Role of the gene regulators TFEB and TFE3 in Macrophages and B cells

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Acknowledgement Page

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II. KEYWORDS AND ABBREVIATIONS

Keywords:

Autophagy: self-degradation of cellular components in response to various kinds of environmental stress/infection.

Immunophagy: autophagy in immune cells

Phagocytosis: the engulfing of microorganisms or other cells and foreign particles by phagocytes.

LPS: lipopolysaccharide: A model endotoxin derived from cell wall of gram-negative bacteria

Abbreviations:

Ab, Antibody

ATG, Autophagy related gene

B, B cell

BCR, B cell Receptor

b-HLH-Zip, basic helix-loop-helix/Leucine zipper

BM, Bone marrow

BR, basic region

BSA, bovine serum albumin

B/T, B and T cell

CD, Cluster of Differentiation

CD40L, CD40 Ligand

CSR, Class Switch Recombination

dko, TFEB, TFE3 double knockout

E. coli, Escherichia coli

ER, Endoplasmic reticulum

FBS, fetal bovine serum

FITC, Fluorescein isothiocyanate

FO, Follicular

GC, Germinal Center

HSC, Hematopoietic Stem Cell

Ig, Immunoglobulin

IFN-γ, interferon-gamma

IL, Interleukin

IP, Intraperitoneal

KLH, keyhole limpet hemocyanin

ko, knock out

LPS, Lipopolysaccharide

LZ, Leucine zipper

M-CSF, macrophage colony stimulating factor

MHC, Major histocompatibility complex

MZ, Marginal Zone

MiT, Microphthalmia Family of Transcription Factors

Mitf, Microphthalmia associated Transcription Factor

Mi, microphthalmia

mTOR, mammalian target of rapamycin

NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells

NLS, nuclear localization signal

ns, not significant

PAMPs, pathogen-associated molecular patterns

PC, Plasma cell

PerC, Peritoneal cavity

PEC, peritoneal exudate cells

PE, Phycoerythrin

RBC, Red Blood Cell

T, T cell

TCR, T cell receptor

TFEB, Transcription factor EB

TFEB ko, conditional TFEB knock out

TFEC, Transcription EC

TFE3, transcription E3

TD/ TI, Thymus-dependent/ Thymus-independent

TDN, Trans dominant negative

TLR, Toll-like receptor

TNF-α, Tumor necrosis factor- alpha

TNP, trinitrophenol

VEGF, Vascular Endothelial Growth Factor

ABSTRACT:

In multicellular organisms, differential regulation of gene expression is necessary to generate cells with specialized functions and to respond to environmental challenges such as metabolic stress and microbial infection. The experiments in this thesis focus on the study of the gene regulators of the MIT family, TFEB TFE3 and Mitf, in the mammalian immune system. These molecules are transcription factors that share sequence homology, virtually identical DNA binding properties and often functionally overlap. Recently, TFEB has been implicated by others to be the "master regulator" of genes involved in autophagy and lysosomal biogenesis [1]. Autophagy is important for the development and function of the immune system: monocytes must up-regulate autophagy in order to differentiate into macrophages, which then need autophagy for efficient phagocytosis, antigen presentation via MHC II, host antiviral defense, antifungal responses and for defense against gram-negative bacteria[2]. Moreover, autophagy is also required for the survival of B1 cells and plasma cells [3, 4].

Given the importance of autophagy to macrophage function, in Aim1 I studied the role of TFEB and TFE3 in autophagy in macrophage development and function. I studied autophagy activated by metabolic stress and immunological stimuli in vitro using transformed mouse RAW 264.7 macrophages and primary macrophages (derived from the bone marrow (BM) and peritoneal cavity) from mice conditionally deficient in TFEB alone or both TFEB and TFE3 (dko). Using these mice, in a complementary in vivo approach, I evaluated macrophage differentiation using established mouse models for phagocytosis, sterile peritonitis and for endotoxin-induced sepsis. I found that TFEB and TFE3 are both dispensable for monocyte differentiation into macrophages and for stereotypical phagocytotic responses to particulates. However, TFEB- and TFE3-deficient macrophages had impaired proinflammatory cytokine responses such as IL-6 and TNF-α to bacterial endotoxin, indicating a critical role for these molecules in host defense.

In Aim 2, I studied antibody responses and phagocytic properties of B cells in which the MiT proteins have been inactivated. Preliminary studies by a previous student in the laboratory showed that

Marginal Zone (MZ) B cells and B1a B cells are reduced in mice in which TFEB and TFE3 are inhibited in the B lineage. MZ B cells are critical for protective antibody responses against encapsulated bacteria, whereas B1 cells provide important antibody protection to barrier sites such as the peritoneal cavity. In both cases, these B cell populations can respond to non-protein containing antigens without relying on help from CD4 T cells, a type of antibody response known as "thymus-independent" (TI) because CD4 T cells require the thymus to develop. B1 cells are known to depend on autophagy for their development and homeostasis, and B1 cells are known to be phagocytic. In contrast, follicular (FO) B cells, the predominant B cell subset, primarily respond to protein antigens in a CD4 T cell dependent manner ("thymus-dependent," antigens, TD). We observed B cell hyper responsiveness and splenomegaly, which we hypothesize, were due to a defect in FO B cells caused by Mitf inactivation. However, the underlying mechanisms to explain the selective loss of MZ and B1 B cells in TFE3/TFEB inhibited mice as well as the FO B cell defect, and the consequences of these losses on antibody responses are unclear. Therefore, in Aim 2, I analyzed antibody responses to TI and TD antigens in mice in which MiT family is inhibited in B cells or in both B and T cells. I also analyzed the phagocytic properties of TFE3/TFEB-deficient B cells in vivo and in vitro using fluorescent latex beads and *E. coli* particles. I found that despite the decrease in MZ B cells caused by MiT inhibition in B cells, unexpectedly, these mice had elevated IgG responses to TI antigens compared to WT mice. However, the IgG isotype produced in response was more reflective of FO rather than MZ B cells. In contrast, all IgG responses were impaired in mice that had MiT family inhibited in both B and T cells, attributed due to a defect in T cell help caused by impaired CD40L expression. Phagocytosis by peritoneal B cells was also impaired, which may explain the reduction in their homeostatic numbers over time.

My studies provide important new information about regulation of proinflammatory responses in macrophages and B cells, which will inform the development of novel therapeutic strategies to combat microbial infections and autoimmune diseases.

INTRODUCTION

Transcription Factors and MiT family:

All the cells in our bodies contain the full set of genes required for all life processes. Spatiotemporal regulation of gene expression is critical for development and function of different cell types. Gene transcription, the process in which the genetic information on the DNA is transcribed into mRNA, is known to be central to tissue-specific gene expression. Transcription factors are the proteins that bind to the DNA and act as "switches" to regulate transcription by either stimulating (activators) or inhibiting (repressors) the synthesis of mRNA. Transcription factors are frequently modular but generally contain two essential functional regions: a DNA-binding domain and a regulatory domain. Transcription factors are typically classified according to the structure of their DNA-binding domains.

Historically, our lab has been interested in a small family of transcription factors, known as the Mit/TFE (MiT) family (Figure 1A) that comprises four molecules related to the microphthalmia transcription factor, MITF, because of their potential importance in the development and function of the immune system. The microphthalmia-TFE (MiT) is a subfamily of basic helix-loop-helix leucine zipper (bHLH-ZIP) transcription factors, and is made up of TFE3, TFEB, TFEC, and Mitf [5]. MiT proteins bind the same cognate μE3 DNA sequence (CANNTG) via nearly identical basic regions that requires homo- or heterodimer formation between MiT family members, some dimers activate gene expression where as others repress it [5]. Dimerization is essential for DNA binding and function. Structural and amino acid sequence similarities as well as co-expression in different cell types explain the known examples of functional redundancy between different MiT family members.

The physiological roles of MiT Family of transcription factors in different systems

TFEB and TFE3 and Mitf have diverse cell specific roles in the organism and in the immune system in particular. These include both autophagy related and non-autophagy related functions. A better understanding of their roles can be gleaned from what is known about them in the context of mouse genetics and human congenital diseases. Mitf is by far the most well studied member of the family. It was first

isolated as the gene responsible for the *microphthalmia* mouse mutant, and was found to be critically important for the development of melanocytes and other pigmented cells like those in the inner ear and retinal epithelial cells [6]. Similarly, in humans, Mitf mutations cause deafness and other pigmentation cell disorders, including Waardenburg Syndrome type 2A (WS2A) and Tietz' Syndrome [7, 8]. Mitf also plays an important role in the development and function of hematopoietic cells like osteoclasts and mast cells, and restrains B cell activation [9]. Results from our lab and others show that there are elevated numbers of activated B cells, plasma cells, and certain autoantibodies in mice deficient in Mitf, all manifestations of a relaxation in the restraint of FO B cell activation [10, 11] In contrast, preliminary data from our laboratory, which are the foundation for the experiments of Aim 2 in this thesis, suggest that TFEB and TFE3 are important for the homeostasis, activation, and/or development of specialized B cell subpopulations that have critical strategic roles in antibody responses. In mice that lack TFE3 and TFEB function, B cell subsets known as Marginal Zone B cells in the spleen, and B1 B cells in the peritoneal cavity, are greatly reduced [11]. These B cells have important roles in barrier defense and against particular microbes such as encapsulated bacteria. How TFEB and TFE3 support the development/function of these B cells is a goal of my thesis research.

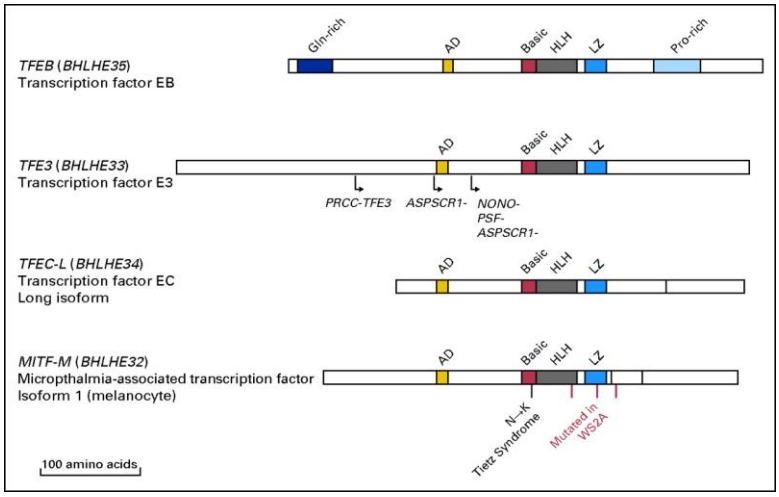
Historically TFEB understanding lagged because, the homozygous TFEB knockout is embryonic lethal due to defects in placental vascularization: the labyrinthine trophoblast cells derived from the TFEB-deficient fetus fail to secrete vascular endothelial growth factor (VEGF) and hence fail to invade the placenta, thus the TFEB-deficient mouse embryos die on day E10 (29). In contrast, TFE3 null mice are phenotypically normal. However, TFE3 and Mitf play redundant roles in the regulation of osteoclast development and function. Inactivation of both leads to severe osteopetrosis in mice [12, 13]. Dominant negative mutations in Mitf also result in similar osteopetrotic phenotype. TFEC null mice are also phenotypically normal, and its genetic absence does not appear to exacerbate defects caused by the loss of other MiT family members. It is known to be expressed in cells of the myeloid lineage like the monocytes

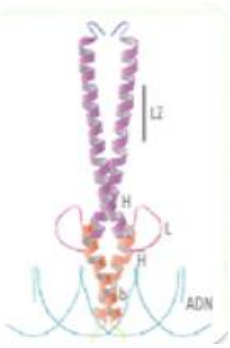
and macrophages, and its expression is responsive to immunological stimuli [14]. However its function still remains unclear.

In T cells, both TFEB and TFE3 jointly control the expression of a gene critical for helper T cell function, CD40L [15]. To reveal this property, an important tool developed in our laboratory was a recombinant trans dominant negative (TDN) protein that lacks the DNA binding basic region and inactivates all four members of the MiT family by forming inactive heterodimers that can no longer bind DNA [15] (Figure 1B). This TDN protein was used to inactivate endogenous MiT proteins in T cells in mice when expressed as a transgene. Such mice developed a hyper IgM-like syndrome due to a defect in CD40L dependent T cell help. This strategy revealed that TFE3 and TFEB were functionally redundant in their control of the expression of the gene encoding CD40L, whereas the inactivation of either gene alone did not cause a defect in CD40L expression.

Together, a growing body of data is starting to show us that the MiT family of transcription factors has diverse and critical roles in the immune system of mammals, but the detailed functions of these transcription factors in macrophages and B cells during the course of an immune response needed to understand the underlying mechanisms remains incomplete and demands further investigation given the importance uncovered so far.

A





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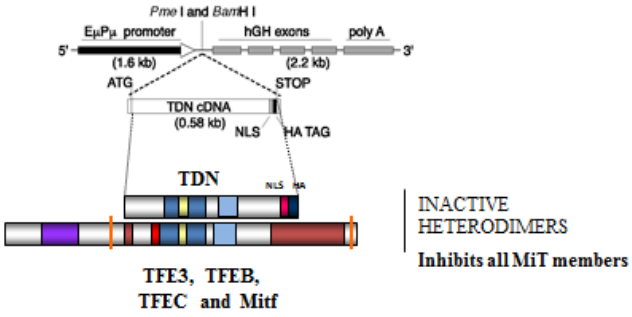


Nature Immunol, 2006, Huan et. al

Figure 1.The s**tructure of the MiT family of transcription factors**. A. Homology among the members of MiT family and Dimerization. Figure adapted from [16] . B. Trans -Dominant Negative transgene (TDN) is a genetic tool to study MiT family function in B cells or T cells or both. It inhibits DNA binding of all MiT proteins by forming inactive heterodimers, thereby addressing redundancy/compensation among the MiT family, and when expressed somatically avoids embryonic lethality of TFEB null mice. Above, the TDN is aligned with a representative MiT member to illustrate the lack of a DNA binding basic region (red) and the presence of the dimerization domains (blue and yellow) that will allow it to form inactive heterodimers. The transgene construct used to express the TDN in lymphocytes is also shown (adapted from [15]).

The immune system and autophagy

An important study published in 2009 advanced the field of autophagy by identifying TFEB as a key transcription factor in some cell types that regulates many genes involved in autophagy and lysosomal biogenesis [17]. Autophagy is a process of self-degradation of cellular components in response to various kinds of environmental stress, in which double-membrane autophagosomes sequester intracellular material "cargo" such as defective organelles (such as mitochondria) or portions of cytosol containing misfolded proteins and pathogens. They then fuse with lysosomes or vacuoles for breakdown by resident hydrolases and the macromolecules are released for recycling. Defective autophagy plays a significant role in human pathologies, including cancer, neurodegenerative diseases, and infectious diseases [18]. Even though autophagy is traditionally defined as a degradative mechanism employed by cells under stress, recent studies have shown that both yeast and mammalian cells utilize autophagic machinery for the "non-classical" secretion of proteins [19, 20].

Autophagy is classified into 1) Macro autophagy, 2) Micro autophagy and 3) Chaperone-Mediated Autophagy (CMA) and 4) non-canonical autophagy. The word "autophagy" in the literature almost always refers to Macro autophagy, and will be used here similarly unless otherwise specified. Micro autophagy refers to the sequestration of cytosolic components directly by lysosomes through invaginations in their limiting membrane. CMA involves direct translocation of unfolded substrate proteins across the lysosome membrane through the action of cytosolic and lysosomal chaperones and integral membrane receptors. Non-canonical autophagy refers to the process that occurs in the absence of some of the key proteins involved in the canonical pathway and is sometimes seen during immune responses [2].

The Basic Steps of the classical autophagy pathway have been well defined through genetics, cell biology, and biochemistry (Figure 2). First, during the initiation step there is the formation of the autophagic isolation membrane, also called the phagophore, which is transiently connected to and derived from the endoplasmic reticulum (ER). The phagophore sequesters the captured cytoplasmic cargo that is destined for

autophagic disposal and, following elongation and closure, an autophagosome is formed. At this stage, the corresponding endomembrane are commonly visualized as double membrane structures. The degradation of the captured cargo begins when the double membrane autophagosome matures into an autolysosome. The fusion of the autophagosome with lysosomes results in the acidification of the autophagosome lumen, the acquisition of lysosomal hydrolases and the degradation of the cargo as well as the inner of the two membranes [2] . Some of these characteristic features of autophagy were used in my studies.

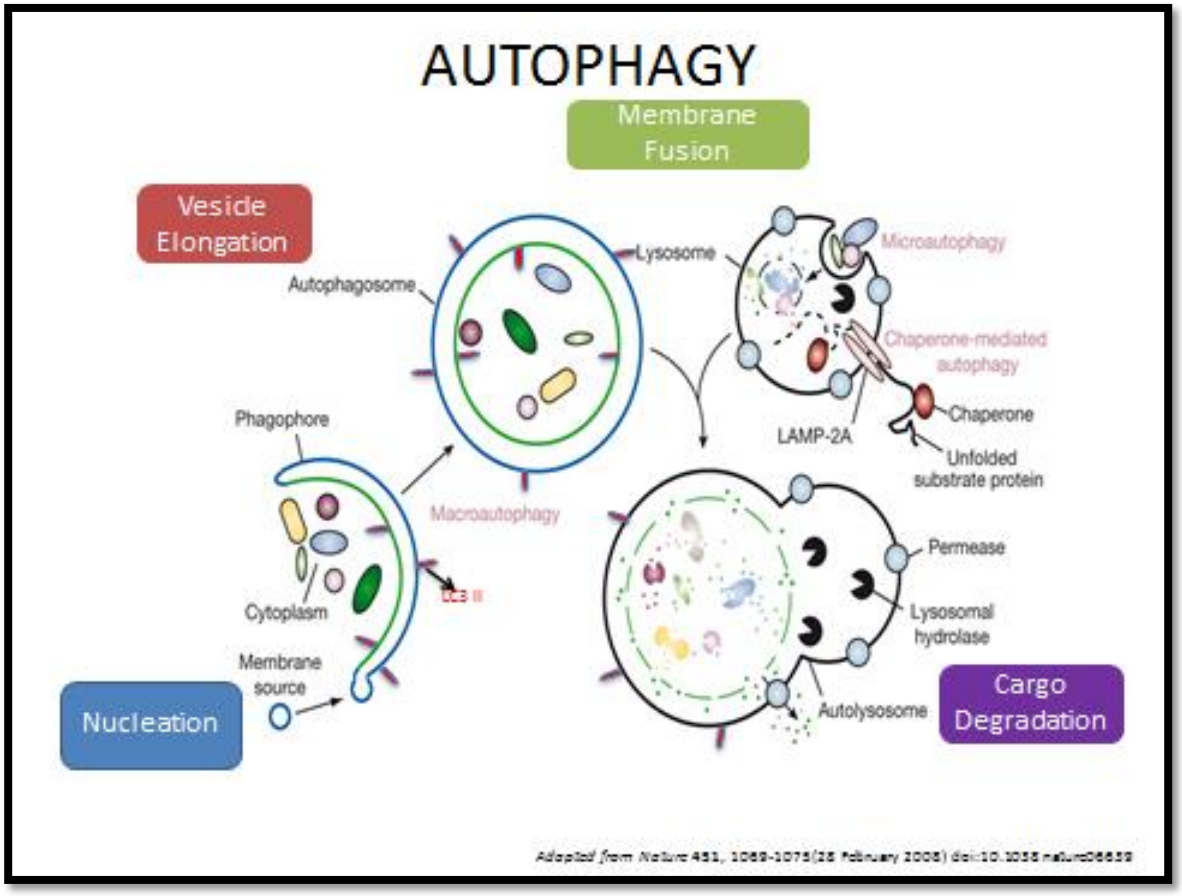


Figure 2. Schematic of steps involved in Autophagy. During the vesicle nucleation step, portions of cytoplasmic cargo get sequestered by a phagophore membrane, which when elongates, and then results in the formation of a double membraned autophagosome. The mature autophagosome then fuses with lysosomes, whereby the cargo then gets degraded by lysosomal enzymes, and the catabolized macromolecules are then released into the cytoplasm for recycling [21]). LC3II, which is an integral membrane protein in the autophagosome, is shown in red.

Autophagy is responsive to various extra- and intra-cellular cues, including those crucial for immune defense, and can either be activated or suppressed. Starvation, pathogen associated molecules like LPS, and cytokines like IFN-γ are known to upregulate autophagy, whereas nutrient abundance, mammalian target of Rapamycin (mTOR) signaling, and Th2 cytokines like interleukins IL-4 and IL-13 are known to suppress autophagy[22].

Autophagy process in immune cells is sometimes referred to as "Immunophagy" [23] and is broadly classified into 1) Type I immunophagy, which refers to the unique and sometimes highly specialized immunological effector and regulatory functions autophagy has in immune cells, such as elimination of bacteria in autophagosomes and processing of antigens derived thereof for MHC presentation [24]; and 2) Type II immunophagy, which refers to general homeostatic functions autophagy has in immune cells and is likely similar to the functions of autophagy in other cell types such as a muscle cell or a neuronal cell [23]; Type II Immunophagy with respect to the development of both B and T lymphocytes and macrophages is important in both innate and adaptive immune responses. In T cells (elaborated more fully below), autophagy seems to have a role in recycling metabolically exhausted mitochondria at specific points in T cell development, such as the exit of T cells out of the thymus. In B cells, B1 cells seem to have the greatest dependency on autophagy, but the underlying mechanism responsible is not clear. Whether TFEB and other MiT factors were involved in autophagy was therefore relevant.

Mouse genetic studies have demonstrated an unambiguously critical role for autophagy in CD4 T cell function and CD8 T cell development [3, 4, 25, 26]. ATG-deficiency in T cells impairs mitophagy and causes functional and developmental defects in both the CD4 and CD8 T cell lineages [25, 27]. ATG-deficient CD4 T cells are somewhat reduced in numbers but fail to secrete cytokines in response to antigen challenge, whereas CD8 T cell development is profoundly impaired [28]. However, published and unpublished studies from our lab have shown that the hallmark manifestations of defects in developmental

autophagy/ type II immunophagy in T cells is not affected in mice in which TFE3 and TFEB, either individually or together, are inactivated in T cells ([15]; unpublished observations). For example, T cells rendered MiT-deficient either by the TDN (TDN-T mice) or by gene deletion, develop normally [15] and cytokine responses appear intact (unpublished). However, TDN-T mice do show a defect in T cell help, which results in an immune deficiency called hyper IgM syndrome caused by a failure of T cell help to B cells [15]. This is because TFE3 and TFEB were found to jointly regulate the expression of the gene encoding CD40L, which is critical for T cells to activate B cells to class switch and produce IgG, IgA, and IgE. This indicates that there are TFEB-dependent functions in lymphocytes that do not involve autophagy. Therefore, the main objective of Aim 2 is to determine whether the importance of TFEB in Marginal Zone and B1a B cells demonstrated in our lab is due to its role in autophagy or some other TFEB-dependent process.

Role of autophagy in Monocyte/Macrophage development and function

Induction of autophagy is essential for monocyte-macrophage differentiation. Hematopoietic stem cells in the bone marrow give rise to myeloid precursors, which differentiate into monocytes to circulate in the blood. Monocytes home to different tissues to become the phagocytic tissue-resident macrophages. During this process of differentiation, monocytes need to upregulate autophagy, and this is essential for their survival so as to avoid "default" apoptosis [29].

Activation of autophagy in macrophages in response to microbial infections can result in host defense in one of many ways. Some of them involve killing of intracellular pathogens. Autophagy can also result in the major histocompatibility complex II (MHC II) presentation of microbial antigens following phagocytosis**.** Such antigens can be processed in the autophagosome and are then loaded on to MHC II molecules for presentation to CD4 T cells [24] IFNγ, which is an antiviral cytokine, upregulates autophagy in macrophages[30]. Toll like receptor (TLR) signaling via TLR1,2,4,and 9 on macrophages in response to pathogen associated molecular patterns (PAMPS) results in more effective immune response involving

autophagy [31]. As autophagic responses in the immune system are primarily aimed at enhancing host protection, pathogens have evolved mechanisms to evade killing by autophagy or hijack autophagic machinery for enhancing their survival and spreading of the infection. Given the findings that autophagy is important for macrophage development and function, and that TFEB is important for transcriptional regulation of autophagy in other cell types, in Aim 1 I studied the role of TFEB in Macrophage development and function. In parallel, I addressed whether TFE3 plays a redundant/compensatory role along with TFEB in these processes given that functional redundancy is a known feature of the MiT family members based on their sequence similarities, virtually indistinguishable DNA binding properties, and that both gene are expressed in macrophages. Thus, these experiments will fill a knowledge gap in our understanding of the role of TFEB/TFE3 in monocyte differentiation and immune response.

Autophagy in B cells:

Most of our knowledge of the involvement of autophagy in B cell development comes from the analysis of mice conditionally deficient ("conditional knockouts") of autophagy genes that are otherwise essential for mouse development, like the Atg5 and ATG7. In the Atg5-CD19 cre mice, in which cre-mediated deletion of the conditional Atg-5 allele occurs only in B cells that express CD19, even though mature B cells develop, there is increased death of the pro-B cells and pre-B cells in the bone marrow[4]. This suggests that autophagy might be protective at distinct developmental stages in the life of a B cell. In addition to that, these mice also have a loss in the maintenance of B1 B cells, which are critical for mucosal immunity. In addition, terminal B cell differentiation into antibody secreting plasma cells relies on autophagy. Conditional $Atg5^{-/-}$ differentiating plasma cells had a larger endoplasmic reticulum (ER) and more ER stress signaling than did their wild-type counterparts in vitro, which led to more antibody secretion. However this enhanced immunoglobulin synthesis was associated with less intracellular ATP and more death of mutant plasma cells. This suggests that autophagy provides a cytoprotective trade-off between immunoglobulin synthesis and viability [3]. *In vivo*, mice with conditional deficiency in Atg5 in B cells had defective antibody responses to T dependent antigens, fewer antigen-specific long-lived bone marrow plasma cells than did wild-type mice, and those surviving plasma cells in the bone marrow had intact alleles of Atg 5 that were not deleted by Cre recombinase . Thus, autophagy is specifically required for plasma cell homeostasis and long-lived humoral immunity.

Interestingly, studies by Vyse, et al showed that B cells from patients with SLE had more autophagosomes compared to the B cells from healthy individuals and that the disease activity correlated positively with autophagosome density in the patients [32]. Inhibiting autophagy in B cells therefore presents a potential therapeutic target in SLE, and may be a clinically relevant mechanism of action of the commonly used immunomodulatory anti-malarial hydroxychloroquine [33]. Hydroxychloroquine inhibits autophagy by raising lysosomal pH and therefore preventing autophagosome–lysosome fusion. It is thought that this

inhibition also impairs APC responses to TLR7/9 signaling – an endosome/lysosome dependent process - that unrestrained can drive a type I IFN response characteristic of lupus.

SPECIFIC AIMS

Given the growing evidence supporting the important roles of the MiT family in the immune system, the goal of the experiments in this thesis was to elucidate the importance of the MiT family in macrophages and B cells specifically, and gain a better understanding of the underlying mechanisms responsible for governing immune responses in these cell types. In macrophages, this is predicated on the known importance of autophagy to their development and immunological effector functions, and that TFEB in other systems controls the expression of genes critical for autophagy. The importance of TFE3 and TFEB in B cells has been demonstrated from preliminary work in our lab showing that Marginal Zone and B1a B cell numbers are reduced when TFE3 and TFEB are inactivated, and that FO B cell responses are abnormal when Mitf is inactivated. However, the underlying mechanisms, and the relevance of autophagy to these phenomena, are unknown. A better understanding of these developmental processes is necessary for enhancing protective immune responses, such as vaccines, and disarming misdirected or self-aggressive immune responses, as in the case of allergy and autoimmune disease, respectively.

AIM 1. Examine consequences of TFEB/TFE3 deficiency in macrophage development and function.

Using a combination of in vitro cell culture and in vivo mouse genetics with mice harboring TFE3 and conditional TFEB gene deletion in macrophages, I studied the role of TFEB and TFE3 deficiency in murine macrophages. Aim 1 can be further sub divided into the following sub aims.

In vitro studies:

AIM 1A. To determine if TFEB is responds to immune and metabolic stimuli that activate autophagy in transformed murine macrophages.

AIM 1B. To determine if conditional deletion of TFEB / TFE3 deletion affects primary macrophage differentiation in culture.

AIM 1C. To determine if conditional deletion of TFEB/TFE3 results in altered immune mediated autophagy in primary BMDMs.

In vivo studies:

AIM 1D. To determine if deletion of TFEB/TFE3 genes in myeloid cells of mice has an effect on macrophage phagocytosis.

AIM 1E. To determine if deletion of TFEB/TFE3 genes in myeloid cells of mice has an effect on macrophage differentiation in vivo, using a model for sterile peritonitis.

AIM 1F.To determine if deletion of TFEB/TFE3 genes in myeloid cells affect proinflammatory responses to low dose endotoxin.

AIM 2. Understand the underlying mechanism and manifestations of defects in FO, MZ and B1 B cell subsets in MiT deficiency.

Using a combination of in vitro cell culture and in vivo mouse genetics for MiT inactivation in B cells only or in both B and T cells, I studied the manifestations of defects in various subsets of B cells caused by MiT inactivation. This aim can be further divided into the following sub aims.

AIM 2A. To analyze antibody responses to TD and TI in mice with MiT inactivation in B cells only (TDN-B) or both B and T cells (TDN-B/T).

AIM 2B. To evaluate antigen trapping and phagocytic properties of B1a B cells in vitro and in vivo, in both TDN-B and TDN-B/T mice.

AIM 2C. To evaluate if the differences observed in antibody responses and B cell activation between TDN-B and TDN-B/T mice are due to impaired CD40L expression in T cells in the TDN-B/T mice.

AIM 1.

Monocytes and macrophages express all members of the MiT family of proteins. Upregulation of autophagy is critical for the survival and differentiation of monocytes to macrophages [29] and macrophages also need to upregulate autophagy for efficient phagocytosis and antigen presentation. Given the role of TFEB/TFE3 in regulating the genes involved in autophagy and lysosomal biogenesis, I tested the hypothesis that TFEB/TFE3 are important for macrophage development and function, such that deficiency of one or both leads to impaired macrophage development and immune function.

BACKGROUND AND SUMMARY

Both human monocytes from blood as well as myeloid precursors murine bone marrow need to upregulate autophagy in order to differentiate into macrophages. During this process of differentiation in the presence of Granulocyte-macrophage colony stimulating-factor (GM-CSF) or Macrophage-colony stimulating factor (M-CSF), monocytes need to upregulate autophagy that is essential for their survival and avoid "default" apoptosis. The suggested mechanisms by which this occurs is that differentiation signal releases Beclin1 from Bcl-2 and by blocking the cleavage of Atg5, both of which are critical for the induction of autophagy during initiation step and elongation of the phagophore[29]. Hence, Induction of autophagy is essential for monocyte-macrophage differentiation.

Activation of autophagy in macrophages in response to microbial infections can result in host defense in one of many ways. Some of them involve killing of intracellular pathogens. An example is the autophagy-mediated killing of intracellular bacteria like *Mycobacterium tuberculosis* [30], *gram positive Listeria monocytogenes* [34] and gram negative *Helicobacter pylori* [35] by macrophages. Autophagy is also important for eliminating intracellular protozoan parasites like *Toxoplasma gondii* [34]. Therefore, autophagy mediated lysosomal killing of pathogens is a protective defense response of the host immune system.

Autophagy can also result in the major histocompatibility complex II (MHC II) presentation of microbial antigens**.** Such antigens can be processed in the autophagosome and are then loaded on to MHC II molecules for presentation to CD4 T cells [24]. IFN-γ, which is an antiviral cytokine, upregulates autophagy in macrophages[30]

Toll like receptor (TLR) signaling in response to pathogen associated molecular patterns (PAMPS) following phagocytosis, results in more effective immune response due in part to activating autophagy. For example, LPS, which binds to TLR4 on macrophages, results in upregulation of the autophagic response. TLR signaling on murine macrophages triggers the rapid recruitment of beclin 1 and activation of phosphoinositide-3-OH kinase activity followed by the formation of LC3 positive phagosome in a manner that depends on the autophagy pathway proteins ATG5 and ATG7 [31].

As autophagic responses in the immune system are primarily aimed at enhancing host protection, pathogens have evolved mechanisms to evade autophagy in different ways. For example, some host-adapted intracellular bacteria like *S. flexneri, L. monocytogenes, B. pseudomallei,* and *S. typhimurium* have developed one or more strategies to avoid entry into the host autophagic pathway. These strategies include damage of sequestering (phagosome or autophagosome) membranes and fusing with plasma membranes to facilitate intercellular spread of infection, "self-masking" to down regulate surface expression of antigens and thereby prevent recognition by the autophagy machinery, effecting transcriptional control of ATGs, and formation of replicative niches in the lysosomes [36].

Some of the key proteins involved in mammalian autophagy during the initiation step for the formation of the phagophore include those of the class III phosphatidylinositol kinase complex, which includes Beclin-1, hVPS34 and autophagy related protein ATG14. During the elongation process, cytoplasmic Microtubule-associated protein 1A/1B-light chain 3 I (LC3 I) protein is lipidated at the C-terminus with Phosphatidylethanolamine to generate LC3 II, which is an integral membrane protein of the autophagosomal membrane. The conversion of LC3 I to LC3 II is a gold standard way to demonstrate autophagic flux, and is a process I used to monitor autophagy in some of my experiments**.** This conversion

process requires various other ATG molecules like the ATG 5, ATG12, etc., to work in concert with the ubiquitination conjugation reaction. The importance of this pathway is evident in that some of the ATG molecules like ATG 5, ATG 7, ATG9 and ATG16L are essential for neonatal survival in mice[37]

It has been established that cells shift their transcriptional program stressed either by nutrient deprivation or by pathogen invasion. Stress induced transcriptional factors regulate important genes involved in the synthesis of the component proteins of the autophagic machinery. This is essential for replenishing the building blocks of autophagic machinery that underwent lysosomal degradation, and also for enhancing the ability of the immune system to obtain microbial peptides for MHC-dependent presentation to T cells and enhanced microbial killing. It has been suggested that TFEB, a member of the MiT family of transcription factors, plays a key role in regulating the genes involved in autophagy and lysosomal biogenesis[1] (Figure 3**)** but its role in macrophages is unknown. Therefore, understanding transcriptional regulation of autophagy in macrophages can inform the development of new anti-microbial strategies to enhance protective immune responses.

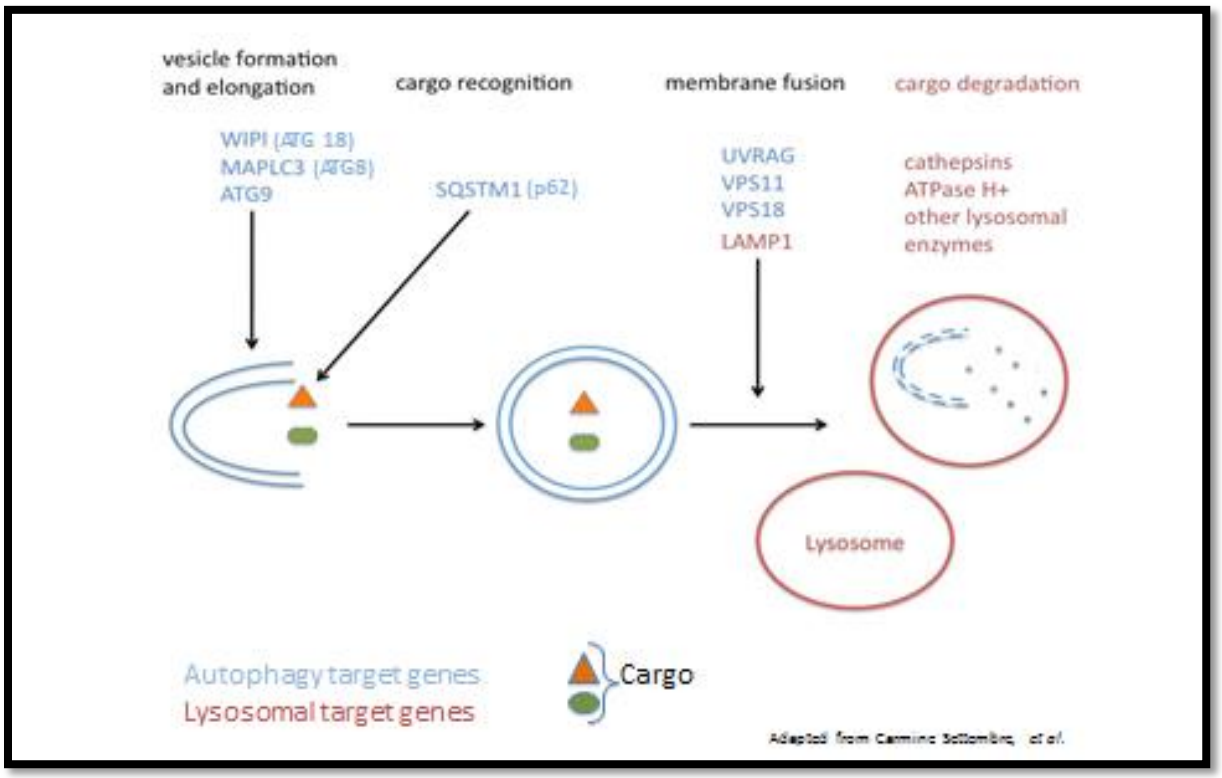


Figure 3. Role of TFEB target genes in sequential steps of autophagy. Putative TFEB target genes implicated in autophagosome (blue) and lysosomal (red) biogenesis. In vitro chromatin immuno precipitation studies have shown that TFEB binds to promoters of genes involved in autophagosome biogenesis, cargo recognition as well as lysosome biogenesis there by transcriptionally regulating various steps of autophagy.

MATERIALS AND METHODS

Cell Cultures RAW 264.7 cells and L-929 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Grand Island, NY) with 10% Fetal Bovine Serum (FBS) and 100 U/ml Penicillin-G and 100 mcg/ml Streptomycin and 2mM L-glutamine (Gemini Bio Products, Woodland CA). All cultures were maintained at 37°C in a humidified atmosphere with 5% CO2.

L-929 Conditioned Medium: To prepare 5X conditioned medium from L-929 cells, the medium was changed when the cells were semi-confluent (10 ml of medium per 100 cm2 dish) and collected after 24 hours. The medium was sterile filtered using .2 µm syringe filter and stored at -20^0 C until further use.

Animals: All the mice are maintained in specific pathogen-free conditions SPF (5 mice per cage and 12 hour light/dark cycle) with sterilized food and water. Mice were euthanized by CO_2 asphyxiation according to DLAR and IACUC standards under approved protocols. TFE 3 null mice have germ-line null mutations where most of the bHLH-Zip domain of each protein was replaced with *pGKNeo* (Nancy Jenkins). The conditional allele of TFEB was created in our lab by insertion of lox P sites flanking the exons encoding the basic region and helix-loop-helix regions; expression of *Cre* deletes these and creates a null allele. Lyz2 cre mice were purchased from Jackson labs. The LyzMcre knock-in allele has a nuclear-localized Cre recombinase inserted into the first coding ATG of the lysozyme 2 gene (*Lyz2*); placing NLS-Cre expression under the control of the endogenous Lyz2 promoter/enhancer elements which is expressed in monocytes, macrophages and neutrophils. Cre mediated deletion was confirmed by PCR.

Primary bone marrow macrophage cultures: BMDM were cultured according to the protocol described by Hailemariam et al. [38]. Bone marrow was obtained from the tibia and femurs of 6-month-old C57BL/6 mice. The mice were sacrificed by CO2 inhalation and dissected under aseptic conditions. Bone marrow cells from both tibia and femurs were collected by flushing the hollow centers of the bone with medium using a 1ml syringe. Cells obtained from one mouse were plated in 4 (100 cm2) dishes. The cells were then grown in DMEM with 10% FBS and 100U/ml Penicillin-G and 100mcg/ml streptomycin and

2mM L-glutamine and 20% L-929 conditioned medium (source of M-CSF). On day 5 after initial culture, new medium was added. BMDM were harvested on day 10.

Preparation of genomic DNA. High molecular weight genomic DNA was extracted from tail samples of 3-week-old mice. Briefly, tail material was incubated in 500μL of 50 mM of NaOH in a 95ºC heat block for 1 hour. After incubation, each tail was vortexed for 10 seconds, followed by neutralization with 50μL of 1M Tris-HCl (pH 8.0) and re-vortex. After vortexing, tails were subjected to centrifugation for 5 minutes at 14,000 RPM and the DNA containing supernatant isolated.

Polymerase Chain Reaction (PCR). Routine PCR analyses were performed to genotype all experimental mice using oligonucleotides that amplify the TDN transgene or MiT allele-specific amplicons and following standard protocols (24). PCR reactions were carried out in a 50μL volume, using 200ng of template DNA in 1 μL of tail lysate, 0.5 U/μL Go Taq polymerase (Promega, Madison, WI), 0.2 mM dNTPs (Roche, Indianapolis, IN), 0.5μM of Forward and Reverse TDN primers (see description below), 5X Green Go Taq Reaction Buffer (Promega, Madison, WI) and HyClone HyPure Molecular biology Grade Water (Thermo Scientific, Logan, Utah).

Cell Suspensions. To isolate PEC, first, the peritoneal cavity cells are remove by a lavage with ice-cold Isolation Buffer (3% FBS in 1x PBS) and cells are collected. Next, the spleen is isolated and weighted. To make splenic single cell suspension, spleen is gently ground using a pestle and suspension is passed through a 100 um cell strainer (Fisher). Bone marrow cells are isolated from femur and tibias of both legs by washing them out with ice-cold 1X PBS using a syringe. Red Blood cells are removed from all cell suspensions by RBC lysis buffer (Sigma, St. Louis, MO). Small aliquots of bone marrow and peritoneal cells are then stained with Trypan Blue 0.4% (Gibco, Grand island, NY) and counted using a hemocytometer.

Antibodies. The following antibodies were used: anti-mouse CD3-PE, CD19 FITC, MHCII-FITC, IgM-PE, IgD- FITC, B220-FITC, CD21-PE, CD23-FITC, CD19-PerCP-Cy5.5 CD5-PE, CD11b-FITC, CD4-FITC, PerCP-Cy5.5, CD3-PE, CD8-PE, CD38-FITC, CD138-PE, Gr-1 PE, TLR4/MD2 PE (BD Biosciences) and F4/80 PE Cy5 (Biolegend).

Flow cytometry. A maximum of 5 x 10^5 cells were used per stain. Cells are incubated on ice and in the dark with an antibody cocktail (FACS buffer + 100 fold dilution of the antibodies of interest). After incubation period, samples are washed twice with FACS Buffer (2% FBS in 1x PBS) and centrifuge. After last centrifugation, cells are suspended in 1x PBS (or fix with 2% paraformaldehyde if not process the same day) followed by FACScan analysis using the Cell Quest Pro software (Version 6.0, BD Biosciences, Mountain View, CA). 10,000 cells are collected for routine analyses. After collection macrophages were electronically gated based on their characteristic scatter properties to exclude large, granular non-lymphocyte cells, cell aggregates and dead cells from further analyses.

Nuclear-Cytoplasmic Fractionation: Briefly, cells were lysed in 0.5% Triton X-100 lysis buffer (50mM Tris-HCl, 0.5% triton, 137.5 mM NaCl, 10% glycerol, 5 mM EDTA supplemented with fresh protease and phosphatase inhibitors. After 15 minutes the lysate was centrifuged. The supernatant represented cytosolic fraction while pellet (nuclear fraction) was washed twice and lysed in 0.5% Triton X-100 buffer with 0.5% SDS and sonicated.

Western blots. 5 X Laemmli buffer was added to the protein samples and denatured by heating them in a dry bath for 5 minutes. The denatured samples were loaded in the wells along with the molecular weight marker. The proteins were electrophoresed for 45 minutes at 200 V. After electrophoresis, the proteins were transferred overnight from the acrylamide gel into an Immobilon-FL Transfer Membrane (Millipore, MA) using the mini-protean system. The membrane is blocked at room temperature with a 5% Dry Milk solution in TBST followed by incubation with primary and secondary antibodies as indicated.

In vitro assessment of phagocytosis : To determine the capacity of phagocytosis of cells obtained from spleen and peritoneal cavity $2*10^5$ cells/well was be plated in 96-well plates(Thermo Fisher Scientific Inc., Waltham, MA, USA) in a final volume of 0.1ml RPMI, supplemented with 5% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin, and incubated for 6h, in a humidified atmosphere of 5% CO2 at 37°C with 1 μm fluorescent beads (Fluoresbrite Yellow Green Microspheres, Polysciences, Warrington, PA, USA) at a cell: bead ratio of 1:10 or with 25 μg pHrodo *Escherichia coli* bio particles for 6h and the non-

ingested beads or bacteria were removed by centrifugation (100 *g* for 10 min at 4°C) over a cushion of 3% BSA (Fisher Scientific) in PBS supplemented with 4.5% D-glucose (Sigma-Aldrich). The cells were incubated with Fc block for 15 min on ice and then washed once and labeled with fluorescent antibodies as described. Control cells were incubated with 30μg/ml of Cytochalasin D for 30 min prior to the addition of beads/*E. coli* particle at 37^0 C. Control cells were incubated on ice for the same time periods and treated similar to experimental samples.

In vivo phagocytosis assay: Individual mice were injected i.p. with pHrodo *E. coli* bio particles (500 μg) or fluorescent 1μm beads ($2*10^9$) in 300 μl PBS. Control mice were injected with 300 μl PBS. After 3 h, PerC cells were isolated and labeled with mAb as described above. Cells were acquired on a FACScan and analyzed using cell quest / Flojo software.

RESULTS

AIM 1A. To determine if TFEB/TFE3 is required for autophagy induced by metabolic stress and immunologic stimuli in transformed murine macrophages

Significance: The major question addressed by this experiment is the physiological role of endogenous TFEB in macrophage function. Transformed murine RAW 264.7 macrophages are a widely used model for understanding normal macrophage biology as they can be activated by various stimuli like LPS and IFN-γ, and respond similarly to mature primary macrophages. When macrophages are subjected to environmental stresses like nutrient starvation or microbial infection, they need to upregulate autophagy in order to survive. Given that RAW 264.7 cells express endogenous TFEB, TFE3 (and also TFEC and Mitf); this is a more physiological model to addresses if TFEB has a role in autophagy as part of a macrophage stress response as well as in response to immune mediated activation.

Experimental Design: In the RAW 264.7 macrophage cell line I induced autophagy by treating the cells with Rapamycin, which is a pharmacological inhibitor of mTOR. mTOR is the nutrient sensing pathway of the cell, and when it is inhibited using Rapamycin the cell interprets that as a lack of nutrients and upregulates autophagy. I also induced autophagy by treating cells with LPS, which is an endotoxin derived from *E. coli* to simulate gram-negative bacterial infection, and with IFN-γ to activate the macrophages. Following induction of autophagy, the nuclear and cytoplasmic levels of TFEB protein, and the expression of autophagy hallmarks, like the conversion of cytoplasmic LC3I to integral membrane protein LC3II present in autophagosomes, were analyzed.

Results: My studies show that in transformed RAW macrophages, TFEB abundance and nuclear localization are responsive to multiple immunological stimuli that activate autophagy, such LPS from *E. coli*, cytokines like IFN-γ, and, pharmacologically, the mTOR inhibitor Rapamycin (Figure 4). Inhibition of mTOR in RAW cells resulted in increased nuclear TFEB and decreased cytoplasmic TFEB (Figure 5),

whereas LPS and IFN-γ increased both cytoplasmic and nuclear TFEB (Figure 6). One explanation of these similar but disparate results is that mTOR inhibition for shorter periods (2 h) may result in the nuclear translocation of the pre-existing cytoplasmic pool of TFEB without replenishment of cytoplasmic stores due to the inhibition of translation, whereas on the other hand, immune stimulation (6 h) may result in both increased nuclear translocation as well as increased protein synthesis in the cytoplasm. This is in line with published observations by Settembre et al., showing that nuclear TFEB can bind to its own promoter and result in increased TFEB transcripts in the cytoplasm as early as 4 h following autophagic stimulation [39]. IFN-γ and LPS treatments both increased autophagosome assembly, as measured by conversion of LC3 I to LC3 (data not shown). Under these culture conditions, TFEB is present in the cytoplasm as well as increased in the nucleus.

In sum, I found that TFEB is responsive to autophagy induced by starvation, LPS and IFN-γ in RAW 247.6 macrophages. These findings are similar to recently published findings by Pastore et al., where they found that nuclear translocation of TFEB and TFE3 are critical for upregulation of autophagy in RAW 247.6 cells and siRNA mediated knockdown of both resulted in a decrease in LC3II. In addition, they also observed a significant reduction of lipidated LC3-II protein and lysosomal membrane protein LAMP-1 following LPS treatment in dko BMDM compared to WT BMDM[40]. These results, along with published reports, show that TFEB is responsive to autophagic stimuli like starvation, endotoxin and viral infection in transformed murine macrophages.

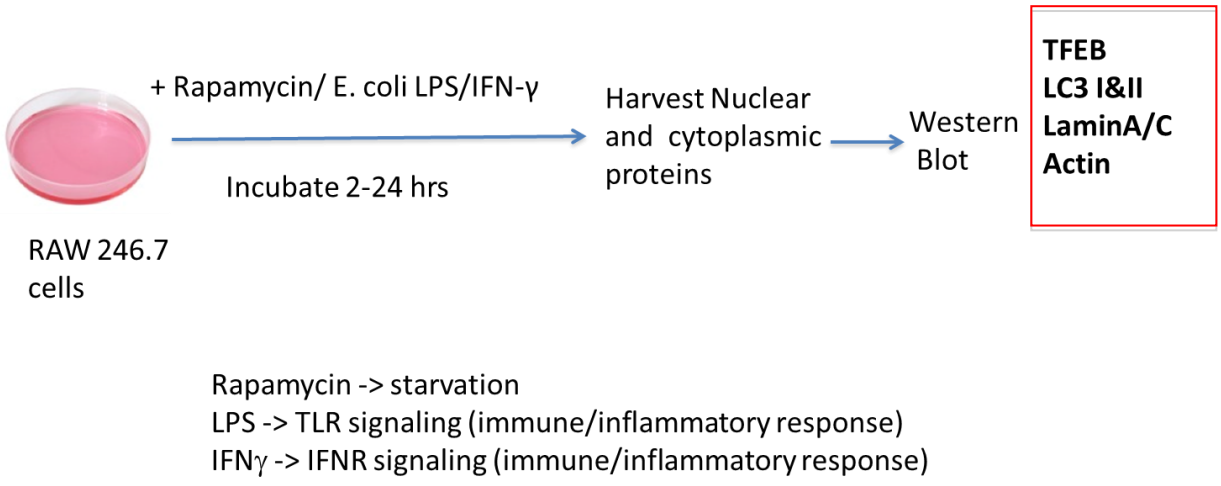


Figure 4. Activating autophagy in RAW 246.7 macrophages: Experimental set up, rationale and read out.

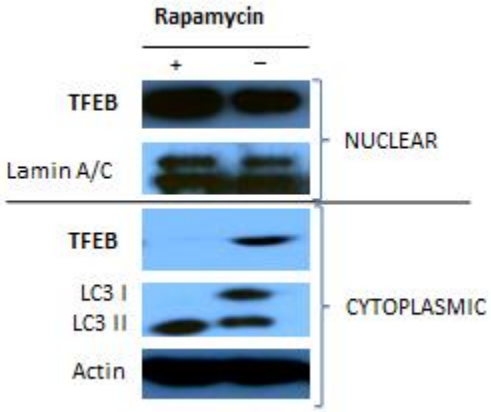


Figure 5. mTOR inhibition in RAW 264.7 cells induces autophagy and nuclear localization of TFEB. Cells were plated, incubated overnight and treated the following day with 50μg/ml of Rapamycin for 2hr; controls received the vehicle (DMSO) for 2 hr. Cytoplasmic protein extracts were made using 0.5% Triton X-100 lysis buffer and nuclear pellets were sonicated in the same buffer with additional 0.5%SDS to extract nuclear proteins; protein expression was detected by western blot. The blots show that upon mTOR inhibition, cytoplasmic TFEB translocated to the nucleus which corresponded with an increase in autophagosome assembly as measured by conversion of LC3 I to LC3 II. Actin was used as a loading control. Data is representative of 3 independent experiments (n=3).

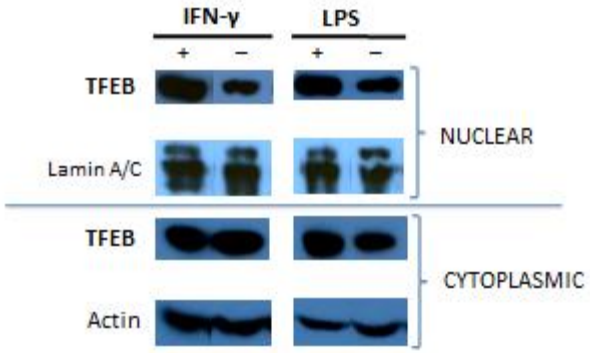


Figure 6. Both IFN-γ and LPS treatments increase nuclear abundance of TFEB in RAW cells: RAW 264.7 cells were plated, incubated overnight and treated the following day with IFN-γ 100U/ml or LPS 100ng/ml for 6hr; controls received the vehicle for the same time periods. As in Figure 5, cytoplasmic protein extracts were made using 0.5% Triton X-100 lysis buffer, and nuclear pellets were sonicated in the same buffer with additional 0.5%SDS to extract nuclear proteins. Shown are representative western blots from the indicated sources. Data is representative of 3 independent experiments (n=3).

AIM 1B. To determine if conditional deletion of TFEB and/or TFE3 deletion affects primary macrophage differentiation in vitro by affecting developmental autophagy.

Significance: Previous studies by Zhang, et al., showed that monocytes must upregulate autophagy in order to differentiate into macrophages and avoid apoptotic death. [29]. This experiment addresses if TFEB and/or TFE3 has a role in developmental autophagy in macrophages using an in vitro differentiation system in culture.

Experimental design: As germline deletion of TFEB is embryonic lethal, I used macrophages from mice with a conditional deletion of the TFEB gene in monocytes. This was accomplished using the Lyz2cre (also called LyzMcre) transgene that expresses the cre recombinase in monocytes and neutrophils. These mice were crossed with TFEB ^{flox} mice to generate conditional TFEB knockouts (Lyz2cre TFEB $^{flox/flox}$), which should only become TFEB $^{-/-}$ in monocyte/macrophages (Figure 7). I also used bone marrow derived macrophages from TFE3 $^{-/-}$ mice as well those with deletions of both TFE3 and TFEB genes (dko). These cells were cultured with the growth factor M-CSF derived from L-929 cells and compared numbers, morphology and cell surface expression of developmental and activation markers using flow cytometery.

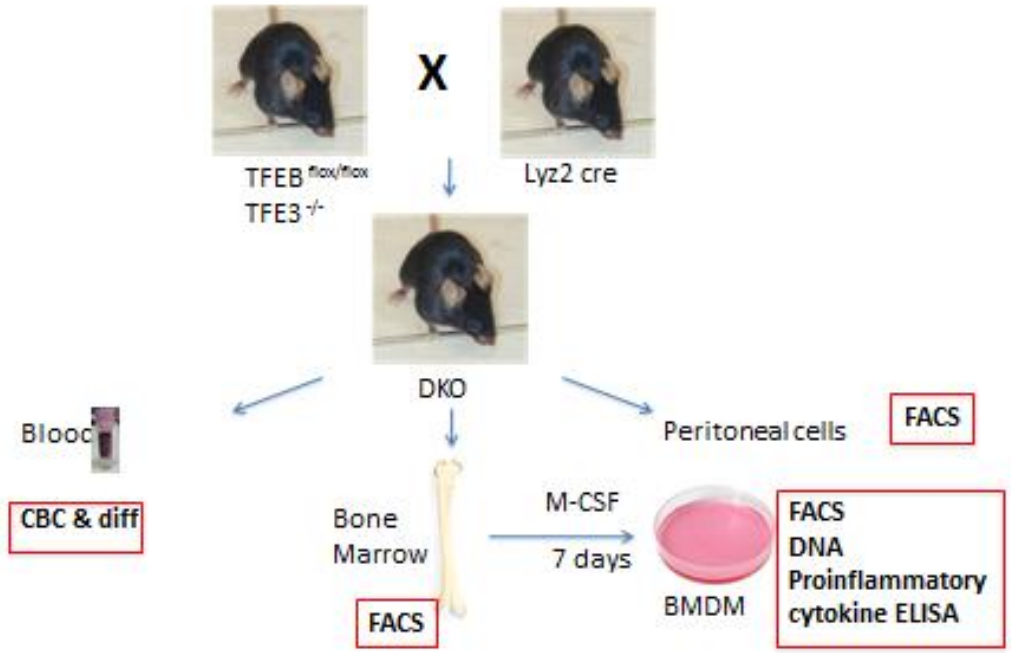


Figure 7. **Schematic depicting generation of TFEB ko, dko mice and BMDM**. Mice carrying the TFEB flox/flox allele were crossed with those carrying Lyz2 cre to generate conditional TFEB ko mice, and these mice were crossed with mice carrying the null allele for TFE3 to generate the conditional dko mice (Lyz2cre TFEB $^{flox/flox}$ TFE3$^{-/-}$). I analyzed Blood collected in purple capped K2 EDTA tubes for complete blood counts (CBC) and differential counts of WBC. I analyzed freshly isolated bone marrow by flow cytometry and also cultured in the presence of M-CSF for 7 days to obtain BMDM which I used for flow cytometry, DNA analysis and proinflammatory cytokine analysis.

Results: My studies show that TFEB- and TFEB/TFE3-deficient macrophages can develop indistinguishably from controls (Figures 8-10). Therefore, I conclude that these MiT factors are not critical for developmental (Type II) autophagy. This conclusion is based on the following findings:

Differential blood counts (CBCs) and peripheral blood smears were indistinguishable among the different genetic cohorts. CBCs were obtained by an automated Siemens Advia hematology analyzer (Dr. Gottesman in the Department of Clinical Pathology). As this machine was calibrated for analyzing human samples and normal mouse blood counts are different from humans, I also performed a manual differential counts to confirm the automated cell counts. Using these complementary approaches, I found there was no significant difference in the number of circulating monocytes (Figure 8). Therefore, the deletion of TFEB/TFE3 in monocytes or neutrophils does not affect the proportions or cell numbers in peripheral blood.

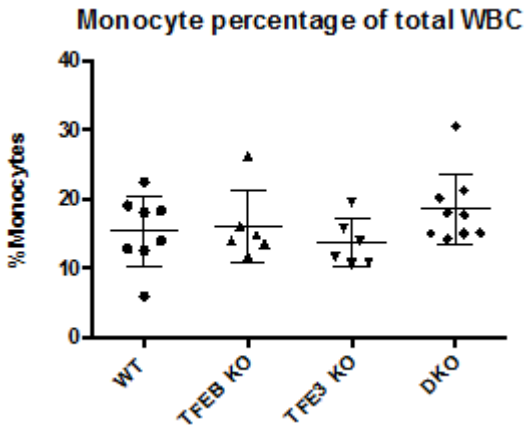
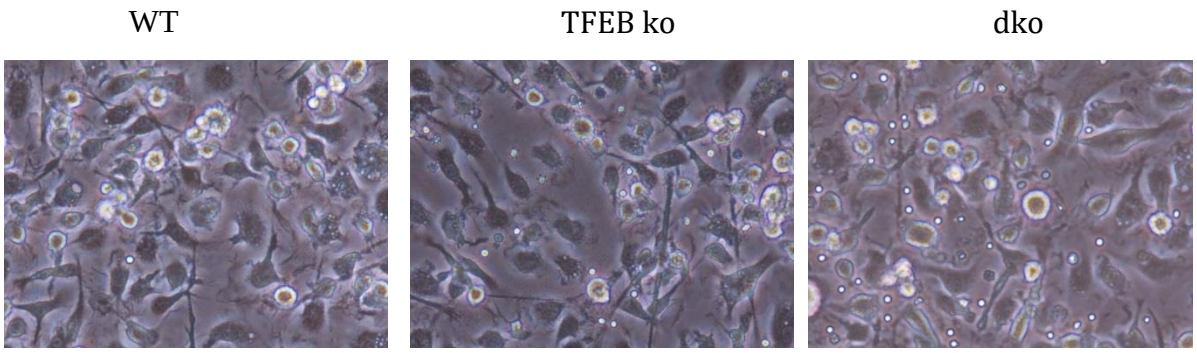


Figure 8. TFEB/TFE3 gene deletion does not affect monocyte proportions in unimmunized mice. Whole blood from 6-8 month old mice is collected by cardiac puncture in K2EDTA tubes and CBC with differential counts were run on an automated cell counter Siemens Advia in the clinical lab; the values were confirmed by peripheral smear analysis. Significance was tested using the non-parametric Mann-Whitney U test; p values were >0.6 between any two groups, which is not significant. Each dot represents one mouse, n=6-9 per group.

In vitro differentiation of bone marrow derived macrophages (BMDM) from TFE3/TFEB-deficient and wild-type controls was indistinguishable between the different genetic cohorts. Freshly isolated bone marrow cells from 6-8 month old Lyz2cre^{+}/ TFEB $^{flox/flox}$ mice, dko and controls were cultured in DMEM with 20% L-929 supernatant for 7-10 days to yield BMDMs. This is a widely used and well-established protocol to generate murine macrophages. Under these conditions, bone marrow precursors were able to differentiate into macrophages in the absence of TFEB and/or TFE3. At the end of the cultures, phase contrast microscopy indicated that the TFEB deleted macrophages, as identified by adherent cells with granular cytoplasm and pseudopods were morphologically indistinguishable from the normal wild type macrophages (Figure 9A).

To confirm that the presence of macrophages in the gene deleted cultures was not due to inefficient TFEB-gene deletion leading to the selective survival of non-TFEB gene-deleted cells, I measured cre mediated deletion of the flox allele of TFEB by analyzing the genomic DNA from the macrophage cultures by PCR and comparing them to the tail DNA samples from the corresponding animals (Figure 9B). The results showed that the cre-mediated deletion was efficient and specific to macrophages, excluding the possibility that there was selective survival of non-TFEB-gene deleted cells.

A. Phase contrast microscopy



B. DNA

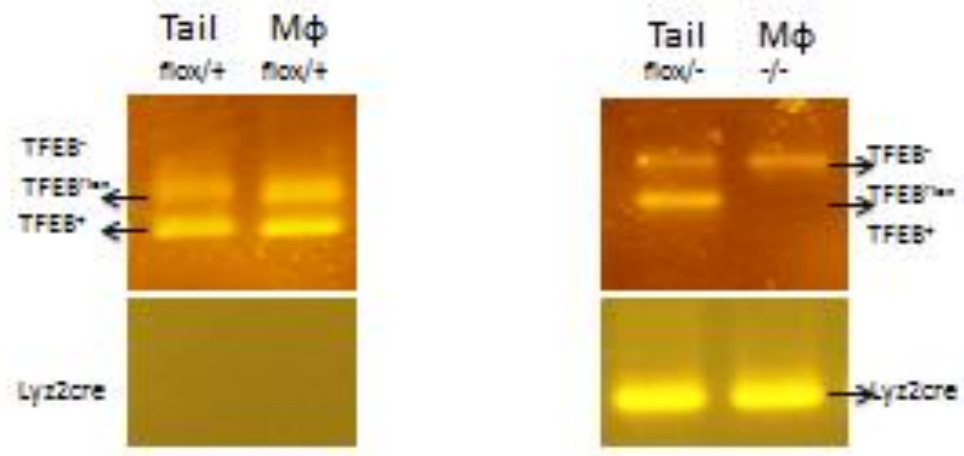


Figure 9. TFEB/TFE3 deleted macrophages are morphologically indistinguishable from WT macrophages. A) Phase contrast microscopy of BM cells incubated for 10 days with M-CSF. Cells derived from WT, TFEB ko and dko mice. The cultures were >95% macrophages as identified by large granular adherent cells with pseudopods. Images representative of cultures one mouse at 400X magnification (n=6-9 per group). B) Genomic DNA PCR from tail samples compared to the DNA from above cultures from the same mouse showing deletion of TFEB in macrophages only.

Analysis of freshly isolated myeloid cells from bone marrow of 6-8 month old Lyz2cre^{+}/ TFEB $^{flox/flox}$ mice, dko and controls using flow cytometry to elucidate myeloid cell numbers, which are F4/80 $^{-}$ and Gr-1 $^{+}$ cells, found that there is no significant difference between the different genotypes (Figure 10A). During the process of differentiation, myeloid precursors and monocytes lose the expression of Gr-1 and gain the expression of F4/80. BMDMs were harvested and stained for macrophage cell surface markers used to discriminate developmental stage, F4/80 and Gr-1, and analyzed by flow cytometry. I found that mature F4/80^{+} Gr-1^{-} macrophages could develop in the absence of TFEB and /or TFE3. This indicates that these transcription factors are dispensable for the development of macrophages from bone marrow derived progenitors in vitro (Figure 10B).

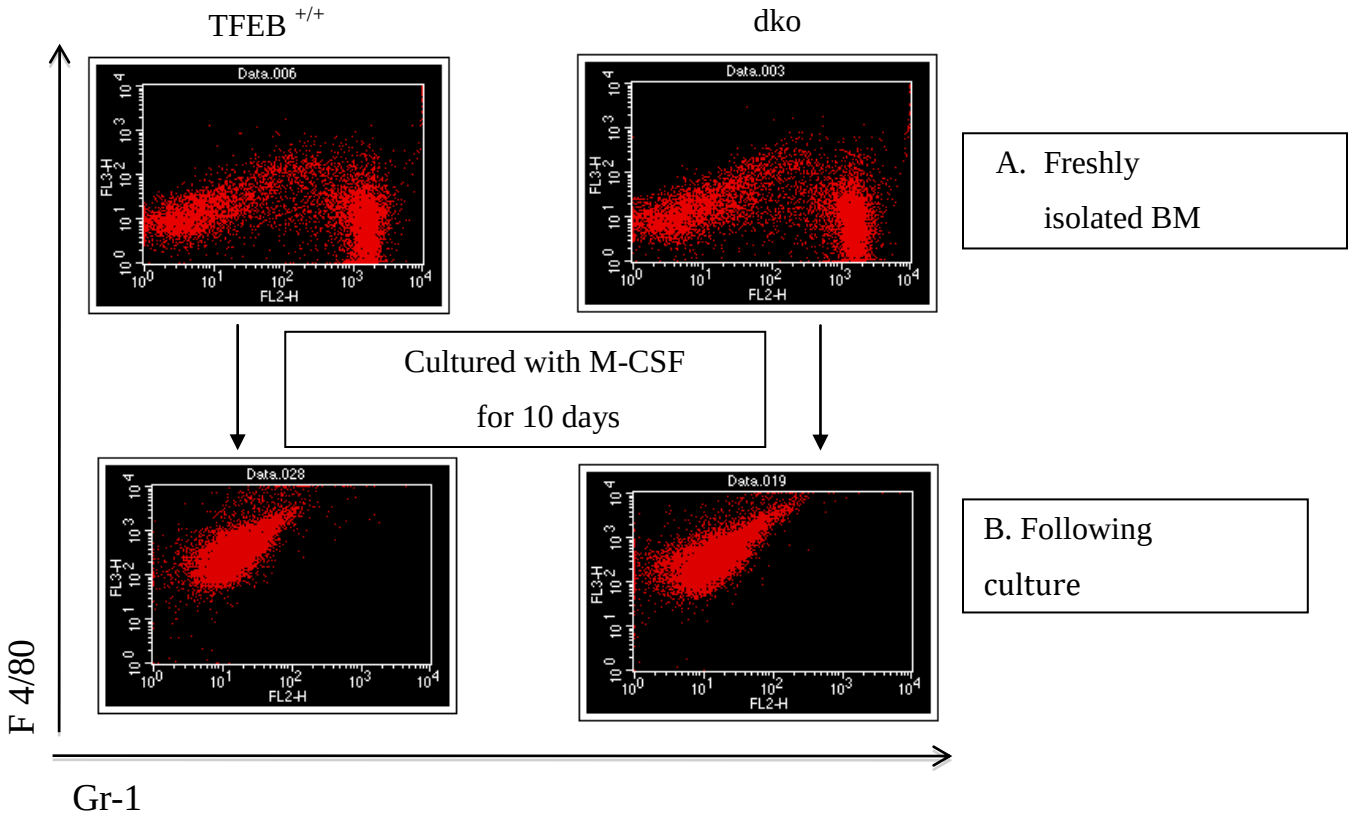


Figure 10. Bone marrow derived macrophages from mice that lack TFEB and/or TFE3 gene function develop in vitro. Representative images of flow cytometric analysis of, A) Freshly isolated bone marrow from WT and dko mice and B) BM cells derived from the same mice cultured in the presence of M-CSF for 10 days to induce macrophage differentiation. Monocytes and their precursors in the bone marrow express high levels of cell surface molecule Gr-1, which is an epitope shared by the closely related molecule Ly6G and Ly6C. During the process of maturation and differentiation into macrophages, monocytes down-regulate and eventually lose the expression of Gr-1 and gain the expression of F4/80. Both WT and dko mice showed similar proportions of F4/80 + macrophages. Dot plots representative of one mouse per group (n=8-10 per group). Significance tested by non-parametric Mann-Whitney U test, $p>0.05$.

AIM 1C. To determine if conditional deletion of TFEB/TFE3 results in altered immune mediated autophagy in primary BMDMs.

Significance: LPS stimulation of mammalian cells occurs through a series of interactions with several proteins including the LPS binding protein (LBP), CD14, MD-2 and TLR4. Upon LPS recognition, TLR4 undergoes oligomerization and recruits its downstream adaptor proteins through interactions with the TIR (Toll-interleukin-1 receptor) domains. TIR domain is critical for LPS response as a single point mutation in the TIR domain of TLR4 can abolish the response [41]. Of the five known adaptor proteins that can interact with TLR4, MyD88 (myeloid differentiation primary response gene 88) is critical as MyD88 knockout mice have impaired cytokine response [42]. Upon LPS stimulation, MyD88 recruits and activates a death domain-containing kinase, IL-1 receptor-associated kinase-4 (IRAK-4). Similar to MyD88 knockout macrophages, IRAK-4 knock out macrophages have impaired cytokine responses to LPS [43]. IRAK-4 along with another adaptor protein TRAF-6 (TNF receptor-associated factor 6), activates TAK1 (transforming growth factor-β-activated kinase 1) [44, 45]. TAK1 then activates downstream IKK (IκB kinase). IKK phosphorylates IκB (inhibitor of κ light chain gene enhancer in B cells) proteins. This phosphorylation leads to the degradation of IκB proteins and the subsequent translocation of the transcription factor NF-κB, which controls the expression of proinflammatory cytokines, in addition to other immune related genes. Activation of macrophages with different TLR ligands or live bacteria was recently shown to result in a robust and persistent accumulation of TFE3 in the nucleus[40]. ChIP-seq analysis confirmed increased binding of TFE3 to the promoters of autophagic/lysosomal genes following LPS stimulation. Accordingly, transcriptional upregulation and secretion of several key cytokines and chemokines are severely reduced in TFEB/TFE3 knockout cells both in vitro and in vivo [40].

Experimental design: I tested the functionality of similarly prepared BMDMs by treating them with low dose LPS (10 ng/ml) for 16 hours as described by Chakraborthy et al [46]. At these “low” levels of LPS, macrophages respond by secreting proinflammatory cytokines into the medium (Figure 11A).

Results: Analysis of the supernatants from WT, TFEB ko reveled that TFEB ko bone marrow derived macrophages secrete higher IL1β but lower IL-12 in response to low dose LPS in vitro. In contrast, IL6 and TNF-α were indistinguishable. These results indicate a differential role for TFEB/TFE3 in proinflammatory cytokine response to endotoxin in murine macrophages (Figure11B).

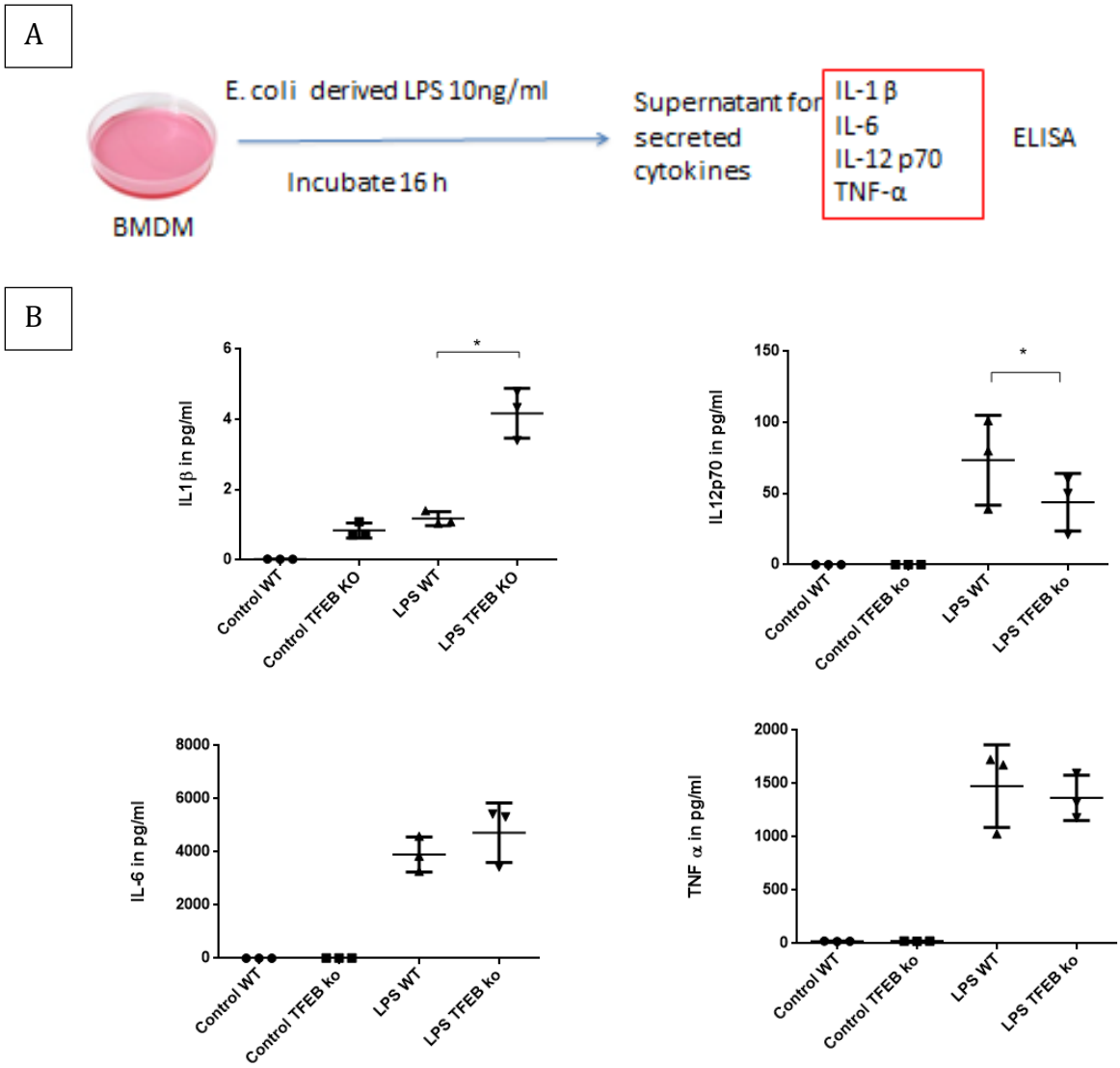


Figure 11. TFEB ko bone marrow derived macrophages secrete less IL-12 and more IL1beta in response to low dose LPS. A) Induction of proinflammatory cytokine responses to endotoxin in BMDM from WT and TFEB ko mice are plated overnight and treated with LPS at 10ng/ml for 16 h and supernatant was analyzed for secreted cytokines by ELISA. Each circle represents one mouse and n=3 per group. Significance tested by paired t-test (n=3) *p<0.05.

Aim 1D: To test the phagocytic function of peritoneal macrophages deficient in TFEB/ TFE3 or both**.**

Significance: The word “Phagocytosis” is derived from Greek (phagein), meaning "to devour", kytos, meaning "cell", and *-osis*, meaning "process". It is an important innate immune response during which a phagocyte engulfs a solid particle or another cell to form a phagosome, which fuses with the lysosome to form a phagolysosome. The particle is then broken down by the digestive enzymes found in the lysosomes. The resulting catabolized material is discharged from the phagocyte by exocytosis. As stated in previous sections, this process is employed by professional phagocytes like macrophages to protect the host by digesting microbes, with the resulting peptide antigens loaded on to MHCII to be presented to CD4 T cells, thus initiating the adaptive immune response. Phagocytosis in mammalian immune cells is activated by to the binding of pathogen-associated molecular patterns (PAMPS), such as LPS, to their corresponding Pattern Recognition Receptors (PRRs), which leads to NF-κB activation. Opsonins such as C3b and antibodies can act as binding sites to complement and Fc receptors, respectively, and activate phagocytosis of pathogens through those receptors [47]. Phagocytosis of *E. coli* particles is mediated by TLR4/MD2 present on the peritoneal macrophages, whereas phagocytosis of non-opsonized fluorescent beads is mediated by the scavenger receptors SR-A and MARCO. Engulfment of material is facilitated by the actin-myosin contractile system and can be inhibited by pre-treating the cells with actin polymerization inhibitor Cytochalasin D [48].

Experimental design for in vivo phagocytosis: WT, TFEB ko, TFE3 null and dko mice were injected with 500μg of pHrodo *E. coli* particles intraperitoneally (IP). These *E. coli* particles are conjugated to red dye that does not emit fluorescence outside the cell but is sensitive to the acidic pH of the phagolysosome and emits red fluorescence when internalized in the phagolysosome. The peritoneal exudate cells (PEC) were harvested 3 hours later, stained with fluorescent Abs and analyzed by flow cytometry. Data was analyzed using Flojo software and significance determined by Mann-Whitney U test.

Experimental design for in vitro phagocytosis in PEC: PEC were harvested from WT, TFEB ko and TFE3 ko mice and plated in RPMI medium at $2*10^5$ cells/well in a 96 well plate. Cells were incubated with *E. coli* particles (25μg/well) or Fluoresbrite beads and incubated at 37^0C and 5% CO_2 for 6 h. Control cells were incubated on ice for the same time period. Phagocytosis was inhibited by treating cells with Cytochalasin D for 30 min prior to the addition of *E. coli* particles.

Results: Internalization of both fluorescent beads and pH sensitive *E. coli* in TFEB ko, TFE3 null and dko peritoneal macrophages is comparable to that of WT mice (Figures 12 and 13).This particle/bead internalization is sensitive to inhibition by Cytochalasin D and incubation on ice (supplementary Figure 1). TFEB/TFE3 is dispensable for phagocytosis of both *E. coli* and beads in vitro and in vivo and for the generation of phagolysosomes.

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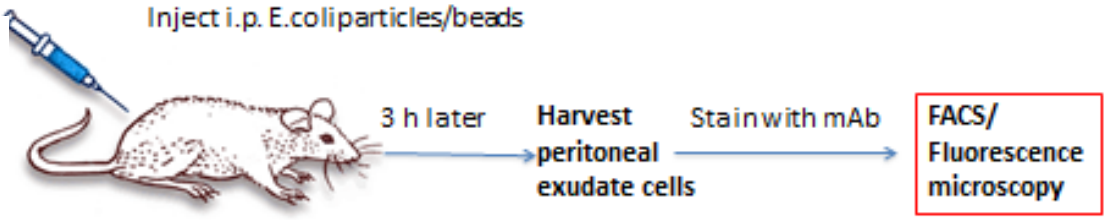

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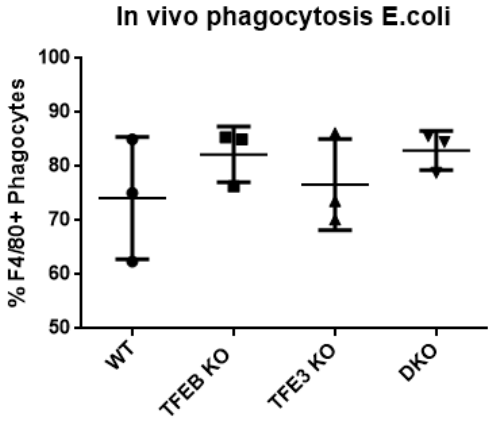

Figure 12. Phagocytosis of *E. coli* particles by peritoneal macrophages in vivo is comparable in WT and TFEB/TFE3 deficient mice. A) Schematic of the experimental set up for in vivo phagocytosis assay: Mice were injected with 500µg of pHRodo E coli particles IP and harvested 3 h later and stained with F4/80 mAb. B) Scatter plots show percentages of F4/80+ cells that ingested the *E. coli* particles. Each dot represents samples from one mouse; n=3 animals per group significance between groups was tested using the non-parametric Mann-Whitney U test $p>0.05$.

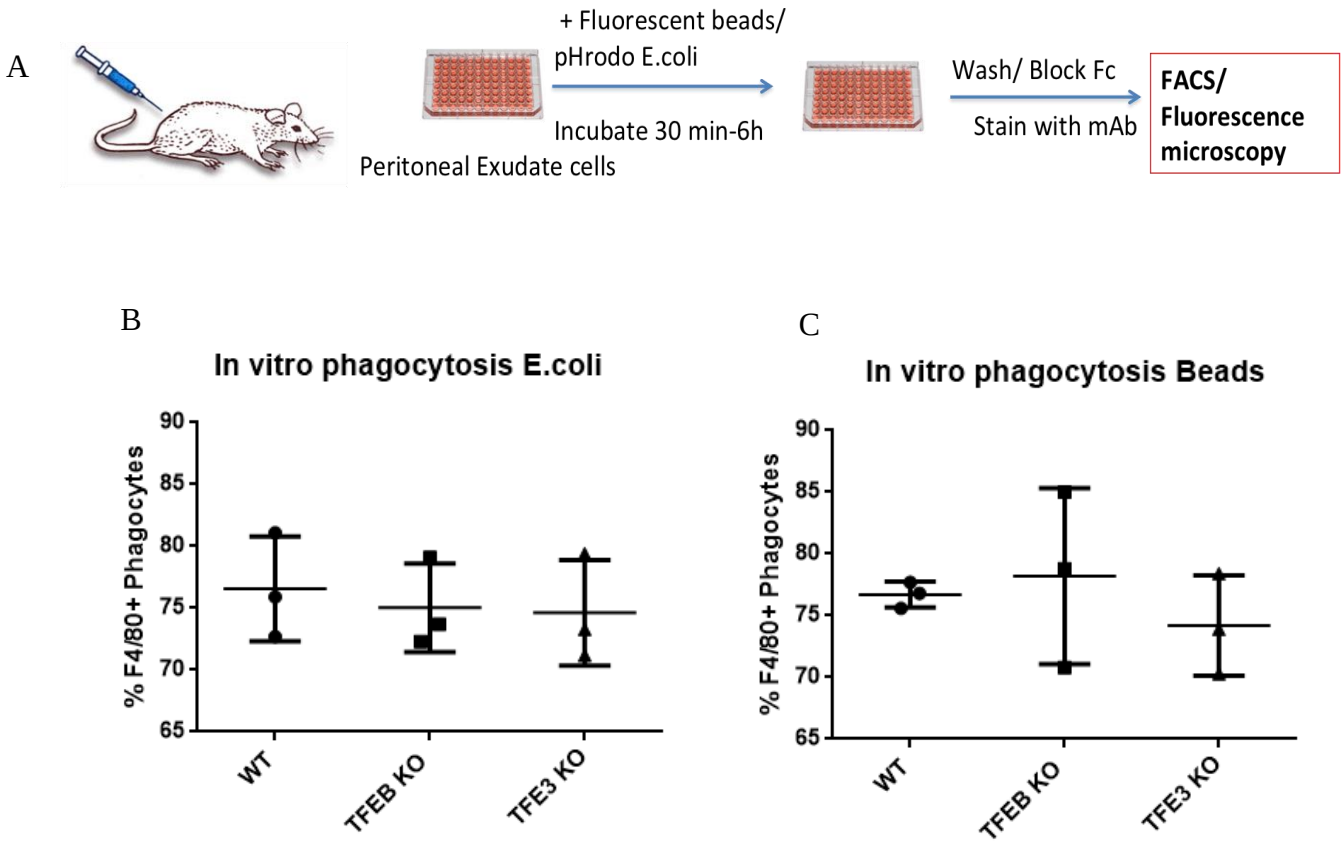

Figure 13. Phagocytosis of *E. coli* and fluorescent beads by peritoneal macrophages in vitro is comparable in WT and TFEB/TFE3 deficient mice. A) Schematic of in vitro phagocytosis assay. PECs were plated, incubated with particulates for the indicated time periods, and then stained for analysis by flow cytometry B) Flow cytometry of peritoneal exudate cells incubated with pHrodo *E. coli* particles for 6 h at 37^0 C and stained and gated for F4/80. C) Flow cytometry of peritoneal exudate cells incubated with Fluoresbrite latex bead particles for 6 h at 37^0 C and stained and gated for F4/80. Each circle represents one mouse, n=3 animals per group and error bars are SD of mean. Significance was tested using the non-parametric Mann-Whitney U test $p>0.05$)

Aim 1E. To determine if conditional deletion of TFEB in myeloid cells of mice has an effect on macrophage differentiation in vivo, using a model for sterile peritonitis.

Significance: Macrophage development, migration, and activation have been shown to depend on autophagy [29]. I tested the importance of TFEB (and TFE3) in this process using an established model for sterile peritonitis. In this model, mice are injected with sterile but aged 4% thioglycollate solution. During the aging process, non-enzymatic reactions between proteins and reducing sugars in thioglycollate broth lead to formation of advanced glycation end-products [49]. These end products are recognized by members of the PRR family, such as the receptor for advanced glycation end-products (RAGE) [50] and the macrophage scavenger receptor (SR-A). Both receptors are expressed on monocytes and macrophages, and when engaged by ligands, cause a robust inflammatory response. The so called "sterile peritonitis" induced by this process attracts monocytes, which, upon entrance into peritoneal cavity, differentiate into macrophages. This has been shown to rely in part on autophagy [29]. This is a well-established protocol to elicit monocytes into the peritoneal cavity and induce in vivo differentiation into macrophages [29] Experimental Design: 6-8 month old Lyz2cre^{+}/ TFEB $^{flox/flox}$ mice, dko and control mice were injected IP with 1 ml of aged, sterile 4% thioglycollate and sacrificed 4 days later at which time the peritoneal exudate cells were collected by lavage with ice-cold PBS +3% serum (Figure 14A). The cells were then stained and analyzed by flow cytometry for markers of macrophages.

A

B

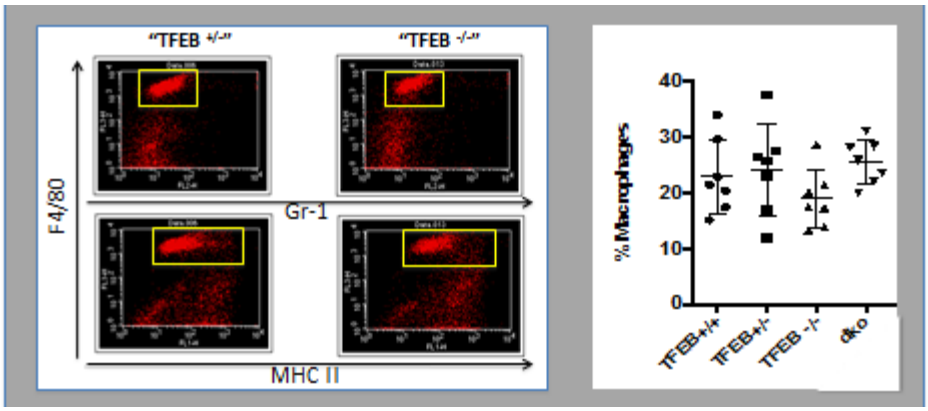

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Figure 14. Monocyte differentiation into Macrophages in vivo proceeds in the absence of TFEB/TFE3. A) Monocyte differentiation to Macrophages in vivo, using a model for thioglycollate induced sterile peritonitis. B) Scatter plot, showing the proportion of macrophages (F4/80 ^{hi} Gr-1^{lo} MHC II^{+}) in PECs; Each dot represents one mouse, n=7 mice per group. Significance was tested using the non-parametric Mann-Whitney U test; p values were between 0.3 and 0.6, which is not significant.

Results: The proportion of macrophages elicited by sterile peritonitis was not affected by the absence of TFEB and/or TFE3, as there was no statistically significant difference between the different groups of mice tested (Figure 14B). Monocyte differentiation into macrophages was not impaired in the absence of TFEB, TFE3 or both.

AIM 1F. To determine if TFEB/TFE3 deleted macrophages can elicit proinflammatory responses to low doses of bacterial endotoxin.

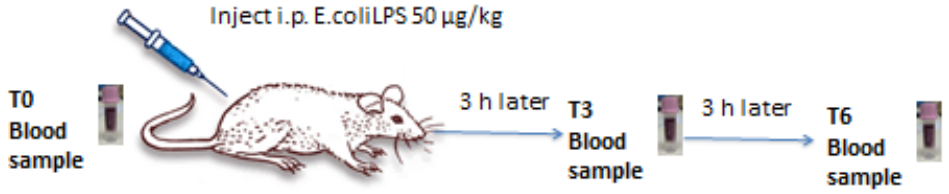
Significance: Every year, severe sepsis strikes more than a million Americans [51]. It's been estimated that between 28 and 50 percent of these people die [52]. "Sepsis" is defined as the inflammatory response to infection and "severe sepsis" is sepsis complicated by severe organ dysfunction [53]. "Septic shock" is sepsis complicated by either hypotension that is refractory to fluid resuscitation or by hyperlactatemia. Both gram negative and gram positive bacteria can cause sepsis by triggering a systemic and pathological inflammatory response. Macrophages respond to bacterial endotoxin derived from gram negative bacteria by secreting proinflammatory cytokines like IL-1, IL-6, IL-12 and TNF-α. Cytokines like these are central to the role of macrophages as sentries and first responders of the innate immune system that help mediate the transition from innate to adaptive immunity. The spatio temporal regulation of these cytokines is critical for mounting an effective immune response but one that is not excessive. For example, TNF–α is responsible for vasodilatation and infiltration of leukocytes to sites of infection to mount a protective immune response, but it is also a potent pyrogen. Dysregulated secretion of TNF-α and along with IL-1 is known to cause septic shock through their effect on the preoptic region of the hypothalamus, resulting in fever, cachexia and stress response.

Experimental Design: The dose of endotoxin needed to induce an interleukin-6 (IL-6) concentration in plasma of ~1,000 pg/ml 2 h after injection was documented to be 2 ng/kg of body weight in humans and 500 ng/kg in mice [54]. In mice the 50% lethal dose of LPS (mice expire from septic shock) ranges from 1.6 mg-25mg/kg body weight depending on the age of the mice [55]. Older mice are more susceptible to lower amounts of LPS. Low doses of LPS 50-100μg/kg body weight in mice, the range used in my experiments, elicit a proinflammatory cytokine response in mice without causing distress (pain or fever).

3-4 month old Lyz2cre^{+}/ TFEB $^{flox/flox}$ mice, dko and control mice were injected IP with 50μg/kg body weight of LPS derived from *E. coli* to elicit macrophage cytokine responses. The T=0 blood samples were collected prior to injecting the endotoxin, and 3 hours later (T3) another blood sample was collected. The mice were euthanized at 6 hours post injection (T6) and final blood sample was collected by cardiac puncture. Serum was collected and analyzed for cytokines IL-1, IL-6, IL-12 and TNF-α by ELISA.

Results: I found that serum titers for secreted cytokines IL-6, IL-1β and TNF-α were decreased in mice with deletion of TFEB and/or TFE3 6 h following LPS injection. These results suggest that both these factors are critical for optimal cytokine response to endotoxin in mice (Figure15).

A



Serum
Cytokines
IL-1 β
IL-6
IL-12p70
TNF-α

B

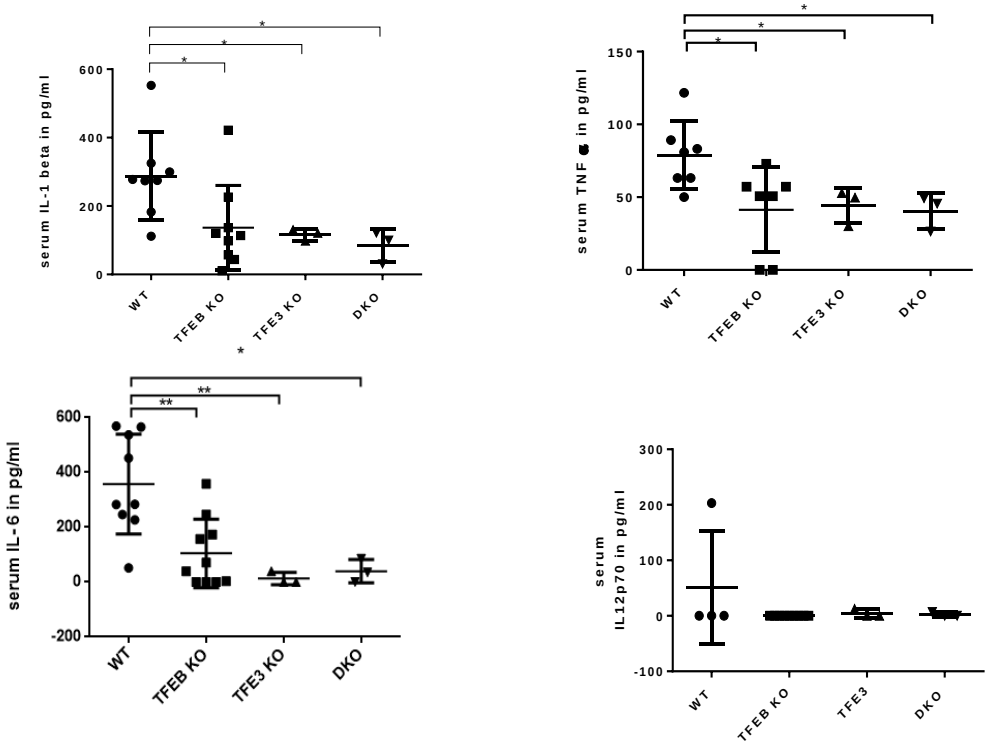


Figure 15. Macrophage dependent IL-6, TNFα and IL-1β cytokine responses to LPS are impaired in TFEB/ TFE3 deficient mice compared to WT: A) Inducing proinflammatory response to Endotoxin in mice: Experimental set up B) Serum analyses for proinflammatory cytokines IL-6, TNF-α, IL-1β, IL-12p70 from conditional TFEB ko, TFE3 ko and dko and littermate control mice 6h after low dose endotoxin challenge are shown. Each dot represents sample from one mouse. Error bars are SD with mean, n=3-8 mice per group. p*<0.05 and p**<0.005.

My studies show that TFEB abundance and nuclear localization is responsive to immunological stimuli that activate autophagy in transformed macrophages. TFEB and TFE3 are not required individually or jointly for the differentiation of bone marrow progenitors into macrophages in the presence of M-CSF in vitro and for the migration and differentiation of circulating monocytes to peritoneal macrophages during sterile peritonitis in vivo. Phagocytosis of *E. coli* particles and beads by macrophages in the absence of TFE3/TFEB was comparable to WT mice both in vivo and in vitro. Yet despite these similarities, proinflammatory cytokine responses by TFE3 and/or TFE3 deficient macrophages to low dose endotoxin was impaired both in vitro and in vivo. These results confirm the critical importance of TFE3 and TFEB to anti-microbial defense, and also mechanistically distinguish processes related to autophagy like phagocytosis and differentiation from PRR-dependent gene expression by the differential importance of TFE3 and TFEB in these processes.

AIM 2

The goal of aim 2 is to understand the underlying mechanism and manifestations of defects in FO, MZ and B1 B cell subsets under conditions of MiT deficiency. In this Aim, I focus on interrogating the function of different B cell populations known as FO, MZ and B1 B cells. Here, I present background information on the immunological importance of these B cells and how they contribute to antibody mediated host defense so that the rationale for the following experiments is clear.

BACKGROUND AND SUMMARY

B cells are the sole producers of antibodies, developing from hematopoietic stem cells in fetal liver and bone marrow in adults. B-cell precursors in the bone marrow are divided into four main populations termed fraction A (pre-pro-B cells), fraction B (pro-B cells), fraction C (pro-B cells) and fraction D (pre-B cells) largely based on the status of Ig gene rearrangement. Differentiation is thought to proceed from fractions A to B to C to D. These fractions are also called Hardy's fractions, first described in the early 90s [56]. Upon successful completion of Ig gene rearrangement and vetting of B cells with autoreactive BCRs, IgM+ immature B cells exit the bone marrow and enter the periphery to complete maturation in secondary lymphoid organs. Mature naïve B cells comprise different subsets characterized by their distinct immune phenotype, anatomical location and function, which can be broadly divided into B1 and B2 cells. B1 B cells are further divided into B1a and B1b subsets, whereas B 2 cells can be further divided into FO and MZ B cells (see table 1). B2 B cells are comprised of follicular (FO) B cells and marginal zone (MZ) B cells. FO B cells are the predominant population and are mostly thought to be involved in generating TD antibody responses to protein antigens. Naïve resting FO B cells express high levels of IgD, and CD23; lower levels of IgM and CD21; and no CD1 or CD5, readily distinguishing this compartment from B1 B cells and marginal zone B-cells. FO B cells organize into the primary follicles of B cell zones focused around follicular dendritic cells in the white pulp of the spleen and the cortical areas of peripheral lymph nodes. In resting

state, they reside in lymphoid follicles adjacent to T cell zones such as the PALS in the spleen. In contrast, MZ B cells in the mice express high levels of IgM and CD 21 and low levels of IgD and CD 23 and are located in between the red pulp and the white pulp of the spleen and are important first responders for blood borne pathogens. MZ B cells can respond to carbohydrate/polysaccharide antigens in a TI manner. To better serve this purpose, these cells have low threshold for activation through their BCRs. MZ B cells can be activated by cells other than T helper cells. [57]. These cells include macrophages, dendritic cells and neutrophils which can help MZ B cells class switch and are often called the 'non-canonical B helper cells' [57, 58]. In the mice MZ B cells are known to secrete mainly IgM and then, can switch to secrete IgG3 and IgG2b in response to TI antigens and to IgG1 in response to TD antigens [59].

B1 B cells are predominantly located in the peritoneal cavity, pleural cavity and payer's patches although a small population can be found in the mouse spleen. B1 B cells are IgM hi, IgD lo, CD43+, which can be further sub divided into B1a and B1b subsets based on the cell surface expression of CD5. B1 B cells are the source of natural IgM and produce polyreactive and autoreactive antibodies.

B cell subset	Anatomical Location	Immunological Purpose	Surface Markers
FO	Follicle of Spleen, Lymph Nodes, Payer's patches	TD, Some vaccines, proteins	IgM^{low} IgD^{high} $CD21^{int}$ $CD23^{+}$
MZ	Marginal Zone and, Marginal Sinus of the spleen	TI (TD) 1^{st} responders to blood borne pathogens. encapsulated bacteria	IgM^{hi} IgD^{low} $CD21^{hi}$ $CD23^{-}$
B1a	Peritoneal Cavity, Payer's patches, Pleural cavity, Spleen	TI, Innate immunity, 1^{st} responders, natural IgM	IgM^{hi} IgD^{low} $CD5^{+}$ $CD43^{+}$
B1b	Peritoneal Cavity, Payer's patches, Pleural cavity, Spleen	TI, Innate immunity, 1^{st} responders, natural IgM	IgM^{hi} IgD^{low} $CD5^{-}$ $CD43^{+}$

Table1. B cell subsets, anatomical location and their immune phenotype.

MiT Family in B cells:

Upon stimulation of the BCR receptor and CD40 engagement from T cells, FO cells proliferate and become GC B cells. Further differentiation of GC B cells into antibody secreting plasma cells involves the transcription factors, Mitf, IRF-4, Blimp-1 and Xbp-1 through the inhibition of Bcl-6. Plasma cell development depends in the expression of Interferon Regulatory factor-4 (IRF-4), which activates B-

lymphocyte induced maturation protein-1 (Blimp-1) [60]. Stanford Peng's group has shown that Mitf is a negative regulator for plasma cell development because B cells lacking Mitf function are more likely to differentiate into plasma cells and secrete autoantibodies [9]. Mitf helps mature B cells to remain in their resting state, and in its absence, IRF4 expression is induced more readily, and as a result, plasma cells form spontaneously and more frequently. Thus, Mitf appears to restrain B cell activation by acting as an inhibitor of IRF4, indirectly inhibiting Blimp-1 expression.

The experiments used by the Peng group studied Mitf function using a well characterized Mitf mutant strain known as *mi*. So called $Mitf^{mi}$ mutation is a dominant negative allele caused by missense mutations in the basic region of Mitf gene that abrogate DNA binding of the Mitf protein but leave dimerization function intact. This mutation in the mouse results not just in albinism due to defects in pigmented cell development, but also in severe osteopetrosis caused by the functional inactivation of both Mitf and TFE3 in osteoclasts because the wild-type TFE3 protein is inhibited by the mutant $Mitf^{mi}$ protein; these results that showed TFE3 has an important role in that cell type. The osteoclast defect results in hostile bone marrow microenvironment and severe lymphopenia. So, Peng's group used adoptive transfer experiments involving the transfer of Mitf*mi/mi* HSCs to syngeneic wild-type irradiated hosts as a means to provide a normal bone marrow environment to study the maturation of Mitf*mi/mi* HSCs. As the dominant mutation is present in all HSCs, it was not possible to ascertain if the B cell defects were intrinsic or extrinsic and caused by other cells lacking MiT. A previous graduate student, Maria Lopez-Ocasio, used complementary genetic approaches to address the role of various MiT members in B cell differentiation and activation [10] See table 2 for various mouse models for studying MiT proteins and the cell types affected.

Mouse Models	MiT Inhibition	Cell type in which MiT activity is affected
TDN-B	All MiT members	B cells
TDN-B/T	All MiT members	B and T cells
CD 40L/TDN B	All MiT members	B cells
Mitf $^{vga/vga}$	Mitf recessive	All cells
TFE3 $^{-/-}$	TFE3	All cells
TFEB $^{+/-}$ TFE3 $^{-/-}$	TFEB/TFE3	All cells
TFEB $^{flox/-}$ CD19Cre	TFEB	B cells only
TFEB $^{flox/-}$ Lyz2cre	TFEB	Monocytes,& Macrophages

.

Table 2. B cell and Macrophage-specific MiT deficient mouse models to study MiT function

MZ and Peritoneal B1a B cells require TFE3 and TFEB

Preliminary studies by Maria Lopez-Ocasio [10] found that MZ B cells, critical for antibody responses against encapsulated bacteria, and peritoneal B1a cells, a major source of immune regulatory natural IgM antibody, both depend on TFEB and TFE3. She observed a decrease in the proportion of MZ B cells and titers of IgG3 antibodies produced by these cells in three different lines of genetically modified mice in which TFE3, TFEB, or both were inactivated. In addition, she also observed decrease in numbers of peritoneal B1a cells as the mice aged from 2-6 months. This was not observed in mice that lacked Mitf function alone. One interpretation of these findings is that TFEB and possibly TFE3 are important because of TFEB's involvement in other cell types in autophagy. It is known that peritoneal B1a cells are maintained

by self-renewal and that autophagy is important for their maintenance, so it is important to investigate if defective autophagy is causing the loss of this B cell subset in the absence of TFEB and TFE3. One important function of B1a B cells is that they are phagocytic, and so it is reasonable to investigate whether the observed defect reflects a defect in their ability to phagocytose. However, other mechanisms unrelated to autophagy may be in operation. The goal of the experiments in Aim2 is therefore focused on understanding whether impaired autophagy in TFE3/TFEB-deficient B1a B cells and MZ B cells explains their reduced numbers, or if other processes are involved.

Significance: The importance of the MZ and MZ B cells is evident in individuals that lack the spleen as a result of surgery or congenital asplenia are at a higher risk of pneumonitis, meningitis and fulminant septic syndrome caused by encapsulated bacteria such as Streptococcus pneumoniae, Haemophilus influenzae and Neisseria meningitidis[61-63]. These infections are generally attributed to an impaired production by MZ B cells of IgM and IgG specific for capsular polysaccharides[61] . A better understanding of antibody responses in MZ B cells can be harnessed in designing improved vaccines for asplenic patients.

Brief overview of TD and TI responses:

B cell antigens can be broadly classified into TD and TI antigens. TD antibody responses are mainly generated by follicular (FO) B cells and require the help of T cells like CD4 T helper cells that in turn require the thymus to develop. These antibody responses were originally defined as such by performing immunizations in intact mice to those in which the thymus was removed at birth. Antibody responses to proteins is TD because peptide antigens derived from those proteins must be presented to cognate CD4 cells via MHCII in order to be mounted. In contrast, TI antigens lack protein and either cannot or do not require CD4 T cells in order to activate an antibody response. TD antibody responses are characterized by a memory response (short lag time, elevated titers, switched and affinity matured IgG) made possible by and long lived memory and plasma cells, whereas those mounted during a TI response do not. Typically, TD responses are largely defined by high affinity IgG in memory responses, whereas most TI responses are low affinity

responses that are primarily IgM but can also be IgG2b and IgG3 in mice depending on the non-proteinaceous immunogen. MZ and B1 B cells are thought to be the main source of TI antibody responses. To better understand the MZ B cell defect observed in TDN-B and TDN-B/T mice (which have impaired CD40L expression and thus impaired T cell help) I elicited TI and TD immune responses in these mice (Figure 16).

Prerequisites	TD antigen	TI-2 Antigen
Type of immunogen	Protein (e.g. KLH)	Polysaccharides (e.g. Ficoll), glycolipids
Requires repeating epitopes	No	Yes
B cell subsets involved	FO B cells	MZ B cells
Need for MHC II	Yes	No
Needs CD4 T helper cells	Yes	No
Immunological Memory	*Yes*	*Occasionally*

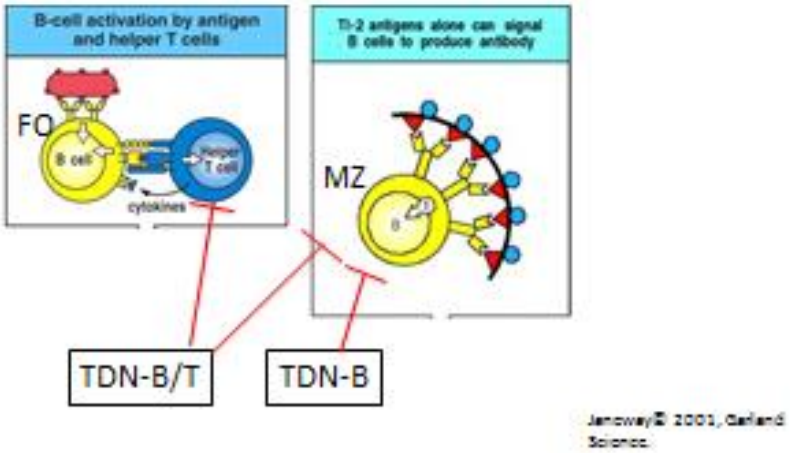


Figure 16. T dependent vs T independent immune responses. The table summarizes characteristics of TD and TI immune responses shown in the cartoon below (adapted from [47]). TD dependent responses are normally generated by FO B cells upon activation and require MHCII presentation of the antigen by cognate CD4 T cells and generate immunological memory, whereas TI antigens are generated by molecules containing repeating epitopes like polysaccharides and glycolipids and are typically generated by MZ B cells independent of T cell help. In the cartoon below, the left side depicts a FO B cell in yellow responding to antigen presentation by MHC II by the CD4 T cell in blue and the right side depicts a MZ B in yellow cell responding to TI-2 antigen. The cartoon also depicts the cell types affected by the transgene in TDN-B and TDN-B/T mice.

MATERIALS AND METHODS

Animals: All the mice are maintained in specific pathogen-free conditions SPF (5 mice per cage and 12 hour light/dark cycle) with sterilized food and water. Mice were euthanized by CO_2 asphyxiation according to DLAR and IACUC standards under approved protocols. All mice used in this aim are 8-10 month old. TDN-B and TDN-B/T mice were developed in our lab by targeting TDN protein expression in the B cell or B plus T cell lines directed by immunoglobulin heavy-chain enhancer and promoter sequences. CD40L mutant mice strain $Cd40lg^{tm1Imx}$ were obtained from The Jackson laboratory and crossed with TDN-B mice to generate double mutant CD40L/TDN-B mice.

Serum and Cell Suspensions. Serum was prepared from blood taken immediately after CO_2 asphyxiation by cardiac puncture. To isolate B cells and their specific subsets such as B-1 and B2 cells from multiple organs and sites, the PerC cells were first harvested by a lavage with ice-cold isolation buffer (3% FBS in 1x PBS). To make splenic single cell suspensions, the spleen was gently ground using a pestle and the suspension is passed through a 100 μm cell strainer. RBCs are removed from all cell suspensions by RBC lysis buffer (Miltenyi Biotech). Recovered spleen and peritoneal cells were then stained with Trypan Blue 0.4% (Gibco, Grand Island, NY) and counted using a hemocytometer/ automated cell counter TC20.

Antibodies. The following antibodies were used: anti-mouse CD19 FITC, MHCII-FITC, IgM-PE, IgD- FITC, B220-FITC, CD21-PE, CD23-FITC, CD19-PerCP-Cy5.5 CD5-PE,CD9 FITC, CD11b-FITC, CD4-FITC, CD38-FITC, CD138-PE and F4/80 PE Cy5 (Biolegend).

Immunizations. To evaluate TI-II responses, wild-type, TDN-B and TDN-B/T were immunized IP with 20 μg of the TI antigen TNP-Ficoll (Biosearch Technologies) in PBS. Mice were be bled before and 7 d after immunization. To determine responses to a TD, wild-type, and TDN-B and TDN B/T mice were immunized IP with 20 μg TNP-KLH (Biosearch Technologies) in a 1:1 homogenate with Alum (Fisher Scientific). Mice were be bled before and 14 and 21 d after immunization. At day 14, a second immunization (boost) with 20 μg TNP-KLH was performed. Serum Abs specific for TNP were measured in Immuron®

plates (Dynex) coated with TNP-BSA (Biosearch Technologies) using HRP conjugated ELISA detection antibodies (Bethyl).

ELISA for TNP specific antibodies. 96-well plates were incubated with TNP-BSA coating antibody, followed by RT incubation for one hour or 4^0 C overnight. Plates were washed with 1x PBS-Tween20 (PBS-T) 5 times. Blocking buffer was then added (1% BSA in 1X PBS) to each well to block any non-specific binding sites on the surface, and incubated for one hour at RT. Sera were diluted in blocking buffer at 1:500, further diluted, IgM (1:10,000) and IgG (1:10,000), added to each well on a 96-well plate, and incubated at RT for 1 hour. Plates were then washed 5 times with PBST. HRP antibody against either IgM or IgG (total and subtypes) was diluted (1:10,000) with blocking buffer followed by a series of dilutions, followed by incubation for one hour. Plates were washed again with 1X TBST. TMB was then added to develop positive signal (blue color). The color reaction was stopped with a 1:10 dilution of H_2SO_4 and read immediately in a plate reader (Novo Star, BMG Labtech).

In vitro assessment of phagocytosis : To determine the capacity of phagocytosis of cells obtained from spleen and peritoneal cavity $2*10^5$ cells/well was be plated in 96-well plates(Thermo Fisher Scientific Inc., Waltham, MA, USA) in a final volume of 0.1ml RPMI, supplemented with 5% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin, and incubated for 6h, in a humidified atmosphere of 5% CO2 at 37°C with 1 μm fluorescent beads (Fluoresbrite Yellow Green Microspheres, Polysciences, Warrington, PA, USA) at a cell: bead ratio of 1:10 or with 25 μg pHrodo *Escherichia coli* bio particles for 6h and the non-ingested beads or bacteria were removed by centrifugation (100 *g* for 10 min at 4°C) over a cushion of 3% BSA (Fisher Scientific) in PBS supplemented with 4.5% D-glucose (Sigma-Aldrich). The cells were incubated with Fc block for 15 min on ice and then washed once and labeled with fluorescent antibodies as described. Control cells were incubated with 30μg/ml of Cytochalasin D for 30 min prior to the addition of beads/*E. coli* particle at 37^0 C. Control cells were incubated on ice for the same time periods and treated similar to experimental samples.

In vivo phagocytosis assay: Individual mice were injected i.p. with pHrodo *E. coli* bio particles (500 μg) or fluorescent 1μm beads ($2*10^9$) in 300 μl PBS. Control mice were injected with 300 μl PBS. After 3 h, PerC cells were isolated and labeled with mAb as described above. Cells were acquired on a FACScan and analyzed using cell quest / Flojo software.

Statistical Analyses. Non-parametrically distributed data sets were analyzed by Mann-Whitney U test and ANOVA using Prism 6-Graph Pad software. Student's t test was be used for parametrically distributed data sets. The level of significance was set at $p < 0.05$.

RESULTS

AIM 2A. Specific Aim: Analyze naïve antibody titers and antibody responses to TD and TI antigens

Significance: TI antibody responses are mainly directed against capsular antigens against blood borne pathogens and are mediated by immunoglobulins IgG3 and IgG2b secreted by the marginal zone B cells. TD antibody responses are mainly generated against proteins by follicular B cells following the germinal center reaction and usually generate IgG1 and IgG2a. The use of trinitrophenol (TNP)-Ficoll as a model antigen to generate TI responses, and trinitrophenol–keyhole limpet hemocyanin (TNP-KLH) as a model antigen for TD antibody responses is well established in the field [15, 64]. TNP is a hapten (an antibody epitope) and ficoll is a high molecular weight polysaccharide, whereas KLH is a carrier protein with a large molecular weight and from which KLH peptides can be derived to present to cognate CD4 T cells. Using these antigens, I immunized the mice of different genetic cohorts and analyzed the serum antibodies generated. Earlier studies from our lab found reduced IgG3 levels (which are TI) in unimmunized mice lacking TFE3 and TFEB function in B cells. Given the reduction in B1 and MZ B cells, I expected to reveal a defect in antibody responses in TFE3 and TFEB deficient mice in response to TI antigen challenge, but that TD would be intact.

Experimental approach: To evaluate TI responses control, TDN-B and TDN-B/T mice were injected IP with 20 μg TNP-Ficoll in PBS for TI responses and bled right before injection and after 7 days post injection. For TD responses, 20 μg TNP-KLH in a 1:1 homogenate with alum as adjuvant was injected IP and a booster dose of 20 μg was administered on day 14. The mice were bled before and 14 and 21 days after immunization. Serum antibody titers specific for TNP (supplementary figure.2) from all animals were measured using ELISA (Figure 17).

Results: My studies show that TI responses in TDN-B mice are intact (Figure.18), which is unexpected given that unimmunized mice had proportionally fewer MZ B cells and lower naïve serum titers of IgG3[11]. splenomegaly before and after immunization was unchanged (supplementary figure3.).

Surprisingly, MZ B cell responses to TI antigens IgG2b and IgG3 were intact and Marginal zone B cell proportions were comparable to WT after immunization (Figures 19 and 20). FO B cell responses as measured by IgG1 and IgG2a were elevated in response to TI antigen suggesting that MiT inhibited B cells have altered immune responses that depended on normal T cells because these isotypes were not elevated in TDN-B/T mice.

My studies also showed that TDN-B/T mice had defective Ig G antibody responses to both kinds of antigens and increased IgM, mimicking our earlier findings in the TDN-T mice and confirming that inactivation of MiT in T cells results in Hyper IgM syndrome (Figures 21 and 22) that indicates the elevated IgG responses to NP-ficoll seen in TDN-B mice relied on T cell help .

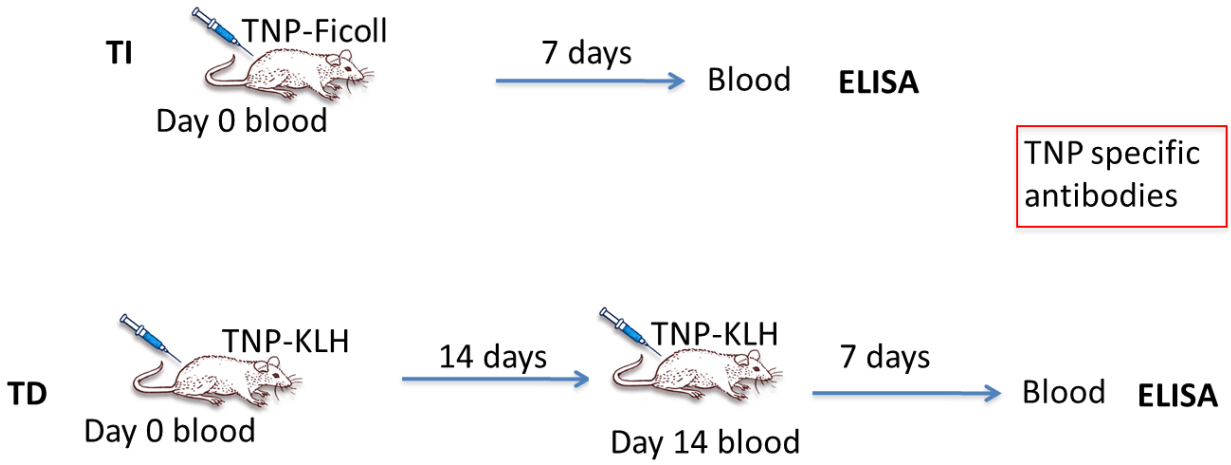


Figure 17. Schematic for studying antibody responses to TI and TD in TDN-B and TDN-B/T mice

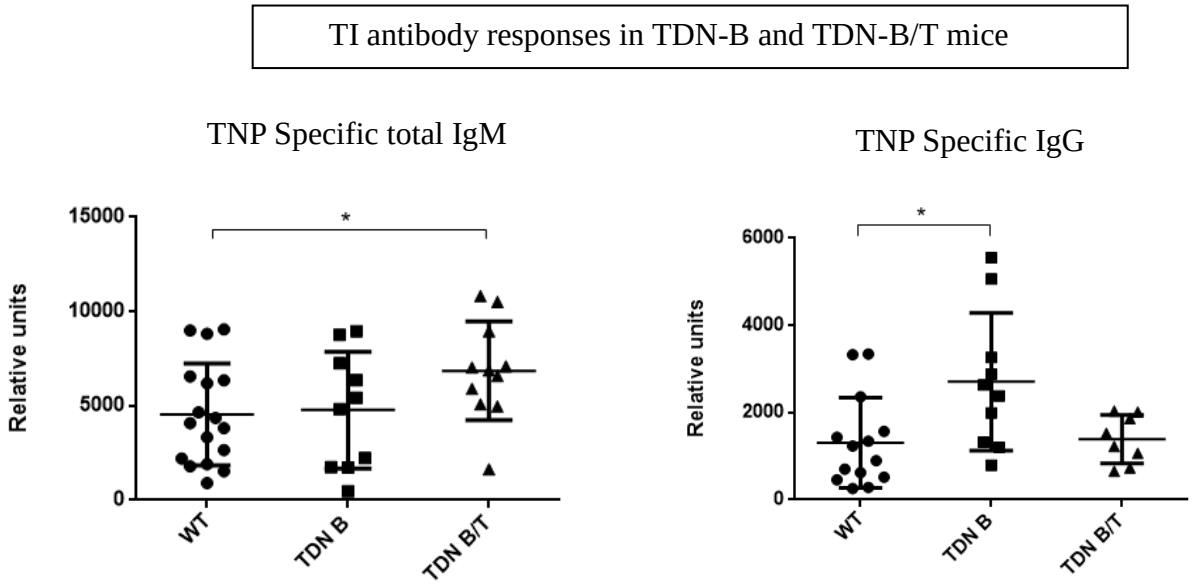


Figure 18. TDN-B mice have increased IgG responses against carbohydrate antigens where as TDN-B/T mice have higher IgM responses Immunoglobulin (IgM and IgG) assays of sera from TNP Ficoll immunized TDN-B, TDN-B/T mice and non-Tg littermates. Each circle represents one mouse. Error bars are SD of mean with n=8-17 per group,*$p<0.05$ and ns, not significant. Significance was measured using the non-parametric Mann-Whitney U test.

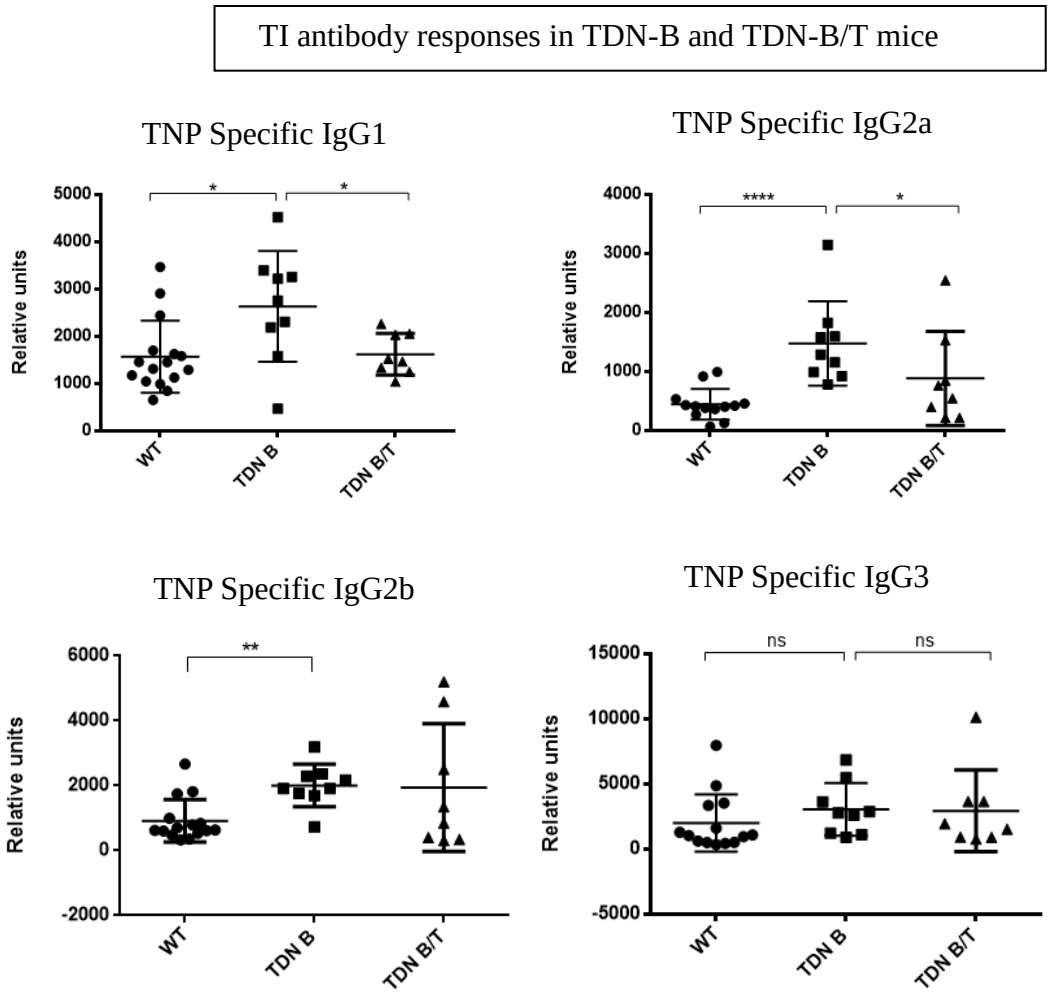


Figure 19. Increased TNP specific total IgG in Ficoll immunized TDN-B mice is due to increase in IgG1, IgG2a and IgG2b isotypes. Immunoglobulin (IgG1, IgG2a, IgG2b and IgG3) assays of sera from TNP Ficoll immunized TDN-B, TDN-B/T mice and non-Tg WT littermates. Each circle represents one mouse. Error bars are SD of mean with n=8-17 per group,*p<0.05, **p<.005 and ****p<.0001 and ns, not significant. Significance was measured using the non-parametric Mann-Whitney U test.

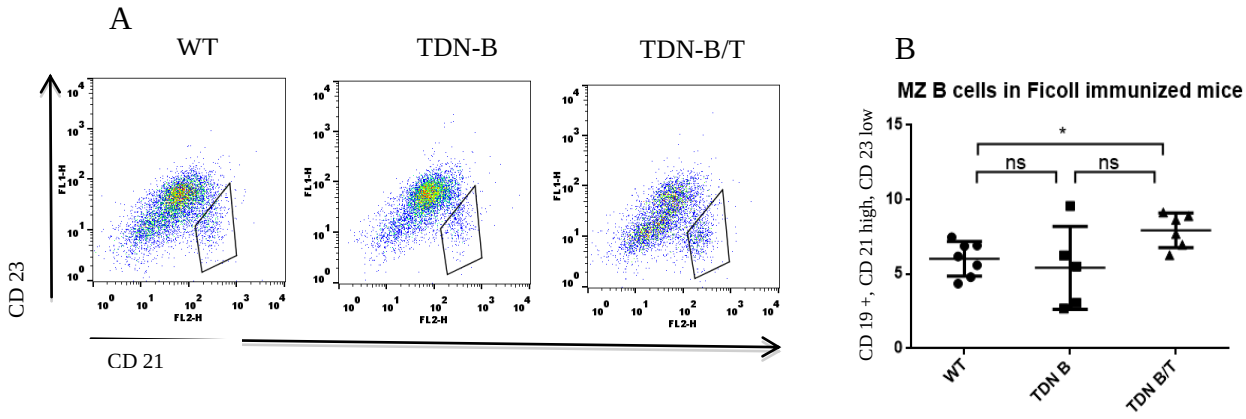


Figure 20. MZ B cells in TNP Ficoll immunized TDN-B and TDN-B/T mice are comparable to that of WT mice. A) Representative flow cytometric analyses of splenocytes from Non-Tg (WT) and TDN-B and TDN-B/T mice are shown. B) Percent of total $CD19^{+}$ splenocytes identified as MZ B cells ($CD21^{high}CD23^{low}CD19^{+}$) from Non-Tg (WT), TDN-B, and TDN-B/T mice groups are shown. Each circle represents a mouse. Error bars are SD of mean with n=5-15 animals per group * $p < 0.05$. Significance was measured using the non-parametric Mann-Whitney U test.

TD antibody responses in TDN-B and TDN-B/T

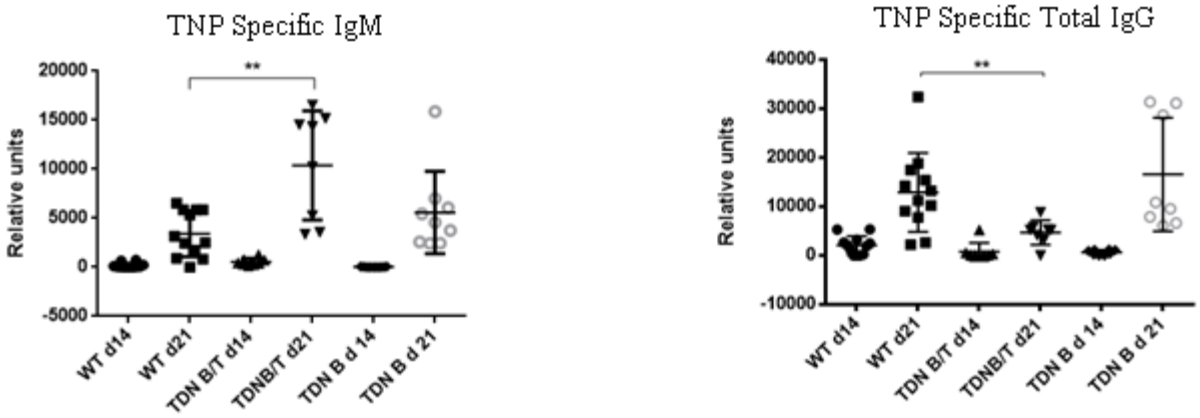


Figure 21. TDN-B/T mice have decreased IgG responses to protein antigens compared to WT mice

Immunoglobulin (IgM and IgG) assays of sera from TNP KLH immunized TDN-B, TDN-B/T mice and non-Tg WT littermates. Each circle represents one mouse. Error bars are SD of mean with n=8-12 per group,*p<0.05, **p<.005. Significance was measured using the non-parametric Mann-Whitney U test.

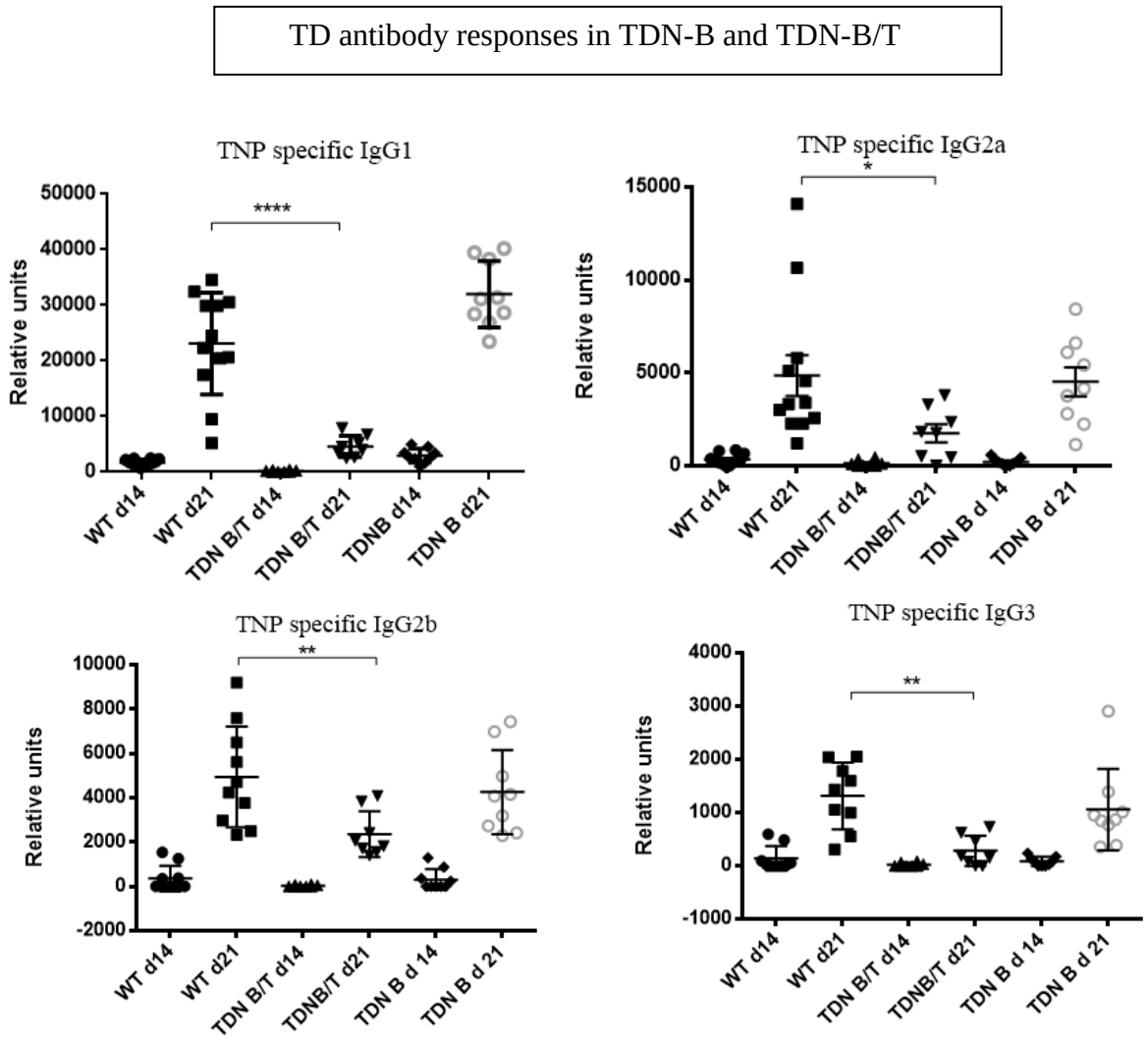


Figure 22. Increased TNP specific IgG subtypes in TD responses in TDN B mice is biased towards IgG1, IgG2a and IgG2b, whereas all IgG isotypes are reduced in TDN-B/Immunoglobulin (IgG1, IgG2a, IgG2b and IgG3) assays of sera from TNP KLH immunized TDN-B, TDN-B/T mice and non-Transgenic WT littermates. Each circle represents one mouse. Error bars are SD of mean with n=8-12 per group,*p<0.05, **p<.005. Significance was measured using the non-parametric Mann-Whitney U test.

AIM 2B. The aim of this experiment is to evaluate antigen trapping and phagocytic properties of MZ B cells and B1a B cells in vitro and in vivo.

Significance: It is known that B cells from early vertebrates have phagocytic function, but only recently it has been found that and peritoneal B1 cells from mice are capable of phagocytosis [48, 65]. Upon internalization by the B cells, the phagosomes matured into phagolysosomes and displayed the ability to kill phagocytosed bacteria. Importantly, B-1a and B-1b cells effectively present antigen recovered from phagocytosed particles to CD4 T cells thus activating the adaptive immune response. Peritoneal B1 B cells are known to rely on autophagy for their survival and maintenance [2]. Thus, in vitro and in vivo analysis of phagocytosis in these cells can provide valuable information about their functional competence. As stated in the experiments with macrophages in Aim 1, fluorescent beads and *E. coli* bio particles are safe and efficient tools widely used to investigate phagocytosis and are readily visualized by flow cytometry or fluorescence microscopy**.** The use of pHrodo bio particles, which emit red fluorescence upon acidification of only ingested particles (acidification occurs within maturing phagosomes), thus helps distinguish phagocytosed particles from those that are attached to the cell surface. All B cell subsets express the receptors known to be important in binding these particles.

Experimental approach for in vitro phagocytosis assay: Briefly PerC cells were plated ($2*10^5$ cells/well) in 96-well plates in a final volume of 0.1 ml complete RPMI, and incubated for 6h, in a humidified atmosphere of 5% CO2 at 37°C with 1 μm fluorescent beads at a cell: bead ratio of 1:10, or with 25 μg pHrodo Escherichia coli bio particles. For inhibition of phagocytosis, needed to help discern non-specific binding, cells were treated with Cytochalasin D 30μg/ml for 30 min before adding beads or *E. coli* particles. After 6 h incubation, non-ingested beads or bacteria were removed by centrifugation (100 g for 10 min at 4°C) over a cushion of 3% BSA in PBS supplemented with 4.5% D-glucose. To block FcRs, cells

were incubated with anti-mouse CD16/32 antibody on ice for 15 min. To analyze phagocytic populations, PerC cells were stained with mAb and analyzed on FACScan.

Experimental approach for in vivo phagocytosis assay**:** Individual mice were be injected IP with pHrodo *E. coli* bio particles (500 μg) or fluorescent 1 μm beads ($2*10^9$) in 300 μl PBS. Control mice were injected with 300 μl PBS. After 3 h, PerC cells were isolated and labeled as described above. The cells were stained for B cell markers analyzed on flow cytometer for internalization of the beads for green fluorescence or *E. coli* for red fluorescence.

Results: I found that CD19+ CD5+ PerC B1 cells from both TDN–B and TDN-B/T had impaired phagocytic responses to beads and *E. coli* in vitro and in vivo (Figures 23 and 24)

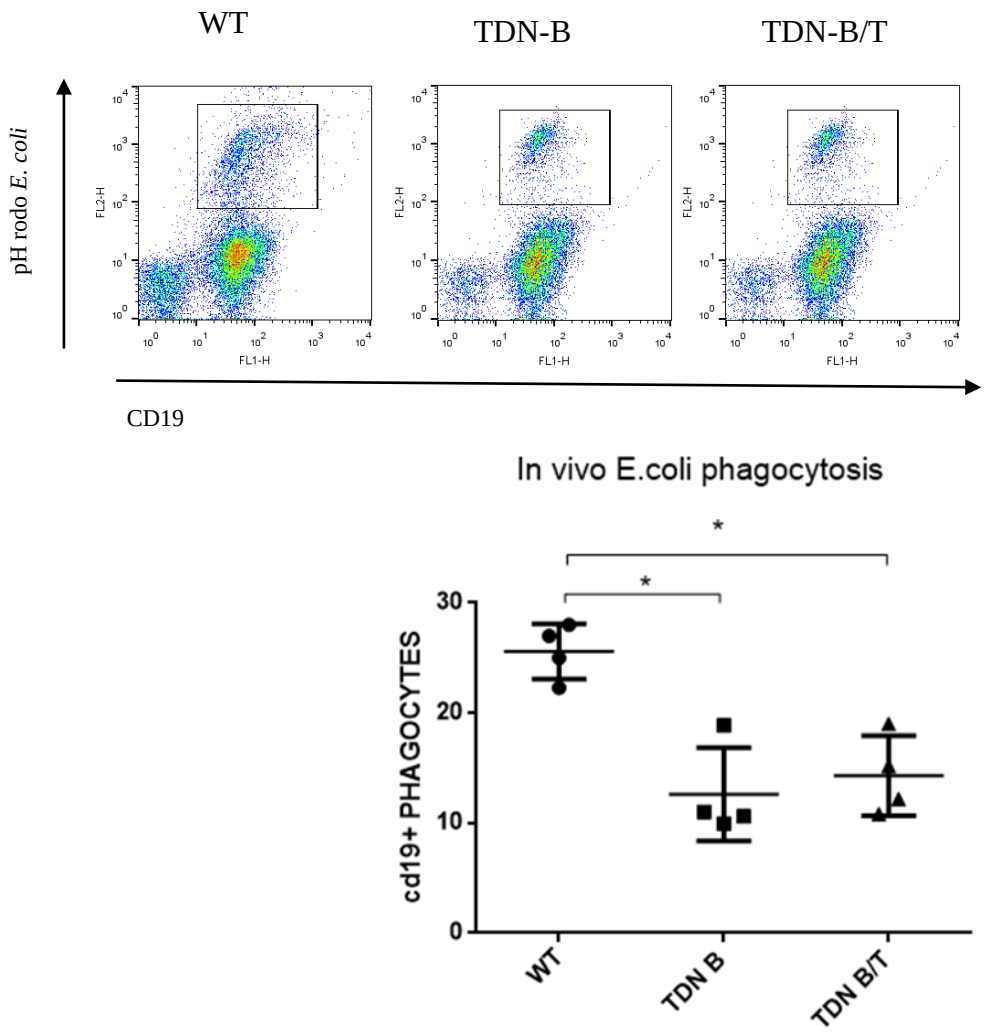


Figure 23. In vivo Phagocytosis of pHrodo *E. coli* by PerC B cells is impaired in both TDN-B and TDN-B/T mice. Mice were injected IP with pHrodo *E. coli* particles and PEC harvested 3 hours later and stained with fluorescent antibodies. Flow cytometry analysis of CD19 gated Peritoneal B1 phagocytes with fluorescent *E. coli* in phagolysosomes. Each dot represents one mouse and n=4 per group and $p<0.05$ and significance is tested using Mann-Whitney U test, error bars are SD of mean.

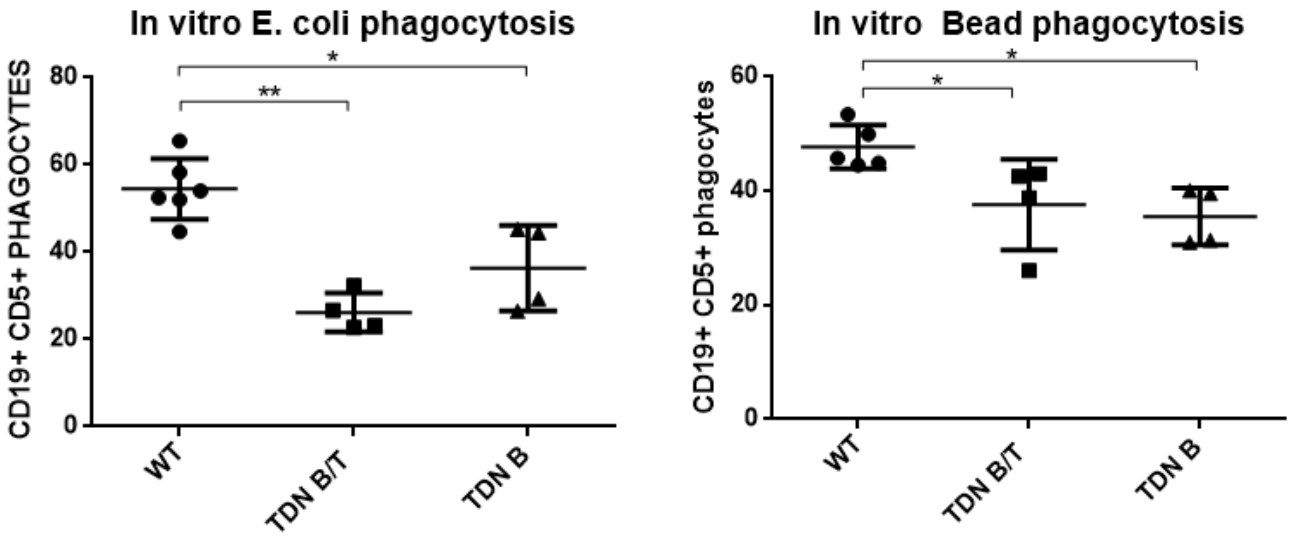


Figure 24. Phagocytosis of pHrodo *E. coli* and Fluoresbrite beads by Per B 1a B cells is impaired in both TDN-B and TDN-B/T mice. Peritoneal exudate cells were incubated in vitro with beads or *E. coli* in vitro for 6 hours. Flow cytometry analysis of CD19, CD5 gated Peritoneal B1a phagocytes with the tabulation of phagocytes with fluorescent beads or *E. coli*. Each circle represents individual mouse sample. n=4-6 per group, error bars are SD of mean and *$p<0.05$ and **$p<0.005$.Significance tested by Mann-Whitney U test.

AIM 2C. To evaluate if the differences seen between TDN-B and TDN-B/T mice are caused due to impaired CD40L in TDN-B/T mice.

STRAIN	MZ and B1a defect by flow	Histological evidence of MZ defect	Reduced IgG3	Increased CD19+, CD5+, CD9 lo, cells	Hyper IgM	Splenomegaly/ spontaneous GC
TDN-B	Yes	Yes	Yes	Yes	No	Yes
TDN-B/T	Yes	Yes	Yes	No	Yes	No

Table 3. MiT deficient mouse models and their B cell phenotype

Significance: TDN-B mice have increased plasma cells, and spontaneous splenomegaly which are not seen in TDN-B/T mice or WT mice. Splenomegaly, spontaneous GC formation and increased plasma cells indicate hyper responsiveness of FO B cells when MiT is inactivated in the presence of normal T cells. TDN-B mice also have increased proportions of subset of splenic B cells which are CD19+, CD5+ and CD9 lo, which may be cells known as splenic B1 a B cells, compared to both WT and TDN-B/T mice.

Both in humans and mice the CD40L gene expressed in CD4 T cells, is carried on the X chromosome and hence male mice carrying the mutation can only be hemizygous ($CD40L^{-/Y}$), whereas female mutant mice can either be heterozygous($CD40L^{+/-}$) or homozygous ($CD40L^{-/-}$). Both male and female CD40L mutant mice have impaired TD immune responses due to defect in class switching by FO B cells whereas the TI responses are intact [66]. To understand the underlying mechanism causing FO B cell abnormalities in TDN-B mice, I developed CD40L/TDN-B mice and observed for signs of B cell hyper responsiveness.

Experimental approach: Spleens were harvested from WT, TDN-B, TDN B/T and CD40L-deficient, TDN-B transgenic (CD40L/TDN-B) mice. Spleens were weighed, single cell suspensions were made from all organs, cell recoveries counted, and flow cytometry was used to enumerate and characterize the different B cell subsets in each mouse.

Results: CD40L/TDN-B mice had greatly reduced splenomegaly, which is similar to the TDN-B/T mice. (Figure 25A). Further analysis of the various B cell subsets by flow cytometry reveled that MZ B cells remained decreased in CD40L/TDN-B mice (Figure 25 B) indicating that the MZ defect caused by TFE3/TFEB inhibition was CD40L independent. Both TDN-B and TDN–B/T mice had a similar defect in MZ B cell proportions compared to WT mice. I also found that the proportions of splenic CD19+,CD5+,CD9 lo B cells in the CD40L/TDN-B mutant mice are comparable to those of both the WT as well as the TDN-B/T mice (Figures 25 C and D). This finding is in contrast to the observed increase in splenic CD19+, CD5+,CD9 lo B cells seen in TDN-B mice and indicates this population was dependent on CD40L expression on helper T cells.

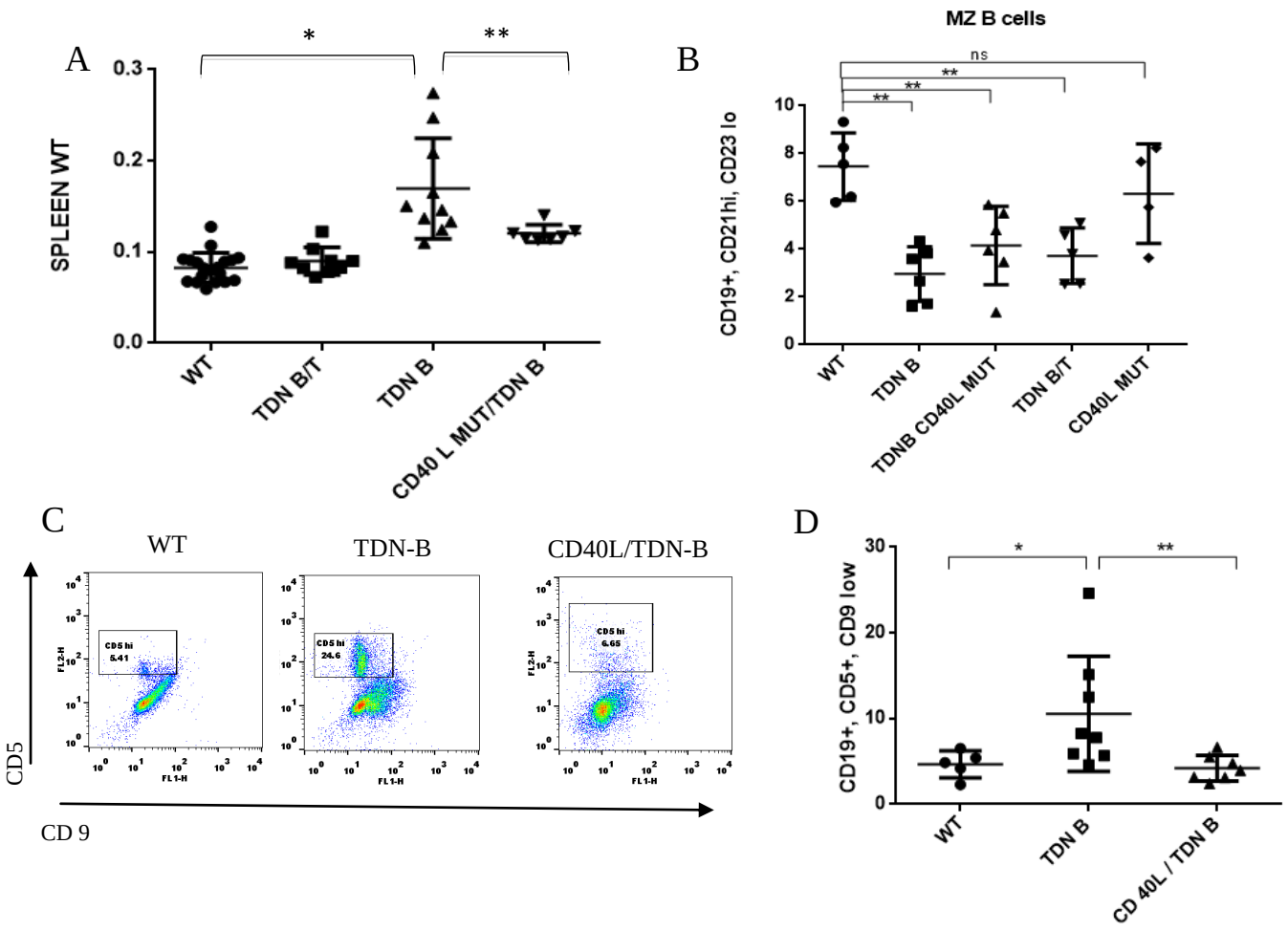
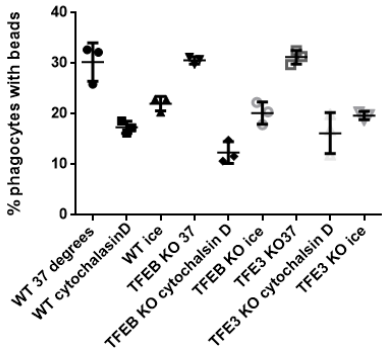


Figure 25.CD40L/TDN-B mice mimic TDN-B/T mice in their B cell phenotypes. A) Spleen weights from 8-10 month old `non-Tg, TDN-B/T, TDN-B and CD40L mutant/TDN-B. Error bars are SD of mean with n=8-20 per group. * $p<0.05$ and **$p<0.005$. B) Percent of total $CD19^{+}$ splenocytes identified as MZ B cells ($CD21^{high}CD23^{low}CD19^{+}$) from Non-Tg (WT), TDN-B, and TDN-B/T mice groups are shown. Error bars are SD of mean with n=5-6animals per group ** $p <0.005$. C) Representative dot plots of staining profiles from WT and TDN-B and CD40L/TDN-B mice from D are shown. D) Scatter plot of percentages of CD19+cells that are identified as splenic B cells which are CD5+ and CD9 low from WT, TDN-B and CD40L/ TDN-B mouse groups. Error bars are SD of mean with n=5-7 animals per group. *$p<0.05$ and ** $p <0.01$. Significance was measured using the non-parametric Mann-Whitney U test.

SUPPLEMENTARY FIGURES

A



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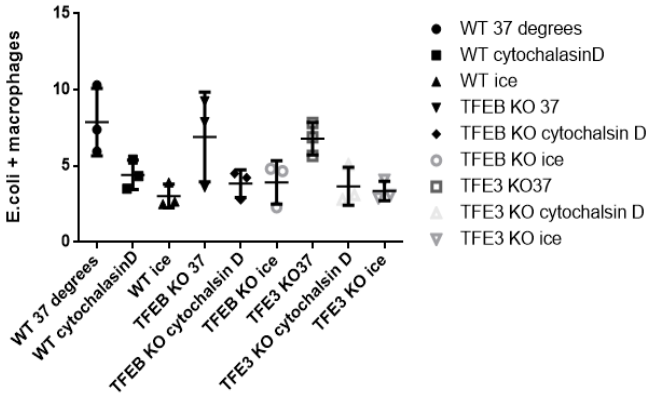

Figure 1. Internalization of the beads and *E. coli* is inhibited by Cytochalasin D pretreatment or by incubation on ice. PerC from WT and TFEB ko and TFE3 ko mice are incubated with beads or *E. coli* particles and % phagocytes are gated on live cells. Each dot represents sample from one mouse. Upon incubation with Cytochalasin D or on ice there is a significant decrease in the % of phagocytes in all the mouse groups tested. Significance tested by paired t test.

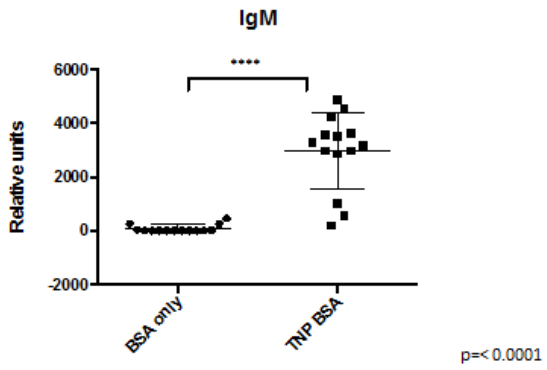

Figure 2. Antibody titers measured in both TI and TD responses are TNP specific. Post immune sera immunized animals comparing BSA vs TNP BSA as capture antibody to show the specificity of the titers measured. Each dot represents sample one immunized mouse. Significance tested by Mann-Whitney U test, error bars are SD of mean. p<0.0001

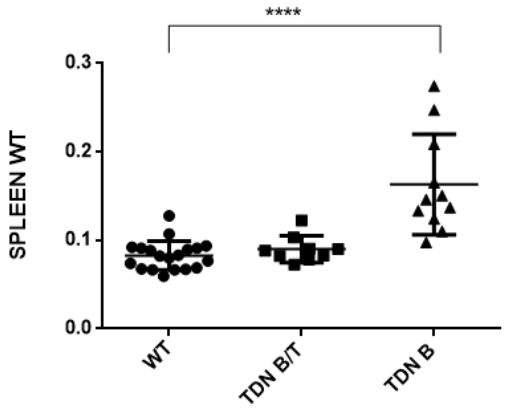

Figure 3. Spleen weights in immunized TDN-B mice are higher compared to WT similar to naive animals. Total spleen weights in TD and TI immunized mice. Each dot represents one mouse and ****p<0.0001 and n=10-20 per group. Significance tested using Mann-Whitney U test.

DISCUSSION

AIM 1

A successful immune response to identify and clear infection is a highly coordinated and regulated response by multiple cell types of primarily hematopoietic origin. Among the critical cell types involved are monocytes/macrophages, and B and T lymphocytes. In the first part of my thesis, I addressed the role of TFE3and TFEB in macrophage development and activation. Monocytes have a half-life of 1 day in mice and 3 days in humans and die by apoptosis in the absence of growth factors or activation signals. Zhang et al. showed that in the presence of growth factors M-CSF and GM-CSF, monocyte differentiation into macrophage depends on the ability to upregulate autophagy [29]. GM-CSF induces JNK activation to mediate the disassociation of Beclin1 and Bcl-2, the induction of autophagy, and monocyte survival. Even though TFEB and most likely TFE3 are important regulators of autophagy, my findings show they are dispensable for the development of macrophages both in vitro and in vivo. However, as macrophages express all the members of the MiT family, follow up studies should examine if Mitf or TFEC, which are also expressed in macrophages, compensate for the lack of TFEB and /or TFE3. TFEC is of particular interest as it is known to regulate the expression of the GM-CSF receptor in macrophages. [14]

In contrast, my findings show that TFEB and TFE3 are each important for mediating proinflammatory cytokine responses to low dose endotoxin in macrophages. Using BMDM in vitro, I showed that TFE3/TFEB-deficient macrophages secreted more IL1β, but lower IL-12 p70 and had comparable level of IL6 and TNF-α in vitro in response to low dose LPS. My studies also showed for the first time that TFEB and or TFE3 deficient mice challenged with low doses of LPS for 6 h had lower serum levels of IL-6, TNF-α and IL-1β in vivo compared to WT. My in vitro studies differ from recently published studies by Pastore et al., in which both dko RAW cells and primary dko BMDMs expressed less mRNA encoding IL-1β, IL-6 and TNF-α [40]. Similarly, Visvikis et al. found that si RNA-mediated knock down of

TFEB expression in BMDM infected with *S. aureus* which is a gram positive bacterium, in vitro resulted in a reduction in IL-1, IL-6 and TNF-α transcripts [67]. The differences in my observation can be explained by 1) I used a 10-100 fold lower dose of LPS used by Pastore et al. 2) the response of macrophages to LPS depends on the species and strain of the source of LPS used, whereas, I used LPS derived from E.coli strain 0111:B4, the species/strains used Pastore et al. is unclear. Moreover, these previous studies focused mainly on the transcriptional regulation of cytokines, which, while being critical to enhancing our understanding of immune responses, do not address the translational regulation, post translational modifications as well as secretion steps of cytokine synthesis. Nevertheless, ChIP-seq analysis of transformed RAW 246.7 confirmed increased binding of TFE3 to the promoters of autophagic/lysosomal genes following LPS stimulation, along with binding to the promoters of numerous immune-related genes [40], suggesting a more direct role of TFE3 in the inflammatory response but it is unknown if TFEB exerts a similar direct transcriptional regulatory role in LPS stimulated primary macrophages.

A better understanding the relationship of TFEB and /or TFE3 to the regulation of secreted cytokine response, as I measured in my studies, is needed to clarify its roles in light of the recent findings that "non-conventional" secretion of cytokines such as IL-1β, which lack the N-terminal signal peptide, use autophagic vesicles for secretion [68] . Inhibition of autophagy using 3 methyl adenine (3MA) or chloroquine resulted in decreased IL-12 and TNF-α cytokine response to LPS and IFN-γ [29]. 3MA is a known inhibitor of PI3kinase and thus inhibits autophagy by preventing vesicle nucleation step, but it also inhibits other kinases and is not the preferred pharmacologic for inhibiting autophagy. Chloroquine, on the other hand, prevents lysosomal acidification, which is the last step in autophagy. Many immune cells use exocytosis of lysosomes and lysosome-related organelles as a mechanism for specialized secretion. For example, cytotoxic T lymphocytes and NK cells store granzymes and perforin in secretory lysosomes and controlled spatio temporal release is critical for eliminating infected host cells. It has been shown that over expression of TFEB or TFE3 is sufficient to induce fusion of lysosomes with the plasma membrane[69, 70] and TFEB-mediated lysosomal exocytosis in osteoclasts and hepatocytes plays an important role in bone

remodeling and copper homeostasis respectively[71, 72]. TFE3 is known to regulate degranulation of mast cells and thus regulate allergic responses. Therefore, it is plausible that TFEB and TFE3 might also (in addition to direct transcriptional activity) regulate immune response by regulating vesicle/lysosomal trafficking involved in the secretion of cytokines, a hypothesis which needs to be tested.

My studies showed that phagocytosis of both *E. coli* particles and fluorescent beads in TFE3 and /or TFEB deleted peritoneal macrophages is comparable to that of WT. Phagocytosis of non-opsonized latex beads in macrophages starts with the binding of the beads to the cell surface scavenger receptor like MARCO and SR-A, whereas *E. coli* particles occurs through binding to TLR4-MD2 complex. This is followed by internalization and formation of phagosome, which is dependent on actin polymerization, and both these processes are sensitive to inhibition of actin polymerization by Cytochalasin D. It is interesting to note that particle binding, internalization and phagolysosome formation appear to be unaffected by the deficiency if TFEB/TFE3 in macrophages. However, the ability of TFEB/TFE3 deficient macrophages to kill internalized bacteria/degrade opsonized particles, and their efficiency in MHC II presentation of the internalized peptide/antigens derived from that internalized material still need to be tested. Understanding the later stages of phagocytosis such as degradation of particle/bacteria is critical, because it relies on the activity of lysosomal hydrolases like v-ATPases, which are known to be regulated by TFEB/Mitf in an mTORC dependent pathway[73, 74]. The mTORC pathway is known to regulate TFEB phosphorylation and thus its sub cellular localization [73]. Antigen presentation to CD4 T cells in phagocytic BMDM deficient in TFE3/TFEB should also be evalutated. This can be done using soluble protein-containing antigens and particulate protein containing antigens and measuring MHCII presentation of peptides derived thereof by macrophages, using proliferation and cytokine secretion by CD4 T cells in co-culture assays.

AIM 2

Previous studies from our lab have shown that MiT inhibition in B cells results in spontaneous splenomegaly, increased proportion of plasma cells in the spleen and higher serum total IgG titers in naïve mice, all indicative of hyper-responsive FO B cell activation [11]. Our studies show that splenomegaly is not observed either in TDN-B/T mice or the CD40L/TDN-B mice, indicating that hyper-responsiveness in MiT inactivated FO B cells is dependent on CD4 T cell help. Peng's group did not report splenomegaly in Mitf $^{mi/mi}$ mice, possibly because in the adoptive transfer model of MiT deficient B cells into Rag2-/- mice, they observed the recipient animals only 8-12 weeks post transfer [9], which maybe insufficient time for this abnormality to develop. The increase in plasma cells in naive mice and elevated IgG levels were most likely due to inactivation of Mitf in B cells, because it was observed in both TDN-B and Mitf $^{vga/vga}$ mice, as well as the Mitf $^{mi/mi}$ transplantation model [9, 11]. The Peng group showed that Mitf represses the transcription factor IRF-4 (interferon regulatory factor) to prevent spontaneous differentiation into plasma cells [9].However, that these abnormalities were observed in TDN-B mice but not in TDN-B/T or CD40L/TDN-B mice indicates that MiT inhibited B cells are hyper responsive in vivo only in the presence of normal T cells [11].

Another B cell abnormality distinct from FO hyper-responsiveness caused by Mitf inactivation, is that naïve mice lacking TFEB and TFE3 function, either via TDN or by genetic inactivation, had fewer MZ B cells and lower naïve serum titers of their main antibody product, IgG3. Surprisingly, in TDN-B mice following immunization with TNP-Ficoll, a model TI antigen, IgG2b titers were higher and IgG3 titers were comparable to WT. However, post-immunization analysis revealed that MZ B cell proportions were then comparable to wild type, which could explain the recovery of IgG3 titers. This, I hypothesize, is likely due to proliferation of the MZ B cells in situ or migration of FO B cells into the MZ zone and changing their cell surface phenotype in response to challenge. It is also plausible that the increased IgG1 and IgG2c titers secreted by TDN-B FO B cells in TI responses, which is uncharacteristic for this type of response, is likely due to a decreased threshold for FO B cell activation caused by the inhibition of MiT, specifically, Mitf. To

further validate this hypothesis, TI immunization studies need to done in Mitf $^{vga/vga}$ mice. If so, these mice should have higher IgG1 and IgG2c.

Studies from our lab showed that unimmunized TDN-B mice had increased splenic CD19+,CD5+, CD9 lo B cells, which may be cells known as splenic B1a B cells. This abnormality was also observed in Mitf $^{vga/vga}$ mice but not observed in TDN-B/T [11]. Splenic B 1a B cells are similar to peritoneal B1a B cells in that both these B cell subsets express CD5, which dampens signaling through the BCR, and proliferate more rapidly when stimulated by LPS compared to B2 cells[75]. Ig from both B1a subsets is both polyreactive and autoreactive. Polyreactive natural Ig is always present and acts as an initial shield against many common infectious agents, particularly during the lag period that precedes the development of adaptive, high affinity antigen-specific antibody produced by germinal center B-2 cells. Self-reactivity in this case is not pathogenic but is rather thought to serve an important role in clearance of self –debris from apoptotic cells to prevent autoimmune inflammatory reactions. Despite these similarities, both B cell subsets are not monolithic. Splenic and peritoneal B1a B cells differ in the expression of other cell surface molecules, transcription factors/chemokines required for proliferation and survival as well as antibody responses generated. Unlike peritoneal B1a B cells, the splenic B1a B cells do not express CD11b, an integrin commonly expressed on myeloid cells [76] . Deficiency of the chemokine ligand, CXCL 13 does not alter the number of splenic B-1 cells, whereas the number of peritoneal B-1 cells is greatly diminished[77] . Splenic B-1 cells and peritoneal B-1 cells differ in expression of the transcription factors, phosphorylated STAT3 as well as Notch [75, 78] . Peritoneal B1a B cells survive ex vivo and secrete higher levels of IgM spontaneously compared to Splenic B 1a B cells [60]. I found that the increase in CD19+, CD5 hi, CD9 lo subset observed in TDN-B mice is not found in CD40L/TDN-B mice, suggesting that this increase observed in the TDN-B mice is extrinsic and relies on T cell help similar to the FO B cell abnormality. However, even though the CD19+,CD5+, CD9 1o cells express cell surface molecules like CD19 and CD5, we are not sure if these are the splenic B 1a B cells described in literature. This is because it is not known if splenic B 1a B cells need CD40L expression from T cells. It would be important to explore this further, because

dysregulated splenic B1 B cells are implicated in the pathogenesis of lupus as they bind to altered self-antigens, such as oxidized lipids and those expressed by apoptotic cells, and are a potential source of pathogenic auto antibodies.

With respect to the peritoneal B1 B cell defect, I found decreased phagocytic ability of peritoneal B1a B cells in both TDN-B and TDN-B/T mice compared WT. Even though I tested phagocytosis in TDN-B and TDN-B/T mice, based in our previous observations [11] we think that the defect is likely due to inactivation of both TFEB and TFE3.This phagocytic ability of the Peritoneal B cells can be explained by the fact that B cells from early vertebrates are phagocytic [79] and the function is conserved evolutionarily. Also, Peritoneal B cells can adopt a macrophage like phenotype and differentiate into mononuclear phagocytes in vitro, displaying an ability to internalize SRBCs and yeast [80]. Some malignant human B cell lines like Ramos (Burkitt's lymphoma derived human B cell line) and hairy cell leukemia are also known to be phagocytic [81, 82]. This supports the functional, developmental and evolutionary relationships between B cells and macrophages.[79]. Parra et al. showed that peritoneal B cells are capable of killing internalized bacteria and presenting antigens to T cells and induce cytokine secretion from T cells, which can occur in the absence of BCR signaling [48]. Like macrophages, peritoneal B 1 cells are known to depend on autophagy for survival and homeostasis[4]; for example, conditional deletion of ATG5 in B cells resulted in the loss of maintenance of peritoneal B1 cells [83]. It is interesting to note that particle binding, internalization and phagolysosome formation appear to be impaired in peritoneal B cells from both TDN-B and TDN-B/T mice. The presence of the defect in both transgenic lines of mice suggests that is caused by the inhibition of MiT (most likely TFEB and TFE3) in B cells is independent of T cells. Intracellular killing of bacteria and antigen presentation to CD4 T cells by phagocytic B cells in TDN-B and TDN-B/T mice needs to be evaluated to determine whether the defect in phagocytosis (innate immune response) translates into a defect in the ability to generate an acquired immune response. As with macrophages, this can be done by exposing soluble and particulate antigens to B cells and measuring MHCII presentation of these antigens to T cells by measuring proliferation and secreted cytokines in CD4 T cell co-culture assays. Thus, impaired phagocytic

responses may explain the decreased homeostasis and survival of peritoneal B 1a cells caused by MiT inactivation.

In summary, my studies demonstrate that the MiT family regulates pro inflammatory cytokine responses in macrophages and humoral responses in B cells. In the future, it will be important to explore further the mechanisms underlying the regulation of cytokine response by TFEB/TFE3 in primary macrophages, that is, whether it is by direct transcriptional regulation of the cytokine genes or an indirect effect caused by the regulation of lysosomes involved in secretion or both. This additional information will be needed to effectively harness TFE3/TFEB in order to therapeutically modulate cytokine responses in sepsis and autoimmune diseases. My studies also showed that MiT inactivated B cells had aberrant IgG responses to TI antigens, this knowledge can be used for developing better adjuvants for prophylactic vaccines against encapsulated bacteria, such as for asplenic patients.

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