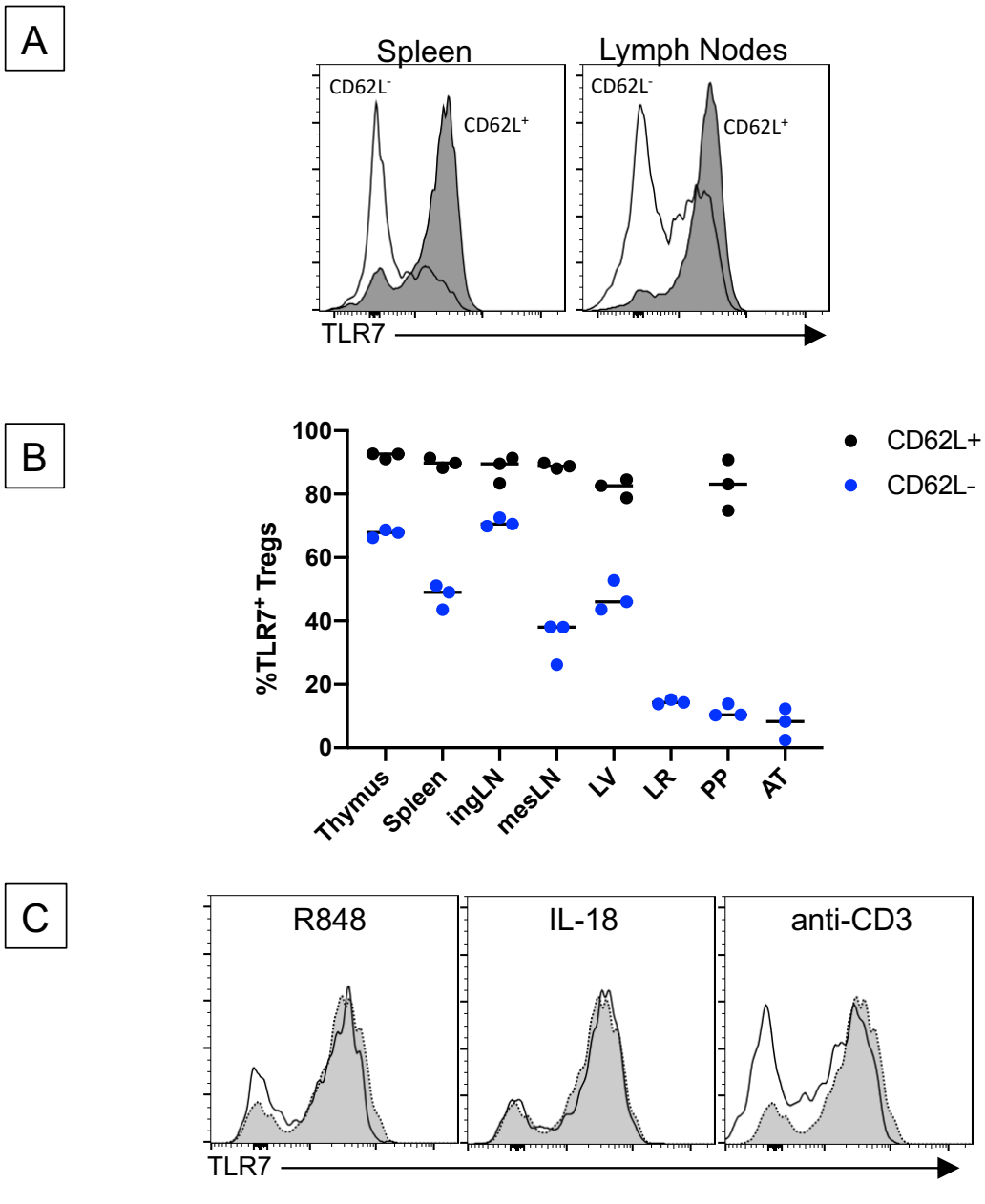


Figure 4. TLR7 expression on Tregs is anticorrelated with activation status. (a) Representative FACS plot of Live,TCRb^{+}CD4^{+}Foxp3^{+} Tregs cells isolated from indicated anatomic location of Foxp3^{GFP}TLR7^{KI} mice, broken down into CD62L+ (shaded) and CD62L^{-} (unfilled). (b) Summary graph of TLR7 expression on Tregs in each anatomical location, broken down by expression of CD62L. Data from three male littermates in same experiment. (c) CD4^{+} T cells from Foxp3^{GFP}TLR7^{KI} mice were isolated and stimulated under the noted conditions for 24 hours. TLR7 expression is shown at Day 0 (shaded) and Day 1 (unfilled). (ingLN=inguinal lymph node, mesLN=mesenteric lymph node, LV=lung vasculature, LR=lung-resident, PP=Peyer's patches, AT=abdominal adipose tissue).

reduced expression of TLR7, while those cultured in the presence of R848 had comparable TLR7 expression to an unstimulated control. **(Fig. 4c).** Therefore, TCR stimulation can drive downregulation of TLR7 on T cells, and likely explains the correlation between activation status and TLR7 expression observed *in vivo*. However, it is worth noting that the degree and rapidity with which TCR stimulation effectuates this change *in vitro* raises an interesting question. Why and how does such a large portion of the $CD44^{hi}CD62L^{low}$ Treg population maintain TLR7 expression, given that these cells have presumably all received TCR stimulation? The strength of TCR signaling could play a role, since the anti-CD3 provides an intense signal, while genuine *in vivo* TCR stimulation varies depending on peptide/MHC:TCR interactions. This hypothesis could be tested by either varying the amount of anti-CD3 in culture or by using one of the well established ova-based systems for creating a gradient of TCR signal strength, although the latter would require significant additional mouse line crosses. An alternative hypothesis is that environmental cues play a role *in vivo*, preventing Tregs from downregulating TLR7 even if a TCR signal is received.

Chapter 3: TLR7 drives Tregs to adopt tissue repair phenotype and boosts activation of CD8$^+$ T cells

The distinct bimodal expression pattern of TLR7 on Tregs, coupled with the lack of consensus regarding the potential role of TLRs on Tregs in the literature, led us to investigate the functional outcome of Treg-intrinsic TLR7 signaling. Foxp3$^+$ Tregs are generally regarded as essential mediators of immune tolerance, capable of preventing undesirable or overexuberant immune responses.[50] The importance of this function is underscored by studies of Foxp3-null mutations in both mice and humans, which invariably leads to severe, fatal autoimmunity at an early age.[50] Treg-mediated suppression is thought to be mediated through some combination of 1) high CD25 expression to "soak up" IL-2 in the environment to prevent activation of other T cells, 2) production of the suppressive cytokines IL-10 and/or TGFβ, 3) expression of CTLA-4 to reduce costimulation of T cells, and 4) additional undiscovered mechanisms. The relative importance of each suppressive mechanism has not been determined, since Treg-specific knockouts of IL-10 and CTLA-4 fail to recapitulate the phenotype of the Foxp3-null mutant, and Tregs lacking CD25 suffer multiple flaws in development and maintenance.[51, 52] Despite the uncertainties regarding the molecular underpinning of Treg-mediated suppression, however, there is no dispute that this function is critical for a properly functioning immune system. Therefore, it was logical to first test whether TLR7 signaling in Tregs alters suppressive capacity.

Treg suppression is commonly measured *in vitro* through co-culture with CD4$^+$Foxp3$^-$ "effector" T cells, wherein anti-CD3 is added to the well to drive division of the CD4$^+$Foxp3$^-$ T cells.[53] The presence of Tregs in the culture inhibits this expansion to some degree, and relative suppressive capacity can be measured by diluting the number of Tregs present until the CD4$^+$Foxp3$^-$ division matches a control well without Tregs. To test the role of TLR7 in Treg-mediated suppression, Tregs were isolated from either wildtype or TLR7^{KO} mice. These two groups of Tregs were cultured in varying dilutions with proliferation dye-stained CD4$^+$ effector T cells in the presence of anti-CD3, with or without TLR7 agonist R848. The CD4$^+$ effector T cells were isolated from TLR7^{KO} mice, to avoid any potentially confounding effect of TLR7 signaling on those cells. As a result, the only cells in the well capable of receiving a TLR7 signal were the Tregs.

TLR7 signaling in Tregs had no impact on suppressive capacity. **(Fig. 5a).** The wildtype Tregs alone, wildtype Tregs in the presence of R848, or TLR7^{KO} Tregs in the presence of R848, all restrained expansion of the CD4$^+$ effector T cells to a near-identical degree regardless of the number of Tregs present in the well. It is worth noting that the assay was successful, as evidenced by the enhanced suppression in conditions with greater numbers of Tregs. There was simply no difference in suppressive capacity as a result of TLR7 signaling. One surprising difference was observed, however.

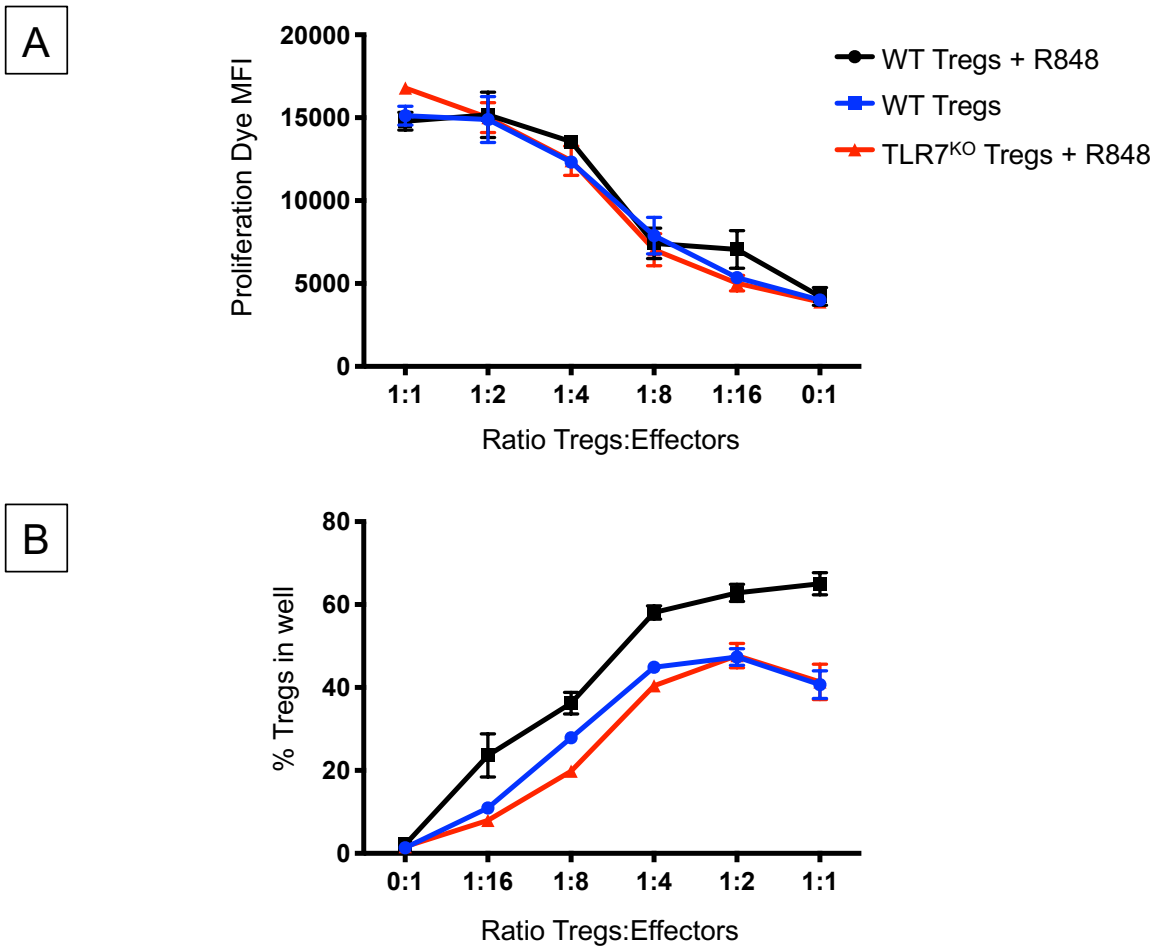


Figure 5. TLR7 signaling does not impact suppressive capacity of Tregs. *In vitro* suppression assay to measure suppressive capacity of Tregs. $CD4^+CD25^-$ T cells were isolated from $TLR7^{KO}$ mice, stained with BD violet proliferation dye 450, and cultured in the presence of anti-CD3 antibody for 3 days in the presence of Tregs of the indicated genotype +/- R848. (a) Division of $CD4^+CD25^-$ T cells after 3 days as indicated by dilution of proliferation dye. (b) Percentage of Tregs (vs. $CD4^+CD25^-$ T cells) under each condition.

Despite all conditions starting with the same number of Tregs per well, the wildtype Tregs in the presence of R848 appeared to have expanded over the course of the three day stimulation when compared to the other conditions. **(Fig. 5b).** This result suggested that Treg-intrinsic TLR7 signaling causes either enhanced survival or proliferation of Tregs, and that these "extra" Tregs have no impact on the suppressive capacity of the population as a whole. Based on these findings we determined that TLR7 signaling does not meaningfully impact suppression by Tregs and turned our focus to further investigating the unexpected expansion caused by TLR7 stimulus.

A) <u>TLR7 signaling causes expansion of Tregs, upregulation of tissue repair markers</u>

To test the apparent TLR7-induced accumulation of Tregs more directly, we moved to a mixed *in vitro* competition assay. $CD4^+$ T cells were isolated from the combined spleens and inguinal lymph nodes, chosen for ease of dissection and high level of TLR7 on Tregs, of B6.SJL and $TLR7^{KO}$ mice. The B6.SJL line is ostensibly wildtype but carries the congenic marker CD45.1 which can be used to distinguish the source of cells in complex experimental settings, in this case competition with the CD45.2-marked $TLR7^{KO}$ cells. The $CD4^+$ T cells from each source were mixed at a 1:1 ratio and cultured for three days either alone, in the presence of R848, or with CpG-B, a TLR9 agonist. CpG-B was selected as a negative control because it stimulates a nucleic acid-sensing TLR, albeit one that is not expressed by T cells. **(Fig. 1).** The mixing and culturing of the two genotypes in the same well is critical to the design of this experiment, and avoids one of the primary pitfalls of previously published studies of TLRs on T cells, because it normalizes the contribution of any other contaminating cells that might also express TLR7.

The results of this experiment were striking, as B6.SJL Tregs cultured in the presence of R848 demonstrated a clear competitive advantage over $TLR7^{KO}$ Tregs in the same well. **(Fig. 6a).** In contrast, the unstimulated and CpG-B conditions ended where they started, with close to identical numbers of B6.SJL and $TLR7^{KO}$ Tregs. This finding unequivocally demonstrates that Treg-intrinsic TLR7 signaling drives expansion. Additionally, B6.SJL Tregs in the mixed culture exhibited highly elevated levels of CD25 and moderately elevated levels of Foxp3, the two primary markers of Treg identity. **(Fig. 6b).** The upregulation of Foxp3 and CD25 indicates that in contrast to inflammatory signals that might drive Treg expansion but reduce stability, TLR7 signaling actually reinforces Treg identity.

The stable expression of classic Treg genes, in combination with the apparent failure to boost suppressive capacity, led us to investigate whether there is a role for TLR7 in a more recently described Treg function - the facilitation of tissue repair. Treg-mediated tissue repair has been described in the context of muscle recovery following acute damage, as well as lung recovery following influenza infection.[48, 49] In both

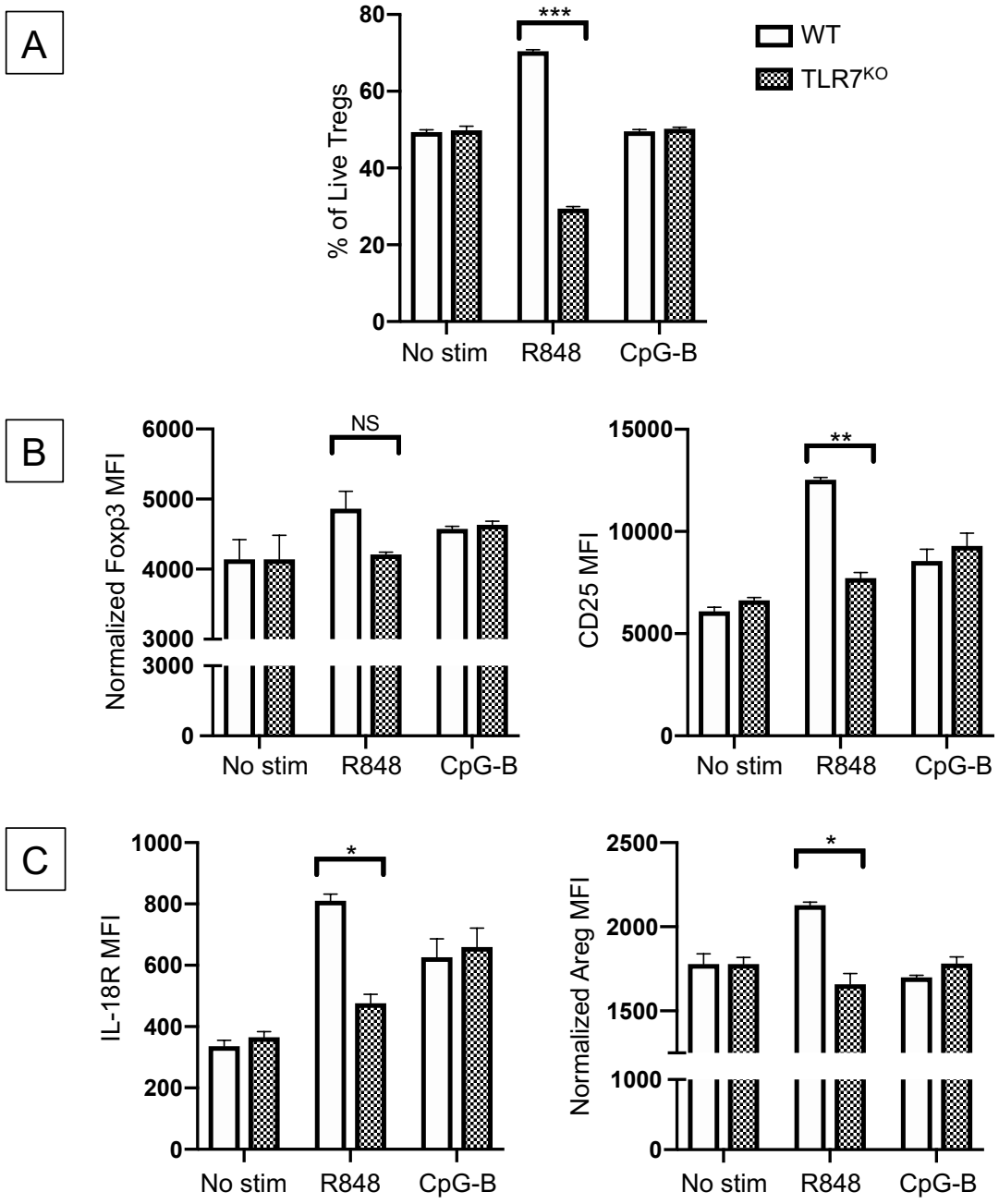


Figure 6. TLR7 signaling drives accumulation of Tregs and adoption of tissue repair phenotype. $CD4^+$ T cells were isolated from the spleens and inguinal lymph nodes of either B6.SJL (CD45.1) or $TLR7^{KO}$ (CD45.2) mice, and then mixed at a 1:1 ratio and cultured for 3 days under the indicated conditions. After 3 days, mixed cultures were subjected to FACS analysis for (a) ratio of genotypes using congenic markers, (b) expression of Treg markers Foxp3 and CD25, and (c) expression of Treg repair markers IL-18R and amphiregulin. *p<0.05; **p<0.005; ***p<0.0005 by unpaired T test.

instances, production of the epidermal growth factor receptor ligand amphiregulin by Tregs was shown to be the essential molecular mediator of repair. It is also worth noting that IL-18r and ST-2 are the two receptors most commonly proposed to trigger Treg production of amphiregulin, and both signal through the signaling adaptor MyD88, which is also required for signaling downstream of TLR7.

Based on these reports, we returned to the competitive *in vitro* stimulation assay to test whether treatment of Tregs with TLR7 alters production of amphiregulin. Using the same experimental setup as described above, we observed that B6.SJL Tregs exhibit elevated levels of both amphiregulin and IL-18r when compared to TLR7^{KO} Tregs in the same well. **(Fig. 6c).** This result indicates that cell-intrinsic TLR7 signaling not only causes Tregs to divide and upregulate classic Treg markers, but also drives conversion to a "tissue repair phenotype."

B) Cell-intrinsic TLR7 signaling on CD8^{+} T cells

Given the convoluted nature of the literature addressing the role of TLRs on CD8^{+} T cells, we also sought to conclusively show that TLR7 on CD8^{+} T cells is functional and to take initial steps toward characterizing its function. Although the focus of this dissertation is the role of TLR7 on Tregs, the success of our mixed *in vitro* stimulation assay led us to conduct a similar experiment with CD8^{+} T cells. CD8^{+} T cells were isolated from the spleens and inguinal lymph of B6.SJL and TLR7^{KO} mice and mixed at a 1:1 ratio. This isolation was followed by a 3 day stimulation with either no stimulus or R848. Each condition was also separately performed in the presence of anti-CD3 TCR stimulation to account for the possibility of a costimulation-like role for TLR7 signaling. Proliferation was measured by addition of proliferation dye, but the staining for amphiregulin performed in our Treg experiments was replaced with staining for the classic CD8^{+} T cell cytokines TNFα and IFNɣ.

In the presence of R848 alone, the B6.SJL CD8^{+} T cells showed a numerical competitive advantage over the TLR7^{KO}, while no difference was observed in the unstimulated condition. **(Fig. 7a).** The most dramatic divergence was observed when TLR7 ligand was combined with TCR stimulation, which caused the B6.SJL CD8^{+} T cells to greatly outpace the TLR7^{KO}. This competitive advantage was accompanied by greatly elevated production of both TNFα and IFNɣ in the B6.SJL T cells. **(Fig. 7b).** It is worth noting that while R848 alone appears to boost cytokine production to some degree, there were far less cells present in the absence of TCR stimulation. Proliferation was also observed with TCR alone, but there was no difference observed between the B6.SJL and TLR7^{KO} genotypes in those conditions. (data not shown). The results of this experiment clearly demonstrate a functional role for CD8^{+} T cell-intrinsic TLR7 signaling that is most dramatic in the presence of TCR signaling. This conclusion generally matches the majority of previous publications that claim a costimulatory-like

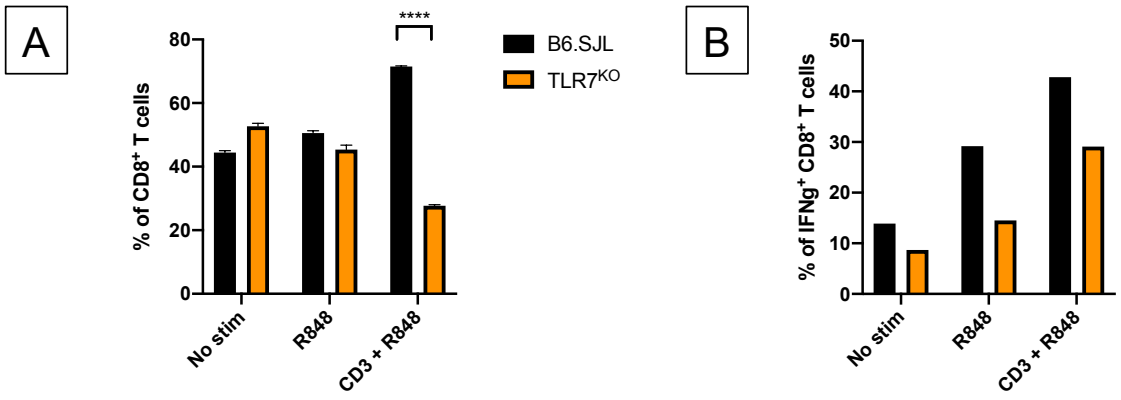


Figure 7. TLR7 signaling drives accumulation of CD8⁺ T cells and production of IFN-γ. $CD8^+$ T cells were isolated from the spleens and inguinal lymph nodes of either B6.SJL (CD45.1) or $TLR7^{KO}$ (CD45.2) mice, and then mixed at a 1:1 ratio and cultured for 3 days under the indicated conditions. After 3 days, mixed cultures were subjected to FACS analysis for (a) ratio of genotypes using congenic markers, (b) production of IFN-γ following 3.5 hour treatment with PMA and ionomycin. ****$p<0.0001$ by 2-way ANOVA. No statistics are shown for b) due to insufficient replicates in the PMA and ionomycin treatment.

role for TLRs on $CD8^+$ T cells, although we have provided clarity by definitely showing that the impact of TLR7 is cell-intrinsic. The role of TLR7 on $CD8^+$ T cells is therefore somewhat less novel than for Tregs, further confirming our decision to focus on the latter population for the majority of this study.

C) Tregs express TLR7 in different anatomical locations that IL-18R and ST-2

TLR-mediated production of amphiregulin by Tregs raises an interesting question regarding the contribution of other MyD88 family members, particularly IL-18R and ST-2, to this same program. Previous reports have suggested that IL-18R and ST-2 might drive Tregs to produce amphiregulin, although this conclusion is based largely on the fact that these receptors tend to be expressed on tissue-resident Tregs. Attempts to induce Treg production of amphiregulin *ex vivo* with these cytokines have proven difficult, especially when using circulating or lymphoid tissue-based Tregs, which tend to express very little IL-18R and ST-2.[48, 54] Nevertheless, if there is a general consensus as to which receptors trigger Tregs to produce amphiregulin, it would be IL-18R and ST-2.

There are at least two explanations for why Tregs would express three different receptors, all of which signal through the same pathway (MyD88) and produce ostensibly the same result (amphiregulin production). The first is that each receptor is responsive to a different type of scenario; for instance IL-18R may be critical when significant inflammasome activation is triggered, ST-2 following damage of epithelial cells, and TLR7 in viral infections. This hypothesis is testable but would require simultaneously testing Treg-specific knockouts of each receptor in carefully orchestrated damage models. One can imagine the difficulty, for example, of trying to measure damage caused by a viral infection that doesn't also impact epithelial layers. A second possibility is that although all three receptors are "expressed on Tregs," there is actually a spatial or temporal difference in expression, such that they are not all expressed on the same Treg at the same time. This theory was relatively simple to investigate using the $Foxp3^{GFP}TLR7^{KI}$ mice, given the availability of reliable antibodies for IL-18R and ST-2.

To assess the relative expression of TLR7, IL-18R and ST-2 on Tregs, a variety of lymphoid and non-lymphoid tissues were collected from $Foxp3^{GFP}TLR7^{KI}$ mice. A range of tissues was sampled because IL-18R and ST-2 are reported to be expressed primarily on activated Tregs resident in non-lymphoid tissues, while our data suggests that TLR7 is expressed primarily (but not exclusively) naive Tregs in lymphoid organs.[55] The results for IL-18R and ST-2 were in accordance with previous reports, in that only 10-30% of Tregs in the spleen and lymph nodes express IL-18R, and less than 20% express ST-2. **(Fig. 8a).** IL-18R was expressed at somewhat higher levels in certain non-lymphoid tissues such as the lung, but still relatively low levels in the adipose

tissue. ST-2 was expressed at high levels in the adipose tissue and moderate levels in the lung. Coexpression of either IL-18R or ST-2 with TLR7 was extremely low, and ST-2 in particular was never coexpressed with TLR7 on more than 10% of Tregs in any anatomical location. **(Fig. 8b).** Therefore, there is significant validity to the hypothesis that TLR7 is expressed on different Tregs than IL-18R and ST-2, which may be a clue to its functional role *in vivo*.

Chapter 4: TLR7 on Tregs is required for tissue repair following lung damage

The TLR7-mediated induction of repair markers in Tregs prompted us to investigate whether this pathway plays a role in recovery from tissue damage *in vivo*. To properly design and execute experiments to test this theory, we took cues from publications in the relatively new field of tissue-resident Tregs. It has become increasingly clear that Treg-intrinsic MyD88 signaling plays a critical role in the differentiation and accumulation of tissue-resident Tregs, suggesting a possible role for TLR7 in the same processes. These studies have identified important roles for Tregs in several different non-lymphoid tissues, including maintenance of homeostasis and facilitation of tissue repair via production of amphiregulin.

A) Introduction to tissue-resident Tregs - maintenance of homeostasis

One of the earliest studies of tissue-resident Tregs arose out of an observation that the adipose tissue of lean mice contained significantly more Tregs than that of obese mice.[56, 57] These Tregs were shown to adopt a transcriptional phenotype distinct from that of non-tissue Tregs, which allows them to control production of inflammatory cytokines and impede the development of insulin resistance. Later publications demonstrated that development of this "adipose tissue" signature was dependent on signaling through ST-2 and MyD88, as well as induction of the essential regulator PPAR-ɣ.[46, 58] Production of IL-33 by adipose stromal cells has recently been described as one of the triggers that drives accumulation and maintenance of Tregs in adipose tissue.[55, 59] Although these studies are perhaps better defined as prevention of tissue damage rather than repair, they represent the first indication that MyD88 signaling in Tregs can promote health in non-lymphoid tissues.

There has also been a long-standing appreciation that Tregs play an important role in maintenance of intestinal homeostasis, and recently this role has been attributed in part to Treg-intrinsic MyD88 signaling.[50] ST-2 was found to be expressed by Tregs in the colon, promoting TGF-β1-mediated differentiation and accumulation/maintenance during inflammation.[60] Tregs have also been shown to express high levels of IL18r1 in the intestine, with signaling through IL-18R promoting expression of Treg effector proteins.[61] Treg-specific knockouts of MyD88 have been reported to cause intestinal dysbiosis and attenuated anti-commensal IgA responses, a result theorized to be caused by impaired sensing of commensal microbes by Tregs.[47]

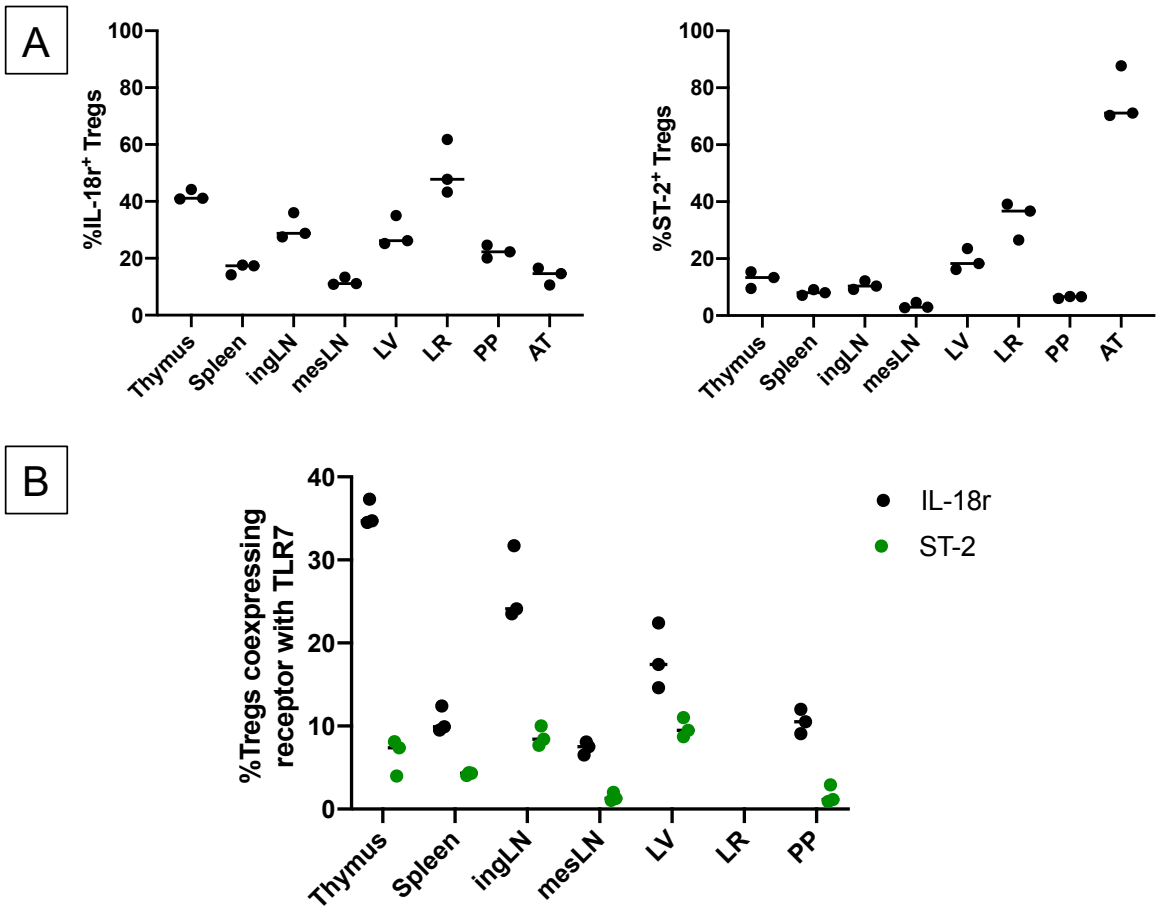


Figure 8. IL-18R and ST-2 rarely expressed on same Tregs as TLR7. (a) Summary of FACS data of Live, TCRb^{+}CD4^{+} T cells isolated from indicated anatomic location of Foxp3^{GFP}TLR7^{KI} mice, stained with antibodies for IL-18R and ST-2. (b) Summary graph of TLR7-positive Tregs in each anatomical location, stained for IL-18 and ST-2, isolated from three male littermates.

B) Introduction to tissue-resident Tregs - repair of tissue damage

While Tregs in the adipose tissue and intestine are generally characterized as important in maintenance of homeostasis and prevention of inflammation, Tregs in other non-lymphoid tissues are thought to play a direct role in mediating repair of damage. This phenomenon was first characterized in a model of acute skeletal muscle damage, whereafter Tregs rapidly accumulate at the site of injury.[49] These repair Tregs produce amphiregulin, a molecule that is widely accepted to promote regeneration of damaged tissue.[62, 63] This initial finding was later linked to MyD88 signaling, by demonstrating that impaired muscle repair in aged mice could be attributed to impaired ST-2 signaling in Tregs.[64] TCR specificity has also been suggested as a driver Treg accumulation in damaged muscle, suggesting an antigen-specific component in adoption of the tissue repair phenotype.[65]

Tregs are also critical players in the repair of lung tissue following injury or infection. In a model of acute lung inflammation, Tregs rapidly accumulate at the site of injury and are required for proper recovery.[66] The molecular mechanisms underpinning this effect are not clear, although Tregs seem to facilitate neutrophil clearance and disposal of apoptotic cells by macrophages.[67] Patients with acute lung inflammation also have elevated numbers of Tregs in the lung, a promising indication that Treg-mediated repair may occur in humans as well. Influenza infection also results in an influx of Tregs to the lung that are necessary for recovery from damage and maintenance of normal lung function.[48] The influenza study also demonstrated, using a Treg-specific deletion, that amphiregulin made by Tregs is the critical mediator of this recovery. IL18R and ST-2 were cited as possible receptors that could trigger Treg production of amphiregulin, although it was not demonstrated that they were required to generate amphiregulin during influenza infection.

The skin is the final anatomical location where Tregs have been shown to play a role in tissue repair. Tregs in the skin were first studied in the context of neonatal mice, where during a crucial developmental window, they are necessary to establish proper tolerance to commensal microbes.[68] It was later shown that skin-resident Tregs localize to hair follicles, and promote follicle regeneration by facilitating stem cell differentiation and proliferation. Notch ligand family member Jagged 1 is highly expressed in skin Tregs, and is important for their impact on hair follicle stem cells.[69] Skin injury results in recruitment of hair follicle stem cells to repair damaged epithelium, where they not only facilitate repair but also prevent development of Th17 inflammatory response.[70, 71]

The above examples conclusively demonstrate that Tregs play a role in maintaining homeostasis and promoting repair in non-lymphoid tissues. There is also strong evidence that the repair function is enabled by production of amphiregulin. The mechanisms that directly cause amphiregulin production, however, are less clear. MyD88 signaling appears to play an important role in muscle repair via ST-2 signaling,

but the connection to other anatomical locations is tenuous at best. Therefore, a major open question in the field is whether additional pathways contribute to amphiregulin production and subsequent tissue repair by Tregs. Based on the results from our *in vitro* experiments described above, TLR7 signaling seemed to be a promising possibility. To properly test the role of TLR7 in promoting tissue repair by Tregs, however, we needed to generate the proper experimental tools.

C) Generation and Validation of TLR7-floxed mouse

The ubiquitous nature of TLR7 expression on most immune cells necessitates the use of a TLR7 floxed mouse line, which would allow for the creation of Treg-specific TLR7 knockout mice that can be tested in the tissue damage models described above. This mouse line had never been published, and to our knowledge did not exist, so we decided to generate a TLR7 floxed mouse line in-house. The Gene Targeting Facility at the UC Berkeley Cancer Research Laboratory (CRL) provides CRISPR/Cas9 gene targeting so that investigators can design gRNAs and constructs, and then pass them along to the CRL for actual generation of novel mouse lines. At the time we were seeking to generate the TLR7 floxed mouse line, CRISPR/Cas9 technology at the CRL had primarily been used to create either frameshift-driven knockout mice, or very small alterations in target genes.

Our task was more complex for multiple reasons. First, by its very nature Cre-Lox technology requires the insertion of two separate 30-40 base pair templates separated by the region to be deleted. Second, the TLR7 coding exon structure is somewhat peculiar, in that almost the entire coding region is contained in the 3.5kb third and final exon, while the first and second exons are small but almost entirely composed of untranslated sequence. Third, CRISPR/Cas9-mediated insertions become increasingly inefficient as the size of the insert increases, to the extent that a 3.5-4kb insertion would make the odds of success vanishingly small. These facts rule out the most straightforward approach to creating the TLR7 floxed mice, which would be to completely replace the coding region with a template that included loxP sites flanking the critical exon. Instead, we sought to increase the odds of successful insertion by inserting the loxP sites one at a time. The downside of this approach was that it required two separate rounds of engineering, the first to generate a mouse line with a loxP site on one side of TLR7 exon 3, and then use of this “single loxP” line as founders for creation of the complete mouse line with loxP sites on both sides of exon 3.

The TLR7 floxed mouse line ($TLR7^{flox}$) was successfully created with one loxP site (along with a SalI restriction enzyme sequence to facilitate genotyping) located in the intron 670 base pairs upstream of TLR7 exon 3, and the second loxP site (along with an AgeI sequence) in the intron 120 base pairs downstream of exon 3. **Fig. 9a).** This construct should result in complete eradication of TLR7 signaling in any cell

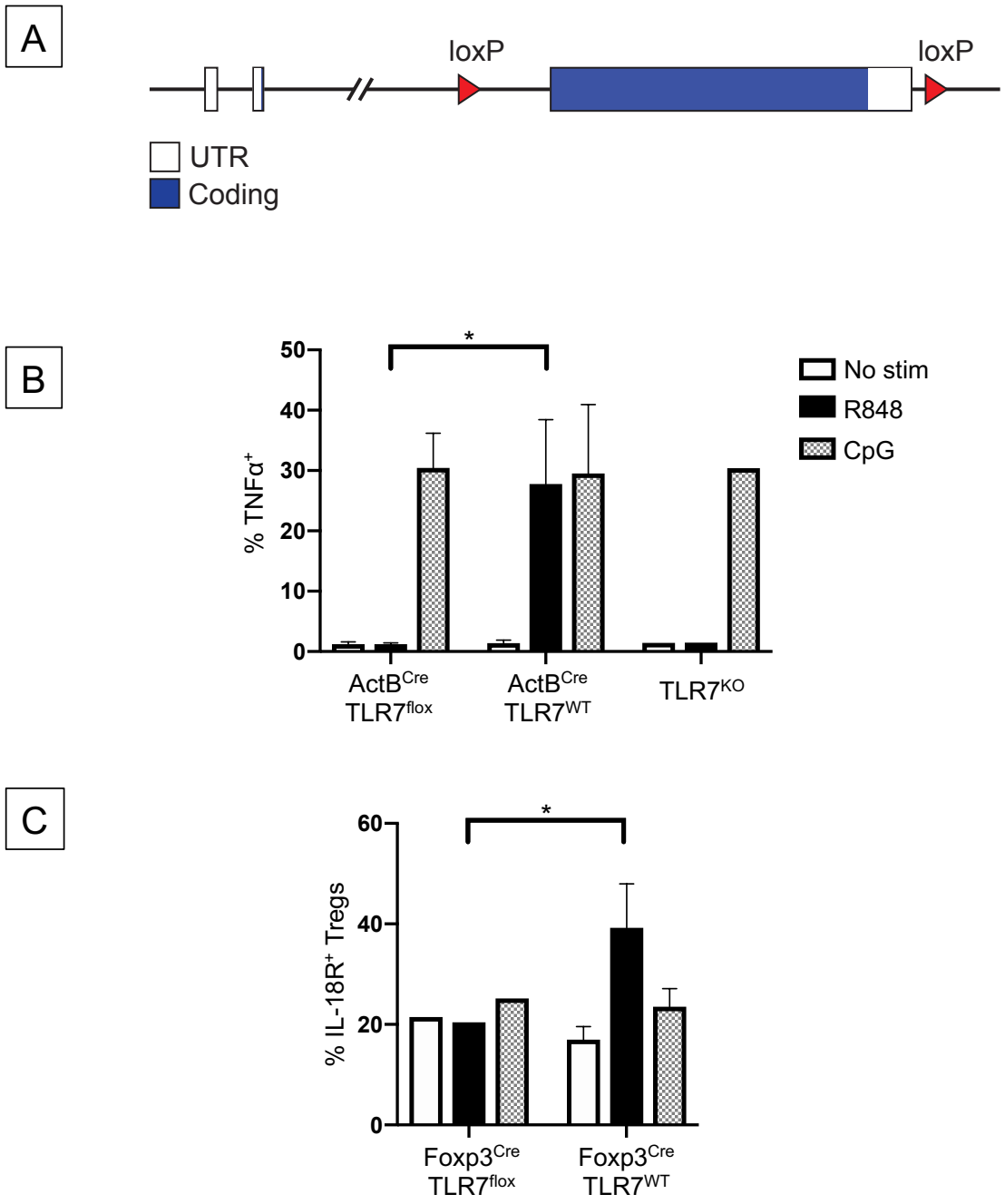


Figure 9. Generation of a novel TLR7 floxed allele. (a) Schematic of TLR7 floxed allele, with loxP sites flanking exon 3. (b) Splenocytes from β-actin^{cre}, β-actin^{cre}TLR7^{flox}, or TLR7^{KO} mice were stimulated for six hours in presence of the indicated ligands. TNFα production was measured by flow cytometry. (c) CD4^{+} T cells from Foxp3^{cre}TLR7^{WT} or Foxp3^{cre}TLR7^{flox} were stimulated for 3 days in presence of the indicated ligands. IL-18R expression was measure by flow cytometry. *p<0.05 by 2-way ANOVA.

expressing Cre recombinase. To confirm the functional deletion of TLR7 in the presence of Cre recombinase we crossed $TLR7^{flox}$ mice to a line expressing Cre under the control of the β-actin promoter. These β-$actin^{cre}TLR7^{flox}$ mice should lack functional TLR7 in virtually every cell, given the ubiquitous role of β-actin in cytoskeletal function. Splenocytes were isolated from these mice along with β-$actin^{cre}TLR7^{WT}$ littermates and $TLR7^{KO}$ negative controls, and stimulated with R848 to measure TLR7-driven production of TNFα. Excitingly, while the β-$actin^{cre}TLR7^{WT}$ splenocytes produced substantial amounts of TNFα in response to R848, the β-$actin^{cre}TLR7^{flox}$ splenocytes produced almost none. **(Fig. 9b).** In fact, the β-$actin^{cre}TLR7^{flox}$ splenocytes responded at a level akin to that of the $TLR7^{KO}$ splenocytes, indicating total ablation of TLR7 signaling. This data demonstrates that our novel $TLR7^{flox}$ mouse line works as expected and can be used to generate cell-specific knockouts of TLR7 when crossed to the proper Cre recombinase drivers.

D) Treg-specific deletion of TLR7 does not cause abnormalities at steady-state

The novel $TLR7^{flox}$ mouse line was crossed to the $Foxp3^{YFP\text{-}cre}$ line, which drives the expression of Cre recombinase using an IRES inserted into the endogenous Foxp3 locus.[72] This cross resulted in $Foxp3^{YFP\text{-}cre}TLR7^{flox}$ mice, which in theory have normal TLR7 expression in all cells except Tregs, which lack TLR7 completely. It is worth mentioning that the $Foxp3^{YFP\text{-}cre}$ mice have certain caveats including hypomorphic expression of Foxp3 and seemingly random ectopic expression that results in Cre expression in non-Tregs.[73, 74] The hypomorph problem can be addressed by always comparing $Foxp3^{YFP\text{-}cre}TLR7^{flox}$ to $Foxp3^{YFP\text{-}cre}TLR7^{WT}$, such that Foxp3 expression is at least consistent across experimental groups. Ectopic expression that results in germline deletion can be revealed through genotyping, so those mice can be removed from any downstream analysis. Ectopic expression that results in only a handful of other cell types expressing Cre is much more difficult to detect, likely requiring the use of a Rosa26 lox-stop-lox RFP or something similar, which would result in the production of RFP in every cell expressing Cre. Despite these limitations of the $Foxp3^{YFP\text{-}cre}$ it is the most well-accepted Treg Cre in the field, and we decided to use it in our study. The only other option is a $Foxp3^{EGFP\text{-}cre}$, which was engineered using a bacterial artificial chromosome, meaning that there is also significant potential for expression in non-Tregs.[75] We also heard anecdotally that some of these mice display characteristics reminiscent of a Foxp3-deficiency, indicating that there may be systematic problems with Treg function that would confound any potential phenotypes.

After generating the $Foxp3^{YFP\text{-}cre}TLR7^{flox}$ mice, we first sought to determine whether loss of TLR7 caused any Treg defects at steady state. To accomplish this baseline phenotyping, we harvested a number of lymphoid and non-lymphoid tissues from $Foxp3^{YFP\text{-}cre}TLR7^{flox}$ males and $Foxp3^{YFP\text{-}cre}TLR7^{WT}$ littermate controls. T cell

populations from each anatomical location were then analyzed by flow cytometry for any potential defect in the Treg population. No differences were observed, with Foxp3$^{YFP\text{-}cre}$TLR7^{flox} mice exhibiting similar numbers and percentages of Tregs as Foxp3$^{YFP\text{-}cre}$TLR7^{WT} mice. **(Fig. 10a).** CD25, IL-18R and ST-2 expression on Tregs also appeared normal in the absence of TLR7. **(Fig. 10b).** Having ruled out a steady-state phenotype for the new Foxp3$^{YFP\text{-}cre}$TLR7^{flox} mice, we turned our attention to investigating the role of TLR7 on Tregs in models of tissue damage.

E) Foxp3^{cre}TLR7^{flox} mice have impaired lung recovery following influenza infection

Our finding that TLR7 drives Tregs to produce amphiregulin, combined with the literature demonstrating a role for amphiregulin in Treg-mediated tissue repair, suggests a role for TLR7 on Tregs in facilitating tissue repair. To properly test this possibility in the Foxp3$^{YFP\text{-}cre}$TLR7^{flox} mice, we needed to select a suitable model of tissue damage. Intranasal influenza infection was an obvious top choice because 1) TLR7 is known to sense influenza, and 2) Treg-mediated repair is required for proper recovery from influenza infection.[48, 76] It is again worth highlighting that TLR7 is capable of sensing both viral and endogenous ssRNA, but use of an ssRNA virus like influenza leaves no doubt about the presence of a ligand.

We obtained the PR8 strain of H1N1 influenza from the Arpaia Lab at Columbia University. The Arpaia Lab regularly uses influenza to study Treg repair function, but the batch we received was new and had not been tested or titered, aside from an observation that a dose of 250 $TCID_{50}$ was lethal for all mice tested and 50 $TCID_{50}$ did not result in observable sickness. As a result, we needed to conduct a series of experiments to determine the best dose at which to test the impact of TLR7 on Tregs. For each dose attempted, we used weight loss and blood oxygen saturation (%SpO_2) as readouts for progression of the infection and lung function. At relatively high doses within the range provided, ranging from 100 $TCID_{50}$ to 200 $TCID_{50}$, both Foxp3$^{YFP\text{-}cre}$ and Foxp3$^{YFP\text{-}cre}$TLR7^{flox} mice lost substantial weight starting at approximately Day 4 post-infection, and declined so severely that they needed to be euthanized in accordance with our animal care protocols. **(Fig. 11a).** These doses did not reveal any difference in %SpO_2 between the genotypes. Intriguingly, however, analysis of lung-resident Tregs at Day 7 post-infection revealed higher numbers in Foxp3$^{YFP\text{-}cre}$ mice when compared to Foxp3$^{YFP\text{-}cre}$TLR7^{flox}, suggesting that TLR7 might help maintain a robust Tregs population during influenza infection. (**Fig. 11b).**

The cellular readout was promising, but we ultimately wanted to test the role of TLR7 on Tregs in tissue repair. %SpO_2 is one of the best methods for measuring tissue repair, because it measures lung function directly - as the infection builds and damage is incurred %SpO_2 drops precipitously, and then eventually returns to near 100% as the infection is cleared and lung tissue repaired. Therefore, we were most interested in

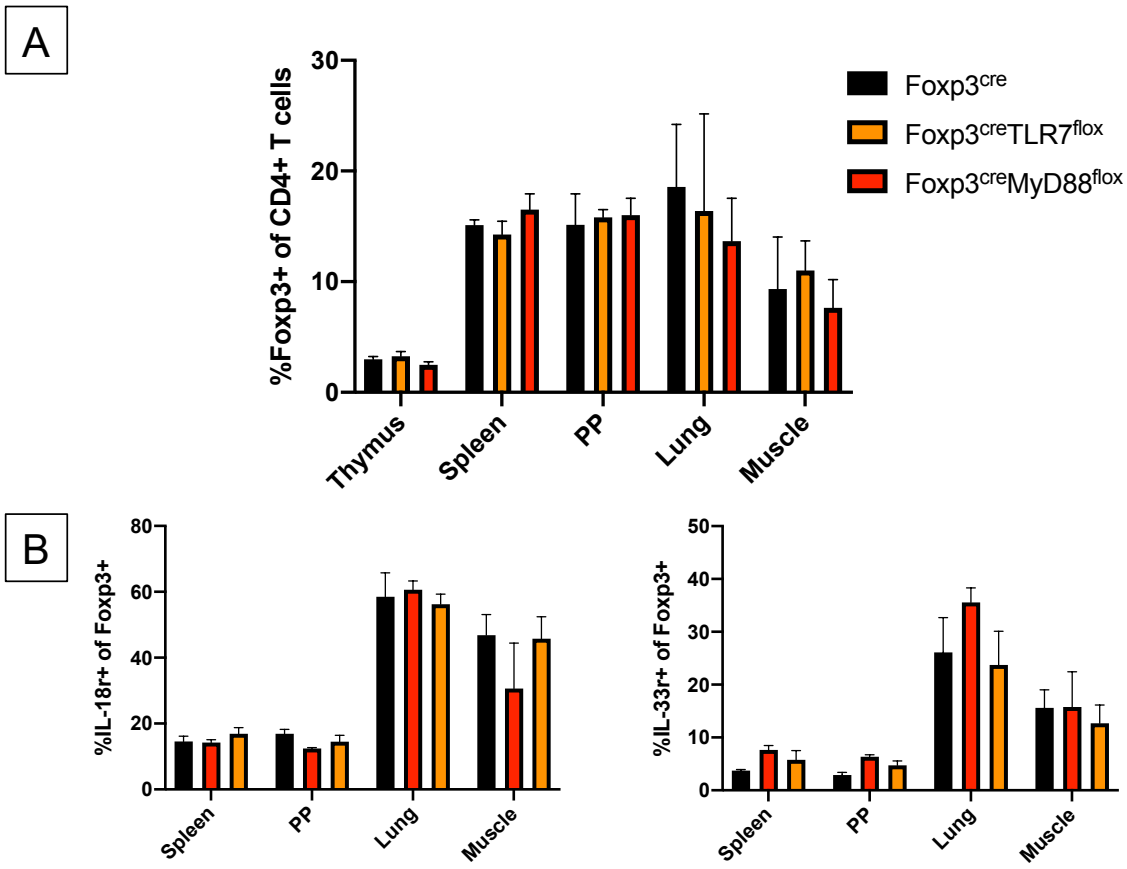


Figure 10. No defect in Tregs from Foxp3^{cre}TLR7^{flox} mice at steady-state. Lymphocytes were harvested from indicated anatomical locations of Foxp3^{cre}, Foxp3^{cre}TLR7^{flox} and Foxp3^{cre}MyD88^{flox} mice and analyzed for composition of T cell compartment. (a) Live,TCRb^{+}CD4^{+}Foxp3^{+} Tregs displayed as a percentage of total live Live,TCRb^{+}CD4^{+} T cells. (b) IL-18R and ST-2 expression on Tregs from (a) was measured by flow cytometry.

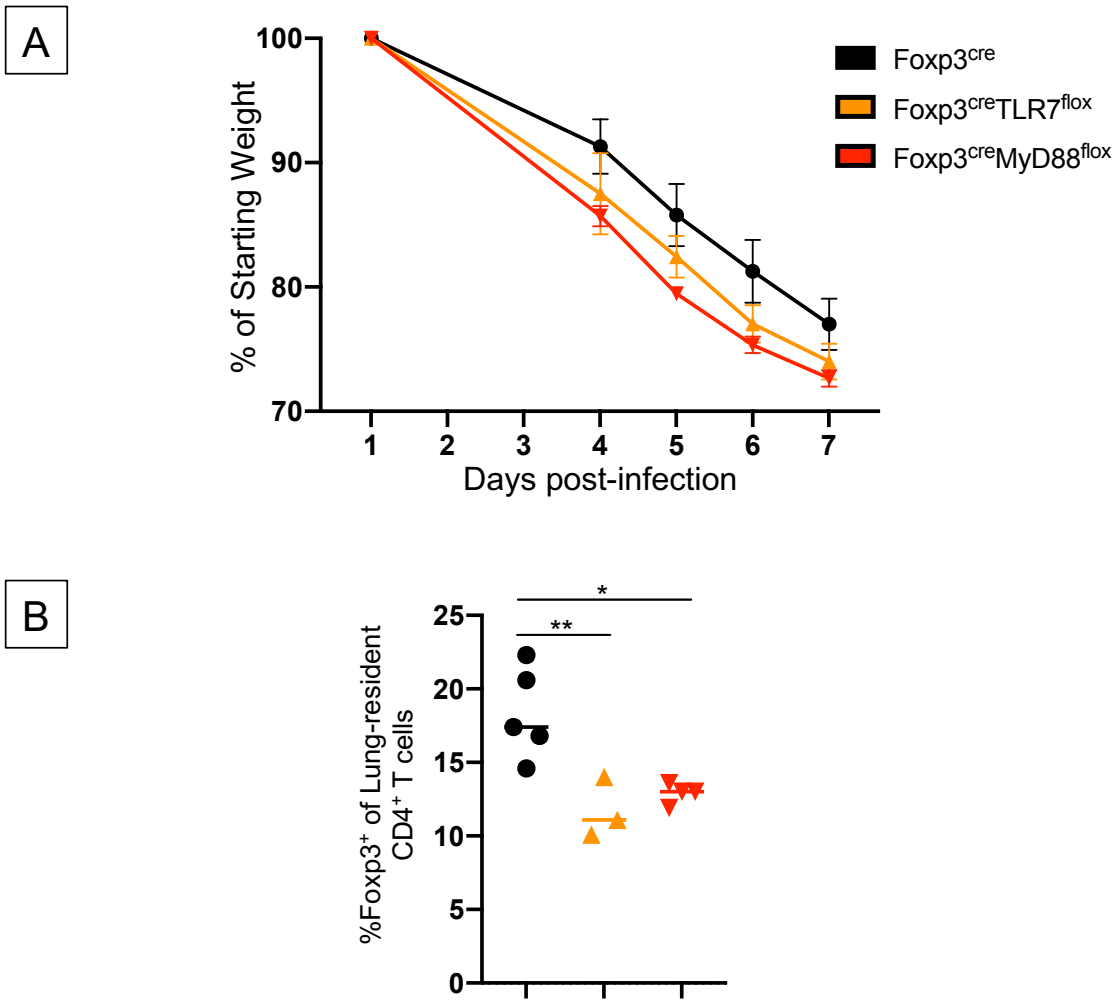


Figure 11. High-dose flu infection causes uniform weight loss but reveals cellular impact of Treg-intrinsic TLR7 signaling. Foxp3^{cre}, Foxp3^{cre}TLR7^{flox} and Foxp3^{cre}MyD88^{flox} mice were intranasally infected with 150 $TCID_{50}$ PR8 H1N1 influenza. (a) Weight loss was tracked for 7 days after which mice were euthanized. (b) Live,TCRb^{+}CD4^{+}Foxp3^{+} Tregs displayed as a percentage of total live Live,TCRb^{+}CD4^{+} T cells at Day 7 post-infection. *p<0.05; **p<0.005 by 2-way ANOVA.

finding a dose at which wildtype mice become moderately sick but eventually recover, so that we could measure the role of TLR7 on Tregs in that recovery. After several additional trials we settled on a dose of 17 $TCID_{50}$, which resulted in mild weight loss and $\%SpO_2$ reduction in wildtype mice. With a suitable dose of 17 $TCID_{50}$ identified, we next infected $Foxp3^{YFP\text{-}cre}$ and $Foxp3^{YFP\text{-}cre}TLR7^{flox}$. The results were striking - while the $Foxp3^{YFP\text{-}cre}$ cohort developed moderate disease, the $Foxp3^{YFP\text{-}cre}TLR7^{flox}$ group experienced a severe decline characterized by accelerated loss of weight and blood oxygen saturation. **(Fig. 12a-b).** The $Foxp3^{YFP\text{-}cre}TLR7^{flox}$ mice were also slow to recover, still exhibiting low blood oxygen saturation 10 days after infection. This result critically demonstrated that Treg-intrinsic TLR7 signaling is required for maintenance of lung function during the resolution of influenza infection.

To date, we have not observed the same differences in Treg numbers between the $Foxp3^{YFP\text{-}cre}$ and $Foxp3^{YFP\text{-}cre}TLR7^{flox}$ at 17 $TCID_{50}$ that we did at higher doses. **(Fig. 12c).** We also have not detected a difference in amphiregulin production between the groups by flow cytometry. **(Fig. 12d).** This is somewhat surprising since the blood oxygen saturation data suggests that there is a defect in the $Foxp3^{YFP\text{-}cre}TLR7^{flox}$ Tregs, but we hypothesized that a lack of difference in Treg numbers or amphiregulin production might indicate potentially important roles for other, previously unidentified genes in Treg-mediated tissue repair. In an attempt to obtain a better appreciation of which Treg genes other than amphiregulin are dependent on TLR7 signaling, we turned to RNA sequencing.

F) <u>Bulk and single-cell sequencing of Tregs during influenza infection</u>

RNA sequencing was selected as a next step in the analysis of the defect in $Foxp3^{YFP\text{-}cre}TLR7^{flox}$ Tregs during influenza infection because it allows for a complete survey of all genes transcribed at a given point in time, rather than relying on a few hand-selected genes to be measured by flow cytometry. The first RNAseq experiment was actually performed prior to our identification of the idealized dose of 17 $TCID_{50}$, and was instead carried out at a higher dose of 100 $TCID_{50}$. $Foxp3^{YFP\text{-}cre}$ and $Foxp3^{YFP\text{-}cre}TLR7^{flox}$ mice were infected and taken out to Day 5 post-infection, which at the higher dose means the mice have lost a moderate amount of weight but are still 2-3 days from the worst physiological consequences. Using the $Foxp3^{YFP\text{-}cre}$ fluorescent marker, we FACS-sorted splenic and lung-resident Tregs from each genotype (n=3), and also sorted Tregs from the spleens of uninfected $Foxp3^{YFP\text{-}cre}$ mice as a “normal” Treg control. RNA was isolated from all samples and submitted to the UC Berkeley Functional Genomics Laboratory (FGL) for library preparation and sequencing. The sequencing data was analyzed by Kathleen Pestal, a postdoctoral scholar in our laboratory.

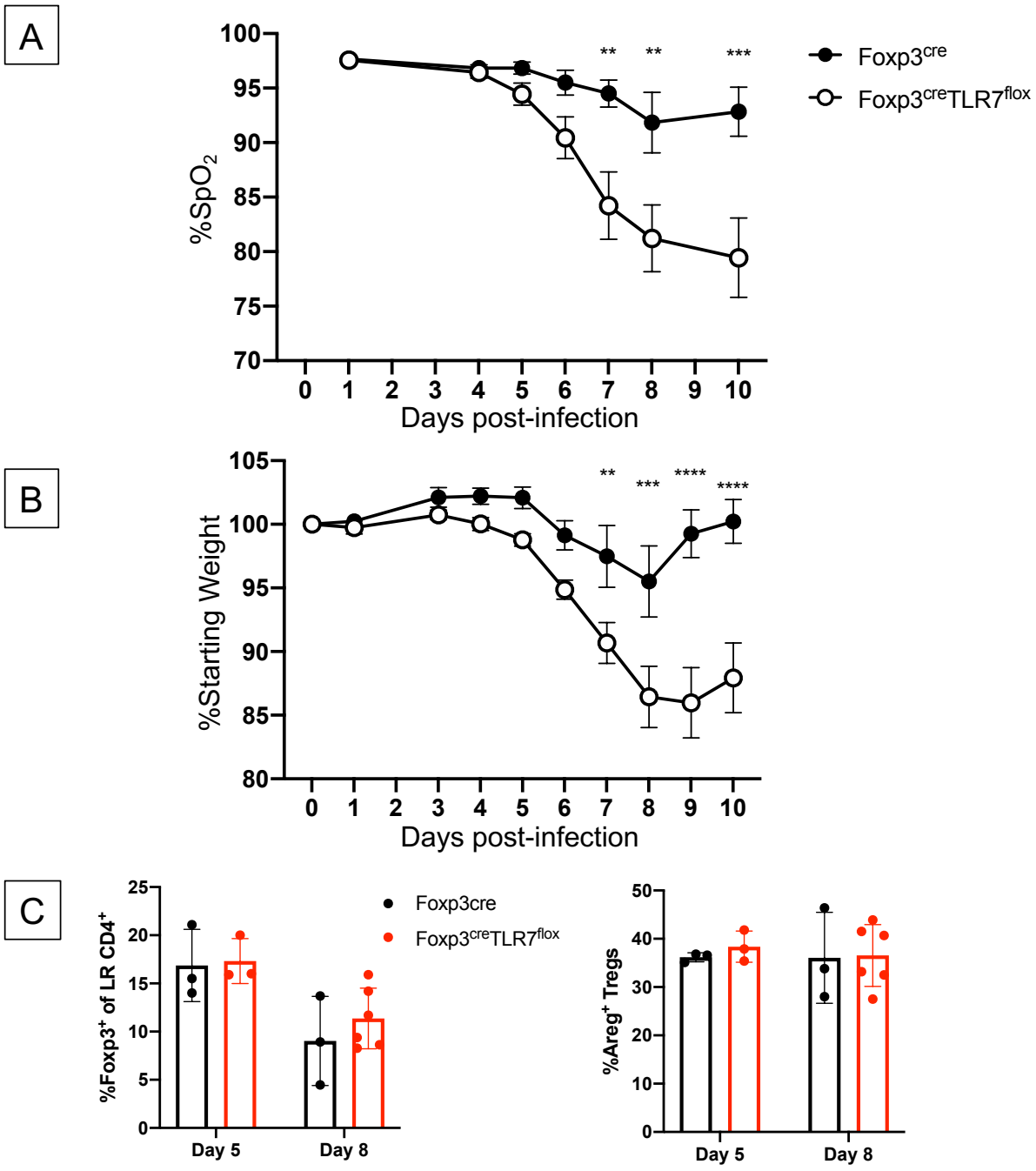


Figure 12. Low-dose flu infection demonstrates requirement for Treg-intrinsic TLR7 signaling. Foxp3^{cre} and Foxp3^{cre}TLR7^{flox} mice were intranasally infected with 17 $TCID_{50}$ PR8 H1N1 influenza. (a) Blood oxygen saturation (%SpO_2) (b) weight loss was tracked for course of infection and recovery. (c) Lung-resident Tregs from each group were analyzed for amphiregulin production at either Day 5 or Day 8 post-infection. **p<0.005, ***p<0.001, ****p<.0001 by 2-way ANOVA.

Although the RNA sequencing appeared to be a technical success, with a clear difference between the splenic and lung-resident Tregs that reflected many classic markers of tissue-residency, there were vanishingly few differentially expressed genes (DEGs) between the genotypes in each location.[77, 78] **(Fig. 13a-b).** In fact, the only DEG between genotypes in the spleen was TLR7, which is a good indication that the floxed mouse was working as expected, but otherwise provided no useful data in support of our hypothesis. **(Fig. 13b).** The lung-resident Tregs yielded a similar result, a similar result, with TLR7 identified as one of the few DEGs, but the other hits did not appear to be clearly tied to tissue repair. The lung-resident DEGs also were not part of any common pathways, and therefore did not provide us with any cohesive hint as to the impact of TLR7 signaling on Tregs during influenza infection. Intriguingly, by setting aside the DEG algorithm and instead looking at normalized read counts of individual genes by hand, we did see a decrease in amphiregulin in the lung-resident $Foxp3^{YFP\text{-}cre}TLR7^{flox}$ group. **(Fig. 13c).** This finding was a promising indication that the TLR7-dependent amphiregulin production observed *in vivo* might be occurring during influenza infection, although it did not get picked out as a DEG.

We next hypothesized that heterogeneity in the lung-resident Treg population during influenza infection could be masking differences between $Foxp3^{YFP\text{-}cre}$ and $Foxp3^{YFP\text{-}cre}TLR7^{flox}$ Tregs in bulk RNA sequencing. To address this possibility, we turned to a single cell RNA sequencing (scRNAseq) approach in collaboration with Nir Yosef's laboratory at UC Berkeley. scRNAseq technology allows for a transcriptional snapshot of every cell analyzed, enabling us to get a better sense of how much heterogeneity exists within the Treg population, and possibly revealing more subtle differences caused by TLR7 signaling. Due to the expense of this technology, we first elected to perform a pilot study comparing 1 sample each of $Foxp3^{YFP\text{-}cre}$ and $Foxp3^{YFP\text{-}cre}TLR7^{flox}$ Tregs at Day 5, as well as 1 sample each at Day 8 post-infection with 17 $TCID_{50}$. The timepoints were selected because at that dose, Day 5 is the very first day that mild weight loss is observed, and Day 8 often represents the most severe weight loss and $\%SpO_2$ prior to recovery. Our goal with this initial experiment was to make sure that we were technically capable of obtaining quality data, and in a best case scenario may give some sense as to differences between genotypes at those populations. For this experiment, we infected the mice and sorted the Tregs, the FGL performed 10X library preparation and sequencing, and Allon Wager from the Yosef laboratory translated the sequencing data into the online Vision platform that enables researchers to analyze data without needing a computer science background.

The pilot scRNAseq experiment was a technical success and yielded multiple interesting leads to new avenues of investigation. The most glaring observation from this experiment was the amount of heterogeneity within the lung-resident Treg population. The strongest clustering differences were between Day 5 and Day 8, meaning that the transcriptional profile of the Tregs changes dramatically over a

A

Lung vs. Spleen

Gene	Log2FC	pvalue	padj
Areg	5.94515246	7.44E-136	1.10E-131
IL1rl1	3.66626344	7.58E-36	2.94E-33
Rora	2.45357911	1.45E-18	1.69E-16
gata3	1.15829002	6.16E-09	2.07E-07
Cxcr3	1.76546289	6.88E-12	3.86E-10

B

Lung KO vs. WT

Gene	Log2FC	pvalue	padj
Tlr8	3.84883863	5.07E-08	0.00059231
Ogfod1	1.74024934	1.57E-05	0.06126853
Arid1b	-0.8752146	1.25E-06	0.00973041
Tlr7	-2.4484854	7.90E-12	1.85E-07
Kcnma1	-3.6330894	2.89E-05	0.09644432
Dnhd1	-4.552526	4.85E-06	0.0226723
LT629154.5	-5.4351541	2.86E-06	0.01668554

Spleen KO vs. WT

Gene	Log2FC	pvalue	padj
Tlr7	-2.4446492	1.60E-10	3.53E-06

C

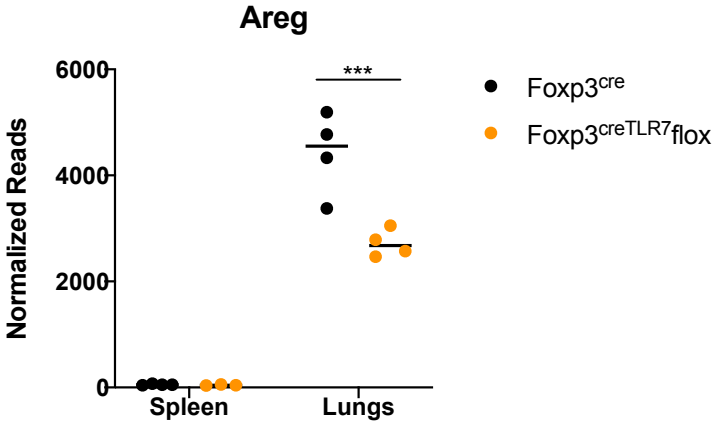


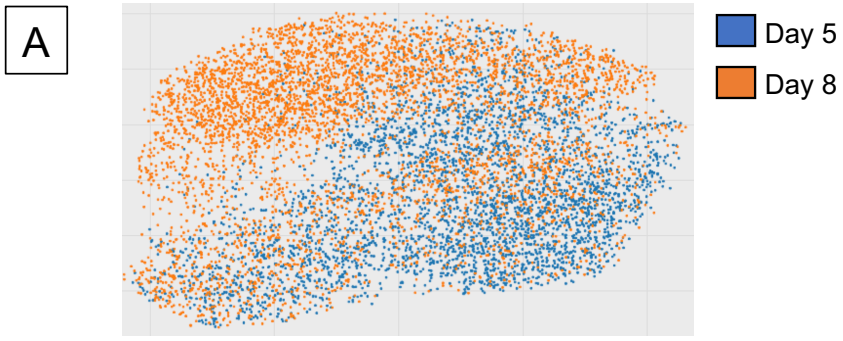
Figure 13. RNA sequencing of Tregs from high-dose influenza infection. Foxp3^{cre} and Foxp3^{cre}TLR7^{flox} mice were intranasally infected with 150 $TCID_{50}$ PR8 H1N1 influenza. Tregs were sorted on Day 5 post infection, RNA extracted, and samples submitted to the UC Berkeley Functional Genomics Laboratory for library preparation and sequencing. (a) Selected differentially-expressed genes (DEGs) in lung-resident Tregs compared to splenic Tregs. (b) All DEGs between Foxp3^{cre} and Foxp3^{cre}TLR7^{flox} Tregs in spleen and lungs. (c) Normalized amphiregulin reads. ***p<0.001 by unpaired T test.

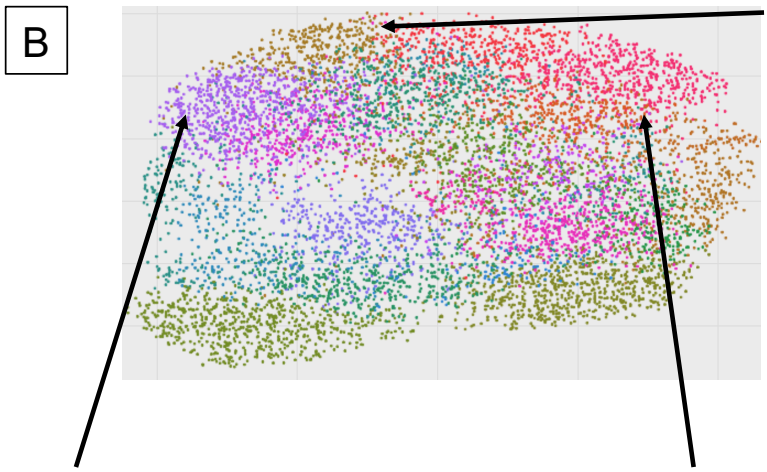
relatively short period of time even within the height of influenza infection. **(Fig. 14a).** Next, we noticed that even when we looked only at Day 5 or Day 8 Tregs, there was still substantial heterogeneity. **(Fig. 14b).** This result would have been extremely surprising several years ago when Tregs were thought of as a relatively homogeneous population, but this heterogeneity is actually in line with that reported by groups performing scRNAseq on tissue-resident Tregs at steady-state.[79] Several of the clusters revealed in our data had transcriptional profiles matching previous publications including "non-lymphoid tissue" or "repair", "non-lymphoid tissue suppressive" and "lymphoid-tissue-like".[79] The amount of overlap between transcriptional profiles in our dataset, from an influenza infection, and published datasets from steady-state is somewhat surprising, suggesting that despite the heterogeneity there may be a finite number of possible Treg phenotypes.

Since our primary intent was to reveal differences between Foxp3$^{YFP\text{-}cre}$ and Foxp3$^{YFP\text{-}cre}$TLR7^{flox} Tregs, we utilized an analysis of differentially expressed clusters performed by the Yosef laboratory. **(Fig. 15a).** This analysis is still in progress, but we identified intriguing differences at Day 8 when looking at a moderately detailed level of clustering (res1.5). The Foxp3$^{YFP\text{-}cre}$ Tregs at Day 8 were highly enriched for cluster "lei7," while the Foxp3$^{YFP\text{-}cre}$TLR7^{flox} Tregs were enriched for clusters "lei5" and "lei6". **(Fig. 15a, black circles).** The lei7 cluster was significantly enriched for both amphiregulin and ST-2 (Il1rl1) when compared with clusters lei5 and lei6, suggesting that TLR7 signaling might be responsible for reinforcing the tissue repair phenotype on a subset of Tregs. **(Fig. 15b).** A slightly less dramatic but similar difference was also observed at Day 5, where one of the clusters enriched in the Foxp3$^{YFP\text{-}cre}$ has increased levels of genes associated with tissue repair. **(Fig. 15a, red circle, data not shown).** Collectively, this data supports our notion that TLR7 facilitates Treg production of amphiregulin, thereby leading to improved tissue repair following influenza infection. These conclusions are somewhat premature given the relatively small scale of this pilot experiment, so we will be conducting a repeat with higher numbers in each group in the coming months. If the results look similar to the pilot, we will have convincing evidence of an altered transcriptional profile that causes the impaired response of Foxp3$^{YFP\text{-}cre}$TLR7^{flox} Tregs during influenza infection.

G) <u>LPS as a non-viral model of lung damage</u>

Since TLR7 is known to be capable of sensing endogenous RNA in addition to viral RNA, our results with influenza could be a result of Tregs sensing: 1) the virus directly, or 2) endogenous RNA released during the course of infection. This latter possibility is somewhat speculative, but it stands to reason that as infection spreads, the immune response builds, and tissue damage accumulates, cell death will result in the release of nucleic acids including RNA. The increased level of RNA is effectively a





Repair

Feature	Type	logFC	AUC	FDR
GATA3	Gene	1.542	0.8834	9.45E-163
IL1RL1	Gene	1.518	0.8668	2.30E-149
AREG	Gene	0.8422	0.7277	1.12E-58

Suppressive

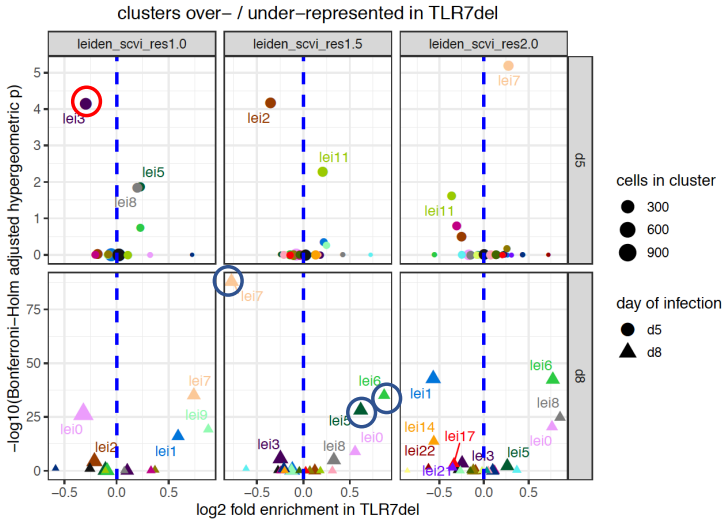
Feature	Type	logFC	AUC	FDR
LAG3	Gene	2.099	0.8769	8.35E-226
GZMB	Gene	0.8575	0.7325	2.30E-87
IL10	Gene	0.707	0.6865	8.08E-57
CXCR3	Gene	0.6353	0.7699	4.68E-117
GATA3	Gene	-0.9155	0.7273	1.41E-83
IL1RL1	Gene	-0.9164	0.6313	5.49E-29
AREG	Gene	-1.029	0.6398	1.22E-32

NLT-like

Feature	Type	logFC	AUC	FDR
KLF2	Gene	2.198	0.9308	2.50E-268
TCF7	Gene	2.04	0.9145	1.17E-248
BCL2	Gene	1.493	0.7644	1.41E-102
NRP1	Gene	1.352	0.8662	8.20E-195
GATA3	Gene	0.3054	0.6417	1.26E-30
IL1RL1	Gene	-0.2868	0.5423	0.000689
AREG	Gene	-1.18	0.6706	1.13E-43

Figure 14. scRNA sequencing of Tregs from low-dose influenza infection. Foxp3^{cre} and Foxp3^{cre}TLR7^{flox} mice were intranasally infected with 17 $TCID_{50}$ PR8 H1N1 influenza. Tregs from 1 mouse of each genotype were sorted on Day 5 and 8 post-infection. Cells were submitted to the UC Berkeley Functional Genomics Laboratory for 10X library preparation and sequencing. Sequencing data was processed for display in the Vision analysis tool by Allon Wagner. (a) Differential clustering of Tregs at Day 5 and Day 8 post-infection, independent of genotype. (b) Detailed clustering of all samples combined, with select known transcriptional signatures highlighted.

A



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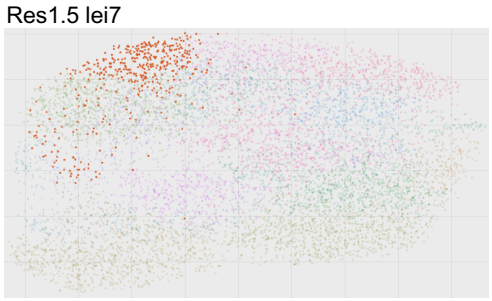
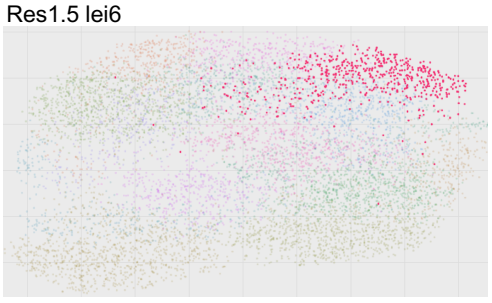
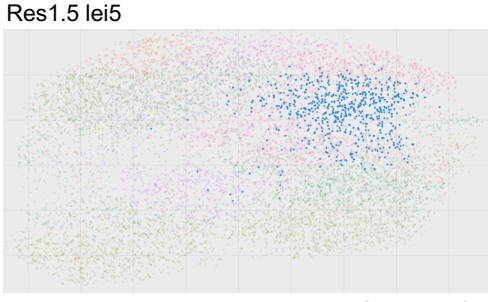




lei7 vs. lei6

Feature	Type	logFC	AUC	FDR
AREG	Gene	2.137	0.8666	4.15E-90
IL1RL1	Gene	0.9195	0.7635	1.82E-47



lei7 vs. lei5

Feature	Type	logFC	AUC	FDR
AREG	Gene	1.502	0.7903	5.21E-59
L1RL1	Gene	0.9605	0.7781	2.56E-54

Figure 15. scRNA sequencing of Tregs from low-dose influenza infection reveals impact of Treg-intrinsic TLR7 signaling. Experimental setup described in Figure 14. (a) Relative representation of each cluster in Foxp3^{cre}TLR7^{flox} compared to Foxp3^{cre}. (b) Clusters overrepresented in Foxp3^{cre} (lei7) or Foxp3^{cre}TLR7^{flox} (lei5,6) with select DEGs displayed.

signal to Tregs that damage has occurred and the repair program should be initiated. If true, TLR7-driven tissue repair by Tregs could be a general response to many infection and injury scenarios, and provide one of the missing links between tissue damage and repair mediated by Tregs.

If TLR7 on Tregs can act as a general sensor of tissue damage, we should be able to detect a similar defect in non-viral models of lung repair that we do following influenza infection. LPS-induced acute lung injury is a well-characterized model of damage where proper recovery is known to depend on Tregs, and any RNA ligands released as a result are necessarily endogenous.[66, 67] Therefore, we decided to subject the $Foxp3^{YFP\text{-}cre}TLR7^{flox}$ mice to intranasal LPS and measure the decline and recovery of lung function.

Similar to influenza infection, high doses of intranasal LPS like those used in previous publication caused severe rapid weight loss and decline in $\%SpO_2$ in both $Foxp3^{YFP\text{-}cre}$ and $Foxp3^{YFP\text{-}cre}TLR7^{flox}$ mice. To find a dose that resulted in a more mild degree of lung injury we conducted a dose titration on wildtype mice, and found that a range of approximately 0.25-1ug LPS per gram of body weight resulted in clear but less severe physiological decline. **(Fig. 16a).** With a proper dose range identified, we again compared the response of the $Foxp3^{YFP\text{-}cre}$ and $Foxp3^{YFP\text{-}cre}TLR7^{flox}$ mice. At 0.25ug/g and 0.5ug/g there was a trend of the $Foxp3^{YFP\text{-}cre}TLR7^{flox}$ losing more blood oxygen saturation, but not to the point of statistical significance. **(Fig. 16b).** When the dose was increased to either 0.75ug/g or 1ug/g the defect became more apparent, with the $Foxp3^{YFP\text{-}cre}$ mice maintaining significantly higher $\%SpO_2$ at days 2 and 3 post-treatment. **(Fig. 16c).** This result indicates that the damage caused by LPS, presumably in the form of RNA released from dying cells, is sensed by Tregs and causes them to facilitate tissue repair.

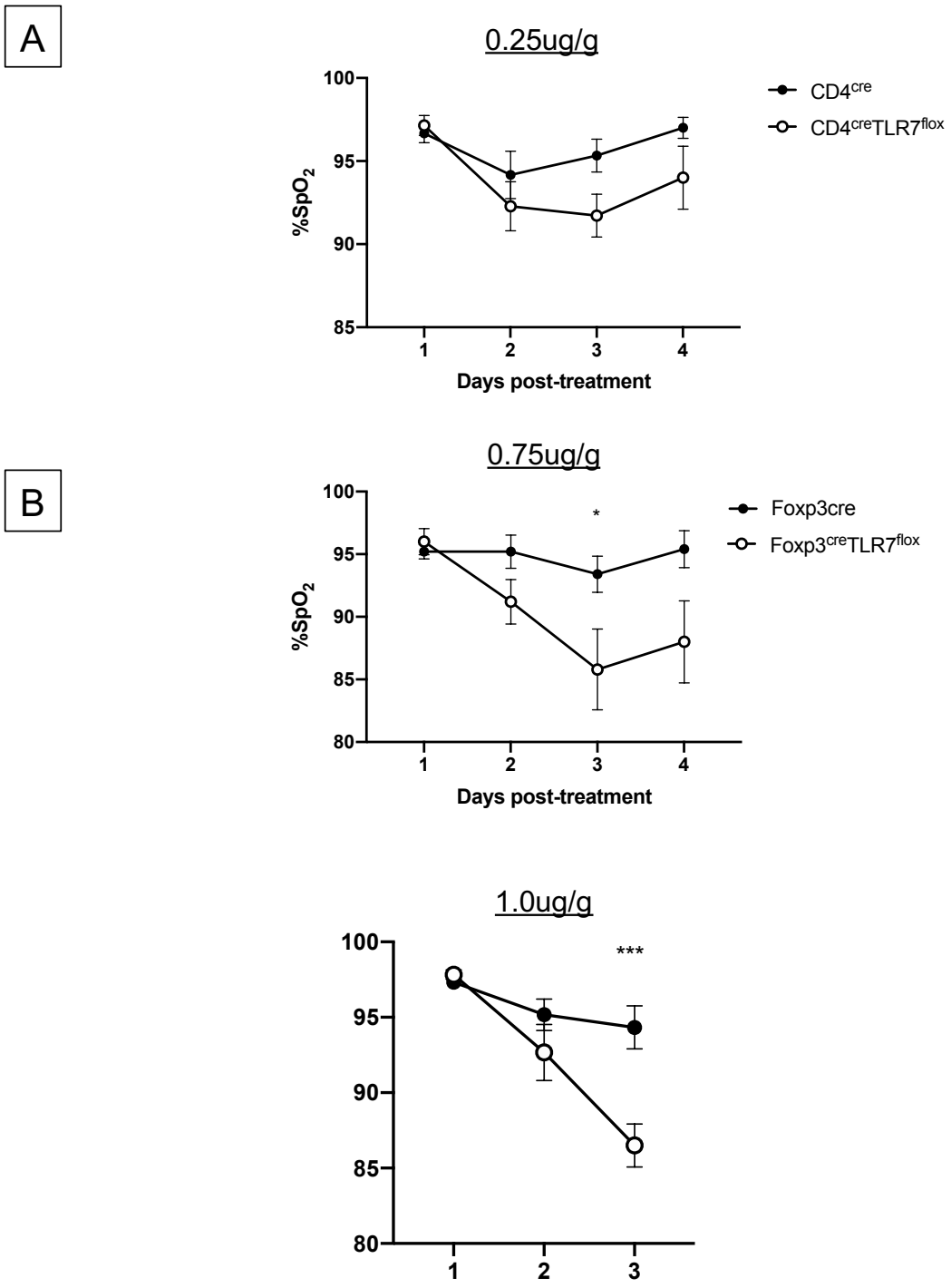


Figure 16. Intranasal LPS treatment expands role for Treg-intrinsic TLR7 signaling beyond viral infections. Foxp3^{cre} and Foxp3^{cre}TLR7^{flox} mice were intranasally treated with either 0.25ug/g (a), 0.75ug/g or 1.0ug/g LPS (b). Blood oxygen saturation (%SpO_2) was tracked for either 3 or 4 days post-treatment. *p<0.05, ***p<0.001 by 2-way ANOVA.

Chapter 5: TLR7/8 stimulation causes human Tregs to adopt tissue repair phenotype

Development of Treg-based adoptive cell therapies has grown increasingly prevalent in recent years, with more than 50 active or completed clinical trials as of 2019.[80] While most of these trials relate to the treatment of autoimmune conditions, there has also been interest in leveraging the tissue repair capacity of Tregs for treatment of human disease. A major barrier to the development of such treatments, however, is that there is no known way to reliably trigger human Tregs to adopt a tissue repair phenotype.[54]

The pathway most commonly associated with Treg tissue repair, as explained above, involves signaling through either IL-18R or ST-2. However, ST-2 is not expressed at meaningful levels on human Tregs located in blood, tonsils, synovial fluid, colon, or lung tissue.[54] This finding was extremely surprising given the prevalence of ST-2 on tissue-resident Tregs in mice. Furthermore, Tregs derived from human PBMCs fail to make amphiregulin in response to stimulation with either IL-18 or IL-33. Modest amphiregulin production can reportedly be transiently achieved upon TCR stimulation, or following transduction of human Tregs with ST-2 and subsequent stimulation with IL-33.[54] Any cell therapy seeking to take advantage of the tissue repair capacity of Tregs, therefore, would seem to 1) require ex vivo transduction of immune receptors that are not natively expressed by human Tregs, and 2) be unlikely to succeed given the low levels of amphiregulin produced following transduction and stimulation with IL-33.

A) TLR7/8 drives amphiregulin production by human Tregs

Our data from the $Foxp3^{GFP}TLR7^{KI}$ mice demonstrates that TLR7 is expressed on nearly all Tregs upon exit from the thymus, and that this expression is maintained on many Tregs in circulation. **(Fig. 3).** In contrast, IL-18R and ST-2 are not generally expressed by mouse Tregs in circulation. **(Fig. 8).** Based on this information we hypothesized that human Tregs derived from PBMCs might be more likely to express TLR7, and conceivably TLR8 (which is more functional in humans compared to mice), than IL-18R and ST-2. Although we do not have a TLR8 reporter mouse line, it is reasonable to expect its expression pattern to be similar to TLR7 given the fact that both recognize ssRNA. If our hypothesis proves true, TLR7 and TLR8 might represent a novel method for driving amphiregulin by human Tregs. To test this theory we obtained blood from 3 healthy human donors and isolated $CD4^+$ T cells to at least 97% purity. **(Fig. 17a).** The $CD4^+$ T cells were then stained with a proliferation dye and either left untreated, stimulated with 1ug/ml R848 (a ligand for both hTLR7 and hTLR8), or stimulated with 5uM CpG (ligand for human TLR9), with timepoints taken at Days 3, 4 and 6 post-treatment. CpG was included in an attempt to rule out, to the extent possible

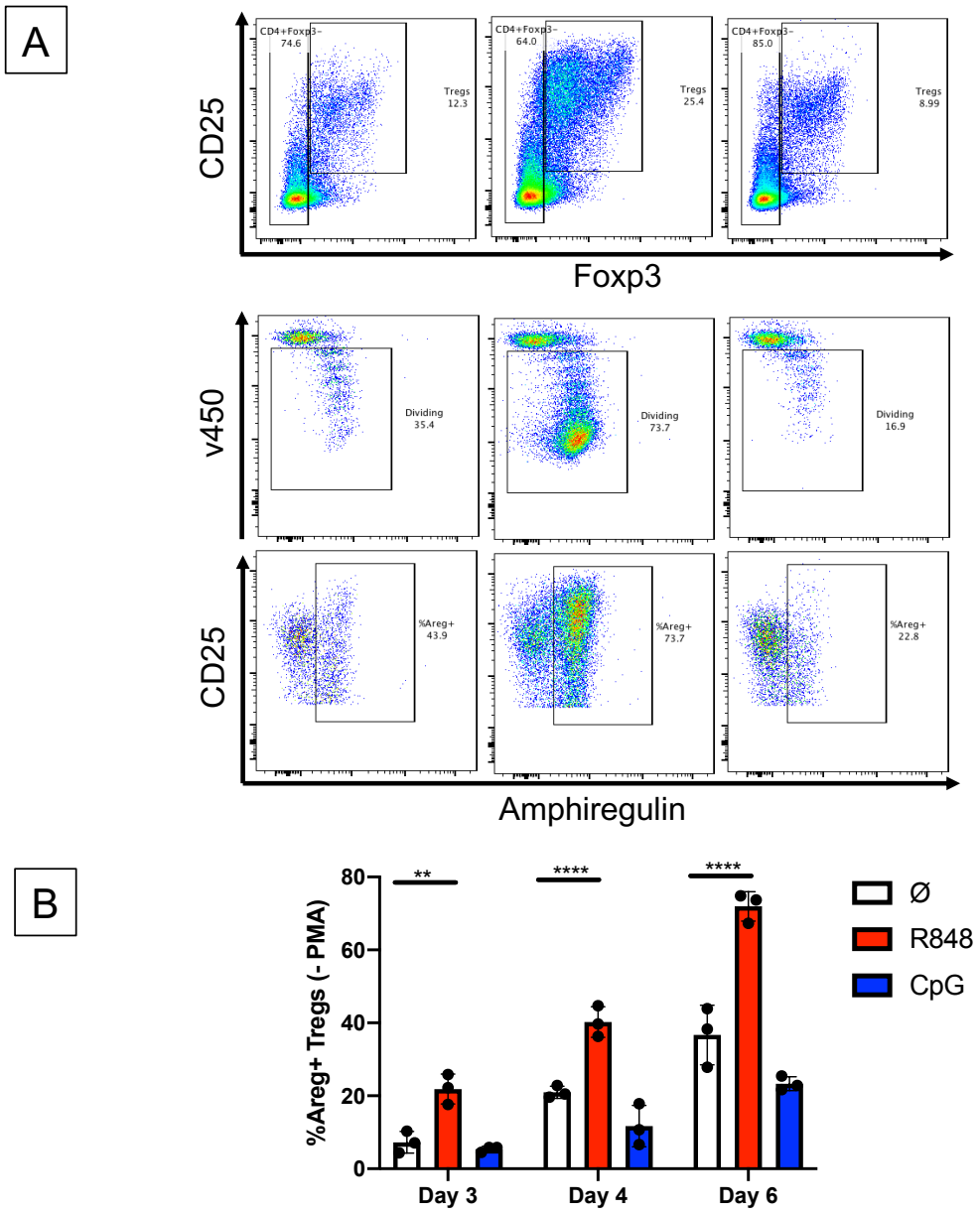


Figure 17. TLR7/8 signaling drives human Tregs to proliferate and produce amphiregulin. Blood from human donors was processed to obtained PBMCs, followed by isolation of $CD4^+$ T cells to >97% purity. Purified $CD4^+$ T cells were then cultured for either 3, 4, or 6 days in the presence of the indicated ligands. (a) FACS plots depicting size of Treg populations, along with proliferation and amphiregulin production by Tregs. (b) Summary graph of amphiregulin production by Tregs by day. **p<0.005; ***p<0.001; ****p<0.0001 by 2-way ANOVA.

in the absence of a genetic knockout, Treg-extrinsic effects from contaminating immune cells.

In samples treated with R848 the Tregs ($CD4^+Foxp3^+CD25^{high}CD127^-$) appeared to be responding in a manner similar to the wildtype mouse Tregs in Figure 7, with enhanced CD25 expression, significant proliferation, and substantial production of amphiregulin. **(Fig. 17b-d).** The kinetics of response from the different donors was somewhat variable, but the end result was the same - TLR7/8 on human Tregs results in the adoption of a tissue repair phenotype. In contrast, neither the unstimulated control nor the CpG-treated samples exhibited notable proliferation or amphiregulin production. This result extends our findings from mouse Tregs to human, and represents the first method of successfully inducing amphiregulin production in human Tregs.

B) Hyperresponsive TLR7 signaling on Tregs as proof of principle for cell-based therapy

Our newly-discovered method for stimulating amphiregulin production in human Tregs makes the development of clinical cell therapies for Treg-mediated repair of tissue damage more feasible. The most obvious method for adapting our findings to humans is to isolate Tregs from a patient, treat with TLR7/8 ligand to drive amphiregulin production, and reimplant the Tregs into the patient. A downside to this method, however, is that the Tregs only receive transient TLR7/8 signaling and then are essentially turned loose in the patient, where they may be subject to a variety of environmental factors that could reduce commitment to the tissue repair phenotype.

An alternative to ex vivo treatment with TLR7/8 is genetic modification of the Tregs to render them permanently more sensitive to TLR7/8 signaling. Previous members of our laboratory have characterized a point mutation in Unc93b1 (the “PKP mutation”), a protein responsible for trafficking and regulation of nucleic-acid sensing TLRs, that results in hypersensitive TLR7 responses.[16] When applied to Tregs the PKP mutation should cause enhanced TLR7 signaling, causing enhanced proliferation and amphiregulin production. In a clinical setting, one could imagine first editing a patient’s Tregs using CRISPR/Cas9 technology to introduce the PKP mutation. The hyperresponsive Tregs could then be transplanted into patients where endogenous TLR7/8 ligands would reinforce commitment to the tissue repair phenotype.

As proof of principle for the therapeutic applications using human Tregs, we sought to determine whether enhanced TLR7 signaling in Tregs actually leads to increased amphiregulin production and/or greater sensitivity to ligands. We therefore returned to the mixed *in vitro* stimulation assay to compare the relative responsiveness of wildtype and $Unc93b1^{PKP/WT}$ Tregs to TLR7 stimulus. This experimental setup is especially crucial when working with a mutation that causes TLR7

hyperresponsiveness, since any contaminating non-Tregs will be even more likely to make a strong response that will confound any attempt to identify a Treg-intrinsic outcome. It is also worth noting that we decided to use Unc93b1$^{PKP/WT}$ mice as the source of Tregs instead of Unc93b1$^{PKP/PKP}$, because mice of the latter genotype have widespread inflammation that might skew the development of Tregs in an unpredictable manner.[16]

The experimental setup was nearly identical to that described above, with spleens and inguinal lymph nodes removed from B6.SJL and Unc93b1$^{PKP/WT}$, followed by isolation of CD4^{+} T cells. The T cells of each genotype were then mixed 1:1, stained with a proliferation dye, and subjected to stimulation with either R848 or CpG-B for 3 days. We also tested a range of R848 doses from 1ug/ml (the concentration used in the B6.SJL versus TLR7^{KO} comparison) down to 1ng/ml, to account for the possibility that Unc93b1$^{PKP/WT}$ are more sensitive at lower doses. This range of doses proved to be important because both B6.SJL and Unc93b1$^{PKP/WT}$ Tregs produced amphiregulin, at roughly equal levels, in the 1ug/ml condition. **(Fig. 18a).** At lower concentrations of R848, however, the Unc93b1$^{PKP/WT}$ Tregs produced significantly more amphiregulin. **(Fig. 18b).** This result indicates that the Unc93b1$^{PKP/WT}$ mutation renders the Tregs hypersensitive, allowing for responses at concentrations of ligand that would not be sufficient to stimulate normal Tregs.

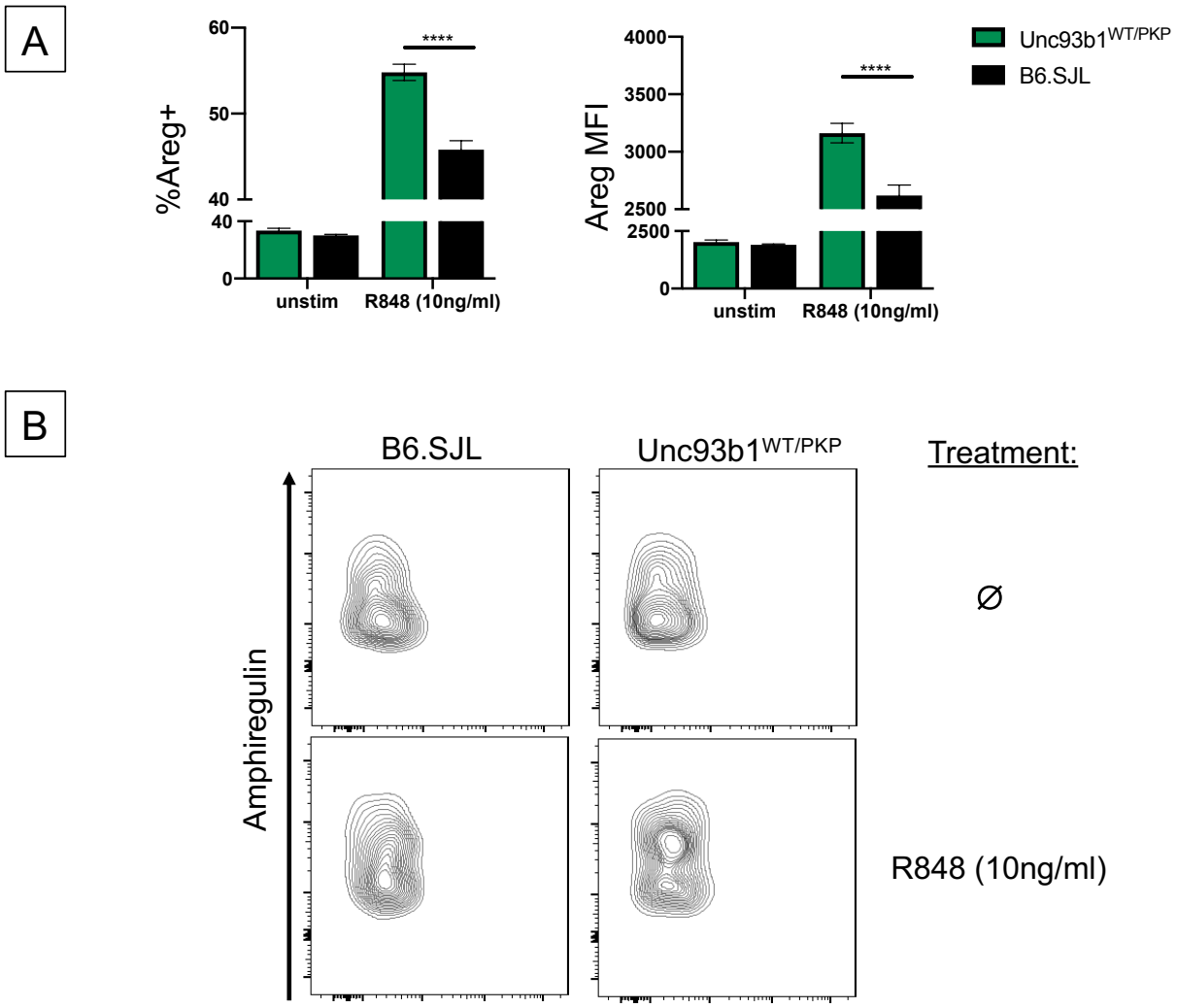


Figure 18. Hyperresponsive TLR7 signaling boosts amphiregulin production by Tregs. $CD4^+$ T cells were isolated from the spleens and inguinal lymph nodes of either B6.SJL (CD45.1) or $Unc93b1^{WT/PKP}$ (CD45.2) mice, and then mixed at a 1:1 ratio and cultured for 3 days under the indicated conditions. After 3 days, mixed cultures were subjected to FACS analysis for amphiregulin production (a). Representative FACS plots shown in (b). ****$p<0.0001$ by unpaired T test.

Chapter 6: Discussion and Future Directions

The data presented above demonstrate that TLR7 is expressed by T cells, and that signaling by TLR7 on Tregs leads to amphiregulin production along with adoption of a tissue repair phenotype. TLR7-mediated tissue repair by Tregs is required for proper recovery from lung damage resulting from influenza infection and intranasal LPS treatment. We have also shown that this pathway functions in a similar manner in human Tregs, with TLR7/8 stimulation driving amphiregulin production. Each of these findings is extremely exciting and represent significant advances in the field, but there are nevertheless numerous additional questions to address.

A) Relative contributions of TLR7, IL-18R and ST-2 to Treg-mediated tissue repair.

While it appears that the MyD88 signaling pathway is the critical driver of amphiregulin by Tregs in response to tissue damage, the relative importance of TLR7, IL-18R and ST-2 is still unclear. Data from our TLR7^{KI} mice suggests that these receptors are generally not expressed on Tregs at the same time, so spatial and temporal variables may provide part of the answer. TLR7 is expressed on most Tregs as they leave the thymus, while IL-18R and ST-2 are much more prevalent on activated Tregs in non-lymphoid tissues. Furthermore, expression of IL-18R and ST-2 varies dramatically between different non-lymphoid tissues, which could provide a clue to their importance in different anatomical locations. Perhaps TLR7 acts as a "default" sensor of tissue damage, while IL-18R and ST-2 are expressed to sense more specific damage signals such as inflammasome activation and damage to muscle or epithelial cells, respectively. These possibilities can be investigated by testing the performance of Tregs from our Foxp3$^{YFP\text{-}cre}$ and Foxp3$^{YFP\text{-}cre}$TLR7^{flox} mice in response to different types of damage, alongside Treg-specific deletions of IL-18R and ST-2. The IL-18R and ST-2 floxed alleles have already been created and would only need to be crossed to the Foxp3$^{YFP\text{-}cre}$ strain to perform these experiments. The amphiregulin floxed allele has also been offered to us by the Arpaia laboratory at Columbia and could serve as an optimal control for Treg-mediated repair. With the proper mice in hand, intranasal influenza, intranasal LPS, cardiotoxin-induced muscle damage, and obesity-driven adipose tissue inflammation would all be informative models to attempt, as they each require one of the MyD88 receptors in Tregs to achieve proper recovery.

B) Anticorrelation of activation status with TLR7 expression

A related open question is why TLR7 is expressed by nearly all naive Tregs, only some activated Tregs, and almost no Tregs in certain non-lymphoid tissues. It is likely that the answer to this question dovetails in some unappreciated manner with the fact

that IL-18R and IL-33R are only present on activated Tregs in certain anatomical locations. The anti-correlation between TLR7 expression and activation status suggests that in some circumstances it is not beneficial for activated Tregs to have the option to undergo TLR7-mediated production of amphiregulin. Since TCR stimulation on Tregs generally facilitates suppressive mechanisms, we have considered that the "repair" and "suppressive" states of Tregs might be mutually exclusive, or at least not completely compatible. We sought to test this hypothesis in an experimental model of induced colitis, where $CD4^{+}Foxp3^{-}$ T cells were transferred into Rag^{KO} mice along with either wildtype or $TLR7^{KO}$ Tregs. If the repair and suppressive programs are mutually exclusive, the $TLR7^{KO}$ Tregs should be better able to suppress colitis. Unfortunately none of the mice in this experiment developed colitis, including an experimental group that received no Tregs, indicating that the microbiota present in our colony does not promote induction of colitis. (data not shown). We have also contemplated testing our $Foxp3^{YFP\text{-}cre}TLR7^{flox}$ mice in a model of experimental autoimmune encephalitis (EAE), to see whether loss of TLR7 on Tregs boosts suppressive capacity. This experiment has not been performed to date. Finally, we have designed but not yet created mice where TLR7 is inserted into the Rosa26 locus following a lox-stop-lox cassette, such that crossing to a $Foxp3^{cre}$ would yield mice where the Tregs are incapable of downregulating TLR7. If TLR7 expression is at odds with suppression in some circumstances, one might predict that these mice would develop detectable signs of autoimmunity.

C) Identification of ligands and how they reach endosome of Tregs

There are a long list of examples of ligands that are capable of initiating TLR7 signaling, but no evidence as to the identity of the genuine *in vivo* ligands. As noted in the introduction TLR7 can be activated by extremely small pieces of ssRNA, in some cases even nucleosides. Other groups have provided clear evidence that endogenous ssRNA can drive TLR7-mediated autoimmunity, and we have now shown that host ssRNA released during tissue damage can be sensed by Tregs via TLR7. Additional preliminary experiments have shown that the ssRNA does not need to be derived from damage, as $TLR7^{WT}$ Tregs outcompete $TLR7^{KO}$ Tregs when co-transferred into Rag^{KO} mice. In other words, there does not seem to be any limitation on the sequence of endogenous that is necessary to trigger TLR7 signaling. Perhaps TLR7 really is capable of sensing almost any endogenous RNA sequence, and this is why it has been preserved as a sensor of tissue damage on Tregs when expression of all other TLRs has been lost. It is interesting to consider that this sensitivity and lack of sequence specificity may explain the web of regulatory mechanisms designed to keep aberrant TLR7 signaling from occurring, the breakdown of which invariably leads to autoimmunity.

We also recognize that Tregs are not phagocytic cells like macrophages or dendritic cells, so it is not immediately clear how ligands reach the endosome to encounter TLR7. Our hypothesis is that the ligands for TLR7 are so small that they are internalized through either pinocytosis or the process of receptor internalization and recycling. There also may be transporters or channels present which facilitate the transport of RNA molecules across the membrane of Tregs.

D) Therapeutic applications of tissue repair Tregs

TLR7/8 signaling drives amphiregulin production by human Tregs, which represents the only known method of initiating the repair program in human Tregs short of artificially transducing ST-2. Can knowledge of this new pathway be utilized for the benefit of human health? There are multiple barriers to entry before this question can be properly addressed. First, there is no evidence that *ex vivo* treatment to generate tissue repair Tregs followed by retransplantation has any beneficial impact on tissue repair, even in murine systems. The initial step towards translating our findings into a treatment for humans would be to establish the benefit of such a treatment in mice. We plan to utilize $Foxp3^{GFP\text{-}DTR}$ mice which are depleted of all Tregs when treated with diphtheria toxin. The depleted Tregs could then be replaced with either $Unc93b1^{WT/PKP}$ (TLR7 hyperresponsive), $TLR7^{WT}$ or $TLR7^{KO}$ Tregs, which would be tested for their ability to contribute to tissue repair following influenza infection. This experiment would be proof of principle that enhanced TLR7 signaling in transferred Tregs can be beneficial in recovery from tissue damage. A similar experiment where Tregs are treated *ex vivo* with TLR7 ligand before transplantation will also likely be attempted, to compare a stronger but transient signaling event with a more physiological response triggered by *in vivo* ligands. Even if these initial experiments in mice are successful, we would need to consider how such a treatment might be beneficial in humans. Although a number of companies are working on cell therapies involving Tregs, we are not aware of any clinical trials currently attempting to take advantage of the tissue repair capacity of Tregs. We would need to consult with clinicians on the best indications to test such a therapy in humans. Severe viral infections or chronic inflammation that impact the lung are obvious candidates, but the ability of TLR7 to drive Treg-mediated repair more generally opens the door to treatment of a wide range of conditions.

Chapter 7: Materials and Methods

Mice: TLR7^{flox} mice were generated using CRISPR/Cas9 with the assistance of the UC Berkeley Cancer Research Lab as described above. The TLR7^{flox} line was bred to Foxp3^{cre} (Jackson Labs 016959) or CD4^{cre} (Jackson Labs 022071) to generate Treg-specific or T cell-specific deletions of TLR7. MyD88^{flox} (Jackson Labs 008888), B6.SJL (Jackson Labs 002014) and C57/BL6 (Jackson Labs 000664) were initially purchased from Jackson Labs and maintained in our colony. The Unc93b1^{PKP} mice were originally generated by Drs. Olivia Majer and Bo Liu from our laboratory and previously published. The TLR reporter mice were generated by Drs. April Price, Allison Roberts and Bettina Lee, and are now available from Jackson Labs. Foxp3$^{GFP\text{-}DTR}$ mice (Jackson Labs 016958) were crossed to the TLR7 reporter mice to generate the Foxp3^{GFP}TLR7^{KI} mouse line. All mouse husbandry and experiments were performed in accordance with protocols approved by the UC Berkeley Animal Care and Use Committee.

Flow cytometry: Desired tissue were harvested from mice, mashed through a 70um filter, centrifuged for 5 minutes at 1600rpm, followed by ACK lysis for 3-4 minutes. Cells were then resuspended in FACS buffer, treated with Fc Block (CD16/CD32) and Live/Dead dye (generally Thermofisher Live/Dead Fixable Aqua) for 10 minutes. Samples were then stained with a master mix of antibodies per the requirements of the experiment. Antibodies used include IL-18R PerCP (Invitrogen P3TUNYA), bv785 CD4 (Biolegend GK1.5), bv711 CD3e (Biolegend 145-2C11,) bv650 CD25 (Biolegend PC61), CD8 Pacific Blue (Invitrogen 53-6.7), IL-33R/ST-2 biotin (MD Bioproducts DJ8), APC-Cy7 CD62L (BD MEL-14). Intracellular staining for Foxp3 (FJK-16s) and amphiregulin (R&D BAF989) was performed following treatment of samples with eBioscience Intracellular Fixation & Permeabilization Buffer Set. Biotinylated amphiregulin antibody required secondary intracellular staining with streptavidin. Amphiregulin detection was performed following *ex vivo* incubation of 2.5-5 hours with Golgi Plug or Golgi Plug+PMA+ Ionomycin.

Detection of lung-resident cells was accomplished by injecting mice I.V. with anti-CD45 (Biolegend 30-F11) antibody 2 minutes prior to the initiation of euthanasia. 15ul antibody + 85ul sterile PBS was given to each mouse. Lung-resident cells are those isolated from lung tissue that are not stained with the anti-CD45 antibody.

Murine *in vitro* T cell stimulation: Spleen and inguinal lymph nodes from mice of desired genotype were harvested and combined, then processed using same procedure noted in Flow Cytometry section. Following ACK lysis, cells were washed and incubated with Miltenyi CD4 (L3T4) or CD8 (Ly-2) MicroBeads for 15 minutes at 4°C. Cells were washed again and run through a magnetic Miltenyi LS Column to isolate desired T cell

population T cells, after which a cell count was performed. If a mixed stimulation of different genotypes was to be performed, T cells from the two samples would be combined at this point. Final cell suspensions were then stained with proliferation dye, either BD Violet Proliferation Dye 450 or Thermofisher CellTrace CFSE. Cells were then plated in a 96-well tissue culture-treated at 100-200 x 10^5 cells per well. All samples received 50U/ml mIL-2. R848 treatment was 1ug/ml unless otherwise stated, CpG-B treatment was 1uM. When used, anti-CD3 and anti-CD28 were used at 1ug/ml and pre-coated on plate for 2 hours at 37°C prior to start of stimulation.

In vitro Treg suppression assay: $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ Tregs were isolated from spleens of mice using either FACS sorting or Miltentyi Columns as noted above. $CD4^+CD25^-$ cells were stained with proliferation dye and plated at 100 x 10^5 cells per well with varying amounts of $CD4^+CD25^+$ Tregs in a ratio ranging from 1:1 to 16:1. All samples received 1ug/ml anti-CD3. When applicable, R848 was used at a concentration of 1ug/ml.

Human *in vitro* T cell stimulation: Donor blood samples were ordered from AllCells, and $CD4^+$ T cells isolated using StemCell EasySep Human CD4+ T Cell Isolation Kit. Isolated T cell were then plated at 200 x 10^5 cells per well in a 96-well plate. R848 was used at 1ug/ml and CpG-B at 5uM. All samples received 100U/ml IL-2.

Intranasal influenza infection: A stock of PR8 H1N1 influenza was a gift from the Arpaia laboratory at Columbia University. Upon receipt, individual use aliquots were immediately made and frozen at -80°C to avoid freeze/thaw cycles. Immediately prior to treatment of mice, aliquots were thawed and diluted in sterile PBS such that desired dose was contained in 40ul volume. Mice were then anesthetized with isoflurane and treated intranasally with the 40ul dose.

Intranasal LPS treatment: Ultrapure LPS (Invivogen LPS-EB Ultrapure) was diluted in sterile PBS such that the appropriate dose could be given in a volume of ~35-60ul. Mice were then weighed to determine the precise volume for each individual. Mice were then anesthetized with isoflurane and treated intranasally.

Pulse oximetry: Blood oxygen saturation (%SpO_2) was determined using MouseOx Plus Pulse Oximeter. Prior to intranasal infect or treatment, hair around neck of mice was removed using a combination of shaving and depilatory cream. For each measurement, mice were placed in an enclosure with MouseOx collar properly affixed to neck. Multiple %SpO_2 readings were taken for each mouse, whenever possible waiting for at least 5-10 seconds of consistent readings.

RNA sequencing and scRNAseq: Tregs to be sequenced were sorted on the FACS Aria using Live/Dead dye, TCRb, CD4 and CD25 antibodies, along with Foxp3$^{YFP\text{-}cre}$ fluorescence. Anti-CD45 pre-euthanasia injection was also used to excluded Tregs in the vasculature. For bulk sequencing, RNA was manually extracted using Trizol and chloroform. Samples were then submitted to UC Berkeley FGL for Bioanalyzer quality check, library preparation and sequencing. For scRNAseq, sorted Tregs were given directly to UC Berkeley FGL for 10X Genomics library preparation and sequencing. Processing and analysis of scRNAseq data was performed by Allon Wagner from Dr. Nir Yosef's laboratory. Cluster analysis was performed using the Vision web-based browser developed by the Yosef laboratory.

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