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Roles of Tnfaip8 Protein in Cell Death, Listeriosis, and Colitis

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Abstract
TNF-alpha-induced protein 8 (TNFAIP8 or TIPE) is a newly described regulator of cancer and infection. However, its precise roles and mechanisms of actions are not well understood. Here I report the generation of TNFAIP8 knockout mice and describe their increased responsiveness to colonic inflammation and resistance to lethal Listeria monocytogenes infection. TNFAIP8 knockout mice were generated by germ line gene targeting and were born without noticeable developmental abnormalities. Their major organs including those of the immune and digestive systems were macroscopically and microscopically normal. However, compared to wild type mice, TNFAIP8 knockout mice exhibited significant differences in the development of listeriosis and experimentally induced colitis. I discovered that TNFAIP8 regulates L. monocytogenes infection by controlling pathogen invasion and host cell apoptosis potentially in a RAC1 GTPase-dependent manner. TNFAIP8 knockout mice had reduced bacterial load in the liver and spleen and TNFAIP8 knockdown in murine liver HEPA1-6 cells increased apoptosis, reduced bacterial invasion into cells, and resulted in dysregulated RAC1 activation. The combined effect of reduced bacterial invasion and increased sensitivity to TNF-alpha-induced clearance likely protected the TNFAIP8 knockout mice from lethal listeriosis. On the other hand, I also discovered that the increased apoptosis sensitized TNFAIP8 knockout mice to dextran sodium sulfate-induced colitis as demonstrated by decreased survival rates and increased body weight loss of TNFAIP8 knockout mice, and enhanced leukocyte infiltration, bacterial invasion, and inflammatory cytokine production in the TNFAIP8 knockout colon. Bone marrow chimeric experiments revealed that TNFAIP8 deficiency in non-hematopoietic cells was responsible for the exacerbated colitis and resistance to listeriosis in TNFAIP8 knockout mice. Taken together, these findings indicate that TNFAIP8 plays an important role in maintaining colon homeostasis, protecting against colitis, and regulating the pathogenesis of L. monocytogenes infection by controlling bacterial invasion and/or cell death of the non-immune cells.

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Thomas P. Porturas

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ABSTRACT

ROLES OF TNFAIP8 PROTEIN IN CELL DEATH, LISTERIOSIS, AND COLITIS

Thomas P. Porturas

Youhai H. Chen

TNFα-induced protein 8 (TNFAIP8 or TIPE) is a newly described regulator of cancer and infection. However, its precise roles and mechanisms of actions are not well understood. Here I report the generation of TNFAIP8 knockout mice and describe their increased responsiveness to colonic inflammation and resistance to lethal *Listeria monocytogenes* infection. TNFAIP8 knockout mice were generated by germ line gene targeting and were born without noticeable developmental abnormalities. Their major organs including those of the immune and digestive systems were macroscopically and microscopically normal. However, compared to wild type mice, TNFAIP8 knockout mice exhibited significant differences in the development of listeriosis and experimentally induced colitis. I discovered that TNFAIP8 regulates *L. monocytogenes* infection by controlling pathogen invasion and host cell apoptosis potentially in a RAC1 GTPase-dependent manner. TNFAIP8 knockout mice had reduced bacterial load in the liver and spleen and TNFAIP8 knockdown in murine liver HEPA1-6 cells increased apoptosis, reduced bacterial invasion into cells, and resulted in dysregulated RAC1 activation. The combined effect of reduced bacterial invasion and increased sensitivity to TNFα-induced clearance likely protected the TNFAIP8 knockout mice from lethal listeriosis. On the other hand, I also discovered that the increased apoptosis sensitized TNFAIP8 knockout mice to dextran sodium sulfate-induced colitis as demonstrated by decreased survival rates and increased body weight loss of TNFAIP8 knockout mice, and enhanced leukocyte infiltration, bacterial invasion, and inflammatory cytokine

production in the TNFAIP8 knockout colon. Bone marrow chimeric experiments revealed that TNFAIP8 deficiency in non-hematopoietic cells was responsible for the exacerbated colitis and resistance to listeriosis in TNFAIP8 knockout mice. Taken together, these findings indicate that TNFAIP8 plays an important role in maintaining colon homeostasis, protecting against colitis, and regulating the pathogenesis of *L. monocytogenes* infection by controlling bacterial invasion and/or cell death of the non-immune cells.

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CHAPTER 1 – Introduction

1.1 Inflammation

Inflammation is a broad term used to describe the body's biochemical and cellular responses to injury. Regulation of this response is critically important in maintaining a healthy homeostasis while simultaneously sustaining the capacity to protect the organism from foreign agents and injury. There are many potential inducers of inflammation, ranging from microbial pathogens to the diet of the organism. Deregulation of the inflammatory response can lead to a wide variety of diseases that include autoimmune diseases, susceptibility to certain pathogens, and even an increased risk of cancer.

The normal response to tissue injury involves the release of pro-inflammatory molecules such as TNFα, IL-1, and IL-6 that activate local leukocytes such as resident mast cells and macrophages that further continue a signaling cascade by releasing histamines that promote blood flow, and chemokines such as IL-8 that attract additional leukocytes to the region (Medzhitov 2008). The leukocytes, primarily neutrophils and antigen presenting cells, attempt to identify pathogenic agents and clear the site of foreign particles and cell debris to clean the wound. If a pathogen is detected, an additional cascade of pro-inflammatory cytokines is released to promote the recruitment of additional phagocytes and the activation of the adaptive immune response through the antigen presenting cells. After the clearance of any pathogens and foreign or cellular debris, anti-inflammatory molecules are released such as IL-4, IL-10, and IL-13 and growth factors such as TGFβ, PDGF, and VEGF from the leukocytes to encourage tissue regeneration and immune suppression (Laskin 2009). After healing is completed, the tissue is restored to homeostasis, and blood flow and leukocyte infiltration is restored to normal.

If the inflammatory response is not resolved, chronic inflammation may persist and cause additional injury. As the immune system attempts to fight off a pathogen, inflammatory molecules

such as cytokines, reactive oxygen species, and apoptotic inducing molecules such as TNFα are released that cause collateral damage within the tissue. This damage may feed back into the inflammatory response creating a persistent cycle of inflammation. This chronic inflammation can be very toxic to the infected organs such as with Hepatitis B and C, which may result in cirrhosis, liver failure and cancer (Caccamo et al. 2014). An exaggerated response may be just as dangerous and may range from mild allergic reactions against external antigens to the often fatal septic shock from overwhelming stimulation of the immune system (Pravda 2014). Besides a persistent chronic or overwhelming infection and response, the immune system may mistakenly target a self-antigen, resulting in an autoimmunity disorder that cannot resolve itself. Autoimmune diseases such as rheumatoid arthritis and inflammatory bowel diseases may be very debilitating and even result in death if untreated as with type 1 diabetes, systemic lupus erythematosus, and multiple sclerosis (Cooper & Stroehla 2003). In addition, the tissue damage resulting from the inflammation can result in genetic instability and promote tumorigenesis.

While too much inflammation may be problematic, a weak response may predispose an organism to infection. The inflammatory environment is very toxic and hostile to pathogens, and therefore pathogenic microorganisms tend to devote significant resources in evading the host immune system. A dulled immune response will, however, allow pathogens free reign over the host tissue that can lead to necrotic cell death and tissue destruction. Even a slightly impaired response may be enough to allow a pathogen to escape the site of injury and gain wider access to the other organs that can lead to sepsis and fatal systemic infection. Immune deficiencies may result from the lack of cellular components such as B cells, T cells, or neutrophils, and a lack of molecular components such as complement proteins or inflammatory cytokines. Each of these deficiencies is associated with a predisposition to certain pathogens that are controlled by that particular aspect of the immune system. A B cell deficiency, for instance, will result in a lack of humoral immunity and predispose the host to certain extracellular pathogens such as *Streptococcus pneumonia*, while a T-cell deficiency will result in susceptibility to intracellular pathogens such as *Listeria monocytogenes* (Bannister et al. 2009).There are many causes of

immunodeficiency such as genetic mutations, immune suppressive drugs, chemotherapy, and HIV infection. The regulation of inflammation is therefore important to making sure the response is sufficient to clear an infection but to keep it under control so as not to cause chronic inflammation or fatal septic shock.

1.2 Inflammation and cancer

Cancer is highly intertwined with inflammation. Tumorigenesis involves tissue injury and necrotic cell death along with the exposure of antigens typically not expressed by normal cells. These events signal the immune system to promote inflammation. The inflammatory response can have two very contrasting effects on the tumor, destroy it and lead to remission or promote vascularization, metastasis, and its growth. Therefore, understanding inflammation is crucial to gaining insight on cancer development and treatment.

Chronic inflammation results in the release of reactive oxygen species and the cyclical destruction and proliferation of tissue that promotes genetic damage, which is the first step to tumor development. Following the escape from cell cycle control, new cancer cells are able to take advantage of released growth factors and inflammatory cytokines such as TNFα to activate molecular survival and proliferation pathways (de Visser et al. 2006). The continued inflammation may accelerate genomic instability through the production of reactive oxygen species, promote angiogenesis to provide blood flow to the developing tumor, and even interfere with immune surveillance that may help fight the cancer. This inflammatory tumor microenvironment exists around most tumors, and while the inflammation is often harmful and ineffective at protecting the host, it is still important in finally clearing the cancer.

The immune system is constantly surveying tissues for not just harmful pathogens and debris, but also for potentially malignant cells. Important cells include natural killer (NK) cells that detect and eliminate distressed cells based on altered surface receptor expression, dendritic cells and macrophages that respond to danger signals and detect and present antigens that are not

normally produced to the adaptive immune system, and cytotoxic T lymphocytes (CTLs) that when activated can target these specific antigens on the cancer cells (Grivennikov et al. 2010). This antitumor response can be inhibited and suppressed by regulatory T cells inhibiting CTLs, and macrophages and neutrophils limiting access to antitumor lymphocytes. A balance may exist between pro and anti-tumor effects from the immune system that if pushed one way can lead to an aggressive cancer and tumor elimination in the other. Macrophages, for example, can be activated into two distinct functional phenotypes, the pro-inflammatory M1 macrophages that contribute to tumor cell killing and autoimmune diseases, and anti-inflammatory M2 macrophages that can suppress the immune response and promote angiogenesis, proliferation and extracellular remodeling that can contribute to tumor growth and development (Hao et al. 2012). Therefore, one of the biggest challenges in cancer therapy is to find ways to modulate the immune system using drugs, adoptive cell therapies, and antibody treatments to destroy cancer cells (Hegmans & Aerts 2014). To develop these novel therapies, a more thorough understanding of both cancer and inflammation is necessary and precisely how these two processes are linked.

1.3 TNFAIP8 is a novel oncogene

Tumor necrosis factor α-induced protein 8 (TNFAIP8 or TIPE), also known as SCC-S2, OXI-α, GG2-1, NDED, and MDC-3.13, is the first described member of the TNFAIP8 family. It is an NF-κB inducible protein upregulated in metastatic head and neck squamous cell carcinoma cell lines and it has been found to protect cancer cells against TNFα-induced apoptosis (Patel et al. 1997; Kumar et al. 2000). By contrast, TNFAIP8 may promote glucocorticoid-induced apoptosis of normal thymocytes in culture (Woodward et al. 2010). Overexpression in tumor cell lines also enhanced proliferation and migration (Kumar et al. 2004). Activated Gαi3 and Karyopherin α2 in PC-3 cells have been identified as potential TNFAIP8 interacting partners to regulating cell death and transformation (Laliberté et al. 2010). TNFAIP8 may not only be involved in disease pathogenesis, but also serve as a novel biomarker for certain inflammatory

diseases and cancers. *TNFAIP8* gene polymorphisms and expression are associated with non-Hodgkin's lymphoma, ovarian, cervical and prostate cancers, psoriasis, and with susceptibility to *Staphylococcus aureus* infection in mice (Ahn et al. 2010; Liu et al. 2013; Romanuik et al. 2009; Shi et al. 2013; Filkor et al. 2013; Zhang, Wang, et al. 2012). However, the precise roles of TNFAIP8 in health and disease remain to be established.

1.4 TNFAIP8 family

TNFAIP8 is the first member discovered of the novel TNFAIP8 family of proteins. TNFAIP8-Like 1 (TIPE1), TNFAIP8-Like 2 (TIPE2), and TNFAIP8-Like 3 (TIPE3) are the other known members and they all share very high amino acid sequence homology to TNFAIP8 (Figure 1). This family of proteins have a very unique structure besides a death effector domain (DED) like sequence, but the crystal structure of TIPE2 revealed this to be a unique fold among DED containing proteins encompassing the entire protein sequence (Lou & Liu 2011; Zhang et al. 2009). TNFAIP8 and TIPE1 are both widely expressed throughout a variety of tissues except for TIPE1 being undetectable in mature lymphocytes (Kumar et al. 2000; Cui et al. 2011). TIPE3 is found predominantly in non-lymphoid tissues while TIPE2 expression is localized to immune cells (Fayngerts et al. 2014; Sun et al. 2008). TIPE1 down-regulation is correlated with worse prognosis for cancer patients and it has been shown to promote cell death by inhibiting the RAC1 pathway (Z. Zhang et al. 2014). In addition, TIPE1 has found to positively regulate autophagy in a neural cell line model of Parkinson's disease (Ha et al. 2014). TIPE2, like TIPE1, has also been shown to have antitumor functionality, and it has been identified as a key negative regulator of inflammation (Y. H. Zhang et al. 2014; Cao et al. 2013; Sun et al. 2008). TIPE3 has only been recently characterized and has been shown to promote tumorigenesis through the regulation of phosphoinositide signals (Fayngerts et al. 2014). All of the members of TNFAIP8 family are involved in tumorigenesis and cell death, and investigating this family may prove crucial to our understanding and treatment of cancer.

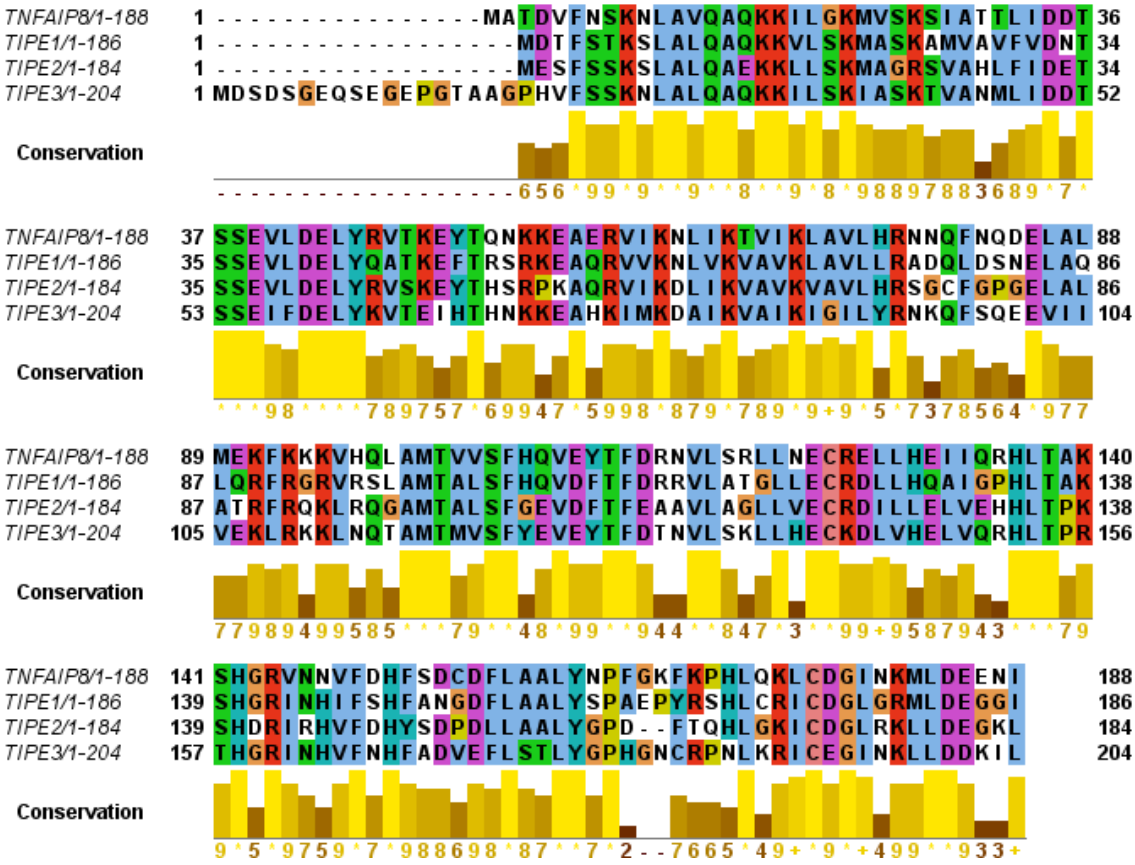

Figure 1. TNFAIP8 family sequence alignment

The TNFAIP8 family shows high homology in its amino acid sequence with a BLOSUM62 score of 15372 and 54% identity. Alignment was calculated by Clustal Omega and PRALINE multiple sequence alignment tools. The image was generated using Jalview.

1.5 TIPE2 in inflammation

TIPE2 is the second most well known member of the TNFAIP8 family. It was discovered in a mouse model for multiple sclerosis by comparing the gene expression profiles of spinal cord tissue from healthy mice with that of mice with induced experimental autoimmune encephalomyelitis (EAE). The inflamed EAE spinal cord tissue expressed large amounts of TIPE2, drawing interest in the gene for further study that eventually resulted in the development of a TIPE2 knockout mouse via germ line gene targeting (Sun et al. 2008). TIPE2 deficient mice develop normally but are prone to many debilitating inflammatory diseases that are characterized by heightened inflammatory cytokine production, sensitivity to septic shock, and premature death. These mice had abnormally high levels of circulating cytokines, and had a larger number of both lymphocytes and monocytes, resulting in significant splenomegaly. In inflammatory diseases, TIPE2 is down-regulated in peripheral blood mononuclear cells in patients suffering from lupus and hepatitis, and it is upregulated in kidneys of diabetic rats (Li et al. 2009; Xi et al. 2011; Zhang et al. 2010). Additionally, recently published work has indicated that TIPE2 may provide a protective function against ischemia/reperfusion injury following a stroke (Zhang, Wei, et al. 2012).

TIPE2 is normally expressed at high levels in all immune cells and shows inducible expression in fibroblast cell lines (Sun et al. 2008). The loss of TIPE2 correlates with increased levels of IκB degradation and increased nuclear localization of NF-κB family members. In TIPE2 overexpression, levels of NF-κB activity in response to TNFα treatment were reduced**.** TIPE2 knockdown also resulted in increased phospho-JNK, phospho-p38, and c-Fos signaling. TIPE2 can associate endogenously with caspase 8 in macrophage cell lines and caspase 8 has been previously shown to activate NF-kB through a BCL10 / MALT1 complex (Su et al. 2005). Knockdown of TIPE2 in EL4 T Cells resulted in resistance to FasL induced cell death, while TIPE2 knockout T Cells are resistant to activation induced cell death. TIPE2 knockout T cells develop normally but produce more tetramer positive CD8+ T cells in response to LCMV infection

and produce more cytokines following CD3/CD28 stimulation. TIPE2 knockout macrophages also produce more cytokines when exposed to TLR ligands. TIPE2 has recently been shown to bind to and inhibit the small GTPase Rac1 resulting in inhibition of phagocytosis in macrophages (Wang et al. 2012). This modulation of the immune system is important during bacterial infections since TIPE2 knockout mice have been shown to be resistant against *Listeria monocytogenes* and *Staphylococcus aureus* infection.

The regulatory role on inflammation by TIPE2 has also been found to be linked to cancer. TIPE2 is an inhibitor of the oncogenic GTPase Ras signaling pathway and has been shown to associate with RGL, an activator of RalA and RalB (Gus-Brautbar et al. 2012). TIPE2 regulates cell migration, exocyst assembly, and Actin dynamics in vitro, and its overexpression inhibited Ras-induced transformation in NIH3T3 cells. Due to the crucial function of TIPE2 in the immune system, inflammation and cancer, and its high homology with TNFAIP8, many functional roles and molecular pathways of TIPE2 may be shared with TNFAIP8. The recently published roles of TIPE2 may reveal the functional roles of TNFAIP8 and shed light on how TNFAIP8 is important to both inflammation and cancer.

1.6 Goals of this research project

The current published works on TNFAIP8 have labeled it as an oncogene, and few have attempted to identify its role outside of the cancer field. Due to the importance of inflammation for tumorigenesis and our lab's published results suggesting that TIPE2 is a critical regulator of inflammation, we began to investigate the roles of TNFAIP8 in inflammation. It is important to note that while TIPE2 is expression is localized to immune cells, TNFAIP8 is widely expressed throughout the body, suggesting a potentially larger impact of gene dysfunction affecting both immune and non-immune cells. Using TNFAIP8 knockout mice, we planned to identify the normal physiological roles of TNFAIP8 in two different models of inflammation, bacterial infection and experimental colitis. After identifying a phenotype, we further investigated the cellular and

molecular role of TNFAIP8 to develop a model that may explain the importance of TNFAIP8 to a wider variety of diseases.

Our overarching hypothesis is that TNFAIP8 regulates inflammation, and this regulation is critical to modulating a variety of diseases. I developed three specific aims to test this hypothesis. The first is to test whether TNFAIP8 affects the outcome of *Listeria monocytogenes* infection in TNFAIP8 knockout mice. The second aim is to determine whether induced experimental colitis is affected by TNFAIP8 deficiency in mice. The third aim is to identify the cellular and molecular role of TNFAIP8 deficiency in knockdown cell models.

This study is one of few that intends to investigate the importance of TNFAIP8 outside of the field of cancer. The information learned may be crucial to understanding a wide variety of inflammatory diseases in addition to gaining a better understanding of how TNFAIP8 is involved in cancer. This understanding may eventually lead to novel medical treatment methods and diagnostic procedures.

CHAPTER 2 – Materials and Methods

2.1 Animals

Wild type (WT) C57BL/6 (B6) and CD45.1^{+} B6 mice were purchased from The Jackson Laboratory. All mice used in this study were housed under pathogen-free conditions in the University of Pennsylvania Animal Care Facilities. All animal protocols used were pre-approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

2.2 Generation and genotyping of Tnfaip8-*/*- mice

The B6 ES cell line with a disrupted *Tipe* gene (NM_134131) was obtained from the Texas A&M Institute for Genomic Medicine (College Station, Texas). The gene-trapping vector that disrupts *Tipe* gene was inserted into the only intron of the gene (Figure 2A). The ES cells were injected into mouse blastocysts to generate chimeras. Six chimeras, one female and five males, were produced. The germline transmission was obtained from one of the male chimeras. A common WT primer 1 (5'-GTTTCATATCCATCTCTTATA-3') was paired with (i) a reverse primer 3(5'-CCAATAAACCCTCTTGCAGTTGC-3') against the sequence on gene trap vector to produce a 178 bp PCR fragment from *Tipe*-deficient allele, and (ii) WT reverse primer 2 (5'-TGGCGTGACTTGAGTGCTTC-3') to generate a 334 bp PCR fragment from the wild type allele. Heterozygous mice for the disrupted *Tipe* locus were bred to produce homozygous *Tipe*$^{-/-}$ animals. Once identified, they were further tested by PCR to ensure that that did not express TIPE mRNA using primers 4 (5'-ACCTGGCCGTTCAGGCACAA-3') and primer 5 (5'-TCACCCTGTACAGCTCATCT-3') (Figure 2B).

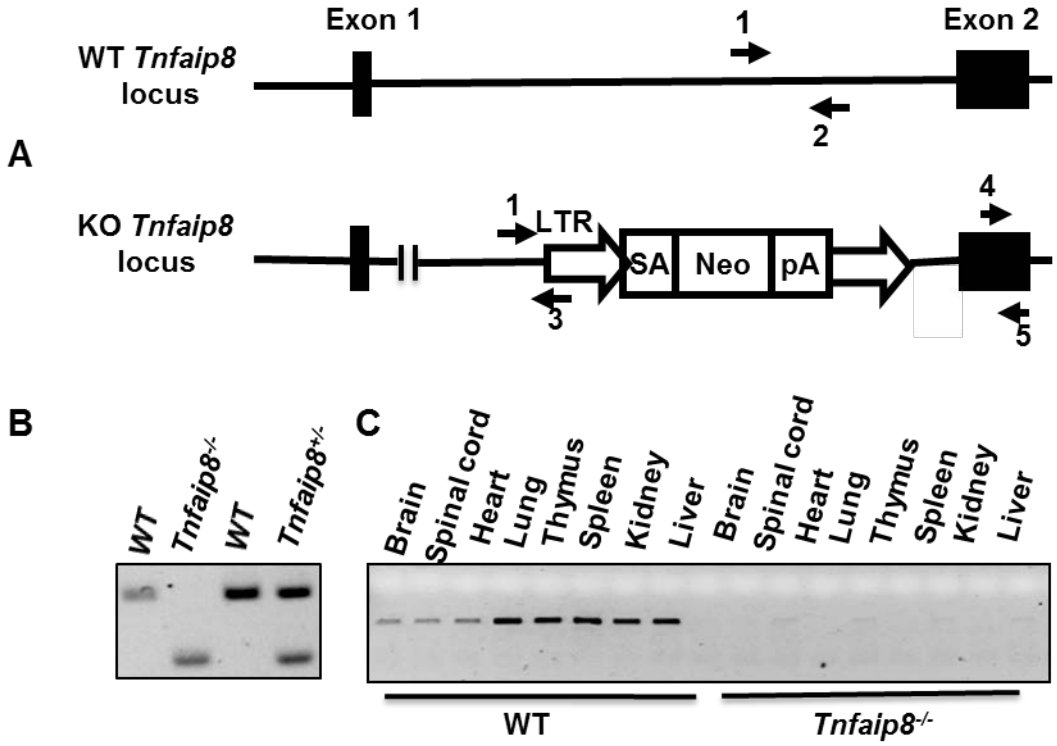

Figure 2. Generation of Tnfaip8-knockout mice by gene targeting

(A) The wild-type *Tipe* (*Tnfaip8*) gene locus and the knockout allele. LTR: viral long terminal repeat; SA: splice acceptor; Neo: Neomycin-resistant gene; pA: poly adenylation sequence. Arrows indicate the locations of PCR primers. (B) Genomic DNA was extracted from mice tails. PCR was performed using primers shown in Panel A (primers 1, 2, and 3). WT band: 334 bp; Tipe knock out band: 178 bp. (C) Total RNA was isolated from the indicated organs of WT and *Tipe*$^{-/-}$ mice; RT-PCR was performed using the *Tipe* primers shown in Panel A (primers 4 and 5).

2.3 Phenotyping of *Tnfaip8*$^{-/-}$ mice

Six-to-eight-week age-matched WT and knockout mice were sacrificed and their immune organs were collected and weighted. Single cell suspensions were prepared from them. Total cell numbers from each organ were determined using the COULTER Counter (Beckman). Flow cytometry was performed after staining cells with anti-mouse CD4, CD8, B220, Gr-1, CD11B, CD11C, NK1.1, CD25, CD44, Foxp3, CD62L and CD69. All the antibodies used were purchased from BD Bioscience.

2.4 Bone marrow chimeras

Bone marrow cells were flushed from the femurs and tibias of donor mice. The red blood cells were lysed with ACK solution (8.29 g NH_4Cl, 1 g $KHCO_3$, and 37.2 mg Na_2EDTA in 1L of water). Cells were washed twice and re-suspended in cold PBS. Recipient mice were sub-lethally irradiated with 500 rads twice separated by 4 hours. The irradiated mice received a total of $10x10^6$ donor bone marrow cells by tail vein injection one or two hours after irradiation. Mice were used seven to eight weeks later for experiments.

2.5 RT-PCR

Total RNA was isolated from brain, spinal cord, lung, liver, heart, spleen, intestine, and mesenteric lymph nodes of wild type and *Tnfaip8*$^{-/-}$ mice with Trizon reagent (Sigma) according to the manufacturer's instruction. The isolated RNA was further purified with RNeasy Mini kits (Qiagen) according to the manufacturer's instruction. 250ng of RNA samples were reversely transcribed with oligo(dT) and SuperScript II transcriptase (Invitrogen). The generated cDNA was

diluted with sterile Milli-Q water (1:4). Real-time quantitative PCR analysis was performed using specific Quantitect Primers for mouse GAPDH, TNFAIP8, TIPE1, TIPE2, TIPE3, IL-6, IL-1β, IL-17, and CXCL2 (Qiagen) in an Applied Biosystems 7500 system using Power SYBR Green PCR Master Mix (Applied Biosystems). Relative levels of gene expression were determined with GAPDH as the control.

2.6 Cell lines and plasmids

The HEK293T, NIH 3T3, and Hepa1-6 cells were cultured in D10 (DMEM with 1% penstrep, 1% L-glutamine, and 10% FCS or 10% CS for 3T3 cells). Full-length TNFAIP8 cDNA was generated by PCR and cloned in frame with an N-terminal Flag into vector pRK5. Human wild-type RAC1, RAC1 T17N, RAC1 Q61L cDNAs were obtained from Addgene and subcloned into pRK5 with Myc or HA tag at the N terminus. Truncated forms of Rac1 lacking the N-terminal amino acids 1–47 and C-terminal amino acids 162–192 or 189–192 were generated by PCR and cloned in-frame with an N-terminal HA tag into vector pRK5. cDNAs encoding TNFAIP8, wild-type RAC1, RAC1 T17N and RAC1 Q61L were subcloned into the pEGFP-N3 plasmid.

2.7 Plasmid DNA transfection and viral infection

293T cells were transfected with plasmid DNA using Fugene 6 (Promega) reagent according to the manufacturer's instructions. For virus production, pLKO.1 (with puromycin resistance) with shRNA-Tnfaip8 (purchased from Open Biosystems) or shScr (a non-specific scramble shRNA purchased from Addgene) fragments and packaging constructs were co-transfected into 293T cells. After 24 and 48 hrs, virus-containing medium was filtered and used to infect Hepa1-6 and 3T3 cell lines in the presence of 6.5 mg/ml of polybrene (Millipore). Infected cells were selected using puromycin (Sigma) to establish shRNA-Scr, shRNA-Tnfaip8-1 (SH1), and shRNA-Tnfaip8-2

(SH2) cell lines. Viral vectors were produced from the pLKO.1 plasmid containing SH1 (AAAGGGATTGTACAAGGCAGC), SH2 (TTGAACTGATTGTTCCTGTGG), or shRNA-scr (CGAGGGCGACTTAACCTTAGG).

2.8 Microbial strains and infection

Wild-type L. *monocytogenes* (10403s) were provided by H. Shen and Y. Paterson (University of Pennsylvania). L. *monocytogenes* and Staphylococcus aureus (ATCC 29213) were grown at 37 °C in brain-heart-infusion medium (Becton Dickinson), and Columbia medium with 2% NaCl, respectively. For all assays, mid-log-phase bacteria were used. Bacterial in vitro infection was assayed in 6-well plates. A total of 7.5×10^5 Hepa1-6 cells were seeded in each well, followed by culturing overnight in DMEM with 10% FBS. Cells were the serum starved for 18-24 hours. Cells were infected with L. *monocytogenes* at multiplicity of infection (MOI) of 50 in antibiotic-free media. Sixty minutes after the inoculation, cells were washed three times with PBS, and fresh medium containing gentamycin (150 μg/mL) was added. At 1.5 hours after infection, cells were lysed with 0.1% Triton in PBS and serial dilutions of the homogenate were plated on brain-heart infusion agar plates (BHI) (Becton Dickinson). The colonies were counted 24 hours later. For in vivo bacterial infection, L. *monocytogenes* was grown in brain-heart-infusion medium until the absorbance at 600 nm reached 0.1 of optical density. S. aureus was grown in Columbia media with 2% NaCl. Six- to 7-wk old WT and TNFAIP8 KO mice were infected intravenously with 2×10^5 L. *monocytogenes* in 200 μL PBS or 2×10^7 S. aureus in 200 μL saline. For measurement of the bacterial burden in liver and spleen, mice were sacrificed 72 h after inoculation, organs were homogenized in 0.1%Triton in PBS, and serial dilutions of the homogenate were plated on BHI agar plates. The colonies were counted 24 h later. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were determined using the Infinity ALT or AST liquid stable reagent (Thermo).

2.9 DSS-induced colitis

Experimental colitis was induced with 3% or 4% (w/v) DSS (MW 36-40kDa; MP Biologicals) dissolved in sterile, distilled water ad libitum for 5 days followed by normal drinking water until the end of experiment. For survival studies, eight-to-ten weeks old mice were treated with 4% DSS for five days and replaced with normal drinking water until day 14. For histology, gene expression and cytokine production studies, mice were treated with 3% DSS for 5 days followed by regular drinking water for two days and euthanized at different time points after the DSS treatment.

2.10 Determination of clinical scores

Body weight, stool consistency, and rectal bleeding were monitored daily [11]. In brief, stool scores were determined as follows: 0, well-formed pellets; 1, soft and semiformed but didn't adhere to the anus; 2, semiformed but adhere to the anus; 3, liquid stool. Bleeding scores were determined as follows: 0, no blood; 1, blood traces in rectum; 2, visible blood in whole rectum; 3, gross rectal bleeding. Weight loss scores were monitored as follows: 0, no body weight change; 1, body weight loss within 5%; 2, body weight loss within 10%; 3, weight loss within 20%; 4. Body weight loss exceeds 20%. DAI (disease activity index) was the total sum of these 3 scores.

2.11 Histopathology and immunohistochemistry

The entire colon was excised to measure the length. The distal colons were washed, fixed in 10% buffered formalin and embedded in paraffin. Tissue sections were stained with hematoxylin & eosin (H&E). For Ki-67 and active caspase 3 staining, mice were treated with DSS and colon were collected, processed, and stained with Ki-67 and active caspase 3 antibody.

2.12 Isolation of colon epithelial cells

Colonic epithelial cells were isolated as described[16]. In brief, colons were dissected, washed with cold PBS, and cut into small pieces. Colon segments were incubated in PBS supplemented with 1mM EDTA and 1mM DTT for 30min at 37^{o}C with gentle shaking. Cells in supernatants were filtered through a 70 μm cell strainer and washed twice. Enrichment for colonic epithelial cells was confirmed by staining cells for the epithelial cell-specific marker EpCam (eBioscience). 70-80% of isolated cells stained positive for EpCam. If needed, the isolated colonic epithelial cells were cultured in epithelial cell culture medium (ECM) containing equal volumes of phenol-red-free DMEM and Ham's F-12 medium (Biowhittaker) with the following additives: 5 μg of insulin (Sigma)/ml, 5x10^{8} M dexamethasone (Sigma), 60 nM selenium (Sigma), 5x10^{8} M triiodothyrenine (Sigma), 5 μg of transferrin (Sigma)/ml, 10 ng of Eidermal growth factor (Sigma)/ml, 20 mM HEPES, 2 mM glutamine, 10% Penicillin/streptomycin, and 2% FBS.

2.13 Colon leukocytes isolation

Colon leukocytes were isolated using a modified version of a previously described protocol[17]. Briefly, colon was washed 3 times and incubated in PBS with 5% FBS, 2mM EDTA, 1mM DTT for 15 min at 37^{o}C with shaking (250 RPM). This was repeated 3 times to remove epithelial cells. The colon samples without epithelial cells were digested in PBS with 5% FBS, 0.5 mg/ml collagenase (Sigma), 0.02 mg/ml Dnase (Roche), and 0.1 mg/ml Dispase (Invitrogen), for 20 minutes at 37℃ with gentle shaking for another 45 min with shaking at 37^{o}C. The mixture then was filtered through 70 μm cell strainer. The flow through contains colon leukocytes.

2.14 Blood cell counts

Before euthanizing the mice, blood was collected, and whole blood cell counts were determined using Drew Hemavet 950FS (Drew Scientific, Oxford, U.K.).

2.15 Bacterial culture

Colon samples were collected and processed as described above, and then homogenized in CelLytic^{TM} M buffer (Sigma) and serially diluted. Different dilutions of the tissue homogenates were plated in triplicates on blood agar (BD Bioscience) and BHI agar. The bacterial colonies formed were counted after incubating at 37^{o}C for 24 hrs.

2.16 Macrophage and neutrophil preparations

To generate bone marrow-derived macrophages (BMDMs), bone marrow cells were flushed from the femurs and tibias of donor mice. The red blood cells were lysed with ACK solution (8.29g NH_4Cl, 1g $KHCO_3$, 37.2mg Na_2EDTA in 1L of water). Cells were washed twice in ice-cold 1xDPBS and cultured for 7 days in 30% L-929 cell culture supernatant and 70% DMEM containing 10% (vol/vol) heat-inactivated FBS, 2 mM L-glutamine, and 100 units/mL penicillin/streptomycin (D10). Cells were washed twice with cold DPBS and collected with 5 mM EDTA in DPBS. After centrifugation, they were resuspended in D10 and rested for 24 h before experimentation. BMDMs were >95% CD11b+ and F4/80+ as determined by flow cytometry. Morphologically mature neutrophils were purified from murine bone marrow by Percoll gradient centrifugation. Briefly, bone marrow cells were harvested from mice using neutrophil isolation buffer (1× HBSS without Ca2+ and Mg2+ containing 0.25% BSA). After RBC lysis, cells were layered on a 62% Percoll gradient. Following centrifugation at 1,200 × g for 30 min at room temperature, pelleted cells were removed and washed once with isolation buffer before being used in the experiment. Neutrophil viability was >95% according to results from trypan blue

staining. Purity was typically 75–85% as assessed by flow cytometry based on forward and side scatter and Gr1 staining.

2.17 Phagocytosis assay.

To prepare apoptotic cells for phagocytosis assay, thymocytes were harvested from 3- to 4-wk-old C57BL/6 mice, loaded with 10 uM carboxyfluorescein succinimidyl ester (CFSE) (Molecular Probes) for 10 minutes at 37°C and washed 2X in PBS. Apoptosis was induced by incubation at 37°C in 5% CO_2 for 5 h in the presence of 5 μM dexamethasone (Sigma-Aldrich). After dexamethasone treatment, cells were washed three times with PBS and resuspended in DMEM with 2% FBS. This treatment routinely yielded over 70% apoptotic thymocytes as measured by annexin V staining. Phagocytosis assay was performed in a 12-well nontissue culture-treated plate (BD Falcon). A total of 1×10^6 BMDMs were seeded in each well, followed by culturing overnight in DMEM with 2% FBS. CFSE+ apoptotic thymocytes were then added at a ratio of 5:1, and centrifugation was performed at 500 × g for 2 min to synchronize binding and internalization. After 30 min of incubation at 37 °C, plates were rapidly washed two times with ice-cold PBS, and cells collected with 5 mM EDTA-PBS. Cells were then fixed with 2% paraformalhyde in PBS, stained with APC-conjugated anti-F4/80, and analyzed by flow cytometry. Gates were set for macrophages in FSC/SSC dot blots. Experiments using fluorescent latex beads (2 μm; Sigma-Aldrich) were performed in a similar fashion. For bacterial phagocytosis assay, L. *monocytogenes* were washed twice with sterile PBS and incubated (at 1×10^9/mL bacteria) in 2 μM CFSE for 20–30 min under constant shaking at 37 °C. CFSE-labeled bacteria were washed three times with PBS before being used. Live bacteria were fed to BMDMs at a ratio of 10:1 in DMEM with 10% serum.

2.18 Chemotaxis assay

Primary neutrophils were loaded with 5 μM Calcein AM in PBS supplemented with 2% FBS for 30 minutes at 37°C. After 3 washes with PBS, neutrophils were resuspended to with 2×10^6 cells per ml of PBS. ChemoTX plates (Neuro Probe) were loaded with 50 μl of cell over each 5.7mm diameter chamber with 5 μm pores. The lower chambers were loaded with 30 μl of PBS containing the specified concentration of IL-8. The Neutrophils were incubated for one hour, and then the top chambers were washed off and the plates were read in a fluorescent plate reader. Chemotactic index was calculated as the number of neutrophils in the lower chambers of chemokine containing wells over the wells with no chemokines.

2.19 ELISA

The colon homogenates and serum were collected and stored at -80^{o}C. Sera were collected from WT and TNFAIP8 KO mice 72 hours after *L. monocytogenes* infection and kept at −80°C. Antibodies used in ELISA were purchased from BD Pharmingen and eBioscience, including purified and biotinylated rat anti-mouse IL-6, IL-17A, IL-1β, and TNFα. Quantitative ELISA was performed using paired mAbs specific for corresponding cytokines according to the manufacturer's recommendations.

2.20 Cell death and proliferation

Colon samples were processed and epithelial cells were obtained from control and colitis WT and *Tipe*$^{-/-}$ mice. The isolated epithelial cells were collected and cell death was determined by 7AAD and Annexin V staining. Alternatively, Tipe knockdown 3T3 cells were treated with 3% DSS for 16 hrs and the cells were harvested for 7AAD and Annexin V staining. The in situ colonocyte

proliferation was assessed by Ki67 staining and cell death was determined by active caspase 3 staining after the colon tissues were collected from DSS-treated mice and fixed in 10% buffered formalin and embedded in paraffin.

2.21 TNFα stimulation

Hepa1-6 cell lines were serum starved for 16 hours in DMEM containing 2mM L-glutamine, and 100 units/mL penicillin/streptomycin. Cells were treated with 10 ng/ml of TNFα for 0, 1, 5, 15, 30, 60 minutes for protein lysates, or with 5 ng/ml TNFα plus 20 ng/ml cyclohexamide (CHX) for 7 or 16 hours. Death was measured by flow cytometry using Annexin V PE and 7-AAD (BD Pharmingen) staining. For measuring death following RAC1 mutant transfections, EGFP positive cells were gated on for comparison. To inhibit RAC1, cells were incubated with inhibitor Z62954982 (Millipore) at 100μM for 30 min prior to experimental treatment.

2.22 Western blot

For Western blot analysis, cells were lysed for 20 min at 4°C in 1% Triton X-100 lysis buffer containing protease and phosphatase inhibitors. Cell debris was removed by centrifugation at 14,000 × g for 20 min at 4°C. For subcellular fractionation, membrane and cytoplasmic proteins were separated using Qproteome Cell Compartment Kit (QIAGEN) according to the manufacturer's instructions. The protein concentration of the lysates was determined by Bradford assay. Equal amounts of total protein were resolved by SDS-PAGE, transferred to membranes, immunoblotted with specific primary and secondary antibodies, and the signals were detected by chemiluminescence (Pierce). Primary antibodies against p-AKT(S473), AKT, Myc, as well as anti-Myc conjugated to HRP, were purchased from Cell Signaling Technology and used according to

the manufacturer's instructions. Anti-TNFAIP8 (1:500) was purchased from ProteinTech Group and Anti-RAC1 (1:500) was purchased from Millipore. Anti-Flag (1:2000), anti-Flag-M2-HRP, anti-β-actin (1:3000) and anti-GAPDH antibody (1:3000) were purchased from Sigma. HRP-conjugated secondary anti-mouse and anti-rabbit IgG (1:1500) were purchased from GE Healthcare. The densitometric quantification of Western blot signals was performed using ImageJ software. Paired *t*-test was used to evaluate the statistical significance of the results.

2.23 Immunoprecipitation

Immunoprecipitation was performed using Dynabeads protein G (Invitrogen). In brief, 1.5 mg protein-G Dynabeads was coated with 5 μg specific antibodies or Ig control for 1 h at room temperature with rotation. After removing unbound antibody, the bead–antibody complex was incubated with 500 μL cell lysate for 4 h at 4 °C with rotation. The captured Dynabead-Ab-Ag complex was washed four times with PBS and boiled in 2× Laemmli buffer. The eluted proteins were fractionated by SDS-PAGE and detected by Western blot.

2.24 Loading of RAC1 with GDP and GTP

The 293T cells were transiently transfected with Myc-tagged RAC1 and Flag-tagged TNFAIP8 for 18 h. Cells were lysed in cell lysis buffer (50 mM Tris, pH 7.5, 10 mM EDTA, 0.2 M NaCl, 0.5% Nonidet P-40, and 1× protease inhibitors mixture) (Roche). A total of 1 mM GDPβS or 0.2 mM GTPγS (Enzo Life Sciences) was loaded to transfected cell extracts. After 20 min incubation at 30 °C, samples were placed on ice immediately and MgCl2 was added to a final concentration of 10 mM to stop nucleotide exchange.

2.25 PAK Pull-Down Assay

Hepa1-6 cells were serum starved for 16 hours, and then treated with 10 ng/ml TNFα for 5 minutes. The cells were washed in PBS and lysed in PBD lysis buffer (50mMTris, pH 7.5, 10 mM MgCl2, 0.2 M NaCl, 2% Nonidet P-40, and 1× protease inhibitors mixture) (Roche). The lysate was incubated with 20 μg of p21-activated kinase (PAK)-GST protein beads (Cytoskeleton) for 30 min at 4 °C. After washing, protein on beads and in total cell lysates was subjected to Western blot to determine the level of active Rac.

2.26 Statistical analysis

Quantitative data are presented as means±SEM of two or three experiments. Survival curves were plotted via the Kaplan-Meier method and compared by the log-rank test. Two-tailed Student *t* test was used for all other results and $p<0.05$ was considered statistically significant. All statistical analysis was performed using Graphpad Prism Software.

Chapter 3 – Listeriosis

3.1 Introduction

TNFα-induced protein 8 (TNFAIP8 or TIPE), also known as SCC-S2, OXI-α, GG2-1, NDED, and MDC-3.13, is the first described member of the TNFAIP8 family. It is an NF-κB inducible protein upregulated in metastatic head and neck squamous cell carcinoma cell lines, and to protect cancer cells from TNFα-induced apoptosis (Patel et al. 1997; Kumar et al. 2000). Overexpression in tumor cell lines also enhanced proliferation and migration (Kumar et al. 2004). TNFAIP8 polymorphisms have been found to be a risk factor for non-Hodgkin's lymphoma in humans and Staphylococcus aureus infection in mice (Ahn et al. 2010; Zhang, Wang, et al. 2012). A few TNFAIP8 interacting partners have been identified, including activated Gαi3 and Karyopherin α2 (Laliberté et al. 2010). TIPE2, a closely related TNFAIP8 family protein, has been reported to interact with RAC1 and to protect against *Listeria monocytogenes* infection (Wang et al. 2012).

Listeria monocytogenes is a predominantly food-borne pathogen with ~10 cases per million inhabitants in most industrialized countries. Despite having a lower incidence of infection than most other food-borne pathogens, listeriosis carries a high risk of mortality ranging between 20-30%, and accounts for 19% of food-borne disease-related deaths in the USA (Mateus et al. 2013; Hernandez-Milian & Payeras-Cifre 2014). *L. monocytogenes* is an intracellular gram-positive bacterium that infects a number of cell types including hepatocytes, neurons, and immune cells. Immune cell-mediated apoptosis of *L. monocytogenes*-infected cells such as hepatocytes is important for resolving infection (Vázquez-Boland et al. 2001). The innate immune system is vital for controlling the infection until the adaptive immune system generates enough cytotoxic T lymphocytes (CTLs) to finally clear the pathogen. Key immune cells are macrophages and neutrophils that release inflammatory molecules and phagocytose extracellular bacteria (Shaughnessy & Swanson 2007; Wing & Gregory 2002; Cousens & Wing 2000). TNFα and IL6

are key inflammatory cytokines that are important in controlling the infection, and the loss of either results in susceptibility to infection (Hoge et al. 2013; Abreu et al. 2013). In this chapter, we sought to characterize *L. monocytogenes* infection in TNFAIP8 knockout mice and we show that TNFAIP8 sensitizes mice to lethal *L. monocytogenes* infection through a mechanism involving the non-immune system. These results may provide new insights into TNFAIP8's regulation of listeriosis and carcinogenesis.

3.2 Results

3.2.1 TNFAIP8 knockout mice are resistant to lethal *Listeria monocytogenes* infection

It has been previously reported that TIPE2-deficient mice are resistant to *L. monocytogenes* infection (Wang et al. 2012). We hypothesized that TNFAIP8-knockout mice might have a similar phenotype. To test this, TNFAIP8 knockout (KO) and wild type C57BL/6 mice were intravenously injected with a lethal dose of *L. monocytogenes*. As expected, TNFAIP8 knockout mice were found to be resistant to death (Figure 3A), and experienced reduced weight-loss during infection (Figure 3B). Next we examined the bacterial load in the liver and spleen 3 days post infection, and found reduced bacterial load in the knockout mice (Figure 4A). We assessed liver damage by measuring serum levels of ALT and AST, but we found no significant differences between wild type and knockout mice (Figure 4B). Serum TNFα and IL-6 levels were also assayed. TNFα and IL-6 are important for controlling *L. monocytogenes*, and mice deficient in either of them have increased bacterial burden (Miura et al. 2000). Although the knockout mice had less TNFα and more IL-6, the differences were not statistically significant (Figure 5). Therefore TNFAIP8 KO mice are resistant to lethal *L. monocytogenes* infection, independent of serum cytokines and hepatocellular toxicity.

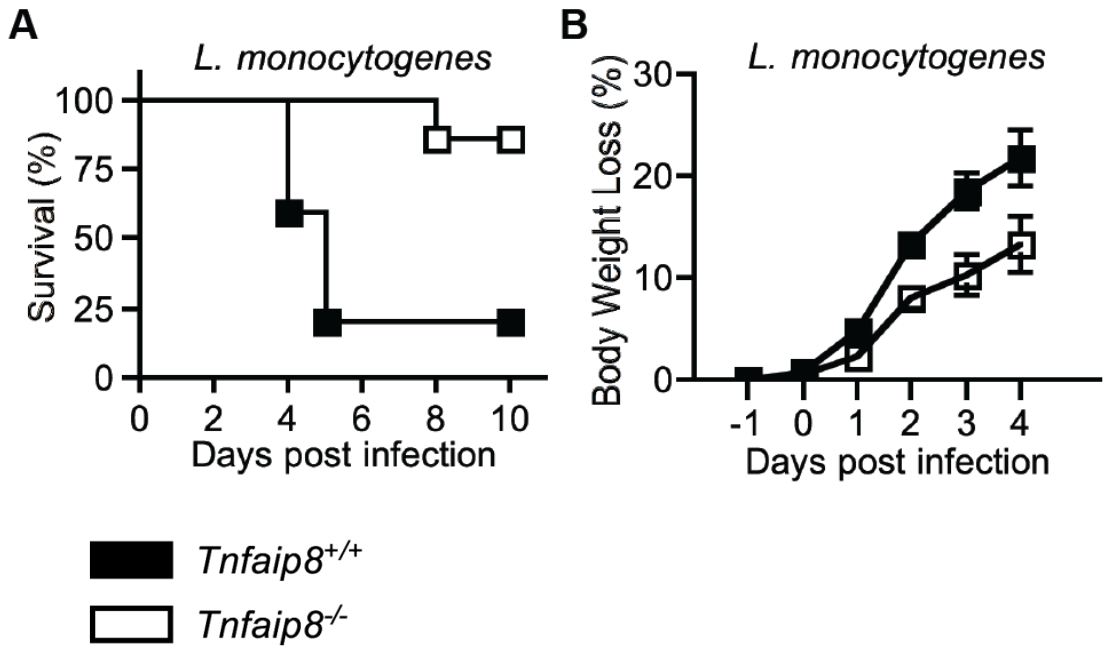

Figure 3. *L Monocytogenes* infection in *Tnfaip8*$^{-/-}$ mice

Wild type (n=5) and TNFAIP8 knockout mice (n=7) were infected with 2x10^5 CFUs of *L. monocytogenes* through the tail vein. (A) Mouse Kaplan-Meier survival curve after *L. monocytogenes* injection (P = 0.01). (B) Changes in body weight following infection (P = 0.04).

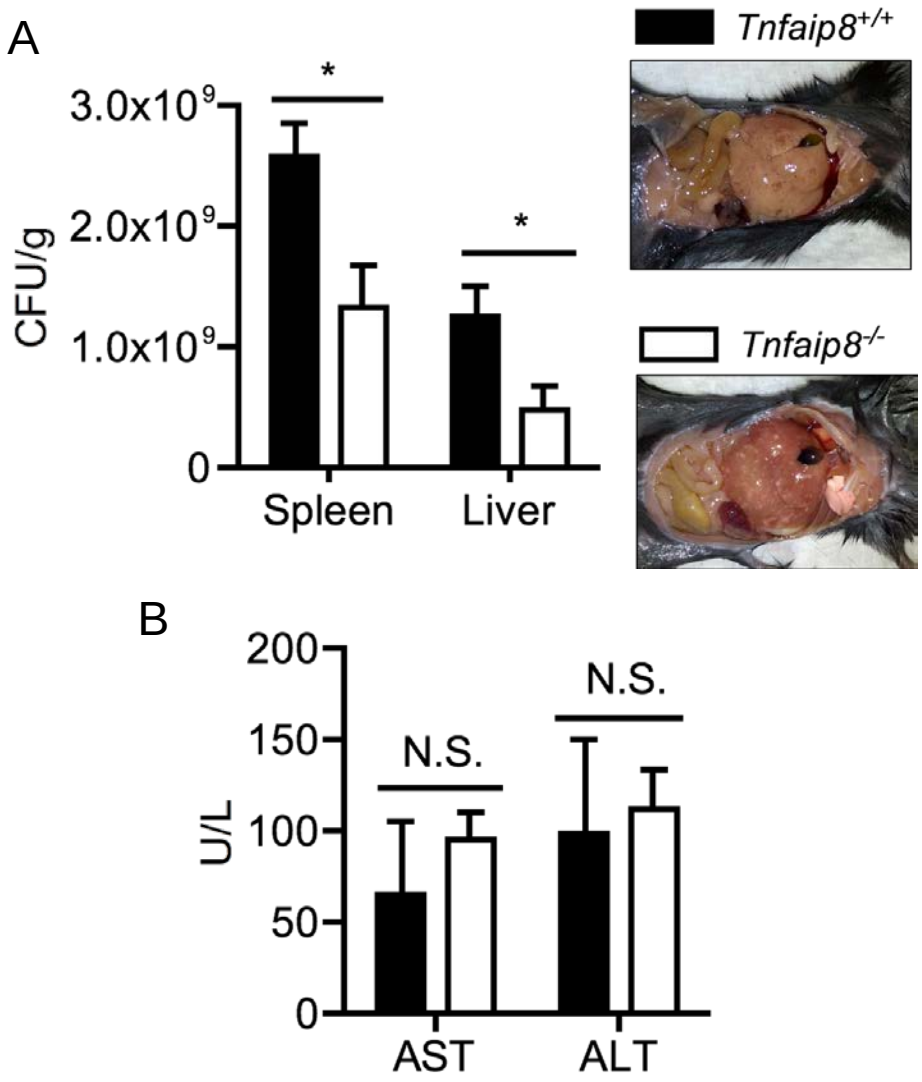

Figure 4. Liver and spleen bacterial load and damage

Wild type (n=5) and TNFAIP8 knockout (n=5) mice were sacrificed at day 3 post infection and their livers, spleens, and sera collected. (A) Spleen and liver bacterial load was measured by a colony-forming assay. (B) Serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT). The results are representative of at least two independent experiments. N.S., not significant; * $P < 0.05$.

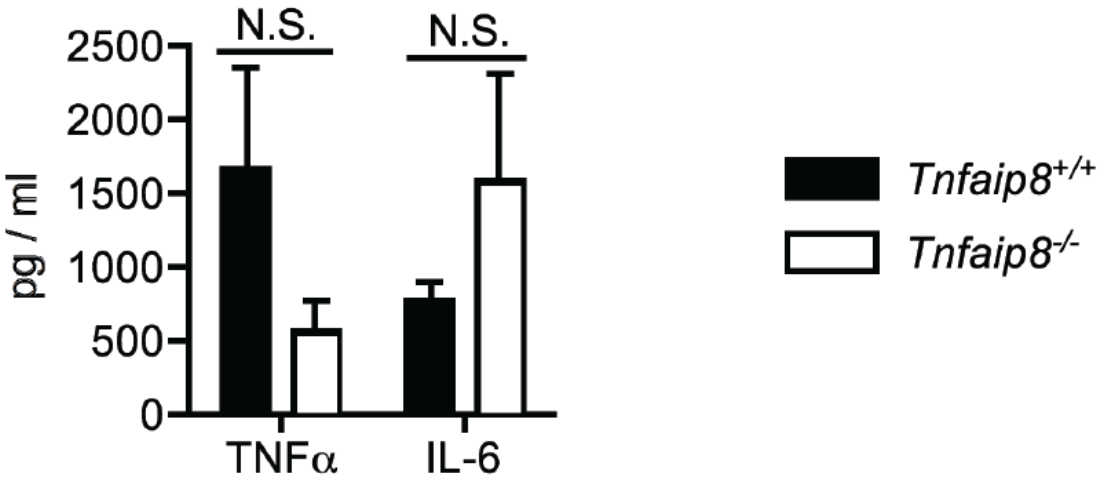

Figure 5. No statistical difference in TNFα and IL-6 in infected mice

Serum TNFα and IL-6 levels were measured in wild type (n=5) and TNFAIP8 knockout (n=5) mice by ELISA 3 days following *L. monocytogenes* infection. The results are representative of at least two independent experiments.

3.2.2 TNFAIP8 deficiency does not protect mice from *Staphylococcus aureus* infection

Next we tested whether there was a difference in mouse survival with another gram-positive pathogen. We chose *Staphylococcus aureus* because susceptibility is associated with the *Tnfaip8* locus in mice (Ahn et al. 2010). TNFAIP8 knockout (KO) and wild type (WT) mice were infected intravenously with a lethal dose of *S. aureus*, but there was no difference in mouse survival (Figure 6). We also tested susceptibility to the gram-negative pathogen *Escherichia coli* and again we found no difference (data not shown). These results suggested that bacterial resistance in TNFAIP8 knockout mice was dependent on the nature of the pathogen, since *L. monocytogenes* is an intracellular parasite while *S. aureus* and *E. coli* are both extracellular.

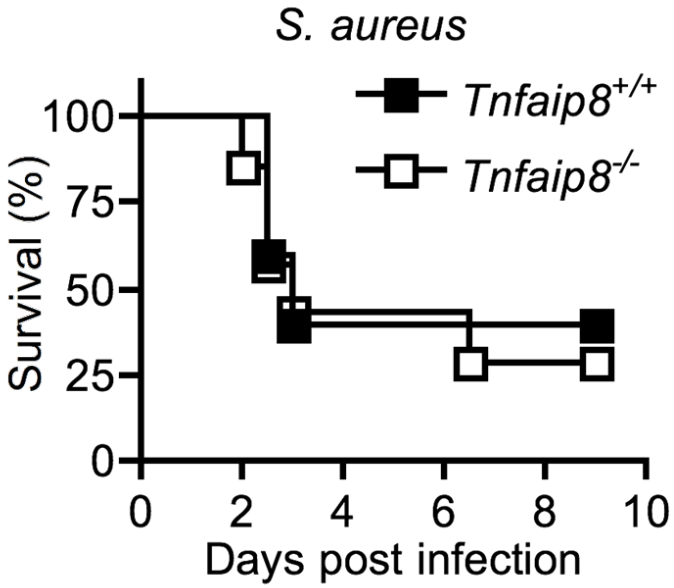

Figure 6. No difference in *S. aureus* infection

Wild type (N=5) and TNFAIP8 knockout mice (N=7) were challenged with $2x10^7$ CFUs of Staphylococcus aureus. The difference in survival between the two groups is not statistically significant.

3.2.3 No difference in immune cell function in *Tnfaip8*$^{-/-}$ mice

To investigate if there were differences in innate immune cell function, we isolated neutrophils from bone marrow and checked if there was a difference in neutrophil chemotaxis. We found no significant difference between WT and KO cells in chemotaxis towards IL-8 in a Boyden chamber assay (Figure 7A). We next studied the phagocytic capacity of bone marrow-derived macrophages and found that there were no differences between WT and KO cells in the phagocytosis of killed CFSE-labeled thymocytes, fluorescently labeled beads, or *L. monocytogenes* (Figure 7B). To determine whether TNFAIP8 deficiency affects the production of TNFα and IL-6 in response to peptidoglycan (PGN), a major component of Gram-positive bacteria, we treated bone marrow-derived macrophages with PGN in vitro and assayed the supernatant. No difference was detected in the production of TNFα and IL-6 upon stimulation with PGN (Figure 8A). Similarly, we injected PGN into WT and TNFAIP8 KO mice, and checked the serum levels of the cytokines. Again, we did not find any significant differences between the two groups (Figure 8B). Taken together, these results suggest that the innate immune system does not play a primary role in the resistance to lethal *L. monocytogenes* infection in TNFAIP8 knockout mice.

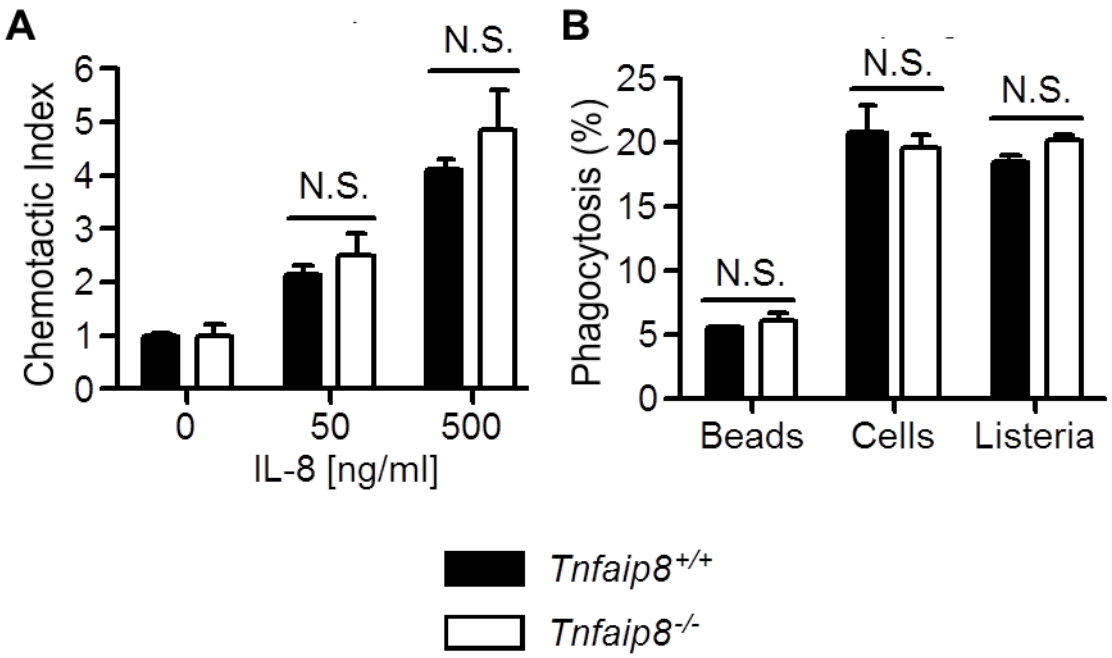

Figure 7. No difference chemotaxis or phagocytosis

(A) Calcein AM-labeled primary neutrophils were incubated for 1 hour in a Boyden chamber filled with or without 50 ng/ml or 500 ng/ml of IL-8. The rate of chemotaxis was determined using a fluorescent plate reader. (B) Bone marrow-derived macrophages (BMDMs) were incubated with 2-μm fluorescent beads at a ratio of 1:20, apoptotic CFSE-labeled thymocytes at a ratio of 1:5, or CFSE-labeled *L. monocytogenes* at a ratio of 1:10 for 30 minutes. The rate of phagocytosis was measured by flow cytometry. The results are representative of at least two independent experiments. N.S., not significant.

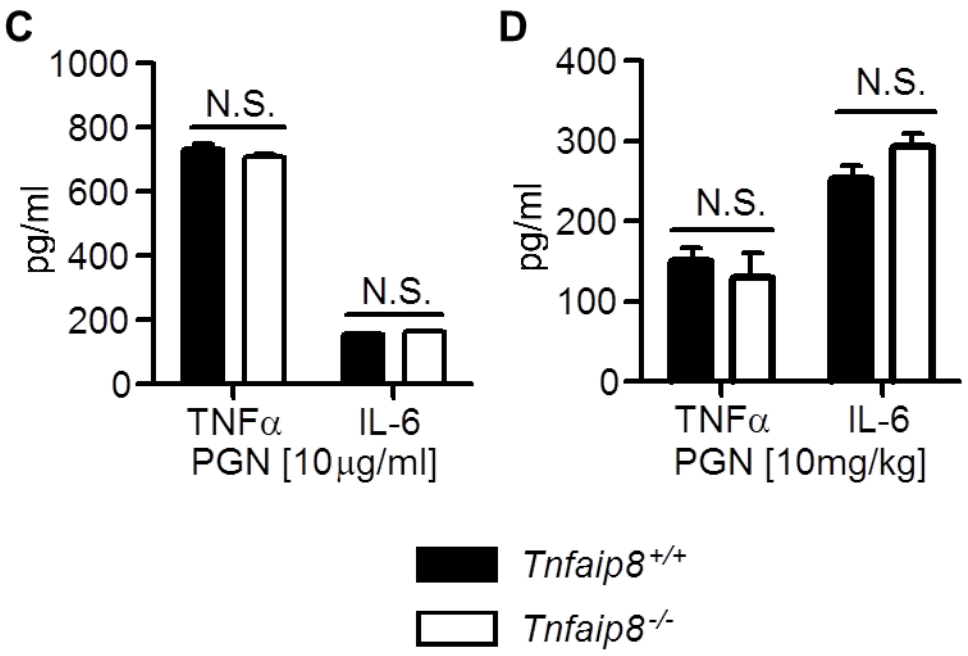

Figure 8. No difference in TNFα and IL-6 production

(A) BMDMs were treated with PGN (10 μg/ml) for 8 hours and TNFα and IL-6 levels were assayed in the supernatant. (B) Wild type and TNFAIP8 knockout mice were intravenously injected with PGN (10 mg/kg) and serum was collected after 8 hours to assay TNFα and IL-6 levels by ELISA. The results are representative of at least two independent experiments. N.S., not significant.

3.2.4 Non-hematopoietic cells may be responsible for resistance to lethal infection

We observed little difference in innate immune cell function between wild type and knockout mice, suggesting that other tissues may be responsible the resistance observed in knockout mice. To test whether TNFAIP8 knockout immune cells are important for the resistance to *L. monocytogenes* infection, we produced bone marrow chimeric mice using both wild type and knockout irradiated mice as recipients of wild type bone marrow (Figure 9). The chimeric mice had their hematopoietic compartment replaced with donor cells, allowing us to test whether the immune cells (derived from the bone marrow) or non-immune cells are responsible for resistance to lethal infection. After the immune cells were replenished, the recipient mice were challenged with a lethal dose of *L. monocytogenes.* TNFAIP8 knockout mice still exhibited significant resistance to lethal infection despite reconstitution with WT bone marrow (Figure 10). This result suggests that the differences in bone marrow-derived immune cells between wild type and knockout mice are not responsible for resistance to lethal L. monocytogenes infection in *Tnfaip8*$^{-/-}$ mice.

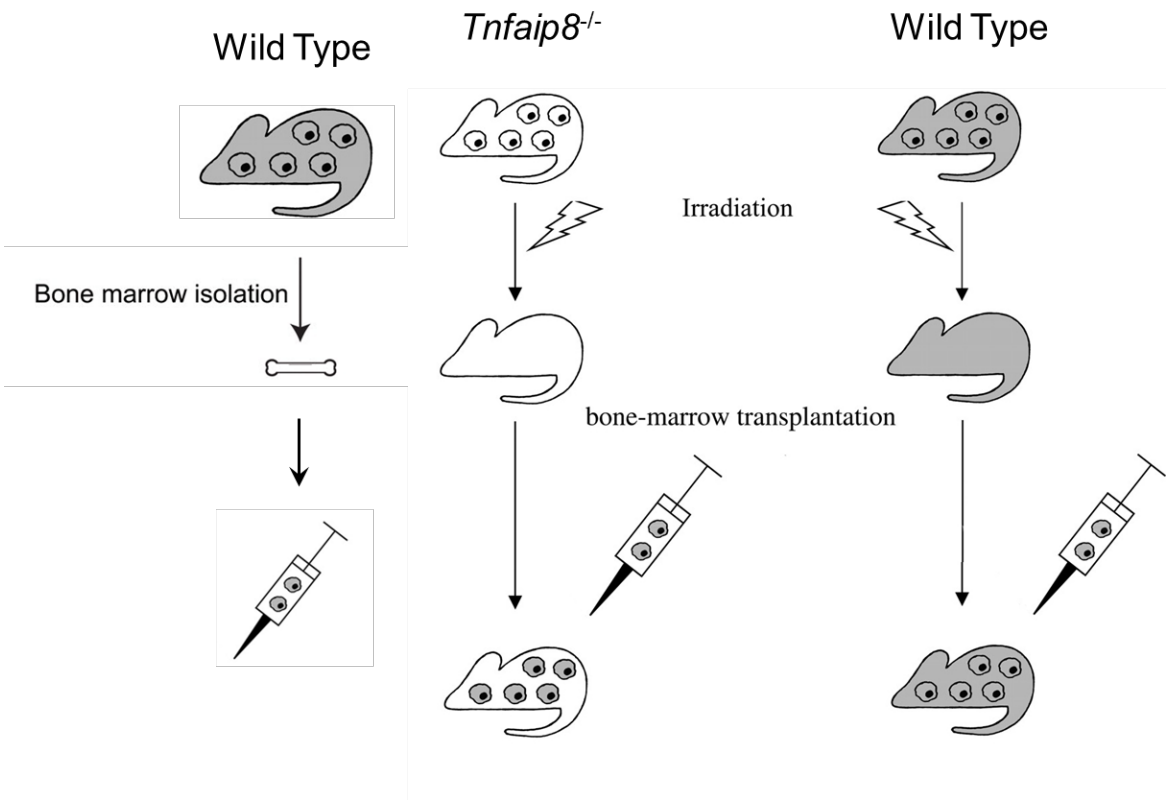

Figure 9. Generation of bone marrow chimeric mice

Bone marrow cells were flushed from the femurs and tibias of donor wild type mice and resuspended in PBS. Recipient wild type and *Tnfaip8*$^{-/-}$ mice were sub-lethally irradiated with 500 rads twice separated by 4 hours. The irradiated mice received a total of 10x10^{6} donor bone marrow cells by tail vein injection one hour after irradiation. Mice were given 8 weeks to reconstitute their immune systems before being challenged with *L. monocytogenes*.

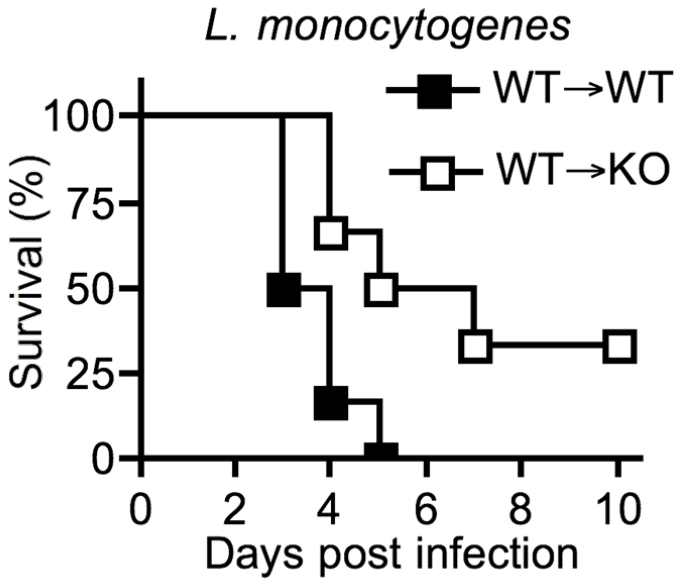

Figure 10. Replacing *Tnfaip8*$^{-/-}$ bone marrow with wild type does not ablate resistance to lethal *L. monocytogenes* infection

Bone marrow chimeras were produced by irradiating wild type (N=6) and TNFAIP8 (N=6) knockout mice and transferring wild type bone morrow into them. Mice were given 8 weeks to reconstitute their immune system before being infected with $2x10^5$ CFUs of *L. monocytogenes*. The difference in survival between the two groups is statistically significant (P = 0.02).

3.3 Discussion

Listeria monocytogenes pathogenesis is dependent on a number of factors related to both immune and non-immune systems. Hepatocytes are quickly invaded by *L. monocytogenes* upon infection, and apoptotic cell death is important in releasing TNFα and other cytokines to trigger an immune response in the infected liver (Pamer 2004; Vázquez-Boland et al. 2001; Rogers et al. 1996; Santos et al. 2005). Neutrophils are attracted to the infected organs to phagocytose and kill bacteria. If the pathogen is not controlled, *L. monocytogenes* will escape to other organs and the organism can die of sepsis. Protecting immune cells from death has been found to be an important aspect in preventing lethal *L. monocytogenes* infection (Zheng et al. 2004; Gurung et al. 2011). But promoting cell death in hepatocytes or blocking cellular invasion might be equally important for the survival of the organism (dos Santos et al. 2011; Santos et al. 2005; Schmeck et al. 2006).

Our results show that TNFAIP8 knockout animals are resistant to lethal *L. monocytogenes* infection with decreased bacterial load in the liver and spleen (Figures 3-4), similar to what has been previously observed with TIPE2 (Wang et al. 2012). However, unlike TIPE2 knockout mice that show heightened immunity to *L. monocytogenes*, there was little difference in phagocytosis, chemotaxis, or cytokine production between WT and TNFAIP8 KO innate immune cells (Figures 5-8). Despite reports suggesting a role for TNFAIP8 in *Staphylococcus aureus* infection, we found no difference in survival between WT and TNFAIP8 KO mice (Figure 6) (Ahn et al. 2010). This suggests that TNFAIP8 knockout mice are not resistant to extracellular pathogens, and that the immune system is no more capable of handling pathogens than that of WT mice. The association of the *Tnfaip8* locus with *S. aureus* resistance may have been a statistical anomaly and an alternative locus or a nearby gene may have been more important to *S. aureus* susceptibility. Rather, the non-immune cells of the TNFAIP8 knockout mice, such as hepatocytes, may be more important in protecting the host against pathogens as has been suggested by the chimeric experiment (Figure 10).

The resistance of TNFAIP8 knockout mice to lethal infection suggests that TNFAIP8 plays an important role during the course of the disease. TNFAIP8 mRNA is found in immune cells of normal mice but the lack of a phenotype in knockout immune macrophages and neutrophils may be due to compensation by TIPE2. TIPE2 is strongly expressed in the immune system and it is a regulator of phagocytosis, cell migration and cytokine production, and any effect from TNFAIP8 deficiency might have been too weak to detect (Sun et al. 2008; Wang et al. 2012). With a phenotype too weak to detect and the fact that the chimeric mice retained resistance to lethal infection despite having a portion of their immune system replaced with wild-type cells, differences in the immune system might not have been important enough to confer resistance to lethal infection in TNFAIP8 knockout mice. The chimeric experiment does not, however, definitively prove that the immune system is not important. A Technical issue with the procedure is that it will not completely ablate the original immune system. Tissue resident macrophages and other non-dividing long lived immune cells will not be completely killed by the irradiation and those cells may be significantly different in knockout mice. As they are not easily replaced during the formation of bone marrow chimeras, there may have been enough TNFAIP8 knockout resident macrophages left in knockout chimeric animals to protect them from *L. monocytogenes*. In addition, due to limitations in the number of mice available for the experiments, chimeric mice reconstituted with knockout bone marrows were not created, so it is not possible to completely rule out a protective effect of the knockout immune system. Another issue is that the chimeric animals had overall increased susceptibility to death by L. monocytogenes infection compared to the mice in figure 3. Possible explanations include the difference in the ages of the mice, the collateral damage of the irradiation on the murine tissues increasing susceptibility to infection, incomplete immune reconstitution resulting in a weaker immune response, and standard error in the experimental results. Despite these limitations, the results support the hypothesis that non-immune cells play an important role in infection. The lack of resistance to extracellular pathogens and the lack of a clear phenotype in immune cells suggest that the resistance to lethal *L. monocytogenes* infection is due to the nature of the

intracellular pathogen and its ability to infect and survive in host cells, and so the attention must turn to alternative explanations involving non-immune cells.

TNFα is critical for protecting mice against lethal *L. monocytogenes* infection, which can block certain aspects of TNFα signaling to protect itself (Miura et al. 2000; Gouin et al. 2010). Both IL-6 and TNFα levels varied greatly depending on the significance of infection within each experimental set, but no statistical difference was observed. A difference may be present and relevant to the resistance found in knockout animals, but a greater sample of animals and more stringent conditions may be necessary to detect it. Despite there being no statistical difference in serum levels of TNFα in infected mice, TNFAIP8 being an inducible gene of TNFα may explain why knockout mice were resistant. TNFAIP8 is protective against TNFα induced death, and differences in apoptosis of bacteria infected cells may explain the differences in survival of the knockout mice. Another potential explanation may be that TNFAIP8 knockout cells are resistant to infection by the bacteria. *L monocytogenes* depends on its ability to invade hepatocytes to evade the immune system. Inflammation is harmful to bacteria, and neutrophils and macrophages are constantly on the lookout for extracellular pathogens but are helpless against invaders hidden within other cells (Vázquez-Boland et al. 2001). By invading a cell and preventing its death, the bacteria can safely proliferate until it is ready to spread to surrounding cells. Therefore, it is logical to form the hypothesis that TNFAIP8-knockout mice are protected against lethal *L. monocytogenes* infection by first reducing hepatocyte invasion and second by increasing apoptosis of infected hepatocytes in response to TNFα, thus blocking a productive infection of the pathogen.

Chapter 4 – Experimental Colitis

4.1 Introduction

Human inflammatory bowel diseases (IBD), including ulcerative colitis and Crohn's disease, are a major health problem in the northern hemisphere and industrialized countries (Fiocchi 1998). These diseases are characterized by chronic colonic inflammation and are mediated by infiltrating inflammatory cells. Ulcerative colitis is characterized by inflammation limited to the large intestine, and has an incidence of 9-12 cases per 100,000 persons in the United States (Adams & Bornemann 2013). Crohn's disease is more common with 20.2 cases per 100,000 persons in North America, and it is characterized by inflammation that can occur anywhere throughout the entire digestive tract (Laass et al. 2014). IBD is known for its high morbidity in patients with any combination of the following symptoms: vomiting, weight loss, severe abdominal pain, diarrhea, and rectal bleeding. It is also associated with an increased risk of osteoporosis and fracture, arthritis, and hypercoagulability (Targownik et al. 2013; Owczarek et al. 2014). Severe IBD resulting in hospitalization is linked with an increased risk of colorectal and breast cancers in Asians populations (Peng et al. 2014; Tsai et al. 2014). In addition, mortality from infection from bacterial pathogens such as *Clostridium difficile* is increased in IBD patients (Kassam et al. 2014). The etiologic factors that trigger these diseases are not well understood, but they are believed to include genetics, diet, psychological factors, immunological factors, infection, drugs, and sleep problems (Ek et al. 2014; Sobczak et al. 2014). There is no effective cure for IBD aside from a colectomy of the entire large intestines in ulcerative colitis but not in Crohn's disease. The goal of treatment is to reduce discomfort, treat complications, and have the disease go into remission, and this is achieved with anti-inflammatory drugs, immunosuppressants, corticosteroids, and antibiotics. It is therefore important to determine the molecular components that regulate these diseases in order to develop more effective treatment therapies.

In mice, oral administration of dextran sodium sulfate (DSS) induces a form of colitis that shares many pathological and clinical features with human inflammatory bowel diseases; therefore DSS-induced colitis is considered to be a valuable experimental model for human IBD (Perše & Cerar 2012; Strober et al. 2007; Diaz-Granados et al. 2000). Increased colon epithelial cell death and decreased proliferation have been implicated in the development of colitis. TIPE2 deficiency in the immune system of mice has been found to be protective against the experimentally induced colitis (Lou et al. 2014). In addition, the NF-κB activator IKK-β is linked to tumorigenesis due to inflammation in the experimental colitis-associated cancer mouse model, and it can regulate both incidence of tumorigenesis and size (Greten et al. 2004). Given the potential roles of TNFAIP8 in regulating these processes, we set out to test the *in vivo* effect of TNFAIP8 deficiency in the development of DSS-induced colitis using our newly generated TNFAIP8 knockout mice. Our data indicates that TNFAIP8 plays a crucial role in maintaining colonic homeostasis during colitis.

4.2 Results

4.2.1 *Tnfaip8*$^{-/-}$ mice are hyper-sensitive to DSS-induced colitis

Tnfaip8$^{-/-}$ mice do not appear to develop more spontaneous diseases than wild type (WT) littermates, unlike *Tipe2*$^{-/-}$ mice. However, after drinking DSS-containing water, they developed more severe colitis than WT mice. Mice began dying after day 6 and all of the *Tnfaip8*-$^{-/-}$ animals died by day 10 while 40% of WT animals were still alive (Figure 11A). The *Tnfaip8*$^{-/-}$ mice suffered greater body weight loss (Figure 11B) and increased overall disease manifestations (Figure 11C). To further assess the severity of colitis, colon length was also measured. The DSS-fed *Tnfaip8*$^{-/-}$ mice, but not wild type or untreated mice, had significantly shorter and lighter colons signifying increased inflammatory damage (Figure 12A, B, and C). Consistent with these findings, a histopathological examination of H&E-stained colons of knockout mice revealed more severe colitis characterized by crypt loss, loss of structure, and more infiltrating leukocytes than in the controls (Figure 12D).

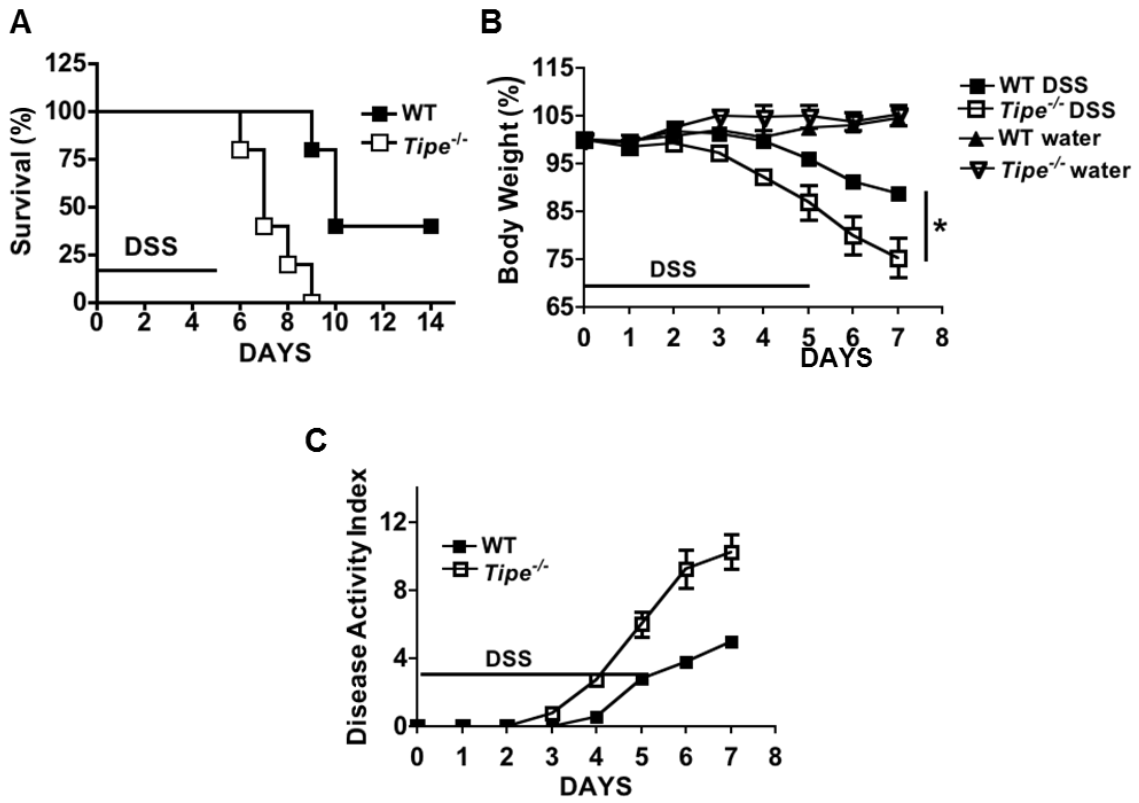

Figure 11. *Tnfaip8*$^{-/-}$ mice are hyper-sensitive to DSS-induced colitis

(A) Wild type (WT) mice (n=5) and Tnfaip8-/- (*Tipe*$^{-/-}$) mice (n=5) were fed with DSS-containing water for five days, followed by regular drinking water. Survival was monitored for two weeks after the start of DSS treatment. (B-E) WT and *Tnfaip8*$^{-/-}$ mice were fed with DSS water for five days, followed by regular drinking water for two days. Body weight (B) and disease score (C) were recorded daily. Disease activity index was the total sum of 3 scores measuring stool consistency, rectal bleeding, and body weight loss. These experiments were performed in collaboration with Honghong Sun and Yunwei Lou. *P<0.05

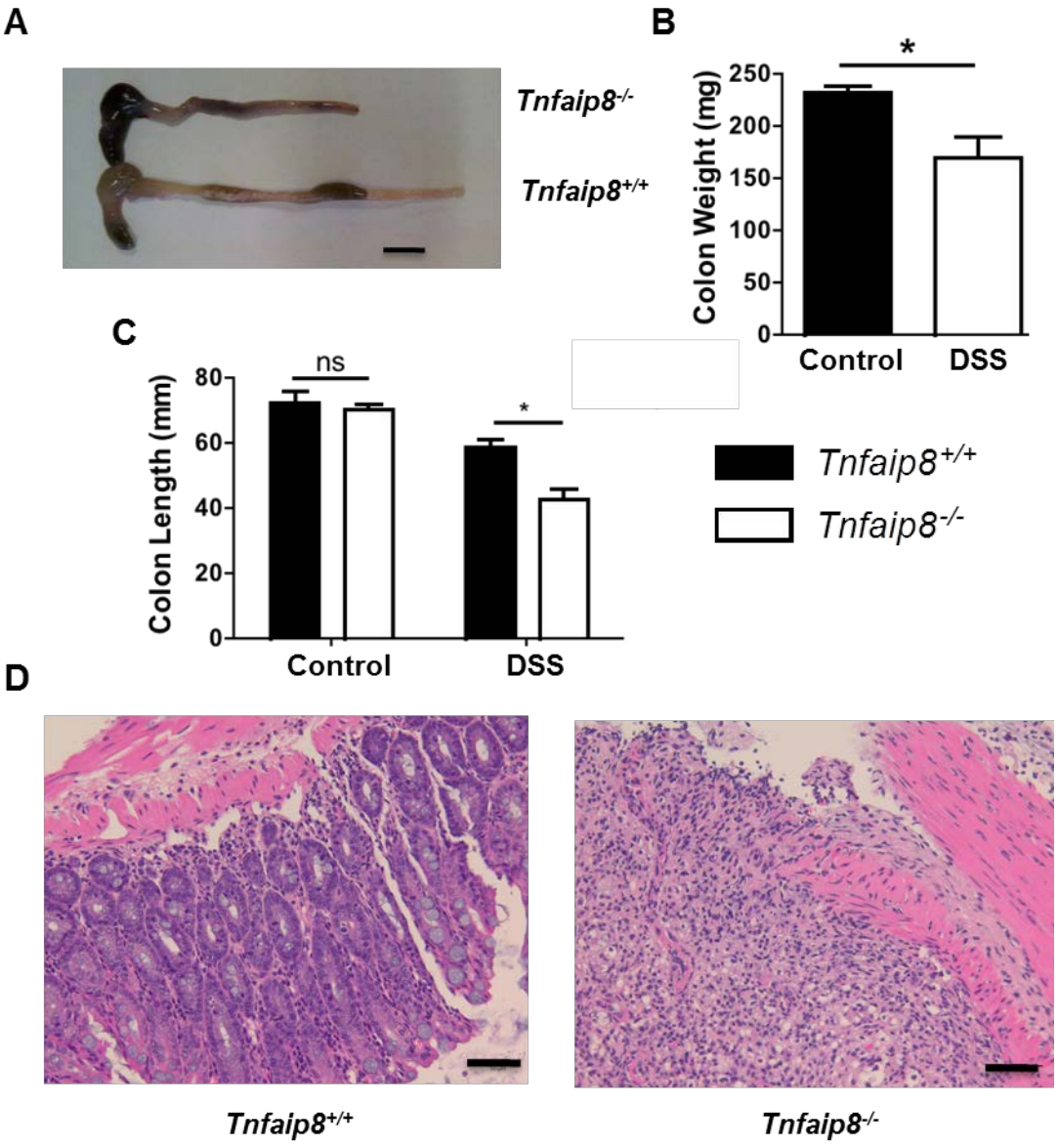

Figure 12. *Tnfaip8*$^{-/-}$ colons are more inflamed

On Day seven, mice were euthanized and colons were harvested (A). Colon weight was measured after DSS treatment in wild type and knockout mice (B), and colon length was compared with or without DSS treatment (C). Histopathological changes (D) in colon tissues were examined by microscopy following H&E staining. Data shown is representative of at least three experiments performed in collaboration with Honghong Sun and Yunwei Lou. Scale bar in A, 1 mm and in D, 50 μm. *P<0.05

4.2.2 Enhanced inflammatory response in *Tnfaip8*$^{-/-}$ mice with colitis

To further define the roles of TNFAIP8 in DSS-induced colitis, we examined the hematological and immunological aspects of the disease in age- and sex-matched *Tnfaip8*$^{-/-}$ mice and WT controls. Significantly increased neutrophil counts were detected in *Tnfaip8*$^{-/-}$ blood samples (Figure 13A). At the same time, the spleens of *Tnfaip8*$^{-/-}$ mice became smaller (Figure 13B) while more leukocytes were found in the colon of these mice (Figure 13C). After dissecting the leukocyte populations in the colon using surface markers, we found that there were increased levels of myeloid derived cells such as macrophages, neutrophils and dendritic cells, and increased B-cell but not T-cell lymphocytes infiltrated into the colon (Figure 14). In contrast, untreated mice had similar spleen weights and cell type composition of the splenocytes (Figure 15). Despite the published role of TNFAIP8 in dexamethasone-induced death of thymocytes, we saw no difference in knockout cells compared to wild type (Figure 16). Enhanced levels of IL-17A and IL-6 were also found in the sera of *Tnfaip8*$^{-/-}$ mice (Figure 17A). Similarly, enhanced levels of IL-6, IL-17A, and IL-1β proteins (Figure 17B) and/or mRNAs (Figure 17C) were detected in the knockout colons. Increased CXCL2 mRNA expression was also detected in *Tnfaip8*$^{-/-}$ colons (Figure 17C). These results point to an enhanced inflammatory response in the *Tnfaip8*$^{-/-}$ colon after DSS treatment that might be responsible for the increased severity of the experimental colitis.

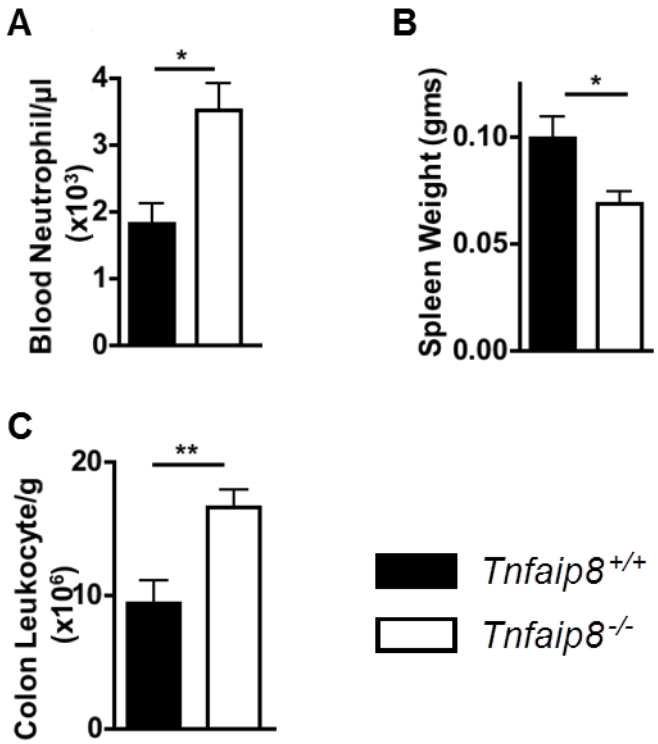

Figure 13. Increased leukocyte mobilization in *Tnfaip8*$^{-/-}$ mice.

Mice (n=4) were treated and sacrificed as described in Figure 11. Increased blood neutrophil counts (A) and decreased spleen weights (B) were observed in *Tnfaip8*$^{-/-}$ mice. The colons from WT and *Tnfaip8*$^{-/-}$ mice were harvested and lamina propria immune cells were isolated (C). These experiments were performed in collaboration with Honghong Sun and Yunwei Lou.*P<0.05; **P<0.01

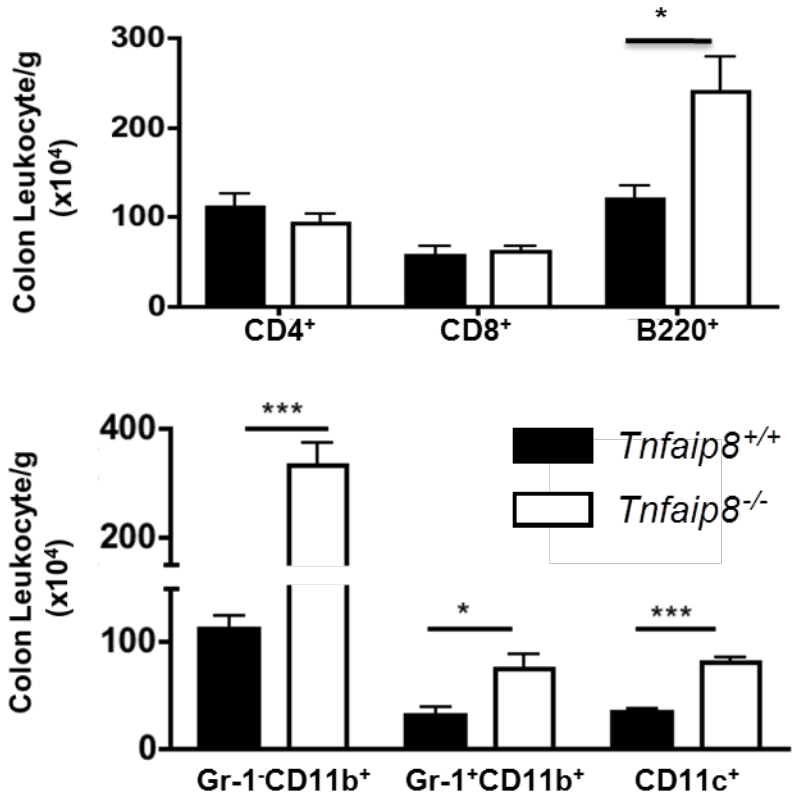

Figure 14. Increased myeloid and B-cell infiltration in *Tnfaip8*$^{-/-}$ colon

The colons from WT and *Tnfaip8*$^{-/-}$ mice were harvested and lamina propria immune cells were isolated. After counting in a hemacytometer, the cells were stained with antibodies to T, B, and myeloid cell markers, and analyzed by flow cytometry. Increases in CD11b, CD11c, and B220 cells were observed. Data shown are representatives of at least three experiments. These experiments were performed in collaboration with Honghong Sun and Yunwei Lou. *P<0.05; **P<0.01; *** P <0.001

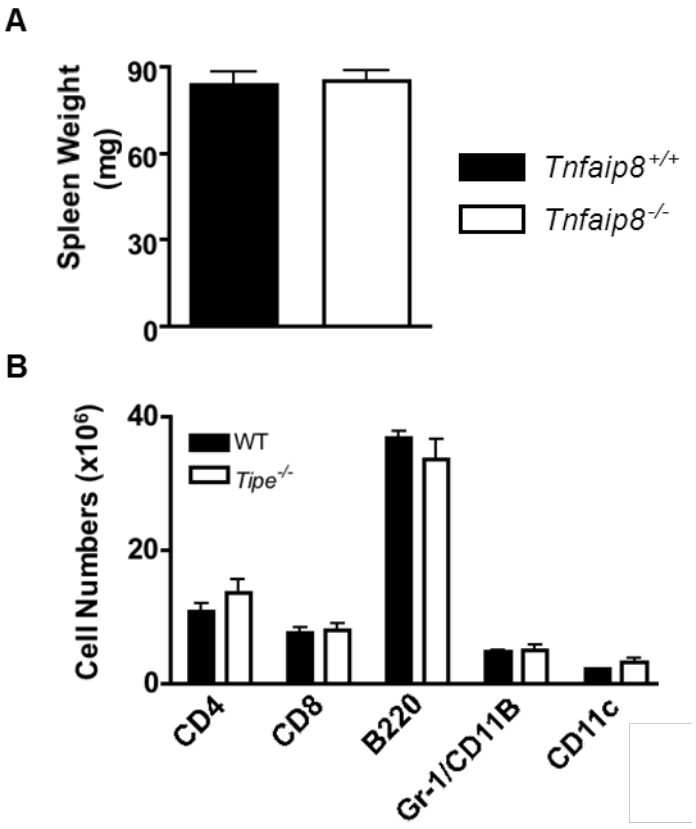

Figure 15. No difference in leukocyte numbers in untreated mice

Eight-week-old wild type and *Tnfaip8*$^{-/-}$ mice (n=4) were sacrificed. (A) Spleens were removed and weighed. (B) Splenocytes were then purified and the cells were stained with antibodies to T, B, and myeloid cell markers, and analyzed by flow cytometry. These experiments were performed in collaboration with Yunwei Lou.

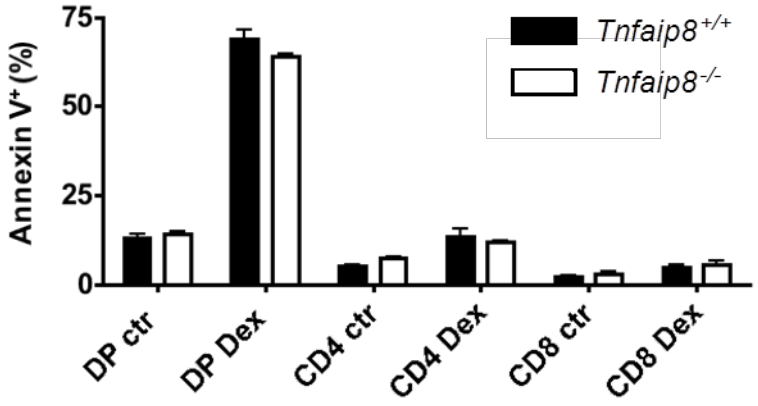

Figure 16. Dexamethasone-induced cell death was not affected in *Tnfaip8*$^{-/-}$ thymocytes

Eight-week-old wild type and *Tnfaip8*$^{-/-}$ mice (n=4) were sacrificed. Percentages of annexin V positive thymocytes treated with dexamethasone (Dex) or without (ctr) in culture for four hours. Challenged cells were further divided into double positive cells for CD4 and CD8 (DP), CD4 single positive, or CD8 single positive cells. Data shown is representative of three independent experiments. These experiments were performed in collaboration with Honghong Sun.

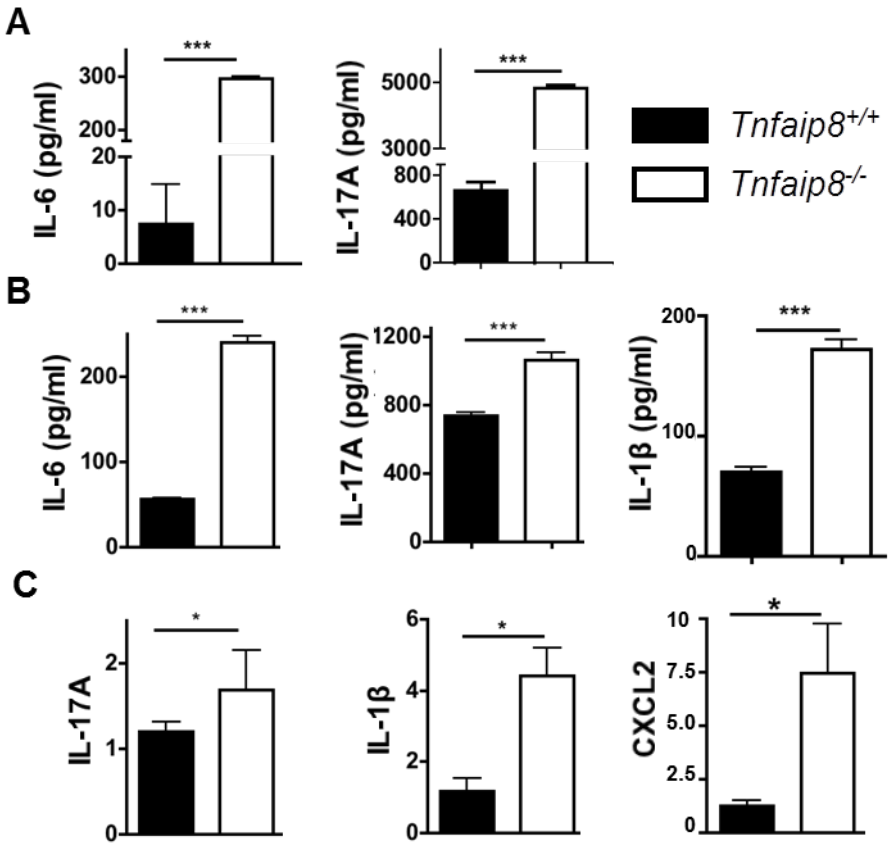

Figure 17. Increased inflammatory cytokines in serum and colon of *Tnfaip8*$^{-/-}$ mice

Wild type and *Tnfaip8*$^{-/-}$ (n=5) mice were treated with DSS water for five days, followed by regular drinking water for two days. Mice were sacrificed and their blood was collected retro-orbitally. Colons were harvested and ½ of them were homogenized in lysis buffer (weight/volume = 1/10). The cytokines in sera and colon homogenates were measured by ELISA. (A) IL-6 and IL-17 -in sera. (B) IL-6, IL-17, and IL-1β in colon homogenates. (C) Total RNA was isolated from the other half of the colon using Trizon reagent. The levels of IL-17, IL-1β, and CXCL2 mRNAs were examined using quantitative real-time PCR. Data shown are representatives of two experiments. These experiments were performed in collaboration with Honghong Sun and Yunwei Lou. **P*<0.05; ***P*<0.01; *** *P*<0.001.

4.2.3 TNFAIP8-deficiency in non-hematopoietic cells exacerbates colitis

Although enhanced inflammatory responses were detected in the DSS-challenged *Tipe*$^{-/-}$ mice, both hematopoietic and non-hematopoietic compartments could contribute to the exacerbated colitis phenotype observed above. To assess which compartment contributes directly to the exacerbation of colitis in *Tnfaip8*$^{-/-}$ mice, we reconstituted sub-lethally irradiated wild type and TNFAIP8 knockout mice with bone marrow cells from wild type or knockout mice. After recovering for eight weeks, mice were then subjected to five days of DSS treatment followed by two days of normal drinking water. As shown in Figure 18, after DSS treatment, the wild type mice receiving wild type or *Tnfaip8*$^{-/-}$ bone marrow cells demonstrated similar changes in body weight, disease index, and colon length (Figure 18A, B, and C) indicating that hematopoietic cells are not major contributors to the exacerbation of colitis observed in *Tnfaip8*$^{-/-}$ mice. By contrast, when wild type bone marrow cells were injected into *Tnfaip8*$^{-/-}$ or wild type recipient mice, after DSS treatment, *Tnfaip8*$^{-/-}$ recipients developed more severe colitis than the wild type recipients further indicating that the non-hematopoietic compartment plays a major role in the disease exacerbation.

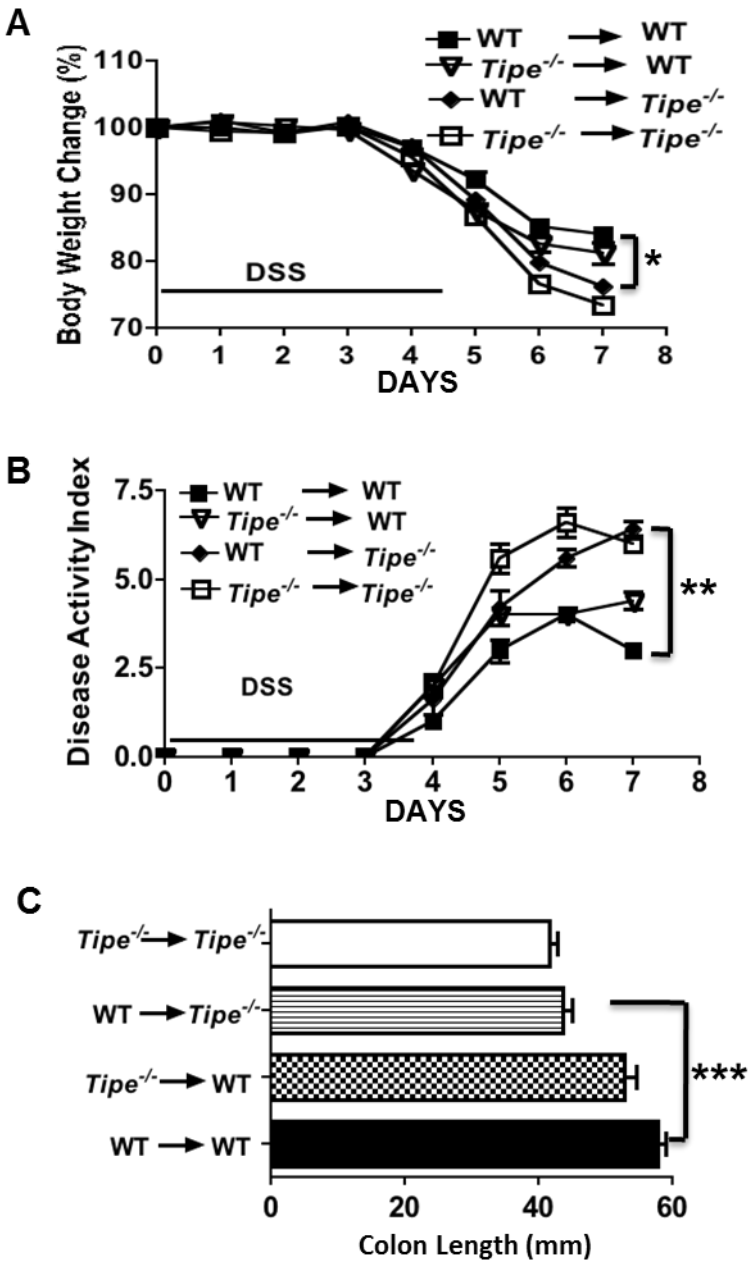

Figure 18. TNFAIP8 deficiency in non-hematopoietic cells contributes to the enhanced colitis in knockout mice

Bone marrow chimeric mice (n=5) were generated as described previously using wild type (WT) and *Tnfaip8*$^{-/-}$ (*Tipe*$^{-/-}$) donor and recipient mice. After the mice were allowed to reconstitute their immune systems for 8 weeks, they were treated with DSS for five days, followed by regular

drinking water for two days. (A) Body weight loss. (B) Disease activity index calculated based on the degrees of weight loss, stool consistency, and rectal bleeding. (C) Colon length at day 7. Data shown are representatives of two experiments. These experiments were performed in collaboration with Honghong Sun and Yunwei Lou. *P<0.05; **P<0.01; *** P <0.001.

4.2.4 Reduced epithelial integrity and increased bacterial infection in *Tnfaip8*$^{-/-}$ mice

TNFAIP8 is expressed in a large number of cell types including colonic epithelial cells. The increased mortality in *Tnfaip8*$^{-/-}$ mice could be explained by increased cell death and/or decreased proliferation of colonic epithelial cells during colitis (Araki et al. 2010).To test whether TNFAIP8-deficiency affects epithelial cell death and proliferation, epithelial cells were isolated from normal and DSS-fed wild type and *Tnfaip8*$^{-/-}$ colons and cell death was determined by 7-AAD and Annexin V staining. Live epithelial cells were negative for both 7-AAD and Annexin V (Figure 19A). Increased cell death was detected in the *Tnfaip8*$^{-/-}$ group as compared to WT controls. In addition, increased activated caspase 3 staining was observed in the *Tnfaip8*$^{-/-}$ colons (Figure 19B), demonstrating apoptotic cell death. Ki67 is an endogenous marker for cell proliferation and histological sections showed significantly reduced Ki67 staining was found in DSS-fed *Tnfaip8*$^{-/-}$ colons compared to those of the wild type colons (Figure 19C and D), suggesting that TNFAIP8 protein is required for epithelial cell proliferation after DSS-induced damage to the colon.

The disruption of mucosal barrier following gastrointestinal epithelial cell injury can lead to commensal bacterial infection and potent inflammatory responses against them, which is believed to be part of the pathogenic mechanisms of DSS-induced colitis (Brown et al. 2013; Brestoff & Artis 2013; Honda & Littman 2012). Therefore we checked the bacterial numbers in the colons of wild type and *Tnfaip8*$^{-/-}$ mice with colitis. We found significantly more bacteria in the colons of *Tnfaip8*$^{-/-}$ mice than in WT mice, indicating enhanced dissemination of the commensal bacteria (Figure 20). Taken together, increased death of *Tnfaip8*$^{-/-}$ epithelial cells combined with compromised proliferation and the accompanying increase in bacterial infiltration may be responsible for the increased mortality in TNFAIP8-deficient mice treated with DSS.

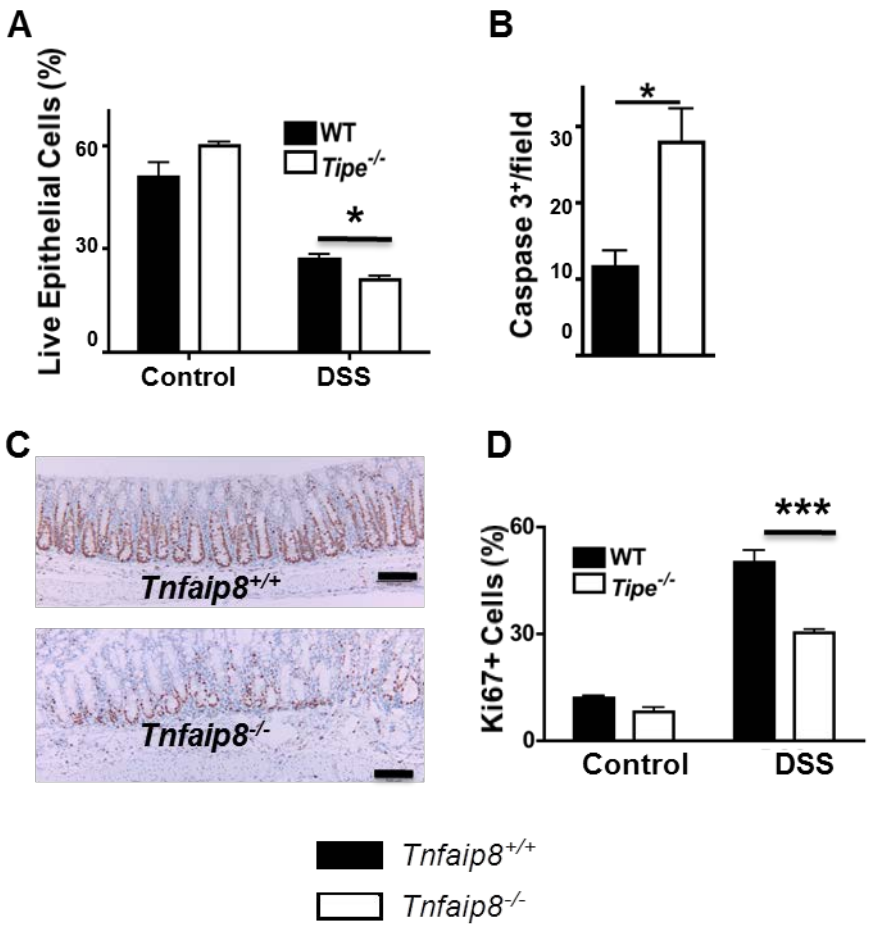

Figure 19. TNFAIP8 in gut epithelial cell death and proliferation

Mice were fed with or without DSS in drinking water for five days followed by regular drinking water for two days, and sacrificed. Colons were collected for epithelial cell isolation or immunohistochemistry. (A) Epithelial cells from normal water-fed and DSS-fed wild type and *Tnfaip8*$^{-/-}$ colons (n=5) were isolated and cell death was determined by 7AAD and Annexin V staining. Colons were washed, fixed, sectioned, and stained for activated caspase 3 or Ki67. (B) Bar graph shows numbers of active caspase3+ cells per high power microscopic field. At least six fields were counted per sample. (C) Ki67 antibody was used to measure colonic epithelial cell proliferation. (D) Bar graph shows percentages of Ki67^{+} cells in the colons of normal water-fed (control) and DSS-fed WT and *Tipe*$^{-/-}$ mice. These experiments were performed in collaboration with Honghong Sun and Yunwei Lou. Scale bar, 100 μm. *P<0.05; **P<0.01; *** p<0.001.

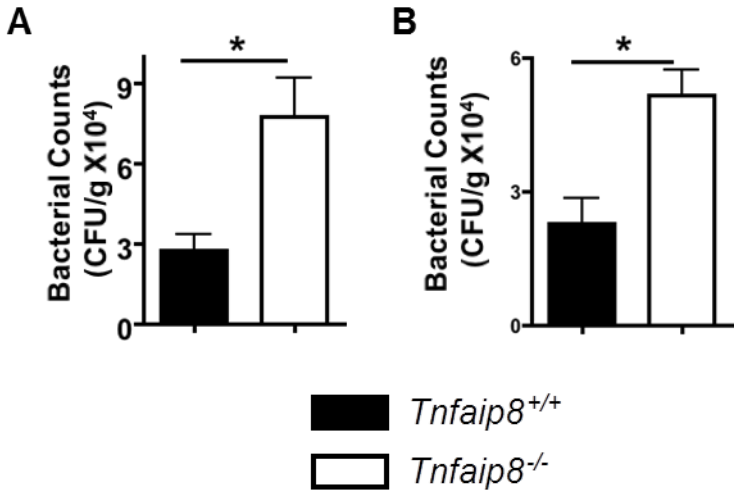

Figure 20. Increased bacterial invasion in *Tnfaip8*$^{-/-}$ colons.

Wild type and *Tnfaip8*$^{-/-}$ mice (n=5) were fed with DSS for five days. Mice were then sacrificed and had their colons collected. Colon homogenates were diluted and plated on blood agar (A) and brain heart infusion (B) plates for determining the bacterial counts. Data shown are representatives of two experiments. These experiments were performed in collaboration with Yunwei Lou. *P<0.05.

4.3 Discussion

We showed here that *Tnfaip8*$^{-/-}$ mice are significantly more susceptible to DSS-induced colitis. The knockout mice suffered from a greater body weight loss, more severe diarrhea and rectal bleeding, and enhanced mortality, indicating a key role of TNFAIP8 in protecting against DSS-induced colon damage. Despite the increased activity of immune cells in DSS treated knockout mice, TNFAIP8 deficiency in immune cells is not responsible for the increased severity of colitis. Consistent with this observation, TNFAIP8 deficiency does not affect T or B cell development or function. TNFAIP8 has been reported as an anti-apoptotic molecule in tumor cells, but knocking down its expression with RNAi protects thymocytes from glucocorticoid-mediated apoptosis (Kumar et al. 2004; Woodward et al. 2010). Although the underlying mechanism is not very clear, GRE (glucocorticoid response element) transactivation and NF-κB transrepression are believed to be involved in multiple myeloma cells (Sharma & Lichtenstein 2008). Our results did not detect any significant differences in apoptosis of TNFAIP8-deficient thymocytes that were treated with dexamethasone (an effective apoptosis-inducing glucocorticoid), anti-CD3, or irradiation from wild type cells (data not shown). It is possible that the dexamethasone-induced apoptosis occurs through alternate pathways not involving TNFAIP8, it is very cell type or condition specific, or alternatively, the RNAi methodology used previously affected other closely related proteins that were responsible for the differences in apoptosis.

The epithelial barrier integrity in the gastrointestinal system is crucial for protecting against environmental insults, including toxins, antigens, and microbes (DeMeo et al. 2002). Increased epithelial cell death and decreased proliferation is associated with increased severity of DSS-induced colitis (Williams et al. 2013). We did observe increased cell death and decreased proliferation in *Tnfaip8*$^{-/-}$ epithelial cells, which is most likely involved with the increased severity of colitis in knockout mice. Increased invasion of commensal bacteria through the epithelium is associated with heightened inflammatory responses and increased immune cell infiltration during

experimentally induced colitis (Xiao et al. 2007). Our results do show increased bacterial counts in the colon. Taken together, these results suggest that the loss of integrity of the epithelium may have allowed increased bacterial infiltration into the colon that may have served to further exacerbate the inflammation and increase the mortality in knockout mice.

What is most surprising about these results is that TNFAIP8 knockout mice behave so differently from TIPE2 knockout mice. TIPE2 knockout mice were protected against DSS induced colitis and had enhanced immune cell function (Lou et al. 2014; Wang et al. 2012). The lack of differences in the TNFAIP8 immune cells may have been due to compensation by TIPE2, so further study may need to be done to elucidate the details. The results from the chimeric experiment, however, suggest that the immune cells are not the primary contributing factor to the differences observed. It is interesting to note that the resistance of TIPE2 knockout mice to DSS induced colitis was determined to be the immune cells by the same kind of chimeric experiment (Lou et al. 2014). The epithelium of TNFAIP8 knockout mice was more sensitive to death in DSS treated mice so the differences in mortality are most likely due to the differences in epithelial cell survival. TIPE2 appears to have pro-apoptotic and inflammatory properties, that when knocked out may have been beneficial in reducing inflammation and prevent cell death in the colonic epithelium (Gus-Brautbar et al. 2012; Sun et al. 2008). Reduced damage to the epithelial layer of TIPE2 knockout animals may have also been responsible for the decreased numbers of bacteria in the colon in DSS treated mice. TNFAIP8, however, has anti-apoptotic properties, and we were unable to observe any increases in cell death from dexamethasone treatment (Kumar et al. 2004). The protective effect of TNFAIP8 may have been necessary to sustain the integrity of the epithelial barrier to protect the mice from the bacterial and subsequent leukocyte infiltration. So despite the high homology shared between TNFAIP8 and TIPE2, they could potential serve as antagonists to one another during cell death resulting opposite phenotypes in DSS treated knockout mice.

Whether or not TNFAIP8 and TIPE2 share the same role, or function in a balance of opposing effects, it is apparent the TNFAIP8 is highly relevant to experimentally induced colitis

and inflammation. Its effect on cell death is widely supported in cancer literature, and it is not surprising that regulating cell death is also highly relevant to the field of inflammation. TNFAIP8 may not yet have a finely defined role in immune cells, but its role in the non-immune system may be just as relevant to inflammation and indeed cancer. Cancer and inflammation are closely linked mechanisms and investigating TNFAIP8's role in inflammation may prove to be crucially important in both the treatment and diagnosis of a wide variety of inflammatory diseases and cancer.

Chapter 5 – Cell death and infection

5.1 Introduction

Listeria monocytogenes is an intracellular gram-positive bacterium that infects a number of cell types including hepatocytes, neurons, and immune cells. Immune cell-mediated apoptosis of *L. monocytogenes*-infected cells such as hepatocytes is important for resolving infection (Vázquez-Boland et al. 2001). In addition, cell death and proliferation of colonic epithelium is considered important in regulating the severity of DSS-induced colitis in mice (Araki et al. 2010). Since no differences in immune cell function were detected, we now hypothesize that the resistance to lethal *L. monocytogenes* infection in TNFAIP8 knockout mice and the increased susceptibility to DSS-induced colitis are the result of the altered function in non-immune cells. TNFAIP8 knockout epithelial cells appear to be sensitized to cell death and have reduced proliferation following DSS treatment. This is supported in cancer literature where TNFAIP8 has been reported to protect cancer cells from TNFα-induced apoptosis and overexpression in tumor cell lines also enhanced proliferation and migration (Kumar et al. 2000; Kumar et al. 2004). Both disease models in TNFAIP8 knockout mice can be potential explained by the enhanced cell death in non-immune cells.

An alternative explanation for increased resistance to lethal *L. monocytogenes* infection is that TNFAIP8 knockout host cells are resistant to invasion by the bacteria. A few TNFAIP8 interacting partners have been identified, including activated Gαi3 and Karyopherin α2, and TIPE2 has been reported to interact with RAC1 and to protect against lethal *L. monocytogenes* and *Staphylococcus aureus* infection (Laliberté et al. 2010; Wang et al. 2012). RAC1 and other RHO GTPases are considered important in *L. monocytogenes* internalization into host cells (Ireton et al. 2014). We sought to determine the importance of RAC1 to the function of TNFAIP8

and whether there is an association or interaction between these two molecules that could explain *L. monocytogenes* infection in TNFAIP8 knockout mice.

To investigate these hypotheses, we created TNFAIP8 knockdown cell lines to test difference in cell death and proliferation, *L monocytogenes* infection, and to determine the molecular pathways affected by TNFAIP8 deficiency.

5.2 Results

5.2.1 TNFAIP8 knockdown cells are sensitized to cell death

Since differences in immune function did not adequately explain the resistance to lethal *L. monocytogenes* infection in TNFAIP8-knockout mice, we investigated the roles of TNFAIP8 in non-immune cells involved in bacterial infection. Hepatocytes are a major target of *L. monocytogenes* infection, so we generated TNFAIP8 knockdown Hepa1-6 murine liver cells. Using a lentiviral vector expressing either a scrambled shRNA sequence or sequences targeting the *Tnfaip8* gene, we created several knockdown cell lines and determined the protein levels of TNFAIP8 and AKT (Figure 21A). We found that shRNA-tnfaip8-1 (SH1) was a nearly complete knockdown, with increased AKT phosphorylation at Serine 473. AKT phosphorylation at Serine 473 is regulated by MTORC2 and PI3K and is important in regulating apoptotic cell death, and PI3K itself is important in *L. monocytogenes* cell invasion (Hatano & Brenner 2001; Jiwani et al. 2012). In addition, we generated TNFAIP8 knockdown NIH3T3 cells to make sure that the cell death phenotypes observed are not isolated to the HEPA1-6 cell line and confirmed it by real time PCR (Figure 21B). We next attempted to confirm reports that TNFAIP8 is a negative regulator of apoptosis and challenged the Hepa1-6 or NIH3T3 cells with either TNFα and cycloheximide or DSS, respectively, and found increased sensitivity in the knockdown cell lines to cell death (Figure 22). Finally, we tested whether proliferation was affected in knockdown cells and we found that TNFAIP8 knockdown cells had a minor increase in doubling time (Figure 23).

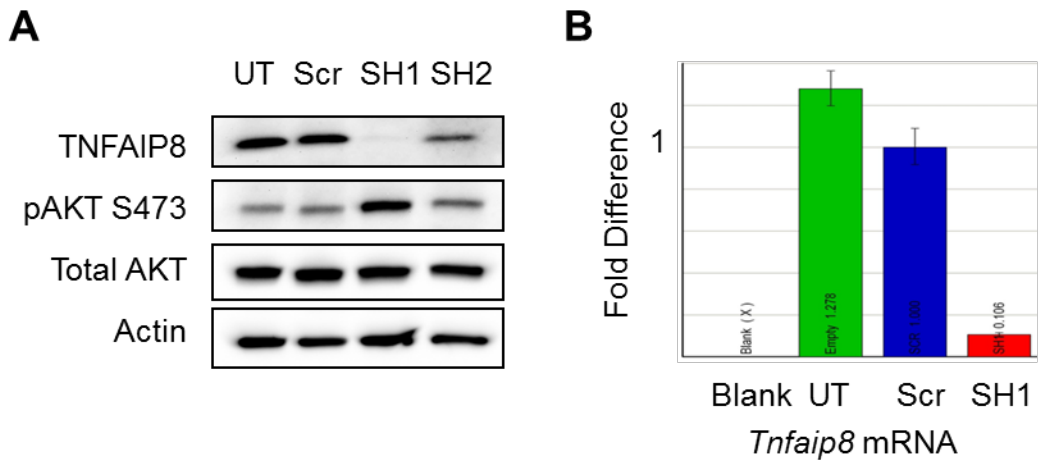

Figure 21. Hepa1-6 and NIH3T3 TNFAIP8 knockdowns

Hepa1-6 and NIH3T3 knockdown cells were generated using the pLKO.1 lentiviral vector to express either a scrambled shRNA (Scr) or one of the two antisense sequences targeting TNFAIP8 (SH1 and SH2). (A) Western blot was performed for the indicated proteins in the lysates of untransfected control (UT) and shRNA-scramble (Scr) or shRNA-Tnfaip8 (SH1 and SH2) Hepa1-6 cells. (B) RT-PCR analysis was used to determine the effectiveness of the knockdown in NIH3T3 cells.

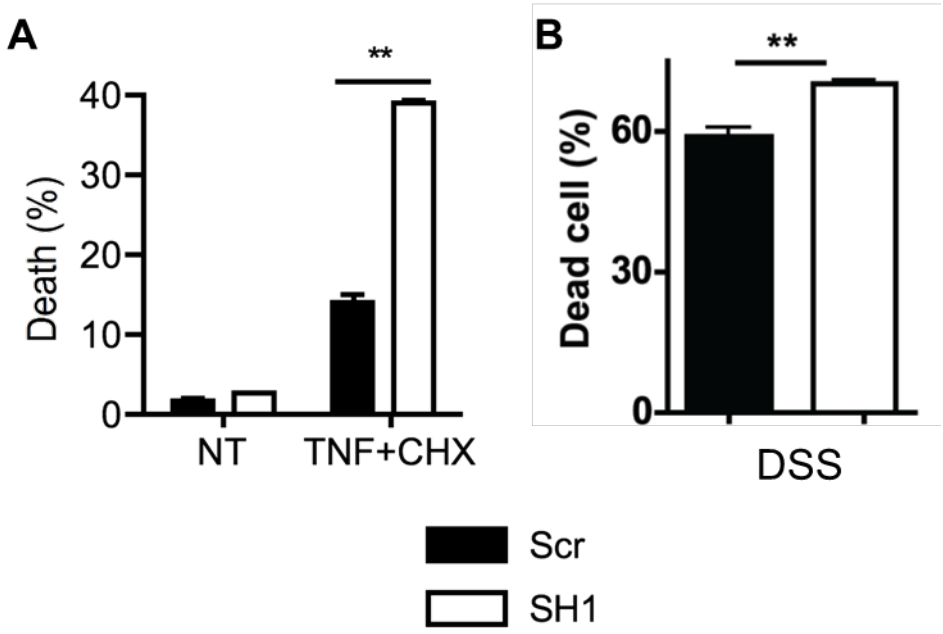

Figure 22. Knockdown cells are sensitized to cell death

(A) Hepa1-6 knockdown and control cell lines were untreated (NT) or treated with 5 ng/ml of TNFα (TNF) and 20 ng/ml of cycloheximide (CHX) for 6 hours. Cell death was determined by flow cytometry after Annexin V and 7-AAD staining. (B) NIH3T3 cells were treated with 3% DSS in culture medium for 16 hours. Cells were then collected and cell death was represented by Annexin V staining. The results are representative of at least two independent experiments. ** $P < 0.01$

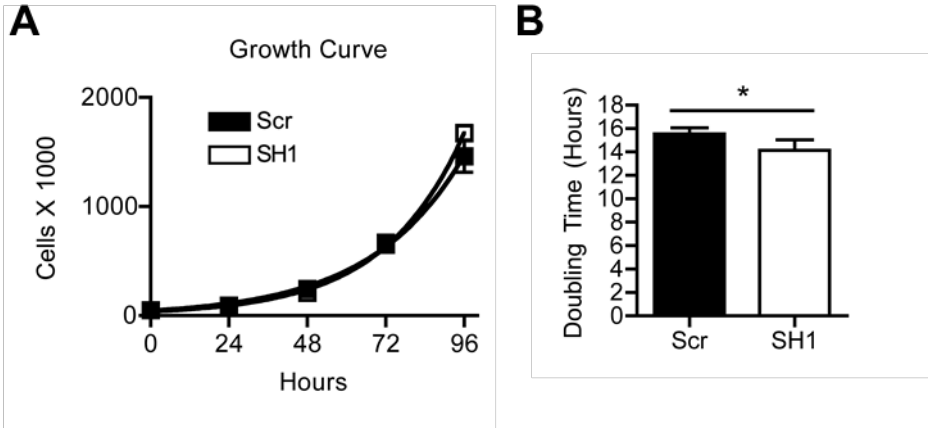

Figure 23. Minor difference in proliferation of knockdown cells

(A). $2.5x10^4$ Hepa1-6 scramble (Scr) and TNFAIP8 knockdown (SH1) cells were plated and counted daily. (B) Doubling time was calculated using four independent experiments. * $P < 0.05$

5.2.2 TNFAIP8 knockdown cells are sensitized to infection and death by *L. monocytogenes*

Next we tested whether *L. monocytogenes* invasion of Hepa1-6 cells was affected by TNFAIP8 depletion. After allowing the bacteria to invade cells for 1 hour, we found that the knockdown cells had reduced bacterial burden, with or without TNFα treatment, and TNFα did not provide any protective effect against bacterial invasion (Figure 24A). TNFα is important in protecting mice against many bacterial pathogens. Hepatocytes produce TNFα upon exposure to *L. monocytogenes*, and its depletion sensitizes mice to death by *L. monocytogenes* (Santos et al. 2005; Miura et al. 2000). Next we tested whether TNFAIP8 knockdown had an effect on *L. monocytogenes* -induced cell death. We incubated the cells with *L. monocytogenes* overnight with or without TNFα and found that the TNFAIP8 knockdown cells showed enhanced sensitivity towards cell death only when treated with both *L. monocytogenes* and TNFα (Figure 24B). Our results suggest that the resistance to lethal *L. monocytogenes* infection in mice could be caused by the resistance of host cells to bacterial invasion and increased sensitivity to cell death following infection, thereby reducing bacterial load in the organism. *L. monocytogenes* evades the host immune system by hiding within cells where phagocytes are unable to reach them. If the host cells are more difficult to infect and are more susceptible to apoptosis, phagocytes will have greater access to the pathogen and bacterial reproduction may be sufficiently impeded to confer a survival advantage to the host.

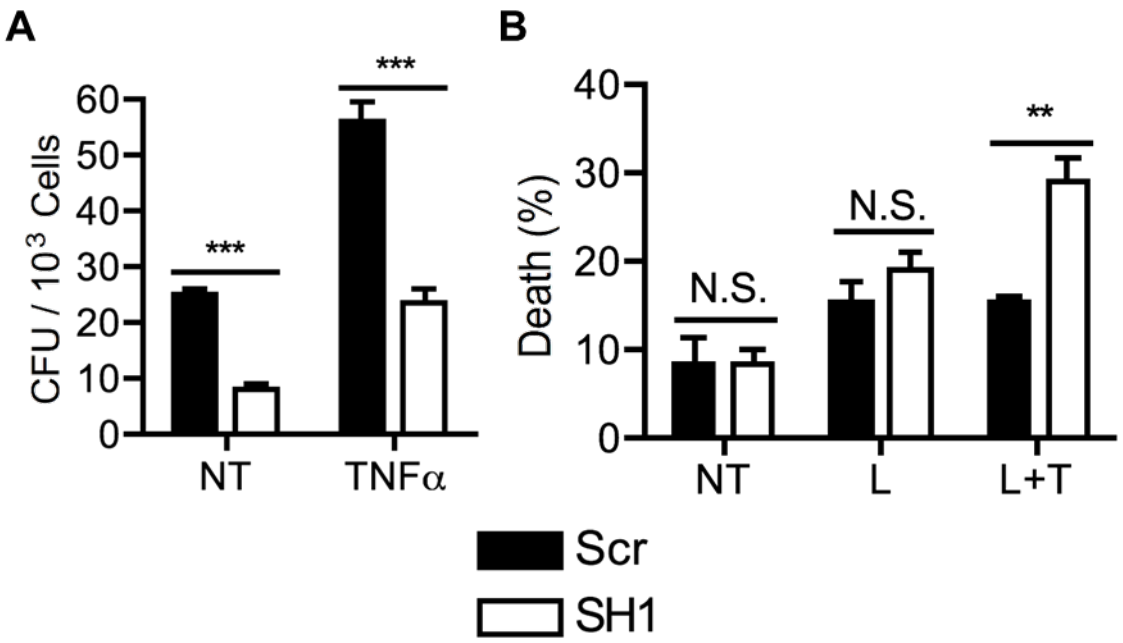

Figure 24. Listeria infection was reduced in TNFAIP8 knockdown cells and cell death was increased

(A) *L. monocytogenes* infection of control and TNFAIP8 knockdown Hepa1-6 cells was measured in a colony formation assay. Cells were infected with 50 MOI of *L. monocytogenes* for 1 hour with 50 ng/ml of TNFα or without (NT). Cells were washed and then treated with 150 μg/ml Gentamycin for 30 minutes to kill extracellular bacteria before being lysed to release the intracellular bacteria. (B) Hepa1-6 TNFAIP8 knockdown and control cells were challenged with *L. monocytogenes* (L) at 60 MOI with or without 50 ng/ml of TNFα (T) for 16 hours. Death was quantified by flow cytometry after Annexin V staining. The results are representative of at least two independent experiments. N.S., not significant; ** $P < 0.01$; *** $P < 0.001$.

5.2.3 TNFAIP8 associates with RAC1 with a higher affinity towards active RAC1-GTP in vitro.

RAC1 is an important regulator of cell death following TNFα stimulation, and TNFAIP8 has been found to protect against TNFα-induced cell death (Jin et al. 2008; Laliberté et al. 2010; Kumar et al. 2004; Kumar et al. 2000; Kim et al. 2007). RAC1 has also been found to regulate *L. monocytogenes* invasion into endothelial cells (Wang et al. 2012; Schmeck et al. 2006). So we tested whether Flag-tagged TNFAIP8 associates with HA-tagged RAC1by co-transfection of their corresponding plasmids. We immunoprecipitated Flag-tagged TNFAIP8 and blotted for HA and found that TNFAIP8 and RAC1 co-immunoprecipitated (Figure 25A). We then tested whether this interaction was dependent on a particular region of RAC1. We created several HA-tagged Rac1 mutants and transfected them into 293T cells along with Flag-tagged TNFAIP8. We pulled down Flag and blotted for HA and found that RAC1 association was impaired with the Δ162-192 RAC1 mutant, which lacks the polybasic region (PBR) and CAAX motif (Figure 25A). The PBR is critical for RAC1 interaction with certain proteins and proper cellular localization (Modha et al. 2008; Williams 2003). The CAAX motif is responsible for post-translational modification and is crucial for the proper function of RAC1 (Casey et al. 1989; Kreck et al. 1994). We then tested whether TNFAIP8 can associate with endogenous RAC1 by transfecting 293T cells with a plasmid encoding Flag-tagged TNFAIP8 and immunoprecipitating cell lysates with anti-RAC1 antibody or control IG and blotted for Flag- TNFAIP8 (Figure 25B). We found that plasmid expressed TNFAIP8 appeared to co-immunoprecipitated with endogenous RAC1 although conclusions about an endogenous interaction cannot yet be reached.

To test whether TNFAIP8-RAC1 interaction is dependent on the activation status of RAC1, we loaded 293T lysate expressing Myc-tagged RAC1 and Flag-tagged TNFAIP8 with non-hydrolyzable forms of GTP (GTPγS) and GDP (GDPβS). We found increased TNFAIP8 association with GTPγS loaded RAC1, suggesting that TNFAIP8 preferentially associates with the active form of RAC1 (Figure 26). These results demonstrate that TNFAIP8 associates with active RAC1 and that this association may be responsible for the TNFAIP8 effect in *L. monocytogenes* infection.

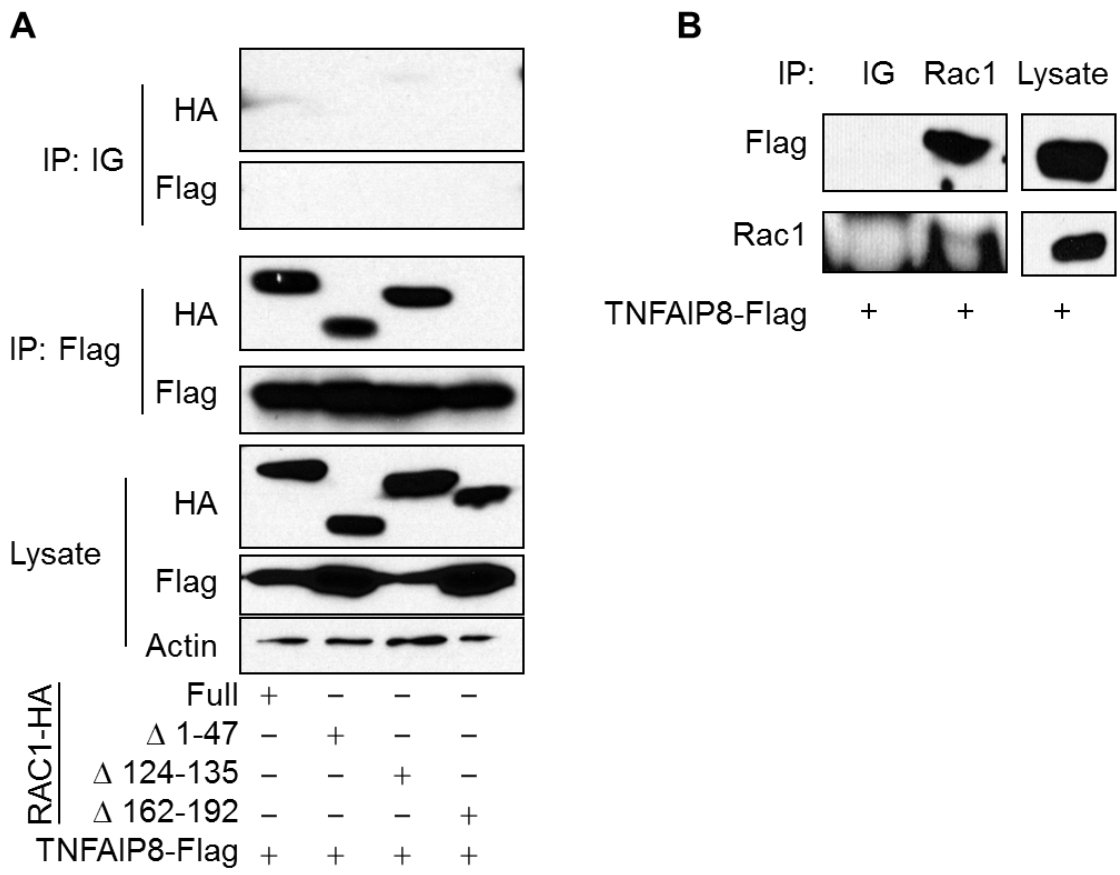

Figure 25. Overexpressed TNFAIP8 associates with RAC1

(A) HA-tagged RAC1 mutants were co-expressed with Flag-tagged TNFAIP8 in 293T cells, and IP was performed using anti-Flag. Western blots were developed using anti-HA antibody to identify RAC1 mutants co-immunoprecipitated with TNFAIP8. (B) 293T cells were transfected with the pRK5 expression plasmids containing Flag-tagged TNFAIP8. Anti-RAC1 antibody or mouse IG was used to immunoprecipitate the Flag-tagged TNFAIP8 and Western blotting was performed using the indicated antibodies. The results are representative of at least two independent experiments.

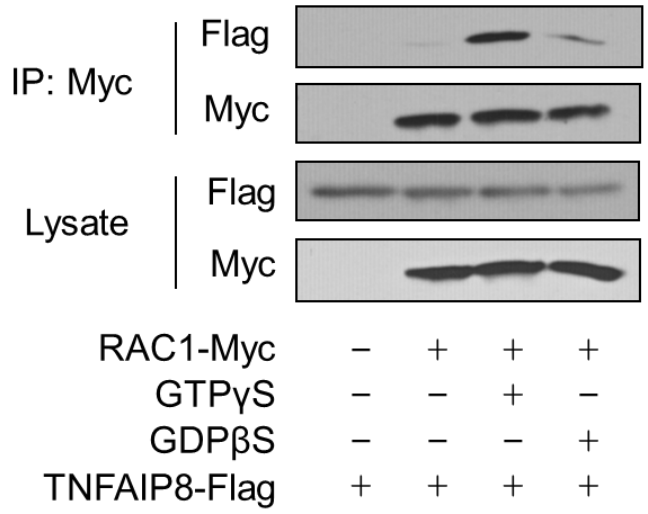

Figure 26.TNFAIP8 has a stronger association with GTPγS loaded RAC1

293T cells were cotransfected with Flag-tagged TNFAIP8 and Myc-tagged RAC1 or empty pRK5 vector. Total cell lysates were then treated with or without GTPγS or GDPβS, and anti-Myc antibody was used to immunoprecipitate RAC1. TNFAIP8 and RAC1 were determined by Western blotting. The results are representative of at least two independent experiments.

5.2.4 TNFAIP8 affects RAC1 activity

To test whether there is a difference in RAC1 activation, PAK-GST beads were used to pull down RAC1. PAK only binds to active RAC1-GTP and is routinely used to assay for RAC1 activity. Knockdown and control cells were stimulated with TNFα for 5 minutes and the amount of RAC1 pulled down with PAK-GST beads was compared (Figure 27A). We found dysregulated RAC1 activation in TNFAIP8 knockdown cells, with higher basal levels of RAC1-GTP, but limited induction following TNFα stimulation. Next we checked whether there were any differences in RAC1 trafficking to the membrane and found impaired RAC1 translocation following TNFα stimulation (Figure 27B). In control cells, TNFAIP8 seemed to primarily localize to the cytosol, but surprisingly, there was more TNFAIP8 on the membrane than in the cytosol in the knockdown cells.

We tested whether the differences in knockdown cell death are RAC1-dependent by transfecting cells with plasmids expressing constitutively active RAC1-61L or inactive RAC1-17N and treated them with TNFα and cycloheximide (CHX) (Figure 28A). We found that the dominant negative mutant partially rescued cell death in both cell lines while the constitutively active mutant greatly sensitized the control cells to death, but had a more limited effect on the knockdown cells (Figure 28B).

We then attempted to determine a working concentration of RAC1 inhibitor that would eliminate the differences in the amount of active RAC1 at basal levels without completely inhibiting RAC1 activity. We pretreated TNFAIP8 knockdown and control cells with varying concentrations of the inhibitor and performed a PAK-GST pull down. We found that the lowest dose tested was effective at reducing the differences at basal levels (Figure 29A). To investigate the relevance of RAC1 signaling to *L. monocytogenes* invasion of the knockdown cells and whether the RAC1 inhibitor will have an effect, we infected TNFAIP8 knockdown and control cells with *L. monocytogenes*, with or without RAC1 inhibitor pretreatment. We found that the inhibitor protected both knockdown and control cell lines from bacterial invasion, and negated any differences between the cell lines at the highest concentration (Figure 29B).

Our results suggest that RAC1 activation is dysregulated in TNFAIP8-deficient cells, which likely contributes to the altered response to *L. monocytogenes* infection and cell death, but the exact mechanism is still unknown.

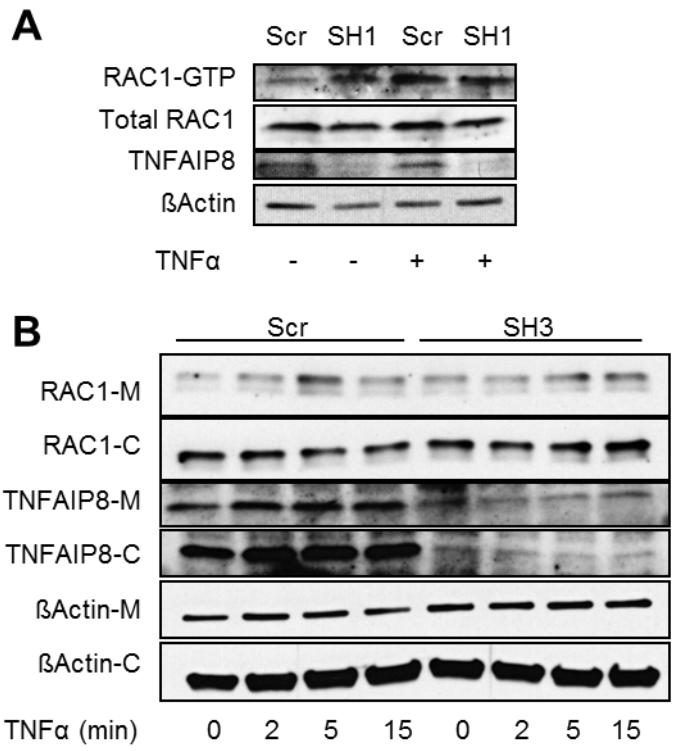

Figure 27. Impaired RAC1 activation in TNFAIP8 knockdown cells

(A) The levels of RAC1-GTP was determined by a PAK-GST pull down assay for control and TNFAIP8 knockdown Hepa1-6 cells stimulated with or without 50 ng/ml of TNFα for 5 minutes. (B) Control and TNFAIP8 knockdown Hepa1-6 cells were stimulated with 50 ng/ml of TNFα for 0, 2, 5, or 15 minutes. Lysates were fractionated into membrane [M] and cytoplasmic [C] portions, and TNFAIP8, RAC1, and actin were detected by Western blot.

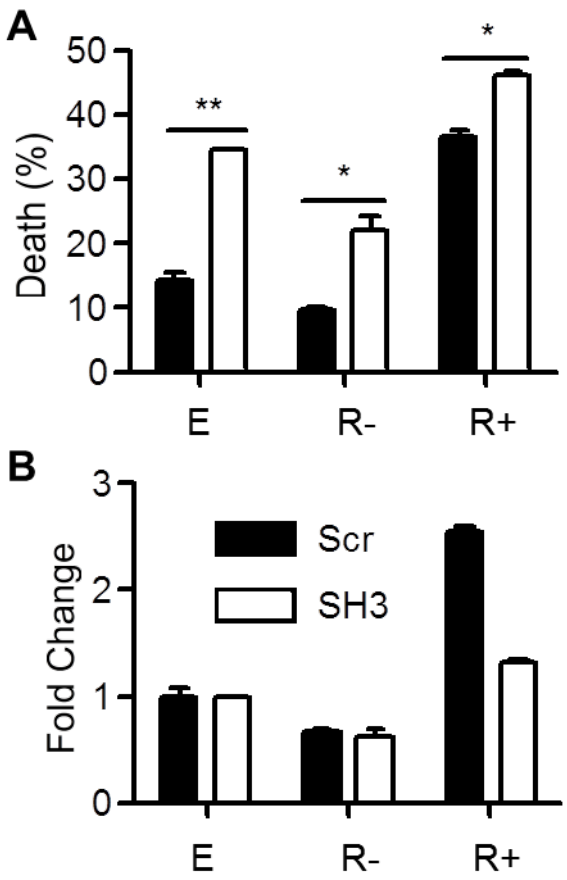

Figure 28. RAC1 mutants did not rescue cell death in TNFAIP8 knockdown cells

(A) Control and TNFAIP8 knockdown Hepa1-6 cells were transiently transfected with pEGFP vectors containing either the EGFP cDNA alone [E], or EGFP plus RAC1-17N [R-] or RAC1-61L [R+] cDNAs. Cells were treated with 5 ng/ml of TNFα and 20 ng/ml of cycloheximide for 6 hours. EGFP positive cells were gated by flow cytometry and cell death was detected by Annexin V and 7-AAD staining. (B) Fold difference in cell death is shown relative to EGFP control. The results are representative of at least two independent experiments. * $P < 0.05$; ** $P < 0.01$

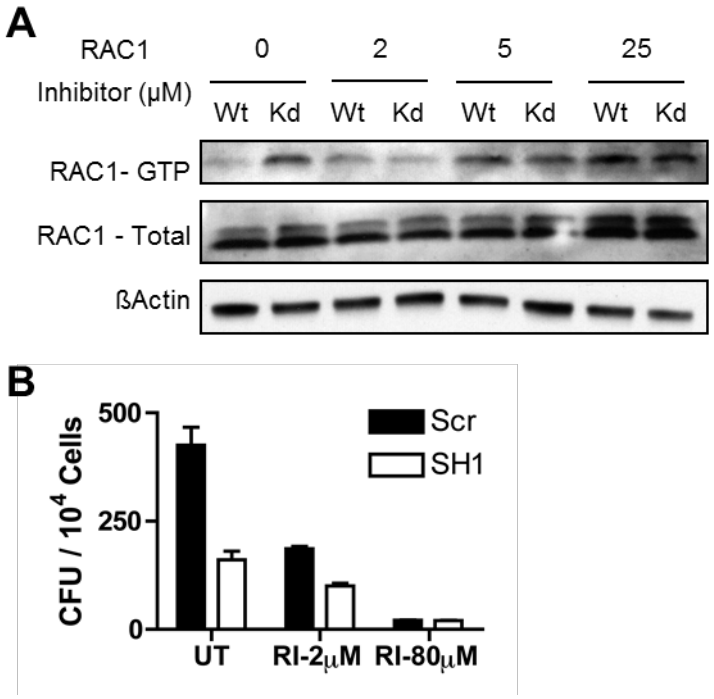

Figure 29. RAC1 inhibition reduced bacterial infection

(A) The levels of RAC1-GTP was determined by a PAK-GST pull down assay for control (Wt) and TNFAIP8 knockdown (Kd) Hepa1-6 cells treated with the indicated amounts of RAC1 inhibitor II for 3 hours. (B) Hepa1-6 control (Scr) and TNFAIP8 knockdown (SH1) cells were infected with 50 MOI of L. Monocytogenes after no pretreatment (UT) or pretreatment with indicated amounts of RAC1 inhibitor II (RI) for 3 hours before infection.

5.3 Discussion

The role of TNFAIP8 in cell death appears to be condition-dependent. In most tumor cells, a protective effect of TNFAIP8 is reported; however in thymocytes, TNFAIP8 has been found to promote gluococorticoid-induced apoptosis (Woodward et al. 2010). The molecular pathways of cell death that are regulated by TNFAIP8 are still largely unknown. Previous work suggests that TNFAIP8 protects against death through its 'death effector domain (DED)', but that hypothesis has been largely debunked by the structural analysis of TIPE2, which revealed a novel fold instead of a DED domain (Kumar et al. 2004; Zhang et al. 2009). Others have suggested that TNFAIP8 works through Gα(i) proteins, and have shown that TNFAIP8 is important for the function of dopamine-D2short receptors in protecting against TNFα-induced cell death (Laliberté et al. 2010). Recently, TIPE1, another TNFAIP8 family member, has been implicated in activating mTOR to protect against oxidative stress in dopamine neurons by blocking autophagy (Choi et al. 2010). The mTOR protein itself plays an important role in promoting cancer, protecting against cell death, and increasing susceptibility to lethal *L. monocytogenes* infection, and has also been found to be regulated by the small GTPase RAC1 (Strimpakos et al. 2009; Weichhart et al. 2008; Saci et al. 2011). We show here that TNFAIP8 associates with RAC1 and regulates TNFα-induced cell death and *L. monocytogenes* infection in Hepa1-6 cells. We propose that it does so by regulating RAC1 signaling.

We observed an increase in phosphorylated AKT at serine 473 in TNFAIP8 knockdown cells, suggesting that mTOR may also be regulated by TNFAIP8. As AKT is important in cell survival and proliferation, we also examined proliferation in TNFAIP8 knockdown Hepa1-6 cells (Zheng et al. 2014; Kim & Chung 2002). Proliferation was slightly increased, which is supported by the AKT results, but the difference was very slight and may have been due to random chance and as a result, an artifact. TNFAIP8's effect on proliferation may be more pronounced in primary cells, as in the knockout epithelium, which actually showed the opposite phenotype in Chapter 4. This discrepancy is likely due to differences in cell type or conditions, or from the limitations of the

knockdown methodology. Similar to the observed increase in epithelial cell death to DSS induced colitis, TNFAIP8 knockdown cells were more susceptible to TNFα induced-death as described previously in cancer cells. Increased AKT phosphorylation did not predict susceptibility and is likely unrelated.

The knockdown cells, while more susceptible to apoptosis, were resistant to infection by *L. monocytogenes*. In addition, while there was no increase in cell death following incubation with the bacteria compared to control cells, TNFα greatly stimulated cell death in knockdown cells but not in controls. Intracellular bacterial pathogens such as *L. monocytogenes, Shigella flexneri,* and *Salmonella typhimurium* have been shown to enhance TNFα-induced cell death in L cells (Klimpel et al. 1990). This suggests that the knockdown cells are sensitized to TNFα-induced cell death following bacterial infection, and it is supported by published data reporting the importance of TNFα function for controlling lethal *L. monocytogenes* infection (Carr et al. 2011; Abreu et al. 2013; Peña-Sagredo et al. 2008). Literature on the pathogenesis of *L monocytogenes* report that its entire lifecycle can occur within host cells and that the bacteria can spread directly to neighboring cells through the formation of pseudopods by the infected cells, and that the apoptotic clearance and isolation of infected hepatocytes by immune cells is important in preventing the spread of the infection (Vázquez-Boland et al. 2001). This led us to hypothesize that TNFAIP8 knockout mice were more resistant to lethal *L. monocytogenes* infection than wild type mice due to a combined effect of reduced bacterial cell invasion and a heightened sensitivity to cell death by inflammatory cytokines of the host cells that helped stall a productive bacterial infection sufficiently to give the knockout mice a survival advantage.

RAC1 has been implicated in regulating mTOR complexes by its interaction with mTORC1 and mTORC2 proteins (Saci et al. 2011). In addition, TIPE2 has been found to associate with RAC1 (Wang et al. 2012). This led us to investigate whether TNFAIP8 effector functions are dependent on RAC1 signaling because both RAC1 and mTOR are implicated in *L. monocytogenes* invasion of cells and apoptosis as well as apoptosis in intestinal epithelium (Weichhart et al. 2008; Schmeck et al. 2006; Jiwani et al. 2012; Jin et al. 2006). We showed that

TNFAIP8 associates with RAC1 by co-immunoprecipitation of plasmid expressed TNFAIP8 with endogenous RAC1. We were forced to use transiently expressed TNFAIP8 tagged with the Flag peptide due to the very weak binding affinity of the commercial TNFAIP8 antibodies. There were difficulties in the immunoprecipitation and visualization of the endogenous RAC1 due to its proximity to the small IGG chain, so it is difficult to come to a definitive conclusion of TNFAIP's endogenous interaction with RAC1, and additional work needs to be done to show that this interaction is not an artifact of the over-expression system. We further found that the TNFAIP8 association is dependent on the C-terminus of RAC1, which contains the PBR and CAAX motif. These two regions are responsible for protein interactions, membrane localization signaling, and post-translational modifications. Our finding that the association is dependent on whether RAC1 is primed with GTP or GDP, suggests that RAC1 interaction with TNFAIP8 is enhanced with RAC1 activation.

After testing several conditions using TNFAIP8 knockdown cells, we were able to conclude that RAC1 is an important mediator of TNFα-induced cell death but we were unable to determine precisely how or whether RAC1 is directly involved with TNFAIP8 during these processes. By introducing the dominant negative mutant of RAC1, TNFα-induced cell death in TNFAIP8 knockdown cells is partially rescued. Furthermore, the constitutively active mutant had a stronger effect on the control cells than they did on the knockdown cells, presumably because the RAC1 signaling was already heightened in knockdown cells. We observed increased RAC1-GTP at basal levels in knockdown cells and enrichment of TNFAIP8 in the membrane fraction when compared to the controls. The localization of TNFAIP8 in knockdown cells suggests that the membrane association is crucial for proper cell function and that it may be related to the increased RAC1 activation. This led us to the hypothesis that TNFAIP8 knockdown cells are more susceptible to TNFα-induced cell death because there is insufficient TNFAIP8 present to inhibit RAC1.

Bacterial entry into knockdown cells is inhibited in spite of increased RAC1 activation and reports that RAC1 activation is necessary for bacterial entry (da Silva et al. 2012; Schmeck et al.

2006). We used a cell permeant RAC1 inhibitor (Z62954982) that functions by blocking the RAC1 interaction with several of its activating GEFs (Ferri et al. 2009). We attempted to titrate the RAC1 inhibitor to a minimal concentration that would reduce the amount of activated RAC1 in TNFAIP8 knockdown cells to the amount present in control cells at basal conditions. The purpose was to find a concentration that would not impede bacterial entry but be sufficient to test the role of RAC1 in *L. monocytogenes* invasion of TNFAIP8 knockdown cells. A surprising result was that the Rac1 inhibitor did not reduce the amount of basal RAC1 pulled down. A possible explanation may be that alternative pathways are activating RAC1 that are stimulated by the inactivation of the classical RAC1 pathway. A more likely explanation is that the inhibitor is more effective at blocking activation as opposed to basal activity states, and published literature for Z62954982 still detects RAC1-GTP at basal levels, and the closely related NSC23766 RAC1 inhibitor is also only effective at inhibiting RAC1 activation but not inhibiting basal states (Yu et al. 2012; Veluthakal et al. 2009; Yuan et al. 2010). The actual effectiveness of the inhibitor concentrations may need to be determined by attempting to stimulate RAC1 activation. The RAC1 inhibitor did, however, inhibit bacterial invasion of both knockdown and control cells and eliminated the differences in bacterial counts, suggesting that RAC1 may be responsible for the differences in bacterial invasion. A possible explanation for the reduced bacterial invasion of knockdown cells in the face of elevated RAC1 activation is that RAC1 is activated nonspecifically throughout the cellular membrane in TNFAIP8 deficient cells, increasing ROS production and activating molecular pathways that do not require localized activation. Functions that require localized activation, like the internalization of a *L. monocytogenes* cell on a host membrane, may be inhibited because there is too much background RAC1 activation to efficiently recruit the required proteins to a specific location and internalize the bacterium. An alternative explanation is that infected TNFAIP8 knockdown cells are dying rapidly, reducing the total number of bacteria protected from the gentamycin treatment. This explanation, however, seems unlikely due to the short incubation time with *L. monocytogenes* for the infection experiments, and that no difference in death induced by *L. monocytogenes* was observed after 24 hours compared to control cells.

In this chapter, we learned that TNFAIP8 regulates cell death and *L monocytogenes* infection in Hepa1-6 cells, and that altered RAC1 function may be responsible. Understanding the molecular interaction of TNFAIP8 with RAC1 is important in identifying therapeutic targets for treating a wide variety of diseases, including inflammatory disease, bacterial infections and cancer, and it should be studied in the future.

Chapter 6 – Discussion and future directions

Listeria monocytogenes pathogenesis is dependent on a number of factors related to both the immune and non-immune systems. A cascade of TNFα and other cytokines is released by infected cells to trigger an immune response to the infected liver (Pamer 2004; Vázquez-Boland et al. 2001; Rogers et al. 1996; Santos et al. 2005). If the pathogen is not controlled by the immune system, *L. monocytogenes* will escape to other organs and the host can die of sepsis. Protecting immune cells from death has been found to be an important aspect in preventing lethal *L. monocytogenes* infection (Zheng et al. 2004; Gurung et al. 2011). But promoting cell death in hepatocytes or blocking cellular invasion might be equally important for the survival of the organism (dos Santos et al. 2011; Santos et al. 2005; Schmeck et al. 2006). In addition, cell death in the colonic epithelium during colitis can exacerbate the inflammatory response and increase susceptibility to infection (Araki et al. 2010; Kassam et al. 2014). The epithelial barrier integrity in the gastrointestinal system is crucial for protecting against environmental insults, including toxins, antigens, and microbes (DeMeo et al. 2002). Increased epithelial cell death and decreased proliferation is associated with increased severity of DSS-induced colitis in mice (Williams et al. 2013).

In chapter 3 we found that TNFAIP8 knockout mice were resistant to lethal *L. monocytogenes* infection, and that this resistance is likely due to the non-immune system. In chapter 4 we found that the TNFAIP8 knockout mice were more susceptible to DSS induced colitis and once again that this was likely due to the non-immune system. More specifically, we determined that it was likely due to the increased death and reduced proliferation of the epithelial cells in the colon. Finally, in chapter 5 we showed that TNFAIP8 regulates cell death and *Listeria* invasion into Hepa1-6 cells and that these processes may be due to the impaired function of RAC1 in TNFAIP8 deficient cells. This led us to the conclusion that the phenotypes in the two disease models can be explained by a common mechanism in inflammation. We therefore

hypothesize that TNFAIP8 is a novel regulator of inflammation, and that the phenotypes observed in the disease models stem from differences in cell death and bacterial invasion into non-immune cells (Figure 30).

Here, we propose that TNFAP8 regulates TNFα-induced cell death by its inhibition of RAC1 (Figure 31). The TNFα signaling cascade activates pro-death pathways through caspase cleavage and ROS production by the NAPDH oxidase complex, and pro-survival pathways through the activation of the NF- κB transcription factor. Inhibition of RAC1 and the associated NADPH oxidase activation has been documented to protect cells against both apoptotic and necrotic cell death following TNFα stimulation (Jin et al. 2008; Kim et al. 2007). TNFAIP8 expression is reported to be up-regulated by TNFα signaling through NF-κB (Kumar et al. 2000; You et al. 2001). Our findings suggest that NF- κB can protect against cell death by preventing ROS production through RAC1 inhibition by the expression of TNFAIP8. Additional work needs to be done to confirm this hypothesis and investigate the other downstream effects of a TNFAIP8 regulated RAC1 pathway, including F-actin polymerization and ROS production. In addition, TNFAIP8 association with other RHO GTPases should also be explored.

We also propose that RAC1 regulation by TNFAIP8 is important for *L. monocytogenes* invasion of host cells. *L. monocytogenes* Internalin A and B surface proteins bind to MET (also known as the hepatocyte growth factor receptor), β-integrin and E-cadherin receptors to activate RAC1 and induce F-actin cytoskeletal remodeling that allow for bacterial entry into the cells (Figure 32) (da Silva et al. 2012). We hypothesize that TNFAIP8 functions to reduce background RAC1 activation, allowing for precise control of RAC1 signaling. Insufficient TNFAIP8 may interfere with this aspect of bacterial infection by preventing localized RAC1 signaling at the site of bacterial entry thus preventing the recruitment of F-actin and inhibiting bacterial internalization (Figure 33). This model needs to be fully investigated and the extent of the RAC1 dysfunction in bacterial invasion of TNFAIP8 deficient cells needs to be scrutinized. Double knockout mice for TNFAIP8 with a conditional RAC1 knockout in non-lymphoid tissues may be used to investigate the importance of RAC1 in TNFAIP8 regulation of listeriosis. RAC1 knockout mice should be

resistant to infection, but if a difference is observed between the TNFAIP8 and RAC1 double knockout mice, and single RAC1 knockout mice, then it is possible that TNFAIP8 is part of an alternative pathway regulating bacterial invasion and cell death in mice. This can be tested in vitro using RAC1 inhibitors and mutants (as has been partially explored in chapter 5), but the results are not as conclusive due to the artificial nature of the systems.

Contrary to the differences observed with *L. monocytogenes* invasion of the Hepa 1-6 cell lines, phagocytosis by TNFAIP8 knockout bone marrow-derived macrophages showed no abnormalities. One possible explanation is that the lack of TNFAIP8 in the knockout macrophages is compensated by the presence of TIPE2. TIPE2 protein is undetectable in Hepa 1-6 cells (data not shown), but is expressed in macrophages where it regulates phagocytosis (Wang et al. 2012). A second potential explanation is that the molecular mechanism for phagocytosis of *L. monocytogenes* in macrophages is sufficiently different from the endocytosis of *L. monocytogenes* in Hepa 1-6 cells that TNFAIP8 deficiency can affect one but not the other. There are several different forms of endocytosis, and *L. monocytogenes* has been shown to take advantage of Clathrin-dependent MET receptor-mediated endocytosis to gain entry into the cell (Veiga & Cossart 2005).

Perhaps the most surprising result from this work is the contrast in the function of TNFAIP8 and its closely related family member, TIPE2. TIPE2 is a novel regulator of inflammation and studies have reported its effects on migration, phagocytosis and oxidative burst in immune cells, resistance to bacterial infections, inflammation, and resistance to experimental colitis (Sun et al. 2008; Wang et al. 2012; Gus-Brautbar et al. 2012; Lou et al. 2014). While both TNFAIP8 and TIPE2 knockout mice are resistant to lethal *L. monocytogenesis* infection, TNFAIP8 knockout immune cells do not exhibit any detectable differences in phagocytosis, cytokine production or chemotaxis, and TNFAIP8 knockout mice do not show any differences in susceptibility to lethal *Staphylococcus aureus* infection. TNFAIP8 knockout mice are also more susceptible to DSS-induced colitis, unlike TIPE2 knockout mice, which are resistant. In addition, TIPE2 bone marrow chimeras suggest that the immune cells as the cause of the resistance of

TIPE2 knockout mice to DSS-induced colitis (Lou et al. 2014). Differences in immune function may be due to compensation of the lack of TNFAIP8 with TIPE2, and their contrasting effects may be due to different expression profiles. Whereas TNFAIP8 is widely expressed throughout the organism, TIPE2 is largely limited to immune cells, and my study here does point to non-immune cells as the culprit to the different outcomes of the disease models in TNFAIP8 knockout mice. On the other hand, TIPE2 is reported to be a promoter of cell death, the opposite of TNFAIP8. These two proteins may potentially function as antagonists to one another to maintain homeostasis within the organism, but this hypothesis needs considerable study and review. Then there are the lesser known TNFAIP8 family members, TIPE1 and TIPE3, which may have other contrasting roles that have yet to be fully understood. What is known for certain, however, is that TNFAIP8 is different from TIPE2, and that both of these molecules are important to inflammatory diseases, and further study is needed on the entire TNFAIP8 family.

The work presented here attempts to describe the importance of TNFAIP8 in inflammation and hopes that understanding the biology might aid studies in treating or abrogating inflammation in patients. Autoimmune diseases are very common and are estimated to occur in 7.6%-9.4% of the population and predispose the patients to certain cancers (Cooper et al. 2009; Mantovani & Balkwill 2006). Treatments typically involve anti-inflammatory drugs, immune-suppressive glucocorticoid steroids, non-steroidal drugs, hormone therapy, intravenous immunoglobulin injections, and novel treatments including antibody depletion of inflammatory cytokines such as TNFα (Dinarello 2010; Danieli et al. 2014; Willrich et al. 2014). Furthermore, the treatment of these disorders often comes at a price of increased susceptibility to microbial infections (Kassam et al. 2014; Abreu et al. 2013). Understanding these processes is necessary to develop novel, more effective and safer treatment methods and it requires the dissection of the molecular pathways involved. What I have uncovered in this study is a novel regulator of inflammation that was once thought to be predominantly important in cancer. I discovered that TNFAIP8 seems to regulate inflammation through non-immune cells and it is important to note that non-immune cells have crucial roles in the inflammatory response. Non-hematopoietic

derived cells such as intestinal epithelial and stromal cells have been shown to be important in regulating inflammation, possibly through the NF-κB pathway, to maintain a healthy immune homeostasis within the mucosa of the intestines that when disrupted could lead to inflammation or increased susceptibility to infection (Zaph et al. 2007; Pasparakis 2012; Owens & Simmons 2013). TNFAIP8, an NF-κB regulated protein, could potentially explain how and why these particular cells are important to inflammation, and lead to further understanding of the impact of other non-immune cells to inflammatory diseases (You et al. 2001). New potential regulators of the inflammatory response, like TNFAIP8, should be thoroughly investigated because they may hold the key to developing novel drugs and treatments for inflammatory diseases, infectious diseases, and even cancers worldwide.

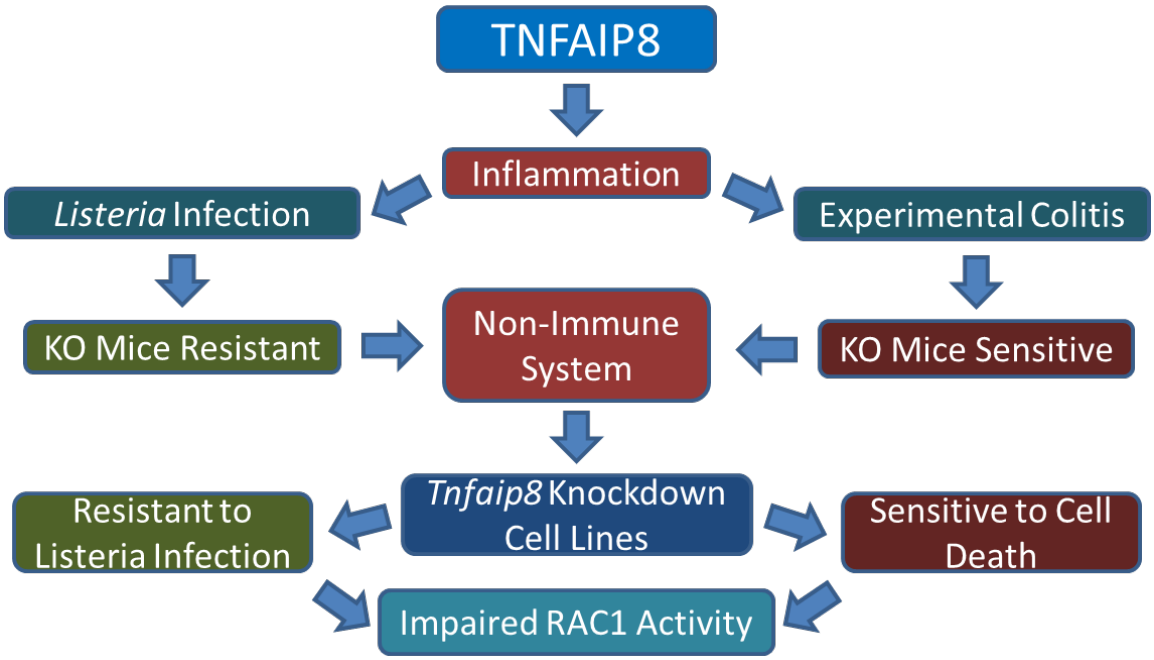

Figure 30. TNFAIP8 project summary

TNFAIP8 is a novel regulator of inflammation. It affects the outcome of two inflammatory diseases in TNFAIP knockout mice, increasing resistance to lethal *Listeria monocytogenes* infection and increasing susceptibility to DSS-induced experimental colitis. Both of these effects appear to be due to differences in the non-immune system, which was investigated in TNFAIP8 knockdown cell lines. It was found that these cell lines were resistant to infection and had increased susceptibility to cell death and that these effects may be due to impaired RAC1 activity. The increased cell death likely lead to exacerbated colitis in knockout mice, and the increased cell death and decreased susceptibility to infection by *L. monocytogenes* probably resulted in increased resistance to lethal *Listeria* infection in knockout mice.

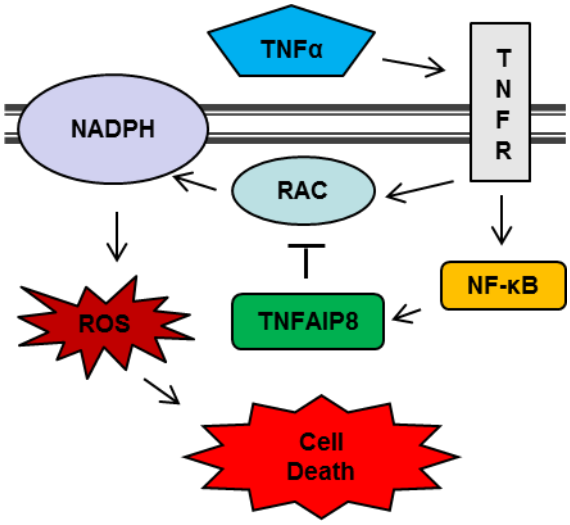

Figure 31. Model of TNFAIP8 molecular function in cell death

TNFα activates the tumor necrosis factor receptor (TNFR), which results in RAC1 and NF-κB activation. RAC1 then activates the NAPDH oxidase complex to generate reactive oxygen species (ROS) that promote cell death. The NF-κB transcription factor promotes TNFAIP8 expression to inhibit RAC1 activation and protect against cell death.

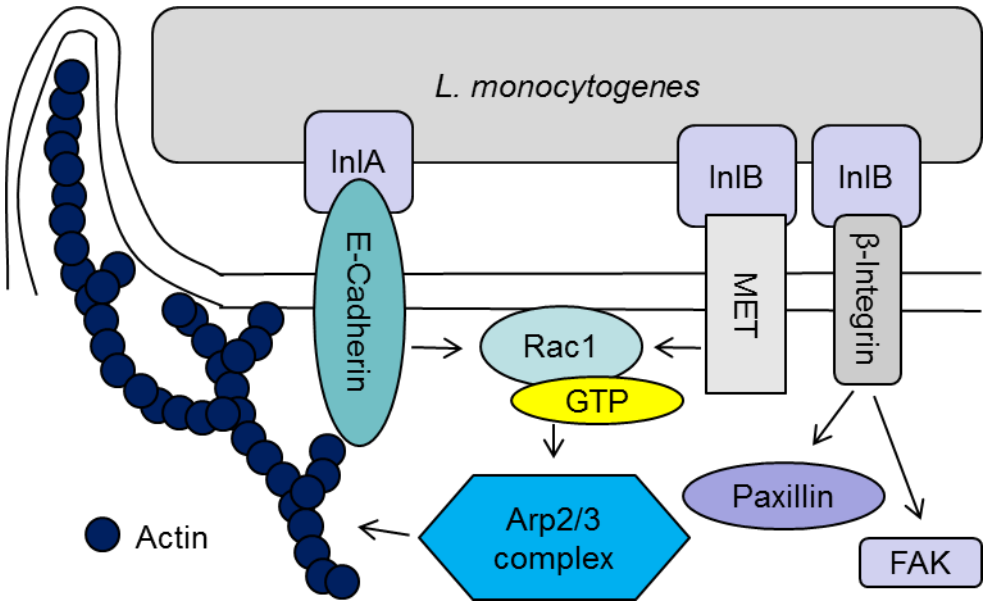

Figure 32. Model of RAC1 molecular function during *Listerial* cell invasion

(A) *Listeria* Internalin A and B (InlA, InlB) surface proteins bind to host cell receptors to activate local cytoskeletal remodeling to allow *L. monocytogenes* to internalize itself into the host. RAC1 activation of the ARP2/3 complex is responsible for actin polymerization that engulfs the bacteria on the cell membrane, thus allowing entry into the cell.

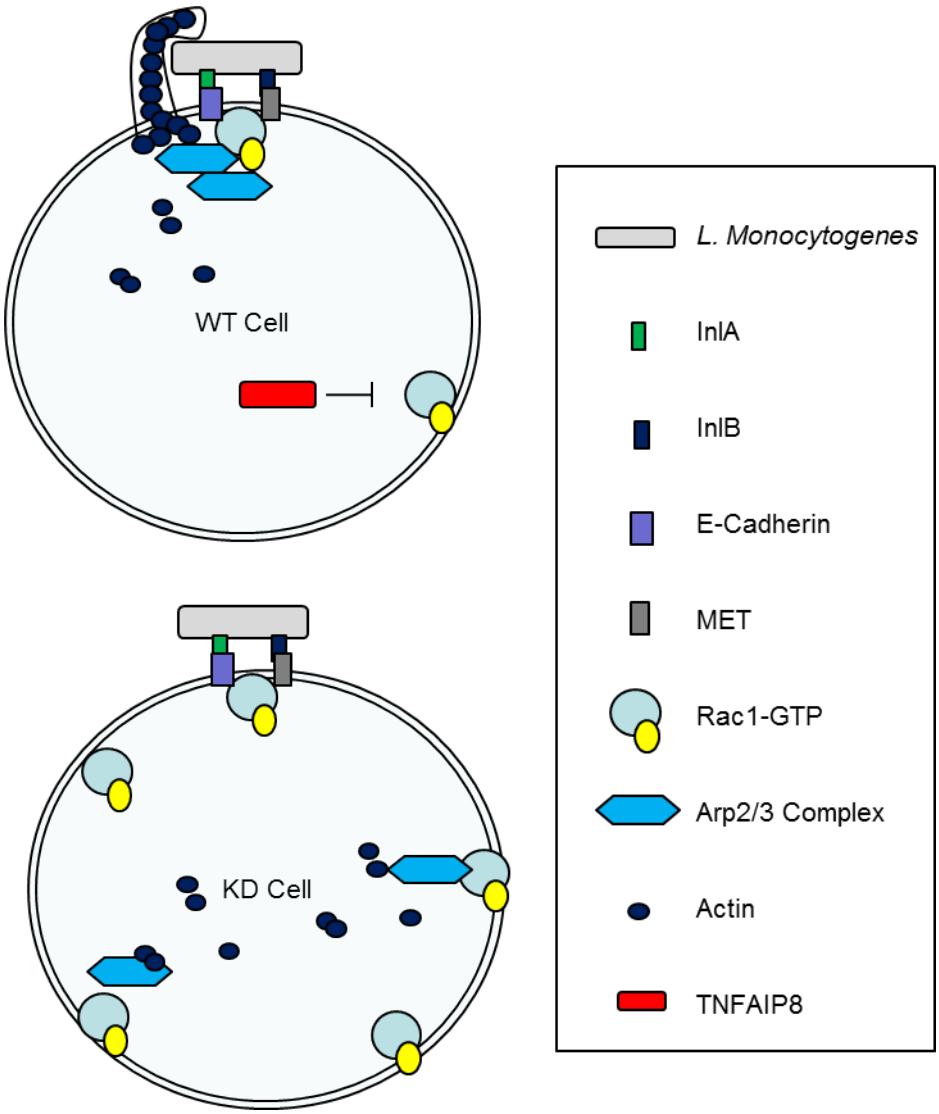

Figure 33. Model of TNFAIP8 role in host cell invasion by *L. monocytogeneses*

TNFAIP8 inhibits nonspecific RAC1 activation in the background of normal cells. *Listeria* attachment to the surface of wild type cells (WT) precisely activates RAC1 on the surface of the membrane allowing efficient entry. TNFAIP8 knockdown cells (KD) have high background levels of RAC1 activation, thereby reducing the efficiency of bacterial entry into the host.

CHAPTER 7 – Publications

The following papers have been published or are submitted for publication in support of this dissertation.

7.1 Crucial roles of TNFAIP8 protein in regulating apoptosis and *Listeria* infection.

Thomas P. Porturas, Honghong Sun, George Buchlis, Yunwei Lou, Xiaohong Liang, Derek S. Johnson, Zhaojun Wang, Youhai H. Chen. Submitted to the Journal of Immunology.
*This paper contains the majority of the work in Chapters 3 and 5 of this dissertation

Abstract

TNFα-induced protein 8 (TNFAIP8 or TIPE) is a newly described regulator of cancer and infection. However, its precise roles and mechanisms of actions are not well understood. We report here that TNFAIP8 regulates *Listeria monocytogenes* infection by controlling pathogen invasion and host cell apoptosis in a RAC1 GTPase-dependent manner. TNFAIP8 knockout mice were found to be resistant to lethal *L. monocytogenes* infection and had reduced bacterial load in the liver and spleen. TNFAIP8-knockdown in murine liver HEPA1-6 cells increased apoptosis, reduced bacterial invasion into cells, and resulted in dysregulated RAC1 activation. TNFAIP8 could translocate to plasma membrane and preferentially associate with activated GTP-RAC1. The combined effect of reduced bacterial invasion and increased sensitivity to TNFa-induced clearance likely protected the TNFAIP8 knockout mice from lethal listeriosis. Thus, by controlling bacterial invasion and the death of infected cells through RAC1, TNFAIP8 regulates the pathogenesis of *L. monocytogenes* infection.

7.2 Exacerbated Experimental Colitis In TNFAIP8-deficient Mice.

Honghong Sun, Yunwei Lou, **Thomas Porturas**, Samantha Morrissey, George Luo, Ji Qi, Qingguo Ruan, and Youhai H. Chen. Submitted to the Journal of Immunology.

*This paper contains the majority of the work in Chapter 4 of this dissertation

Abstract

The TNF-α-induced protein 8 (TNFAIP8 or TIPE) is a risk factor for lymphoma genesis and bacterial infection, and is upregulated in a number of human cancers. However, its physiological and pathological functions are unclear. Here we report the generation of TIPE-deficient mice and describe their increased responsiveness to colonic inflammation. TIPE-deficient mice were generated by germ line gene targeting and were born without noticeable developmental abnormalities. Their major organs including lymphoid organs and intestines were macroscopically and microscopically normal. However, after drinking dextran sodium sulfate-containing water, TIPE-deficient mice developed more severe colitis than wild type mice, as demonstrated by decreased survival rates, increased body weight loss, and enhanced leukocyte infiltration, bacterial invasion, and inflammatory cytokine production in the colon. Bone marrow chimeric experiments revealed that TIPE deficiency in non-hematopoietic cells was responsible for the exacerbated colitis in TIPE-deficient mice. Consistent with this result, TIPE-deficient intestinal epithelial cells had increased rate of cell death and decreased rate of proliferation as compared to wild type controls. Taken together, these findings indicate that TIPE plays an important role in maintaining colon homeostasis and in protecting against colitis.

7.3 Critical roles of TIPE2 protein in murine experimental colitis.

Yunwei Lou, Honghong Sun, Samantha Morrissey, **Thomas Porturas**, Suxia Liu, Xianxin Hua, and Youhai H. Chen. (2014). J Immunol 193(3), 1064-1070.

Abstract

Both commensal bacteria and infiltrating inflammatory cells play essential roles in the pathogenesis of inflammatory bowel disease. The molecular mechanisms whereby these pathogenic factors are controlled during the disease are not fully understood. We report here that a member of the TNFAIP8 (tumor necrosis factor-a-induced protein 8) family called TIPE2 (TNFAIP8-like 2, or TNFAIP8L2) plays a crucial role in regulating commensal bacteria dissemination and inflammatory cell function in experimental colitis induced by dextran sodium sulfate (DSS). Following DSS treatment, TIPE2-deficient mice, or chimeric mice that are deficient in TIPE2 only in their hematopoietic cells, lost much less body weight and survived longer than wild type controls. Consistent with this clinical observation, TIPE2-deficient mice exhibited significantly less severe colitis and colonic damage. This was associated with a marked reduction in the colonic expression of inflammatory cytokines such as TNF-α, IL-6, and IL-12. Importantly, the ameliorated DSS-induced colitis in *TIPE2*$^{-/-}$ mice was also associated with reduced local dissemination of commensal bacteria and the systemic inflammatory response. Combined with our previous report that TIPE2 is a negative regulator of anti-bacterial immunity, these results indicate that TIPE2 promotes colitis by inhibiting mucosal immunity to commensal bacteria.

Chapter 8 – References

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