UNIVERSITY OF CALIFORNIA, SAN DIEGO

Toll-like Receptor 4 Signaling on CD4+ T cells Plays a Regulatory Role in Inflammatory Bowel Disease

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by

Jason Kumhung Law

Committee in charge:

Professor Eyal Raz, Chair
Professor Stephen Hedrick, Co-Chair
Professor Michael David

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Chair

University of California, San Diego
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ABSTRACT OF THE THESIS

Toll-like Receptor 4 Signaling on CD4+ T cells Plays a Regulatory Role in Inflammatory Bowel Disease

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Jason Kumhung Law

Master of Science in Biology

University of California, San Diego, 2010

Professor Eyal Raz, Chair

Professor Stephen Hedrick, Co-Chair

Toll-like Receptors (TLRs) are a set of pathogen recognition receptors which are well characterized in dendritic cells and macrophages as important mediators of the innate immune system. With TLRs being recently identified on T cells, we show that TLR4 stimulation on CD4+ T cells regulates their ability to provoke intestinal inflammation. Using the IL-10 deficient model of colitis we show that *Il10*$^{-/-}$*/Tlr4*$^{-/-}$ mice develop spontaneous colitis as early as 8 weeks while *Il10*$^{-/-}$ mice do not develop colitis even by 8 months. Similar results were obtained using the adoptive transfer model of colitis in which we transferred *Il10*$^{-/-}$*/Tlr4*$^{-/-}$ or *Il10*$^{-/-}$ naïve CD4+ T cells into *Rag1*$^{-/-}$ recipients. *Il10*$^{-/-}$*/Tlr4*$^{-/-}$ naïve CD4+ T cells induced more colitis than their

Il10$^{-/-}$ counterparts. Mechanistically, LPS stimulation of TLR4 induces MKP-3 which inactivates ERK, resulting in less potent T cell activation. Collectively, our data suggests that TLR4 signaling plays an inhibitory role on subsequent T cell receptor (TCR) signaling and ultimately regulates activation of CD4+ T cell.

1. Introduction

1.1 Inflammatory Bowel Disease: Clinical Pathology

Inflammatory bowel disease (IBD) consists of a group of diseases which causes inflammation and ulceration in the small and/or large intestine. This disease currently affects approximately 1.4 million Americans. The two main forms of IBD are ulcerative colitis (UC) and Crohn's disease (CD). Ulcerative colitis results in inflammation of the colon in an uninterrupted pattern (Abraham and Cho, 2009), and is usually only limited to the mucosa and leads to rectal bleeding and diarrhea (Sandborn, 2008). Crohn's disease on the other hand is characterized by mucosal ulceration and transmural inflammation of the distal small intestine and/or colon. CD usually results in patches of inflammation or "skip lesions" over the small and/or large intestine (Abraham and Cho, 2009). Over time, CD can cause the formation of fistulas and abscesses.

1.2 Inflammatory Bowel Disease: Predisposition

The specific cause for IBD is currently unknown. However, there is a plethora of evidence suggesting both genetic and environmental factors contribute to both CD and UC. Both factors point to aberrant interactions between the host and the commensal bacteria found throughout the colon (Sandborn, 2008). An over aggressive immune response from the host to the commensal flora found in the colon is implicated as the cause of IBD. Whether this over activation of immune cells is due to a lack of regulation or an over activation of immune cells is still unknown. Some

genetic factors associated with IBD include genes encoding for interleukin-10 (IL-10) and interferon-γ (IFN-γ), which will both be discussed later. Environmental factors such as the use of nonsteroidal anti-inflammatory drugs, which alter the intestinal barrier, can increase the risk of IBD. Conversely the use of antibiotics and probiotics have beneficial effects for specific subgroups of patients (Sandborn, 2008) showing the relationship between the bacteria and colon homeostasis.

1.3 Disregulation of Colon Homeostasis

Starting from birth, an intestinal microbiota is acquired and changes rapidly throughout the first year of life but remains relatively stable afterwards. This population of bacteria contains at least 395 different bacterial phylotypes and colonizes the colon at an estimated 10^{11} bacteria per gram of colon content (Zenewicz et al., 2009). The microbiota supplies the host with nutrients, aids in the development of the intestinal immune system and modulates energy metabolism (Backhed et al., 2005). Because the microbiota is in constant contact with the host's immune cells found in the intestinal lamina propria, the lamina propria contains a variety of immune cells which balance the need for tolerance to the commensal flora and the need to protect the host from invading pathogens. Genetic defects which result in the lack of tolerance toward the gut flora or disruption can result in IBD.

1.4 The role of CD4+ T cells in IBD

There are currently four identified subtypes of CD4+ T cells. These four subtypes consist of type 1 helper T cells (Th1), type 2 helper T cells (Th2), regulatory

T cells (Tregs) and the more recently discovered type 17 T helper T cells (Th17). Naïve CD4+ T cells, T cells which have not encountered their specific antigen can be differentiated into one of the four T cell subsets. This requires stimulation of the T cell receptor (TCR) and engagement in a cytokine milieu. While patients with CD predominantly have both Th1 and Th17 T cells subsets, those with UC predominantly have both Th2 and Th17 T cell subsets. The Th1 subset of T cells can be identified with lineage-specifying transcription factor T-bet, and its overexpression results in the production of the prototypic Th1 cytokine, IFN-γ (Dong and Flavell, 2000). In vitro it has been shown that both the cytokines Interleukin-12 (IL-12) and IFN-γ, initially produced by macrophages elicits the activation of T-bet (Di Cesare et al., 2009). IFN-γ plays an important role in the clearance of viruses and intracellular bacteria through the activation of macrophages and CD8+ T cells to phagoctyose or lyse infected cells, respectively. However, when the adaptive immune system is disregulated, excessive amounts of IFN-γ are produced and this causes excessive inflammation and infiltration of immune cells into the submucosa of the colon. Hence in both patients with CD (Hommes et al., 2006) and more prominently in certain murine models of colitis (Powrie et al., 1994) the blocking of IFN-γ with neutralizing antibodies can improve colitis. The other T cell subset, Th17, involved in CD produces the prototypic cytokines, IL-17A and IL-17F along with other cytokines such as IL-21 and IL-22. IL-17A is implicated in neutrophil recruitment to the site of infection for pathogen clearance (Korn et al., 2009). Because the induction of one T cell subset inhibits the differentiation of other T cell subsets, there has been a lot of debate over whether Th1 or Th17 is primarily responsible for CD. Although there is no definite answer yet, it

seems that both play a critical role in CD. As for murine models, the contribution of each subset of T cells is dependent on the model of colitis utilized.

1.5 The IL-10 Deficient Model of Experimental Colitis

IL-10 is an anti-inflammatory cytokine which is important for preventing inflammatory and autoimmune pathologies (Saraiva and O'Garra). IL-10 is able to downregulate the expression of costimulatory markers and the production of pro-inflammatory cytokines in dendritic cells and macrophages (Roncarolo et al., 2006). Furthermore it can also prevent antigen presenting cells from inducing a Th1 response while increasing the effectiveness and proliferation of Tregs which also produce Il10, allowing for a positive regulatory loop (Roncarolo et al., 2006). Because of the essential role IL-10 plays in limiting inflammatory responses, mice lacking IL-10 spontaneously develop colitis. This colitis is characterized as having excessive regenerative hyperplasia of the mucosa causing a thickening of the intestinal wall. The crypts in the colon are also abnormal as they are enlarged and branched. This is compounded with excessive lymphocyte, plasma cell, macrophage, neutrophil infiltration into the lamina propria (Kuhn et al., 1993). This model of colitis is dependent on the interaction of the immune system and the intestinal flora in the gut as *Il10*$^{-/-}$ mice in germ free conditions do not develop colitis (Sellon et al., 1998). The essential role of IL-10 secretion from CD4+ T cells in the prevention of spontaneous colitis in *Il10*$^{-/-}$ mice has been shown using CD4+ T cell specific *Il10*$^{-/-}$ mice which develop spontaneous colitis similar to that found in *Il10*$^{-/-}$ mice (Roers et al., 2004).

1.6 The Adoptive Model of Colitis

The adoptive transfer model of colitis is another CD4+ T cell dependent model which is used to delineate the specific role of CD4+ T cells in colitis. In this model, naïve CD4+ T cells ($CD4^{+}CD25^{-}CD45RB^{high}$) from a donor mouse are injected into mice lacking adaptive immune cells such as *Rag1*$^{-/-}$ mice. When these T cells are presented with bacterial antigens from antigen presenting cells (APCs) from the commensal flora in the gut, they are activated but do not maintain tolerance to the flora because of the lack of natural Treg cells (Aranda et al., 1997). The colitis has been shown to be mediated through induction of Th1 phenotype with the subsequent overexpression of IFN-γ found in the colon (Read and Powrie, 2001).

1.7 Involvement of Toll-like Receptors

Toll-like receptors (TLRs) are pattern recognition receptors which recognize common bacterial and viral components. Traditionally TLRs are associated only with the innate immune system. Activation of TLRs causes dendritic cells and macrophages to upregulate costimulatory molecules and release proinflammatory cytokines allowing them to prime T cells (McAleer and Vella, 2008). More recently however, it has been shown that T cells also express TLRs (Fukata et al., 2008). Specifically, TLR4, which is shown to be present on CD4+ T cells respond to lipopolysaccharide (LPS), a common component of the cell wall of gram-negative bacteria. Although extensive work has not been done in this field, it has been shown that activation of certain TLRs in CD4+ T cells has been shown to increase survival and act as

costimulatory molecules along with TCR activation (Gelman et al., 2004). There has also been evidence showing that activation of specific TLRs such as TLR2 can trigger cytokine release from mature T cells (Imanishi et al., 2007). However, the role of TLR4 on effector T cells has not been shown.

1.8 Signaling Pathway of TLR4

In order for TLR4 to detect bacterial LPS, several proteins including LPS binding protein (LBP), CD14 and MD-2 must interact with each other before TLR4 signaling can occur. Initially LPS binding protein (LBP), a soluble protein, must bind to LPS. LBP aids in the association of LPS to CD14, which is a soluble glycosylphosphatidylinositol-anchor protein. This complex is then transferred to the TLR4/MD-2 complex. MD-2 is a soluble protein which aids in the adhesion of the two complexes. Upon binding, TLR4 undergoes oligomerization and recruits its downstream adaptors (Lu et al., 2008). TLR4 signaling recruits two pairs of adaptor molecules using its Toll-interleukin-1 receptor (TIR) domain found on its cytoplasmic face to elicit its effects. The more studied pair of adaptor molecule consist of the myeloid differentiation protein 88 (MYD88) and TIR domain-containing adaptor protein (TIRAP). Their binding to TLR4 results in the activation of the mitogen-activated protein kinases (MAPKs) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). The other pair of adaptor molecules are TIR-domain-containing adaptor-inducing interferon-β (TRIF) and TRIF related adaptor molecule (TRAM), results in the activation of the interferon regulatory factors (IRFs) as well as the MAPKs and NF-κB (Kenny and O'Neill, 2008). The MAPKs are a group of

cytoplasmic kinases which phosphorylate downstream targets ultimately leading to activation of transcription factors that initiate an inflammatory cascade. As a member of the MAPK family, extracellular signal-related kinase (ERK), has been shown to be over-expressed and found in its phosphorylated active form in immune cells in IBD patients (Broom et al., 2009). This activation is suggested to influence the inflammatory process of IBD patients. The other two MAPKs, JNK (c-Jun N-terminalkinase) and p38 are also implicated in the inflammatory process and inhibition of either of these MAPKs have been shown to reduce the production of pro-inflammatory cytokines (Broom et al., 2009). Finally, NF-κB is a nuclear transcription factor which is also highly expressed in colons of patients with IBD. In unstimulated cells, NF-κB remains as an inactive dimer in the cytoplasm and retained in the cytoplasm with three small inhibitory molecules, IκBα and IκBβ. Upon stimulation of cells with LPS which activates TLR4, IkB kinases (IKK) will be activated and phosphorylate IkB and target it for degradation, allowing for NF-κB to enter into the nucleus (Atreya et al., 2008). In the nucleus, NF-κB targets a variety of proinflammatory genes such as IFN-γ and TNF-α (Pahl, 1999).

1.9 T-cell Receptor Signaling Cascade

TCR stimulation occurs when an antigen presenting cell presents a peptide recognized by a specific T cell. This recognition along with stimulation by costimulatory markers found on the APC to the T cell initiate an immune response. This stimulation can be mimicked using αCD3 antibodies for the TCR and αCD28 antibodies for the costimulatory receptor. This results in activation of downstream

pathways, many which are shared with TLR4 such as MAPKs (Cronin and Penninger, 2007; Rincon et al., 2000), and NF-κB (Burbach et al., 2007). There are also pathways which do not overlap with the TLR4 signaling cascade such as the NFAT pathway (Burbach et al., 2007). Ultimately these pathways lead to the activation of T cells signified through their production of IL-2 and other proinflammatory cytokines along with upregulation of costimulatory molecules (Fietta and Delsante, 2009).

1.10 Regulation of MAPK signaling

The MAPK signaling cascade is activated through phosphorylation on multiple levels. The cascade has a three tier level of activation. With the MAPKKKs phosphorylating the MAPKKs which finally phosphorylate the MAPKs (Turjanski et al., 2007). In order for ERK1/2 to be activated, MEK1 and MEK2, both of which are MAPKKs, are necessary as they are upstream dual specificity kinases that are capable of phosphorylating both the tyrosine and threonine residues in the ERK1/2 activation loops allowing for ERK activation (Raman et al., 2007). In order to inactivate MAPKs, phosphoprotein phosphatases are used to remove the phosphates from the activation loop. Phosphoprotein phosphatases which are capable of removing both threonine and tyrosine residues are known as dual specificity phosphatases (DUSPs). Of these DUSPs, those which act selectively on the MAPKs are known as MAPK phosphatases (MKPs) (Liu et al., 2007). These MKPs are different from each other in that they have different localizations and different preferences for their targets. Specifically, MKP-3 is found in the cytosol and has a preference for ERK over the other two MAPKs. On the other hand, MKP-1 is found in the nucleus and has a

preference for P38 and JNK. Although not much work has been done on MKPs in T cell biology, there have been numerous papers documenting their expression in T cells (Jeffrey et al., 2006; Marti et al., 2001; Zhang et al., 2009).

1.11 Hypothesis

With the observation that *Il10*$^{-/-}$/*Tlr4*$^{-/-}$ mice develop more severe colitis than *Il10*$^{-/-}$ mice, we hypothesize that TLR signaling can regulate subsequent TCR activation since they share the same signaling pathways (ie. MAPKs). Furthermore, we believe that this phenotype is due to CD4+ T cells and not innate immune cells because of their essential nature in the *Il10*$^{-/-}$ model of colitis.

2. Materials and Methods

2.1 Mice

Six to ten week old mice were used for all experimental procedures. C57BL/6J (B6) mice were purchased under specific pathogen-free conditions from Harlan. *Rag1*$^{-/-}$, *Il10*$^{-/-}$, and OT-II mice on a B6 background were purchased from The Jackson Laboratory and subsequently bred in our vivarium under specific pathogen-free conditions. *Tlr4*$^{-/-}$ and *Tlr9*$^{-/-}$, *MyD88*$^{-/-}$ mice were provided by S. Akira (Osaka University, Osaka, Japan), and *LPS-2* mice on a B6 background were provided by B. Beutler (The Scripps Research Institute, San Diego, California, USA). Both TLR4 and TLR9 deficient strains were backcrossed to *Il10*$^{-/-}$ mice for 10 generations in order to generate double-KO mice. All experimental procedures were performed in accordance with IACUC of UCSD.

2.2 Reagents

LPS was isolatated from *Salmonella Minnesota* Re595 using the protocol described in (Mathison et al., 1988). The ERK specific inhibitor UO126 was purchased from Promega. Chicken OVA and dual specificity phosphatase inhibitor NSC 95397 were purchased from Sigma-Aldrich. CD3, CD4 and TLR4 antibodies (MTS510) for FACs analysis was purchased from eBioscience.

2.3 Evaluation of Colitis

The entire colon was excised, opened longitudinally and then feces were removed. The colon was then rolled onto a wooden stick and fixed in Bouin's solution overnight and transferred into 70% ethanol the following day. The samples were later embedded in parafilm. Five micrometer tissue sections were prepared, deparaffinized, and stained with H&E. Inflammation was evaluated based on crypt length and inflammatory cell infiltration. Crypt length was measured based on the average of the two longest crypts in two different samples. Inflammatory cell infiltration was determined on manual cell counting of high magnification and normalized to every mm^2 of tissue.

2.4 Culture and Stimulation of CD4+ Cells

RP10 media consisting of RMPI 1640 medium from Irvine Scientific supplemented with 10% heat-inactiaved FCS (Life Technologies), 2 mM L-glutamine (Cellgro), 100 U/ml penicillin, and 100 μg/ml streptomycin (Cellgro) was used to culture the cells. CD4+ T cells were isolated from the spleen and mesentery lymph nodes using CD4+ isolation kits from Miltenyi Biotec. Enriched FACS sorted CD4+ T cells were further isolated through staining with anti-CD3 and anti-CD4 monoclonal Abs from eBioscience. They were then sorted with a MoFlow Cytometere equipped with Summit Software from Dako. Purity of the CD4+ T cell population was over 99% for all experiments. The sorted T cells were then cultured in RP10 medium with 5 μg/ml plate bound anti-CD3 antibody along with 1μg/ml soluble anti-CD28

antibody. Supernatants were collected twenty-four hours later and cytokines were analyzed by ELISA (eBioscience). All ELISA experiments were run in accordance with the provided protocol. In experiments requiring LPS prestimulation, cells were treated with 100 ng/ml of LPS or left untreated in complete RMPI 1640 media for 2 hours. Afterwards, they were stimulated with 5 μg/ml of plate bound anti-CD3 antibody and 1 μg/ml anti-CD28 antibody.

For the inhibition of ERK1/2 phosphorylation, splenic CD4+ T cells from *Il10*$^{-/-}$ mice were incubated for 30 minutes with 10 μM UO126 or DMSO (vehicle) before stimulation with anti-CD3 and anti-CD28 antibodies. Cells were then collected at different time points and lysed for RNA and analyzed with quantitative RT-PCR.

2.5 Dendritic Cell Co-culture System

Splenic CD4+ T cells from transgenic OT-II mice which have CD4+ T-cells that are specific for ovalalbumin were isolated with the protocol above. Bone marrow dendritic cells (BMDCs) were isolated from B6 mice. Tibia and Femur of B6 mice were removed with RP10 media and plated on bacterial Petri dish from Fisher Scientific at 2 x 10^5 cells/ml with fresh complete RPMI 1640 media and 5 ng/ml of GM-CSF (BD PharMingen). On day 3, the same medium in equal volume was added. The nonadherent cells were then harvested on day 7. This protocol was modified from previous papers (Datta et al., 2003; Lutz et al., 1999). The BMDCs were then loaded with 2 ug/ml of I-A^d –restricted OVA peptide ($OVA_{323-339}$:ISQAVHAAHAEINEAGR; PeptidoGenic Research) 2 hours before the addition of OT-II CD4+ T cells to the culture. After 5 days of co-culture, the CD4+ T cells were

collected and restimulated with plate bound anti-CD3 and soluble anti-CD28 antibodies for twenty four hours. The supernatant was then collected and analyzed by ELISA.

2.6 Isolation of RNA and quantitative RT-PCR

RNA was isolated from colonic tissue and CD4+ T cells using RNeasy Mini Kits from QIAGEN in accordance with the manufacturer's protocol. After isolation, the RNA sample was treated with DNase 1 (Invitrogen) to digest contaminating DNA. One microgram of RNA was then used for the reverse transcriptase reaction using Superscript III First-Strand Synthesis (Invitrogen) in order to make cDNA. Quantitative RT-PCR was run using SYBR Green Master Mix (Applied Biosystems) at half reactions on an AB7300 RT-PCR system (Applied Biosystems). GAPDH was used as an internal reference for all experiments. RT-PCR primers were designed based on their reported sequences and synthesized by IDT Technologies.

2.7 Culture of Colonic Explants

Small pieces (~5 mm) of the mid-colon were excised. The piece of colon was then weighed and washed in RMPI 1640 with 200 U/ml penicillin, and 200 μg/ml streptomycin. The colon explants were then cultured in complete RMPI 1640 media for 24 hours at 37°C and 5% CO_2. Culture supernatants were the collected and cytokines levels were measured by ELISA (eBioscience).

2.8 CD4+ T cell Depletion

Four week old *Il10*$^{-/-}$/*Tlr4*$^{-/-}$ mice were injected intraperitoneal with 1 mg of anti-CD4 antibody (GK1.5, BioExpress) or PBS. Mice were sacrificed two weeks later and then analyzed.

2.9 Adoptive Transfer of CD45RB^{high} CD4+ T cells

CD4+ T cells from spleens of *Il10*$^{-/-}$, *Il10*$^{-/-}$/*Tlr4*$^{-/-}$, and B6 donor mice were first isolated using immunomagnetic bead separation mentioned above. Cells were then stained with PE-Cy5-anti-CD4 (L3T4) from eBioscience, PE-anti-CD25 (PC61), and FITC-anti-CD45RB (16A) from BD Biosciences-Pharmingen. These cells were sorted using a MoFlow Flow Cytometer (Dako) using summit software for naïve CD4+CD45RB^{high} and regulatory CD4+CD45RB^{low}CD25+ populations with a purity of over 98%. Seven to nine week old sex matched *Rag1*$^{-/-}$ mice were then used as recipients. To eliminate the risk of infection by pathogens in the recipients, we added an antibiotic cocktail containing amoxicillin (0.6 mg/g food), clarithromycin (0.1 mg/g food), metronidazole (0.2 mg/g food), and omeprazole (0.004 mg/g food) (Bio-Serv) for two weeks before the T cell transfer. After antibiotic treatment, mice were transplanted with 5 x 10^5 CD4+CD45RB^{high} naïve T cells from *Il10*$^{-/-}$ or *Il10*$^{-/-}$/Tlr4$^{-/-}$ mice through an i.p injection. For the cotransfer experiments, 2 x 10^5 CD4+CD45RB^{low}CD25+ Tregs from B6 mice were coinjected with the naïve CD4+ T cell population. Diseased animals were sacrificed for analysis between 8 and 10 weeks

after transfer. Crypt length and infiltration of cells into the lamina propria were determined as described above.

2.10 Isolation of Lamina Propria Lymphocytes and Interepithelial Lymphocytes

Isolation of lamina propria lymphocytes (LPLs) and interepithelial lymphocytes (IELs) was performed following the protocol found in (Weigmann et al., 2007). Briefly, colons were excised from mice and cut longitudinally. They were then washed with PBS and cut into 1-cm pieces. The intestinal pieces were then incubated in HBSS with 5 mM EDTA and 1 mM DTT solution for 20 minutes at 37°C in a shaker. After this predigestion step, the pieces were filtered through a 100-μm cell strainer, and the flow through containing the IELs was collected in a fresh tube. The IELs were then washed with fresh PBS to remove remaining EDTA. The tissue was then cut into even smaller pieces (1-2mm) and digested with PBS containing 0.5 mg/ml collagenase D (Roche), 0.5 mg/ml DNase I (Sigma-Aldrich), and 3 mg/ml Dispase II (Roche) for 20 minutes at 37°C in a shaker. This step was repeated three times. After digestion, LPLs were collected using a 40-μm cell strainer. Finally, the IELs and LPLs were isolated by centrifugation with 40/80 Percoll (Sigma-Aldrich) gradient for 20 minutes at 1,000g at 20°C without brakes. IELs and LPLs were then cultured in RP10 media and stimulated with anti-CD3 and anti-CD28 antibodies as described above.

2.11 EMSA and Immunoblotting

Translocation of NF-κB into the nucleus was measured by EMSA using consensus NF-κB oligonucleotides (Santa Cruz Biotechnology Inc.). For immunoblotting the following antibodies were used: anti-p-ERK, anti-p-p38, anti-p-JNK, anti-p38, and anti-ERK (Cell Signaling Technology); anti-IkBα, anti-MKP-1, and anti-MKP-3 (Santa Cruz Biotechnology); anti-NFAT-1 (Abcam); anti-β-actin (Sigma-Aldrich).

2.12 Administration of Anti-TLR4 Neutralizing Antibody

Mice were injected with 100 μg of anti-TLR4/MD2 antibody clone MT5510 (eBioscience) every week for 2 weeks. 100 μg of Rat igG2a isotype control clone eBR2a (eBioscience) was used as a control. Mice were monitored for signs of inflammation twice a week and sacrificed after the 2 weeks. CD4+ T cells from spleen and MLN were isolated and stimulated as described above.

2.13 siRNA Gene Silencing

Purified CD4+ T cells from the MLNs were transfected by electroporation using a mouse T cell Nucleofactor kit (Amaxa) in accordance with the manufacturer's protocol. Briefly, CD4+ cells were resuspended in mouse T cell nucleofection solution at a density of 2×10^6 to 3×10^6 cells per 100 μl. For each transfection, 100 μl of cell suspension was mixed with 500 nM of either control siRNA or MKP-3 siRNA (Santa Cruz Biotechnology Inc.) The siRNA sequences are as follows: strand 1,

GAAGGUGGCUUCAGUAAGU; strand 2, GAACGAUGCUUACGACAUU; strand 3, GGACAUCCAUCCAGUAGA.

The material and method chapter, in part, is published in TLR4 Signaling in Effector CD4+ T Cells Regulates TCR Activation and Experimental Colitis in Mice. 2010. Gonzalez-Navajas JM, Fine S, Law J, Datta SK, Nguyen KP, Yu M, Corr M, Katakura K, Eckman L, Lee J, Raz E., Journal of Clinical Investigation, 2010. Jose M. Gonzalez-Navajas is the primary investigator and author of this material. Other co-authors include Sean Fine, Sandip Datta, Kim Nguyen, Mandy Yu, Maripat Corr, Kyoko Katakura, Lars Eckman, Jongdae Lee and Eyal Raz.

3. Results

3.1 TLR4 deficiency exacerbates IL-10 dependent colitis

Since TLRs were recently identified on CD4+ T cells, we wanted to investigate the role of these receptors on a CD4+ T cell dependent model of colitis. To study this, we crossed *Tlr4*$^{-/-}$ or *Tlr9*$^{-/-}$ mice with *Il10*$^{-/-}$ mice to create double knock-out mice. We then followed these mice for two months and assessed their colons for signs of inflammation. While *Il10*$^{-/-}$ and *Il10*$^{-/-}$*/Tlr9*$^{-/-}$ mice did not develop colitis by even 8 months, the *Il10*$^{-/-}$*/Tlr4*$^{-/-}$ spontaneously developed severe signs of colitis by week 8. These mice had thickened intestinal walls, decrease colon lengths and enlarged spleens (figure 3.1.1).

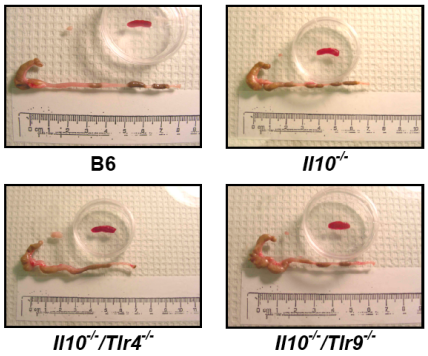

Figure 3.1.1 Representative colons and spleens from 8 week old B6, *Il10*$^{-/-}$, *Il10*$^{-/-}$*/Tlr4*$^{-/-}$, and *Il10*$^{-/-}$*/Tlr9*$^{-/-}$ mice.

To further assess the inflammation, histological analysis was done to reveal that *Il10*$^{-/-}$*/Tlr4*$^{-/-}$ mice colons develop much more severe colitis confirming our previous observation (figure 3.1.2). Their colons had a high degree of epithelial crypt

hyperplasia and mononuclear cell infilitration of the colonic lamina propria (figure 3.1.3).

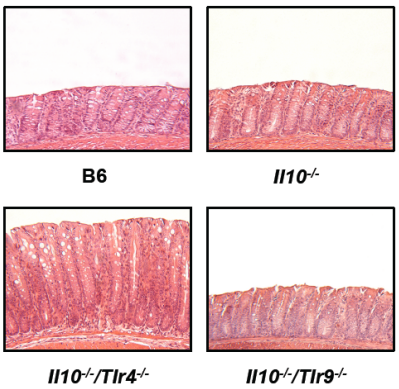

Figure 3.1.2 Histological analysis of 8 week old B6, *Il10*$^{-/-}$, *Il10*$^{-/-}$*/Tlr4*$^{-/-}$, and *Il10*$^{-/-}$*/Tlr9*$^{-/-}$ mice

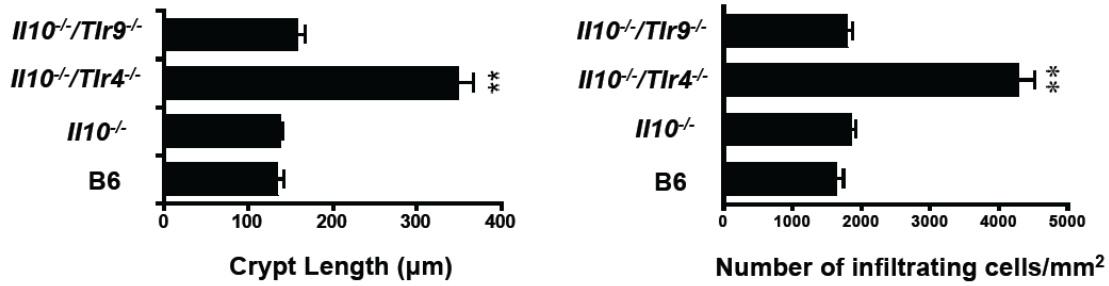

Figure 3.1.3 Quantitative measurements of crypt length and cellular infiltration in the different groups of 8 week old mice

Since the colitis in the *Il10*$^{-/-}$ model of colitis is largely dependent on the gut flora, a difference in microbiota between the different strains of mice could have caused the phenotypic difference. In order to address this issue, we co-housed both *Il10*$^{-/-}$ and *Il10*$^{-/-}$*/Tlr4*$^{-/-}$ mice. Co-housing mice in the same cages allows for colonization of the mice with the same microflora (Ivanov et al., 2008). The *Il10*$^{-/-}$ mice co-housed with *Il10*$^{-/-}$*/Tlr4*$^{-/-}$ mice were then compared with *Il10*$^{-/-}$ mice housed in a separate cage to see if there was a difference in colitis due to differences in

microflora. Under these conditions, there were no differences between the two groups of *Il10*$^{-/-}$ mice (figure 3.1.4-5).

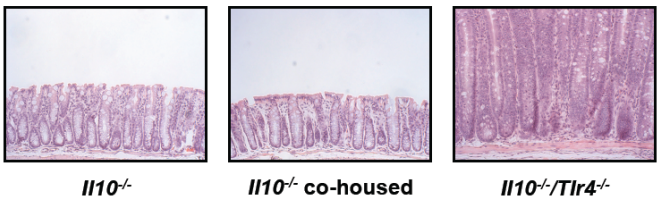

Figure 3.1.4 Histological analysis of 4 week old *Il10*$^{-/-}$ mice co-housed with or without *Il10*$^{-/-}$/*Tlr4*$^{-/-}$ mice

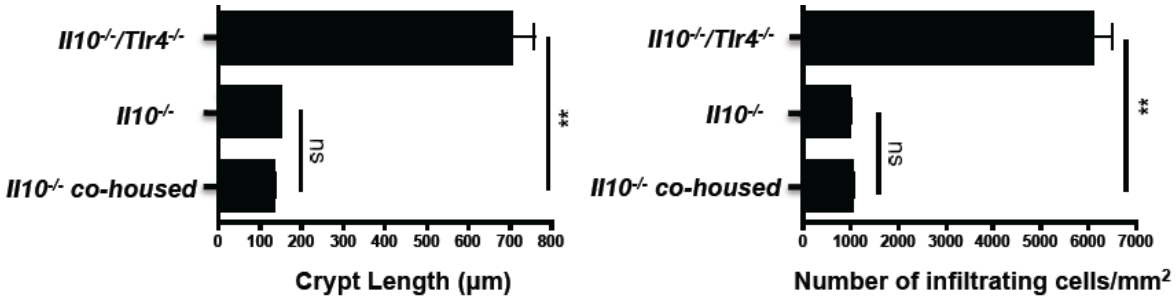

Figure 3.1.5 Quantitative measurements of crypt length and cellular infiltration in co-housed and non-co-housed mice.

This indicates that it was truly the lack of TLR4 which exacerbates intestinal inflammation in the *Il10*$^{-/-}$ host.

3.2 Characterization of the Inflammatory Profile in *Il10*$^{-/-}$ */Tlr4*$^{-/-}$ mice

To assess the inflammation in *Il10*$^{-/-}$/*Tlr4*$^{-/-}$ mice, we compared colons from *Il10*$^{-/-}$/*Tlr4*$^{-/-}$ mice and *Il10*$^{-/-}$ mice. We excised colon from 8 week old mice and measured mRNA levels of cytokines, chemokines and cell markers from total colon homogenate. Consistent with the proinflammatory phenotype, we found elevated

transcription levels of inflammatory cytokines such as IL-6, IFN- γ, IL-1b and IL-17A. Chemokines such as KC (keratinocyte chemoattractant), MIP1a (macrophage inflammatory protein 1a), MIP1b, CCL22 and CXCL10, and cellular markers such as F4/80 (macrophages), MPO (neutrophils) and CD3d/CD4 (T cells) were also increased (figure 3.2.1).

qRT-PCR analysis of RNA samples isolated from colon of mice of the different genotypes

	B6	*Il10*$^{-/-}$	*Il10*$^{-/-}$*Tlr4*$^{-/-}$	*Il10*$^{-/-}$*Tlr9*$^{-/-}$
Cytokines				
IL-6	0.54 ± 0.11	1 ± 0.22	6.33 ± 0.50^{A}	1.49 ± 0.48
Il12p40	0.22 ± 0.16	1 ± 0.18	20.8 ± 1.39^{B}	0.91 ± 0.11
IFN-γ	0.68 ± 0.13	1 ± 0.32	11.8 ± 0.24^{B}	1.88 ± 0.38
IL-1β	0.41 ± 0.21	1 ± 0.14	17.8 ± 0.58^{B}	1.08 ± 0.09
IL-23p19	0.38 ± 0.08	1 ± 0.81	2.11 ± 0.32^{A}	0.43 ± 0.37
TNF-α	0.51 ± 0.38	1 ± 1.03	3.06 ± 0.08^{A}	0.95 ± 0.71
IL-17	0.26 ± 0.31	1 ± 0.08	7.61 ± 0.58^{B}	0.61 ± 0.55
Chemokines				
KC	0.48 ± 0.21	1 ± 0.31	24.1 ± 0.71^{B}	1.11 ± 0.19
MIP1α	0.71 ± 0.13	1 ± 0.08	8.09 ± 0.22^{B}	2.07 ± 0.36
MIP1β	0.57 ± 0.46	1 ± 0.18	13.9 ± 0.28^{B}	1.59 ± 0.52
CCL19	0.59 ± 0.15	1 ± 0.09	7.38 ± 0.39^{B}	1.74 ± 0.74
CXCL10	0.71 ± 0.28	1 ± 0.54	3.73 ± 0.41^{A}	1.03 ± 0.08
CCL22	0.62 ± 0.31	1 ± 0.35	1.95 ± 1.73	1.14 ± 0.48
Cell markers				
F4/80	0.49 ± 0.39	1 ± 0.27	2.52 ± 0.23^{A}	0.91 ± 0.21
MPO	1.01 ± 0.18	1 ± 0.09	10.0 ± 0.39^{B}	1.91 ± 3.16
CD3d	0.75 ± 0.11	1 ± 0.11	13.7 ± 0.38^{B}	2.67 ± 0.24^{A}
CD4	0.68 ± 0.09	1 ± 0.12	3.64 ± 0.28^{A}	1.04 ± 0.08

Results were normalized to those obtained from *Il10*$^{-/-}$ mice, which were designated as 1 unit. Data are expressed as mean ± SD of 6 mice/group. $^{A}P < 0.05$. $^{B}P < 0.01$.

Figure 3.2.1 Total colon homogenate from 8 week old B6, *Il10*$^{-/-}$, *Il10*$^{-/-}$/*Tlr4*$^{-/-}$, and *Il10*$^{-/-}$/*Tlr9*$^{-/-}$ mice analyzed for proinflammatory cytokines, chemokines and cell markers by qRT-PCR

Consistent with the increase in colon inflammation, lymphocytes from the colon of *Il10*$^{-/-}$/*Tlr4*$^{-/-}$ mice also showed an increase in the production of

proinflammatory cytokines. Both intraepithelial lymphocytes (IELs) and lamina propria lymphocytes (LPLs) were isolated from the colon and stimulated with anti-CD3 and anti-CD28 antibodies for 24 hours. ELISA was then used to check the supernatant for cytokines. These colonic lymphocytes showed an increase production of IL-6, IL-17A, TNF-α, and IFN- γ when compared with colonic lymphocytes from *Il10*$^{-/-}$ mice.

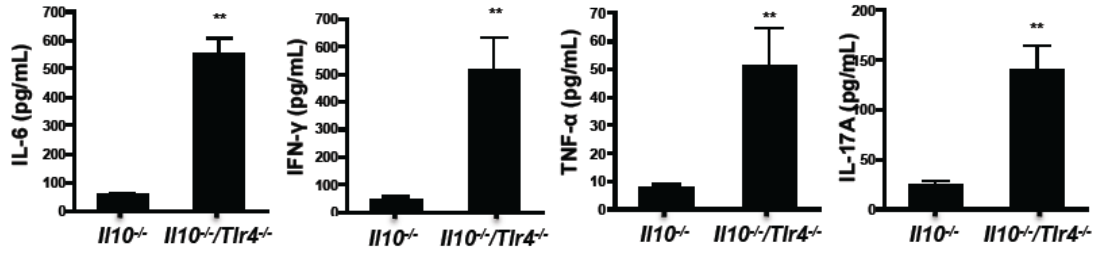

Figure 3.2.2 Cytokine levels obtained from LPLs isolated from *Il10*$^{-/-}$ and *Il10*$^{-/-}$/*Tlr4*$^{-/-}$ mice and stimulated with anti-CD3 and anti-CD28 for 24 hours

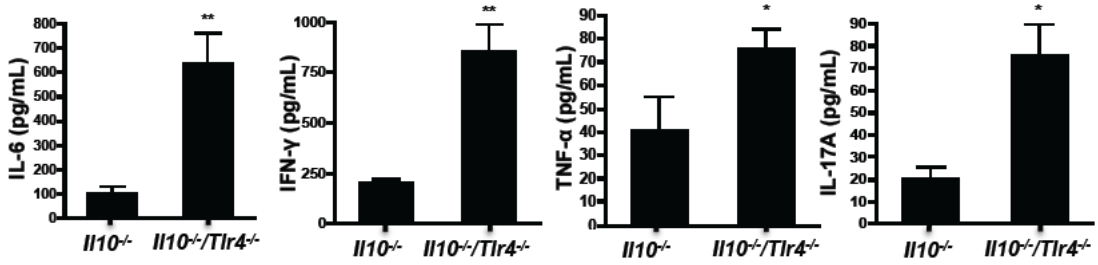

Figure 3.2.3 Cytokine levels obtained from IELs isolated from *Il10*$^{-/-}$ and *Il10*$^{-/-}$/*Tlr4*$^{-/-}$ mice and stimulated with anti-CD3 and anti-CD28 for 24 hours

To ensure that these findings were not due to developmental defects from the loss of TLR4, an anti-TLR4 blocking antibody was injected into mice every week for two weeks. The excised colons from anti-TLR4 antibody treated mice compared to a

group of mice treated with an isotype antibody had elevated levels of IL-6, IL-17A, TNF-α and IFN- γ.

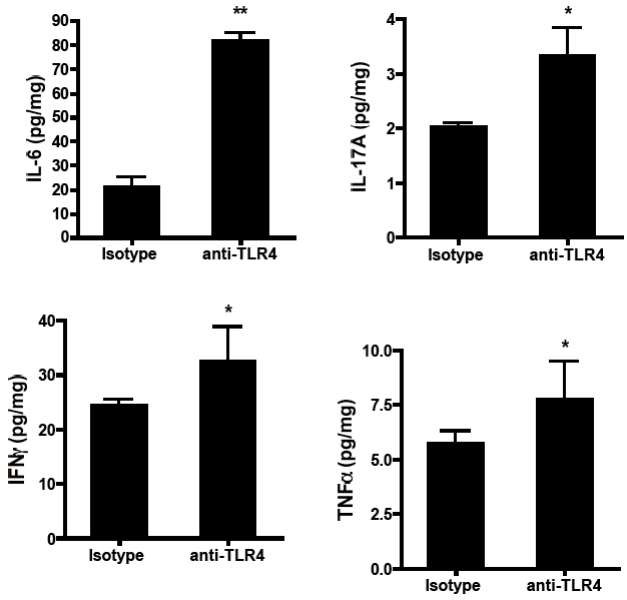

Figure 3.2.4 *Il10*$^{-/-}$ mice treated using weekly intraperitoneal injections of 100 μg of anti-mouse TLR4 antibodies or IgG2a isotype for two weeks. Cytokines were measured from whole colon cultured for 24 hours.

The lymphocytes from the MLN also had produced elevated levels of IL-6, IL-17A, TNF-α and IFN- γ when treated with anti-TLR4 blocking antibody.

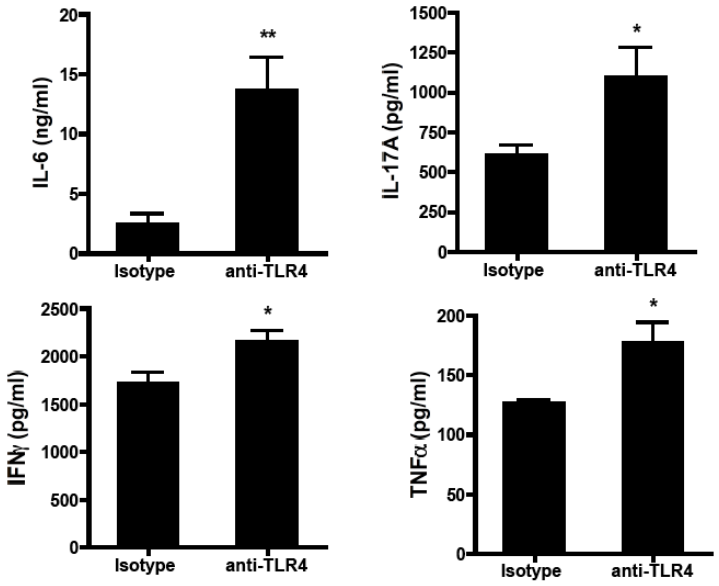

Figure 3.2.5 *Il10*$^{-/-}$ mice treated using weekly intraperitoneal injections of 100 μg of anti-mouse TLR4 antibodies or IgG2a isotype for two weeks. Cytokine levels from CD4+ T cells isolated from MLN and stimulated with anti-CD3 and anti-CD28 for 24 hours.

3.3 CD4+ T cells express functional TLR4

The expression of TLR4 was recently shown on naïve CD4+ (CD4+CD45RB^{high}) T cells through flow cytometry (Fukata et al., 2008). In addition, the expression of TLR4 is also found in colitic CD4+ T cells found in the lamina propria (Tomita et al., 2008). As expected, we detected TLR4 expression in CD3+CD4+ cells from the spleen of both B6 and *Il10*$^{-/-}$ mice but not in *Il10*$^{-/-}$*/Tlr4*$^{-/-}$ mice.

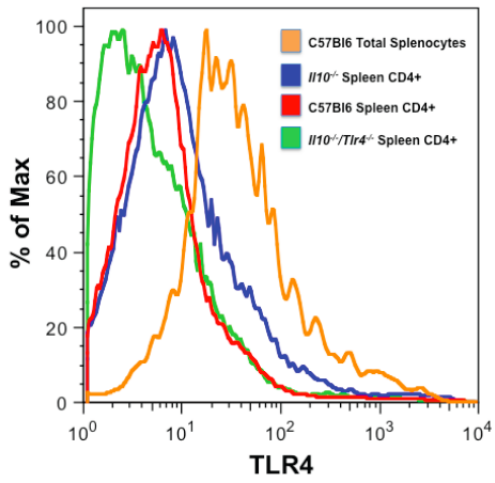

Figure 3.3.1 Samples from total spleen of B6 mice or CD4+ T cells from spleen of B6, *Il10*$^{-/-}$, *Il10*$^{-/-}$*/Tlr4*$^{-/-}$ mice were stained for CD3, CD4 and TLR4. TLR4 expression was checked in the gated CD3 and CD4 positive population

3.4 TLR4 expression on CD4+ T cells restrains colitis in *Il10*$^{-/-}$ mice

To determine whether the accelerated colitis in *Il10*$^{-/-}$*/Tlr4*$^{-/-}$ mice is dependent on the lack of TLR4 in CD4+ T cells, two experiments were performed. In the first experiment, CD4+ T cells were depleted using a CD4+ depleting antibody (figure 3.4.1). In the second experiment, we performed an adoptive transfer by injecting naïve CD4+ T cells from either *Il10*$^{-/-}$ mice or *Il10*$^{-/-}$*/Tlr4*$^{-/-}$ mice into *Rag1*$^{-/-}$ mice. In the first experiment, depletion of CD4+ T cells in *Il10*$^{-/-}$*/Tlr4*$^{-/-}$ mice was done over a two week period. Compared to PBS injected mice, mice given CD4+ depleting antibody had significantly less hyperplasia, mononuclear cell infiltration (figure 3.4.2) and production of proinflammatory cytokines such as IL-6, IL-17A, TNF-α and IFN- γ (figure 3.4.3).

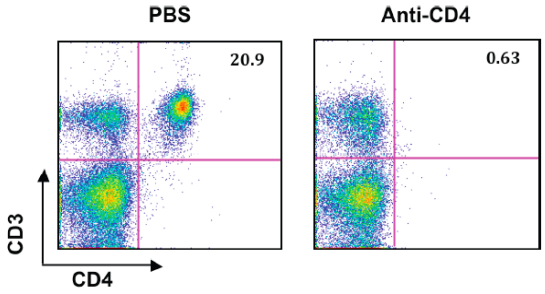

Figure 3.4.1 FACs analysis of CD3 and CD4 populations of T cells isolated from spleen of *Il10*$^{-/-}$/*Tlr4*$^{-/-}$ CD4 neutralized mice

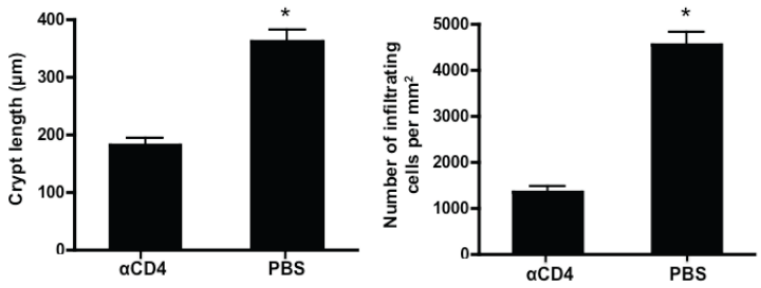

Figure 3.4.2 Quantitative measurements of crypt length and cellular infiltration in CD4 neutralized and control groups

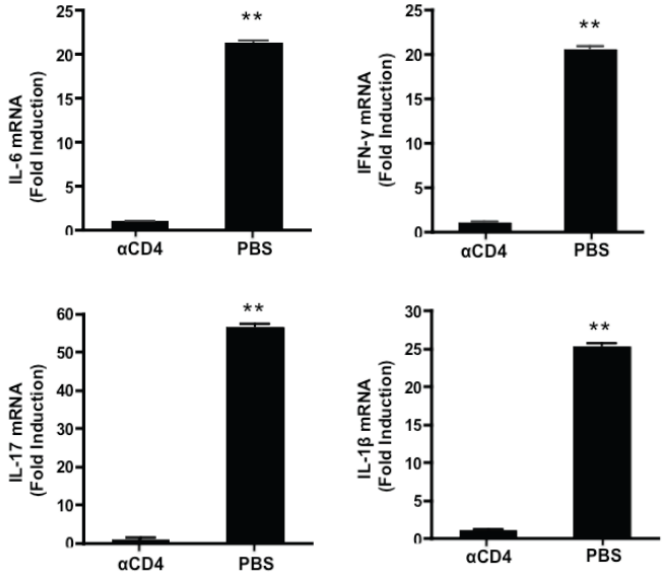

Figure 3.4.3 Transcription levels of proinflammatory cytokines in CD4 neutralized and control colons analyzed by qRT-PCR

This indicates that CD4+ T cells play an essential role in the induction of colitis in *Il10*$^{-/-}$/*Tlr4*$^{-/-}$ mice, which is consistent with the essential role of CD4+ T cells in the *Il10*$^{-/-}$ model of colitis.

To determine whether the lack of TLR4 specifically on CD4+ T cells was necessary to exacerbate inflammation we performed an adoptive transfer. In this experiment, we transferred naïve CD4+ (CD4+CD45RB^{high}) T cells from *Il10*$^{-/-}$ or *Il10*$^{-/-}$/*Tlr4*$^{-/-}$ donor mice into *Rag1*$^{-/-}$ recipient mice. As a control we cotransferred Treg cells (CD4+CD45RB^{low}) from B6 mice with naïve CD4+ T cells from either *Il10*$^{-/-}$ or *Il10*$^{-/-}$/*Tlr4*$^{-/-}$ mice. This ensures that the mice were not developing colitis or any other pathologies due to other types of infections. We observed that naïve *Il10*$^{-/-}$/*Tlr4*$^{-/-}$ T cells induced greater body weight loss and greater inflammation in their recipients when compared to recipients receiving only *Il10*$^{-/-}$ naïve T cells. As expected, the cotransfer controls did not have a drop in body weight.

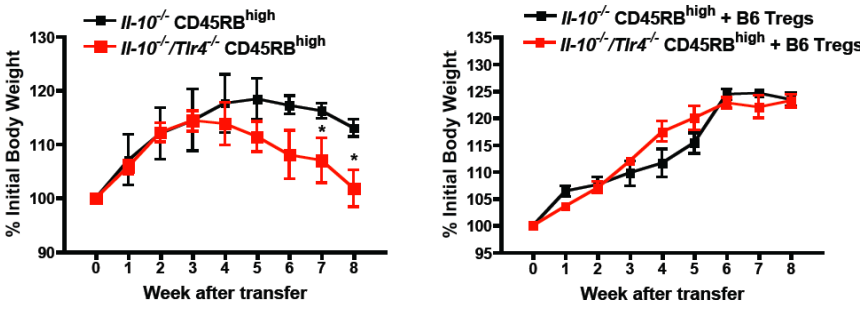

Figure 3.4.4 Percentage of initial body weight of *Rag1*$^{-/-}$ recipients transferred with *Il10*$^{-/-}$ or *Il10*$^{-/-}$/*Tlr4*$^{-/-}$ FACS-sorted naïve CD4+CD45RB^{high} T cells with or without the regulatory CD4+CD45RB^{low}CD25+ Treg population

Upon histological examination, *Il10*$^{-/-}$/*Tlr4*$^{-/-}$ naïve T cell recipients had elongated crypts and increased mononuclear cell infiltration.

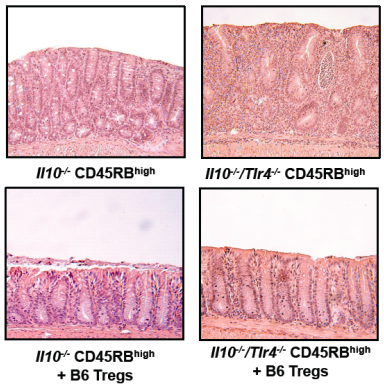

Figure 3.4.5 Histological analysis of colon of *Rag1*$^{-/-}$ recipients transferred with *Il10*$^{-/-}$ or *Il10*$^{-/-}$/*Tlr4*$^{-/-}$ FACS-sorted naïve CD4+CD45RB^{high} T cells with or without the regulatory CD4+CD45RB^{low}CD25+ Treg population

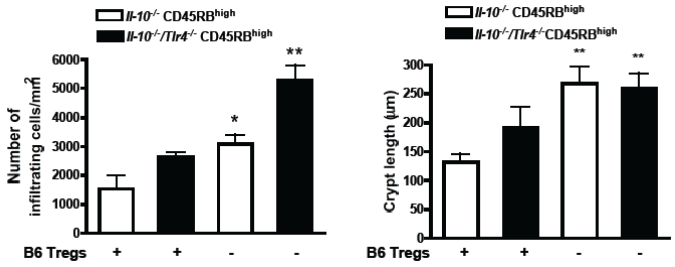

Figure 3.4.6 Quantitative measurements of crypt length and cellular infiltration of colon of *Rag1*$^{-/-}$ recipients transferred with *Il10*$^{-/-}$ or *Il10*$^{-/-}$/*Tlr4*$^{-/-}$ FACS-sorted naïve CD4+CD45RB^{high} T cells with or without the regulatory CD4+CD45RB^{low}CD25+ Treg population

In accordance with the increased inflammation, there was increased proinflammatory cytokine production in colonic explants taken from recipients transferred with *Il10*$^{-/-}$ /*Tlr4*$^{-/-}$ naïve CD4+ T cells (figure 3.4.7). We then analyzed different CD4+ T cell populations from different organs to assess their contribution to the colitis. While there was no significant difference in the cytokine production of CD4+ cells found in the spleen, CD4+ T cells isolated from the MLN, IELs, LPLs showed an increase in proinflammatory cytokines after stimulation with anti-CD3 and anti-CD28 antibodies for 24 hours. The CD4+ T cells from the MLN produced more IFN- γ while the CD4+

T cells from the IELs and LPLs showed a significant increase in IL6, IFN- γ and IL-17A (figure 3.4.8).

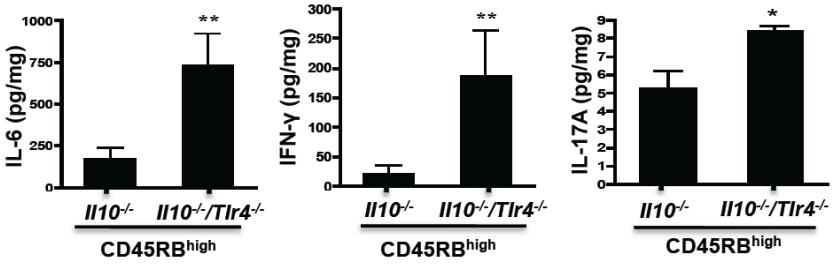

Figure 3.4.7 Cytokine levels in colonic explants after 24 hours of culture from colons of *Rag1*$^{-/-}$ recipients transferred with *Il10*$^{-/-}$ or *Il10*$^{-/-}$/*Tlr4*$^{-/-}$ FACS-sorted naïve CD45RB^{high} T cells

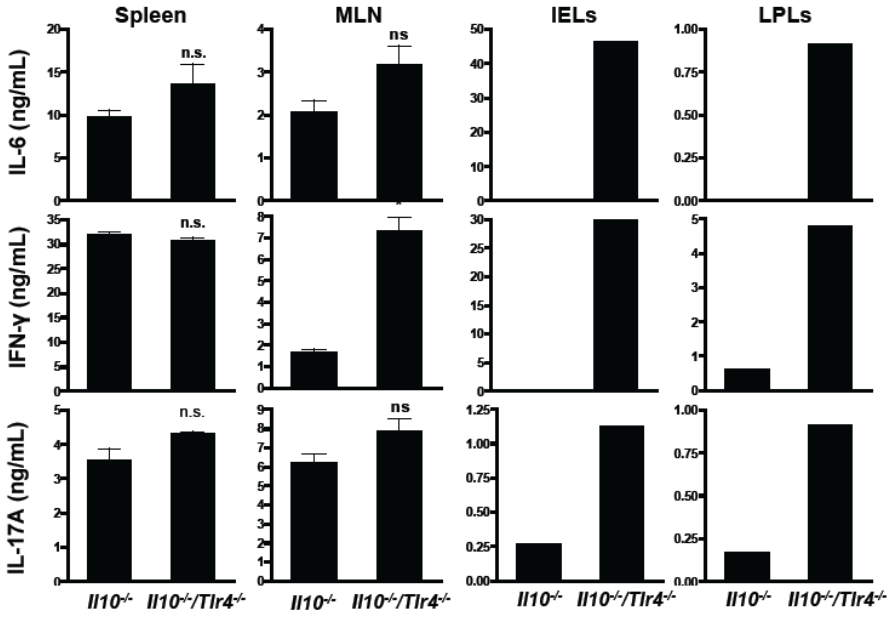

Figure 3.4.8 Cytokine levels of CD4+ T cells isolated from the colon (IELs and LPLs), spleen and MLNs and stimulated with anti-CD3 and anti-CD28 antibodies for 24 hours.

3.5 TLR4 is expressed in CD4+ T cells from various sites

Because CD4+ T cells produce different inflammatory phenotypes depending on the site they were isolated, we hypothesize that expression of TLR4 may be site specific or an activation-induced regulation. We therefore analyzed the TLR4 expression in FACS-sorted CD4+ T cells isolated from the spleen, MLN , IELs and LPLs of *Il10*$^{-/-}$ mice by quantitative RT-PCR (qPCR). The purity of the CD4+ population was over 98% (data not shown). There was indeed different levels of TLR4 expression in the CD4+ T cells isolated from the various sites with the lowest expression found in CD4+ T cells isolated from the IELs and LPLs.

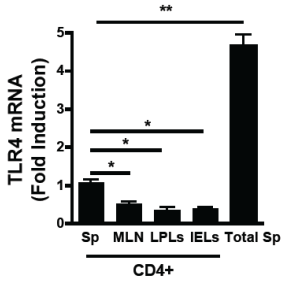

Figure 3.5.1 qRT-PCR analysis of TLR4 expression in unstimulated CD4+ T cells from spleen, MLNs, LPLs, ad IELs of *Il10*$^{-/-}$ mice

Also consistent with a previous report (Gelman et al., 2004), we found that TCR stimulation downregulates TLR4 expression.

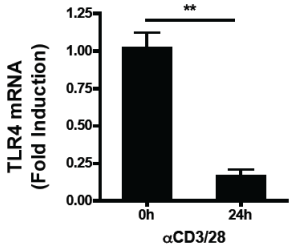

Figure 3.5.2 qRT-PCR analysis of TLR4 expression in spleen CD4+ T cells isolated from *Il10*$^{-/-}$ mice before and after 24 hour anti-CD3 and anti-CD28 antibody stimulation

Because TLR4 deficiency in CD4+ T cells affected their inflammatory phenotype in vivo, we explored the TLR4 signaling cascade in CD4+ T cells. LPS stimulation of CD4+ T cells from MLN activated NF-κB as well as the MAPK family, ERK, P38 and JNK.

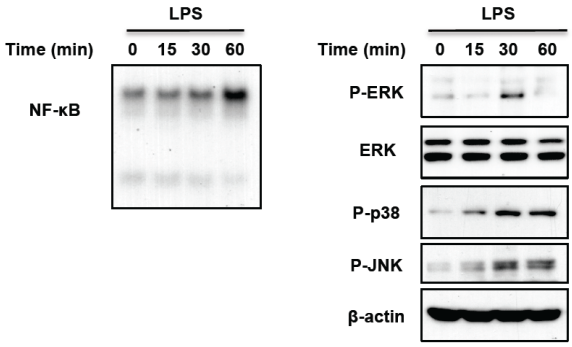

Figure 3.5.3 MLN CD4+ T cells from *Il10*$^{-/-}$ mice were treated with LPS for the indicated times. Nuclear lysates were examined for NF-κB activation (EMSA) and cytoplasmic extracts were tested for ERK1/2, p38 and JNK activation by immunoblotting with phospho-specific antibodies

Also as expected CD4+ T cells from *Il10*$^{-/-}$*/Tlr4*$^{-/-}$ mice did not show an activation of NF-κb or ERK upon LPS stimulation.

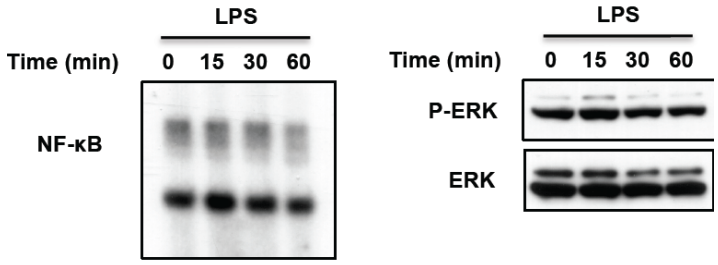

Figure 3.5.4 MLN CD4+ T cells from *Il10*$^{-/-}$ mice were treated with LPS for the indicated times. Nuclear lysates were examined for NF-κB and cytoplasmic extracts were tested for ERK1/2.

3.6 TLR4 signaling inhibits TCR production of INF-γ

Since LPS is a soluble mediator, its interaction with TLR4 on CD4+ T cells does not need to occur in lymphoid organs unlike the interaction of T cells with APCs. We therefore hypothesized that at the intestinal mucosal sites, there are considerable opportunities of having TLR4-stimulated CD4+ T cells prior to TCR engagement. This hypothesis would remain consistent with the downregulation of TLR4 upon TCR stimulation. To further explore the role of LPS signaling on CD4+ T cell phenotype, we isolated splenic CD4+ T cells from OVA-transgenic (OT-II) mice. We pretreated these OT-II CD4+ T cells with LPS or media for two hours before washing them and coculturing them with OVA-loaded bone marrow dendritic cells (BMDCs) from B6 mice for 5 days. LPS pretreatment significantly reduced the production of IFN- γ by CD4+ T cells while increasing the production of IL-17A. The levels of other cytokines such as TNF-a or IL-2 remained unaffected by LPS pretreatment following TCR activation. (data not shown)

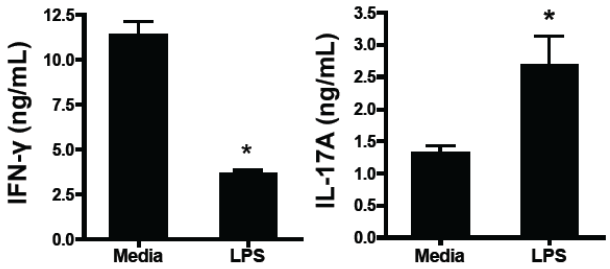

Figure 3.6.1 Splenic CD4+ T cells from OT-II mice were incubated for 2 hours in the presence of 100 ng/ml LPS or medium alone. The cells were then washed and cultured for 5 days with OVA-load BMDCs. After coculture the CD4+ cells were stimulated with anti-CD3 and anti-CD28 antibodies for 24 hours and cytokine levels were measured

Similar data was observed in CD4+ T cells isolated from various sites and pretreated with LPS following anti-CD3 and anti-CD28 TCR stimulation.

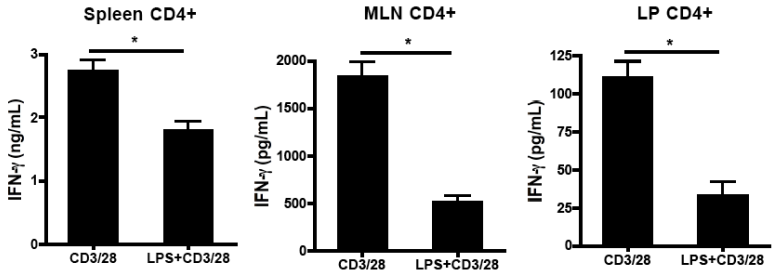

Figure 3.6.2 IFN-γ production from CD4+ cells from the spleen, MLN or LP treated with 100ng/ml of LPS before anti-CD3 and anti-CD28 stimulation for 24 hours. Cytokines were then measured.

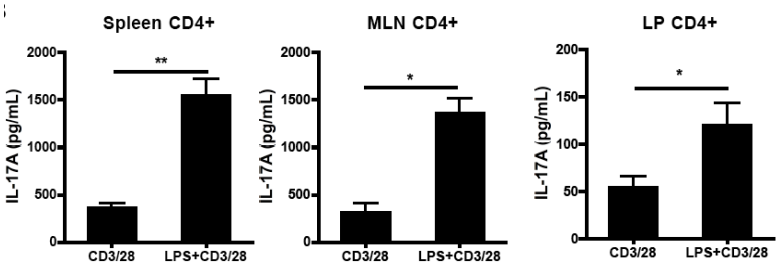

Figure 3.6.3 IL-17A production from CD4+ cells as in figure 3.6.2

3.7 TLR4 signaling downregulates IFN-γ production via ERK inhibition

To elucidate the mechanism behind this observation, we looked into pathways shared between TLR4 and TCR signaling. Two of the major pathways shared include the NF-κB (Burbach et al., 2007; Kenny and O'Neill, 2008), and MAPKs (Cronin and Penninger, 2007; Lu et al., 2008; Rincon et al., 2000) signaling pathways. In spite of LPS signaling being able to activate NF-κB, LPS prestimulation is not able to modulate the TCR signaling of NF-κB (figure 3.7.1). On the other hand, LPS signaling did modulate TCR signaling activation of the MAPKs. LPS prestimulation following TCR activation, resulted in a reduced phosphorylation of ERK when compared to TCR alone (figure 3.7.2). However, this seems to be specific activation since, the phosphorylation of p38 remains relatively unchanged with LPS prestimulation. As a control, the expression of NFAT was not affected by LPS prestimulation as expected (figure 3.7.3).

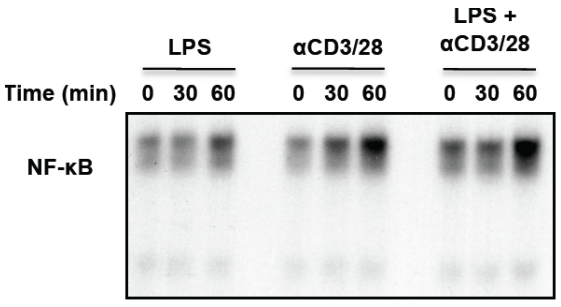

Figure 3.7.1 NF-κB activation of MLN CD4+ T cells stimulated with LPS alone, anti-CD3 and anti-CD28 alone or two hour LPS presitmulation following anti-CD3 and anti-CD28 stimulation for the indicated time points

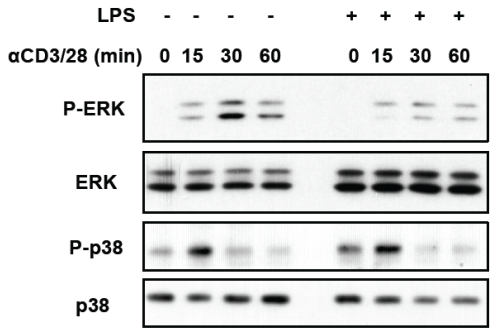

Figure 3.7.2 MLN CD4+ T cell cytosolic lysates were immunoblotted for MAPK protein activation after anti-CD3 and anti-CD28 antibodies for the indicated time points with or without 100ng/ml of LPS two hours prior

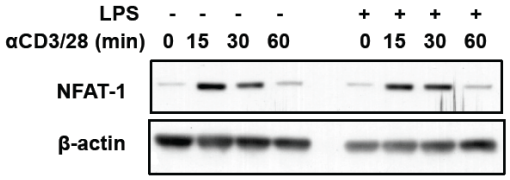

Figure 3.7.3 MLN CD4+ T cell nuclear lysate was immunoblotted for the activation of NFAT-1 as in figure 3.7.2

Because of the downregulation of both IFN- γ and ERK upon LPS prestimulation, we asked if the two events were linked. To explore this possibility, we stimulated CD4+ T cells with the MEK1/2 specific inhibitor, UO126 before TCR activation. The ensuing transcription of IFN- γ following TCR activation was diminished.

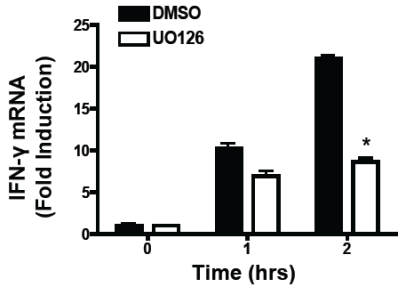

Figure 3.7.4 qRT-PCR analysis of CD4+ T cells incubated with the MEK/ERK1/2 inhibitor UO126(DMSO) or vehicle before being stimulated with anti-CD3 and anti-CD28 antibodies for the indicated time points

However, transcription levels of other pro inflammatory cytokines such as IL-17A were not significantly altered by MEK inhibition.

3.8 LPS signaling regulates subsequent TCR signaling events through the induction of MKP-3

The lack of ERK1/2 activation due to LPS prestimulation could be explained as either a lack of phosphorylation by MEK1/2 or an increase in dephosphorylation by phospho-ERK 1/2 specific phosphatases such as the MKPs. To explore the possibilities, we first checked for activation of MEK1/2. LPS stimulation did not downregulate TCR's activation of MEK1/2, indicating that the lack of ERK phosphorylation is most likely due to increased dephosphorylation.

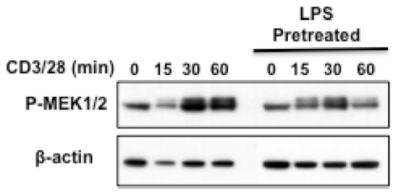

Figure 3.8.1 MLN CD4+ T cell cytosolic lysates were immunoblotted for Phospho-MEK1/2 protein activation after anti-CD3 and anti-CD28 antibodies for the indicated time points with or without 100ng/ml of LPS two hours prior

In order to explore the second possibility, we looked into the DUSPs which also encompasses the family of MKPs. This family of phosphatases can dephosphorylate phosphates on both threonine and tyrosine residues found on activated ERK1/2 allowing them to single-handedly inactivate the MAPKs. Because many of these MKPs are expressed in T cells, we checked if induction of MKPs could explain the

reduction in ERK1/2 phosphorylation following TLR4 stimulation. This was indeed the case, as LPS stimulation of *Il10*$^{-/-}$ CD4+ T cells leads to an increase in MKP-3 induction. However, other phosphatases such as SHIP-1 and MKP-1 were not upregulated.

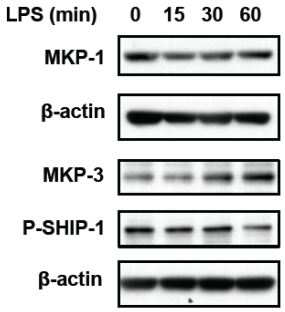

Figure 3.8.2 MLN CD4+ cells immunobloted for of MKP-1, MKP-3 and phospho-SHIP-1 after 100ng/ml LPS stimulation for the indicated time points

In accordance with this data, *Il10*$^{-/-}$ CD4+ T cells freshly isolated from the MLN showed a strong expression of MKP-3 while those isolated from *Il10*$^{-/-}$*/Tlr4*$^{-/-}$ showed a reduced expression. SHIP-1 and MKP-1 were also again not affected.

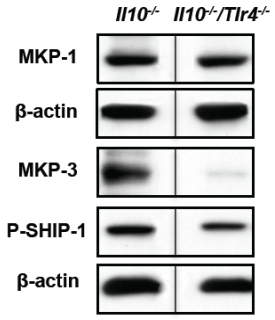

Figure 3.8.3 Unstimulated MLN CD4+ cells isolated from *Il10*$^{-/-}$ or *Il10*$^{-/-}$*/Tlr4*$^{-/-}$ mice and immunobloted for MKP-1, MKP-3 and p-SHIP-1

More importantly, TCR stimulation of *Il10*$^{-/-}$ CD4+ T cells did not induce MKP-1, MKP-3 or SHIP-1 activation. In contrast when preceded by LPS stimulation, both

MKP-1 and MKP-3 were noticeably activated while SHIP-1 had a very slight activation.

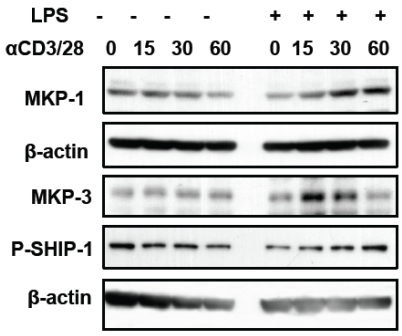

Figure 3.8.4 MLN CD4+ T cells stimulated with anti-CD3 and anti-CD28 antibodies for the indicated time points with or without 100ng/ml of LPS 2 hours prior. The cells were then lysed and checked for MKP-1, MKP-3 and p-SHIP-1

Since MKP-3 was not highly expressed in *Il10*$^{-/-}$*/Tlr4*$^{-/-}$ mice and it was further activated with LPS prestimulation, we wanted to explore its potential role in regulating TCR signaling and subsequent T cell activation. We used MKP-3 siRNA to assess its role. As shown in figure 3.8.5, the siRNA greatly reduced MKP-3 expression.

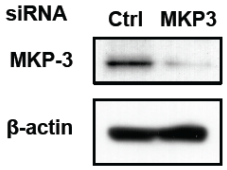

Figure 3.8.5 Analysis of the siRNA knockdown in MLN CD4+ T cells 24 hours after transfection with either MKP-3 or control (Ctrl) siRNA

Knockdown of MKP-3 abrogated the LPS dependent downregulation of ERK1/2 activation in vitro, as determined by FACS-based intracellular staining.

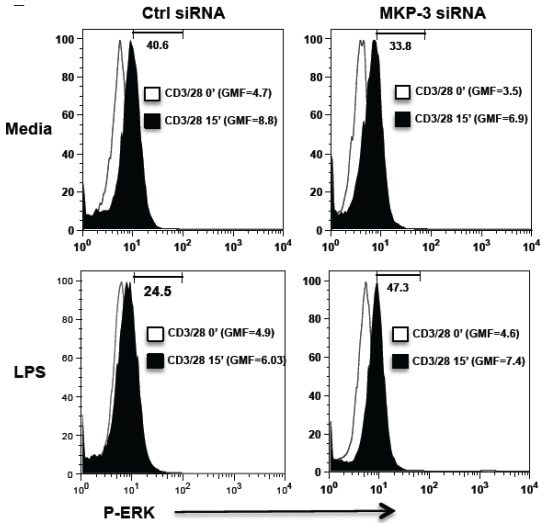

Figure 3.8.6 TCR dependent phosphorylation of ERK1/2 24 hours after transfection with MKP-3 or control siRNA. Number in histogram denotes percentage of phospho-ERK1/2 cells in stimulated cells compared to control cells.

LPS prestimulation resulted in a decrease in ERK1/2 phosphorylation while it was unable to exhibit this inhibitory effect in MKP-3 knockdown CD4+ T cells. MKP-3 knockdown was also able to inhibit the LPS downregulation of INF-γ production by anti-CD3 and anti-CD28 stimulation but not affect IL-17A production (figure 3.8.7). This suggests that LPS mediates its inhibitory effect on T cell activation through MKP-3.

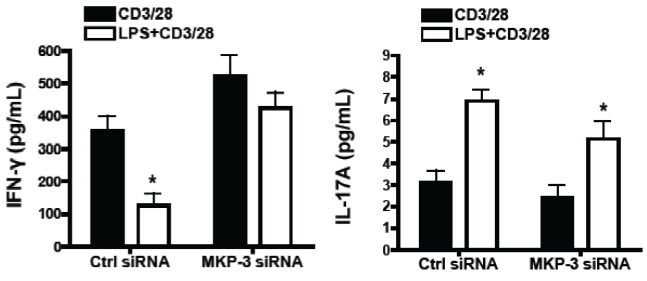

Figure 3.8.7 Cytokine levels measured from 24 hour supernatant of cells treated as in figure 3.8.6

In order to validate these findings in vivo, we used a quinone-based inhibitor for MKP-1 and MKP-3, NSC 95397 on *Il10*$^{-/-}$ mice. Injection of a single dose of this compound resulted in significant body weight loss (figure 3.8.8) and diarrhea compared to control 3 days later (figure 3.8.9).

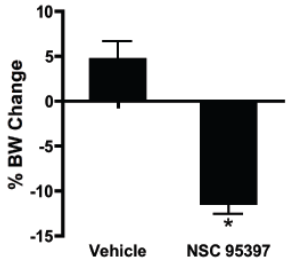

Figure 3.8.8 Percent body weight change in mice given a single dose (4mg/kg) of MKP-3 inhibitor NSC 95397 or vehicle after 3 days

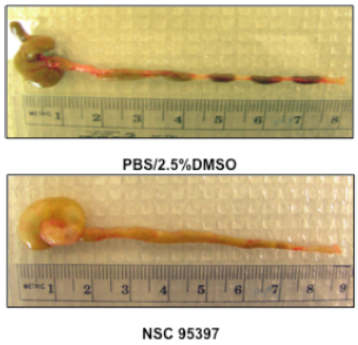

Figure 3.8.9 Representative colons from mice treated as in figure 3.8.8

In addition, CD4+ T cells isolated from the MLN of *Il10*$^{-/-}$ mice treated with inhibitor showed a marked increase in ERK phosphorylation compared to vehicle.

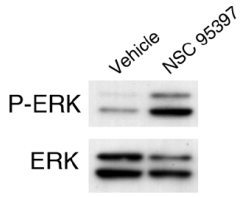

Figure 3.8.10 p-ERK1/2 expression in freshly isolated CD4+ cells from *Il10*$^{-/-}$ mice treated with NSC 95397 or vehicle

Upon isolation of CD4+ T cells from the MLN and stimulated with anti-CD3 and anti-CD28 for 24 hours, the mice treated with inhibitor produced more proinflammatory cytokines such as IFN- γ, IL-17A and IL-6.

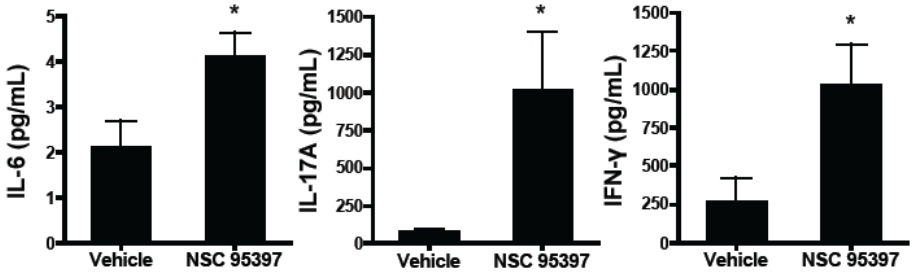

Figure 3.8.11 MLN CD4+ T cells were isolated from NSC 95397 or vehicle treated mice after three days. CD4+ T cells were then stimulated with anti-CD3 and anti-CD28 antibodies for 24 hours and the supernatant was checked for cytokines.

Finally, in vivo blockade of TLR4 using anti-TLR4 antibodies resulted in a decrease of expression of both MKP-1 and MKP-3 in CD4+ T cells isolated from the spleen and MLN. This data suggests that TLR4 signaling in colitogenic CD4+ T cells regulates their activation mainly through the induction of MKP-3 that restrains p-ERK levels upon subsequent TCR stimulation.

Collectively, the data indicates that TLR4 signaling in colitogenic CD4+ T cells regulate their activation mainly through the induction of MKP-3 which restrains p-ERK upon subsequent TCR stimulation.

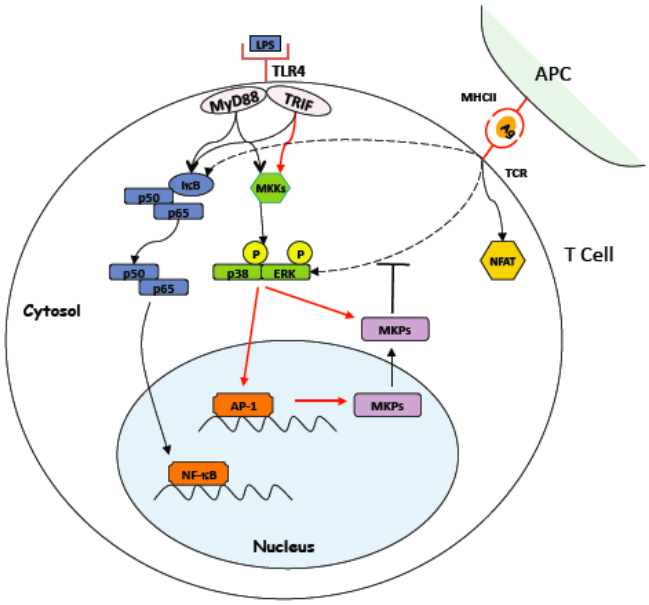

Figure 3.8.12 Proposed mechanism for TLR4 regulation of subsequent TCR activation

The results chapter, in part, is published in TLR4 Signaling in Effector CD4+ T Cells Regulates TCR Activation and Experimental Colitis in Mice. 2010. Gonzalez-Navajas JM, Fine S, Law J, Datta SK, Nguyen KP, Yu M, Corr M, Katakura K, Eckman L, Lee J, Raz E., Journal of Clinical Investigation, 2010. Jose M. Gonzalez-Navajas is the primary investigator and author of this material. Other co-authors include Sean Fine, Sandip Datta, Kim Nguyen, Mandy Yu, Maripat Corr, Kyoko Katakura, Lars Eckman, Jongdae Lee and Eyal Raz.

4. Discussion

TLR activation of APCs leads to an upregulation of costimulatory markers and the release of proinflammatory cytokines. These changes modulate the conditions upon which CD4+ T cells are activated and lead to different responses (Medzhitov, 2001). In this classical model of T cell activation, TLR ligands only play an indirect role in T cell activation. More recently, there has been evidence that certain TLRs are also expressed on CD4+ T cells (Fukata et al., 2008). This suggests that their ligands may also directly affect T cell function. There has been recent evidence that this is the case. TLRs have been shown to act as costimulatory receptors on T cells (Rahman et al., 2009) and in some cases can even act on mature CD4+ T cells without simultaneous TCR stimulation (Imanishi et al., 2007). Here we show that TLRs signal even before TCR stimulation and regulates subsequent TCR activation.

Although *Il10*$^{-/-}$ mice usually develop spontaneous colitis, vivarium conditions affect the age at which colitis develops and its severity (Strauch et al., 2005). In our vivarium, *Il10*$^{-/-}$/*Tlr4*$^{-/-}$ mice develop spontaneous colitis by week 8 while *Il10*$^{-/-}$ mice and *Il10*$^{-/-}$/*Tlr9*$^{-/-}$ mice do not develop colitis even by 8 months. Although, TLR4 deficiency does play a major role in innate immune responses, *Il10*$^{-/-}$ has been shown to be a CD4+ T cell dependent model, and we therefore utilized the adoptive transfer model of colitis to delineate the specific role of TLR4 on CD4+ T cells. This resulted in *Rag1*$^{-/-}$ recipients suffering more severe colitis from receiving naïve CD4+ T cells from *Il10*$^{-/-}$/*Tlr4*$^{-/-}$ as opposed to those receiving naïve CD4+ T cells from *Il10*$^{-/-}$mice.

The colitis in *Il10*$^{-/-}$ mice was originally described as being Th1 mediated inflammation through the production of both IFN-γ and IL-12. Recently, there has been evidence that Th17 cells also play a major role in inducing colitis through their production of IL-17A (Yen et al., 2006). The current literature suggests that proinflammatory cytokines from both these T cell subsets contribute to the development of colitis. Although we have shown a decrease in IFN-γ accompanied by an increase in IL-17A secretion with the prestimulation of CD4+ T cells with LPS, it has recently been shown that IFN-γ is required for the initiation of colitis, whereas IL-17A is required for the perpetuation of colitis (Montufar-Solis et al., 2008).

There has been a recent publication addressing the role of Treg function in *Il10*$^{-/-}$*/Tlr4*$^{-/-}$ mice in a model of intestinal inflammation mediated by *Helicobacter hepaticus* (Matharu et al., 2009). In accordance with our data, the authors reported an increase in intestinal inflammation in *Il10*$^{-/-}$*/Tlr4*$^{-/-}$ mice and increased proinflammatory cytokines in the colon. This was attributed to an increase in IFN-γ production from Foxp3+ T cells lacking TLR4. This suggests that TLR4 also enhances regulatory function. It is most likely that both the effects of increasing the inhibitory capabilities of Tregs and inhibiting TCR activation can be attributed to TLR4 signaling which ultimately regulates colon homeostasis.

In summary, our results demonstrate that LPS stimulation modulates subsequent TCR signaling and this limits the induction of a proinflammatory phenotype, independent of TLRs on innate immune cells. Both TLRs and TCR share certain signaling pathways such as the MAPKs and therefore can affect each others signaling. This expands the thought of LPS tolerance to not only innate immune cells

but now also to adaptive immune cells. This data indicates the importance of a term we coined conditional activation of CD4+ T cells. Because T cells are constantly in contact with cytokines and microbial ligands that use certain pathways shared by TCR, these molecules can affect T cell activation and proliferation that can only be seen after TCR activation. This mechanism therefore can explain the dissimilar activation of CD4+ T cells isolated from different organs (figure 3.4.8) and other studies and underlines the physiological role of conditional activation in the regulation of T cell responses.

The discussion chapter, in part, is published in TLR4 Signaling in Effector CD4+ T Cells Regulates TCR Activation and Experimental Colitis in Mice. 2010. Gonzalez-Navajas JM, Fine S, Law J, Datta SK, Nguyen KP, Yu M, Corr M, Katakura K, Eckman L, Lee J, Raz E., Journal of Clinical Investigation, 2010. Jose M. Gonzalez-Navajas is the primary investigator and author of this material. Other co-authors include Sean Fine, Sandip Datta, Kim Nguyen, Mandy Yu, Maripat Corr, Kyoko Katakura, Lars Eckman, Jongdae Lee and Eyal Raz.

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