

ROLE OF TULA-FAMILY PROTEINS IN T CELL DRIVEN RESPONSES

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ABSTRACT

Role of TULA-family Proteins in T cell Driven Responses

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The TULA-family consists of two proteins implicated in cellular regulation. TULA-1 is expressed in T-cells and is involved in apoptosis. TULA-2 is a ubiquitously expressed phosphatase that suppresses receptor-mediated signaling. T cells from mice lacking TULA-1 and 2 (double knockout, or dKO) are hypersensitive to TCR stimulation. This may be due to these proteins having a similar function working synergistically or dissimilar functions having a convergent effect. To understand functional interaction of these proteins we have characterized TULA-family knockout mice without and during an immune challenge. We show that CD4⁺ T cells of dKO mice have a characteristic CD45RB distribution, and that within the CD45RB^{low} subset effector/memory T cells are expanded only in dKO, but not in single knockouts (sKO) of either TULA-1 or TULA-2. However, CD4⁺ T cells of sKO and wild-type (WT) mice respond differently to TCR stimulation as seen using signaling and responses *in vitro*. To evaluate consequences of TULA deficiency *in vivo*, we utilized two mouse models of inflammatory bowel disease: TNBS-induced colitis and colitis induced by the adoptive transfer of CD45RB^{high} CD4⁺ T cells. Studies utilizing TNBS indicate that deficiency of any TULA-family protein exacerbates TNBS-induced colitis. Likewise, dKO CD45RB^{high} CD4⁺ T cells were significantly more colitogenic than cells from WT mice in the transfer model. Taken together, our data indicate that TULA-family proteins are key to the physiological regulation of T-cell reactivity that drives intestinal inflammation.

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LIST OF ABBREVIATIONS

7-AAD	7-Aminoactinomycin D
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ADTC	Adoptive transfer colitis
Alexa488	Alexa Fluor dye 488
APC	Allophycocyanin
APC	Antigen-presenting cell
APC-Cy7	Allophycocyanin-Cyanine 7
BSA	Bovine Serum Albumin
cc	cubic centimeter
cDNA	complementary DNA
DAPI	4',6-diamidino-2-phenylindole
DEPC	Diethyl pyrocarbonate
dH ₂ O	Deionized H ₂ O
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
DSS	Dextran sulfate sodium
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
G	gauge

GM-CSF	Granulocyte macrophage colony-stimulating factor
GWAs	Genome-wide association studies
HBSS	Hank's Balanced Salt Solution
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA	Human leukocyte antigen
IACUC	Institutional Animal Care and Use Committees
IBD	Inflammatory Bowel Disease
IFN γ	Interferon-gamma
IL-	Interleukin
IP-10	Interferon gamma-induced protein 10
KC	Keratinocyte-Derived Chemokine
kDA	kiloDalton
MCP-1	Monocyte chemotactic protein-1
MIG	Monokine induced by IFN-gamma
MIP1 α	Macrophage Inflammatory Protein-1alpha
MLNs	mesenteric lymph nodes
mRNA	Messenger RNA
NES	Nuclear Export Sequence
NLS	Nuclear Localization Sequence
OCT	Optimum Cutting Temperature
PBS	Phosphate buffered saline
PCCM	Primary cell culture Medium
PCR	polymerase chain reaction

PE	R-phycoerythrin
Pe-Cy7	R-phycoerythrin-Cyanine 7
PerCP	Peridinin-chlorophyll-protein complex
PerCP-	
Cy5.5	Peridinin-chlorophyll-protein complex-Cyanine 5.5
PGM	phosphoglycerate mutase
PMSF	phenylmethanesulfonylfluoride
PTK	Protein tyrosine kinases
PTP	Protein tyrosine phosphatases
pY	phospho-Tyrosine
RAG	recombination activating genes
RBCs	Red blood cells
RNA	Ribonucleic acid
RNAi	RNA interference
RPMI1640	Roswell Park Memorial Institute medium 1640
SCID	Severe combined immunodeficiency
SDS-	sodium dodecyl sulfate polyacrylamide gel
PAGE	electrophoresis
SH3	Src homology domain-3
TBS	Tris-Buffered Saline
TCR	T cell receptor
TEM	Effector Memory T cells
TNBS	2,4,6-trinitrobenzene sulfonic acid

TNF α	Tumor necrosis factor-alpha
Treg	Regulatory T cells
TRIS	tris(hydroxymethyl)aminomethane
UBA	Ubiquitin-association domain
VEGF	Vascular endothelial growth factor

CHAPTER 1

INTRODUCTION

TULA-family of Proteins

The TULA-family of proteins have recently emerged as novel regulators of cellular signaling. There are two members of the TULA-family of proteins TULA-1 and TULA-2. The discovery of these proteins is attributed to the work of several groups, and therefore there are various names for both TULA-1 and TULA-2. The names given are indicative of the manner in which the proteins were characterized (described in detailed below). For simplicity sake these proteins will always be referred to as TULA-1 and TULA-2.

Initial Characterization of TULA-Family Proteins

The initial identification of TULA-1 was made in an effort to find candidate genes for Down syndrome phenotype or monogenic disorders that map to chromosome 21q22.3 (Wattenhofer et al., 2001). Investigators focused on the autosomal recessive nonsyndromic deafness locus (DFNB10), a region critical for this disease, which contained a few candidate genes. One of these genes was shown to encode for a protein containing UBA and SH3 domains, hence it was assigned the name *UBASH3A*. This study excluded *UBASH3A* as a candidate for autosomal recessive nonsyndromic deafness (Wattenhofer et al., 2001).

The TULA-2 protein was first characterized as an interactor of the Jak2 protein tyrosine kinase (Carpino et al., 2002). Investigators used affinity purification to identify proteins that bind to a specific tyrosine in Jak2. Among others, a 70-kDa protein was identified and termed p70. Investigators found that p70 had a SH3 domain and a domain that was 46% similar to the catalytic domain of phosphoglycerate mutase (PGM).

Later, several groups published data on structure and function of these proteins. Carpino et al. developed mice that were deficient in proteins that group termed STS-1 and STS-2. They found that individual knockouts of STS-1 and STS-2 had no observed phenotype. However, when mice were doubly deficient in both STS-1 and STS-2, responses of their T cells were altered (San Luis et al., 2011) Independently, Feshchenko et al. and Kowanetz et al. identified and defined the TULA/STS family of proteins. Feshchenko et al identified the gene product of UBASH3A and termed it TULA, T-cell Ubiquitin LigAnd. They described TULA as a novel protein that binds to c-Cbl via its SH3 domain and to ubiquitin via its UBA domain. Using yeast two-hybrid system Kowanetz et al. cloned TULA/STS-2 and termed it Clip-4 (Cbl-interacting protein 4) as it binds to the proline-rich region of Cbl (Feshchenko et al., 2004; Kowanetz et al., 2004).

Structure of TULA-Family Proteins

TULA is a unique family of proteins. This is the only mammalian family of proteins that contain an Src-homology domain 3 (SH3), an Ubiquitin-associated domain (UBA), and a phosphatase domain. TULA-1 and TULA-2 are 43-45% identical/59-62% similar; the exact number of the homology percentage depends on whether the long or the short isoform of TULA-1 is used for alignment (Tsygankov, 2008). TULA-1 short

isoform is a result of alternative splicing that excludes exon 5 from the mRNA sequence; this isoform is prevalent (Feshchenko et al., 2004).

Ubiquitin Associated Domain – UBA

UBA domains are the first described ubiquitin-binding domains. There are now several ubiquitin binding domains, the largest group is the helical domain group to which UBA belongs. The UBA domain has a compact three-helix bundle to which mono- and polyubiquitin chains bind. The process of forming the covalent bond between an ubiquitin moiety and a protein is known as ubiquitylation. Initially ubiquitylation was found to be associated with cellular regulation by protein degradation, now ubiquitylation is associated with several processes from receptor endocytosis to DNA repair (Hurley et al., 2006). The UBA domain of TULA proteins is located near the N-terminus of the protein. The homology between the UBA domain of TULA-1 and TULA-2 is 50% identity/66% similarity (Tsygankov, 2008). The UBA domain in TULA proteins mediates binding of these proteins to ubiquitin and ubiquitylated proteins, including TULA-family proteins themselves (Feshchenko et al., 2004; Hoeller et al., 2006a; Kowanetz et al., 2004).

Src Homology Domain 3 – SH3

The SH3 domain is a versatile, well-characterized domain of ~60 amino acid, which binds ligands containing proline-rich motifs (PxxP). This domain folds into 5-strand β sheet with surface loops that binds to proline-rich motifs (Kaneko et al., 2008).

TULA proteins have a centrally located SH3 domain (contained within amino acids 280-335 of TULA-1 and 211-271 of TULA-2) that mediates their binding to c-Cbl (Feshchenko et al., 2004; Kowanetz et al., 2004) and dynamin (Bertelsen et al., 2007). As in the case of the UBA domain of TULA proteins, homology within the SH3 domain of TULA-family proteins is substantial (58% identity/72% similarity) (Tsygankov, 2008).

Phosphatase Domain

When the TULA-1 coding sequence was initially described, it was noted that the protein encoded by *UBASH3A* contains a domain of unknown function with homology to Hypothetical proteins found in *C. elegans* and *Drosophila*, which was termed HCD (Wattenhofer et al., 2001). Later, Carpino et al. described TULA-2's (which they termed p70 at that point) C-terminal half as having 46% homology to a catalytic domain of phosphoglycerate mutase (PGM) (Carpino et al., 2004). Since the original description of TULA-family proteins, much work has been done to uncover the structure and function of this domain with homology to PGM.

Proteins with the PGM domain belong to the super family of histidine phosphatases known as 2H phosphatases. The 2H-family of phosphatases has an essential histidine residue within the catalytic site (Davies et al., 2007). Also included in the 2H phosphatase superfamily are the acid phosphatases, co-factor dependent PGMs, fructose-2,6-bisphosphatase, TIGAR proteins, and TULA-family proteins. The 2H phosphatases typically dephosphorylate substrates in a 2-step fashion. The histidine in the signature motif of all 2H's (RHGE) attacks the phosphorus atom in the phosphate group of a phosphorylated substrate; the phosphorus atom forms a covalent bond with an active site

histidine residue, and then the dephosphorylated substrate is released. Subsequently an activated molecule of H₂O hydrolyzes the phosphorus atom; the phosphate group is released, and the phosphatase returns to its pre-reaction state (Ostanin and Van Etten, 1993; Taga and Van Etten, 1982). It is important to note that there are several proteins in the 2H family that are not phosphatases, but mutases instead as is, for example, PGM. The catalytic domain of this family features a conserved catalytic core with the α/β structure, in which a β -sheet core is surrounded by α helices. 2H phosphatases can hydrolyze a set of diverse substrates, including phosphoproteins, phosphopeptides, and low-molecular substrates, such as para-nitrophenyl phosphate (pNpp).

Interest in the potential phosphatase activity of the PGM-like domain of TULA-family proteins developed based on a study describing the hyper-responsive nature of T cells from mice lacking TULA-family proteins (dKO) (Carpino et al., 2004). The hyperactivity of dKO T cells seemed to be the result of increased phosphorylation of proteins involved in TCR signaling (Carpino et al., 2004). Later, TULA-family protein phosphatase activity was further explored, and it was reported that endogenous TULA-1 and TULA-2 could hydrolyze pNpp, albeit TULA-1 phosphatase activity is much lower than that of TULA-2 (Carpino et al., 2009b; Chen et al., 2009; Mikhailik et al., 2007; San Luis et al., 2011). Also, TULA-1 and TULA-2 cannot hydrolyze pSer or pThr. Hence the PGM-like domain was termed a phosphatase domain, and will be referred to it as such.

The crystal structure of the phosphatase domain of TULA-family proteins revealed that TULA-1 and TULA-2 share conserved catalytic residues, Arg379/365 His380/366, Arg462/448, His565/551 (TULA-1/TULA-2). His380/366 is a nucleophilic residue of the active site and its mutation results in an inactive phosphatase. However,

TULA-1 is less active as a phosphatase than TULA-2, so much so that TULA-1 dephosphorylates pNpp at a rate that is similar to that of TULA-2 with the essential histidine mutated. The dephosphorylation activity of TULA-1 toward pNpp is reduced as compared to that of TULA-2, in part, because the pH optimum of TULA-1 phosphatase activity is pH 5. While there is an increase in the K_{cat}/K_m values for TULA-1 and TULA-2 under pH of 5, this modestly increased TULA-1 activity does not compare to that of TULA-2 activity at either pH 5 or pH 7 (Chen et al., 2009). The difference in phosphatase activity between TULA-family members may be due to residues outside the active site. In the three-dimensional structure opposite of the nucleophilic His residue there are sequence variations between TULA-1 and TULA-2. In TULA-1 Gln372, Glu481, and Ser582 are opposite to the nucleophilic His residue, while in TULA-2 these are Val386, Val495, and Tyr596. It is possible that the differences between these residues result in an alternate conformation of the phosphatase domain that prohibit movement of key catalytic residues and/or change charges within the catalytic domain that interfere with the transition state during catalysis (Chen et al., 2009).

It has been determined that the phosphatase domain of TULA-2 prefers to act on substrates that have certain sequences of residues on the N-terminal and C-terminal side of the phosphorylated tyrosine residue. On the N-terminal side of the phosphorylated tyrosine TULA-2 prefers two distinct sequence profiles termed Class I and Class II. Substrates of Class I have a proline residue at the Tyr(P) -1 position followed by a hydrophilic residue in the Tyr(P) -2 position (Ser, Asn, Asp, Gly, or His), then an aromatic hydrophobic residue in the Tyr(P) -3 and -4 position (Trp, Phe, or Tyr). Class II substrates have acidic (positions -2 and -3) and aromatic hydrophobic (-4 and -5)

residues. All Class II N-terminal sequences were found to have at least one aromatic residue (most have two). On the C-terminal side of the phosphorylated tyrosine the sequences are less specific as compared to that of the N-terminal side sequences. C-terminal sequences that are preferred by TULA-2 phosphatase contain acidic and aromatic residues (Chen et al., 2010). Furthermore, both N-terminal and C-terminal-side substrate sequences contain very few basic residues. The only exception from this rule is N-terminal Class I substrate sequences, in which basic residues are permissible in the positions -4 and -5 (Chen et al., 2010).

Using this substrate specificity profile, it was determined that several known signaling proteins contain the pY-centered motif that should be actively dephosphorylated by TULA-2. Of these proteins, tyrosine 352 of Syk was predicted to be the best substrate site for TULA-2. Consistent with this prediction, it was shown that phosphotyrosine 352 of Syk is a much better substrate for TULA-2 than phosphotyrosine 525 of Syk, which is surrounded by not-so-ideal sequences. Accordingly, stimulated platelets from mice deficient in TULA-2 have an increase in the phosphorylation of Syk on tyrosine 317, 346, and 519/520 (corresponds to human tyrosine 323, 352 and 525/526 respectively) and tyrosine 346, which is predicted to be the best substrate, shows in these experiments the highest increase in phosphorylation (Chen et al., 2009).

Nuclear Localization Signal (NLS) & Nuclear Export Signal (NES Motifs)

The TULA-family proteins have a Nuclear Localization Signal (NLS), which directs traffic of proteins to the nucleus. TULA-1 contains two putative NLS sequences 400PRRSRGL406 and 463KKIK466 (Feshchenko et al., 2004; Wattenhofer et al., 2001).

In addition to the two putative NLS sequences, a putative Nuclear Export Signal (NES) (454LILEELKLEK463) has been found within the C-terminal portion of TULA-1. Regions homologous to TULA-1 NLS are not present in TULA-2. Both TULA-1 and TULA-2 are present in nuclear cell fractions, but most of TULA-1 and TULA-2 is found within cytoplasm (Feshchenko et al., 2004).

Post-translation modifications

It has been shown that the TULA-family proteins undergo some post-translational modifications. TULA-1 proteins are monoubiquitylated at Lys-202, and ubiquitylation at this site is dependent on an intact UBA domain (Hoeller et al., 2006a). The second post-translational modification is phosphorylation of Tyr-19. TULA-2 is phosphorylated on Tyr-19 in response to CD3 stimulation; however, with CD28 co-stimulation this phosphorylation is reduced by 25% (Kim and White, 2006)

Expression of TULA Family Proteins

While TULA-family proteins share structural similarities they are differentially expressed. TULA-1 is expressed in lymphoid cells; both in T and B cells in humans, however, to a less extent in B cells (Carpino et al., 2004; Feshchenko et al., 2004; Kowanetz et al., 2004; Wattenhofer et al., 2001). However, in mice TULA-1 expression is practically limited to T-cells. TULA-2 is expressed ubiquitously, although platelets have the highest level of TULA-2 expression known to date (Thomas et al., 2010)

Function of TULA Family Proteins

UBA and SH3 Mediated Functions

Receptor endocytosis as a means of regulation is a well known and characterize process that was first observe in *C. elegans* where SLI-1 (Cbl orthologue) suppresses signaling of LET-23 (EGFR orthologue) (Jongeward et al., 1995; Sternberg et al., 1995; Yoon et al., 1995). In general, the mechanism of regulation via receptor endocytosis occurs in phases. After the receptor has been engaged the ligand receptors and co-receptors form a complex were a series of autophosphorylation events occur. Phosphorylation of the receptor recruits several proteins that perpetuate a downstream signaling cascade that ultimately results in a cellular response. After the signaling cascade has been initiated the receptor is removed from the cell surface via endocytosis, E3 ligases, like c-Cbl mediate ubiquitylation of these receptors (Hoeller et al., 2006a; Hoeller et al., 2006b). Monoubiquitylation may result in the recruitment of UBA binding proteins; however, if the receptor is polyubiquitylated it will be targeted to degradation via the 26S proteasome. In either case, when the receptor is removed from the cell surface the signaling events initiated by ligand binding are dampened (reviewed in (Schmidt and Dikic, 2005).

TULA-family proteins were found to be involved with processes whereby c-Cbl mediates degradation of the receptor protein tyrosine kinases (PTKs). Specifically, TULA-family proteins where shown to block c-Cbl mediated degradation of the epidermal growth factor receptor (EGFR) after ligand stimulation (Feshchenko et al., 2004; Kowanetz et al., 2004). After stimulation of receptor PTKs, c-Cbl is recruited to

the receptor complexes. When TULA is present it does not inhibit c-Cbl binding or ubiquitylation of receptor PTKs. However TULA-family proteins do inhibit removal of EGFR from the cell surface, formation of vesicles containing EGFR, activation of pathways downstream of receptor PTK signaling, and transcription factors (Feshchenko et al., 2004; Kowanetz et al., 2004).

Additionally, when mice are deficient in TULA-family proteins TCR-induced phosphorylation of a subset of ubiquitylated proteins is significantly increased suggesting that TULA-family proteins may target ubiquitylated phosphorylated proteins (Carpino et al., 2009a). The UBA domain of the TULA-family proteins has another function independent of their interaction with c-Cbl. In an effort to identify proteins that bind to TULA-1, it was found that both TULA-family proteins bind to ABCE-1 (ATP-binding cassette proteins family E member 1) (Kerr, 2004), also termed previously human protein 68kDa and RNase L inhibitor (HP68 and RLI). ABCE-1 was found to be essential for HIV-1 capsid assembly, and to negatively regulate RNase L, an anti-viral enzyme. TULA-1 and -2 were shown to dampen viral replication. While the binding of TULA proteins to ABCE-1 was not dependent on the UBA or the SH3 domain of the TULA-family proteins, the suppressive effect on HIV-1 replication was dependent on functional UBA domain. TULA-1 and TULA-2 both suppress HIV-1 replication (Smirnova, 2008).

In the same mass spectrometry-based study that demonstrated binding of TULA-1 to ABCE-1, we also showed that TULA-1 binds to the Apoptosis Inducing Factor (AIF), a protein known to induce a specific type of apoptosis (Collingwood et al., 2007a). This binding was specific for TULA-1, since TULA-2 was not found in association with AIF.

Currently, cell death is classified into several types, including apoptosis, autophagy, necrosis, secondary necrosis, pyroptosis, and mitotic catastrophe (reviewed in (Green et al., 2009)). Apoptosis can be induced in a caspase-dependent and/or -independent manner. AIF was the first protein shown to induce apoptosis in the presence of a caspase inhibitor, indicating that the apoptosis induced by AIF is caspase-independent (Lorenzo et al., 1999). AIF is normally located in the mitochondria, however, under conditions of cellular stress AIF translocates to the nucleus, where it causes chromatin condensation and DNA degradation (reviewed in (Green et al., 2009)). AIF induces cell death in response to specific cellular stresses including those related to neuronal excitotoxicity, DNA alkylation damage, and ischemia/growth factor withdrawal (reviewed in (Joza et al., 2009)).

The study by our group demonstrated that TULA-1 overexpression increases basal level of apoptosis as well as that induced by growth factor withdrawal, while RNAi-mediated depletion of TULA-1 reduced apoptosis. As hypothesized initially, facilitation of apoptosis by TULA-1 overexpression was dependent on AIF; RNAi depletion of AIF reduced the pro-apoptotic effect of TULA-1. Finally, the role of TULA-1 domains was characterized; TULA-1 binds to AIF via its N-terminal half and the mutant lacking this region was unable to facilitate apoptosis. Mutants of TULA-1 lacking either the UBA or SH3 domain were capable of interacting with AIF, but unable to facilitate apoptosis; this result suggests that TULA-1 facilitates apoptosis by simultaneous binding to AIF and other proteins (Collingwood et al., 2007a).

Phosphatase Mediated Functions

Since the initial characterization of phosphatase domain of TULA-family proteins much has changed. It is now termed a phosphatase domain, because it belongs to the superfamily of 2H-histidine phosphatases and consistent with this classification exhibits phosphatase activity. TULA-2 is a very active phosphatase. TULA-1 is dramatically less active, although its weak phosphatase activity can be increased under acidic conditions (Chen et al., 2010; Chen et al., 2009; Mikhailik et al., 2007). For this reason we will focus on TULA-2 in this section.

The first indication that TULA-2 may function as a negative regulator of cellular signals was provided by the work done in TULA-family knockout (mice doubly deficient in both TULA-1 and TULA-2 are termed double knockout mice (dKO), those deficient only in TULA-1 or TULA-2 are termed single knockout-1 or single knockout-2 (sKO1 or sKO2). Carpino et al. developed mice that were deficient in either TULA-1 or TULA-2 and observed no overt change in their phenotype. However, dKO resulted in hyperactive T cells; when these T cells were stimulated *in vitro*, their proliferation and cytokine responses were increased significantly as compared to those of WT counterpart cells (Carpino et al., 2004). The mechanism underlying this hypersensitivity of dKO T cells to TCR engagement was an increase in the phosphorylation of signaling proteins downstream of TCR, such as Zap-70. These data suggested that TULA-family proteins have a role in dampening cellular signals after stimulation. Accordingly, dKO mice were more sensitive to experimental autoimmune encephalomyelitis than WT mice. Later the same group determined that the hyperactivity of the dKO T cells was the dependent on

the phosphatase activity of TULA-2 (Mikhailik et al., 2007). TULA-2 has been shown to be a protein tyrosine phosphatase capable of dephosphorylating such proteins as Zap-70 and Src (Mikhailik et al., 2007), EGFR (Mikhailik et al., 2007; Tsygankov, 2008), c-Cbl (Raguz et al., 2007), Syk and Fyn (Agrawal and Carpino, 2008). Using a library of pY peptides, classes of peptides with certain sequences of residues were predicted to dephosphorylate by TULA-2 were characterized (in more detail above). Based on these predictions and the PhosphoSitePlus data for convulxin-induced platelets it was found that Syk contains the best known substrate site and that several other proteins are likely to be targets of TULA-2 in platelets, including PEAR1, Erk1/2, Src, focal adhesion kinase, BTK, and PKC (Chen et al., 2010).

All these data indicate that TULA-2 is a protein tyrosine phosphatase that down regulates cellular signals induced by receptor-linked PTKs. It is not known how TULA-1's PGM domain contributes to the overall effect seen in T cells deficient in both TULA-1 and TULA-2. We do know that the activity of TULA-1 is increased under acidic conditions, however this activity is still much lower than that of TULA-2. It has been suggested, that either TULA-1 functions in a phosphatase-independent manner or TULA-1 dephosphorylates unknown signaling proteins that are key for TCR signaling.

TULA Family Deficiency

It was observed that there were high levels of TULA-family proteins in hematopoietic cells. However, there was no difference in the numbers of red blood cells, hematocrit or hemoglobin levels between dKO and WT mice. Nor was a difference in the number of bone marrow cells, peripheral blood leukocytes, B220 positive B cells, and

Thy1.2 positive T cells or the ratio of thymic single positive CD4 and CD8 T cells between dKOs and WT. Additionally, the ratio of single positive CD4 and CD8 in the spleen and lymph were comparable between dKO and WT.

Interestingly, the number of total splenocytes was increased in dKOs (20%) as compared to the number of WT splenocytes. Also, there was an increase in the percent of mature B cells (~20%) in the spleen, and a similar phenomenon was observed in lymph nodes. T cells from dKO mice, as compared to WT, were hyper-responsive to TCR stimulation as seen by increases in proliferation, production of cytokines (IL-2, IL-4, IL-5, IL-10, and IFN γ) and phosphorylation of signaling proteins downstream of TCR. Accordingly, Carpino et al. observed that mice doubly deficient in TULA-family proteins are susceptible to experimental autoimmune encephalomyelitis (Carpino et al., 2004).

Inflammatory Bowel Disease

IBD in Brief

Crohns disease, one of the two major forms of IBD, can be traced back to 1761; Morgagni was the first to describe ulcerative colitis and Crohns disease among several other diseases (Morgagni, 1761, 1762). In 1932, Crohns, Ginzburg, and Oppenheimer were the first to publish a clear and comprehensive description of regional ileitis (Crohn et al., 1932).

IBD is an umbrella term that describes its two major manifestations: Crohns disease and ulcerative colitis. No single agent has been demonstrated to be the root cause of IBD, which is clearly a multifaceted condition with several contributing factors. The hygiene hypothesis put forth by David Strachan in 1989, suggests that prevalence of

autoimmune disorders like IBD is a relatively new phenomenon. Furthermore, it was suggested by Strachan that the lack of intense infection in industrialized countries owing to improved hygiene, vaccination, and the use of antibiotics might alter the human immune system in such a way that it responds inappropriately to innocuous substances as in the case of IBD (Strachan, 1989). Currently, the general consensus is that an overly aggressive, unregulated immune response to intestinal flora causes IBD.

Clinical Manifestation of IBD

IBD develops during the second to fourth decade of life. Patients suffer from chronic and relapsing bouts of intestinal inflammation that result in abdominal pain, bloating, rectal bleeding, diarrhea, fatigue, malnutrition and weight lost. In Crohns disease patients may suffer from fistulae. Fistulae are obstructive strictures of the bowel or that can also occur between segments of bowel or between the bowel and skin and other organs. In addition to the symptoms caused by intestinal inflammation, patients with IBD typically suffer from another autoimmune disorders like primary sclerosing cholangitis, ankylosing spondylitis and psoriasis (Loftus, 2004). Crohns disease can affect the entire gastrointestinal track, while inflammation typical of ulcerative colitis is found only with the colon. Histologically inflammation associated with Crohns disease is transmural and discontinuous, while inflammation associated with ulcerative colitis is superficial and continuous throughout the colon (Cho, 2008; Strober et al., 2007; Xavier and Podolsky, 2007).

Factors that Contribute to the Development of IBD

Microbes and genetic disposition are thought to be the major contributors to the development of IBD, while no one microbe can be defined as a causative agent IBD does not develop in a germ free environment. I will describe in the contribution of each in more detail below.

Microbial Contributions to IBD

The role of microbial flora in the development of IBD is not entirely understood in part because the composition of this flora is complex. It is thought that commensal flora has a fundamental role in the development and the progression of IBD. The indirect support for this argument is provided by the studies showing that the patients with IBD have more enteric bacteria when compared to the control healthy group (Barnich et al., 2007; Darfeuille-Michaud et al., 1998). Additionally, probiotics have been shown to ameliorate IBD in a subset of patients. This may be mediated by stimulation of IL-10 production by certain probiotic bacteria (Gionchetti et al., 2003; Lammers et al., 2003). More direct evidence to support the crucial role of bacteria in the development of IBD is provided by the studies demonstrating that certain bacteria can induce colitis in certain mouse strains (Sellon et al., 1998). Most noteworthy are the studies indicating that mice in which colitis develops spontaneously under specific pathogen-free conditions do not develop colitis when housed in gnotobiotic (pathogen-free) facilities (Elson et al., 2005; Onderdonk et al., 1977).

Genetic Factors

There are several lines of evidence supporting that contribution of genetic factors to the development of IBD. Epidemiological studies that suggest a genetic contribution by showing IBD is more common in industrialized countries with the highest prevalence in North America, Europe, and the United Kingdom averaging 100-200 cases per 100,000 (Bernstein and Blanchard, 1999; Bernstein et al., 1999; Loftus, 2004; Loftus et al., 1998, 2000). Americans of European decent, especially those of Jewish decent, have a higher prevalence of IBD (Cho, 2008). The importance of genetic contribution to IBD is supported by the finding that 5-20% of IBD patients have a family history of this disease, especially in the case of Crohns disease (Binder, 1998). Accordingly, studies involving twins show that there is a higher prevalence of IBD among monozygotic twins as compared to dizygotic (Bernstein and Blanchard, 1999; Halfvarson et al., 2003; Orholm et al., 2000; Thompson et al., 1996; Tysk et al., 1988).

The use of genome-wide association studies (GWAs) has also provided strong evidence that there are genetic factors contributing to the development of IBD. The genes identified in GWAs have been characterized based on the known function of that gene and how it is altered in IBD patients. Polymorphisms in genes are typically categorized as those having a role in intestinal barrier functions, innate immunity, adaptive immunity, or regulatory immune functions (Illustration 1).

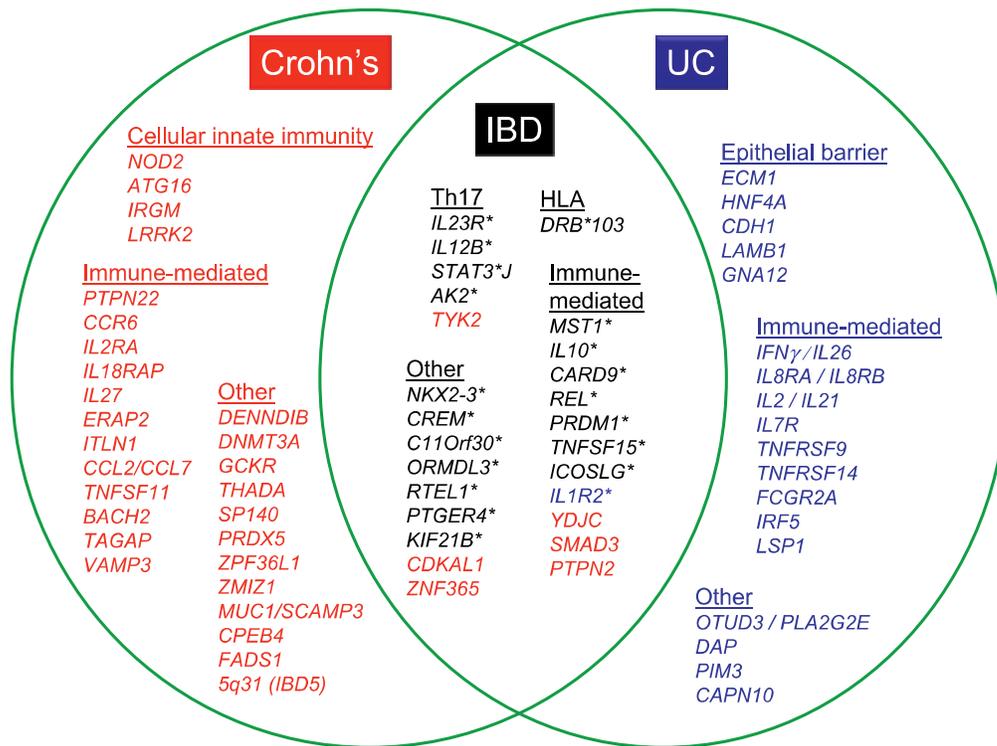


Illustration 1

IBD susceptibility loci. The loci (depicted by lead gene name) attaining genome-wide significance are shown for Crohn's disease (red where $p < 5 \times 10^{-8}$), ulcerative colitis (blue where $p < 5 \times 10^{-4}$) and IBD (black where $p < 5 \times 10^{-8}$) (Lees et al., 2011).

Barrier function. A single layer of epithelial cells provides a physical barrier between the contents of the gut and the intestine. Tight junctions between epithelial cells in this layer physically maintain the integrity of the intestinal barrier. Tight junctions inhibit large molecules and bacteria from freely passing into intestine. In addition to epithelial cells there are goblet cells and paneth cells, both of which contribute to protection of the epithelial barrier. Goblet cells provide a protective layer of mucus, while Paneth cells secrete anti-microbial defensins, both work to prevent pathogens from colonizing the intestinal mucosa. Early evidence that suggested that barrier function is affected in IBD patients were provided by the studies done in first degree relatives of Crohns disease patients, which showed that these individuals had abnormal intestinal permeability (Irvine and Marshall, 2000; May et al., 1993; Soderholm et al., 2002). Genome-wide association studies found that the gene that encodes for the prostaglandin receptor EP4 is associated with Crohns disease (Libioulle et al., 2007). This receptor is expressed on epithelial cells and directly regulates barrier function. Consistent with these observations, EP4 knockout mice are susceptible to DSS-induced colitis (Kabashima et al., 2002). Goblet cells, paneth cells and epithelial cells play an important role in maintenance of the epithelial barrier, defects in genes related to the epithelial barrier lead to increased sensitivity to the development of colitis (Mashimo et al., 1996; McVay et al., 2006; Van der Sluis et al., 2006; Wehkamp et al., 2004; Wehkamp et al., 2005). Of the 99 current susceptibility loci/genes that have been associated with the development of IBD, 71 loci are associated with Crohns disease and 47 are associated with ulcerative colitis. Among these loci/genes few have known function. Among the genes with known roles, many affect epithelial and/or barrier function and are associated with ulcerative

colitis. These genes are ECM1, HNF4A, CDH1, LAMB1, and GNA12 (Anderson et al., 2011; Barrett et al., 2009; McGovern et al., 2010; Rioux et al., 2009).

Innate immune function. The innate immune function as it pertains to IBD is to sample microbes from the lumen and, if needed, initiate an effector immune response. It is crucial that the immune system maintains a state of hypo-responsiveness known as tolerance to avoid inflammatory response to commensal bacteria (Xavier and Podolsky, 2007). The innate response is orchestrated by a number of cells lying beneath the epithelial-cell barrier that screen luminal contents for pathogens. M cells, macrophages, and especially dendritic cells are responsible for collecting and presenting antigen to T cells and initiating an appropriate and specific response (Coombes and Maloy, 2007; Zaph et al., 2007). The collection of antigen is mediated by a vast system of receptors that recognize molecular microbial patterns including 11 toll-like receptors, 23 NOD-like receptors, glycan-binding receptors. While there are several studies stressing the role of each of these cell types and their respective receptors in the pathogenesis of IBD, the major genetic factor that effects innate immune responses is the polymorphism in an IBD susceptibility locus IBD1 or NOD2. Dendritic cells, macrophages, and to a lower extent epithelial cells and T cells express NOD2 (Hugot et al., 2001; Ogura et al., 2001).

NOD2, nucleotide-binding oligomerization domain protein, is a pattern recognition receptor that functions as an intracellular sensor of bacterial peptidoglycan or MDP (Girardin et al., 2003a; Girardin et al., 2003b; Inohara et al., 2003). The 3 most common polymorphisms are Arg702Trp, Gly908Arg, and Leu107fsinCys (frame shift). All of these polymorphisms are associated with Inflammatory Bowel Disease (Croucher

et al., 2003; Leong et al., 2003; Yamazaki et al., 2002). The mutations are typically within the leucine-rich repeat region, which mediates the sensing of bacterial proteins (Abraham and Cho, 2006). These polymorphisms are found primarily in people of European descent and not among people of African or Asian descent (Kugathasan et al., 2005). However, the three most common polymorphisms result in a 40-fold increase in the likelihood of developing Crohns disease in any person regardless of the ancestry (Xavier and Podolsky, 2007).

How exactly NOD2 polymorphisms contribute to the development of Crohns disease is not fully understood, but there are several studies that shed light on this. Lamina propria mononuclear cells of Crohns disease patients have an increase in NF- κ B activity (Hampe et al., 1998). The activation of NOD2 by MDP leads to the activation of NF- κ B through receptor-interacting serine-threonine kinase-2 (RIPK2) signaling pathways (Inohara et al., 2003; Kobayashi et al., 2005). The activation of NOD proteins are regulated, and their interaction with SUGT1 and HSP90 during signaling serve to transmit activating and desensitizing signals, suggesting that the NOD2/MDP interaction is involved in the development of tolerance (da Silva Correia et al., 2007; Mayor et al., 2007). Additionally, NOD2 knockout mice have been shown to have a decrease in the defensin response. Accordingly, Crohns disease patients with a NOD2 polymorphism have a decrease in alpha-defensins (Bevins et al., 2009; Voss et al., 2006; Wehkamp et al., 2004; Wehkamp et al., 2005) suggesting that NOD2 facilitates the production of anti-microbial substances from Paneth cells. Another possibility is that NOD proteins synergize with TLRs to promote the production of IL-23, which has been shown to be the major mediator of intestinal inflammation. Interestingly, recent studies have linked

NOD2 function to the function of ATG16L1, which is encoded by the gene strongly associated with IBD as shown by genome-wide association studies. In fact, NOD2 and ATG16L1 are both strongly associated with Crohns Disease specifically (Croucher et al., 2003; Hampe et al., 2007; Hugot et al., 2001; Hugot et al., 2007; Leong et al., 2003; Ogura et al., 2001; Prescott et al., 2007; Wehkamp et al., 2004). NOD2 recognizes MDP and recruits ATG16L1 to the bacterial entry site following autophagy mediated by RIPK2, ATG5, ATG7 (Fritz et al., 2011). Accordingly, dendritic cells from Crohns disease patients who have NOD1 and NOD2 or ATG16L1 polymorphisms have defective bacterial processing and autophagy (Cooney et al., 2010; Travassos et al., 2010). The intestine should be in a hyporesponsive immune state this mediated by TLRs and NLRs signaling within innate immune cells. Chronic stimulation of TLRs will result in tolerance and this should also be the case for NLRs, however in Crohns Disease patients, chronic stimulation does not induce tolerance (Hedl et al., 2007; Watanabe et al., 2008).

Current research suggests that dysfunction in microbial processing by APCs is the central to the etiology of Crohns disease. Many genes including NOD1/2, ATG16, IRGM, and LRRK2 are associated with Crohns disease specifically (Lees et al., 2011).

Adaptive immune function. Dendritic cells are among the multiple innate cell types that can prime the adaptive immune response. Likewise, it is known that CD4⁺ T cells are involved in the development of IBD, in fact historically Crohns disease and Ulcerative colitis have been distinguished based on their association with Th1 and Th2 responses, respectively (Illustration 2). Innate immune cells will present antigen to naïve CD4⁺ T cells and produce cytokines that will mediate naïve CD4⁺ T cell differentiation

into T helper subsets. The production of IL-12 promote the activity of transcription factor T-bet and mediates the differentiation of naïve T cells into Th1 cells, which are classically associated with the pathogenesis of Crohns disease. However if IL-4 is produced by innate cells it will induce activation of the transcription factor GATA3 resulting in the development of Th2 from naïve CD4⁺ T cells, Th2 cell are typically associated with the Ulcerative colitis. Another subgroup of T-helper cells is Th17, which is involved in the development of both Ulcerative colitis and Crohns disease. Th17 T cells differentiate from naïve T cells when both IL-6 and TGFβ dominate the cytokine milieu; these cytokines induce signaling that leads to the activation of the transcription factor RORγt (Kastelein et al., 2007; Mosmann et al., 1986; Murphy, 2005; Weaver et al., 2007).

Dendritic cells and macrophages secret IL-23 (Kastelein et al., 2007; Schnurr et al., 2005). IL-23R is highly regulated and expressed on activated Th17 cells (Cho and Weaver, 2007). IL-23-stimulated cells produce, IL-17A/F, IL-6, IL-22, TNFα, and CXCL1 (Kastelein et al., 2007; Schnurr et al., 2005). IL-23 binds to its receptor on Th17 cells and initiates the activation of Jak2, which results in autophosphorylation of Jak2 and the phosphorylation of IL-23R by Jak2, which lead to the recruitment, phosphorylation and homodimerization of STAT3, which then translocates to the nucleus where it induces the transcription of genes related to Th17 (Parham et al., 2002).

There are several polymorphisms within the IL-23 pathway that are associated with IBD and one specific polymorphism is uncommon in Crohns disease patients and is thought to provide protection, it is known as Arg381Gln mutation (rs11209026), where the glutamine is protective. IL-23Rα, one of the two subunits of IL-23R, has tyrosines in

its cytoplasmic tail that mediate IL-23R signaling. HuIL23R α , which encodes for IL-23R α , has 24 alternative spliced variants. The dominant spliced variant excludes exon 9 (accounts for 20% of total HuIL23R α protein) and results in the IL-23R α protein with an intact extracellular domain, but lacking the membrane anchor and signaling domain. The soluble Δ 9 splice variant of IL-23R α is secreted and sequesters IL-23, thus inhibiting its actions on Th17 cells (Yu and Gallagher, 2010). It is theorized that the dominance of the protective spliced variant of IL-23R α is secreted from the cells and sequesters IL-23, thus preventing it from binding to the IL-23R and providing intrinsic regulation of IL-23R signaling in Th17 cells.

IL-23 is essential for the maturation, expansion and maintenance of Th17 cells (Manel et al., 2008; Mangan et al., 2006; Veldhoen et al., 2006; Volpe et al., 2008; Yang et al., 2008; Zhou et al., 2007). The importance of these cells to the development of IBD is supported by several functional and genome-wide association studies. In fact, genome-wide association studies have linked 5 genes involved in the IL-23 pathway to the development of IBD (IL-23R, IL-12B, JAK2, STAT3 and TYK2) (Anderson et al., 2011; Barrett et al., 2008; Barrett et al., 2009; Fisher et al., 2008; Franke et al., 2010; Parham et al., 2002). Furthermore, genome-wide association studies have linked IL-23 polymorphisms with several other autoimmune diseases, some of which have clinical overlap with IBD, including psoriasis, ankylosing spondylitis, Bechet's disease (Illustration 3) (Burton et al., 2007; Cargill et al., 2007; Mizuki et al., 2010; Remmers et al., 2010).

Regulatory immune function. The effector functions of immune cells are actively regulated by a subset of T cells known as regulatory T cells. IBD patients and experimental models have shown that there is a significant role of regulatory T cells and indicates dysfunction in these cells leads to the development of IBD.

The best-described regulatory T cells are those that express a transcription factor known as Forkhead Box P3 (FoxP3). There are two major types of FoxP3-expressing regulatory T cells, naturally occurring (nTregs) and inducible (iTregs), which are derived in the thymus and the periphery, respectively. nTregs are mainly self-antigen specific, while iTregs develop from naïve CD4⁺ T cells and have a similar TCR repertoire. While the mechanism of regulation employed by nTregs and iTregs are considered to be similar, overall nTregs function to prevent autoimmunity, while iTregs suppress immune responses to microflora and food antigens (Horwitz et al., 2008). In addition to regulatory T cells that express FoxP3, another well-characterized regulatory T cell is the IL-10 producing type 1 regulatory T cell named Tr1. Less is known about Tr1 than about the FoxP3-expressing regulatory T cells, but we do know that these cells developed from naïve CD4⁺ T cells that encounter their cognate antigen on tolerogenic APC and/or in the presence of IL-10 (Allan et al., 2008).

The mechanisms by which regulatory T cells suppress immune functions are: production of IL-10, TGFβ, IL-35 and/or granzyme/perforins, deprivation of IL-2, degradation of ATP via ectonucleotides, dampening APC cell function via CTLA-4 (reviewed in (Shevach, 2009)). Currently, volume of data supporting the importance of regulatory T cells in the development of IBD is less than that of adaptive or innate immune cells, however the research is extremely significant. The best experimental

model describing the importance of these cells is one that stresses the *in vivo* consequences of regulatory depletion. Powrie and colleagues developed a model in which effector CD4⁺ T cells are sorted based on CD45RB expression. CD45RB highly expressing cells alone can cause colitis in an immunocompromised host, such as RAG or SCID mice. Inflammation in this model is suppressed by CD4⁺ CD25⁺ T cells, which are present, for example, in the CD45RB low expressing population. Anti-IL-10 or anti-TGFβ antibodies administered *in vivo* blocked suppression of inflammation induced by the CD45RB low expressing cells. Additionally, it was found that CD45RB^{low} cells from IL-10 deficient mice could not suppress colitis in this model. These data suggest that suppression in this model is dependent on IL-10 and TGFβ (Asseman et al., 1999; Powrie et al., 1994b; Uhlig et al., 2006). IL-10 and TGFβ were also found to be essential for the development of colitis in a *Helicobacter hepaticus*-induced-T cell transfer model (Maloy et al., 2003). Furthermore, studies in humans have shown that regulatory T cells migrate from the periphery into inflamed tissues in IBD patients (Brand, 2009; Eastaff-Leung et al., 2010; Himmel et al., 2008; Maul et al., 2005; Saruta et al., 2007; Takahashi et al., 1990; Uhlig et al., 2006). Additionally IBD patients have an increase in SMAD7 expression, which blocks TGFβ expression, thus inhibiting suppression of immune responses by regulatory T cells (Eastaff-Leung et al., 2010). The most convincing evidence that regulatory T cells are important in the development of IBD are the genome-wide association studies that reveal an association of polymorphisms in genes encoding FoxP3 and IL-10R in Ulcerative colitis and Crohn's disease patients. Polymorphisms in FoxP3 result in a disease known as Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked (IPEX). IPEX is characterized by severe intestinal inflammation.

Accordingly, polymorphism in the IL-10R results in primary immunodeficiency characterized by a severe IBD-like phenotype within the first year of life (Franke et al., 2008; Franke et al., 2010; Maul et al., 2005). These studies directly link the role of regulatory T cells to human disease and strongly suggest the importance of regulatory T cells in the development of IBD and emphasize the role of the intestinal immune system in maintaining a hypo-responsive state.

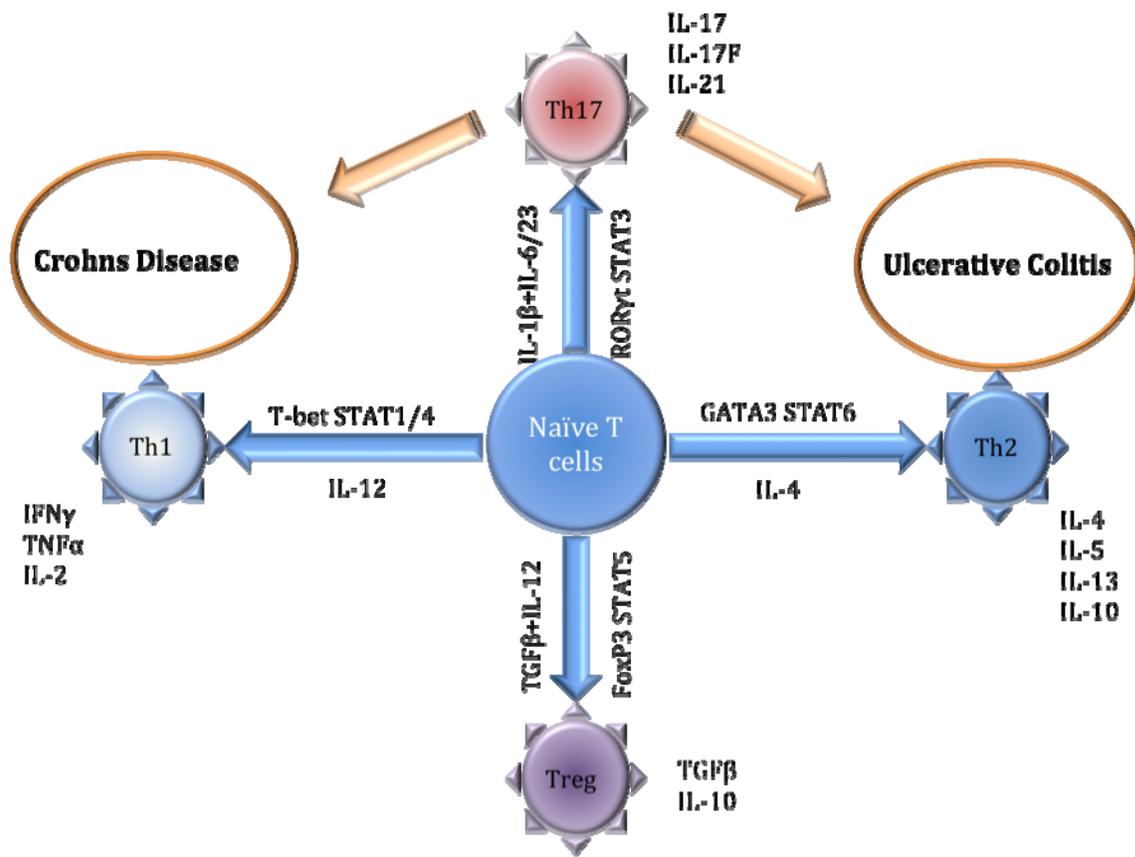


Illustration 2

Helper T cell differentiation and associated cytokines and transcription factors.

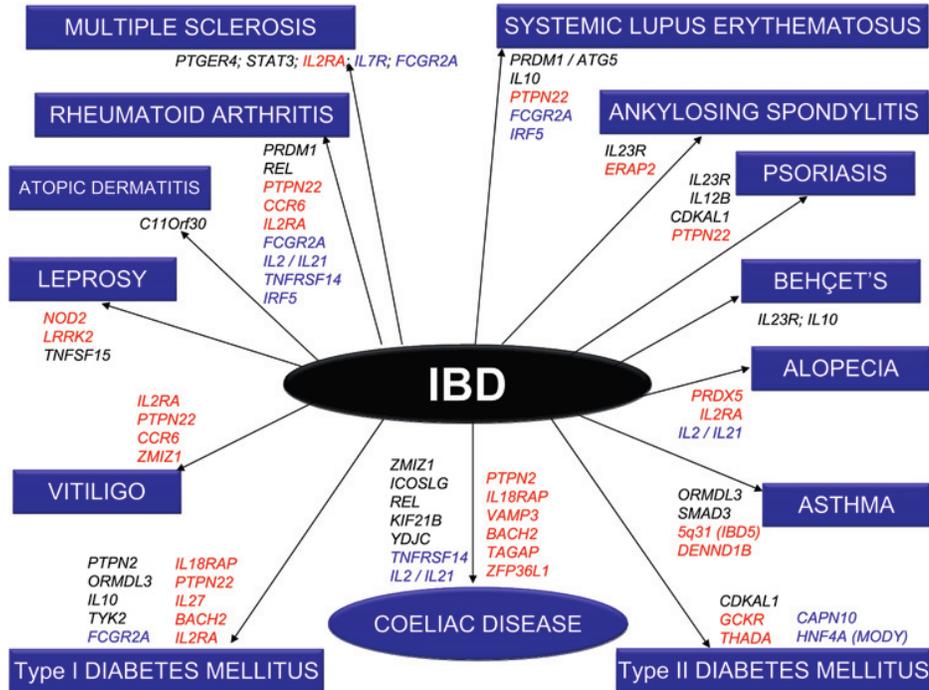


Illustration 3

IBD Disease Overlap. To ensure clarity of present data as well as presentation, only those genes that have attained genome-wide levels of significance in all diseases are depicted here. IBD loci (Crohn's disease and ulcerative colitis) are depicted in black, Crohn's disease (CD) only loci in red and ulcerative colitis (UC) only loci in blue (Lees et al., 2011).

Murine Models of T cell-mediated IBD

There are several animal models used to study IBD-like diseases. Generally these models are characterized based on whether they depend on innate or adaptive immune cells to mediate inflammation. There are genetic alterations that will mediate the development of colitis, for example knockout mice, transgenic mice, and congenic strains. Chemical models of inflammation are also used to induce colitis in genetically susceptible strains. Also there are models where by the transfer of T cells can directly cause colitis (Illustration 4).

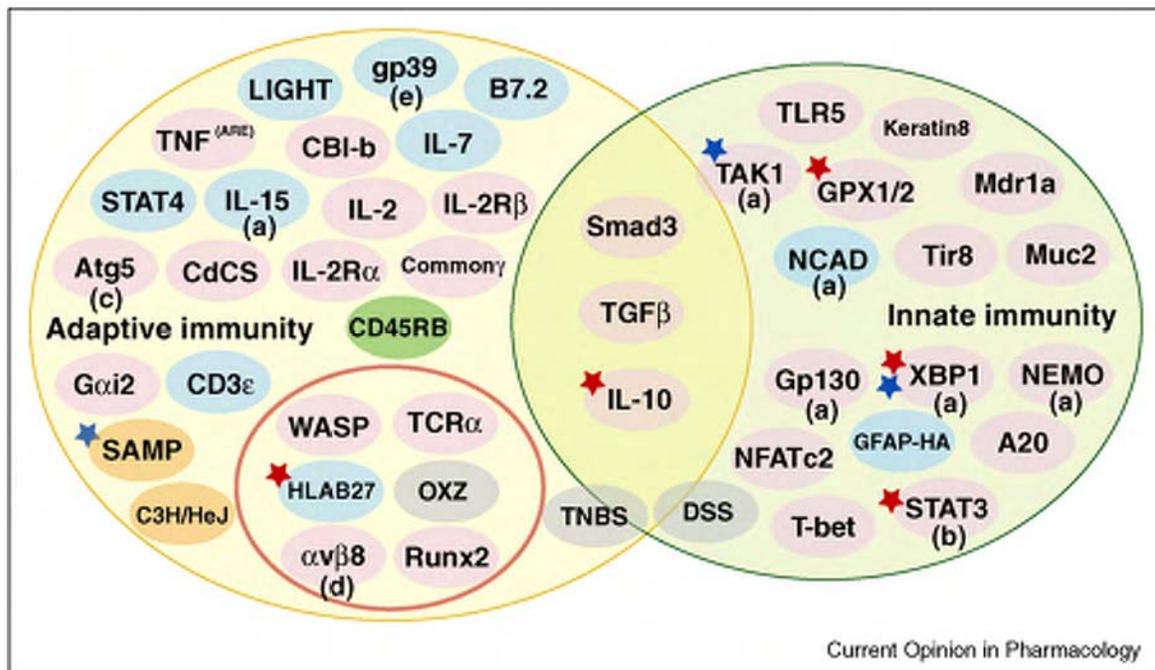


Illustration 4

Animal models of IBD. Many animal models of IBD are currently available to use, including knockout mice (pink), transgenic mice (blue), congenic mice (orange), chemically induced models (gray), and cell-transfer model (green). There are genetically engineered mice that have specific deletion of a target molecule in epithelial cells (a), epithelial cells/macrophages (b), thymic epithelial cells (c), dendritic cells (d), or B cells (e). A red circle surrounds Mouse models, which have been used for studying UC. Blue stars indicate Mouse models, which develop ileitis spontaneously. Mouse models, which lack an IBD-associated gene, are highlighted by red stars. The models used that rely on the adaptive immune response are to the left (orange circle) and innate immune response on the right (green circle) (Mizoguchi and Mizoguchi, 2010).

Genome-wide association studies revealed new susceptibility loci/genes and provided further validation of previously known susceptibility loci/genes. The majority of transgenic mice engineered based on IBD susceptibility loci do not develop IBD-like condition spontaneously, however, mice deficient in IL-10, STAT3, XBP1 and GPX1/2 mice and human HLA-B27-expressing rats spontaneously develop colitis or ileitis. Other transgenic mice are susceptible to the development of IBD under certain circumstances.

There are three major models of chemically induced colitis: dextran sulfate sodium induced colitis (DSS), oxazolone-induced colitis, and 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis. In DSS-induced colitis, 3 to 10% DSS is added to drinking water. This causes acute epithelial damage leading to bloody diarrhea, wasting, shortening of the colon, and histological inflammation. Inflammation in DSS colitis is thought to be facilitated by mechanisms within the innate immune system, because it can be induced in SCID mice lacking T or B cells (Mizoguchi and Mizoguchi, 2010). Oxazolone-induced colitis is a model where the rectal administration of oxazolone dissolved in ethanol causes intestinal inflammation characterized by wasting, death, and histological inflammation similar to that in IBD (Mizoguchi and Mizoguchi, 2010). T cells from the oxazolone model mice, when stimulated *ex vivo*, secrete a set of cytokines characteristic for Th2 dominance. Therefore, this model is typically used to study Th2-mediated mechanisms of intestinal inflammation (Elson et al., 2005; Mizoguchi and Mizoguchi, 2010; Strober and Fuss, 2006; Uhlig and Powrie, 2009; Wirtz et al., 2007).

TNBS-induced colitis is the main model used throughout this work, and therefore it will be described in more detail. TNBS-induced colitis is a model of intestinal inflammation that is initiated by the rectal installation of TNBS dissolved in ethanol. TNBS is a hapten that turns intestinal and microbial proteins immunogenic, thus causing an immune response mediated by Th1/Th17 cells. This model requires genetic susceptibility and can only be induced in certain strains of mice (Table 1). However, transgenic mice on a TNBS-resistant background may allow us to test whether mutation of the gene of interest results in susceptibility of the previously resistant strain. Histologically, mice subjected to TNBS-induced colitis develop thickening of the submucosa, dense infiltration of mononuclear cells, including lymphocytes and macrophages, loss of crypt structures and goblet cells. This is a practical and reproducible model that allows for the indirect assessment of T cells involvement in colitis (Elson, 1996; Elson et al., 2005; Mizoguchi and Mizoguchi, 2010; Scheiffele and Fuss, 2001; Strober and Fuss, 2006; Uhlig and Powrie, 2009; Wirtz et al., 2007).

The adoptive transfer colitis (ADTC) model allows for the direct

Table 1		
Mouse strain susceptibility to TNBS-induced colitis (Scheiffele and Fuss, 2001)		
Strain	Haplotype	Degree of Susceptibility
ASW/Sn	H-2 ^s	Intermediate
Balb/c	H-2 ^d	Susceptible
C3H/He ^{ou}	H-2 ^k	Intermediate
C57/B6	H-2 ^b	Resistant
C57/B10	H-2 ^b	Resistant
DBA/2	H-2 ^d	Resistant
SJL	H-2 ^s	Highly Susceptible

assessment of the contribution of effector and regulatory T cells to the development of colitis. This model was introduced by Powrie and colleagues, who showed that isolated CD4⁺ cells are divided into two functionally distinct populations based on the expression of CD45RB. The CD4⁺ T cells that expressed CD45RB at a high level are naive T cells; upon injection into an immunocompromised host (SCID or RAG mice), they induce

development of colitis in a recipient mouse. In contrast, the CD4⁺ cells with low expression of CD45RB, when injected, do not induce colitis themselves and suppress colitis induced by CD45RB^{high} cells. This suppression was shown to be caused by FoxP3⁺ regulatory T cells, which are present in the population of CD4⁺CD45RB^{low} T cells (Maloy et al., 2003; Mottet et al., 2003; Powrie et al., 1996; Powrie et al., 1994a; Powrie et al., 1993; Powrie et al., 1994b; Uhlig and Powrie, 2009). This model is unique in that it is completely dependent on the adaptive immunity and allows us to directly examine the involvement of both effector and regulatory T cells in the pathogenesis of IBD.

Hypothesis

The pathogenesis of Inflammatory Bowel Disease (IBD) has been described as an inappropriate immune response to usually harmless commensal intestinal bacteria. This response is largely perpetuated through overly active and/or persistent T cells. Recently, the novel family of T-cell Ubiquitin Ligand (TULA) proteins has been identified. The two members of this family TULA-1 and TULA-2 have been shown to function in the facilitation of caspase-independent apoptosis and suppression of T-cell signaling, respectively. The role of TULA-family proteins as it pertains to T-cell-driven immune responses such as those seen in disorders that involve a strong inflammatory and/or autoimmune component, such as IBD have yet to be defined. Accordingly, we are interested in the role of TULA family proteins in pathogenesis of IBD. To elucidate the role of TULA-family proteins in the pathogenesis of IBD we planned to assess the development of colitis in murine models using TULA knockout mice. We hypothesize that the TULA-1 and TULA-2 suppress T-cell-driven intestinal inflammation.

CHAPTER 2

MATERIALS AND METHODS

Antibodies

Antibodies for the Characterization of Cell Populations in TULA-family KO and WT Mice using Flow Cytometry

To identify T cells, NK T cells, NK cells, B cells, granulocytes and monocytes and to determine their fractions in various cell populations, following cell surface markers were used: CD3 (T cells), NKG2D (NK and NK T cells), B220 (B cells), GR-1 (granulocytes), CD11b (granulocytes and monocytes) (see Table 2). To distinguish CD3⁺ T cells from NK and NK T cells, cells were gated based on CD3 and NKG2D: T cells were CD3⁺/NKG2D⁻, NK T cells were CD3⁺/NKG2D^{dull}, and NK cells were CD3⁻/NKG2D⁺. To distinguish B cells from granulocyte/monocytes cells were gated using B220 and GR-1 staining. B cells were identified as B220⁺/GR-1⁻, while granulocytes and monocytes are B220⁻/CD11b⁺. To subsequently distinguish granulocytes and monocytes in the B220⁻/CD11b⁻ cell population, we gated them based on the expression of GR-1. Granulocytes are GR-1⁺, while monocytes are GR-1⁻.

To characterize the percent of CD4⁺ and CD8⁺ T cells we first gated the cells based on CD3, then assessed the percent of CD4⁺ and CD8⁺ among the CD3⁺. The CD45RB expression was then evaluated among T cells expressing CD4 or CD8.

To characterize the presence of antigen-naïve and antigen-experienced cells (also referred to as activated or effector/memory) in the CD4⁺ cell population, we labeled cells with antibodies to CD4, CD45RB, CD44 and CD62L. CD4⁺ staining was used for the

initial gating; then CD4⁺ cells were gated based on the intensity of CD45RB expression into the CD45RB^{high} and CD45RB^{low} sub-population. To characterize various phenotypic populations we used antibodies against CD44 & CD62L and CD25 & FoxP3. Effector Memory T cells (T_{EM}) were then gated as CD4⁺ CD45RB^{low} CD44⁺ CD62L⁻. Regulatory T cells were gated as CD4⁺ CD45RB^{low} FoxP3⁺ or CD4⁺ CD45RB^{low} FoxP3⁺ CD25⁺ double positive.

Table 2			
Antibodies for Flow Cytometry			
Antigen	Fluorophore	μL (per 106 cells)	Company
Splenic Immune Cell Population Staining			
CD3	APC	1	Biologend, San Diego, CA
NKG2D	PE	1	Biologend, San Diego, CA
B220	PECy7	1	Biologend, San Diego, CA
GR-1	PerCPCy5.5	1	Biologend, San Diego, CA
Cd11b	FITC	1	Biologend, San Diego, CA
T cell Population Staining			
CD3	APC	1	BD Bioscience, San Diego, CA
CD8	PE	1	BD Bioscience, San Diego, CA
CD4	PerCP	1	Biologend, San Diego, CA
CD45RB	FITC	1	BD Bioscience, San Diego, CA
Memory T cell Population Staining			
CD4	PerCP	1	Biologend, San Diego, CA
CD45RB	FITC	1	BD Bioscience, San Diego, CA
CD62L	PE	1	BD Bioscience, San Diego, CA
CD44	APC	1	BD Bioscience, San Diego, CA
Regulatory T cell Population Staining			
CD4	PerCP	1	Biologend, San Diego, CA
CD45RB	APCCy7	1	BD Bioscience, San Diego, CA
CD25	PE	1	BD Bioscience, San Diego, CA
FoxP3	Alexa488	5	BD Bioscience, San Diego, CA

Antibodies for Western Blotting

To characterize the phosphorylation state of proteins downstream of T-cell receptor (TCR), we used a panel of primary antibodies listed in Table 3. The secondary antibody used with all the listed primary antibodies was goat-anti-rabbit labeled with a near-infrared fluorophore (Li-COR, Lincoln, NE). It was used at a concentration of 1:20,000 for all primary antibodies.

Table 3				
Antibodies for Western Blotting				
Antigen	Concentration	Host Species	Reactive with species	Company
ZAP-70	1:2000	Rabbit	Hu, M	Cell Signaling, Danvers, MA
Phospho ZAP-70 (319)	1:1000	Rabbit	Hu, M	Cell Signaling, Danvers, MA
Phospho ZAP-70 (493)	1:1000	Rabbit	Hu, M, R	Cell Signaling, Danvers, MA
ERK1/ERK2	1:1000	Rabbit	Hu, M, R, Mk, Pg, Sc, Hm, B, Mi, Z,	Cell Signaling, Danvers, MA
Phospho ERK1/2 (Thr202/Tyr204)	1:2000	Rabbit	Hu, M, R, Mk, Pg, Sc, Hm, B, Dm, Z, Dg	Cell Signaling, Danvers, MA
PLC γ	1:1000	Rabbit	Hu, M, R	Cell Signaling, Danvers, MA
Phospho PLC γ (Try783)	1:1000	Rabbit	Hu, M, R	Cell Signaling, Danvers, MA
LAT	4ug/ml	Rabbit	Hu, M	Upstate/ Millipore, Billerica, MA
Phospho LAT (Tyr191)	1:1000	Rabbit	Hu	Cell Signaling, Danvers, MA
TULA-1	1:250	Rabbit	M	
TULA-2	1:250	Rabbit	M	

Antibodies for Stimulation of T cells

T cells were stimulated either in culture for production of cytokines and chemokines or stimulated to assess phosphorylation of TCR signaling proteins.

Unconjugated anti-CD3 and anti-CD28 antibodies were used to stimulate cells in culture. Biotin-conjugated antibodies were used to stimulate cells to assess phosphorylation of TCR signaling proteins (see Table 4).

Table 4		
Antibodies for the Stimulation of T cells		
Antibody	Assay	Company
Anti-CD3	Stimulation for cytokines	BD Bioscience, San Diego, CA
Anti-CD28	Stimulation for cytokines	BD Bioscience, San Diego, CA
Anti-CD3-Biotin	Stimulation for phosphorylation	BD Bioscience, San Diego, CA
Anti-CD28-Biotin	Stimulation for phosphorylation	BD Bioscience, San Diego, CA

Preparation of GK1.5 antibody

Anti-CD4 GK1.5 monoclonal antibody (mAb) was prepared from culture supernatant of the GK1.5 hybridoma cell line. Cells were cultured in media (see below cell culture). Cells were grown at 37°C in 5% CO₂. To initially assess production of antibody from hybridoma a competition assay was performed between GK1.5 hybridoma culture supernatants and a GK1.5 antibody conjugated to fluorophore. The supernatant was then collected via centrifugation and GK1.5 antibody was purified from culture supernatant using Protein G affinity chromatography (GE Healthcare, Piscataway NJ). Eluted antibody was then dialyzed in PBS overnight 4°C. Dialyzed antibody was then stored at -20°C until used. To deplete CD4⁺ T cell from mice 400µg of the purified antibody was given intraperitoneally to each mouse one day prior to sensitization and induction phases of the TNBS experiments.

General Techniques

Cell Culture

Primary and hybridoma cells were cultured in an incubator at 37°C in with 5 percent CO₂. Primary cell culture media (PCCM) is RPMI-1640 supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Carlsbad, CA), 2mM L-glutamine, 100IU penicillin, 100µg/mL of streptomycin, 30µg/mL of gentamycin, 20mM 2-[4-(2-hydroxyethyl) piperazin-1-yl]ethanesulfonic acid (HEPES) buffer (Fisher Scientific, Hampton NJ) and beta-mercaptoethanol (Life Technologies, Carlsbad, CA). Hybridoma was cultured in Dulbecco's Modified Eagles Medium (DMEM) (Fisher Scientific, Hampton, NJ) supplemented with supplemented with 10% FBS (Life Technologies, Carlsbad, CA), 2mM L-glutamine, 100IU penicillin, 100µg/mL of streptomycin, 20mM HEPES buffer (Fisher Scientific, Hampton NJ).

Preparation and Administration of Anesthesia

A 1:1 mix of xylazine and ketamine (Fisher Scientific, Hampton, NJ) was prepared and loaded into a 1cc syringe with a 22G needle (BD Biosciences Carlsbad, CA). This solution (20µL) is given to each mouse intra-peritoneally. Mice are monitored visually for 1 hour.

Single Cell Suspension

The spleen and/or MLNs were removed and placed into a 40µm cell strainer over a 50-mL conical tube that was pre-wet with PBS or HBSS without Ca²⁺ and Mg²⁺ with

2% fetal bovine serum (Life Technologies, Carlsbad, CA) and 30µg/mL gentamycin (Fisher Scientific, Hampton, NJ). A 3cc syringe plunger was used to push tissue through the strainer using an up and down movement thus dissociating the tissue. The single cell suspension was washed from the strainer with 10-20mL PBS or HBSS without Ca²⁺ and Mg²⁺ with 2% fetal bovine serum and 30µg/mL gentamycin. The cells were collected in the 50mL conical tube were washed by centrifugation (300 X g for 5 minutes at 4°C) and re-suspended in an appropriate media.

Lysis of Red Blood Cells (RBCs)

In the case that single cell suspensions are made from spleen the RBCs were lysed. The red blood cells were lysed using an AKC lysis buffer. 5mL of AKC lysis buffer was added to the cell suspension of one spleen then allowed to incubate at room temperature for 1 minute with gentle shaking. After the incubation the cell suspension was diluted 10-fold with PBS or HBSS without Ca²⁺ and Mg²⁺ with 2% fetal bovine serum and 30µg/mL gentamycin. The cells are centrifuged at 300 X g for 5 minutes at 4°C. Cells were then resuspended in PBS or HBSS without Ca²⁺ and Mg²⁺ with 2% fetal bovine serum and 30µg/mL gentamycin and kept on ice, while their density is being determined.

Isolation of CD4⁺ T cells from Single Cell Suspensions

T cells were isolated from single cell suspensions using CD4⁺ T cell enrichment kit (Stem Cell Technologies, Vancouver, Canada). Single cell suspensions were re-

suspended in a 5mL polystyrene tube at a concentration of 10^8 cells/mL in PBS with 2 percent FBS and 5 percent Normal Rat Serum (provided by the kit). 50 μ L of Stem Cell CD4⁺ T cell Enrichment Cocktail was added per milliliter of cell suspension, mixed and incubated for 15 minutes at 4°C. Stem Cell Biotin-Selection Cocktail was added at 100 μ L per milliliter of cell suspension. Again, the cells were incubated as in previous steps. Stem Cell magnetic nanoparticles were mixed via vortexing immediately before adding 50 μ L per milliliter of cell suspension. Again, the cells were incubated as in previous steps. The volume of the cell suspension was brought up to 5mL using PBS with 2 percent FBS, then placed in a the Stem Cell Bucket magnet and incubated there for 5 minutes before the CD4⁺-enriched fraction was poured off into another sterile culture tube. Cell density was determined, and cells were assessed for purity using flow cytometry.

In-vitro Stimulation of T cells

CD4⁺ T cells isolated from spleens or MLNs using magnetic separation (Stem Cell Technologies, Danvers, MA.) were resuspended in PCCM. Cells were placed in antibody-coated 96-well plates at 0.4×10^6 cells per well. The wells were coated with anti-CD3 and/or anti-CD28 antibodies for 2 hours at 37°C at a concentration of 5 μ g/ml and 10 μ g/ml for anti-CD3 and anti-CD28, respectively. Cells were incubated at cell culture conditions for 24-48 hours. Then culture supernatant was collected and stored at -20°C.

Histology

Paraffin embedded colons were cut into 5 μ m sections and placed on to slides. Slides were then placed in a 60°C incubator for an hour to mediate adherence of the tissue section to the positively charge slide. The slides were then de-paraffinized in xylene for 3 minutes (three changes). The slides were rehydrated in 100 percent ethanol for 1 minute (two changes), then 95 percent ethanol for 1 minute (two changes), then 70 percent ethanol 1 minute. Then the slides were rinsed with dH₂O for 5 minutes (2 changes). Once rehydrated the slides were stained with hematoxylin and eosin. The staining procedure was as follows: 3 minutes in hematoxylin, 3 minute tap water rinse, 1 minute in defined acid alcohol, 1 minute in tap rinse, 1 minutes in lithium carbonate, 1 minute in tap water rinse, 1 minute in 70 percent ethanol, 1 minute in eosin, 1 minute in 70 percent ethanol, 1 minutes in 95 percent ethanol (2 changes), and 1 minute in 100 percent ethanol (2 changes) and 1 minute in xylene (3 changes). The tissue sections on the slide where covered with micromount-mounting medium and a cover slip was placed over the section.(Leica, Buffalo Grove, IL).

Colons used for frozen-sectioning were embedded in optimal cutting temperature medium (OCT). Embedded colons were cut into 20 μ m sections at -30°C and placed on positively charged slides. These slides were then allowed to dry for 1 hour at room temperature. Colon tissues where fixed on the slide with ice-cold acetone for 10 minutes. The slides were then transferred into a coplin jar containing TRIS-buffered saline (TBS) for 20 minutes at room temperature on a shaker. The slides were then transferred into a coplin jar containing TBS plus 5 percent bovine serum albumin (BSA) and 0.1 percent Tween-20. Again, the slides were incubated for 20 minutes at room temperature on a

shaker. Slides were incubated with an anti-CD4-FITC antibody or the isotype control (Biolegend, San Diego, CA) for 1 hour at room temperature. The slides were washed in TBS/BSA/Tween-20 3 times for 5 minutes on shaker. Slides were allowed to dry briefly before the slides were covered with Vectasheild DAPI-mounting medium (Vector Labs, Burlingame, CA) and cover slips were placed over the sectioned tissues. FITC staining of the colon tissues was assessed via confocal microscopy (Leica, Buffalo Grove, IL).

RNA Isolation from Colon Tissue, Reverse Transcription, and Quantitative PCR

RNA was isolated from total colon by homogenizing flash frozen tissue with a mortar and pestle. 50-100mg of tissue was further homogenized with a glass homogenizer in 1mL of Trizol (Life Technologies, Carlsbad, CA). Trizol-treated tissues were further incubated at 15-30°C for 5 minutes after glass homogenization. To homogenized tissue 200µL of chloroform per 1mL of Trizol was added. The mix was shaken for 15 seconds and then incubated at 15-30°C for 2-3 minutes. Samples were then centrifuged at 12,000 X g for 15 minutes at 4-8°C. The clear aqueous layer containing the RNA was removed without disturbing the interphase and then transferred into a new tube. The RNA was then precipitated by adding 500µl of 100% isopropyl alcohol and incubating the mix for 10 minutes at 15-30°C. Again, the samples were centrifuged at 12,000 X g at 4-8°C for 10 minutes. Isopropyl alcohol was removed from the tube, and the RNA pellet was washed with 1mL of 75% ethanol per 1mL of Trizol added. The RNA was centrifuged at 7500 X g for 5 minutes at 4-8°C, and the resulting pellet was air-dried and then resuspended in diethylpyrocarbonate (DEPC) DEPC water.

To reverse transcribe RNA into cDNA, 1µg of RNA was added to 16µL DEPC water and heated for 5 minutes at 65°C and then placed on ice. The following reaction cocktail was prepared: 1.5µL of Reverse Transcriptase (Promega, Madison WI), 1.5µL of RNase inhibitors (Promega, Madison WI), 6µL of 5X MMLV buffer (Promega, Madison WI), 1.0µL of Random Primers (Promega, Madison WI), 1.5µL of 10mM dNTP (Promega, Madison WI) 3µL of 1% BSA, and 16µL of heated RNA/DPEC H₂O. These components were mixed and heated to 42°C for 1.5 hours.

The reverse transcribed cDNA was used to perform qPCR (volume of cDNA for the specific cytokine primers sets are listed in Table 7). The following cocktail was prepared for qPCR reactions: 0.05µL of 100mM Forward Primer, 0.05µL of Reverse Primer, 10µL Sybr Green (Life Technologies, Carlsbad, CA), cDNA and DEPC water up to 20µL. The qPCR was performed using an Eppendorf Mastercycler® realplex (Eppendorf Hamburg, Germany), with the following cycles: 95°C 10 minutes (1 cycle); 95°C 30 seconds, 55°C 1 minute, 72°C 30 seconds (40 cycles); 95°C 1 minute (1 cycle); 55°C 30 seconds (1 cycle); 20 minute ramp time; 95°C 30 seconds end.

Table 5		
Primer Sets and cDNA volumes for qPCR		
CYTOKINE	FORWARD	REVERSE
IL-4	ACGGAGATGGATGTGCCAAACG	TGCATGATGCTCTTTAGGCTTTCC
IL-10	CAGCCGGGAAGACAATAACTG	CCGCAGCTCTAGGAGCATGT
IL-17	CTGAGAGCT GCC CCT TCA CT	CCACACCCACCAGCATCTTC
IFN γ	AGCTCATCCGAGTGGTCCAC	GCTACGACGTGGGCTACAG
TNF α	CCCTCACACTCAGATCATCTTCT	CTGCCACAAGCAGGAATGAG
B actin	TCCACCACCACAGCTGAGAGG	CAG CTT CTC TTT GAT GTC ACG
IL-2	AAAGGACTAGCCCACACCCT	TGGATGTGAGAGAAAGCACG
IL23R	ACATTGGACTTTTGTCTGGGAA	AAAATCGGCAACATG

Statistical Analysis

Statistical analysis was performed using Prism software provided by Graph Pad, La Jolla, CA. The figure legends indicate the type of statistical analysis used. The symbol “*” indicates statistical significance. Three “*” the p value is less than 0.001. Two “**” the p value is less than 0.01. One “*” the p value is less than 0.05. When the p value is greater than 0.05, a difference is deemed not statistically significant (ns).

Characterization of Cell Populations using Flow Cytometry

We characterized the cell populations of TULA-family KO mice and compared them to those of wild-type mice using flow cytometry. Single-cell suspensions of spleens or MLNs from each mouse were cleared of red blood cells, and 0.5 to 1×10^6 cells were labeled with antibodies to identify T cells, B cell, NK T cells, NK cell, granulocytes, and monocytes. Furthermore, we characterized the CD4⁺ and CD8⁺ subpopulations of CD3⁺ T cells. CD4⁺ T cells were also characterized for the distribution of CD45RB expression, and the ratio of high and low expressers was determined. Among the CD4⁺ CD45RB^{low} population, we characterized the percent of cells displaying phenotypic markers for Effector Memory T cells and Regulatory phenotype using antibodies to CD44/CD62L (Effector Memory T cells) and CD25/FoxP3 (Regulatory T cells).

For extracellular staining, cells were resuspended in 100 μ l phosphosphate buffered saline (PBS) with 2% FBS and kept on ice. Cells were treated with an antibody to block Fc receptor (1 μ l Fc blocker per 100 μ l of cell suspension) (BD Bioscience, San Diego, CA) 5 min before to labeling with a primary antibody (0.5 -1 μ L of primary

antibody per 100 μ l of cell suspension) for 30 min in the dark on ice. Cell suspension volume was brought up to 1mL with ice-cold PBS. Cells were centrifuged for 5 min at 350 X g at 4°C and then washed once with ice-cold PBS and centrifuged again. Cells were finally resuspended in 300 μ L of ice-cold PBS and analyzed using a BD Canto flow cytometer and BD Aria software. One-way ANOVA was performed using the Prism software.

Intracellular staining for FoxP3 was done after extracellular staining of other markers using the Mouse Treg Flow Kit (Biolegend, San Diego, CA). Extracellularly stained cells were washed with 1ml of staining buffer and centrifuged as described before. The 1mL 1X FIX/PERM solution (Biolegend, San Diego, CA) was added to the cells, which were then incubated for 20 minutes at room temperature in the dark. Cells were washed, resuspended in 1mL of 1X permeabilization buffer (Biolegend, San Diego, CA) and incubated for 15 minutes in the dark at room temperature. The cells were centrifuged and then resuspended in 100 μ l of permeabilization buffer (Biolegend, San Diego, CA) to which 5 μ l of anti-FoxP3 antibody was added. Cells were then incubated for 30 minutes at room temperature in the dark. Cells were finally resuspended in 300 μ L of ice-cold PBS and analyzed using the BD Canto and BD Aria software (BD Biosciences, San Diego, CA.).

Apoptosis Assays

Splenic single cell suspensions were prepared and resuspended in at a density of 1.6 X10⁶ cells/ml in primary cell culture media (PCCM). The cells were plated in 24-well plate at 0.4 X 10⁶ per well in triplicate. The cells were then treated in 3 ways to induce

apoptosis: (1) cells were cultured in PCCM without IL-2, (2) with PCCM with 0.5% FBS (instead of 10% FBS), and (3) cells were cultured in wells pre-coated with anti-CD3 alone (at 1µg/mL) without co-stimulating antibody (incomplete stimulation). Viability of these cells was compared to the following control cell culture conditions: PCCM with 40ng/mL of IL-2 and PCCM with 5µg/mL anti-CD3 and 10 µg/mL of anti-CD28 antibodies. The cells were cultured in and samples were taken every 10 hours and viability was assessed using 7-Amino-Actinomycin D (7-AAD). To the 750µL of single cell suspension of no more than 1×10^6 cells 0.25µg of 7-AAD (5µL of solution provided by BD Bioscience, San Diego CA) was added. The mix was incubated for 10 minutes at room temperature in the dark. Cells were washed with 5mL of cold PBS and then centrifuged at 350g for 3 minutes. Cell pellets were resuspended in 100µL of PBS, and the percent of 7-AAD- and 7-ADD⁺ cells was assessed using flow cytometry.

Measurement of Cytokines and Chemokines in Culture Supernatant and Serum

The concentrations of cytokines and chemokines in serum and culture supernatants of stimulated cell cultures were measured using Luminex or ELISA.

ELISA

Concentration of IL-2 and IL-17 in cell culture supernatants was assessed using ELISA. The capture antibodies (BD Bioscience, San Diego, CA) were diluted in coating buffer and plated into a 96-well plate at 100µL per well and incubated overnight at 4°C or at room temperature for 4 hours. The plates were washed with ELISA wash buffer and

then blocked with FBS blocking buffer for 1 hour at room temperature and repeatedly washed after blocking. The standards and samples were then placed into the wells at a total volume of 100µL per well and incubated overnight at 4°C or at room temperature for 4 hours. The detection antibody (BD Bioscience, San Diego, CA) was diluted in FBS blocking buffer and placed in the wells for 1 hour at room temperature. The plates were washed 5 times with ELISA wash buffer. Avidin-horseradish peroxidase (BD Bioscience, San Diego, CA) was diluted to the working concentration (Table 6) with BSA blocking buffer and placed into wells (100µL/well) for 30 min at room temperature. 2,2'-Azino-bis(3-Ethylbenzthiazoline-6-Sulfonic Acid (ABTS) substrate solution was then added to the wells (100µL/well) for 5 min at room temperature. Absorbance was read using a Polarstar Omega plate reader (BGM Labtech, Offenburg, Germany) at 405 nm.

Table 6			
ELISA Antibody and Standard Concentration			
		IL-2	IL-17
Capture antibody		2µg/ml	2µg/ml
Detection antibody		1µg/ml	1µg/ml
Standards dilution	2-Fold	500pg/ml to 4pg/ml	1600pg/ml to 12.5

Table 7		
ELISA Buffers		
Buffers	Recipe	Protocol notes
Coating Buffer	0.1 NaHPO ₄ pH 9	
Wash Buffer (PBS/Tween)	0.5mL of Tween-20 in 1L PBS	
FBS Blocking Buffer	10 % FBS in sterile PBS	
BSA Blocking Buffer	1% BSA in sterile PBS	Filtered to remove particulates
ABTS	150mg of ABTS in 500mL of 0.1 anhydrous citric acid; pH 4.35	store -20°C 11mL aliquots prepared fresh and add 100uL of 3% H ₂ O ₂ per 11mL of ABTS
3% H ₂ O ₂	10mL of 30% H ₂ O ₂ to 90mL of H ₂ O.	

Luminex

FGF basic, GM-CSF, IFN- γ , IL-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p40/p70, IL-13, IL-17, IP-10, KC, MCP-1, MIG, MIP-1 α , TNF- α , and VEGF in sera and culture supernatants were measured using the Luminex mouse 20-plex kit according to the manufacturer's protocol (Life Technologies, Carlsbad, CA). In brief, the protocol requires that 25 μ L of 1X antibody-coated beads and 200 μ L wash solution be added to a pre-wet plate. The plate was washed once with 200 μ L of wash solution. Then 50 μ L samples were diluted 1:1 with assay diluent. The diluted samples and standards were further diluted with Luminex® incubation buffer and incubated with the Luminex® beads for 2 hours at room temperature in the dark. The plates were washed twice as described from the previous steps, and 100 μ L Luminex® biotinylated detector antibody was added per well for 1 hour at room temperature in the dark. The plates were washed two times, and streptavidin-R-phycoerythrin was added at 100 μ L/well for 30 minutes at

room temperature in the dark. The plate was then washed three times. The beads were finally resuspended in 100 μ L of wash solution, and their fluorescence was measured to determine the concentration of the analytes using the Luminex $\text{\textcircled{R}}$ 200 TM System with xPONENT $\text{\textcircled{R}}$ Software (Life Technologies, Carlsbad, CA).

Analysis of stimulation-induced phosphorylation

To stimulate naïve T cells, 20 x 10 6 CD4 $^+$ splenic cells were purified (Stem Cell Technologies, Vancouver Canada) from each type of mice (TULA-family KO or WT mice) for each type of stimulation condition (for analysis of phosphorylation via western blotting). Stimulation of T cells for analysis of phosphorylation via flow cytometry on 1 0.5-1 million splenocytes were used for each condition. The conditions were as follows: no stimulation, anti-CD3 stimulation with anti-CD3 alone, stimulation with anti-CD3 and anti-CD28, and pervanadate stimulation. CD4 $^+$ splenic T cells were incubated on ice for 20 minutes with 20 μ g/mL of biotinylated anti-CD3 (BD Biosciences) alone or along with biotinylated anti-CD28 (BD Biosciences), then washed with ice-cold PBS with 2% FBS and centrifuged at 350 x g for 5 minutes at 4 $^{\circ}$ C. To crosslink antibody-treated cells, the cells were resuspended in 100 μ L of PBS containing 20 μ g/mL of streptavidin and incubated on ice for 20 minutes. As a positive control cells were treated with pervanadate, which was prepared by mixing 0.5mL of 10mM sodium vanadate with 3.1 μ L of 30% H $_2$ O $_2$. The mix was incubated for 10 minutes at room temperature. Then 10 μ L of the sodium vanadate solution was added 100 μ L of ice-cold cells. All stimulation cell samples were placed into a 37 $^{\circ}$ C water bath for 3 minutes, the cell were then lysed or fixed with paraformaldehyde (12 μ L of 36% solution for every 100 μ L sample).

Cell Lysis and Protein Concentration Determination

Cells were washed and resuspended in TNE lysis buffer (25 mM Tris-HCl [pH 7.5], 150 mM NaCl, 5 mM EDTA) containing sodium vanadate (1mM), aprotinin and leupeptin (10 μ g/mL), phenylmethanesulfonylfluoride (PMSF, 1mM), sodium fluoride (10mM). The cells were incubated in the lysis buffer for 30 minutes on ice, then centrifuged at 16,000 x g for 15 minutes at 4°C. The supernatant was transferred to a fresh tube, and the protein concentration was determined.

To determine the protein concentration, the lysates were diluted in a 96-well plate. 3.3 μ L of sample lysate was diluted with 30 μ L of H₂O. This sample dilution was further diluted 1 to 5 in 10 μ L of H₂O in duplicate. A standard curve was generated using BSA. An aliquot of 1, 2, 3, 4, 5 μ L of the 0.2 μ g/ μ L BSA standard is placed in the 96 well plate in duplicate and diluted with 10 μ L H₂O. To all wells excluding the initial sample dilutions 250 μ L of Coomassie protein assay reagent (Pierce, Rockford IL) was added. Absorbance was read using a Polarstar Omega plate reader (BGM Labtech, Offenburg, Germany) at 570nm.

SDS-PAGE and Western Blotting

Proteins from cell lysates were treated with 2X loading buffer and heated 95°C for 3 minutes. Samples were cooled and loaded in 10-12% polyacrylamide gel to be separated using standard electrophoresis techniques. The gel was run for ~800 volt hours. The separated proteins were transferred onto nitrocellulose membrane using a sandwich method (Whatman paper, polyacrylamide gel, nitrocellulose membrane, Whatman paper)

at 42 volts for 3 hours. The nitrocellulose membrane, containing transferred proteins was then incubated in blocking buffer (Li-cor, Lincoln, NE) for at least 1 hour at room temperature on a shaker. The membrane was washed with TPBS three times for five minutes each on a shaker. The membrane was incubated with the indicated primary antibody for either 1 hour at room temperature or overnight at 4°C (anti-phosphosite-antibodies). The membrane was incubated with the appropriate secondary antibody for 30 minutes. The membrane was washed as in previous steps and protein detection was performed using the Odyssey (Li-Cor, Lincoln, NE).

Description of Animals

Mice used in all experiments were on the C57/BL6 genetic background. Five different genotypic groups of mice were used, including wild-type (WT), which express both TULA-1 and TULA-2; single knockout mice, which lack one of the two TULA-family-encoding genes, either TULA-1 or TULA-2 (sKO-1 and sKO-2, respectively); and double knockout mice, which lack both TULA-1- and TULA-2-encoding genes (dKO). In the adoptive transfer model the B6.CB17-Prkdc^{scid/SzJ} (Jackson Labs Bar Harbor, ME) mice were used as an immunocompromised host. All mice were housed according to an IACUC protocol in the specific pathogen-free environment of the Temple University Central Animal Facility.

Adoptive Transfer of CD4⁺ CD45RB^{high} Splenic T cells induced Colitis (ADTC)

For adoptive transfer CD4⁺ CD45RB^{high} cells from 10-12 weeks old donor WT, sKO-1, sKO-2, and dKO mice were used. Spleens were removed, single-cell suspensions were prepared, and splenocytes were counted and used to isolate CD4⁺ cells by magnetic separation (see Isolation of CD4⁺ cells). CD4⁺ cells were stained for CD4 and CD45RB using fluorescence-labeled antibodies and prepared for cell sorting (see labeling of cell surface markers for flow cytometry and cell sorting). The top 30% of CD45RB expressing cells among CD4⁺ cells were sorted, counted and resuspended in PBS at a density of 8×10^5 cells/ml. The cell suspension is loaded into a 1cc syringe with a 28G needle attached (BD Bioscience San Diego, CA). 0.5mL of the cell suspension was injected intraperitoneally or intravenously into a recipient SCID mouse (6-8 weeks old). The weight of each recipient mouse on the day of transfer is recorded and then continuously (weekly for 49 days, then every other day up to day 69). On day 69, the animals were anesthetized and sacrificed. The colon is removed and prepared for histology. MLNs and spleens are also removed, and single-cell suspensions are made. The cell suspensions are subsequently labeled CD4 and CD45RB fluorescent antibodies, and the reconstitution of donor cells in the recipient mouse is assessed using flow cytometry.

Trinitrobenzene Sulfonic Acid-Induced Colitis (TNBS)

TNBS is a compound that haptenizes proteins making them immunogenic, thus this model is based on direct application of this compound to tissue. In this model of colitis a sensitization phase is required to prime the immune system. Subsequent direct

application of TNBS to the intestinal tract causes local inflammation in genetically susceptible strains of mice.

Sensitization

In order to develop an adaptive immune response in this model we give low dose treatment of TNBS to sensitize the animal. Sensitization solution is prepared in advance and kept on ice until use. Sensitization solution is made by mixing 4 volumes of the acetone: olive oil mix (4:1) with 1 volume of 5% TNBS in H₂O. For negative control, 5% TNBS solution is replaced with dH₂O.

An area of mouse skin (~1 cm²) is shaved between the shoulder blades. When an animal is anesthetized the sensitization solution (150µl/per mouse) is placed on the shaved area. On day 7 after sensitization, the induction phase is initiated.

Induction

All mice are starved prior to induction for 24-30 hours. Colitis is induced by slow intra-rectal instillation of 5% TNBS solution in 50% ethanol (120µl/mouse). Control mice are treated identically with 50% ethanol. After delivering the enema mice were kept upside-down for ~ 1 minute, then placed horizontally to recover from the anesthesia. Their weight and health status is monitored every day for the duration of an experiment, which was typically 4 days.

Preparation of Syringe-Enema

A G28 ½1cc insulin syringe (BD Bioscience, San Diego, CA.) is fitted with ~5-6cm plastic microbore tubing 0.10inch x 0.30inch X 0.01inch tubing on the needle (Saint Gobain PPL, Bridgewater, NJ). Once the tube has been firmly attached to the needle, the tubing was to cut to 4.0cm.

Preparation of 50% TNBS and Control Solution

The 5% solution (w/v) of TNBS was distributed in 60µL aliquots corresponding to individual doses of 3mg TNBS per mouse. To each aliquot an equal volume of absolute ethanol was added. The solutions are mixed well and kept on ice until administration via enema. In the control solution, TNBS is replaced with sterile deionized H₂O.

Application of the TNBS/ethanol Enema

Mice are starved for 24 hours prior to the application of the enema. The control or TNBS solution was pulled through the tubing into the prepared syringe. The tubing was lubricated with K-Y® lubricant (Johnson & Johnson, New Brunswick NJ). The mice were inverted and stabilized by taping its tail to a vertical surface (e.g., the cage). And the tube was slowly inserted it into the anus of a mouse up to the tip of the needle. No force was used if the colon was blocked instead some of the solution was pushed out to facilitate entry. The mouse was kept inverted for ~1 minute.

Depletion of T cells in the TNBS-induced colitis

T cells were depleted from TULA-family KO mice using the GK1.5 anti-CD4 mAb twice: one day prior to sensitization and one day prior to induction. Each time 400 μ g of GK1.5 was given per mouse, since 400 μ g of GK1.5 antibody was determined to be a sufficient amount to deplete T cell from the spleen and MLNs in preliminary experiments. Depletion was assessed in the MLNs and in the spleen of the TNBS experimental animals upon termination of the experiment.

Termination of Animal Models and Assessment of Disease Progression

We terminate colitis models on days 4 and 69 post induction for TNBS and ATDC, respectively. In brief, the animals in these models are euthanized using CO₂ inhalation unless specified otherwise, and several parameters are measured to assess the disease severity.

Blood Collection and Serum Separation

The animal is anesthetized (as stated previously) and covered in 70% ethanol. Then using a 22G needle and 1cc syringe the heart was punctured and slowly 500-1000 μ L of blood was removed. The blood was transferred from the syringe into an eppendorf tube and placed in an incubator at 37°C for 30 minutes. Once the clots have formed, the tube is placed at 4°C overnight. The following day the tubes are centrifuges at 14,000 rpm for 30 second, and the serum is removed from the top without disturbing the clot.

Collection and Stimulation of Splenocytes and Mesenteric Lymph Node (MLN) Cells

Spleen and MLNs were stored in ice-cold PBS or Hank's balanced salt solutions (HBSS) without Ca^{2+} and Mg^{2+} with 2% fetal bovine serum and 30 $\mu\text{g}/\text{mL}$ gentamycin until all tissues are collected (1 hour, on average). Single cell suspensions are made from the spleen and MLNs, and the RBCs are lysed (spleen only). Cell suspensions from spleens and MLN's are analyzed using flow cytometry to assess the percent of CD4^{+} T cells. In ADTC, this was done to evaluate re-population of the recipient mice immune system. In TNBS model studies, CD4^{+} T cells were isolated from spleen and MLN single-cell suspensions and stimulated *ex vivo* with antibodies to TCR (as stated above) for 48 hours. Cell culture supernatants were collected and assessed for the IL-2 and IL-17.

Removal of Colon for Histology and Photography

The caecum was located and the attached large intestine and colon were then disconnected from connective tissues along the length of the colon from the caecum to the anus. The colon was freed from the animal by clipping at the base of the anus and pulling by the caecum. The colon was stored in ice-cold PBS or HBSS without Ca^{2+} and Mg^{2+} with 2% fetal bovine serum and 30 $\mu\text{g}/\text{mL}$ gentamycin. When all the colons were collected from experimental mice, the colons were removed from buffer, photographed and then processed for frozen or paraffin embedding (see above). Feces were removed from the colon by flushing it with PBS or HBSS without Ca^{2+} and Mg^{2+} with 2% fetal bovine serum and 30 $\mu\text{g}/\text{mL}$ gentamycin. Flushed colons were then cut into sec

tions: one 2 cm section proximal to the caecum is cut and frozen on dry ice and was later stored at -20°C and processed for RNA isolation, reverse transcription, and subsequent qPCR (see above). Another 50mm section of colon proximal to the anus is prepared for paraffin or frozen sectioning. Colon sections that were to be paraffin-embedded were removed, placed in formalin and kept at room temperature overnight to fix, then transferred into 70% ethanol overnight at room temperature, then stored in 70% ethanol at 4°C until processing and embedding and sectioning and staining (see above). Colons that were used for frozen sections were embedded immediately after flushed into optimum cutting temperature medium (OCT) and stored in it at -80°C. Both frozen and paraffin- embedded colons are processed and embedded such that the position that is 15mm from the anus is oriented to be sectioned. Sectioned and H&E stained colons were photographed, and severity of inflammation was assessed by a histologist (Dr. Fayez Safadi) using the criteria listed in Table 8.

Table 8	
Histological Score of Inflammation	
Score	Histological Changes
0	No evidence of inflammation
1	Low level of inflammation with scattered infiltrating mononuclear cells (1-2 foci)
2	Moderate inflammation with multiple foci
3	High level of inflammation with increased vascular density and marked wall thickening
4	Maximal severity of inflammation with transmural leukocyte infiltration and loss of goblet cells

CHAPTER 3

RESULTS

Characterization of TULA deficiency

Previous characterization of mice deficient in both TULA-1 and TULA-2 indicated that the number of splenocytes was increased and that T cells were hyper-responsive to TCR-mediated stimulation *in vitro* (Carpino et al., 2004). Based on the first published results, T cells appeared to be the only cell type affected by this deficiency (Carpino et al., 2004). In an effort to better understand the *in vivo* consequences of TULA-family deficiency, we first characterized phenotypic subsets of cells in the spleen using extracellular and intracellular markers. Among immune cell subsets tested, T cells, granulocytes, and monocytes seem to be affected by TULA deficiency. More specifically, mice lacking both TULA-1 and TULA-2 (dKO) show a small decrease in the percent of T cells however these differences are not statistically significant. Likewise, the percent of cells that are CD11b positive (granulocytes or monocytes) are increased in spleens of dKO and sKO2 mice (data not shown), however these differences were not found to be significant. Furthermore, the ratio of granulocytes and monocytes among CD11b positive cells is comparable between all groups (figure 1).

Given the nature of TULA-family proteins and previously observed effects seen in mice deficient in both TULA-family proteins, we focused our further efforts on the phenotypic subsets of T cells in mice deficient in TULA-family proteins. Analysis of T cells revealed that the percent of CD4⁺ cells among T cells varies slightly, but

significantly; it is the highest in dKO splenocytes, while sKO1 mice show a decrease in the percent of CD4⁺ T cells as compared to both dKO and WT mice. In agreement with the data on CD4⁺ cells, the percent of CD8⁺ cells was the highest in sKO1, lower in WT, and the lowest in dKO and sKO2 (figure 2).

The fractions of CD45RB^{low} and CD45RB^{high} cells among CD4⁺ T cells in WT mice are ~25% and ~75%, respectively. The fraction of CD45RB^{low} cells was significantly increased in dKO to ~50% of all CD4⁺ cells. Effects of individual knockouts on CD45RB expression were differential; the examined ratio in sKO1 and sKO2 was similar to that in WT and dKO, respectively (figures 3 and 4).

Considering that CD4⁺ CD45RB^{low} population is expected to contain TEM and Treg cells (Morrissey et al., 1993; Powrie et al., 1993; ten Hove, 2004), we analyzed markers characterizing effector/memory versus naïve cells as well as Treg. The experiments indicated that CD45RB^{low} populations of CD4⁺ T cells contained a considerable proportion of CD44⁺CD62L⁻ cells (figure 5), while CD45RB^{high} populations contained no more than 5% of CD44⁺CD62L⁻ cells (data not shown). The fraction of antigen-experienced, CD44⁺CD62L⁻ cells among splenic CD4⁺ T cells in dKO was more than 2-fold higher than that in WT. Percent of CD44⁺CD62L⁻ cells among CD4⁺ T cells in both sKOs exhibited a modest increase over the WT level, but this increase was not statistically significant (figure 5).

Another considerable fraction of CD45RB^{low} population was constituted by FoxP3⁺ CD4⁺ T cells (Treg); the percent of FoxP3⁺ cells among the total CD4⁺ T-cell population was in the range between ~8 and ~18%, while among the CD4⁺ CD45RB^{low}

cells the fraction of FoxP3⁺ was between ~21 and ~32% (figure 6). The fraction of FoxP3⁺ cells was affected by knockouts in a way similar to the effect on the fraction of CD44⁺CD62L⁻ cells; namely, the fraction was increased in dKO mice, but not in sKO mice (figure 6). However, FoxP3⁺ CD4⁺ T cells in dKO demonstrated lower expression of CD25 (46% compared to 62% in WT data not shown) on their surface, so no substantial difference was seen between the percent of CD25⁺FoxP3⁺ cells among CD4⁺ CD45RB^{low} in dKO and all other mice (figure 6). Since low levels of CD25 (the high-affinity IL-2 receptor subunit) may reduce their responses IL-2 and/or their ability to sequester IL-2 (Pandiyana et al., 2007) our findings suggest that Treg cells in dKO may be functionally deficient.

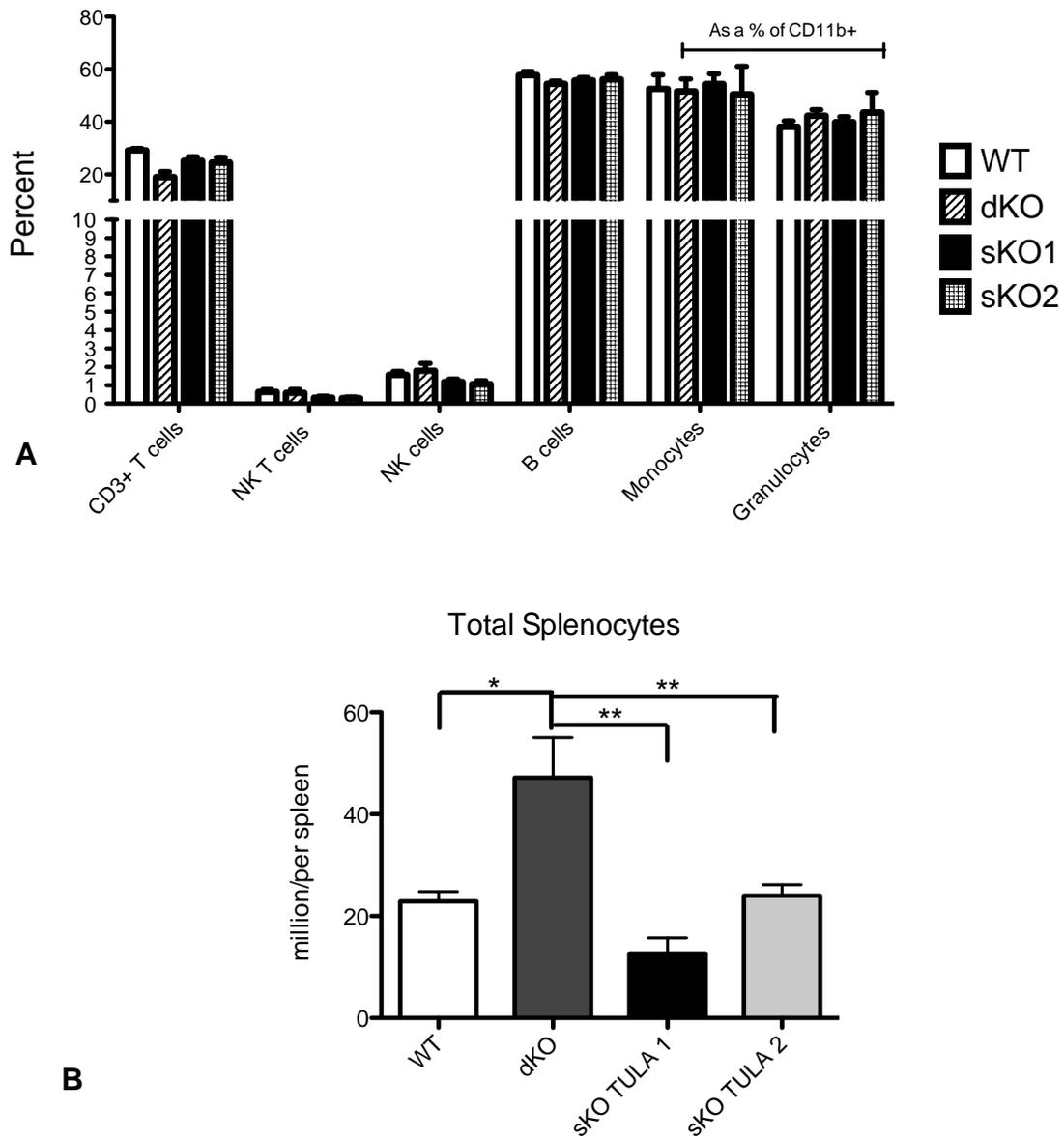


Figure 1

Characterization of splenic cell populations of TULA-family knockout. Spleens of 6-8 week old mice were stained with antibodies to determine the percents of phenotypic immune cell populations, monocytes and granulocytes are present as a percent of CD11b⁺ population. Means and SEM \pm are plotted. Two-way Anova statistical analysis was performed (no statistically significant between groups). (a). The total number of splenocytes among the WT and TULA-family KO mice are represented in Means and SEM \pm are plotted. One-way Anova statistical analysis was performed WT was not statistically significant from the TULA-single knockout mice (b)

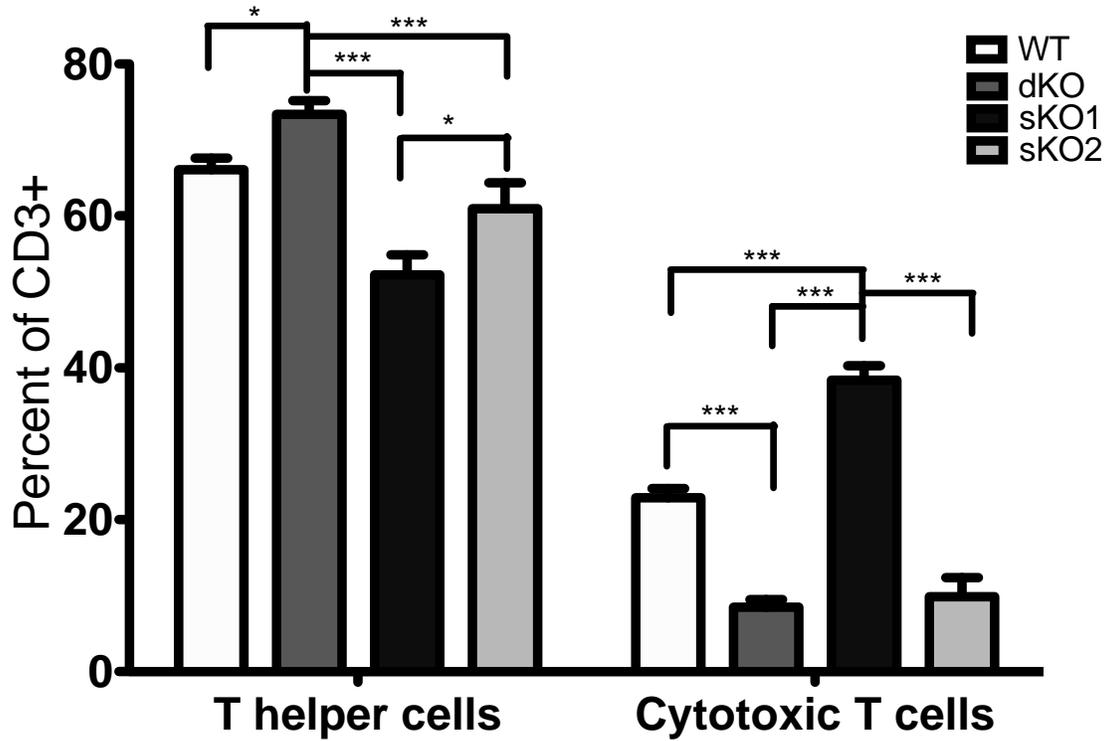


Figure 2

Characterization of splenic T cells of TULA-family knockout mice. Splens of 6-8 week old mice were stained with antibodies against CD3, CD4 and CD8. T helper cells were gated as CD3⁺ and CD4⁺. Likewise cytotoxic T cells are gated based on expression of CD3⁺ and CD8⁺. Mean and SEM^{+/-} are plotted. Two-way Anova statistical analysis was performed.

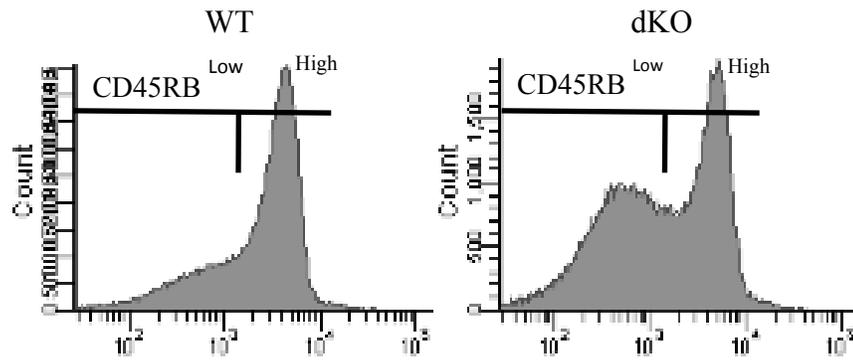


Figure 3

Histogram of CD45RB distribution of WT and dKO splenic CD4⁺ T cells. Cells were stained with antibodies against CD4 and CD45RB. CD4 positive cells were subsequently gated based on CD45RB expression. Above is a histogram of the splenic CD45 distribution among CD4⁺ population from WT and dKO mice the black bar distinguishes the low from the high population.

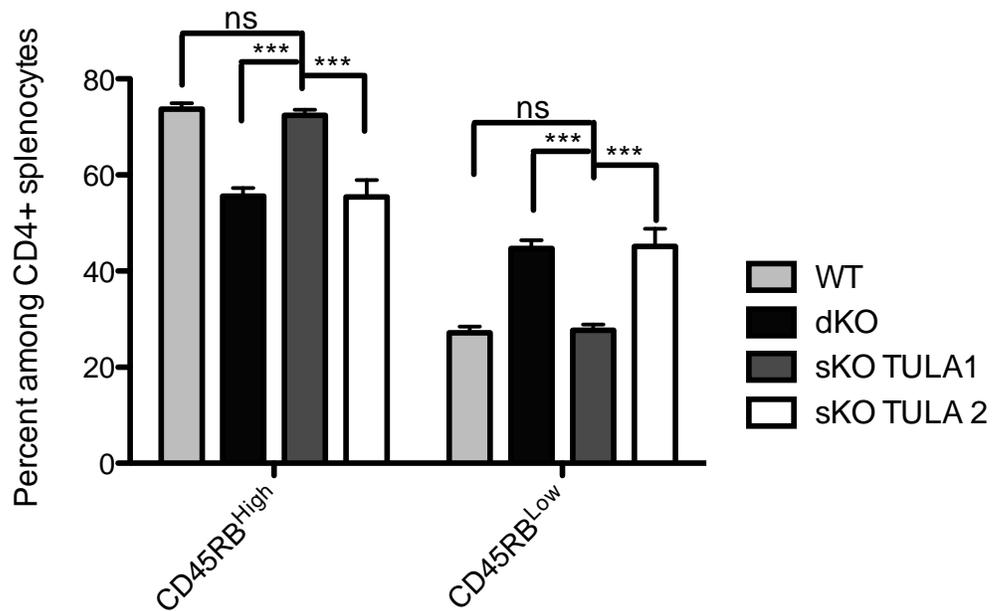


Figure 4

CD45RB distribution of CD4⁺ splenic T cells from TULA-family knockout mice. Splenocytes from WT and various TULA KO mice were stained with cells were stained with antibodies against CD4 and CD45RB. CD4 positive cells were subsequently gated based on CD45RB expression. Mean and SEM^{+/-} are plotted. Two-way Anova statistical analysis was performed. The difference between both dKO and sKO2, on one hand, and WT, on the other hand, is statistically significant (***).

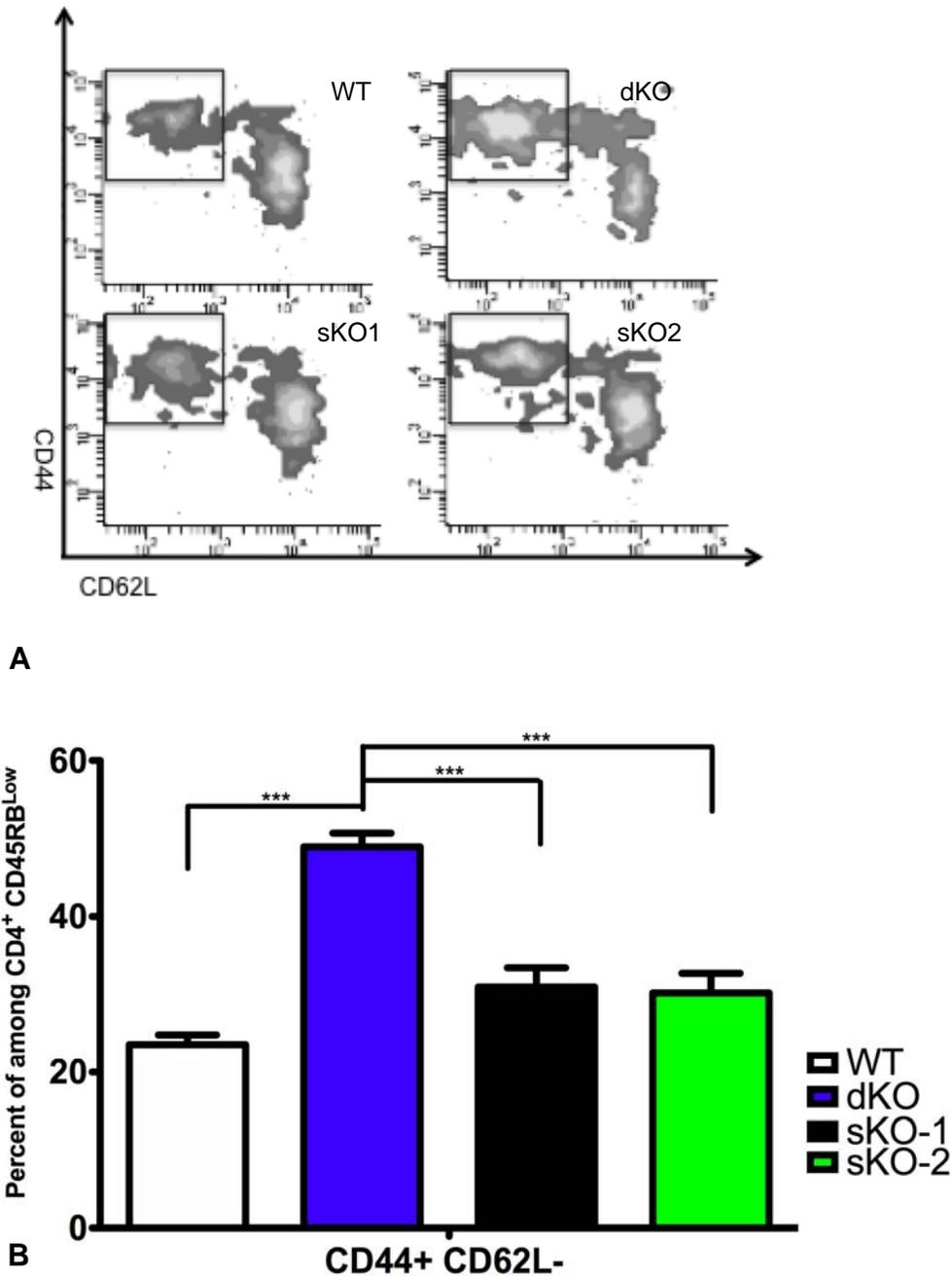


Figure 5

Characterization of T cells expressing Memory markers. Splenocytes were stained with antibodies against CD4, CD45RB, CD44, and CD62L. CD4⁺ CD45RB^{Low} were first gated then subsequently gated for CD44 and CD62L expression. The percent of CD44⁺ and CD62L⁻ cells are shown in the square box (a) and quantified in the bar graph (b). Means and SEM^{+/-} are plotted. One-way Anova statistical analysis was performed. Difference between WT and either sKO1 or sKO2 is not statistically significant.

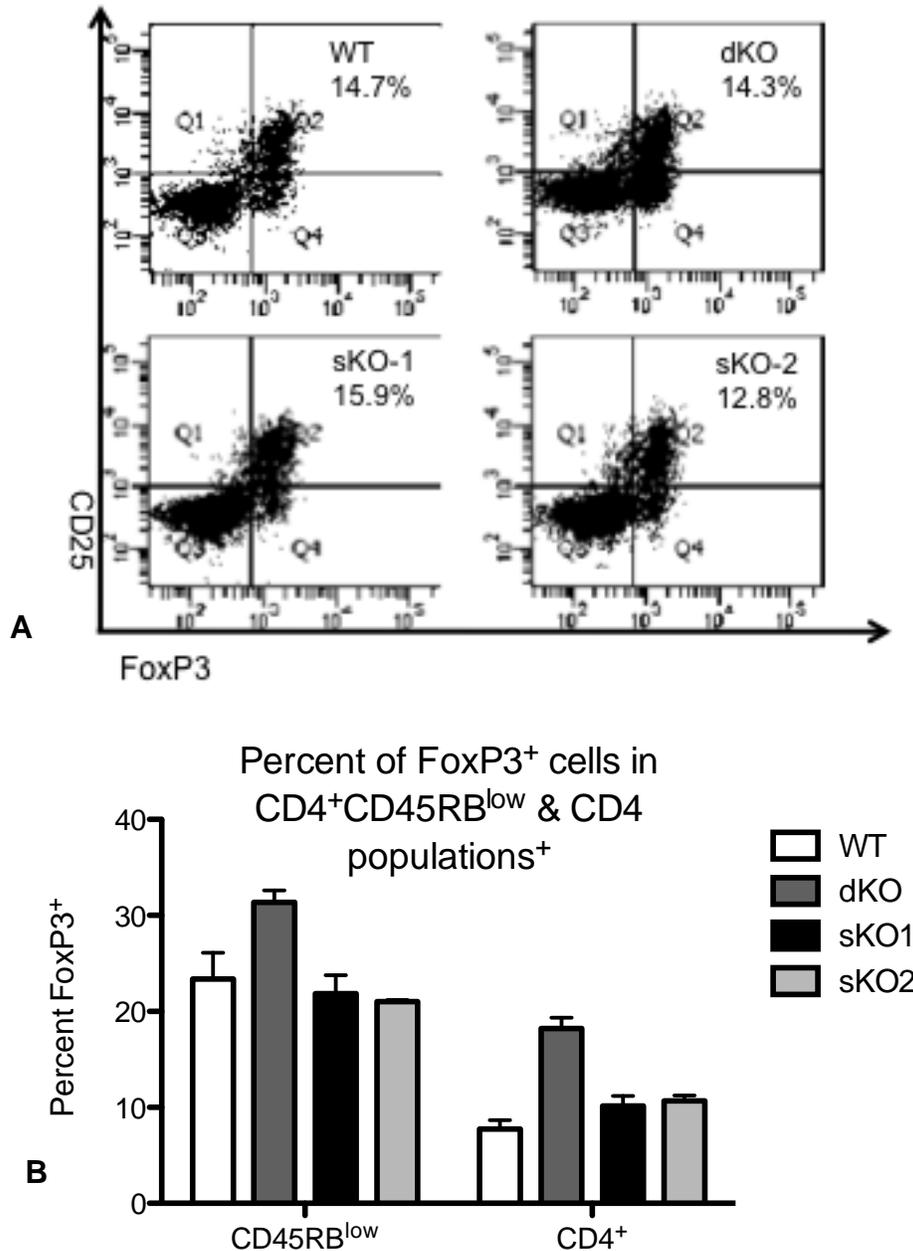


Figure 6

Characterization of CD4⁺ T cells expressing regulatory markers. Spleens of 6-8 week old mice were stained with antibodies against CD4, CD45RB, and CD25. CD4⁺CD45RB^{low} were first gated then subsequently gated for CD25 and FoxP3 expression (a). Double positive cells are within quadrant 2 (Q2). To the left, the percent of FoxP3⁺ (Q2 & Q4) cells within the CD45RB^{Low} and CD4⁺ populations are quantified (b). Means and SEM^{+/-} are plotted. Two-way Anova statistical analysis was performed. Difference between dKO and each WT, sKO1 and sKO2 is statistically significant (***)

Consequences of TULA deficiency - Functional Assays

TULA-family proteins structurally are very similar, however previous research indicates that their functions differ. TULA-2 is quite active as a PTP, whereas TULA-1 exhibits just a small fraction of TULA-2's activity (<0.01) (Chen et al., 2009; Mikhailik et al., 2007). In contrast, TULA-1 has been shown to facilitate apoptosis in Jurkat cells, while TULA-2 lacks this ability (Collingwood et al., 2007b). These findings suggested that TULA-1 and TULA-2 might play their roles in the regulation of T-cell activation through different mechanisms.

TCR stimulation initiates a cascade of signaling events that ultimately induce various cellular responses, including cytokine production. Both TULA-family proteins possess phosphatase domains, and TULA-2 has been shown to be capable of dephosphorylating key proteins of TCR-mediated signaling, such as Zap-70 (Carpino et al., 2004; Chen et al., 2010; Chen et al., 2009). However, the individual roles of TULA-1 and TULA-2 in the regulation of TCR signaling events and subsequent cellular responses still are poorly understood. To determine if deficiency in either TULA-family protein can alter cellular responses to TCR stimulation, we assessed cytokine production by CD4⁺ T cells from spleens of WT and various KO mice in response to stimulation with anti-CD3 and anti-CD28 antibodies. In order to be able to detect differential responses of WT and KO cells in the situation when at least some KOs are anticipated to increase responses, we selected 24 hours, an early time point for collecting supernatants. As expected, the cytokine production by WT CD4⁺ T cells in response to both anti-CD3-only and anti-CD3⁺anti-CD28 at 24 hours post-stimulation was not much higher than for non-stimulated controls. CD4⁺ T cells from dKO, sKO1 and sKO2 mice activity produced

cytokines in response to both CD3 and CD3/CD28 stimulations, but their responses clearly differed.

dKO CD4⁺ T cells responded to anti-CD3 by production of almost all cytokines measured (figure 7). Their response to anti-CD3/CD28 was lower than that to anti-CD3 alone, but it was notably significant for IL-17. Other cytokine responses of dKO CD4⁺ T cells to the CD3/CD28 stimulation were not statistically significant, but a trend is clearly seen, especially for MIP-1 α and IFN γ (figure 7). The patterns of responses to anti-CD3 were very similar for dKO and sKO1 CD4⁺ T cells, while no response of sKO1 cells to anti-CD3/CD28 was detected (figure 7). In contrast, sKO2 CD4⁺ T cells responded in this system only to anti-CD3/CD28, but not to anti-CD3 alone (figure 7). Thus, it appears that sKOs exert specific effects on *in vitro* responses of primary CD4⁺ T cells from spleen; the lack of TULA-1 facilitates responses to incomplete, suboptimal stimulation with anti-CD3 alone, while the lack of TULA-2 facilitates responses to stimulation through both TCR and CD28 co-receptor. The response pattern of dKO CD4⁺ T cells resembles a combination of the two sKO-specific patterns.

Overall, these results indicate that the loss of each TULA-family member exerts a specific effect on T-cell reactivity *in vitro*, arguing that TULA-1 and TULA-2 play independent roles in the suppression of T-cell activation. Although these findings do not point out the mechanisms through which the individual TULA-family members are involved in the suppression of T-cell activation, the body of data related to TULA-2 suggest that this family member functions by dephosphorylating signaling proteins, most likely Zap-70 (Carpino et al., 2009a; Carpino et al., 2004; Chen et al., 2010). It cannot be ruled out that TULA-1 also act as a PTP, as it was suggested before (San Luis et al.,

2011), but it is also possible that its role is to promote apoptosis of T cells, thus modulating T-cell responses by reducing the number of activated T cells.

In fact, processes inducing cell death are among well-known mechanisms of cellular regulation. Thus, IBD patients have T cells that are resistant to therapies that induce cell death, suggesting that pathological activation of T cells correlates with a decrease in their sensitivity to death induction (Lugering et al., 2006). We performed functional assays to assess how T cells of TULA-family knockout mice respond to conditions that induce apoptosis: growth factor withdrawal (complete medium with 10% serum, but without IL-2) anti-CD3-only “incomplete” TCR stimulation, and serum deprivation (medium with 0.5% serum). Viability was determined using staining with 7-AAD followed by flow cytometry as the percent of 7-AAD negative cells in culture at 10 and 40 hours. The experiments indicated that dKO CD4⁺ T cells are more viable than WT and sKO2 T cells (figure 8). However, sKO1 CD4⁺ T cells also exhibit increased survival when they are cultured in the presence of anti-CD3 only without any co-stimulation, a stimulation that causes T-cell apoptosis and anergy (Chai and Lechler, 1997; Radvanyi et al., 1996).

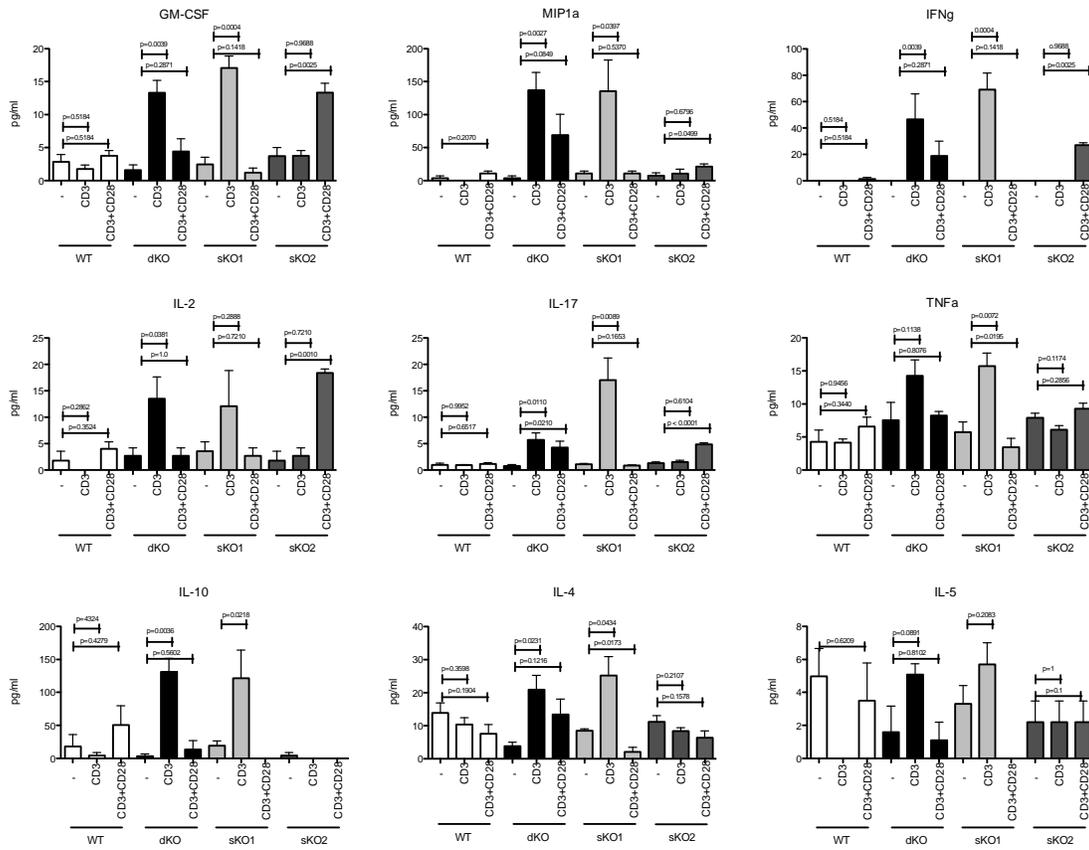


Figure 7.

Response of CD4⁺ Splenic T cells to TCR stimulation. 4 X 10⁵ CD4⁺ splenocytes from 6-8 week old mice were incubated in cell culture conditions with no stimulation (-), anti-CD3 antibodies (CD3), or anti-CD3 and anti-CD28 antibodies (CD3 + CD28) for 24 hours. Culture supernatants were assayed for the presences of cytokines and chemokines using Luminex multiplex systems. Means and SEM^{+/-} are plotted. Student's t Test statistical analysis was performed.

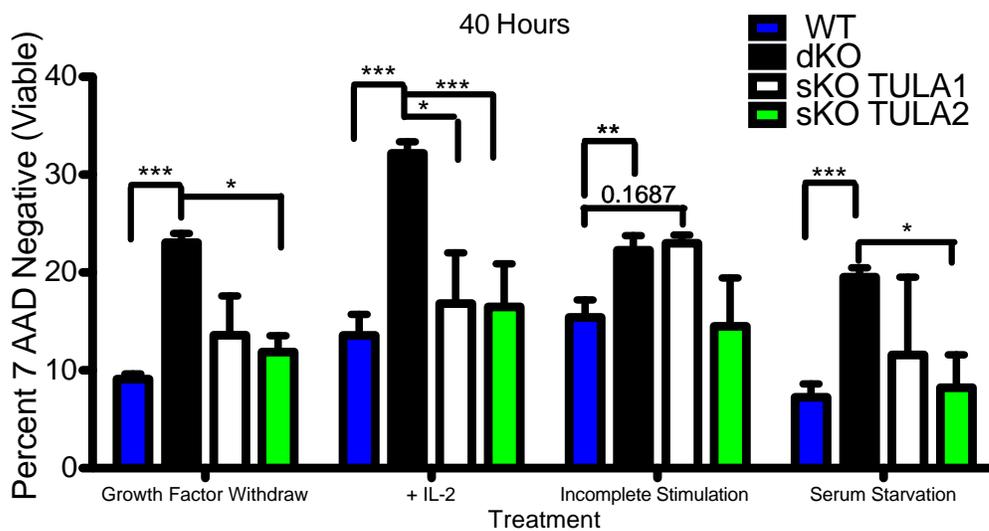
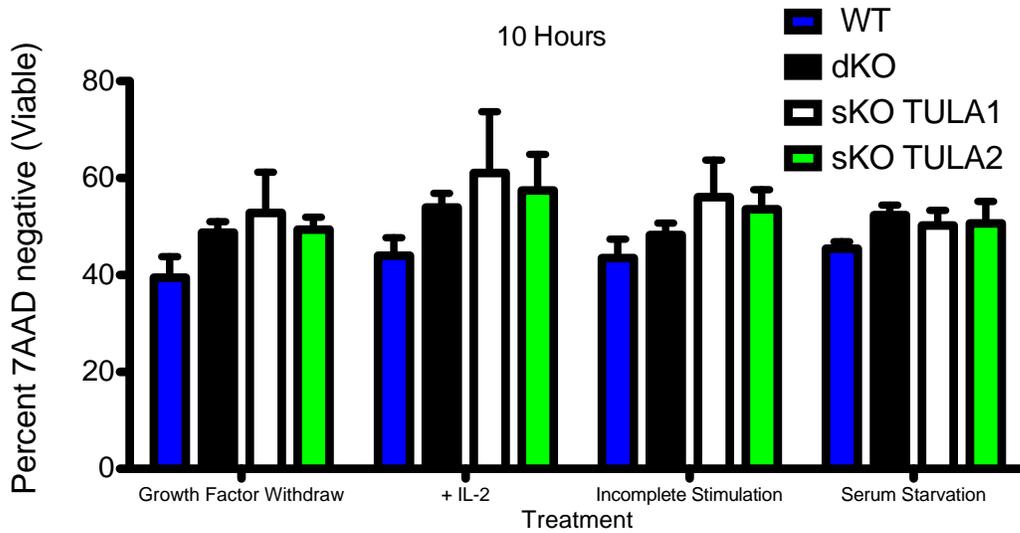


Figure 8

TULA-1 deficiency confers resistance to apoptosis. 4×10^5 CD4⁺ T cells from spleens of 6-8 week old mice were cultured under the following conditions: PCCM without IL-2 (growth factor withdrawal), PCCM plus IL-2 (growth factor), PCCM plus low dose of anti-CD3 (“incomplete stimulation”), and PCCM with 0.5% serum (serum starvation) for 10 or 40 hours. Cells were then stained with 7-AAD and analyzed using flow cytometry to determine the fraction of 7-AAD positive and negative cells. Means and SEM^{+/-} are plotted. Two-way Anova was performed.

TULA deficiency leads to susceptibility to TNBS-induced colitis

The results shown above indicated that both TULA-1 and TULA-2 sKOs modify T-cell responses *in vitro* and that the effect of dKO on T-cell responses appeared to combine effects of sKOs. However, it remained unclear whether the lack of TULA-family proteins can promote inflammatory processes *in vivo* and, if so, what are the individual roles of TULA-1 and TULA-2 in these phenomena. To examine contribution of TULA-family proteins in regulation of T cell-driven inflammation, we used animal models that simulate inflammatory bowel disease (IBD). One such model is TNBS-induced colitis, since inflammation in this model is known to involve CD4⁺ T cells (Elson, 1996; Elson et al., 2005). Mice of different genetic backgrounds are differentially sensitive to TNBS-induced colitis susceptibility; C57BL/6 mice are considered refractory to this treatment. Indeed, WT C57BL/6 mice responded very poorly under the conditions used in our work, while mice deficient in TULA-family proteins developed immune pathology at the level that was a stark contrast to that seen in WT mice (see below).

Once TNBS-induced colitis is initiated in dKO, sKO1 and sKO2 mice, they develop wasting (weight loss), which is typical of the model and increased as compared to the WT group. This response peaks in dKO mice given an enema of TNBS/ethanol on day 1 post-induction at 21 percent difference compared to the ethanol control animals. Individual knockouts develop wasting as well, that peaks on day 3 and day 2 at 13 and 24 percent difference compared to the ethanol control for sKO1 and sKO2, respectively. Compared to the 8% percent weight that was lost by the WT mice that received TNBS, KO mice develop wasting that is more severe (figure 9). In addition to an increase in wasting, KO mice show an increase in TNBS-induced mortality. Only 65, 52, and 33

percent of dKO, sKO1, and sKO2 mice, respectively, survived 3 days post-induction as compared to 82 percent of WT TNBS-treated mice (figure 10). The observed weight loss was transient; the response of dKO mice to TNBS peaked on day 1 or 2 post-induction and then gradually decreased to the control level (data not shown).

Overall, vital parameters of animals in the TNBS model are dependent on TULA-family proteins. Whereas WT mice retain high viability and normal weight following induction, dKO mice exhibit a significant weight loss and mortality. Responses of sKO are specific for individual KOs: sKO1 shows high mortality, but loses weight not so profoundly and slower than dKO, while sKO2 exhibits weight loss similar to that of dKO, but no significant increase in mortality ($p=0.08$). Interestingly, administration of the CD4⁺ cell-depleting GK1.5 antibody (see below, figures 9 and 10) to mice prior to their TNBS sensitization and treatment did not significantly reduce their physiologic responses to TNBS (data not shown, figure 2 table).

Upon termination, TNBS-experimental animals were examined for signs of intestinal inflammation. As expected, colons of WT mice exposed to TNBS have little to no inflammation as seen anatomically and histologically (figures 11 and 12, inflammation score is shown in figure 16). However, analysis of colon anatomy and histology revealed severe inflammation in the colons of TNBS-treated knockout mice. Anatomically, the colons of TNBS-treated knockout animals lack well-formed stool, inflamed and necrotic (especially in the distal colon). Inflammation seen in the colon of dKO is more severe than that in other knockout mice receiving TNBS. Histologically, the overall architecture of the colons was destroyed in knockout animals receiving TNBS. The muscularis mucosa and submucosa were thick and filled with infiltrating mononuclear cells. Crypts,

if present, were ill formed, and goblet cells were generally absent. Luminal epithelial barrier of the colon was broken in most cases (figures 13-15). Although GK1.5 did not completely ameliorate inflammation in KO mice, it caused a moderate decrease in the inflammation score in TNBS-treated dKO and sKO1 mice (figure 16). Anti-inflammatory effect of GK1.5 seemed to be most effective in sKO1 mice. Also noteworthy, the inflammation score of all KO mice receiving TNBS were comparable and did not differ significantly (figure 16).

First, the ability of GK1.5 to deplete CD4⁺ cells was assessed in mice that were not treated to induce colitis. Mice were euthanized 24 hours after GK1.5 injection, mesenteric lymph nodes (MLN) and spleens were removed and single cell suspensions of these organs were stained with anti-CD4 and anti-CD8 to show specific depletion of CD4⁺ cells, which was found to be complete (figure 17). Furthermore, upon termination of experimental groups in TNBS colitis experiments CD4⁺ cell depletion was assessed using flow cytometry and immunofluorescence; colons were harvested and flash frozen in OCT medium, sections of the colons were prepared and stained with fluorescence-conjugated anti-CD4 to detect CD4⁺ T cells. Together, these experiments indicated that CD4⁺ T cells were almost completely depleted from spleen and MLN, but remained present in the colonic tissue (figures 18-21).

To further evaluate severity of inflammation in experimental animals in our model, we determined production of cytokines and chemokines in (a) serum, (b) tissue extract from total colon, and (c) cell culture of CD4⁺ T cells from MLN and spleen.

The systemic effect of TNBS was evaluated by measuring the levels of cytokines and chemokines in the serum of experimental mice. Of the 20 cytokines and chemokines IL-6, MIP-1 α , MIG, IP-10, IL-4, VEGF, KC and IL-13 were detected in the serum of TNBS experimental groups. In general TNBS induced an increase in the levels of these proinflammatory cytokines. Especially for IL-6, IP-10 and VEGF that were increased in response to TNBS with the highest increase seen in sKO1 mice (figure 22).

The findings that the level of mostly pro-inflammatory cytokines and chemokines are increased in the serum of TNBS-treated mice and that the effect of TNBS is more profound in KO mice (figure 22) corresponded well with the findings that TNBS induces inflammation in KO mice (see above). Furthermore, an increase in the serum levels of cytokines and chemokines indicated that the effect of TNBS in this system may be systemic. However, production of pro-inflammatory cytokines by immune cells does not have to increase the serum levels of these cytokines in order to drive inflammation, since the effects of cytokines are mostly local. Indeed, the results shown in figure 22 indicate that the effect of TNBS on serum cytokines in KO mice follow the order: sKO1>dKO>sKO2. This order corresponds neither to the severity of inflammation determined histologically nor to the intensity of *in vitro* responses of splenic CD4⁺ T cells (see above). Therefore, it is quite possible that sKO1 mice have a unique characteristic rendering the effect of TNBS on cytokine levels systemic (see Discussion).

Therefore, we assessed the effects of TNBS on cytokine expression in the colon using RT-qPCR, an independent approach, which determines the levels of the corresponding mRNA in the colon. These experiments shown in figure 23 demonstrated, first, that TNBS had a very weak effect on WT mice; most of the cytokine messages

tested did not increase in these mice. In contrast, dKO mice exhibited a clear increase in the levels of most cytokine messages determine. Consistent with the findings that dKO CD4⁺ T cells are more responsive to TCR-mediated stimuli than are sKO CD4⁺ T cells (Carpino et al., 2002; San Luis et al., 2011), the increase in TNBS-induced cytokine expression is not seen in sKO mice. Third, an increase in expression is more profound for pro-inflammatory cytokines; IL-4, a typical Th2-type cytokine, is not upregulated, while pro-inflammatory ones (TNF α , IFN γ , and IL-17) are. IL-10 is also upregulated, but its role is complex and not simply that of an anti-inflammatory cytokine (reviewed in (Ouyang et al., 2011)). Fourth, the role of IL-17 suggested by a dramatic increase in IL-17 mRNA in dKO mice upon TNBS treatment was further analyzed by measuring mRNAs of IL-23R and ROR- γ t, key regulatory components of the IL-17 inflammatory axis. Consistent with the idea of the critical involvement of IL-17 in the effect of TULA-family KO *in vivo*, levels of both IL-23R and ROR- γ t messages were increased in sync with that of IL-17. Finally, administration of anti-CD4 GK1.5 dramatically reduced effects of TNBS. This result is in agreement with the key role of CD4⁺ T cells in the inflammatory responses in our TNBS model. Overall, findings made using RT-qPCR on RNA samples from the colons of experimental animals indicate that dKO exert the strongest effect on T-cell responses to TNBS *in vivo*, as anticipated based on previous results, and that IL-17 expression appears to be critical for the effects of KO on inflammation.

Considering that mRNA expression is not necessarily indicative of cytokine secretion, we verified the findings arguing in favor of the involvement of IL-17 in the effect of dKO (figure 24) by evaluating *in vitro* responses of CD4⁺ T cells isolated from

spleens and MLNs of WT and dKO mice treated with TNBS or control solution. Cells were stimulated *ex vivo* with only anti-CD3 or anti-CD3 plus anti-CD28 antibodies as described above (see figure 24), and culture supernatants were analyzed for IL-2 and IL-17 using ELISA. First, T cells from both WT and dKO mice produce IL-2 in response to stimulation, albeit differentially; a response to TCR stimulation is seen in WT cells from spleen, but not from those from MLN, which show detectable secretion of IL-2 even in non-stimulated culture, the opposite is true for dKO MLN cells respond to TCR stimulation, while spleen dKO cells secrete even in the absence of stimulation. Second, TNBS does not exert a significant effect on IL-2 production. Likewise, IL-17 is produced by WT and dKO cells from both spleens and MLNs. However, production of IL-17 is much higher for MLN dKO cells. Most importantly, TNBS does not facilitate IL-17 production of spleen/MLN WT CD4⁺ T cells, whereas it greatly facilitates IL-17 secretion by MLN dKO T cells in response to anti-CD3 alone, which represents a sub-optimal stimulation (figure 24). Interestingly, TNBS reduced responses of dKO spleen T cells. However, it should be noted that the colonic immune response appears to be regulated by cells in MLNs, not spleen (Powrie, 2004). One possible explanation for this finding is preferential localization of TNBS-sensitized T cells to the MLN and colon.

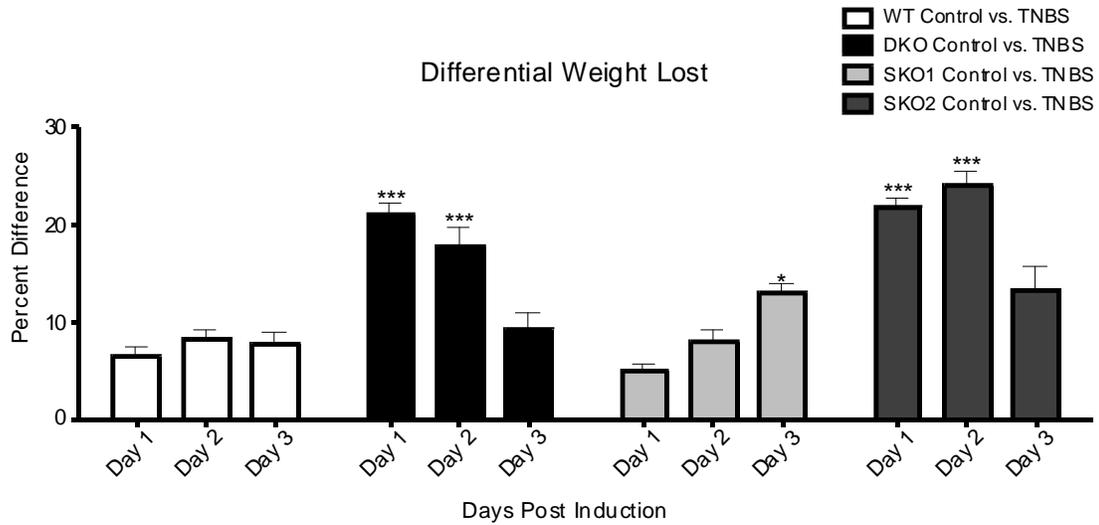


Figure 9

Mice deficient in TULA-family proteins develop wasting disease in response to intra-rectal administration of TNBS. Initial weight of mice was measured one day before intra-rectal instillation of TNBS/ethanol solution or ethanol. The weight was monitored and the difference between the TNBS and ethanol groups was plotted. There was no statistically significant difference between TNBS-treated mice with and without administration of GK1.5 mAb (not shown). Means and SEM^{+/-} are plotted. Two-way Anova statistical analysis was performed stars indicate a difference compared to WT on that day.

Statistical Significance of Survival Curves (Mantel-Cox Test)			
TNBS GROUP	n=		P=
WT EtOH vs. WT TNBS	44	62	0.1084
WT TNBS vs. dKO GK.1.5	62	11	0.0056
WT TNBS vs. dKO TNBS	62	65	0.0207
WT TNBS vs. sKO1 GK1.5	62	17	0.0407
WT TNBS vs. sKO1 TNBS	62	25	0.0021
WT TNBS vs. sKO2 GK1.5	62	9	0.0002
WT TNBS vs. sKO2 TNBS	62	15	<0.0001
dKO EtOH vs. dKO GK1.5	46	11	<0.0001
dKO EtOH vs. dKO TNBS	46	65	<0.0001
dKO TNBS vs. dKO GK1.5	65	11	0.2698
dKO TNBS vs. sKO1 TNBS	65	17	0.2579
dKO TNBS vs. sKO2 TNBS	65	15	0.0288
sKO1 EtOH vs. sKO1 GK1.5	18	17	0.0027
sKO1 EtOH vs. sKO1 TNBS	18	25	0.0007
sKO1 TNBS vs. sKO1 GK1.5	25	17	0.5479
sKO1 TNBS vs. sKO2 TNBS	25	15	0.2746
sKO2 EtOH vs. sKO2 Gk1.5	8	9	0.1005
sKO2 EtOH vs. sKO2 TNBS	8	15	0.0813
sKO2 TNBS vs. sKO2 GK1.5	15	9	0.8087

Survival of TNBS Experimental Groups

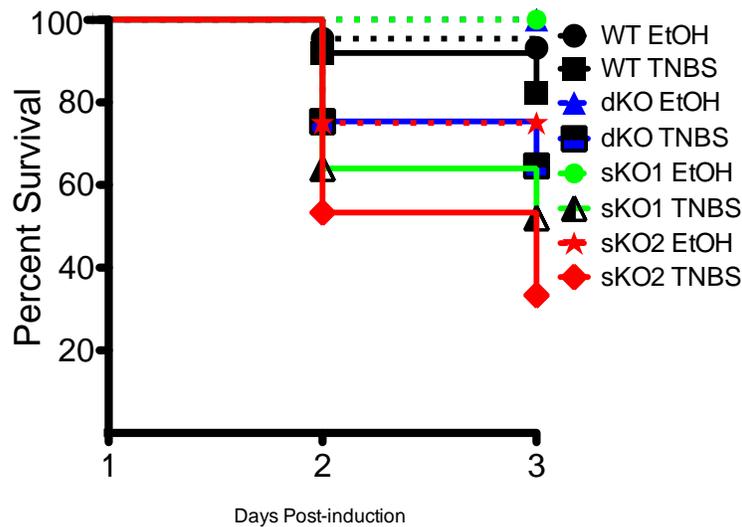


Figure 10

Mice deficient in TULA-family proteins have a decreased viability in the TNBS-induced colitis model. Mice were exposed to TNBS/ethanol or ethanol, and their survival was monitored for 3 days post-induction. There was no statistically significant difference between GK1.5 and TNBS groups, thus only TNBS and ethanol control groups are shown above (left). The statistical significance between all experimental groups is shown on the right.

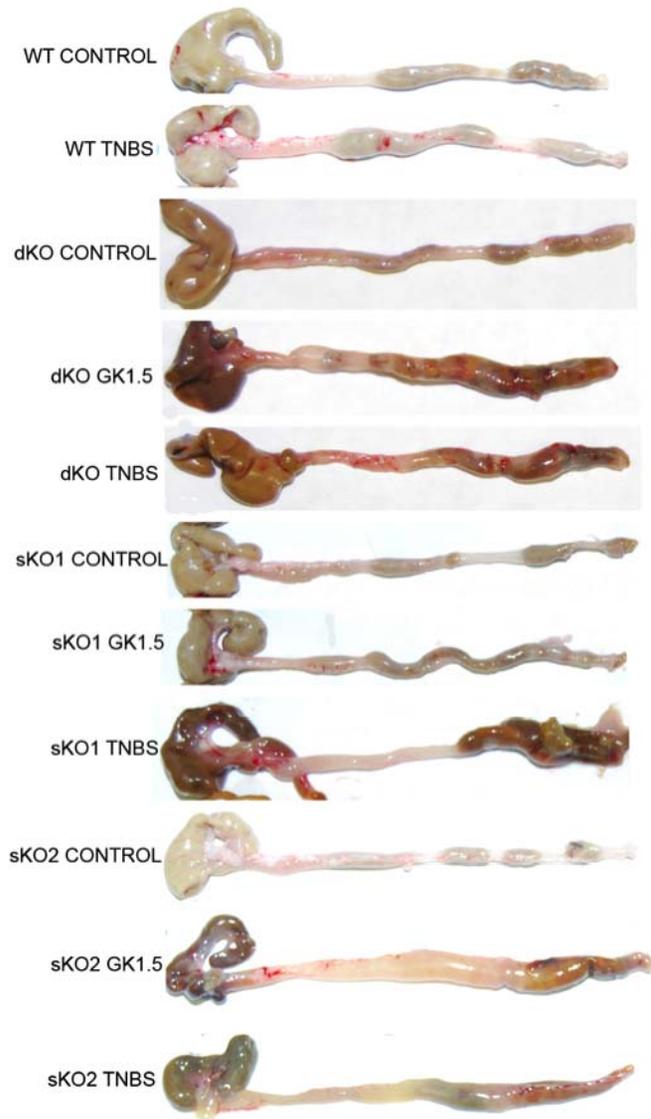


Figure 11

TNBS induces anatomical changes in TULA-family knockout mice. Intestine of TNBS experimental mice was removed from the caecum to the anus as described in the Methods and Materials. A representative photograph of the colons is shown above.

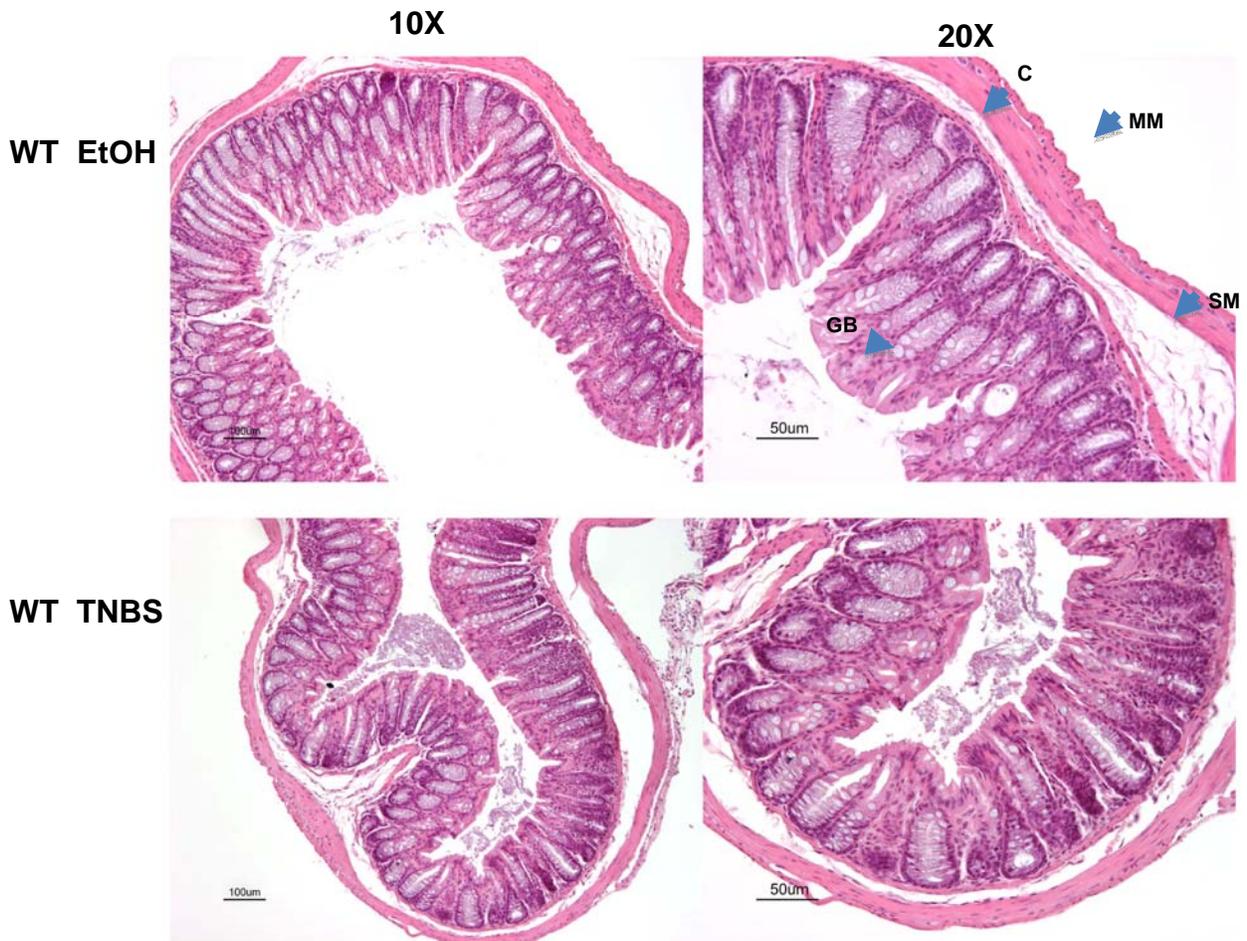


Figure 12

Hematoxylin and Eosin staining of WT colons reveals no inflammation in TNBS treated animals as compared to ethanol control animals. Mice were treated with TNBS or ethanol (EtOH) as indicated and euthanized 3 days post-instillation. The colons were removed and fixed in formalin. Fixed colons were embedded in paraffin, sectioned, and stained with H & E. Key elements of the colon architecture are shown: muscularis mucosa (MM), the submucosa (SM), crypts (C), and goblet cells (GC).

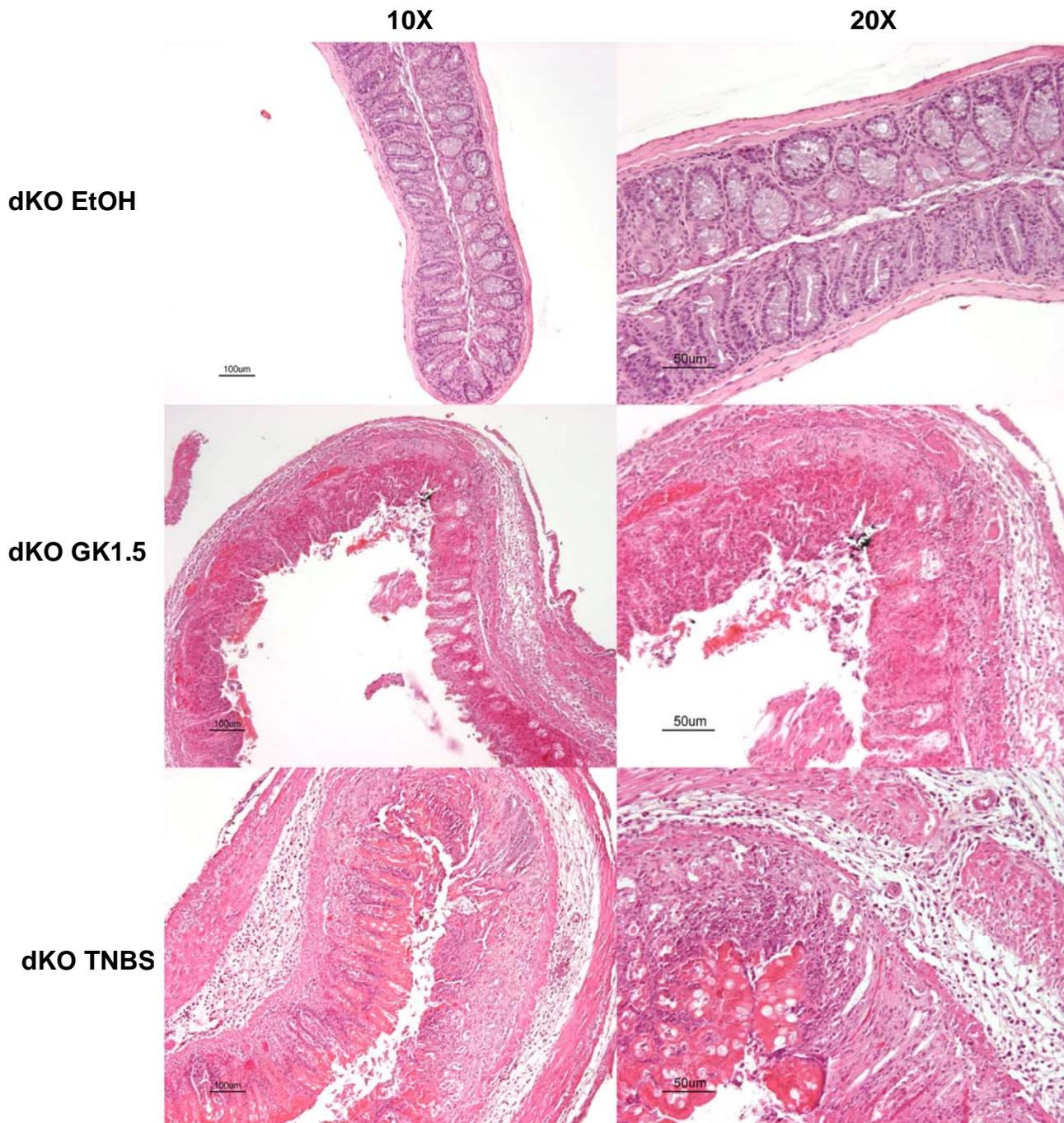


Figure 13

Hematoxylin and Eosin staining of dKO colons reveals severe inflammation in TNBS treated animals as compared to ethanol control animals. dKO mice were treated and their colons were examined as described in the legend to Fig. 11. Images of colons from GK1.5-treated mice, which were also treated with TNBS, are also shown.

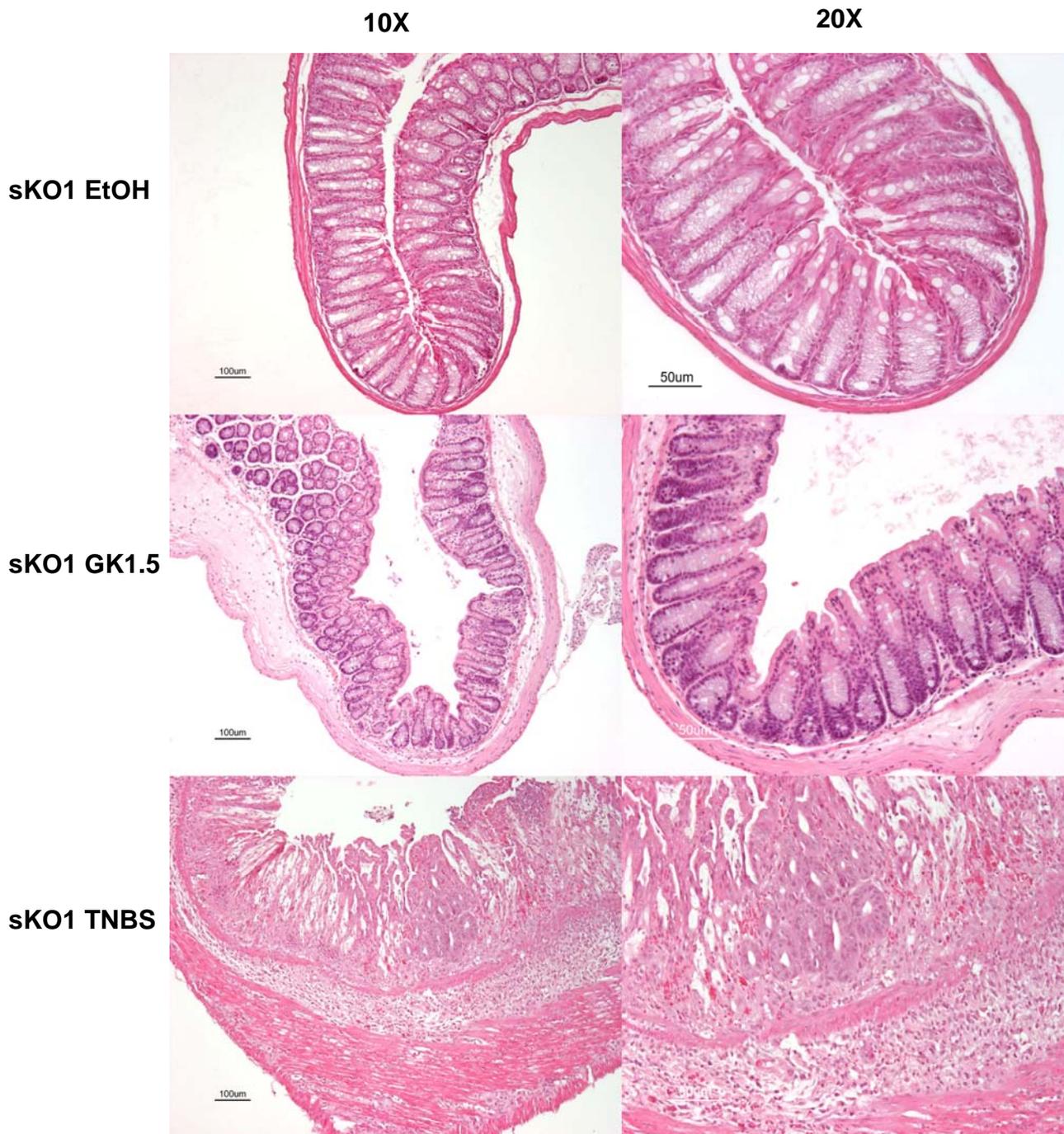


Figure 14

Hematoxylin and Eosin staining of sKO1 colons reveals severe inflammation in TNBS treated animals as compared to ethanol control animals. sKO1 mice were treated and their colons were examined as described in the legend to Fig. 11. Images of colons from GK1.5-treated mice, which were also treated with TNBS, are also shown.

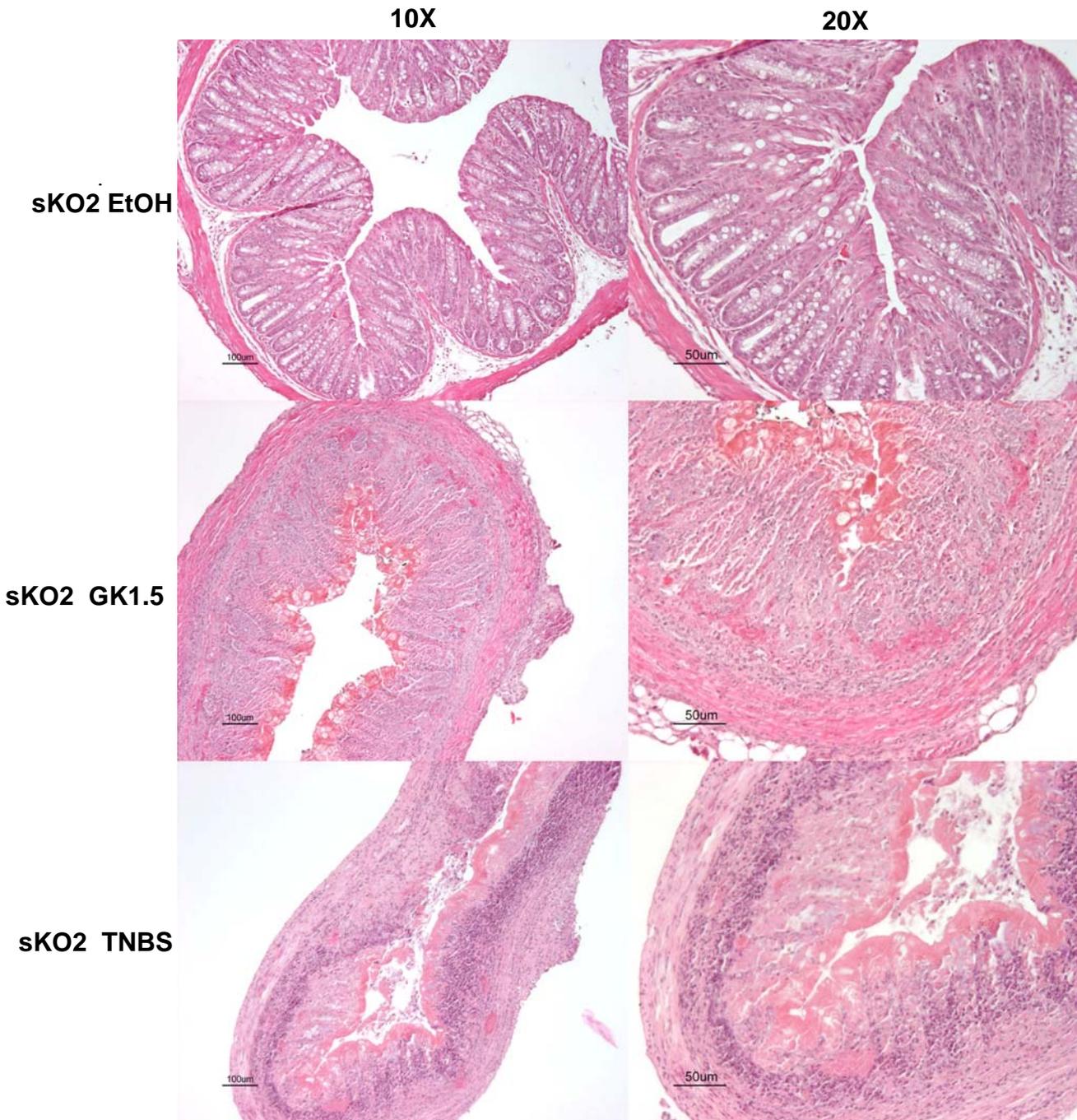


Figure 15

Hematoxylin and Eosin staining of sKO2 colons reveals severe inflammation in TNBS-treated animals as compared to ethanol control animals. sKO2 mice were treated, and their colons were examined as described in the legend to Fig. 11. Images of colons from GK1.5-treated mice, which were also treated with TNBS, are shown as well.

Histological Score of Inflammation in TNBS Induced Colitis

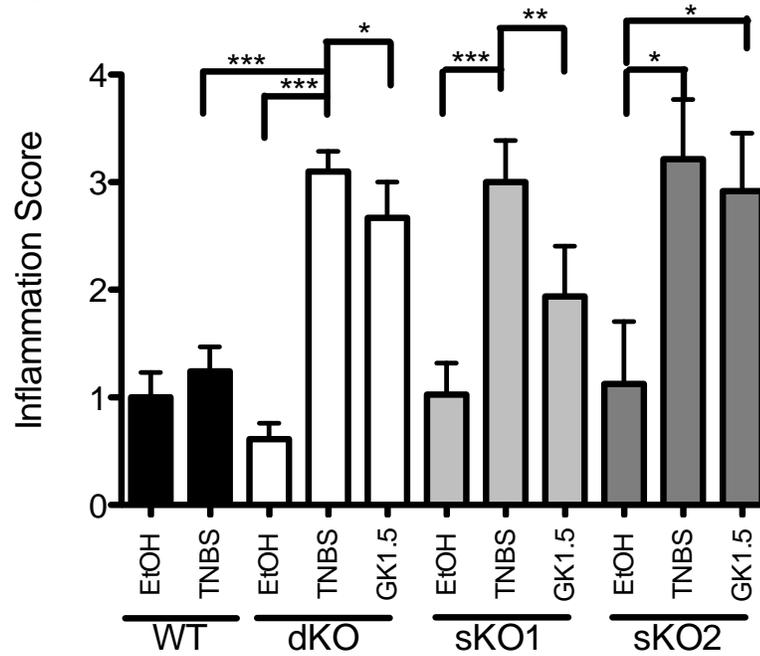


Figure 16

TULA-family deficiency leads to an increased in colonic inflammation in response to intra-rectal administration of TNBS. Each H & E stained sectioned colon was scored based on the severity of inflammation as described in detail in Table 7. The above bar graphs show the means of these score for each TNBS groups with standard error. Means and SEM^{+/-} are plotted. Two-way Anova statistical analysis was performed.

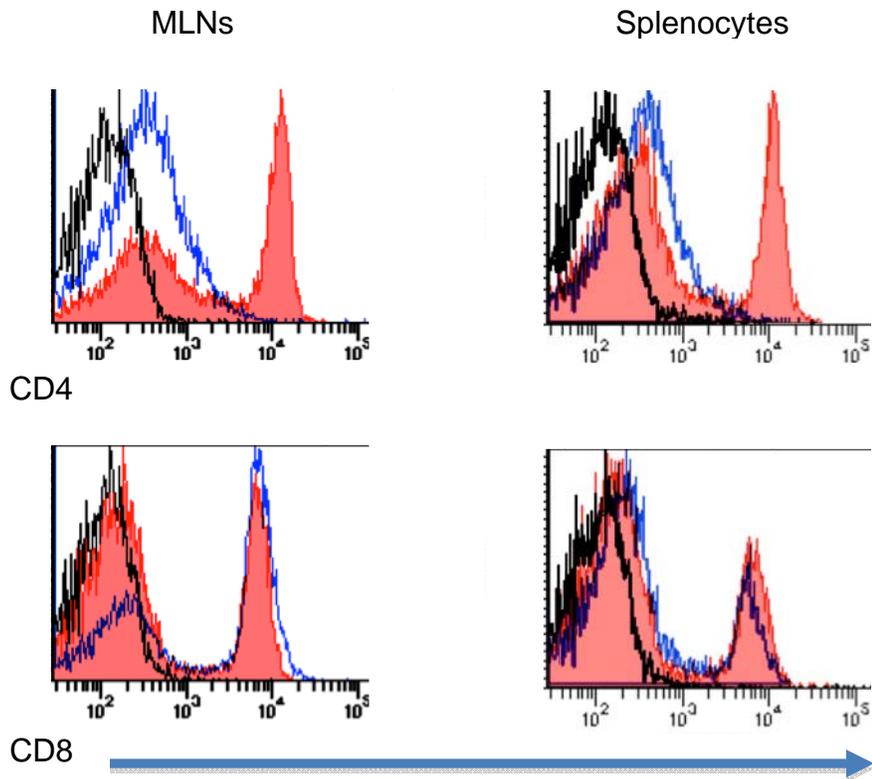


Figure 17

Depletion of CD4+ cells from spleen and mesenteric lymph nodes (MLNs) of dKO TNBS experimental mice. Mice were given 400 μ g of GK1.5 antibody 1 day prior to sensitization and induction phases of the TNBS-induced colitis. dKO mice that were treated with PBS (red) or GK1.5 (blue line) were sacrificed upon termination of the experiment and splenocytes (right panel) and MLNs (left panel) were collected and stained with antibodies against CD4 (top panel) and CD8 (bottom panel) and isotype control antibody for each (black line) show specific depletion of CD4 expressing cells from the spleen and MLNs.

DAPI (blue)+Isotype Control(green) DAPI (blue)+CD4(green)

WT EtOH

WT TNBS

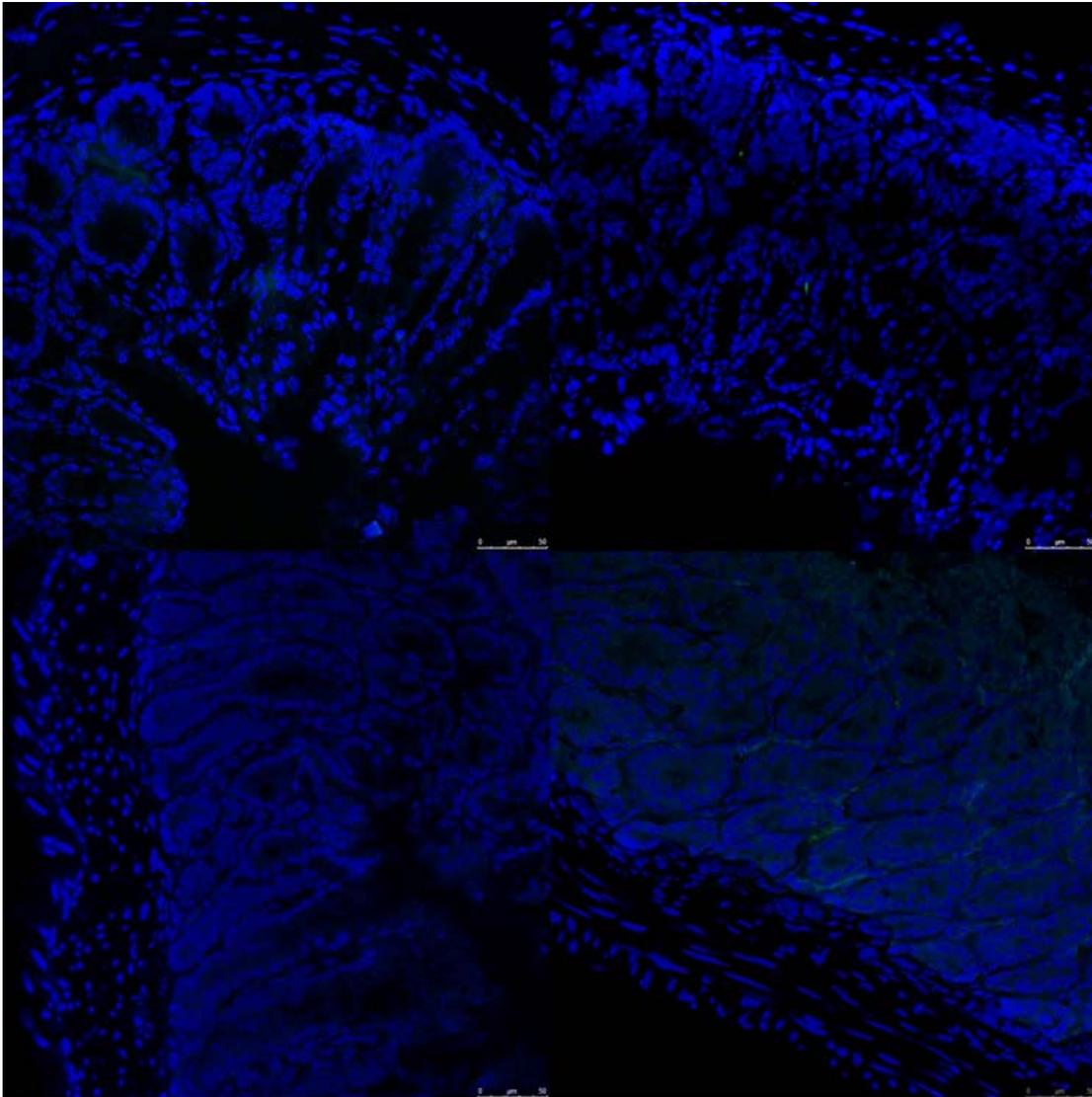


Figure 18

Immunohistochemistry staining for CD4+ T cells within the colon of WT. Colons of animals were embedded in OCT. Colons were cut into 20 μ m sections. Acetone fixed on slide, and stained with antibodies for CD4⁺ or the respective isotype control.

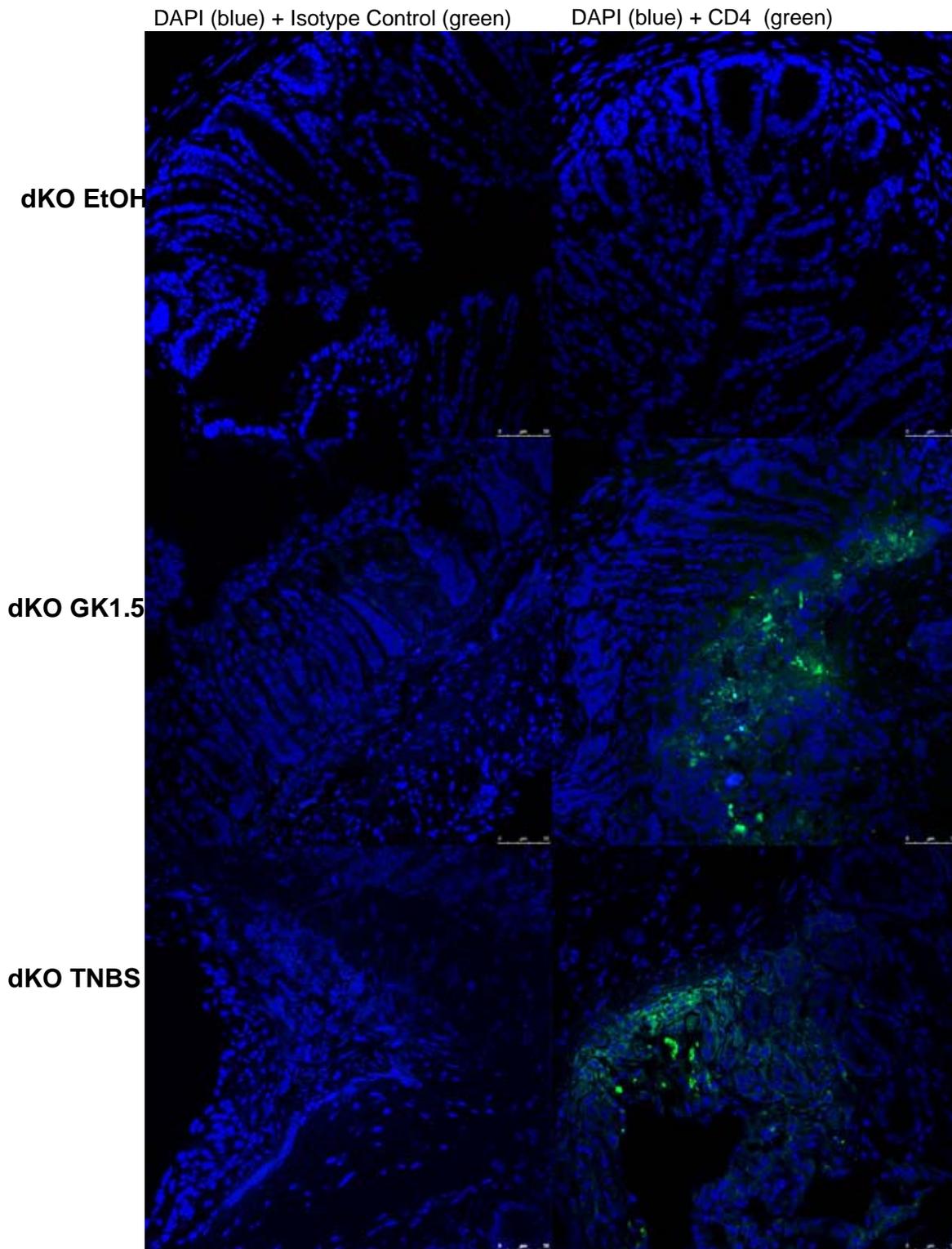


Figure 19

Immunohistochemistry staining for CD4⁺ T cells within the colon of dKO. Colons of animals were embedded in OCT. Colons were cut into 12- μ m sections. Acetone fixed on slide, and stained with antibodies for CD4⁺ or the respective isotype control.

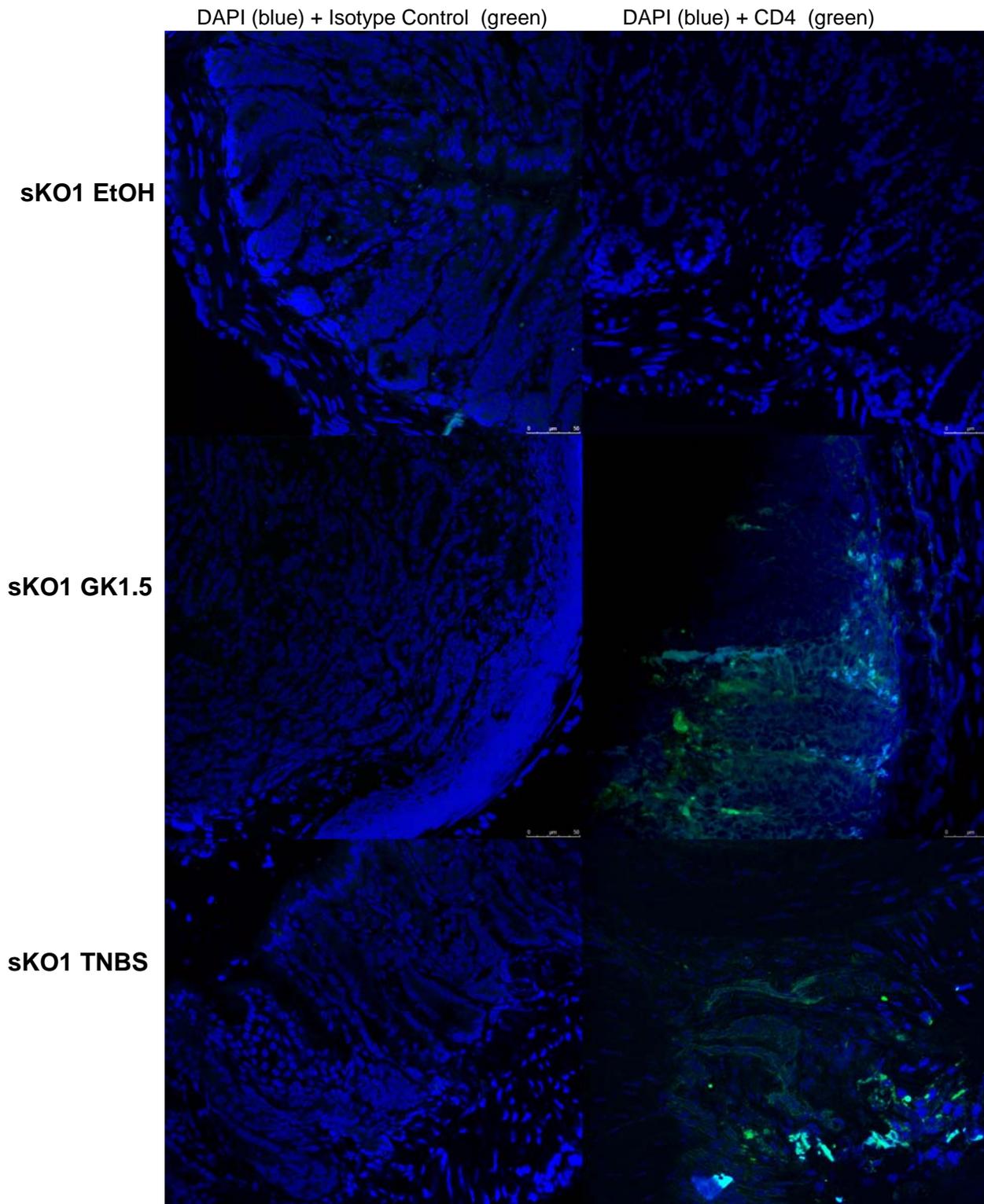


Figure 20

Immunohistochemistry staining for CD4⁺ T cells within the colon of sKO1 mice. Colons of animals were embedded in OCT. Colons were cut into 12- μ m sections. Acetone fixed on slide, and stained with antibodies for CD4⁺ or the respective isotype control.

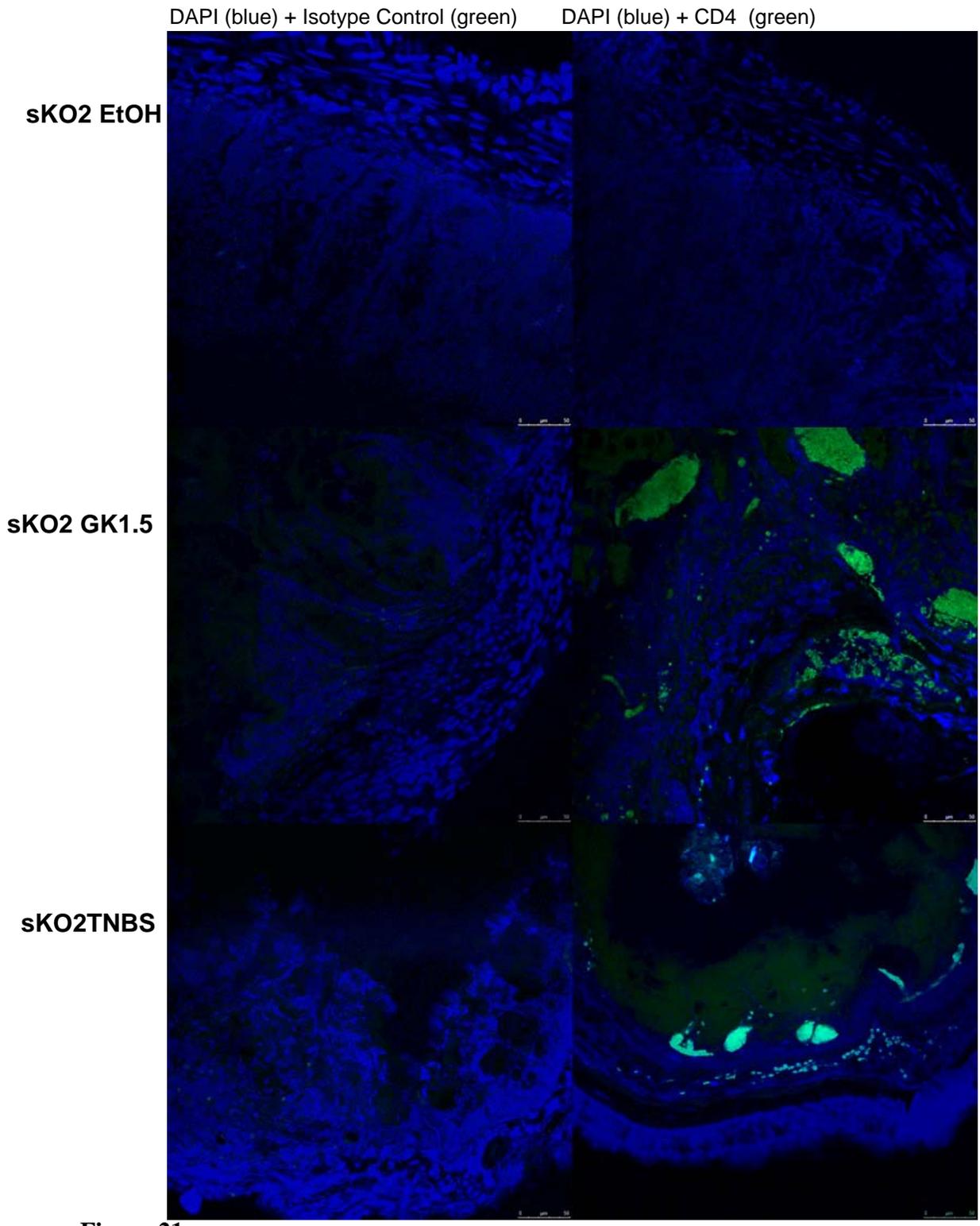


Figure 21

Immunohistochemistry staining for CD4⁺ T cells within the colon of sKO2 mice. Colons of animals were embedded in OCT. Colons were cut into 12- μ m sections. Acetone fixed on slide, and stained with antibodies for CD4⁺ or the respective isotype control.

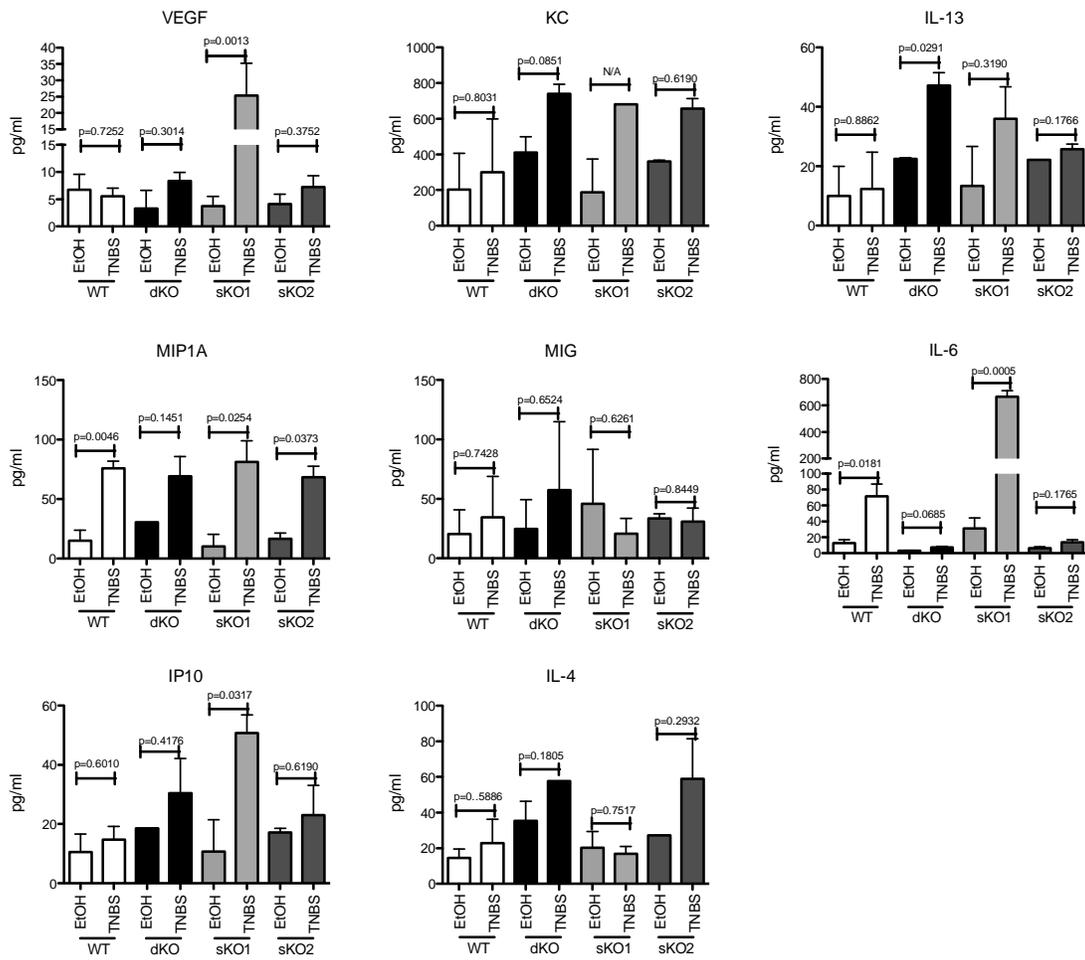


Figure 22

TNBS treatment increased the levels of cytokines and chemokines in mouse serum. Three days post-induction blood was taken from TNBS experimental mice via cardiac puncture. Cytokines and chemokines concentration were determined in the serum using Luminex systems. Means[±]-SEM are plotted. Student's t Test statistical analysis was performed.

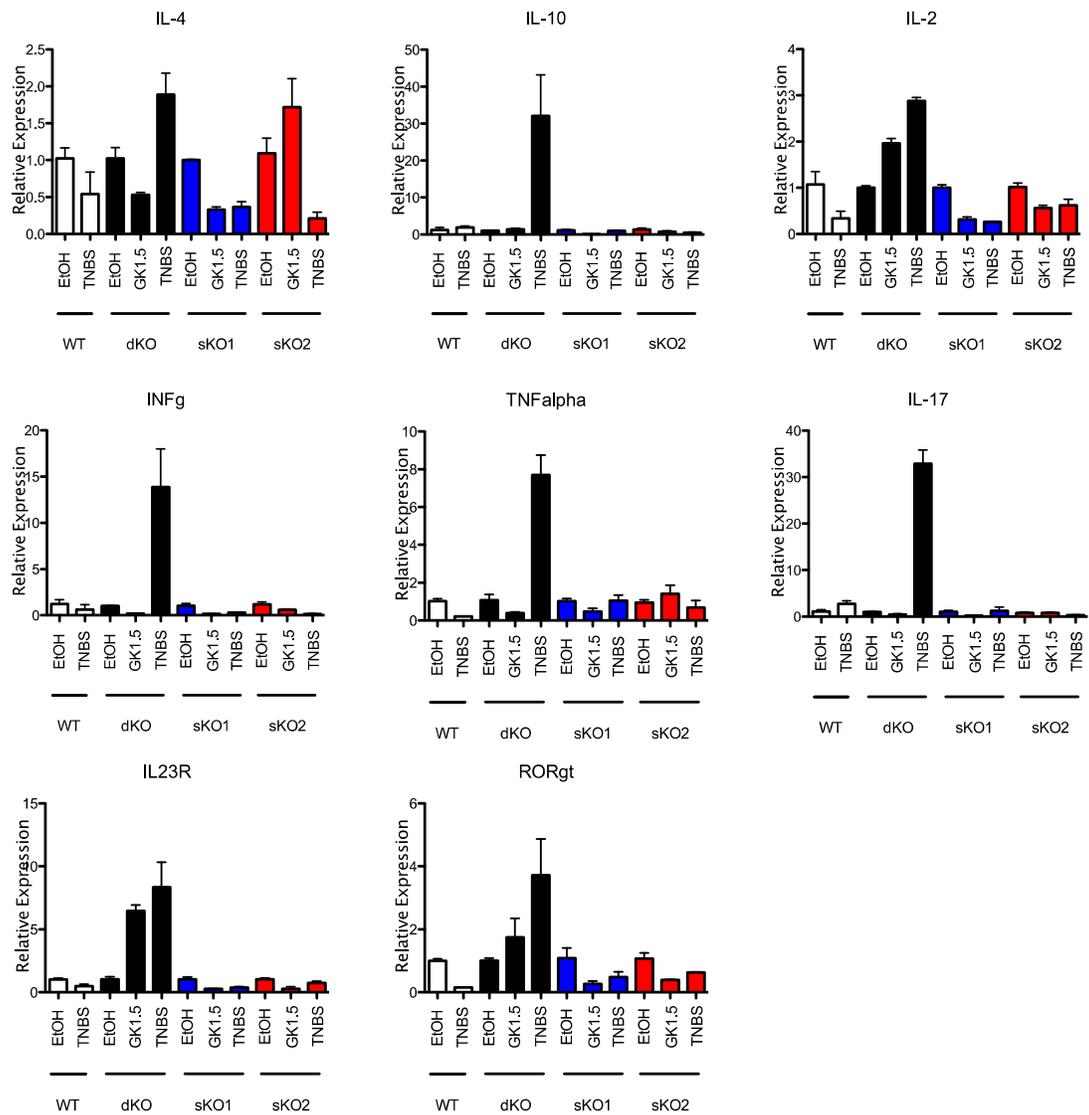


Figure 23

Expression of pro-inflammatory mRNA within colons of TNBS experimental groups. RNA was extracted from colons of TNBS experimental groups, reverse transcribed into cDNA. Primers for the indicated markers were used to amplify cDNA using qPCR. Means[±]-SEM are plotted. One-way Anova statistical analysis was performed in all cases dKO TNBS groups are significantly higher than all other groups.

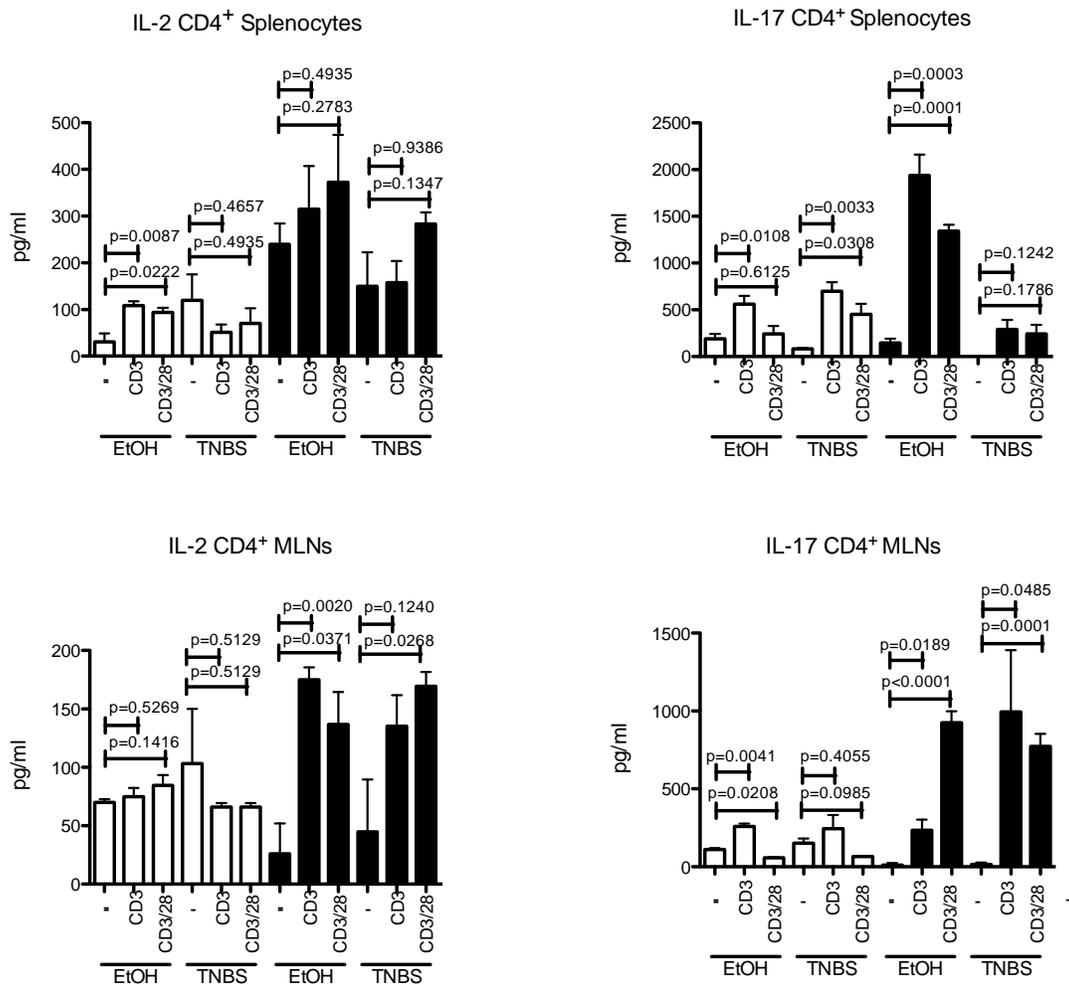


Figure 24

CD4⁺ T cells from spleen and mesenteric lymph nodes produce cytokines in response to TCR stimulation *ex vivo*. 4 X 10⁵ CD4⁺ T cells from spleen or MLNs from WT (white bars) and dKO (black bars) were incubated in cell culture conditions with no stimulation (-), anti-CD3 (CD3), or anti-CD3 and anti-CD28 (CD3⁺CD28) for 48 hours. Culture supernatants were assayed for the presences of IL-17 and IL-2 via ELISA, Means ⁺/₋SEM are plotted. Student's t Test statistical analysis was performed.

TULA deficiency leads to increased ability of T cells to cause colitis in an adoptive transfer model

The development of colitis in the TNBS model is multi-factorial and involves several types of immune cells. Therefore, it is difficult to precisely define the contribution of CD4⁺ cells to the exacerbation of inflammation clearly seen in KO mice in this model. To assess the effect of TULA-deficiency on T cell-driven inflammation *in vivo*, we used another model of colitis. In this model CD4⁺ CD45RB^{high} T cells from donor mice are transferred into immunocompromised recipient mice lacking T cells, but maintaining normal expression of TULA-family proteins. This system, which we refer to as Adoptive Transfer Colitis (ADTC), allows us to directly assess the effect of TULA-family proteins on colitogenic activity of T cells. In this model, we evaluate disease by monitoring weight of recipient mice, while inflammation is developing, and by scoring histological signs of colonic inflammation in mice euthanized upon termination of the experiment. Consistent with the highest degree of TCR hyper-responsiveness of dKO T cells, mice that received dKO T cells demonstrate a significant weight loss earlier than those receiving WT, sKO1 or sKO2 T cells. Weight loss seen in SCID recipients of sKO1 and sKO2 T cells is not significantly different from that seen in the SCID recipients of WT T cells (figure 25). Accordingly, histological analysis of colons shows that recipients of WT, sKO1 and sKO2 have a similar moderate degree of inflammation with few loci of infiltrating cells and the overall structure of colon maintained. However, SCID mice that received dKO T cells have far more infiltrating cells and altered structure of the colon (crypts and goblet cells are no longer present). However, ADTC does not involve the destruction of the luminal epithelial barrier (figure 26), unlike it happens in the TNBS model. Hence, while histological scoring of colons from SCID mice that received WT,

dKO, sKO1, and sKO2 T cells have been increased as compared to that in PBS control (SCID recipients received PBS without cells), dKO recipients show the strongest increase in inflammation (figure 27).

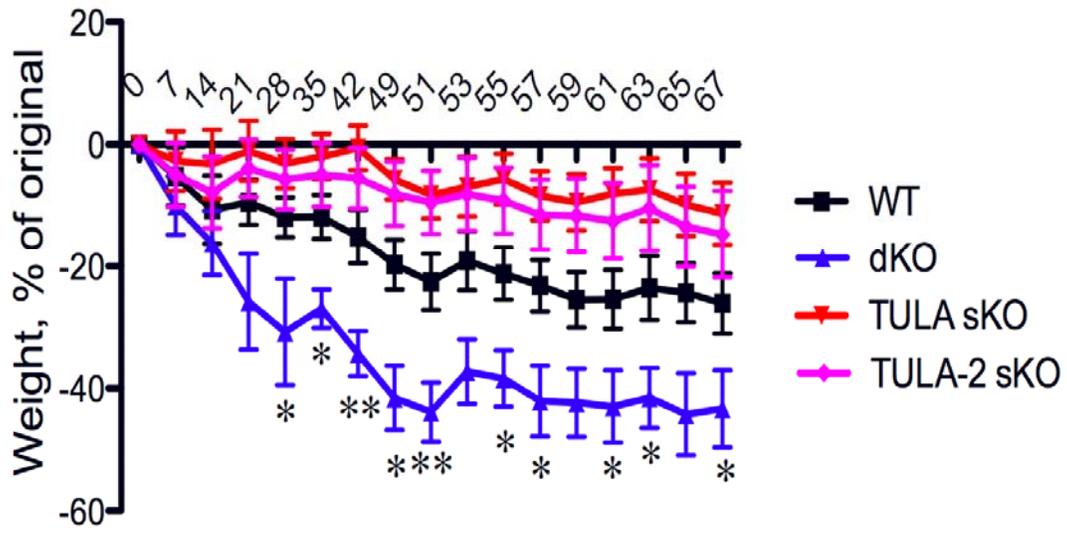


Figure 25

T cells from dKO mice have the highest propensity to cause weight lost. Weight lost was monitored in SCID mice receiving donor CD45RB^{High} CD4⁺ T cells, and the weight difference between each experimental group and PBS control was plotted as percent of the original weight for each group. PBS control mice gained ~20% weight in the course of this experiment (not shown). Means[±]SEM are plotted. Student's t Test statistical analysis was performed.

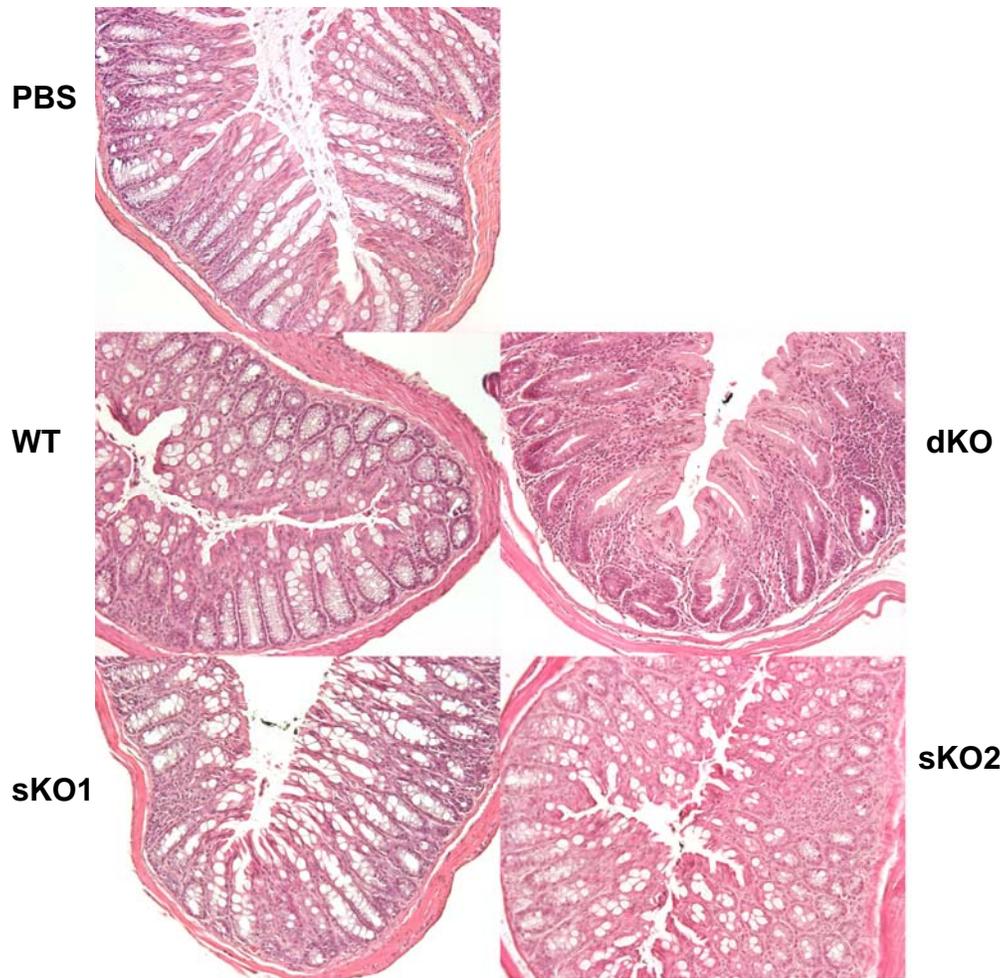


Figure 26

Hematoxylin and Eosin staining of colons from mice in the ADTC model. Colons from SCID mice of ADTC experimental groups, these animals were euthanized after 70 days, colons were removed and processed for paraffin embedding, sectioning, and stained with H & E. A representative image for each group is shown as indicated.

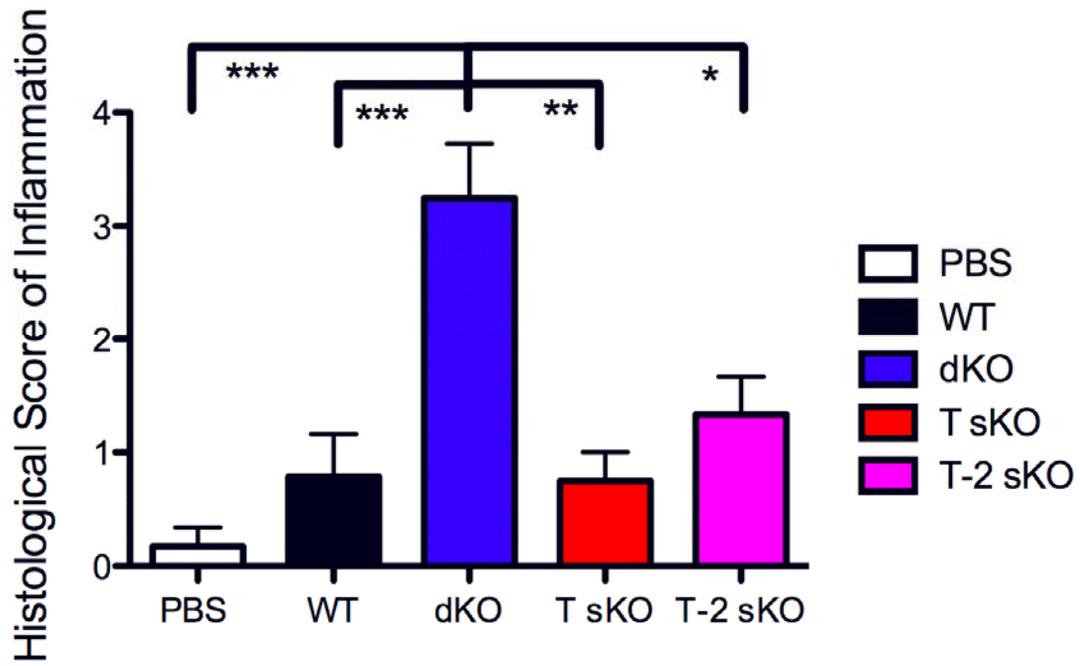


Figure 27

Histological scoring of inflammation in the colons of ADTC experimental groups. Histological scoring of sections shown in figure 2 was carried out as described in the method section and plotted as mean \pm SEM. One-way anova was performed.

CHAPTER 4

DISCUSSION

TULA-family proteins play an important role in regulating cellular processes that influence cell differentiation

Regulation of cellular differentiation in the immune system is a well-orchestrated constellation of signaling events where a single defect can affect the fate of a cell. Studies of key players in the regulation of these signaling events have led to better understanding of cell differentiation and autoimmune disorders. Protein tyrosine phosphatases (PTP) are known to be relevant in several regulatory processes, and thus deficiency in key PTPs can affect immune cell differentiation. Previous data implicated that only dKO mice had an observable phenotype and this phenotype was restricted to T lymphocytes. We set out to determine if there were individual phenotypes that could be observed in the mice deficient in only one TULA-family protein. Our data support the early observations that T cell are primarily affected by TULA-deficiency; we show that among immune cells in the spleen TULA-family protein deficiencies cause little changes in the ratio of CD3⁺ T cells, NK T cells, NK cells, monocytes and granulocytes (figure 1). Consistent with the idea that T cells are strongly affected by TULA-family deficiencies our data also indicate that these defects influence the CD4/CD8 cell ratio (figure 2). Mice deficient in both TULA-1 and TULA-2 have an increase in the percent of CD4⁺ T cells. Among other factors, TCR signaling and co-receptor signal strength in double positive thymocytes can modulate the expression of GATA (Hernandez-Hoyos et al., 2000). The upregulation of GATA expression is dependent on the TCR signal strength perpetuated

by Lck (Hernandez-Hoyos et al., 2003; Hernandez-Hoyos et al., 2000). It may be likely that strong signaling seen in T cells of dKO causes the differences seen in the percent of CD4⁺ T cells in spleen. Accordingly, weaker signals are thought to drive the differentiation of CD8⁺ thymocytes (Hernandez-Hoyos et al., 2000). It is interesting that mice expressing TULA-1 (with low phosphatase activity), but lacking TULA-2 (high phosphatase activity) have a decrease in CD8⁺ T cells and an increase in CD4⁺ T cells as compared to the mice lacking only TULA-1. It would be interesting to test the hypothesis that while TULA-2 is much more efficient at dephosphorylating most of the substrates than TULA-1, the latter regulates a substrate that perpetuates a signal that drives CD4⁺ selection.

How exactly TULA-family proteins work to regulate TCR signaling is not completely understood. It was uncovered previously that hyperactivity of T cells to TCR stimulation seen in mice deficient in both TULA-1 and TULA-2 is partially due to the phosphatase activity of TULA-2 (Carpino et al., 2004; Chen et al., 2010; Chen et al., 2009; Mikhailik et al., 2007). T cells from sKO2 mice have an increase in the phosphorylation of TCR signaling proteins in response to TCR stimulation. Interestingly, the hyper-phosphorylated state seen in sKO2 T cells is not as strong as that in stimulated dKO T cells (San Luis et al., 2011). This suggests that TULA-1, either directly or indirectly, is also responsible for dephosphorylation of TCR signaling proteins and that the lack of both TULA-1 and TULA-2 contribute to dampening TCR signaling by dephosphorylation of signaling proteins. TCR activation and co-stimulation of naïve CD4⁺ T cells leads to their activation and differentiation into different effector subsets. To define the individual contributions of TULA-1 and TULA-2 to the regulation of T-cell

activation we examined the *in vivo* activation status of CD4 cells in WT and various KO mice based on CD45RB expression, since CD45RB is a major marker that distinguishes naïve and antigen-experienced T cells (Morrissey et al., 1993; Powrie et al., 1993; ten Hove, 2004). Naïve CD4 T cells express CD45RB, a protein tyrosine phosphatase, at a high level, but upon their activation, CD45RB expression is down regulated. We observed that deficiency in both TULA-1 and TULA-2 results in bi-modal CD45RB distribution among CD4⁺ T cells; the number of cells expressing CD45RB at a low level is significantly increased. Likewise, this is true for mice lacking TULA-2 only. These findings argue that TULA-2 is important for suppressing T-cell activation that results in CD45RB down regulation *in vivo*. Previously it was shown that in neonatal mice and in mice housed in germ-free facilities CD4⁺ T cells uniformly express CD45RB at a high level, but the subpopulation of CD45RB^{low} expressing cells appears among CD4 T cells after mice were housed in non-germ-free (specific pathogen-free) facilities (Birkeland et al., 1992). It is likely therefore that an increase in the fraction of CD45RB^{low} CD4⁺ T cells may be caused by hyper-responsiveness of CD4 T cells to antigens in dKO and sKO2 mice due to the lack of negative regulation that TULA-2 provides.

After responding to antigen naïve CD4⁺ T cells down regulate CD45RB expression and differentiate into T helper cells. When T helper cells have carried out their effector function a small portion evade cell death and further differentiate into memory T cells or inducible regulatory T cells (iTreg). CD45RB is likely to contribute to immune cell responses and differentiation as do other CD45 isoforms. It is known that CD45 positively regulates immune responses by dephosphorylating the negative regulatory sites of Src-family kinases (Penninger et al., 2001), down regulating selectin

(CD62L) expression (Stibenz et al., 1996; Wroblewski and Hamann, 1997), regulating integrin function (Allan et al., 2008; Roach et al., 1997), and cytokine receptor signaling via Jak kinase (Irie-Sasaki et al., 2001). Given previous data showing bi-modal expression of CD45RB on CD4⁺ T cells and known functions of CD45, we examined whether the lack of TULA-family proteins affects the generation of effector memory T cells. There are two types of memory T cells: effector memory T cells (T_{EM}) which are thought to be active and located in the periphery, and central memory T cells (T_{CM}), which are resting (Sallusto et al., 1999). TEM cells are phenotypically characterized based on CD44 and CD62L expression. CD44 is upregulated in activated T cells during clonal expansion and its expression is maintained (reviewed in (Mitchell and Williams, 2010)). CD44 has been shown to promote trafficking and adhesion, activation of T cells, and delivery of survival and cell death signals. Recently, CD44 expression has been associated with FAS-mediated activation-induced cell death (reviewed in (Mitchell and Williams, 2010)). Baaten et al. observed that in Th1 cells, but not in Th2, Th17, or CD8⁺ T cells, expression of CD44 prevented FAS-mediated cell death. CD62L is a selectin that mediates T-cell migration to secondary lymphoid organs (Baaten et al., 2010a; Baaten et al., 2010b). High expression of CD44 and low expression of CD45RB and CD62L are commonly used to define TEM cells. We observed that mice deficient in both TULA-1 and TULA-2 not only have an expanded population of CD4⁺ T cells with low CD45RB expression, but also have an expanded population of CD44⁺CD62L⁻CD4⁺ cells. Deficiency in only TULA-2 resulted in the expansion of CD45RB^{low} cells, but not of TEM cells. This finding indicates that the regulatory roles of TULA-1 and TULA-2 are not identical; TULA-2 may regulate TCR signaling that affects CD45RB expression,

whereas TULA-1 and TULA-2, while working in concert, regulate T-cell differentiation that follows antigen exposure. Interestingly, there is evidence that supports the idea that Th1 cells evading activation-induced cell death go on to differentiate into TEM cells (Baaten et al., 2010b). Previous data from our lab show that TULA-1 interacts with Apoptosis Inducing Factor to induce apoptosis in Jurkats cells (Collingwood et al., 2007b). Data presented here imply that T cells from sKO1 and dKO mice are resistant to cell death under certain cell culture conditions. Together these data support the idea that the expansion of TEM among dKO mice may in part be due to the lack of TULA-1.

As seen with the expanded TEM populations, dKO mice also show an increase in the percent of cells that express phenotypic markers characteristic of regulatory T cells (Tregs). [There are several types of Tregs however there are only two types that are functionally well established, they are inducible Tregs (iTregs) and natural T regs (nTregs). The increase seen in the fraction of Treg cells in dKO mice is likely due to generation of inducible Treg (iTreg). Like memory cells, iTregs evolve from antigen-exposed CD4⁺ T cells and show low CD45RB expression. It has long been established that CD4⁺CD25⁺ (Treg) are responsible for suppressing CD4⁺CD25⁻ cells and that selective depletion of CD4⁺CD25⁺ cells results in severe autoimmunity (Sakaguchi et al., 1995). However, the key marker of Treg cells is the transcription factor FoxP3, which is essential for the suppressor function and differentiation of Treg (Fontenot et al., 2003; Hori et al., 2003). Expression of the high-affinity IL-2 receptor CD25 on Tregs is thought to facilitate suppression of CD4⁺CD25⁻ T cells. For instance, expression of IL-2 on the surface of Tregs appears to facilitate IL-2 consumption from the microenvironment, thus sequestering IL-2 from CD4⁺CD25⁻ T cells (Pandiyani et al., 2007). Treg cells are likely

to employ several means of suppression, but regardless of the exact mechanism, reduced expression of CD25 on CD4⁺FoxP3⁺ cells may render these Tregs non-functional. Our data show that there is an increase in the percent of cells expressing FoxP3 in mice lacking both TULA-1 and TULA-2. However, FoxP3⁺ cells in dKO mice have lower expression of CD25 than do FoxP3⁺ cells in WT or sKO mice. The notion that Treg cells in dKO mice are functionally deficient is consistent with the previous research showing that dKO mice are more sensitive to experimental autoimmune encephalomyelitis than are WT mice and on our data presented here demonstrating that dKO renders mice more susceptible to TNBS-induced colitis colitis. Taken together, our findings support the view that TULA-family proteins play an important role in regulating processes that influence cell differentiation.

TULA-family proteins respond differently to TCR stimulation

Regulation of different signaling pathways can be redundant or very specific. Although it is not completely clear how TULA-family proteins regulate multiple signaling cascades induced by TCR engagement, we can outline several possibilities. First, TULA-family proteins have an SH3 domain that may mediate scaffolding of TCR signaling complexes. Furthermore, TULA-family proteins have an UBA domain, which may be involved in targeting activated TCR complexes to ubiquitin-mediated degradation. Lastly, TULA-family proteins have a phosphatase domain. It has been shown that TULA-2 mediated dampening of TCR signaling via its phosphatase activity. However, TULA-1 has a phosphatase activity that is quite low as compared to that of TULA-2. Hence, it is unclear whether TULA-1 suppresses T-cell activation acting as a

phosphatase or acting in a phosphatase-independent manner (Carpino et al., 2009a; Carpino et al., 2002; Carpino et al., 2004; Chen et al., 2010; Chen et al., 2009; Mikhailik et al., 2007; San Luis et al., 2011). We found that CD4⁺ T cells from dKO and sKO1 mice survive suboptimal stimulation with anti-CD3 antibodies that is expected to cause cell death or anergy (Chai and Lechler, 1997) (Figure 8), suggesting that TULA-1 is involved in regulation of TCR-induced cell death. This notion is consistent with our previous finding that TULA-1 interacts with the AIF protein and plays a role in inducing apoptosis in Jurkat cells. This effect of TULA-1 is known to depend on the presence of AIF, but the exact mechanism underlying it remains unknown.

In order to further elucidate individual roles of TULA-1 and TULA-2 and outline mechanisms through which they play their roles, we studied how the lack of TULA-family proteins modified responses of T cells to TCR stimulation *in vitro*. Our findings indicated that CD4⁺ T cells from different TULA-family KO mice respond to TCR stimulation differently (figure 7). The response of CD4⁺ T cells from sKO1 mice to CD3, but not to CD3/CD28 stimulation, is significantly higher than that of WT CD4⁺ T cells, whereas sKO2 CD4⁺ T cells demonstrate an increase in responses to anti-CD3/CD28, but not to anti-CD3 alone. These data suggest that TULA-family proteins may have different means of regulating TCR signaling and/or responses to TCR signaling. In CD4⁺ T cells lacking TULA-1 co-stimulation via CD28 is not necessary for the production of a cellular response. These data suggest that TULA-1 may regulate signaling events that are initiated by CD3 stimulation and not by CD28 co-stimulation. These data also suggest that TULA-2 may be involved in regulating signaling events induced both by CD3 and CD28 stimulation. Interestingly, sKO1 seems to have a more

prominent effect on the production of cytokines and chemokines in response to TCR stimulation than does sKO2. In nearly all cases, CD3-stimulated sKO1 CD4⁺ T cells produced more cytokines and chemokines tested than did sKO2 CD4⁺ T cells in response to CD3/CD28 stimulation (figure 7). The reason for this discrepancy remains unclear. The observation that TULA-1 deficiency not only increases response to CD3 stimulation, but also reduces responses to CD3/CD28 stimulation, suggests that TULA-1 may promote signaling events or responses initiated by CD28 engagement. There are known proteins having phosphatase domain lacking activity that are necessary for proper TCR signaling, like CD45, which has two phosphatase domains, one inactive and one active, that are both necessary in regulating T-cell responses positively and negatively (Owada et al., 2010). It is possible that TULA-1 exerts both positive and negative effects on T-cell signaling, a situation reported for other regulatory proteins, such as Cbl (Tsygankov, 2008). However, even if this is the case, TULA-1's negative regulatory role dominates over its positive effects. Furthermore, whatever means of regulation TULA-1 is responsible for, it is clear that in dKO mice its effect is more profound as compared to that of TULA-2, because dKO CD4⁺ T cells and sKO1 have similar cytokine production profiles in response to CD3 and CD28 stimulation (figure 7) except for IL-17 production.

Previous data suggested that only dKO T cells have differential responses to TCR stimulation (Carpino et al., 2009a; Carpino et al., 2002; Carpino et al., 2004; Mikhailik et al., 2007). However, our data show that there are differences in sKO's T cells responses to TCR stimulation. The initial early responses to TCR stimulation that TULA-1 and TULA-2 are possibly regulating are independent of each other. Our data show that at an early time point post TCR stimulation suggest that these proteins independently affect

TCR regulation, and the pathways in which they affect are also different. Further studies should focus on the signaling events that are specific for CD3 or CD28 to elucidate how TULA-family proteins function. Consistent with our data are the results indicating that the response of sKO2 CD4⁺ cells to anti-CD3 is not as intense as what is seen in dKO CD4⁺ T cells, suggesting again that TULA-1 contributes to the regulation of TCR stimulation in dKO. TULA-1 may affect late TCR signaling events, because among all the signaling events analyzed, ERK1/2 is the only protein that is hyper-phosphorylated in sKO1 CD4⁺ T cells (San Luis et al., 2011). Furthermore, data presented here (figures 9-23) and in other studies indicated that deficiency in either TULA-1 and TULA-2 will result in the development of an autoimmune immunopathology (Carpino et al., 2004; Concannon et al., 2008; Fei et al., 2009; Jin et al., 2010; Zhernakova et al., 2011).

TULA-family proteins regulate processes that predispose a host to developing an autoimmune disorder

Previous work by Carpino et al. established that deficiency in both TULA-1 and TULA-2 increases susceptibility of mice to experimental autoimmune encephalomyelitis (EAE). However, it was not shown that susceptibility to EAE could be induced in mice lacking only one TULA-family protein. Furthermore, mice used in these experiments were of mixed genetic background. Both problems were addressed in our studies. Consistent with the results obtained with EAE, we have shown that mice deficient in both TULA-family proteins are more susceptible to the development of TNBS-induced colitis than WT animals, which are refractory to this treatment, as seen by weight loss, survival and multiple signs of intestinal inflammation. However, we have shown for the first time that deficiency in either TULA-family member also exacerbates inflammation. Mice

lacking only TULA-1 or TULA-2 developed TNBS-induced colitis, and the severity of inflammation in these animals is comparable to that in mice lacking both TULA-1 and TULA-2, thus suggesting that the individual roles of these proteins in regulating immune responses are significant. The effects of TULA-family KOs on colonic inflammation in mice are likely mediated by T cells. First, mouse TULA-1 is expressed only in T cells and, therefore, it is likely that T cells represent the only cell type affected by sKO1. Hence, an increase in TNBS-induced inflammation in TULA-1 sKO is likely to be perpetuated by hyperactivated T cells. In contrast, TULA-2 is ubiquitously expressed, and thus various cell types may play a role in facilitating TNBS-induced colitis in sKO2. On the one hand, the inflamed colons of TNBS-treated sKO2 mice show infiltration with CD4⁺ cells, which is similar to that seen in dKO and sKO1 mice. On the other hand, depletion of CD4⁺ T cells moderately, but significantly reduced TNBS-induced inflammation in dKO and sKO1 mice, but not in sKO2 mice. Interestingly, the effect of GK1.5-mediated CD4⁺ T-cell depletion in sKO1 mice was more profound than that seen in dKO mice; this result is consistent with the notion that the lack of TULA-2 exerts an effect on inflammation through non-T cells. Thus, the effect of sKO2 on TNBS-inflammation may involve both T cell-dependent and -independent phenomena. Together, our results suggest that TULA-family proteins are involved in the regulation of processes that drive T cell-mediated inflammation.

In an attempt to clarify the individual roles of TULA-1 and TULA-2 in suppression of inflammation and to further elucidate the underlying mechanisms, we examined cytokine production *in vivo* in control and TNBS-treated WT and knockout mice. First, we determined the levels of various cytokines and chemokines in serum of

experimental animals. An increase in serum cytokines/chemokines seen in knockout mice was generally consistent with the effect of these knockouts on inflammation. IL-6, MIP-1 α , MIG, Ip-10, IL-4, VEGF, KC and IL-13 were at detectable levels in the serum of TNBS experimental groups. IL-6 and MIP-1 α were present in the serum of WT and TULA-family knockout mice, and TNBS increased serum levels of these proteins. Serum levels of MIP-1 α are comparable in all groups. Serum levels of IL-6 are increased in response to TNBS in all groups, however the amount present in the serum of different groups is significantly different. Serum levels of IL-6 in WT animals treated with TNBS are 20- and 10-fold higher than that in dKO and sKO2 animals treated with TNBS, respectively. Likewise, serum levels of IL-6 in sKO1 animals treated with TNBS are ~100 and ~50-fold higher than that in dKO and sKO2 animals treated with TNBS, respectively (figure 22). TNBS treatment caused a marked increase in the VEGF serum level of sKO1 mice as compared to the ethanol control group. This increase is only seen in mice lacking TULA-1, this may be a link as to how TULA-1 independent of TULA-2 regulated inflammation. Increased VEGF in the serum has long been associated with the IBD (reviewed in (Danese, 2011)) VEGF serves to mediate leukocyte adhesion and migration through microcirculation of the inflamed colon. (Danese, 2011; Rutella et al., 2011). IP-10 is another chemokine associated with the development IBD in humans (Ugucioni et al., 1999). In experimental models IP-10 facilitates inflammation in the IL-10 $^{-/-}$ model of spontaneous colitis and anti-IP-10 antibodies block DSS-induced colitis (Sasaki et al., 2002; Singh et al., 2003). Furthermore, a human antibody that blocks IP-10 is currently in phase 2 clinical trials (reviewed in (Nishimura et al., 2009)). Given the importance of IP-10 in the development of human disease it is likely that it is also

important to the development of colitis in TULA-family knockout mice and should be investigated further. Mice deficient in both TULA-1 and TULA-2 have increases in IL-13 in the serum of TNBS treated mice as compared to their controls this is also true for sKO1 mice, albeit a lower level of IL-13 is produced. IL-13 can have pro- and anti-inflammatory properties promoting Th2 mediated inflammation and suppressing Th1/Th17 mediated inflammation. It known that in patients with Crohns Disease IL-13 can suppress Th1/Th17 mixed populations (Wilson et al., 2011). However, IL-13 is associated with driving Th2 mediated ulcerative colitis (Fuss et al., 2004). In experimental animal models, IL-13 facilitates the Th2-mediated model of colitis induced by oxazolone and the chronic model of TNBS induced colitis (Fichtner-Feigl et al., 2008; Heller et al., 2002). Interesting is that sKO2 mice have an increase in the IL-13 in the serum as compared to WT groups, however there is no effect of TNBS (figure 22). TULA-1 and dKO mice have an increase in the level of IL-13 in response to TNBS. These levels are substantially higher than that seen in sKO2 and WT. In general, serum levels of cytokines can be indicative of a specific T helper cell type, our data suggest that the inflammation is Th2 seen in TNBS treated animals maybe Th2 mediated because none of the classically Th1 cytokines were present at a detectible levels in the serum. It is likely that Th1 cytokines are produced are an earlier time point and it no longer presenting the serum because other data presented here suggest a strong contribution of Th1 and Th17 cells (figures 23 and 24). It is also possible that inflammation induced by TNBS is mediated by Th1, Th2 and Th17 helper T cells. In fact, new research suggests that plasticity of T helper cell types may mediate existence Th1, Th2, or Th17 at different sections of the intestine perpetuating inflammation (Murphy and Stockinger, 2010). Also,

noteworthy is the presence of IL-6 in the serum of mice lacking only TULA-1 treated with TNBS and a complete absence of it in the serum of dKO and sKO2 mice treated with TNBS. Both WT and sKO1 mice have an increase in serum levels of IL-6 as a response to TNBS. As expected, the increase in IL-6 in the serum is much higher in sKO1. However, IL-6 is nearly completely absent in the serum of dKO and sKO2 mice treated with TNBS or ethanol. IL-6 is well established to be the one of the two cytokines responsible for Th17 differentiation and thus is closely associated with several autoimmune disorders as it drives the IL-17-mediated inflammation. It is odd that IL-6 serum level in sKO1 is not comparable to those in dKO and sKO2 mice where inflammation is comparable. IL-6 trans signaling with its secreted IL-6 receptor may provide us with an idea as to why IL-6 levels are so reduced in dKO and sKO2 mice. IL-6 can bind to a secreted IL-6 receptor, this receptor-ligand complex can then bind to cells that do not express the IL-6R and initiate signaling. Blocking of IL-6-sIL-6 complex with neutralizing antibody to IL-6R ameliorates experimental models of colitis (Atreya et al., 2000). It may be possible that IL-6 is sequestered in T cells of dKO and sKO2 mice, and this results in a decrease in the level of IL-6 in serum. Trans signaling through the IL-6/sIL-6R complex may perpetuate inflammation seen in sKO2 and dKO mice via Th17-mediated mechanism, since other data strongly suggest that inflammation seen in dKO is mediated in part by Th17 cells (figures 23 and 24).

However, the observed increase in serum cytokines/chemokines did not correlate well with the intensity of inflammation assessed based on histological parameters; the increase in serum cytokines was the highest in sKO1, lower in dKO mice, and the lowest in sKO2 mice, while “histological” inflammation was similar in all KO mice.

Considering that an increase in the level of pro-inflammatory cytokines in serum does not necessarily reflect their levels and effects at the site of inflammation, we assessed the relative expression of IL-4, IL-2, IL-10, TNF α , INF γ , IL-17, ROR γ t and IL-23 in the colon of TNBS experimental and ethanol control groups using qRT-PCR. Consistent with an increase in presence of pro-inflammatory cytokines in the serum, dKO mice that were treated with TNBS expressed all the mRNA of pro-inflammatory cytokines with in the colon. However, colons from TNBS-treated sKO mice did not show an increase in the expression of inflammatory cytokines (figure 23). One possibility to explain this finding is that the inflammation seen in sKO1 and sKO2 mice is not mediated by any of the cytokines analyzed, which is unlikely. It is more likely that at the time of termination mRNAs encoding for these cytokines are no longer present in the colon. The TNBS model of inflammation is acute, and if TNBS-treated animals survive, they typically recover around day 5. Therefore, a reason for this discrepancy may be that the inflammation seen in the colon of dKO mice treated with TNBS is persistent, while that in sKO mice is transient, and these data are consistent with all observations of our work presented here.

There is overwhelming pool of evidence that supports the involvement of Th17 cells in the development of IBD, whether Ulcerative Colitis or Crohns Disease. Given the data presented thus far regarding IL-17 production from naïve T cells of dKO, sKO1 and sKO2 CD4⁺ T cells (figure 7), IL-6 levels in the serum of TNBS experimental groups (figure 22), and the expression of mRNAs for IL-17, IL-23R, and ROR γ t in dKO mice in response to TNBS (figure 23), we decided to determine the production of IL-17 and IL-2 from the splenocytes and MLNs of TNBS experimental mice. T cells migrate from the

spleen to other secondary lymph organs upon immune challenge. Thus, TNBS exposure may promote migration from the spleen to the mesenteric lymph nodes. When we assess production of IL-17 and IL-2 from CD4⁺ T cells from spleens and MLNs of TNBS experimental groups. Interestingly, only IL-2 production from splenic CD4⁺ T cells of dKO mice treated with TNBS are at levels that were much higher than those for WT mice (figure 24). In fact, when splenic cells from dKO TNBS were stimulated *ex vivo* they produce less IL-17 than T cells from their respective ethanol group, and T cells from both WT control and WT TNBS groups. It seems that IL-17 producing CD4⁺ T cells were less present in the spleen and present more so in the MLNs as seen by IL-17 levels with the culture supernatants of MLN-derived T cells, suggesting that dKO T cells migrate more easily from the spleen to the colon draining lymph nodes in response to TNBS, compared to WT T cells. This is consistent with previous data showing that dKO mice treated with TNBS express mRNA of pro-inflammatory cytokines TNF α , IL-17 and IFN γ . Also, the expression of ROR γ t and IL-23R was increased in colons of TNBS-treated dKO mice suggesting the presence of mature Th17 cells. It is likely that the absence of similar effects in the colons and CD4⁺ T cells of sKO1 and sKO2 mice is due to the nature of TNBS-induced colitis. The assessment of cytokine production is done 3 days post-induction, while on day 5 within this model surviving animals begin to recover. The inflammation seen in dKO mice is seemingly more persistent than that of single knockouts. It is clear from our data that deficiency in either TULA-1 or TULA-2 will result in susceptibility to the development of an immune pathology and that these processes are reliant on T cells and production of pro-inflammatory cytokines. The TNBS model of colitis is known to be driven in part by T cells (Elson, 1996; Elson et al.,

2005). Here we show that TNBS-induced inflammation seen in mice deficient in either TULA-family member involves T cells as they are present in the colon of these animals. T cell-associated cytokines and chemokines are present in the serum, expressed in the colon, and produced by T cells of TULA-family knockout mice.

As stated previously, while T cells are quite possibly the major cell type perpetuating disease in this model, this model is multifactorial, and inflammation seen in this system is likely to be perpetuated by non-T-immune cells. Thus, this model does not allow us to directly assess the role of T cells in inflammation exacerbated by TULA-family deficiency. We have to consider the possibility that TULA-1 is present in a non-T cell type yet to be identified and the fact that TULA-2 expression is not limited to T cells. In fact, the highest level of TULA-2 was found in platelets. To further elucidate the individual roles of TULA-family proteins in the development of inflammation we employed the adoptive transfer model, which is based on the finding that the CD4⁺ CD45RB^{High} population of T cells lacking Treg can induce intestinal inflammation in an immunocompromised recipient. Using this model in SCID recipient mice, we determined that dKO CD4⁺ CD45RB^{High} T cells induced an onset of wasting earlier and to a larger extent than did WT and sKO CD4⁺ CD45RB^{High} T cells. Inflammation was most severe in recipients of dKO cells than in other recipient groups. In contrast, sKO CD4⁺ CD45RB^{High} T cells were no more colitogenic than were WT cells. Overall, the data obtained in the ADTC model argue that TULA-1 and TULA-2 work in concert to regulate T-cell responses. In the case where the entire immune system is intact, and non-T-immune cell are contributing the immune process, deficiency in a single TULA-family protein is sufficient to drive the development of an immune pathology (i.e. the TNBS

model). However, the ADTC model is different in that the only factor contributing to the development of disease is the presence of T cells responding to normal flora (mice are housed in specific pathogen-free facilities). SCID mice do not have T or B cells, however cells of the innate immune system are present. It is likely that the contribution of non-T-immune cells are necessary for sKO's to develop an immune pathology, because only deficiency in both of TULA-1 and TULA-2 proteins can result in the development of an immune pathology that is unlike, and more severe than the WT mice. This suggests that while these proteins can independently cause disease, they may also be working in concert to render T cells more colitogenic. Consistent with this notion is the fact that in every circumstance presented in this work, deficiency in of both TULA-1 and TULA-2 results in more dramatic phenotypic difference as compared to sKO's and WT. The mechanisms mediating the effects of TULA-1 and TULA-2 on T-cell responses and T cell-driven inflammation remain unclear. However one possible mechanism to explain hyperactivity seen in dKO mice lies with the dimerization sequence of these proteins. It is possible that heterodimerization of TULA-1 and TULA-2 mediates recruitment to TCR that perpetuates regulation by both TULA-1 and TULA-2.

Finally, the notion of the role played by TULA-family proteins in the regulation of T cell-driven inflammatory responses was supported by recent findings that polymorphisms in the genes encoding for these proteins are associated with the development of several autoimmune diseases. TULA-1 is associated with Type 1 diabetes (Concannon et al., 2008), celiac disease (Zhernakova et al., 2011), rheumatoid arthritis (Zhernakova et al., 2011), and generalized vitiligo (Jin et al., 2010). TULA-2 is

associated with Beçhets Disease (Fei et al., 2009). The existence of links between individual TULA-family genes and human autoimmune/inflammatory conditions supports the view that deficiency in TULA-1 and TULA-2 can individually drive immune pathology, which is likely mediated by abnormal T-cell responses. In general, in animals with an intact immune system deficiency in TULA-1 and TULA-2 can individually drive an immune pathology (TNBS experimental models and GWAs studies in humans). This support our data with the TNBS model, if entire immune system is intact deficiency of a single TULA-family protein is sufficient to drive an immune pathology.

Overall, TULA-family proteins appear to represent a novel class of cellular regulators. Like other cellular regulators and protein tyrosine phosphatase, deficiency of these proteins may result in autoimmune diseases. This work not only sheds light on the contribution of TULA-family proteins to the regulation of T cell-driven inflammatory response, it provides a systems whereby one can study the individual contribution of each TULA-family protein independently (TNBS), and how these proteins work together (ADTC) to drive T cell-driven immune responses. Research using these models may provide useful in studying these proteins in general, and how these proteins may function in diseases that they are currently associated with.

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