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Resident Macrophages are Locally Programmed for Silent Clearance of Apoptotic Cells

By

Allison Whitney Roberts

A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Molecular and Cell Biology

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University of California, Berkeley

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Abstract

Resident Macrophages are Locally Programmed for Silent Clearance of Apoptotic Cells

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Doctor of Philosophy in Molecular and Cell Biology

University of California, Berkeley

Professor Gregory M. Barton, Chair

Although apoptotic cells (ACs) contain nucleic acids that can be recognized by Toll-like receptors (TLRs), engulfment of ACs does not initiate inflammation in healthy organisms. In this dissertation I explore the mechanisms that help maintain this homeostasis. I discuss nucleic acid sensing TLRs and their regulation. I also provide an introduction to the clearance of ACs from the body. I discuss how I identified and characterized macrophage populations that continually engulf ACs in several distinct tissues. These macrophages share characteristics compatible with immunologically silent clearance of ACs, including high expression of AC recognition receptors, low expression of TLR9, and reduced TLR responsiveness to nucleic acids. When removed from tissues these macrophages lose many of these characteristics and generate inflammatory responses to AC-derived nucleic acids, suggesting that cues from the tissue microenvironment are required to program macrophages for silent AC clearance. I show that KLF2 and KLF4 control expression of many genes within this AC clearance program. Coordinated expression of AC receptors with genes that limit responses to nucleic acids may represent a central feature of tissue macrophages that ensures maintenance of homeostasis

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List of abbreviations

AC	Apoptotic cell
AIM2	Absent in melanoma 2
ANA	antinuclear antibody
AP-1	Activator protein 1
APC	Antigen presenting cell
BAI1	Brain-specific angiogenesis inhibitor 1
BMM	Bone marrow derived macrophage
CD	Cluster of differentiation
cGAS	Cyclin GMP-AMP synthase
CXCL3	Chemokine (C-X-C motif) ligand 3
DAI	DNA-dependent activator of IFN-regulatory factors
DC	Dendritic cell
DOCK	Dedicator of cytokinesis
dsDNA	Double-stranded DNA
DUSP1	Dual specificity protein phosphatase 1
EGF	Epidermal growth factor
ELMO	Engulfment and cell motility
ERK	Extracellular signal regulated kinase
FAS1	Fasciclin-like
Gas6	Growth arrest specific gene 6
GPCR	G-protein-coupled receptor
Hes1	Hairy and enhancer of split-1
HMGB1	High mobility group box 1
IFN	Interferon
IκB	Inhibitor of κB
IKK	IκB kinase
IRAK	Interleukin-1 receptor-associated kinase
IRF	Interferon regulator factors
JNK	c-Jun N-terminal kinases
LOX-1	Lectin-like oxidized low-density lipoprotein receptor 1
LRP1	Low density lipoprotein receptor-related protein 1
MAPK	Mitogen activated protein kinase
MARCO	Macrophage receptor with collagenous structure
MFG-E8	Milk fat globule-EGF Factor 8
MyD88	Myeloid differentiation primary response gene 88
NEMO	NF-kB essential modulator
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PAMP	Pathogen-associated molecular pattern
PI3K	Phosphoinositide 3-kinase
PRR	Pattern recognition receptor
PS	Phosphatidylserine
RAGE	Receptor for advanced glycation endproducts

RIG-I	Retinoic acid-inducible gene-I
SCARF1	Scavenger receptor class F membrane 1
SHP-1	Src homology region 2 domain-containing phosphatase-1
SLE	Systemic lupus erythematosus
SOCS	Suppressor of cytokine signaling
ssRNA	Single-stranded RNA
STING	Stimulator of interferon genes
TAB1	TGF-beta activated kinase 1 (MAP3K7) binding protein 1
TAK1	TGF-beta-activated kinase 1
Tim-4	T-cell immunoglobulin mucin protein 4
TIR	Toll/interleukin-1 receptor
TIRAP	TIR domain containing adaptor protein
TLR	Toll-like receptor
TLT2	TREM-like protein 2
TNF	Tumor necrosis factor
TRAF6	TNF receptor associated factor
TRAM	TRIF-related adaptor molecule
TRIF	TIR domain containing adaptor inducing interferon-beta

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Chapter 1: Nucleic acid sensing Toll-like Receptors

The immune system consists of a variety of cells and molecules that sense and eliminate microbial infections. The vertebrate immune system has two branches, the innate immune system and the adaptive. The adaptive immune system is comprised of T and B cells that recognize individual antigens and generate specific responses. Each of these cells recognizes an individual antigen using a specialized receptor that is generated during cell development. These receptors are generated by gene segment rearrangement, which allows each individual lymphocyte to express a unique receptor. In this way the adaptive immune system collectively expresses a large repertoire of receptors, allowing for recognition of virtually any pathogen. When T and B cells recognize their antigen they mount a defense through secretion of cytokines, antibodies, or other cell-mediated responses. Some adaptive immune cells activated during an immune response differentiate into memory cells, allowing for long lasting immunity. Although the responses of the adaptive immune system can be highly specific and effective, initiation of these responses can take several days. The innate immune system, which is comprised of a variety of cells including macrophages, dendritic cells, and neutrophils, is able to initiate responses immediately upon infection and acts as a first line of defense against pathogens. The innate immune system also has the vital role of initiating adaptive immune responses.

PRRs and their role in the immune system

Although the effector functions of the immune system can protect the host and eliminate pathogens, there is also the potential for harm through inappropriate or excessive responses. In order to avoid mounting effector responses against the host the immune system must be able to distinguish between microbes and self derived antigens. As first outlined by Charles Janeway in his introduction to the 1989 Cold Spring Harbor Symposium, much of the burden of distinguishing microbes from self is mediated by the innate immune system[1]. To achieve this discrimination the innate immune system recognizes conserved structural features of microbes, which have been termed pathogen-associated molecular patterns (PAMPs), which are largely absent in the host. The receptors that recognize PAMPs, which have been termed pattern recognition receptors (PRRs), are distinct from the antigen receptors of the adaptive immune system in that they are not generated by gene segment rearrangement. Instead, PRRs are germline encoded and therefore shaped by the microbes encountered during evolution.

PRR engagement by a PAMP can lead to a variety of outcomes depending on the cell type and PRR involved. Often when the cells of the innate immune system recognize a PAMP they produce inflammatory cytokines and chemokines leading to the recruitment of additional innate immune cells. PRR engagement also initiates processes that directly kill microbes, such as engulfment and the generation of toxic agents. Another vital outcome of PRR engagement is the activation of the adaptive immune

response. Although the adaptive immune system can specifically recognize antigen it lacks contextual information. Since the antigen receptors of the adaptive immune system are generated by random gene rearrangement they have the potential to recognize self derived ligands. Therefore, in order to be activated the cells of the adaptive immune system require not only engagement of their antigen receptor but also a second signal from the innate immune system alerting them to the presence of microbes [2]. The classic example of this second signal is the upregulation of the co-stimulatory molecules CD80 and CD86 on the surface of antigen presenting cells (APCs) upon activation of their PRRs. In order to be activated by recognition of its cognate antigen a T cell must also receive a co-stimulatory signal, such as recognition of CD80 or CD86. Without PRR stimulation, the APC will not upregulate co-stimulatory molecules and even if a T cell recognizes its antigen it will not become activated. In this way PRR recognition of PAMPs provides the information that a microbe is present. Through secretion of different cytokines as well as other cues the innate immune system can also inform the adaptive immune system about the type of microbe it has detected.

Toll-like Receptors

One of the most well characterized families of PRRs is the Toll-like receptors (TLRs). This family of receptors recognizes a variety of PAMPs. TLRs can be broadly divided into two categories – those that recognize nucleic acids and those that recognize other microbial structures such as lipopolysaccharide (LPS), lipopeptides, or proteins. Many TLRs recognize ligands and signal from the cell surface. However, the nucleic acid sensing TLRs are only functional in endosomes. TLRs are expressed by many cell types including dendritic cells (DCs) cells, macrophages, neutrophils, and B cells [3].

TLRs consist of an N-terminal extracellular domain containing a series of leucine-rich repeats that fold into a horseshoe-like shape, a single transmembrane spanning region, and a C-terminal cytoplasmic domain that includes a Toll-Interleukin-1 receptor (TIR) domain that mediates recruitment of signaling components. Ligand binding is mediated by the leucine-rich repeat regions of TLR homo-dimers or hetero-dimers. Crystal structures of TLR-ligand interactions have demonstrated that each TLR binds to its ligand using distinct sites and modes of binding [4-9].

Ligand recognition results in the recruitment of TIR domain-containing adaptors that initiate downstream signaling. TLR signaling adaptors include MyD88 and TRIF, as well as the bridging adaptors TIRAP and TRAM. All TLRs except TLR3 utilize MyD88 for signaling, while TLR3 and TLR4 engage TRIF [10]. Ligand binding and the subsequent conformational changes allow for recruitment of MyD88, likely as an oligomer, which in turn recruits the serine/threonine kinase IRAK4 [11]. IRAK4 recruitment leads to association with IRAK1 or IRAK2. This large oligomer of multiple MyD88 and IRAKs has been termed the Myddosome. Myddsome assembly allows for IRAK4 autoactivation and phosphorylation of IRAK1 or IRAK2 [12]. Activation of these IRAKs allows recruitment and activation of the E3 ubiquitin ligase TRAF6 [13]. TRAF6 then forms a complex with

TAK1, TAB1 and TAB2/3. This complex then activates the IKK complex. The IKK complex (consisting of IKKα, IKKβ, and NEMO) phosphorylates IκB (the inhibitor of NF-κB,) leading to its degradation [14]. Once released from IκB, NF-κB is able to translocate to the nucleus and initiate transcription of a variety of genes including proinflammatory cytokines such as TNF, IL-6, and IL-12p40 [15]. TAK1 also activates the MAPKs ERK1, ERK2, p38 and JNK, which activate transcription factors including AP-1 [16]. MyD88 signaling can also activate the transcription factor IRF7 via phosphorylation by IRAK1 [17]. IRF7 activation leads to the production of type I interferon (IFN). The TRIF signaling pathway includes activation of TRAF3 that results in activation and dimerization of a TBK1 and IKKi complex, which leads to phosphorylation and activation of the transcription factor IRF3.

Nucleic acid sensing TLRs in pathogen recognition

Induction of inflammatory responses upon recognition of nucleic acids is a critical aspect of the innate immune system. Nucleic acids are recognized by multiple PRRs. For instance, TLR9 recognizes DNA [18], TLR3 recognizes double-stranded RNA, TLR7 and TLR8 recognize single-stranded RNA, and TLR13 recognizes bacterial ribosomal RNA [19-23]. This strategy affords broad recognition of multiple pathogen classes, and its failure can render the host susceptible to infection by a variety of pathogens. While nucleic acid sensing TLRs localize to endosomes, there are also nucleic acid sensing PRRs in the cytosol. For instance, the RIG-I like receptors recognize atypical RNA species in the cytosol [24], [25]. There are also a variety of cytosolic receptors that recognize DNA including cGAS, DAI, and AIM2 [26-29]. Many of these cytosolic DNA sensors lead to activation of STING, which induces type I IFN [30].

Nucleic acid sensing TLRs are particularly important for recognition of viruses, which use host cell machinery for replication and thus generate few truly foreign molecular structures. A variety of viruses have been shown to activate endosomal TLRs. For instance, mice deficient in TLR9 are highly susceptible to Ectromelia virus, a DNA virus that is the causative agent of mousepox [31], [32]. STING is also needed for effective resistance to mousepox, highlighting the need for diverse DNA sensing PRRs. TLR9 is also needed for survival and efficient type I IFN production during infection with the DNA virus murine cytomegalovirus [33], [34]. Although murine cytomegalovirus is a DNA virus, TLR7 also appears to sense and defend against this virus, most likely due to RNA intermediates generated during viral replication [35]. Single-stranded RNA viruses have also been shown to stimulate TLR7 [20], [21]. In addition to viruses, nucleic acid sensing TLRs have been demonstrated to protect against bacteria and parasites [18], [36], [37].

Regulation of nucleic acid sensing TLRs

The cost of the broad recognition of pathogens by nucleic acid sensing TLRs is the potential for inappropriate responses to self-derived nucleic acids, which can lead to

autoimmunity or autoinflammatory diseases [38]. Multiple mechanisms function to limit the likelihood of responses against self by TLRs that recognize nucleic acids.

Some nucleic acid sensing TLRs preferentially detect features that are enriched in microbial nucleic acids. For instance, TLR9 preferentially recognizes DNA that contains unmethylated CpG dinucleotides, and these motifs are more frequent in microbial DNA than mammalian DNA [39-41]. However, mammalian derived DNA is also capable of stimulating TLR9. TLR7 has recently been demonstrated to use two independent binding sites to recognize guanosine and a ssRNA oligonucleotide consisting of at least 3 bases [5], [42]. These molecules are present in the steady state of hosts, highlighting the need for additional mechanisms to regulate signaling.

As mentioned earlier nucleic acid sensing TLRs are located in endosomes. The chaperone UNC93B1 is required for trafficking of these TLRs to the endosome [43], [44]. As these receptors traffic through the cell they remain in a full-length form that is not able to initiate signaling. It is only upon reaching the endosome that these receptors are processed into a cleaved functional form by acid-dependent endosomal proteases [45], [46]. This endosomal localization limits access to extracellular self-derived DNA and RNA while allowing responses to pathogens that access the endosome [47]. Mice expressing mutant TLR9 that responds to extracellular DNA die from systemic inflammation [48].

All TLRs are regulated by a variety of molecules that negatively control signaling. Negative regulators prevent excessive TLR activation that could disrupt homeostasis. There are several categories of negative regulators. Some inhibit the TLR signaling cascade. For instance, SHP-1 suppresses IRAK1 and IRAK2 activities [49]. DUSP1 dephosphorylates and inactivates the MAPKs ERK1, ERK2, JNK1, and p38 [50]. Other negative regulators induce degradation of TLR signaling components. For example SOCS1 and SOCS3 can induce degradation of TRAF6 [51]. And finally some negative regulators prevent transcriptional responses. The transcriptional repressor Hes1 can repress transcription of TLR-induced cytokines [52], [53].

Nucleic acid sensing TLRs in autoimmunity

Despite the many ways that nucleic acid sensing TLRs are regulated there are pathological conditions in which these receptors contribute to autoimmune and autoinflammatory diseases. One mechanism that can facilitate pathology is bypassing endosomal compartmentalization of nucleic acid sensing TLRs. For example, the generation of immune complexes containing nucleic acids can lead to Fc receptor-mediated uptake of endogenous nucleic acids, activation of endosomal TLRs, and subsequent autoimmune responses [54-56].

Nucleic acid sensing TLRs play roles in systemic lupus erythematosus (SLE). In murine models of the disease mice deficient in MyD88, Unc93B1, both TLR7 or TLR9, or TLR7 alone display reduced disease manifestations [57-61]. Much of the pathological effects of TLR signaling in these models are believed to be mediated by B cells. One characteristic of SLE is the elevated expression of anti-nuclear antibodies. B cell intrinsic expression of nucleic acid sensing TLRs can contribute to the generation of

these antibodies [62]. This may become a feed forward cycle as B cell receptor mediated uptake of endogenous nucleic acids can stimulate B cell proliferation [55]. Surprisingly the role of TLR9 in SLE is more complex; in many models of the disease TLR9 deficient mice actually develop more severe disease manifestations [57], [63]. This may be due to TLR7 becoming hyperresponsive. Mice that express a mutant version of Unc93B1 that skews TLR trafficking, making TLR7 hyperresponsive and TLR9 hyporresonsive, develop TLR7 dependent systemic inflammation [64]. However, TLR9 does seem to contribute to the development of other auto-immune diseases such as arthritis and experimental autoimmune encephalomyelitis [65], [66].

TLR induced autoimmune disease has been demonstrated by mice that overexpress TLR7. Mice that harbor the Y-linked autoimmune accelerator (*Yaa*) locus are predisposed to a lupus like disease. Studies of this locus revealed that it contained a duplication of the *Tlr7* gene [67], [68]. A subsequent study confirmed the duplication of *Tlr7* was necessary for disease progression. The authors also generated several transgenic mouse lines that overexpressed TLR7 to differing degrees, and found that increased TLR7 alone could induce systemic autoimmunity disease with disease severity correlated with TLR7 copy number [69]. Clearly strict control of nucleic acid sensing TLR responses is vital to prevent autoimmune pathologies.

Chapter 2: Clearance of apoptotic cells

Apoptosis is an ongoing process required for tissue homeostasis. It has been estimated that millions of cells die by apoptosis in the human body every day [70]. A variety of cells mediate the clearance of these apoptotic corpses including professional phagocytes, such as macrophages and DCs, as well as other cell types such as epithelial cells [71].

When a cell undergoes caspase-dependent apoptosis the cell beings to shrink, chromosomal DNA is degraded into nucleosome units, the chromatin condenses, and the plasma membrane beings to form blebs [72]. However, the plasma membrane remains intact, preventing intracellular components from being released. As the cell dies it releases soluble molecules, termed find-me signals, which attract phagocytes. These find-me signals include a cleaved form of CXCL3, sphingosine-1-phosphate, and the nucleotides ATP and UTP [73-75]. The dying cell also displays markers on its surface, termed eat-me signals, that are recognized by phagocytic receptors. Once a phagocyte recognizes an AC, receptor signaling leads to activation of the small GTPase Rac, resulting in actin polymerization and cytoskeletal rearrangement at the phagocytic interface[76].

AC recognition receptors

Phosphatidylserine (PS) receptors: The most well studied eat-me signal is phosphatidylserine (PS). In a healthy cell PS is asymmetrically distributed to the inner membrane by flippases that actively move PS from the outer membrane to the inner. Once a cell begins to undergo apoptosis the flippases become inactivated and scramblases randomize the distribution of PS [77], [78]. In addition to its exposure on ACs, PS is exposed on activated T cells although at lower surface densities [79]. A variety of receptors have been demonstrated to recognize PS and can be broadly divided into those that direct bind PS and those that use bridging molecules.

Direct PS recognition:

Tim-4 – Tim-4 is a cell surface receptor with an N-terminal immunoglobulin-like domain, a mucin domain, a single transmembrane domain, and a short cytoplasmic region. Tim-4 has been demonstrated to directly bind to PS but not other phospholipids and is sensitive to the surface density of PS on membranes, which may allow it to effectively distinguish ACs [80], [81]. Tim-4 is exclusively expressed on APCs including some macrophage and DC populations [82]. Tim-4 does not mediate direct signaling, as it has been demonstrated that the cytoplasmic tail of Tim-4 is dispensable for Tim-4 mediated AC engulfment [83]. In addition, Tim-4 seems to be a tethering receptor that requires additional AC recognition receptors for engulfment. For instance when Tim-4 is transfected into a non –phagocytic cell line it can bind ACs but engulfment of the AC requires an additional receptor such as integrin $\alpha_v\beta_3$ or MerTK [84], [85]. Tim-4 family

members Tim-1 and Tim-3 have also been demonstrated to bind to PS, however these receptors are preferentially expressed on T cells and seem to primarily regulate T cell responses [86]. However, there is evidence that Tim-3 on DCs can mediate cross-presentation of AC-associated antigen [87].

BAI1 – BAI1 is a GPCR that directly binds PS as well as other phospholipids [88]. BAI1 is highly expressed in the brain and testes although lower expression in macrophages has been reported [89]. BAI1 signaling recruits the ELMO-DOCK180 complex that functions as a guanine nucleotide exchange factor for the small GTPase Rac leading to corpse engulfment. BAI1 has other functions such as inhibiting angiogenesis.

Stabilin-2 – Stabilin-2 is a large transmembrane protein that contains seven FAS1 domains and four EGF-like repeats. Stabilin-2 directly binds PS but not other phospholipids [90]. Stabilin-2 is expressed by endothelial cells in many tissues and also functions as a hyaluronan receptor [91]. Stabilin-2 has also been shown to bind bacteria.

CD300f – CD300f is a transmembrane protein that contains an IgV-like extracellular domain and an extended cytoplasmic tail with an ITIM motif. CD300f is primarily expressed in myeloid cells. CD300f binds directly to PS with preference for PS over other phospholipids [92]. CD300f directly signals as cells expressing mutant receptors lacking the cytoplasmic tail engulf fewer ACs. The cytoplasmic tail of CD300f recruits the p85α regulatory subunit of PI3K eventually leading to activation of Rac and AC engulfment [93]. Association of SHP1 with CD300f negatively regulates AC engulfment.

TLT2 (Treml2) – Thioglycollate elicited peritoneal macrophages were shown to displayed enhanced AC engulfment after stimulation with poly I:C, and this effect can be partially blocked with anti-TLT2 antibodies or soluble TLT2 extracellular domain [94]. In addition, TLT2 transfection can enhance AC-engulfment by a non-phagocytic cell line. TLT2 directly binds PS but not other phospholipids.

RAGE – Soluble RAGE has been demonstrated to bind to ACs and to directly bind PS but not other phospholipids [95]. RAGE deficient macrophages display a small defect in AC engulfment *in vitro*. And Rage deficient mice demonstrate some enhancement in lung inflammation after LPS exposure. However, as RAGE has been demonstrated to bind other ligands including HMGB1, the role of RAGE as a PS receptor remains less clear.

PS recognition through bridging molecules:

MerTK, Axl, and Tyro3 – Tyro3, Axl, and MerTK (TAM) are receptor tyrosine kinases that consist of an ectodomain with two Ig-like domains and two fibronectin type III domains, a single-pass transmembrane domain, and a cytosolic region that includes a tyrosine kinase domain as well as an ITIM-like motif [96]. TAM receptors are expressed

on myeloid cells, NK cells, NKT cells, platelets, neurons, epithelial cells, and endothelial cells. However, TAM receptors are not thought to be expressed by B or T cells [97]. Upon ligand binding TAM receptors display dimerization and initiation of phosphorylation cascades. The TAM receptors bind PS through the bridging proteins Gas6 and Protein S. MerTK deficient thioglycollate elicited macrophages demonstrate reduced AC-engulfment [98]. It has also been demonstrated that MerTK and Axl expression varies based on the inflammatory environment and cell type; in resting bone marrow derived macrophages (BMMs) MerTK is highly expressed and Axl expression is low, while in resting bone marrow derived DCs (BMDCs) Axl is highly expressed and Mer expression is low. LPS and poly(I:C)stimulation increases Axl expression and decreases MerTK expression in BMMs; under these conditions Axl mediates AC engulfment [99]. Mice deficient in all three TAM receptors develop systemic inflammation [97]. However, in addition to AC clearance TAM receptors are thought to detect PS on the surface of activated T cells and modulate T cell responses, so it is unknown how much of the inflammatory phenotype is due to defects in AC clearance.

Integrin $\alpha_v\beta_5/\alpha_v\beta_3$ – In addition to their roles in cell adhesion and migration integrins $\alpha_v\beta_5/\alpha_v\beta_3$ bind PS through the bridging protein MFG-E8, which binds PS on ACs [100], [101]. As discussed above $\alpha_v\beta_3$ may cooperate with Tim-4 to engulf ACs. MFG-E8 has a preference for PS over other phospholipids. MFG-E8 is known to be expressed and secreted by thioglycollate elicited macrophages, follicular DCs, BMDCs, and Langerhans cells [102], [103].

Calreticulin receptors: Calreticulin is a Ca^{2+} binding protein that typically resides in the lumen of the endoplasmic reticulum. Calreticulin has been demonstrated to be upregulated on ACs [104], [105]. Thioglycollate-elicited macrophages demonstrate reduced engulfment of calreticulin deficient ACs. This uptake is thought to mediated by the receptor LRP1/CD91 on phagocytes that recognizes calreticulin on ACs. However, the role of calreticulin in AC clearance is complex as calreticulin has also been suggested to cooperate with LRP1 in phagocytic cells to function as a complex that binds C1q that has opsonized to ACs [106].

Oxidized LDL-like receptors:

MARCO – MARCO is a scavenger receptor that binds a variety of ligands including modified low-density lipoproteins. A non-phagocytic cell line transfected with MARCO demonstrated increased capacity to bind ACs. Interestingly, SLE prone mice and human SLE patients have elevated serum antibodies against MARCO, suggesting these antibodies may block AC clearance by interfering with MARCO function [107]. In addition, expression off MARCO has been shown to be reduced in the BXSB autoimmune prone mouse stain, and BMMs derived from these mice demonstrate reduced uptake of ACs. Although MARCO blocking antibodies reduced AC uptake by control BMMs they had not effect on the BSXB derived BMMs [108].

LOX-1 – LOX-1 is a C-type lectin-like receptor that recognizes oxidized low-density lipoprotein; ACs contain large amounts of native or oxidized fatty acids. Oxidized low-density lipoprotein can compete with ACs for phagocytosis, implying it could bind the same receptors. Non-phagocytic cells transfected with LOX-1 bound ACs, but not live cells [109]. LOX-1 was found to be upregulated in IFN-α treated monocyte derived DCs. IFN-α treated DCs had increased capacity to engulf ACs, a process that was blocked by αLOX-1 antibodies [110].

Complement involvement in AC clearance: C1q and other complement components are thought to opsonize late ACs. The receptors LRP1/CD91, CD93, and complement receptors 3 and 4 are thought to be involved in complement mediated engulfment of ACs. [111]. In addition the receptor SCARF1 has also been demonstrated to bind to ACs through C1q [112]. SCARF1 is also known to bind acetylated low-density lipoprotein and heat-shock proteins.

Processing of AC-derived cargo

Once engulfed, ACs must be degraded. Since an AC can be of similar size to the phagocyte, engulfed cargo can significantly increase the amount of protein, lipids, and carbohydrates in a cell. Professional phagocytes often engulf many ACs in quick succession. DNase II degrades DNA in lysosomes. Failed digestion of this DNA leads to activation of the STING pathway of cytosolic DNA sensing [113]. Of note, it has recently been demonstrated that DNase II processing is required for TLR9 to sense DNA. This requirement illustrates why DNase II deficient mice do not generate TLR9 dependent inflammatory responses [114]. It has been demonstrated that phagocytes upregulate UCP2 upon AC recognition and UCP2 is needed for efficient AC engulfment [115]. UCP2 is a mitochondrial protein that uncouples oxidative phosphorylation from ATP synthesis and may be needed to maintain ATP levels within an appropriate range while degrading AC-derived cargo. It has also been demonstrated that expression of ABCA1, a cholesterol efflux transporter, is upregulated upon recognition of ACs[116]. ABCA1 dependent efflux of cholesterol presumably helps the phagocyte maintain homeostasis despite uptake of AC-derived cholesterol. In addition, several ligand activated transcription factors that sense lipids and are involved in lipid homeostasis have been demonstrated to sense ACs and help maintain efficient AC engulfment [117-120]. Activation of these transcription factors is likely needed to effectively manage lipids accrued during AC engulfment. These transcription factors have also been suggested to be involved in avoiding inflammatory responses; this aspect of AC clearance will be discusses in more detail below.

LC3-associated phagocytosis (LAP) is a recently characterized method of phagocytosis that involves recruitment of autophagy machinery to a single-membrane phagosome, and has been implicated in engulfment of pathogens, immune complexes, and ACs [121], [122]. LAP has been suggested to facilitate phagosome maturation after engulfment of ACs. LAP deficient BMMs produce increased levels of IL-1β and IL-6 when fed ACs [123]. Whether, this cytokine production is mediated by TLRs has not

been addressed, and the release of IL-1β could suggest involvement of a cytosolic PRR. Mice deficient in LAP machinery in LysM+ cells display defective degradation of engulfed ACs and develop autoimmune symptoms later in life with increased antinuclear antibodies (ANAs) and serum IL-1β, IL-6, and IL-12p40 [124]. However, once again the PRRs involved in this inflammation are unknown.

Avoidance of Inflammation

AC-derived nucleic acids have the potential to activate TLRs [125], [126], yet AC clearance is typically an immunologically silent process. This silence is vital to prevent autoimmunity in response to the millions of ACs cleared everyday. However, the mechanisms mediating this avoidance of inflammatory responses remain unclear with many conflicting reports.

There have been many reports of ACs exerting immunosuppressive effects on phagocytes. Incubation of ACs with mouse BMMs, thioglycollate elicited peritoneal macrophages, or macrophage cell lines can reduce responses to exogenously added TLR stimulation [118-120], [127-134]. A similar effect has been demonstrated in human PBMCs and human monocyte derived macrophages [117], [133], [135-137]. However, the signaling pathways linking AC recognition and suppression of phagocyte responses are unclear. Using approaches such as blocking antibodies or inhibitors of lipid mediators, some reports attribute the immunosuppressive effect of ACs to the production of the anti-inflammatory cytokines TGFβ and/or lipid mediators [129], [131], [136]. However, others report that soluble TGFβ and IL-10, or lipid mediators do not mediate the immunosuppressive effects of ACs [127] [128], [133], [134]. Cell autonomous signaling pathways downstream of AC recognition receptors have also been implicated in reducing inflammatory responses [137]. The most well characterized inhibitory pathway downstream of AC recognition is that of the TAM receptors. TAM receptor signaling has been shown to use IFNAR/STAT1 to signal and induce upregulation of SOCS1 and SOCS3, which then negatively regulate TLR signaling [138].

Several lipid sensing transcription factors have also been implicated in maintaining homeostasis during AC clearance. Members of the PPAR family of transcription factors are activated by a variety of ligands, including unsaturated fatty acids, and regulate fatty acid storage and glucose metabolism. PPARδ and PPARγ have been shown to influence AC clearance in very similar ways [117], [118], [120]. Macrophages lacking either PPAR demonstrate reduce AC engulfment. Thioglycollate elicited macrophages fed ACs upregulate expression of several genes including C1q, Tgfb1, and Mertk and macrophages lacking either PPAR demonstrate significantly reduced upregulation of these genes. In addition, the immunosuppressive effect of ACs in responses to LPS stimulation was reduced in macrophages lacking either PPAR. However, no response to ACs alone was observed. Mice lacking either PPAR in LysM+ cells develop ANAs, glomerular inflammation, and demonstrate reduced engulfment of injected ACs. Initially PPARδ (but not PPARγ) was shown to be upregulated in BMMs in response to ACs [118]. However, recently PPARγ (but not PPARδ) expression and its subsequent influence on AC clearance was shown to be upregulated in response to

erythropoietin signaling in thioglycollate elicited macrophages. Erythropoietin secretion was induced by the find-me signal sphingosine 1-phosphate [139].

Liver X receptors (LXRs) sense elevated cholesterol and trigger cholesterol efflux. Similar to the reports on the PPARs, thioglycollate elicited macrophages from mice lacking both LXRα and LXRβ demonstrate reduced AC engulfment. LXR-deficient mice demonstrate reduced clearance of ACs *in vivo*. Upregulation of Mertk, Il10, Tgfb1, and the cholesterol efflux transporter Abca1 in thioglycollate elicited macrophages in response to ACs was dependent on the LXRs. And the immunosuppressive effect of ACs in responses to LPS stimulation was reduced in macrophages lacking the LXRs. However, once again no response to ACs alone was observed. LXR deficient mice develop systemic autoimmunity, including splenomegaly, ANAs, and kidney inflammation. LXR agonist treatment could ameliorate autoimmune disease in the autoimmune prone lpr/lpr mouse stain [119]. It is interesting that AC clearance is similarly influenced by deficiencies in either PPAR and the double LXR knock out; despite their similar effects all seem to be required. However, mice lacking both PPARs and LXRs have not been examined and they may have some uncharacterized redundant roles in AC clearance.

<u>Consequences of defective AC clearance:</u>

If an ACs is not engulfed it will undergo a process known as secondary necrosis in which the plasma membrane breaks down. Patients with SLE demonstrate an accumulation of uncleared ACs, suggesting uncleared ACs may be involved in SLE disease progression [140]. Monocyte derived macrophages from SLE patients also demonstrate reduced phagocytosis of ACs [141]. Mice that have been injected with a mutant MFG-E8 that masks PS and inhibits AC engulfment develop ANAs. Interestingly, the injection of enough ACs alone can also induce low levels of ANAs [142].

Mice deficient in some AC recognition receptors demonstrate accumulation of ACs and autoimmune phenotypes. Mice lacking MFG-E8 demonstrate reduced uptake of ACs by tingible body macrophages and display splenomegaly, ANAs, and kidney inflammation [143]. The level of autoimmunity may correlate with the amount of uncleared ACs since mice lacking both MFG-E8 and Tim-4 produced increased ANAs [144]. CD300f deficient mice demonstrate enhanced disease in the Fcgr2b-/- model of systemic autoimmunity with increased splenomegaly, ANAs, and kidney inflammation; CD300f deficiency alone does not confer pathology [93]. SCARF1 deficient mice also display increase autoantibody levels and spontaneous inflammation [112].

However, in some of these models reports of autoimmune phenotypes vary, likely due to mouse colony housing and microbiota differences. For instance, mice that lack Tim-4 expression have been described in some cases to develop ANAs, increased serum cytokines, and kidney damage [124]. However, in other reports Tim-4 deficient mice do not display these pathologies [144], [145].

Chapter 3: Identification of apoptotic cell – engulfing phagocytes and characterization of their endosomal TLR responses

Background:

Avoidance of self nucleic acid recognition during clearance of ACs presents many challenges. First, the volume of cargo that must be cleared is immense; it has been estimated that millions of cells die by apoptosis in the human body every day [70]. If clearance is disrupted, accumulation of ACs can lead to immune stimulation and, eventually, autoimmune disease [140], [142], [143]. Second, professional phagocytes, such as macrophages and DCs, which engulf ACs express TLRs capable of nucleic acid recognition. Third, after recognition by a variety of phagocytic receptors, ACs traffic to phagosomes, the same organelles that house nucleic-acid sensing TLRs. Thus, the compartmentalization of TLR9 and TLR7 is not sufficient to explain the lack of response to the nucleic acids within ACs. Nevertheless, AC-derived nucleic acids do not typically initiate inflammatory responses. As discussed above, this avoidance is generally attributed to AC-induced expression of anti-inflammatory mediators. Largely through *in vitro* studies, it has been shown that ACs can induce anti-inflammatory cytokine production as well as cell autonomous anti-inflammatory signaling pathways in phagocytes [131], [136-138]. However, *in vivo* AC clearance is a constant process, and it remains unclear how the innate immune system balances induction of anti-inflammatory responses while maintaining the ability to respond to pathogens.
It has been proposed that tissue-resident macrophages are important mediators of AC clearance [70]. During inflammation, tissue-resident macrophages, rather than recruited monocyte-derived macrophages, engulf ACs injected into the peritoneum [130]. Tingible body macrophages have been demonstrated to use MFG-E8 to engulf apoptotic B cells in germinal centers of the spleen [143]. After intravenous injection of ACs, or after an inflammatory response, Kupffer cells in the liver have been demonstrated to engulf ACs [146], [147]. The metallophillic macrophages and marginal zone macrophages in the spleen have been shown to colocalize with intravenously injected ACs [148], [149]. Depletion of these splenic macrophage populations can result in delayed clearance of injected ACs followed by generation of anti-dsDNA antibodies. Recently it has been demonstrated that in a diphtheria toxin induced model of intestinal epithelial cell apoptosis a CD103+ DC subset as well as a CD103+CD11b+ macrophage and CD103-CD11b+ macrophage population engulf ACs. After induction of intestinal epithelial cell apoptosis the phagocyte populations that had engulfed ACs demonstrated changes in gene expression profiles with all three populations downregulating genes involved in inflammatory responses and upregulated negative regulators of the immune response, although the specific genes differed between the populations [150].

The identities of the cells that clear ACs from most tissues in the steady state remain unclear. In addition, the majority of studies examining suppression of inflammatory responses to ACs have used thioglycollate-elicited macrophages, BMMs, or macrophage cell lines. In recent years it has become apparent that macrophages from different tissues are heterogeneous [151]. It remains unclear whether there is also

heterogeneity in the ability of different resident macrophage populations to clear ACs, or regulate responses to ACs.

In this chapter I present our work identifying macrophage populations in several tissues that efficiently engulf ACs *in vivo* at steady state. We explore the expression of nucleic acid sensing TLRs in these macrophages and find that they lack expression of TLR9. When forced to express TLR9 in AC-engulfing macrophages mice generate subtly more inflammation during an induced model of lupus.

Results:

Identification of macrophage populations that clear apoptotic cells *in vivo*

To investigate how tolerance to AC-derived ligands is maintained we first sought to identify the cells that clear ACs under homeostatic conditions *in vivo*. We generated mixed bone marrow chimeric mice: C57BL/6 (WT) bone marrow expressing the congenic marker CD45.2 was mixed with bone marrow expressing the congenic marker CD45.1 and the fluorescent protein tdTomato and injected into irradiated WT recipients (Figure 3.1 A). In these chimeric mice the only cells that genetically encode for tdTomato also express CD45.1. After ten weeks of reconstitution, tissues from these mice were harvested and analyzed by flow cytometry. After gating out CD45.1$^+$ cells that express TdTomato, we identified a population of tdTomato$^+$ cells that was not present in control chimeras generated with CD45.1$^+$ bone marrow lacking tdTomato (Figure 3.1 C). Since the CD45.1$^-$ cells do not genetically encode for tdTomato themselves, we reasoned that they had engulfed apoptotic tdTomato$^+$ cells. Immunoflourescence microscopy of cells from the tissues of chimeric mice confirmed the presence of cells containing tdTomato$^+$ AC corpses, as shown by the colocalization of tomato and DNA within WT cells (Figure 3.1 B).

After analyzing several tissues, we identified three tissue-resident macrophage populations as the primary AC-engulfing cells in their respective tissues: peritoneal macrophages that express the AC recognition receptor Tim-4, pleural cavity macrophages that express Tim-4, and lung alveolar macrophages, which do not express Tim-4 (Fig 3.2 A and B). Resident peritoneal macrophages have previously been shown to engulf ACs injected into the peritoneum [130], [145]; our analysis now demonstrates that these macrophages engulf ACs at steady state *in vivo.* In other tissues analyzed, we did not identify a primary cell population that appeared to be specialized for AC clearance. However, we did identify several macrophage populations that engulf ACs to a lesser extent than the three populations described above: CD11b$^+$ interstitial macrophages in the lung, red pulp macrophages in the spleen, and Kupffer cells in the liver (Figure 3.2 A and B). We did not identify a macrophage population that engulfed ACs in skin draining lymph nodes, but note that CD11c^{mid} migratory dendritic cells represented about 25% of AC-engulfing cells, suggesting they may acquire ACs in the skin and then migrate to the lymph nodes (Figure 3.2 A).

To confirm that the identified macrophage populations possess an enhanced ability to engulf ACs, we compared AC engulfment by Tim-4$^+$ peritoneal macrophages (pMacs) and bone marrow derived macrophages (BMMs). Tim-4$^+$ pMacs engulfed more ACs than BMMs, consistent with our *in vivo* findings (Figure 3.2 C).

AC-engulfing macrophage populations do not express TLR9

After identifying macrophage populations that are adept at engulfing ACs we sought to determine how these populations avoid generating responses to AC-derived nucleic acids. As a first step we wanted to analyze expression of nucleic acid sensing

TLRs in these macrophages. Examining endogenous TLR expression is notoriously challenging due to a lack of reliable antibodies. To enable analysis of endogenous TLR expression levels the lab has generated reporter mice that contain C-terminal epitope tags and fluorescent protein driven by internal ribosome entry sequences downstream of the TLR coding sequence. When we began these studies the lab had already generated a TLR9 reporter mouse, which expresses an HA tagged TLR9 followed by an IRES GFP (Figure 3.3 A). I generated a TLR7 reporter, which expresses a FLAG tagged TLR7 followed by an IRES tdTomato (Figure 3.3 B). We engineered a targeting vector that in which the endogenous stop codon was replaced by a FLAG tag with by a stop codon followed by a floxed neomycin resistance gene and then an IRES - tdTomato. We successfully targeted one clone of male C57BL/6 - derived embryonic stem cells. The targeting was confirmed by southern analysis of BamHI digested ES cell DNA using a probe that bound within the coding region of TLR7 (Figure 3.3 B and C). Since TLR7 is located on the reverse strand of the X chromosome only one copy of the tlr7 locus is present in male ES cells. Although attempts to confirm the 3' side of the knock in by southern were unsuccessful, PCR amplification and sequencing of the region targeted by the 3' homology arm of the vector confirmed correct integration. These mice were crossed to EIIA-cre transgenic mice to remove the neomycin resistance gene. Western blot was used to confirm the expression of FLAG tagged TLR7 in BMMs derived from TLR7KI mice (Figure 3.3 D).

Intriguingly, while we detected TLR7 expression in all macrophages studied, the three AC-engulfing macrophage populations described above did not express TLR9 (Figure 3.4 A). Other macrophage populations, such as red pulp macrophages, Kupffer cells, and lung $CD11b^+$ interstitial macrophages did express TLR9. We confirmed that the three AC-engulfing macrophage populations lack TLR9 by stimulating macrophages *ex vivo* with different TLR ligands. In agreement with the results from the reporter mice, these macrophages produced TNF in response to stimulation with LPS (TLR4 ligand) or R848 (TLR7 ligand) but not CpG oligonucleotides (ODN), a synthetic TLR9 ligand (Figure 3.4 B). $CD11b^+$ interstitial macrophages and red pulp macrophages generated robust responses to TLR9 stimulation. Interestingly, while Kupffer cells in the liver did express TLR9 and generated responses to TLR9 stimulation, these responses were muted compared to responses to other TLR ligands. Kupffer cells also represented a larger percent of the AC-engulfing cells in the liver compared to red pulp macrophages or lung interstitial macrophages (Figure 3.2 B). Therefore, Kupffer cells are intermediate in both engulfment of ACs and responses to TLR9 stimulation, suggesting that there may be an inverse correlation between AC engulfment ability and expression of TLR9. To further confirm that AC-engulfing macrophages do not express TLR9, we isolated $Tim\text{-}4^+$ pMacs from TLR9KI mice and analyzed levels of TLR9 protein by immunoblot against the HA tag encoded by the knock-in allele of TLR9. TLR9 levels were significantly lower in $Tim\text{-}4^+$ pMacs than in BMMs (Figure 3.4 C). The very small amount of TLR9 that we did detect is likely due to contaminating cells in the preparation of $Tim\text{-}4^+$ pMacs. These data lead us to hypothesize that strict regulation of TLR9 expression is one mechanism used to avoid responses to self-derived DNA.

Forcing expression of TLR9 in AC-engulfing macrophages subtly enhances inflammation during pristane-induced lupus

To investigate whether inhibiting TLR9 expression in AC-engulfing macrophages is needed to maintain self-tolerance we forced these macrophages to express TLR9. We generated mice that ectopically express TLR9 in all cells (rosa-TLR9) or in LysM^{+} cells (LysMcre$^{+/-}$Rosa$^{stop\text{-}flox\text{-}TLR9}$) by crossing mice in which TLR9 is inserted behind a floxed stop cassette in the Rosa26 locus to EIIA-cre or LysM-cre mice, respectively. Tim4^{+} pMacs, pleural cavity macrophages, and alveolar macrophages from LysMcre$^{+/-}$Rosa$^{stop\text{-}flox\text{-}TLR9}$ mice responded to CpG ODN (Figure 3.5 A). This result confirms that AC-engulfing macrophages control responses to TLR9 ligands through TLR9 expression and rules out additional mechanisms such as altered receptor trafficking or repression of cytokine production downstream of signaling. However, we were surprised to find that despite this forced TLR9 responsiveness, Tim-4^{+} pMacs from rosa-TLR9 mice did not respond to ACs *in vitro* (Figure 3.5 B). This lack of response was not due to reduced AC engulfment (Figure 3.5 C).

To determine if expression of TLR9 in AC-engulfing macrophages disrupts homeostasis we aged rosa-TLR9 mice for one year then compared them to age matched WT mice. We saw no evidence of myeloid cell expansion in any organs analyzed. Cell numbers were not affected by overexpression of TLR9 (Figure 3.5 D). Serum cytokine levels were not significantly upregulated in rosa-TLR9 mice (Figure 3.5 F). We also analyzed serum anti-nuclear antibodies (ANA). Although more rosa-TLR9 mice had ANAs as tested by nuclear staining of HEp-2 slides, these mice did not have elevated anti-dsDNA antibodies (Figure 3.5 E and F). Altogether, there was little evidence of inflammation resulting from TLR9 expression in AC-engulfing macrophages. Although more rosa-TLR9 mice had ANAs these mice overexpress TLR9 in all cells, and the absence of other indicators of inflammation may suggest that increased TLR9 expression in B cells can induce higher ANA levels.

To rule out B cell intrinsic overexpression of TLR9 and more specifically study macrophages we examined LysMcre$^{+/-}$Rosa$^{stop\text{-}flox\text{-}TLR9\ mice}$. When housed unperturbed in our specific pathogen-free mouse facility, LysMcre$^{+/-}$Rosa$^{stop\text{-}flox\text{-}TLR9}$ mice showed no evidence of inflammatory disease over twelve months (data not shown), demonstrating that forced expression of TLR9 in AC-engulfing macrophages is not sufficient to disrupt homeostasis. We next considered whether restricted expression of TLR9 might be critical under conditions where there is increased cell death *in vivo*. To this end we employed the pristane-induced model of lupus, in which intraperitoneal injection of pristane oil induces substantial apoptosis in the peritoneal cavity followed by recruitment of inflammatory cells and development of a lupus-like disease, including production of anti-nuclear antibodies [152]. It has previously been demonstrated that TLR7 and TLR9 play roles in the pathogenesis of this disease model [60], [153]. We compared LysMcre$^{+/-}$Rosa$^{stop\text{-}flox\text{-}TLR9}$ and LysMcre$^{-/-}$Rosa$^{stop\text{-}flox\text{-}TLR9}$ littermates at two weeks and nine months post injection. LysMcre$^{+/-}$Rosa$^{stop\text{-}flox\text{-}TLR9}$ mice had small increases in the number of cells recruited into the peritoneum at two weeks, including neutrophils and Ly6C^{+} monocytes (Figure 3.6 A). After nine months LysMcre$^{+/-}$Rosa$^{stop\text{-}flox\text{-}TLR9}$ mice still

demonstrated a small increase in peritoneal cell numbers, including CD11b$^+$ cells, T cells, and neutrophils (Figure 3.6C). Sera from these mice also contained increased anti-dsDNA IgG (Figure 3.6 B). Altogether, the overexpression of TLR9 in LysM$^+$ cells seems to slightly enhance inflammation in the pristane model of lupus.

Discussion:

Here we identified three resident macrophage populations that are the primary AC-engulfing population in their tissue. Intriguingly, these populations do not express TLR9, suggesting the possibility that this lack of expression is vital to maintain tolerance to self derived DNA. However, we found that forcing expression of TLR9 in these macrophages had minimal impact. Continual responses to endogenous DNA would induce inflammation that would be damaging to the host. Thus, additional mechanisms must limit tissue macrophage responses to nucleic acids within ACs.
Interestingly, the three AC-engulfing macrophage populations we identified are motile and reside in cavities. Many other tissue-resident macrophage populations are immobilized [154]. A recent report demonstrated that peritoneal macrophages can be recruited directly from the peritoneal cavity into the liver to repair damage after liver injury [155]. It has also been shown that ACs release find-me signals that recruit phagocytes to dying cells [73], [75]. Perhaps these macrophages are particularly adapted to engulf ACs since they can be recruited to dying cells in their cavities as well as neighboring organs.

Our data demonstrate that TLR9 expression correlates with the functions of different murine macrophage populations. Cell type specific expression of endosomal TLRs has also been demonstrated in human myeloid cells. It has been shown in human peripheral blood mononuclear cells that TLR7 and TLR9 are exclusively expressed in pDCs, while TLR8 is more broadly expressed in macrophages and conventional DCs [156], [157]. These data have often been cited to suggest that no human macrophages express these endosomal TLRs. However, given our growing appreciation of tissue macrophage heterogeneity, this concept is worth revisiting. It has not yet been explored whether human tissue-resident macrophages are programmed by environmental cues. Assuming a similar degree of heterogeneity is present in humans as is seen in mice, it would be very interesting to examine TLR expression in different human macrophage populations. For instance, human decidual macrophages have been shown to express TLR3, TLR7, TLR8 and TLR9 in addition to TLR2 and TLR4 [158]. It will be important to expand our understanding of nucleic acid sensing TLRs in human tissue-resident macrophages with different functions, especially those that adeptly engulf ACs.

The lack of significant inflammation in mice that overexpress TLR9 in every cell is potentially surprising given previous studies that examined mice that overexpress TLR7 [67], [69]. However, as discussed before, the role of TLR9 in autoimmune disease is more complex with mice deficient for TLR9 being protected in many models of SLE but TLR9 contributing to other diseases such as SLE [57] [63] [66]. It has been suggested that the complexity of TLR9s role in autoimmunity may be due to competition with TLR7 for association with Unc93b1 [64]. Overexpression of TLR9 in our system may limit TLR7 responses and blunt potential autoimmunity. It should also ne noted that although macrophages from rosa-TLR9 mice generate robust responses to TLR9 stimulation, the TLR9 expressed from the rosa locus is HA tagged, which reduces signaling capacity. The differing roles of TLR9 and TLR7 in autoimmunity may also be explained by the differences in their respective ligands. Crystal structure data of TLR9 and TLR7 binding

to their ligands has demonstrated that TLR7 binds two small ligands, a guanosine and oligonucleotide as short as three nucleotides long with a U in the middle position [5]. TLR9 binds a longer oligonucleotide [4]. Perhaps smaller endogenous TLR7 ligands more readily access the endosome. In addition, as endogenous DNA contains very few unmethylated CpG dinucleotides, it likely is a weaker TLR ligand than endogenous RNA.

The inverse correlation between expression of TLR9 and AC-engulfment ability is very intriguing and may be one important mechanism limiting autoinflammatory responses during AC-clearance. However, our data examining TLR9 overexpressing mice clearly indicate that redundant mechanisms must also be involved. Given the importance of maintaining self-tolerance it is not surprising that there would be many levels of regulation.

Materials and Methods:

Mice

Mice were housed under specific pathogen-free conditions at the University of California, Berkeley. All mouse experiments were performed in accordance with the guidelines of the Animal Care and Use Committee at UC Berkeley. Unless noted mice were analyzed at 6-12 weeks of age. C57BL/6, C57BL/6 CD45.1^{+} (stock #002014), rosa26 $^{\text{stop-flox-tdTomato}}$ (stock #007909), LysM-cre (stock #004781), and EIIa-cre (stock #003724) mice were obtained from Jackson laboratories. A previous graduate student in the lab generated the TLR9 reporter mice. TLR9KI mice were generated using a construct encoded an HA tag on the 3' end of the TLR9 gene followed by an IRES-EGFP sequence. Rosa$^{\text{stop-flox-TLR9}}$ mice, in which a floxed region (containing eGFP, a Neo cassette, and a transcriptional stop sequence) followed by an HA tagged TLR9 was inserted into the Rosa26 locus, were obtained from the Shlomchik lab at the University of Pittsburgh.

Chimeric mice were generated by irradiation of C57BL/6 with 1000rads. Mice were reconstituted with $1x10^{7}$ bone marrow cells (80% C57BL/6 and 20% CD45.1^{+} tdTomato^{+}). Mice were analyzed 10-12 weeks after cell transfer.

Generation of TLR7KI mice

TLR7 reporter mice were generated by constructing a targeting vector encoding a FLAG tag on the 3' end of the TLR7 gene followed by IRES-tdTomato sequence. This vector was electroporated into C57BL/6-derived embryonic stem cells by the Mouse Biology Program at UC Davis. The vector also introduced a loxP-flanked neomycin resistance cassette. Targeting was assessed by southern blot and correctly targeted ES cells were injected into ICR/CD1 blastocysts. Chimeric males were mated with C57BL/6 background EIIA-cre females to remove the neomycin resistance cassette.

Tissue harvest

Cells from the peritoneal and pleural cavities were recovered by lavage with ice cold PBS. Spleens and lymph nodes were digested with collagenase XI (Sigma #C9697) with DNAse I (Sigma #D4513) for 30min and single cell suspensions were generated by mechanical disruption. Perfused lungs were digested with collagenase XI with DNAse I for 45min, single cell suspensions were generated by mechanical disruption through a 100um filter, cells were resuspended in 44% isotonic percoll (GE healthcare #17-0891-01), underlayed with 67% percoll, and spun at 1550xg without brake. Cells from the interface were collected for analysis. Perfused livers were digested with collagenase VIII (Sigma #C2139) with DNase I for 45min, cells were resuspended in Hank's buffered salt solution and centrifuged at 30xg for 3min. After low speed centrifugation cells in suspension were collected and underlayed with a solution of 25% isotonic Percoll and 50% Percoll, then spun for 15min at 1800xg without brake. Cell counts were obtained using Count Bright absolute counting beads (Life technologies #C36950).

Isolation of Tim-4^{+} peritoneal Macrophages

Peritoneal cells were recovered by lavage with 5ml of ice cold PBS. For RNAseq and western blot experiments B cells were first depleted using anti-CD19-biotin antibody and biotin binder dynabeads (ThermoFisher #11047). Tim-4^{+} cells were isolated using anti-TIM-4 antibody (clone RMT4-54, BioLegend) and anti-rat IgG microbeads (Miltenyi #130-048-501).

Cell culture
BMMs were differentiated for seven days in RPMI complete media (RPMI-1640 supplemented with 10% (vol/vol) fetal calf serum, L-glutamine, penicillin-streptomycin, sodium pyruvate, and HEPES pH 7.2) supplemented with M-CSF containing supernatant from 3T3-CSF cells. Peritoneal, lung, and pleural cells were harvested as described above and plated on non-tissue culture treated plates in RPMI complete media.

Apoptotic cell generation and engulfment
Thymi were harvest from WT or CD45.1^{+} rosa-tdTomato mice and single cell suspensions were generated by mechanical disruption through a 70um filter. Cells were irradiated with 600rad and incubated in RPMI complete media at 37°C for 4 hours. Apoptotic cells were then incubated with macrophages at the indicated ratios. For experiments examining AC engulfment capabilities macrophages were allowed to engulf ACs for 60min.

Stimulations
Cells were plated in RPMI complete media. Tissue macrophages were stimulated directly *ex vivo*, or after overnight culture. Cells were stimulated with TLR ligands (LPS, CpG-B ODN 1668, R848 all from Invivogen), or ACs. For analysis of secreted cytokines, supernatant was collected 7 hours after stimulation. For intracellular cytokine staining, 30 minutes after stimulation brefeldin A (GolgiPlug, BD Biosciences) was added to cells before incubation for another 4 hours.

Flow cytometry and Antibodies
Dead cells were excluded using a fixable live/dead stain (Life technologies) or DAPI (Life Technologies) and all stains were carried out in PBS containing 2% FBS (v/v) and 2mM EDTA including anti-CD16/32 Fc blocking antibody (2.4G2, UCSF monoclonal antibody core) and normal mouse serum (Sigma). Cells were stained for 20min at 4°C with antibodies (see table 1). For intracellular TNF staining cells were permeabilized with Fix/Perm buffer (BD) for 20min at 4°C. Cells were then stained with antibodies. All cells were analyzed on an LSRII or LSR Fortessa (BD Biosciences), and data was analyzed with FlowJo (TreeStar).

Pristane
Mice were intraperitoneally injected with 0.5ml of pristane (Sigma #P2870) that had been filtered through a 0.22um filter. Mice were analyzed after two weeks or after nine months.

Western Blot Analysis
Cells were lysed in RIPA buffer (50mM Tris pH 7.4, 150mM NaCl, 1mM EDTA, 0.5mM EGTA, 1% NP-40, 1% DOC, 0.1% SDS) containing protease inhibitors (Roche #05 892 791 001). Cell lysates were immunoprecipitated with anti-HA matrix (Roche #11573000). After washing in RIPA buffer, matrix beads were boiled in SDS-PAGE loading buffer. Protein was run on a 4-15% gel (Biorad #4561083) and transferred to Immobilon-FL membrane (Millipore #IPFL00010). After blocking, membranes were probed with anti-HA antibody, followed by anti-rat-680 secondary (Life Technologies #A21096) or probed with anti-tubulin, followed by anti-mouse-800 secondary (Li-Cor #926-32210). Images were scanned using a Licor Odyssey.

Enzyme-linked immunosorbent assay (ELISA) and CBA assay
Cytokines were measured using a Cytometric Bead Array assay according to the manufacturer's instructions (BD Biosciences).

For anti-dsDNA ELISAs NUNC Maxisorp plates (eBioscience) were coated first with poly-l-lysine (Sigma #P-6516) at 50µg/ml overnight at -20°C, washed, then coated with 100µg/ml dsDNA (Calf thymus DNA Sigma #D45220) overnight at 4°C. Plates were then blocked with PBS + 1% BSA (w/v) + 5% goat serum (v/v) at room temperature for four hours before serum samples diluted 1:500 (unless otherwise noted) in PBS + 1% BSA (w/v) + 5% goat serum (v/v) were added and incubated overnight at 4°C. Secondary anti-IgG all-biotin (Jackson #115-065-205) was used at 1:10000 followed by Streptavidin-HRP (BD Biosciences #554066). Plates were developed with 1mg/ml OPD (Sigma #P6912) in Citrate Buffer (PBS with 0.05M NaH2 PO4 and 0.02M Citric acid) with HCl acid stop.

HEp-2 slide staining
Mouse serum was diluted 1:40 in PBS. 30ul of diluted serum was added to wells of HEp-2 slide (MBL #AN-1016) and incubated at room temperature for 30 minutes. Wells were washed twice in PBS. Secondary anti-mouse IgG-488 (Jackson #115-546-146) was diluted 1:300 (from stock of 1.5mg/ml) in PBS. 30ul of secondary was added to wells of slide and incubated at room temperature for 30 minutes. Slides were washed twice in PBS and mounting media with DAPI was used. Slides were imaged using a Zeiss Axio Scan.Z1 slide scanner.

Statistical Analysis
Statistical analysis was performed with the Prism software (GraphPad software). P-values were determined using unpaired two-tailed Students's *t*-test. Where noted *t*-tests were performed on log transformed data to account for the non-normal distribution of the data. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Table 3.1: Antibodies

Antibody	Clone	Vendor
B220	RA3-6B2	eBioscience
CD11c	N418	BioLegend
CD19	6D5	BioLegend
CD3ε	145-2C11	Tonbo
CD11b	M1/70	BioLegend
CD45.1	A20	BioLegend
CD45.2	104	BioLegend
CD64	X54-5/7.2	BioLegend
F4/80	BM8	Tonbo
F4/80	CI:A3-1	Biorad
Ly6C	HK1.4	BioLegend
Ly6G	1A8	BioLegend
MHC II	M5/114.15.2	eBioscience
Siglec-F	E50-2440	BioLegend
Tim-4	RMT4-54	BioLegend
TNF	MP6-XT22	BioLegend
GATA6	D61E4	Cell Signaling
pERK1/2 biotin	D13.14.4E	Cell Signaling
HA antibody	3F10	Roche
tubulin	DM1A	Calbiochem

Figures:

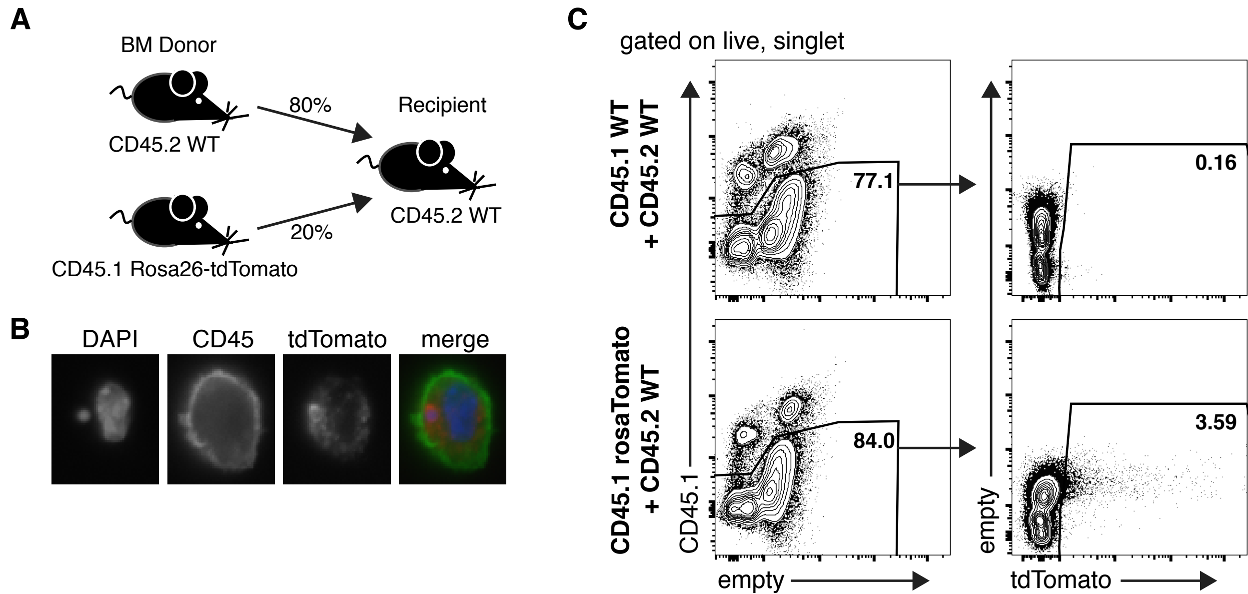


Figure 3.1: Bone marrow chimera system used to identify populations that clear apoptotic cells *in vivo*. (A) Diagram of bone marrow chimeras used to identify AC-engulfing cells at steady state. Congenically marked CD45.1$^+$ bone marrow cells expressing tdTomato were mixed with wild type CD45.2$^+$ bone marrow cells and injected into an irradiated wild type recipient. Tissues were analyzed after 10 weeks of reconstitution. **(B)** Detection of cells that contain tdTomato$^+$ cell corpses. Representative immunofluorescence picture of AC-engulfing cell isolated from chimeras depicted in (A). Cells were stained with DAPI and antibody against CD45. **(C)** Example gating strategy to identify AC-engulfing cells. Control bone marrow chimeras generated with WT CD45.1$^+$ bone marrow were compared to chimeras generated with tdTomato$^+$CD45.1$^+$ bone marrow.

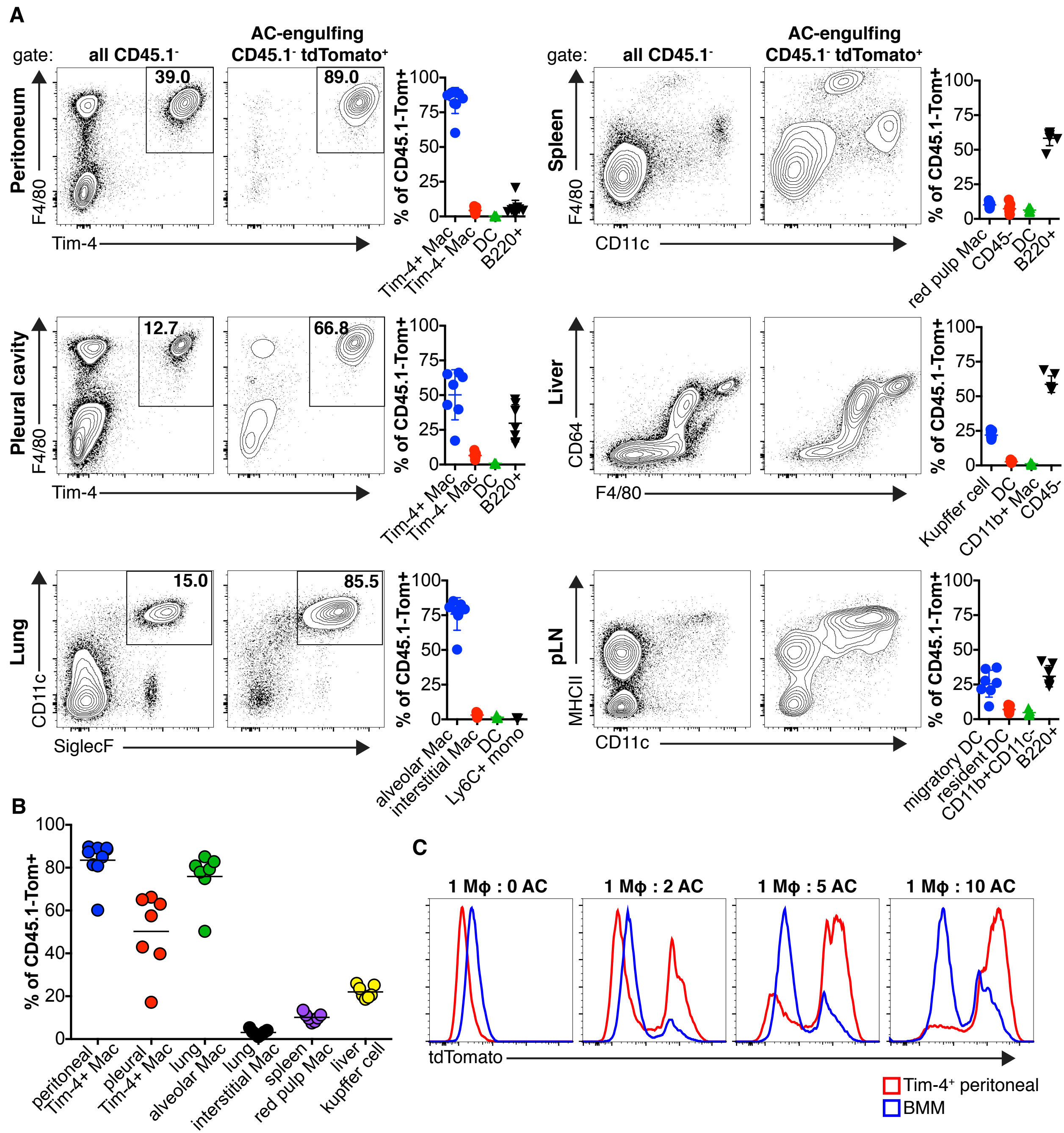


Figure 3.2: Identification of macrophages that clear apoptotic cells *in vivo*. (A) Tim-4$^+$ pMacs, alveolar macrophages, and Tim-4$^+$ pleural cavity macrophages are adept at engulfing ACs *in vivo.* Representative flow cytometric analyses of tissues from bone marrow chimeras. Cells were gated on CD45.1$^-$ cells or AC-engulfing cells (CD45.1$^-$ tdTomato$^+$), as indicated. Data are representative of at least three independent experiments. Quantification of AC-engulfing (tdTomato$^+$CD45.1$^-$) cells in different tissues. Data are the combined results from three independent experiments.

(B) Quantification of AC engulfment by the indicated macrophage populations in tissues. Data are the combination of three independent experiments. **(C)** Tim-4$^+$ pMacs are adept at engulfing ACs. CD45.2$^+$ Tim-4$^+$ pMacs and CD45.2$^+$ BMMs were incubated with CD45.1$^+$ tdTomato$^+$ ACs at indicated ratios and analyzed by flow cytometry. Data are representative of three independent experiments.

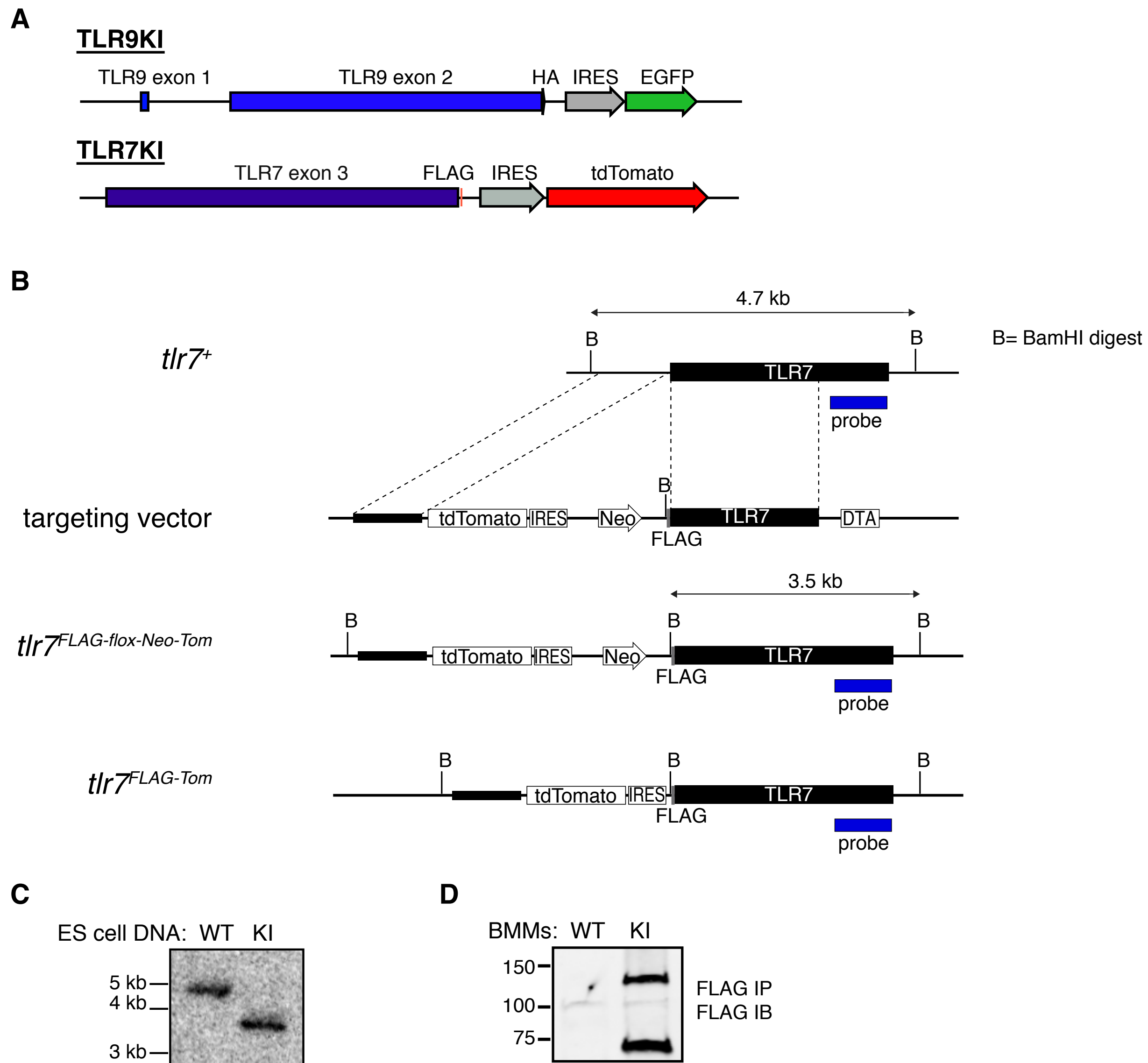
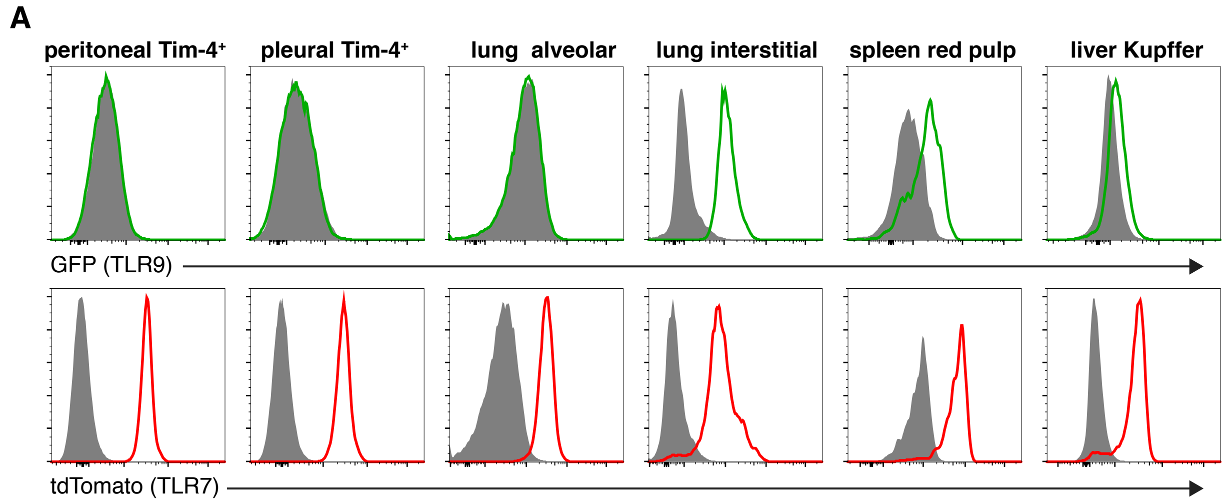


Figure 3.3: TLR7 and TLR9 reporter mice. (A) Schematic of reporter TLR9HA-GFP and TLR7FLAG-tdTomato reporter mice. **(B)** Targeting strategy for $tlr7^{FLAG\text{-}tdTomato}$ knock-in at the tlr7 endogenous locus. The endogenous tlr7 locus is on the reverse strand of the X chromosome. BamHI digestion strategy for southern analysis. Blue box represents probe 5' of targeted locus. Expected band sizes are indicated after initial knock-in in ES cells ($tlr7^{FLAG\text{-}flox\text{-}Neo\text{-}Tom}$). Neomycin resistance cassette was removed after crossing with EIIA-cre mice. **(C)** Southern analysis on WT and targeted ES cell DNA from male mice. **(D)** Representative western blot on BMMs derived from TLR7KI mouse or littermate control. Cell lysates were immunoprecipitated with anti-FLAG resin and eluted protein was analyzed by anti-FLAG western. The two bands represent ER localized TLR7 (larger band) and endosomal cleaved TLR7 (smaller band)

A



TLR9HA-IRES GFP
TLR7FLAG-IRES-tdTomato
WT

B

LPS
R848
CpG
peritoneal Tim-4+
pleural Tim-4+
lung alveolar
lung interstitial
spleen red pulp
liver Kupffer
TNF
stimulated
unstimulated

C

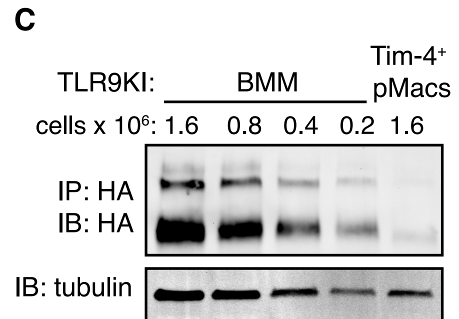
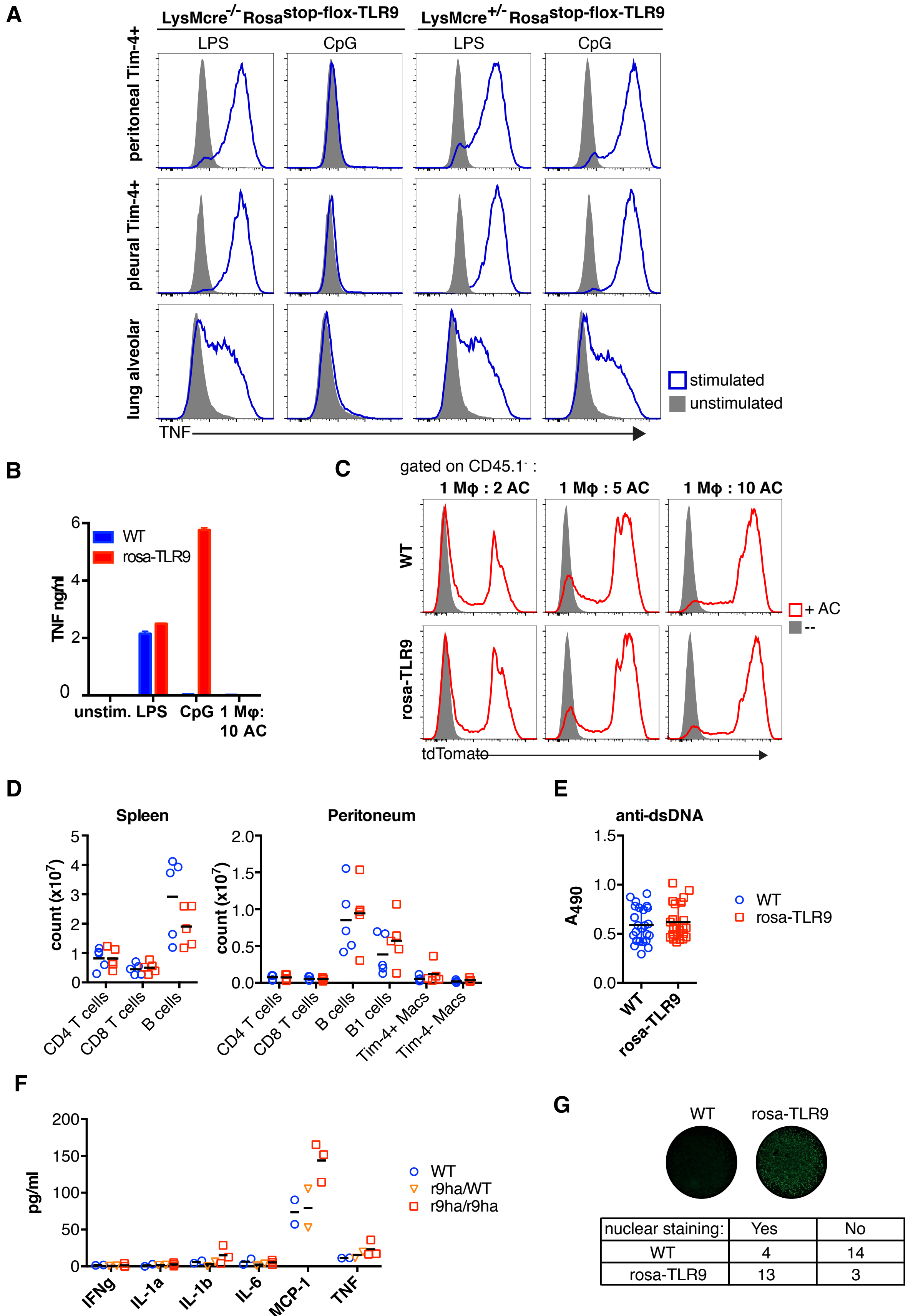


Figure 3.4: AC-engulfing macrophages do not express TLR9. **(A)** AC-engulfing macrophages do not express TLR9. Representative histograms of fluorescent protein expression by gated macrophages harvested from TLR9KI, TLR7KI, or WT mice. Peritoneal and pleural Tim-4^{+} macrophages are gated as live F4/80^{+}Tim-4^{+} cells. Alveolar macrophages are gated as live SiglecF^{+}CD11c^{+}CD64^{+}CD11b^{-} cells. Lung interstitial macrophages are gated as live CD11b^{+}F4/80^{+}MHCII^{+} cells. Red pulp macrophages are gated as live F4/80^{hi}CD11b^{-} cells. Kupffer cells are gated as live CD45^{+}CD64^{+}MHCII^{+} cells. Data are representative of at least three independent experiments. **(B)** AC-engulfing macrophages do not respond to TLR9 ligands. Macrophages from WT mice were stimulated *ex vivo* with TLR ligands. Macrophages were gated as in (A). TNF production was measured by ICS. Data are representative of at least three independent experiments. **(C)** Tim-4^{+} pMacs and BMMs from TLR9KI mice were analyzed for TLR9 protein expression. Cell lysates were immunoprecipitated with anti-HA resin and eluted protein was analyzed by anti-HA western. The two bands represent ER localized TLR9 (larger band) and endosomal cleaved TLR9 (smaller band). An anti-tubulin immunoblot was performed on the same lysates. Data are representative of at least three independent experiments.



nuclear staining:	Yes	No
WT	4	14
rosa-TLR9	13	3

Figure 3.5: Forcing expression of TLR9 in AC-engulfing macrophages does not induce inflammation in the steady state. (A) When forced to express TLR9 AC-engulfing macrophages respond to CpG ODN. Macrophages from LysMcre$^{+/-}$Rosa$^{stop\text{-}flox\text{-}TLR9}$ or littermate control LysMcre$^{-/-}$ Rosa$^{stop\text{-}flox\text{-}TLR9}$ mice were stimulated *ex vivo* with TLR ligands. TNF production was measured by ICS. Data are representative of at least three independent experiments. **(B)** Tim-4^{+} pMacs forced to express TLR9 do not generate responses to ACs. Isolated rosa-TLR9 Tim-4^{+} pMacs were stimulated with TLR ligands and ACs. TNF was measured by CBA. Data are representative of at least three independent experiments. **(C)** Rosa-TLR9 macrophages are adept at AC engulfment. Tim-4^{+} pMacs were isolated from WT and rosa-TLR9 mice. Macrophages were cultured overnight then incubated with CD45.1^{+}tdTomato^{+} ACs at indicated ratios and analyzed by flow cytometry. **(D-G)** Mice that overexpress TLR9 in all cells develop ANA but no other signs of inflammation in the steady state. WT and rosa-TLR9 mice were compared after one year of age. (D) Cells were harvested from spleen and peritoneum and analyzed by flow cytometry. Data are representative of at least three experiments. (E) Serum samples were collected, diluted 1:100, and analyzed by ELISA for anti-dsDNA IgG + IgM. Data are the combined results of four independent experiments. (F) Cytokine levels in serum from WT, heterozygous rosa-TLR9, and homozygous rosa-TLR9 mice was analyzed by CBA. (G) Representative picture and quantification of results from HEp-2 slides stained with serum diluted from WT or rosa-TLR9 mice.

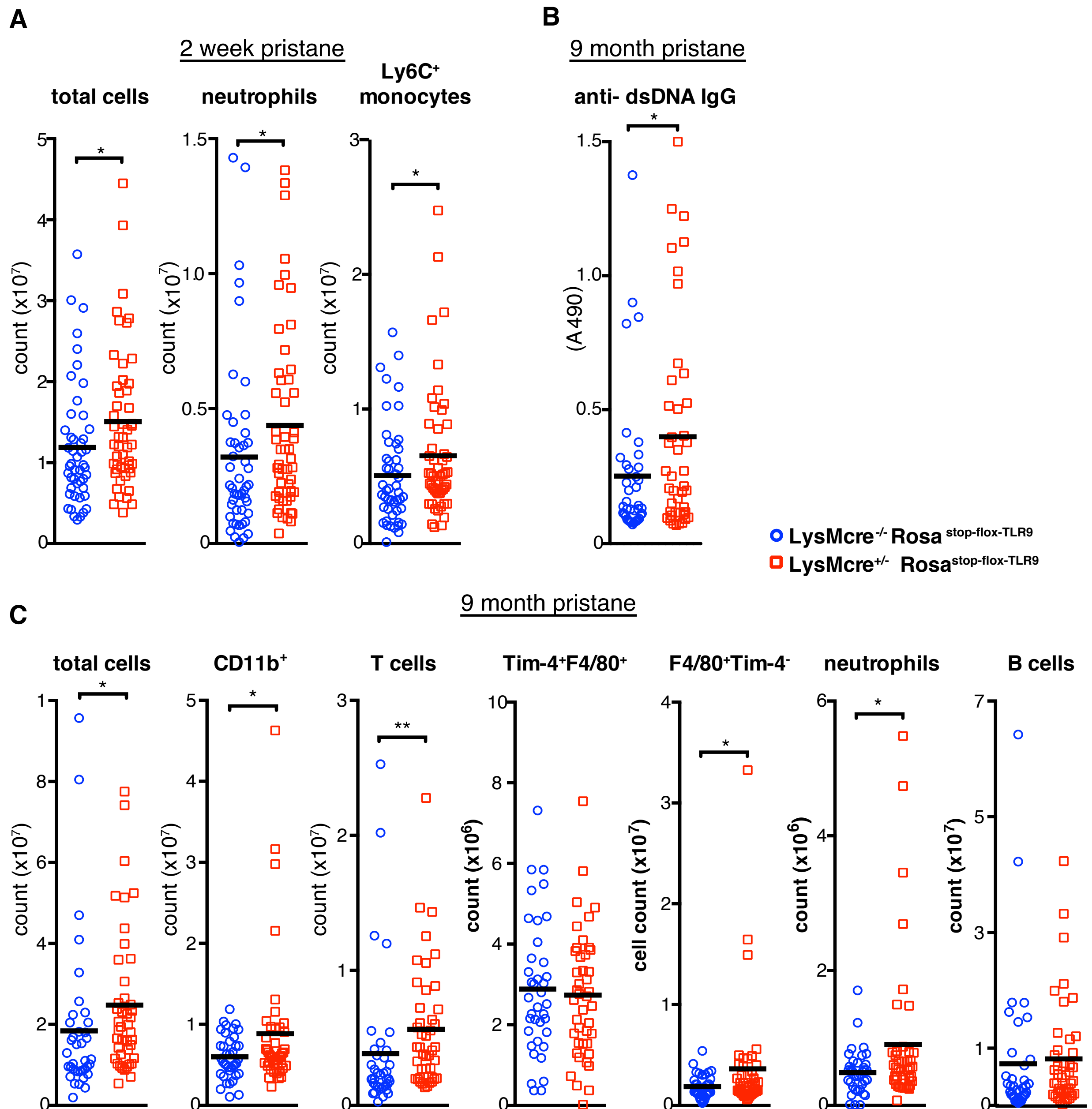


Figure 3.6: Forcing expression of TLR9 in AC-engulfing macrophages induces a small increase in inflammation during pristane-induced lupus. LysMcre$^{+/-}$Rosa$^{stop\text{-}flox\text{-}TLR9}$ or littermate control LysMcre$^{-/-}$ Rosa$^{stop\text{-}flox\text{-}TLR9}$ mice were injected intraperitoneally with pristane. **(A)** Mice overexpressing TLR9 in LysM^{+} cells have slightly enhanced cell recruitment 14 days after pristane injection. 14 days after injection peritoneal cells were harvested and analyzed by flow cytometry. The number of total peritoneal exudate cells, neutrophils (CD11b^{+}Ly6G^{+}Ly6C^{mid}), and Ly6C^{+} monocytes (CD11b^{+}Ly6C^{+}Ly6G^{-}) are shown. Data are the combined results of five independent experiments. **(B and C)** Mice overexpressing TLR9 in LysM^{+} cells have slightly enhanced cell counts and anti-dsDNA IgG 9 months after pristane injection. (B) Serum samples were collected and tested by ELISA for anti-dsDNA IgG. (D) Peritoneal cells were harvested and analyzed by flow cytometry. The number of total peritoneal exudate cells, CD11b^{+} cells, T cells (CD3ε^{+}CD11b^{-}CD19^{-}) Tim-4^{+} macrophages, F4/80^{+}Tim4^{-} macrophages, neutrophils (CD11b^{+}Ly6G^{+}Ly6C^{mid}), and B cells (CD19^{+}CD3ε^{-}CD11b^{-}) are shown. Data are the combined results of four independent experiments. All p-values were determined by t-test performed on log-transformed data to account for the non-normal distribution of the data.

Chapter 4: Environmental programming of AC-engulfing macrophages

Background:

As discussed in chapter three, we identified three populations of macrophages that were particularly adept at AC clearance. These three macrophage populations do not express TLR9, the TLR that recognizes DNA. However, TLR9 overexpression in macrophages had only a modest effect during an induced lupus model *in vivo*, and was not sufficient to induce TNF production in response to ACs *in vitro*. We therefore reasoned that these cells must employ additional mechanisms to prevent responses to ACs. Many recent studies have demonstrated that tissue-resident macrophages are programmed by cues from their environment to express specific transcription factors that shape their identity and function [151], [159-161]. We hypothesized that AC-engulfing macrophages may be subject to similar programming of which inhibition of TLR9 expression is only one facet.

During development macrophage precursors in the fetal liver express a core macrophage transcriptional program. However, as macrophages colonize tissues they diversify [162]. Much of this diversity is controlled by locally derived tissue signals that induce gene expression and dictate the phenotype and function of resident macrophages [151], [159], [163]. For instance, resident peritoneal macrophages are influenced by signals from the omentum that induce expression of peritoneal macrophage signature genes (i.e., genes with expression much higher in pMacs relative to their expression in other tissue macrophages). Retinoic acid generated in the omentum induces a number of genes in pMacs, including the transcription factor GATA6, which in turn induces the expression of many other pMac signature genes [160], [164]. Heme, which is released during degradation of erythrocytes but red pulp macrophages, induces expression of the transcription factor SPI-C, which is required for development of splenic red pulp macrophages and bone marrow macrophages [165]. GM-CSF secreted by lung epithelial cells induces expression of PPARγ, which is required for differentiation of alveolar macrophages [166]. Norepinephrine, released by neurons innervating the gut, signals to β2 adrenergic receptors expressed on macrophages located in the gut muscularis layer and influences their gene expression [161].It remains unclear whether environmental programming induces heterogeneity in the ability of different resident macrophage populations to clear ACs, or influences responses to ACs.

In this chapter I present data demonstrating that AC-engulfing macrophages are programmed by their tissue environment to limit responses to low doses of nucleic acid TLR ligands and inhibit expression of TLR9. We identify two transcription factors, KLF2 and KLF4, as critical regulators of this AC-clearance program. KLF2 and KLF4 induce expression of genes that facilitate AC uptake as well as negatively regulate TLR signaling.

Results:

Tissue environments program resident macrophages for silent clearance of ACs

To investigate the possibility that AC-engulfing macrophages are programmed by their tissue environments, we compared isolated Tim-4$^+$ pMacs that had been cultured *ex vivo* overnight to macrophages that had been cultured *ex vivo* for three days. Remarkably, the macrophages cultured for three days not only gained responsiveness to CpG ODN but also began responding to ACs (Figure 4.1 A). This altered response corresponded with increased expression of TLR9 protein (Figure 4.1 D). Similar results were obtained with alveolar macrophages and Tim-4$^+$ pleural macrophages (Figure 4.1 B and C). This altered signaling was not due to changes in AC uptake, as Tim-4$^+$ pMacs cultured for three days engulfed ACs at levels equivalent to macrophages cultured overnight (Figure 4.1 G). We refer to this change in macrophage responsiveness as "deprogramming".

As discussed above retinoic acid along with other signals derived from the omentum induce a number of genes in pMacs, including the transcription factor GATA6, which in turn induces the expression of many other pMac signature genes [160], [164]. To test whether these previously described environmental cues were responsible for the AC-clearance program in Tim-4$^+$ pMacs, we supplied macrophages undergoing deprogramming with omentum culture supernatant, which has been demonstrated to induce pMac signature genes [160]. While this treatment improved macrophage survival, it did not prevent the responses to CpG ODN or ACs observed after a three-day culture (Figure 4.2 A, and data not shown). Therefore, for the remainder of these studies we cultured pMacs in omentum culture supernatant during deprogramming to maintain similarity to programmed macrophages and improve their survival *ex vivo*. Next, we directly tested whether GATA6 could induce the AC-clearance program. While deprogrammed macrophages did express reduced levels of GATA6 (Figure 4.2 B), GATA6 overexpression in resident Tim-4$^+$ pMacs by *in vivo* lentiviral transduction did not prevent responses to CpG ODN or AC stimulation (Figure 4.2 C). These results agree with previous work demonstrating that the omentum and GATA6 participate in the establishment of the peritoneal macrophage identity, but these signals are not sufficient to maintain the AC-clearance program in *ex vivo* tissue macrophages.

To more thoroughly compare programmed and deprogrammed Tim-4$^+$ pMacs we analyzed gene expression by RNA sequencing. Expression of mRNA for Tim-4 was reduced over ten fold in deprogrammed macrophages; however, protein expression had not decreased enough to affect AC engulfment (Figure 4.1 G and 4.3 D). The expression of many previously identified pMac signature genes decreased during the deprogramming period, further demonstrating that deprogrammed macrophages were losing aspects of their tissue-programmed identity even in the presence of omentum culture supernatant (Figure 4.3 A, [151]). However, expression of macrophage core genes (i.e., genes expressed by tissue-resident macrophage populations but not DCs) did not show a similar reduction (Figure 4.3 B). In addition, although the expression of some genes previously associated with classical (M1) macrophages increased during

the deprogramming period, others decreased. Similarly, expression of some alternatively activated (M2) macrophage associated genes increased while others decreased. Thus, there was no coordinated change in gene expression indicative of a shift from M2 to M1 or vice versa ([167], Figure 4.3 C).

We also used RNAseq to obtain a more complete picture of the responses generated by programmed and deprogrammed macrophages upon stimulation with ACs. Programmed Tim-4$^+$ pMacs generated a limited transcriptional response to ACs that did not include the production of pro- or anti- inflammatory cytokine mRNA. No genes were significantly upregulated more than 5 fold by programmed macrophages in response to ACs. Deprogrammed Tim-4$^+$ pMacs generated a robust response to ACs including production of mRNA for many inflammatory cytokines such as *Tnf, Il1a, Il1b*, and *Cxcl2* (Figure 4.1 E). Deprogrammed macrophages did not demonstrate enhanced basal activation, in fact basal expression of mRNA for several cytokines decreased over the deprogramming period.

To determine if the responses to ACs were dependent on nucleic-acid sensing TLRs we tested Tim-4$^+$ pMacs from UNC93B1-deficient mice. UNC93B1 is a trafficking chaperone required for endosomal TLR function [43], [168]. Deprogrammed Tim-4$^+$ pMacs from UNC93B1$^{-/-}$ mice did not generate responses to ACs, demonstrating that this response is dependent on nucleic-acid sensing TLRs (Figure 4.1 F). The absence of responses to ACs by deprogrammed UNC93B1$^{-/-}$ macrophages was not due to a defect in AC engulfment (Figure 4.1 G).

Next, we tested directly whether environmental cues can confer the AC-clearance program. To this end we examined how gene expression changes when macrophages are transplanted into a tissue. Congenically marked BMMs differentiated with M-CSF for seven days *in vitro* were injected into the peritoneum or administered intranasally. Strikingly, after three weeks in the peritoneum, about 25% of the BMMs upregulated Tim-4 (Figure 4.4 A). Similarly, after five and a half weeks in the lungs, many BMMs upregulated Siglec-F, a marker of alveolar macrophages (Figure 4.4 B). Furthermore, when harvested and stimulated with TLR ligands, both populations of transplanted BMMs failed to respond to CpG ODN (Figure 4.4 C and D). Thus, the TLR responses of transferred BMMs were more similar to those of resident macrophages in these tissues than to those of uninjected BMMs.

These results suggest that environmental cues induce a program in certain populations of tissue macrophages that facilitates silent clearance of ACs. This program induces high expression of AC recognition receptors and low expression of TLR9. Without this programming, the endosomal TLRs of AC-engulfing macrophages robustly recognize and respond to AC-derived nucleic acids.

Programmed AC-engulfing macrophages have a higher activation threshold for endosomal TLR responses

We next sought to determine why tissue macrophages forced to express TLR9 do not respond to ACs. We hypothesized that programmed macrophages fail to initiate TLR signaling responses to AC-derived nucleic acids. Alternatively, programmed

macrophages may recognize these ligands and initiate upstream signaling, but other mechanisms, such as transcriptional repressors, may prevent further downstream transcriptional responses. To distinguish between these possibilities, we added ACs to programmed and deprogrammed macrophages and examined TLR signaling responses. Interestingly, while deprogrammed macrophages generated a small but consistent phospho-ERK signal in response to ACs, programmed macrophages did not generate this response (Figure 4.5 A). This result indicates that TLR signaling in programmed macrophages is impaired at a step prior to phosphorylation of ERK.

To further investigate how programmed macrophages avoid responses to ACs we analyzed gene expression by programmed and deprogrammed Tim-4$^+$ pMacs by RNAseq. One notable change was the downregulation of several inhibitors of TLR signaling in deprogrammed macrophages (Figure 4.5 B). Some of these inhibitors, such as SOCS3, DUSP1, and SHP-1, affect TLR signaling prior to ERK phosphorylation [169].

Due to the small signaling response observed in deprogrammed macrophages fed ACs, as well as the reduced expression of several inhibitors of TLR signaling, we hypothesized that to avoid responses to host-derived nucleic acids AC-engulfing macrophages require a higher threshold of engagement to activate endosomal TLR signaling. This high threshold could prevent responses to weak ligands, such as those derived from ACs. Several aspects of AC-derived nucleic acids make them weak ligands for endosomal TLRs. For instance, mammalian DNA contains very few unmethylated CpG dinucleotides, and upon induction of apoptosis, DNA and RNA are actively degraded into smaller fragments [39], [41], [72], [170], [171]. To test the activation threshold for TLR signaling in programmed and deprogrammed macrophages, we measured responses to low doses of TLR ligands. Deprogrammed Tim-4$^+$ pMacs generated a more robust response to low levels of TLR7 ligand (Figure 4.5 C). Notably, deprogramming only minimally affected TLR7 expression (Figure 4.3 D). After deprogramming, Tim-4$^+$ pMacs forced to express TLR9 generated significantly enhanced responses to low doses of CpG (Figure 4.5 D). Although there were small differences in response to low doses of TLR4 ligand, these were less notable (figure 4.5 E). Deprogrammed Tim-4$^+$ pleural and alveolar macrophages also demonstrated an increased response to low dose TLR7 stimulation (Figure 4.5 F and 4.5 G). Altogether, these data are consistent with the model that macrophages programmed by environmental cues maintain a higher threshold for TLR7 and TLR9 activation and cannot generate responses to the less stimulatory ligands derived from ACs.

KLF2 and KLF4 imprint an AC-clearance program on macrophages

We reasoned that environmental cues would induce expression of one or more transcription factors that control the AC-clearance program by upregulating TLR inhibitory genes, downregulating TLR9, and upregulating AC recognition receptors. Expression of many transcription factors was lower in deprogrammed Tim-4$^+$ pMacs (Figure 4.6 A). These transcription factors included GATA6 and RARβ that have previously been described to influence peritoneal macrophage identity. In addition, the

transcription factors Kruppel-like factor 2 (KLF2) and KLF4 were significantly downregulated after deprogramming. These transcription factors contribute to gene regulation in a variety of biological contexts, including vascular biology, cell self-renewal, and, most relevant to our study, macrophage polarization and repression of myeloid cell activation [172-175].

To examine whether these transcription factors contribute to the AC-clearance program we first assessed their importance for inhibition of TLR9 expression in the peritoneal environment. To this end we used Cas9 genome editing to generate BMMs lacking each transcription factor (Figure 4.6 B). These BMMs were injected into the peritoneums of congenically marked mice. Despite low recovery of injected BMMs, we could demonstrate that after two weeks *in vivo* the BMMs transduced with empty vector or with guides for GATA6 or RARβ lost the ability to respond to TLR9 stimulation, similar to what we observed previously for transferred untransduced BMMs (Figure 4.6 C and D). In contrast, BMMs expressing guides specific for KLF2 continued to respond to TLR9 stimulation, and KLF4 targeted BMMs had an intermediate response (Figure 4.6 C and D). These data suggest that KLF2, potentially with contributions from KLF4, inhibits TLR9 responses in resident pMacs.

To further investigate the role of these transcription factors in macrophage programming we overexpressed KLF2, KLF4, and GATA6 in BMMs using retroviral vectors. We confirmed overexpression of these transcription factors by examining mRNA levels (Figure 4.7 C and D). KLF2- and KLF4-overexpressing macrophages showed a dramatic reduction in responses to TLR9 but not TLR4 stimulation, while GATA6 overexpression had little effect (Figure 4.7 A). KLF2 and KLF4 overexpressing BMMs expressed much less TLR9 protein than empty vector transduced BMMs, indicating that these transcription factors inhibit TLR9 expression (Figure 4.7 B). Gene expression analyses confirmed this result as BMMs overexpressing KLF2 and KLF4 expressed reduced levels of TLR9 mRNA (Figure 4.7 C). Remarkably, KLF4 and KLF2 upregulated expression of several known inhibitors of TLR signaling including *Hes1, Socs3, Sigirr, Usp4,* and *Nlrx1* (Figure 4.7 E). Expression of several of these negative regulators was reduced in deprogrammed Tim-4$^+$ pMacs (Figure 4.5 B). In addition, KLF2 or KLF4 overexpression led to upregulated mRNA expression of several receptors known to recognize ACs: *Timd4*, which is expressed by pMacs and pleural macrophages, *Marco*, which is expressed by both pMacs and alveolar macrophages, and *Olr1*, which is expressed by alveolar macrophages [107], [109] (Figure 4.7 F). Expression of *P2ry2,* an ATP receptor demonstrated to aid in recruitment of phagocytes to ACs, was also induced by KLF2 and KLF4 expression [75]. KLF2 and KLF4 also induced expression of other peritoneal macrophage signature genes (*Fn1*, *Rarb*, and *KLF9*) as well as genes upregulated in both pMacs and alveolar macrophages (*Nt5e* and *Plaur*) (Figure 4.7 E and G, [151]). In contrast, GATA6 overexpression induced expression of known GATA6 dependent genes *Ltbp1* and *Tgfb2*, but not AC recognition receptors or negative regulators of TLR signaling (Figure Figure 4.7 G) [160].

These data demonstrate that the transcription factors KLF2 and KLF4 control a program in macrophages that could aid in silent clearance of ACs. These transcription factors induce a series of coordinated changes, including the downregulation of TLR9

and the upregulation of inhibitors of TLR signaling and receptors that recognize ACs. Notably, while alveolar macrophages express low levels of KLF2, their expression of KLF4 is quite high (data from the Immunological Genome Project), suggesting that KLF4 may play a more prominent role in programming alveolar macrophages while KLF2 plays a similar role in pMacs.

Discussion:

Here we demonstrated that AC-engulfing macrophages are programmed by signals from their environment to silently engulf ACs. We show that these signals inhibit expression of TLR9 and induce a higher threshold of activation for endosomal TLRs. We identify KLF2 and KLF4 as transcription factors critical for induction of this AC-clearance program in macrophages. We demonstrate that KLF2 and KLF4 induce expression of AC recognition receptors as well as negative regulators of TLR signaling. In this way these transcription factors establish an AC-clearance program that links enhanced AC-engulfment ability with reduced TLR signaling. By linking these two features, KLF2 and KLF4 may ensure that macrophages with enhanced abilities to engulf ACs do not generate inappropriate responses to self nucleic acids. A previous study demonstrated that mice lacking KLF2 in $LysM^+$ cells display increased levels of proinflammatory cytokines at steady state in their sera and show enhanced inflammation during infection [174]. Perhaps dysregulation of macrophage AC engulfment ability and endosomal TLR responses contributes to this inflammatory phenotype.

KLF2 and KLF4 expression is not restricted to AC-engulfing macrophages (data from the Immunological Genome Project). Therefore, it is likely that other transcriptional regulators induced by the tissue microenvironment are involved in induction of the AC-clearance program. Although some transcription factors involved in environmental programming are restricted to a single macrophage population (e.g., GATA6 in peritoneal macrophages) others have more complex expression profiles. The transcription factor PPARγ is required for the development of alveolar macrophages [166]. Without PPARγ pre-alveolar macrophages fail to express many alveolar macrophage signature genes. However, PPARγ was also demonstrated to be important for the development of splenic red pulp macrophages and these macrophages do not express PPARγ-dependent alveolar macrophage signature genes [176]. Clearly, transcriptional regulation in tissue-resident macrophages is complex and gene induction by tissue-induced transcription factors is influenced by interactions with other transcriptional regulators as well as by differences in chromatin structure established by pioneer transcription factors in different populations. The other transcriptional regulators involved in imprinting the AC-clearance program remain to be identified.

It remains to be determined how tissue environments impart an AC-clearance program. A recent study demonstrating that KLF4 expression is upregulated after macrophage precursors colonize the lung and differentiate into alveolar macrophages, suggests that KLF4 expression is induced by the lung microenvironment [162]. We were unable to demonstrate a role for the recognition of ACs as the initiating signal (data not shown). However, it may be difficult to recapitulate *in vitro* the continual engulfment of ACs that occurs *in vivo*. It remains possible that continual recognition of ACs could program AC-engulfing macrophages. It is interesting to note that KLF2 and KLF4 expression is induced in endothelial cells following exposure to extracellular ATP released in response to shear stress [177]. ATP is also a known find-me signal released by ACs that induces phagocyte recruitment [75]. Perhaps continual exposure of AC-

engulfing macrophages to ATP can induce expression of KLF2 and KLF4. We demonstrated that KLF2 and KLF4 expression upregulates P2ry2, the receptor that recognizes this find-me signal, suggesting there could be feed forward regulation of ATP detection and expression of KLF2 and KLF4. Alternatively, signals resulting from macrophage motility could provide the signal. Movement of macrophages is mediated by interactions between adhesion molecules (such as integrins), other cells, and the extracellular matrix [178]. Integrins are also signaling receptors, and it is possible that integrin signaling during macrophage movement can induce the AC-clearance program.

Programmed AC-engulfing macrophages have a higher activation threshold for endosomal TLR signaling. This feature likely prevents responses to less stimulatory self ligands, while allowing responses to microbial nucleic acids. Although we saw minor differences in programmed and deprogrammed macrophage responses to low doses of surface TLR ligands, the difference in responses to low doses of endosomal TLR ligands was more significant. Perhaps endosomal localization of negative regulators of TLR signaling could explain this result. It is also possible that trafficking of endosomal cargo is different in programmed versus deprogrammed macrophages. LC3-associated phagocytosis (LAP) has previously been demonstrated to facilitate phagosome maturation and degradation of internalized pathogens and ACs [122], [123]. Mice with deficiencies in LAP components demonstrate defective clearance of ACs and develop an autoinflammatory disorder [124]. Differences in the ability of deprogrammed macrophages to induce LAP may enable responses to AC-derived cargo.

Overexpression of KLF4 or KLF2 upregulated several negative regulators of TLR signaling in BMMs. KLF2 and KLF4 can function as transcriptional repressors or activators [179]. Whether these transcription factors directly mediate repression of TLR9 or upregulation of TLR signaling regulators remains unclear. It is interesting to note that KLF2 has previously been shown to upregulate expression of integrin β7 [180]. By RNAseq we observed over twenty-fold downregulation of integrin β7 in deprogrammed macrophages. Previous studies have demonstrated that integrin signaling can inhibit TLR responses [181]. For instance, integrin β2 signaling has been demonstrated to induce expression of several negative regulators of TLR signaling including SOCS3 and Hes1, which we demonstrated were downregulated in deprogrammed macrophages and upregulated in BMMs overexpressing KLF4 [182]. Perhaps KLF2 or KLF4 induce integrin expression, and signaling from these integrins induces expression of negative regulators of TLR signaling, leading to a higher activation threshold for endosomal TLRs.

Our work suggests coordinated expression of receptors that promote AC clearance with genes that limit innate responses to the nucleic acids within ACs may represent a central feature of tissue macrophages that ensures maintenance of homeostasis in the face of constant AC clearance.

Materials and Methods:

Mice

Mice were housed under specific pathogen-free conditions at the University of California, Berkeley. All mouse experiments were performed in accordance with the guidelines of the Animal Care and Use Committee at UC Berkeley. Unless noted mice were analyzed at 6-12 weeks of age. C57BL/6, C57BL/6 CD45.1^{+} (stock #002014), rosa26 $^{stop\text{-}flox\text{-}tdTomato}$ (stock #007909), LysM-cre (stock #004781), and EIIa-cre (stock #003724) mice were obtained from Jackson laboratories. Unc93B1$^{-/-}$ mice were generated by the trans-NIH Knock-Out Mouse Project (KOMP) and obtained from the KOMP Repository (www.komp.org). Rosa$^{stop\text{-}flox\text{-}TLR9}$ mice were obtained from the Shlomchik lab at the University of Pittsburgh. A previous graduate student in the lab generated the TLR9 reporter mice. TLR9KI mice were generated using a construct encoded an HA tag on the 3' end of the TLR9 gene followed by an IRES-EGFP sequence.

Tissue harvest

Cells from the peritoneal and pleural cavities were recovered by lavage with ice cold PBS. Perfused lungs were digested with collagenase XI with DNAse I for 45min, single cell suspensions were generated by mechanical disruption through a 100um filter, cells were resuspended in 44% isotonic percoll (GE healthcare #17-0891-01), underlayed with 67% percoll, and spun at 1550xg without brake. Cells from the interface were collected for analysis.

Isolation of Tim-4^{+} peritoneal Macrophages

Peritoneal cells were recovered by lavage with 5ml of ice cold PBS. For RNAseq and western blot experiments B cells were first depleted using anti-CD19-biotin antibody and biotin binder dynabeads (ThermoFisher #11047). Tim-4^{+} cells were isolated using anti-TIM-4 antibody (clone RMT4-54, BioLegend) and anti-rat IgG microbeads (Miltenyi #130-048-501).

Cell culture

HEK293 cells were cultured in DMEM supplemented with 10% (v/v) fetal calf serum, L-glutamine, penicillin-streptomycin, sodium pyruvate, and HEPES pH 7.2 (all supplements and media were purchased from Gibco). BMMs were differentiated for seven days in RPMI complete media (RPMI-1640 supplemented with 10% (vol/vol) fetal calf serum, L-glutamine, penicillin-streptomycin, sodium pyruvate, and HEPES pH 7.2) supplemented with M-CSF containing supernatant from 3T3-CSF cells.

Unless noted peritoneal macrophages *ex vivo* for 60 hours, as well as overnight controls, were cultured in 25% (v/v) omentum supernatant in RPMI complete media. Omentum supernatant was generated by harvesting omenta from C57BL/6 mice and culturing the omenta in RPMI complete media at 1ml/omenta overnight. Supernatant was collected, filtered through a 0.22um filter, and frozen at -80°C for future use. Lung

and pleural cells were harvested as described above and plated on non-tissue culture treated plates in RPMI complete media.

Apoptotic cell generation and engulfment

Thymi were harvest from WT or CD45.1$^+$ rosa-tdTomato mice and single cell suspensions were generated by mechanical disruption through a 70um filter. Cells were irradiated with 600rad and incubated in RPMI complete media at 37°C for 4 hours. Apoptotic cells were then incubated with macrophages at the indicated ratios. For experiments examining AC engulfment capabilities macrophages were allowed to engulf ACs for 60min.

Stimulations

Cells were plated in RPMI complete media. Tissue macrophages were stimulated directly *ex vivo*, after overnight culture, or after 60 hours in culture. Cells were stimulated with TLR ligands (LPS, CpG-B ODN 1668, R848 all from Invivogen), or ACs. For analysis of secreted cytokines, supernatant was collected 7 hours after stimulation. For intracellular cytokine staining, 30 minutes after stimulation brefeldin A (GolgiPlug, BD Biosciences) was added to cells before incubation for another 4 hours.

Flow cytometry and Antibodies

Dead cells were excluded using a fixable live/dead stain (Life technologies) or DAPI (Life Technologies) and all stains were carried out in PBS containing 2% FBS (v/v) and 2mM EDTA including anti-CD16/32 Fc blocking antibody (2.4G2, UCSF monoclonal antibody core) and normal mouse serum (Sigma). Cells were stained for 20min at 4°C with antibodies (see table 3.1).

For intracellular TNF or GATA6 staining cells were permeabilized with Fix/Perm buffer (BD or eBioscience respectively) for 20min at 4°C. Cells were then stained with antibodies. For pERK staining cells were immediately fixed after stimulation with 1.6% PFA at room temperature, then washed and resuspended in ice-cold methanol. After overnight incubation at -20°C, cells were stained with p-ERK1/2 biotin followed by Steptavidin-BV421 (BioLegend). All cells were analyzed on an LSRII or LSR Fortessa (BD Biosciences), and data was analyzed with FlowJo (TreeStar).

BMM transfers

For peritoneal transfer experiments 1e6 differentiated CD45.1$^+$ BMMs were injected into the peritoneum of CD45.2$^+$ mice. Peritoneal cells were harvested 2 or 3 weeks after cell transfer and stimulated with TLR ligands as described above. For lung transfer experiments differentiated 2e6 CD45.1$^+$ Tomato$^+$ BMMs were intranasally administered on three consecutive days. Lungs were harvested, as described above, 5.5 weeks after cell transfer.

Transcription factor overexpression

For retroviral transduction of BMMs a mouse stem cell virus (MSCV)-based retroviral vector was used that expressed the gene of interested followed by IRES- puromycin

resistance gene-T2A-EGFP. VSV-G-pseudotyped retrovirus was made in GP2-293 packaging cells. GP2-293 cells were transfected with retroviral vectors and pVSV-G using Lipofectamine LTX reagent (ThermoFisher #15338100). 24 hr post-transfection, cells were incubated at 32°C. 48 hr post-transfection viral supernatant was used to infect bone marrow cells in RPMI complete media containing M-CSF. Bone marrow cells and viral supernatant were spun at 1250xg for 90 min at 32°C then incubated overnight at 32°C. Bone marrow cells were re-transduced with fresh viral supernatant the following day. Two days after last transduction puromycin selection was added for three days.

For lentiviral overexpression a vector with a pHAGE backbone was used that expressed GATA6 followed by IRES-mCherry. Lentiviral particles were produced in 293T cells by transfecting with the lentiviral plasmid and the helper constructs psPAX2 and pCMV-VSV-G. Lentivirus was concentrated by filtering cell supernatant with 0.45µm filter then centrifuging at 47,850xg. Viral pellets were resupended in PBS. 0.5e6 transduction units were injected per mouse. One week after injection peritoneal cells were harvested.

Cas9 genome editing of BMMs

The lentiviral gRNA plasmid pKLV-U6gRNA(BbsI)-PGKpuro2ABFP (Addgene #50946) was used. The sequences of the guide RNA target sites are as follows with the protospacer adjacent motif underlined: GATA6 - ACCGCCTCGGCGTCGAGCTG<u>CGG</u>, KLF2 guide 1 – TTCGCCAGCCCGTGCGAGCG<u>CGG</u>, KLF2 guide 2 - CTGGCCGCGAAATGAACCCG<u>AGG</u>, KLF4 – CTCCACGTTCGCGTCCGGCC<u>CGG</u>, RARβ - TATGGCGTCAGTGCCTGCGA<u>GGG</u>. BMMs from rosa-Cas9 mice (Jackson Laboratory) were transduced with lentivirus and selected with puromycin.

Western Blot Analysis

Cells were lysed in RIPA buffer (50mM Tris pH 7.4, 150mM NaCl, 1mM EDTA, 0.5mM EGTA, 1% NP-40, 1% DOC, 0.1% SDS) containing protease inhibitors (Roche #05 892 791 001). Cell lysates were immunoprecipitated with anti-HA matrix (Roche #11573000). After washing in RIPA buffer, matrix beads were boiled in SDS-PAGE loading buffer. Protein was run on a 4-15% gel (Biorad #4561083) and transferred to Immobilon-FL membrane (Millipore #IPFL00010). After blocking, membranes were probed with anti-HA antibody, followed by anti-rat-680 secondary (Life Technologies #A21096) or probed with anti-tubulin, followed by anti-mouse-800 secondary (Li-Cor #926-32210). Images were scanned using a Licor Odyssey.

CBA assay

Cytokines were measured using a Cytometric Bead Array assay according to the manufacturer’s instructions (BD Biosciences).

RNA sequencing

Total RNA was prepared from directly *ex vivo* or deprogrammed (60 hour cultured) Tim-4^{+} pMacs using RNAzol (Molecular Research Center #RN 190), DNAse treated (Turbo

DNase from Ambion #AM2238) and purified using RNA clean and concentrator columns (Zymo #R1015). 1 ug of total RNA was subsequently used for rRNA depletion using the RiboZero depletion kit to prepare RNA-Seq library by using the TruSeq RNA sample prep kit (Illumina) according to manufacturer's instructions. 50-cycle single-end RNA sequencing was performed on a HiSeq 4000 (Illumina). Sequenced reads were quality-trimmed (cut-off of 30) with cutadapt and aligned to the mouse transcriptome (GRCm38, release 79) with Kallisto [183]. Using R and Bioconductor packages (see list in table), counts for each transcript were extracted using Sleuth, collapsed at the gene level, and analyzed for differential expression using DESeq2. Q-values (Benjamini-Hochberg correction) lower than 0.05 were considered significant. Heat maps were generated using MeV (Multiple Experiment Viewer) software.

For the AC stimulation experiment AC derived RNA was analyzed as well as RNA from macrophages ± ACs. Peritoneal macrophages were stimulated with ACs immediately *ex vivo* (programmed) or after 60 hours in culture (deprogrammed). Two hours after addition of ACs RNA was prepared as above. Genes that were less than two fold upregulated in macrophage + AC compared to AC alone were considered not elevated by AC stimulation.

Quantitative PCR

Cells were lysed in RNAzol (MRC) and RNA was purified according to manufacturer's instructions and concentrated using a column (Zymo #R1015). cDNA was prepared with iScript cDNA synthesis kit (Biorad), and quantitative PCR was performed with SYBR Green (Biorad) on a StepOnePlus thermocycler (Applied Biosystems). Primer sequences for GATA6 and KLF4 were obtained from PrimerBank and synthesized by IDT.

For analysis of cas9 genome editing genomic DNA from BMMs was prepared using a genomic DNA isolation kit (Biorad #732-6340). Primers were designed to bind the cut site and normalized using control primers that bind an unrelated site (see table).

mRNA transcript count using Ncounter

The expression levels of a set of genes associated with tissue macrophages, AC clearance, or TLR signaling were analyzed using the NanoString nCounter Analysis System (NanoString Technologies). Raw counts of samples were normalized according to the manufacturer's recommendations using reference genes as internal controls (Cltc, Gapdh, Gusb, Hprt, Pg1, and Tubb5). Normalization was performed using nSolver Analysis Software v3.0 (NanoString Technologies)

Statistical Analysis

Statistical analysis was performed with the Prism software (GraphPad software). P-values were determined using unpaired two-tailed Students's *t*-test. Where noted *t*-tests were performed on log transformed data to account for the non-normal distribution of the data. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Table 4.1: qPCR primers

Primer	Sequence
KLF4 F	GTG CCC CGA CTA ACC GTT G
KLF4 R	GTC GTT GAA CTC CTC GGT CT
GATA6 F	GCC GGA GGA AAT GTA CCA GAC
GATA6 R	CCC CTT GAA GGT AGG GCA G
Gata6 guide test F	AGTGGATGGCCTTGACTGAC
Gata6 guide test R	GTCGAGCTGCGGCGTCCT
KLF2 guide 1 test F	GTGCGAGCGCGGCCTCCAG
KLF2 guide 1 test R	TCTAACAACTAGGCCCTCAA
KLF2 guide 2 TEST F	GAAATGAACCCGAGGCGG
KLF2 guide 2 TEST R	GCTCGGCCTTCACTAGCC
KLF4 guide 1 test F	CGGCCCGGCGGGAAGGG
KLF4 guide 1 test R	ACACGCACTTAAGGCCAACT
Rarb guide 2 test F	CAGTGCCTGCGAGGGGT
Rarb guide 2 test R	GTTTGCCCTTGGTCACTCAT
Cas9 editing ctl F	TGCAATCGCAAGAGGAAAG
Cas9 editing ctl R	AGGCTATTGATTTTCCCGGT

Figures:

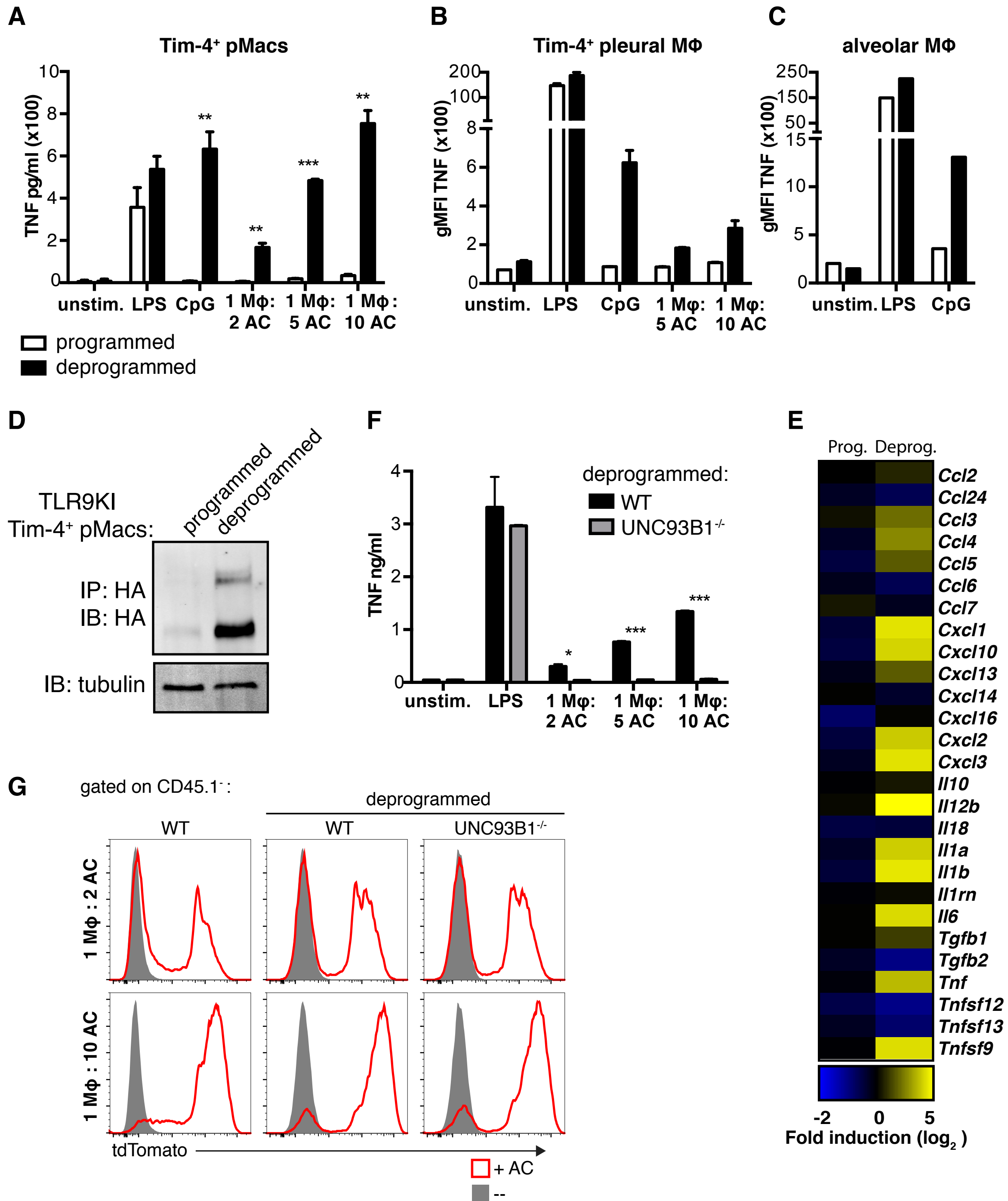


Figure 4.1 AC-engulfing macrophages are programmed for silent AC-clearance by their tissue environment. (A) Tim-4$^+$ pMacs removed from their tissue environment gain responsiveness to CpG and ACs. Isolated Tim-4$^+$ pMacs were cultured overnight (programmed) or for 60 hours (deprogrammed) before stimulation with TLR ligands and ACs. TNF was measured by CBA. Data are representative of at least three independent experiments. **(B)** Tim-4$^+$ pleural macrophages removed from their tissue

environment gain responsiveness to CpG and ACs. Pleural cells from WT mice were harvested and incubated overnight or for 60 hours before stimulation with TLR ligands and ACs. TNF production was measured by ICS. Data are representative of at least three independent experiments. **(C)** Alveolar macrophages removed from their tissue environment gain responsiveness to CpG. Lung cells from WT mice were harvested and incubated overnight or for 60 hours before stimulation with TLR ligands. TNF production was measured by ICS. Data are representative of at least three independent experiments. **(D)** Tim-4$^+$ pMacs removed from their tissue environment gain expression of TLR9. Lysates from programmed and deprogrammed Tim-4$^+$ pMacs were immunoprecipitated with anti-HA resin and eluted protein was analyzed by anti-HA western. An anti-tubulin immunoblot was performed on each lysate as a reference. Data are representative of three independent experiments **(E)** Deprogrammed macrophages upregulate inflammatory cytokine mRNA expression in response to ACs. Programmed and deprogrammed Tim-4$^+$ pMacs were stimulated with ACs. RNA was isolated from unstimulated and AC stimulated cells and analyzed by RNA sequencing. Data are presented as fold stimulated over unstimulated and are results from one experiment with technical duplicates averaged so only one value is shown. **(F)** Deprogrammed macrophage responses to ACs are dependent on endosomal TLRs. Deprogrammed Tim-4$^+$ pMacs from WT or UNC93B1$^{-/-}$ mice were stimulated with TLR ligands and ACs. TNF was measured by CBA. Data are representative of at least three independent experiments. **G)** Deprogrammed macrophages are adept at AC engulfment. Isolated Tim-4$^+$ pMacs were cultured overnight or for 60 hours then incubated with CD45.1$^+$tdTomato$^+$ ACs at indicated ratios and analyzed by flow cytometry.

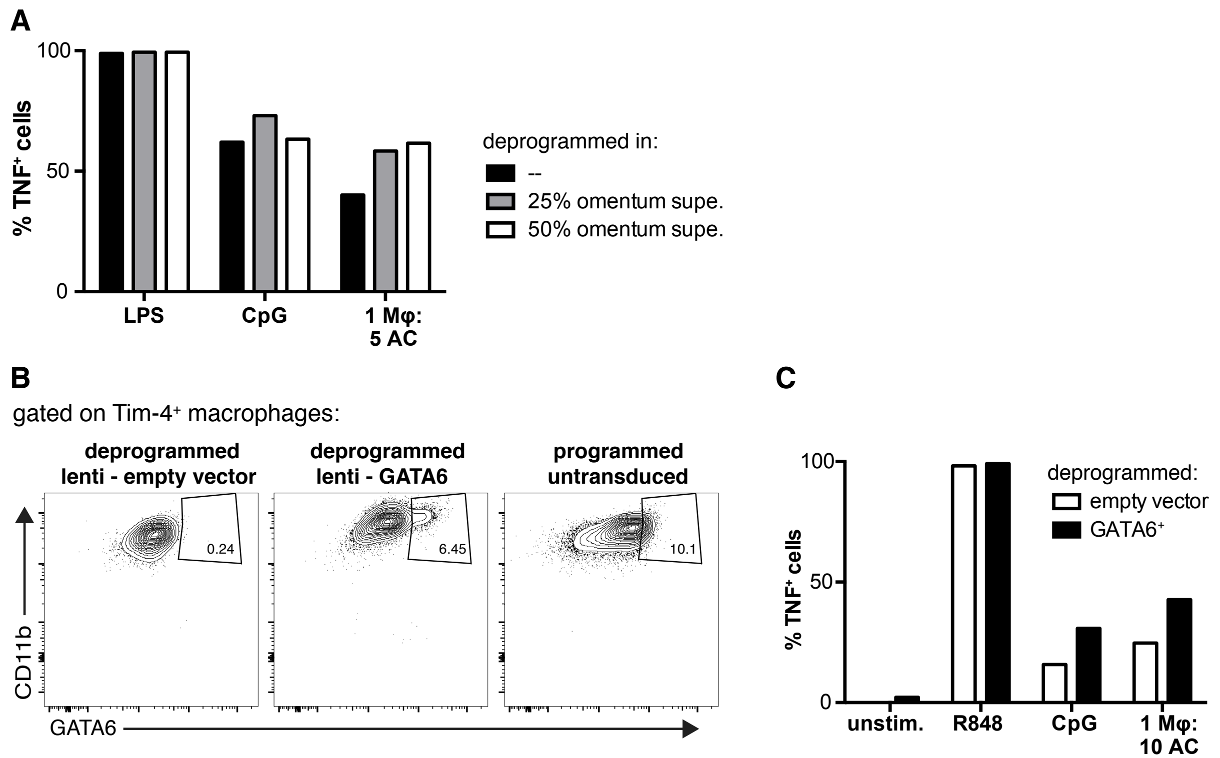
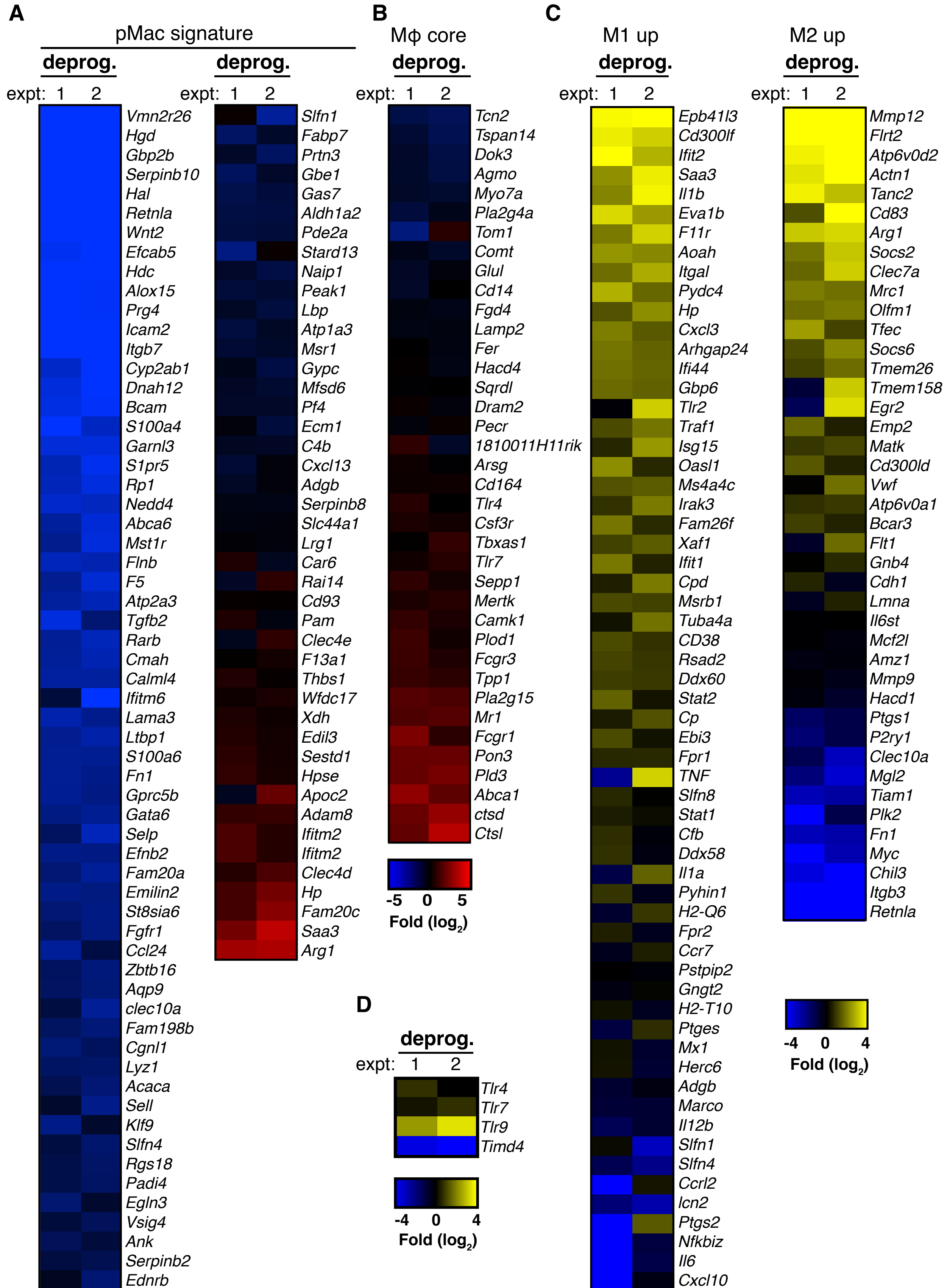


Figure 4.2 Previously described signals do not prevent deprogramming of Tim-4+ pMacs. (A) Omentum-derived factors do not prevent deprogramming of pMacs. Peritoneal cells were cultured in complete media alone or complete media plus 25% or 50% omentum supernatant for 60 hours before stimulation with TLR ligands or ACs. TNF production was measured by ICS. Data are representative of at least three independent experiments. **(B and C)** GATA6 overexpression does not prevent deprogramming of pMacs. Mice were injected intraperitoneally with lentivirus expressing GATA6 or empty vector. One week after injection peritoneal cells were harvested and cultured for 60hrs before stimulation with TLR ligands or ACs. (B) GATA6 expression was compared to directly *ex vivo* pMacs. (C) TNF production was measured by ICS. Data are representative of two experiments.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pMac signature
deprog.
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expt: 1 2
expt: 1 2
Vmn2r26
Hgd
Gbp2b
Serpinb10
Hal
Retnla
Wnt2
Efcab5
Hdc
Alox15
Prg4
Icam2
Itgb7
Cyp2ab1
Dnah12
Bcam
S100a4
Garnl3
S1pr5
Rp1
Nedd4
Abca6
Mst1r
Flnb
F5
Atp2a3
Tgfb2
Rarb
Cmah
Calml4
Ifitm6
Lama3
Ltbp1
S100a6
Fn1
Gprc5b
Gata6
Selp
Efnb2
Fam20a
Emilin2
St8sia6
Fgfr1
Ccl24
Zbtb16
Aqp9
clec10a
Fam198b
Cgnl1
Lyz1
Acaca
Sell
Klf9
Slfn4
Rgs18
Padi4
Egln3
Vsig4
Ank
Serpinb2
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Fabp7
Prtn3
Gbe1
Gas7
Aldh1a2
Pde2a
Stard13
Naip1
Peak1
Lbp
Atp1a3
Msr1
Gypc
Mfsd6
Pf4
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C4b
Cxcl13
Adgb
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Car6
Rai14
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Pam
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F13a1
Thbs1
Wfdc17
Xdh
Edil3
Sestd1
Hpse
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Tspan14
Dok3
Agmo
Myo7a
Pla2g4a
Tom1
Comt
Glul
Cd14
Fgd4
Lamp2
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Hacd4
Sqrdl
Dram2
Pecr
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Arsg
Cd164
Tlr4
Csf3r
Tbxas1
Tlr7
Sepp1
Mertk
Camk1
Plod1
Fcgr3
Tpp1
Pla2g15
Mr1
Fcgr1
Pon3
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Epb41l3
Cd300lf
Ifit2
Saa3
Il1b
Eva1b
F11r
Aoah
Itgal
Pydc4
Hp
Cxcl3
Arhgap24
Ifi44
Gbp6
Tlr2
Traf1
Isg15
Oasl1
Ms4a4c
Irak3
Fam26f
Xaf1
Ifit1
Cpd
Msrb1
Tuba4a
CD38
Rsad2
Ddx60
Stat2
Cp
Ebi3
Fpr1
TNF
Slfn8
Stat1
Cfb
Ddx58
Il1a
Pyhin1
H2-Q6
Fpr2
Ccr7
Pstpip2
Gngt2
H2-T10
Ptges
Mx1
Herc6
Adgb
Marco
Il12b
Slfn1
Slfn4
Ccrl2
Icn2
Ptgs2
Nfkbiz
Il6
Cxcl10
M2 up
deprog.
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Mmp12
Flrt2
Atp6v0d2
Actn1
Tanc2
Cd83
Arg1
Socs2
Clec7a
Mrc1
Olfm1
Tfec
Socs6
Tmem26
Tmem158
Egr2
Emp2
Matk
Cd300ld
Vwf
Atp6v0a1
Bcar3
Flt1
Gnb4
Cdh1
Lmna
Il6st
Mcf2l
Amz1
Mmp9
Hacd1
Ptgs1
P2ry1
Clec10a
Mgl2
Tiam1
Plk2
Fn1
Myc
Chil3
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Tlr7
Tlr9
Timd4
-4 0 4
Fold (log2)

Figure 4.3 Gene expression changes in deprogrammed Tim-4+ pMacs. RNA sequencing results from two independent experiments are represented as heat maps of expression of the indicated genes in deprogrammed relative to programmed Tim-4$^+$ pMacs. Results from replicates are averaged so only one value per experiment is shown **(A)** Deprogrammed Tim-4$^+$ pMacs downregulate many peritoneal macrophage signature genes **(B)** Deprogrammed Tim-4$^+$ pMacs do not downregulate macrophage core genes. **(C)** Deprogrammed Tim-4$^+$ pMacs expression of classical (M1) and alternative (M2) macrophage associated genes. **(D)** Deprogrammed Tim-4$^+$ pMacs upregulate *Tlr9* and downregulate *Timd4*.

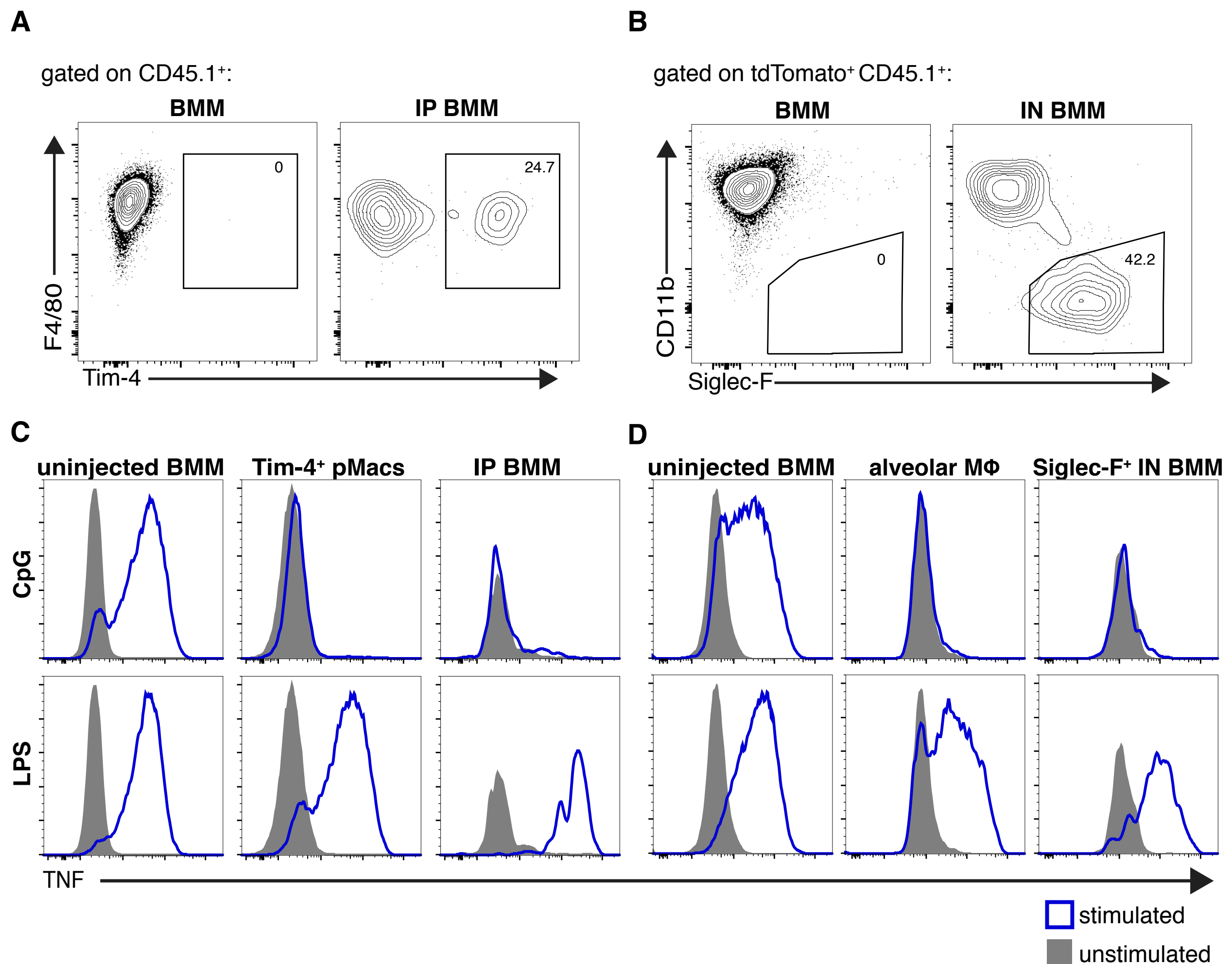


Figure 4.4 Tissue environments impart an AC-clearance program on BMMs. (A) In the peritoneal environment BMMs gain Tim-4 expression. Representative flow cytometric analysis of CD45.1$^+$ BMMs injected into the peritoneum. After three weeks injected cells were compared to uninjected BMMs. Cells were gated on CD45.1$^+$. Data are representative of at least three independent experiments. **(B)** In the lung environment BMMs gain Siglec-F expression. Representative flow cytometric analysis of intranasally administered CD45.1$^+$tdTomato$^+$ BMMs. After five and a half weeks administered cells were compared to uninjected BMMs. Cells were gated on CD45.1$^+$tdTomato$^+$. Data are representative of two independent experiments. **(C)** In the peritoneal environment BMMs lose responsiveness to TLR9 ligands. CD45.1$^+$ WT BMMs were injected into the peritoneum. After three weeks peritoneal cells were harvested and stimulated with TLR ligands. TNF production was measured by ICS. Data are representative of at least three independent experiments. **(D)** In the lung environment BMMs lose responsiveness to TLR9 ligands. CD45.1$^+$ tdTomato$^+$ cells were intranasally (IN) administered. After five and a half weeks lung cells were harvested and stimulated with TLR ligands. Shown are SiglecF$^+$ IN administered BMMs. TNF production was measured by ICS. Data are representative of two independent experiments.

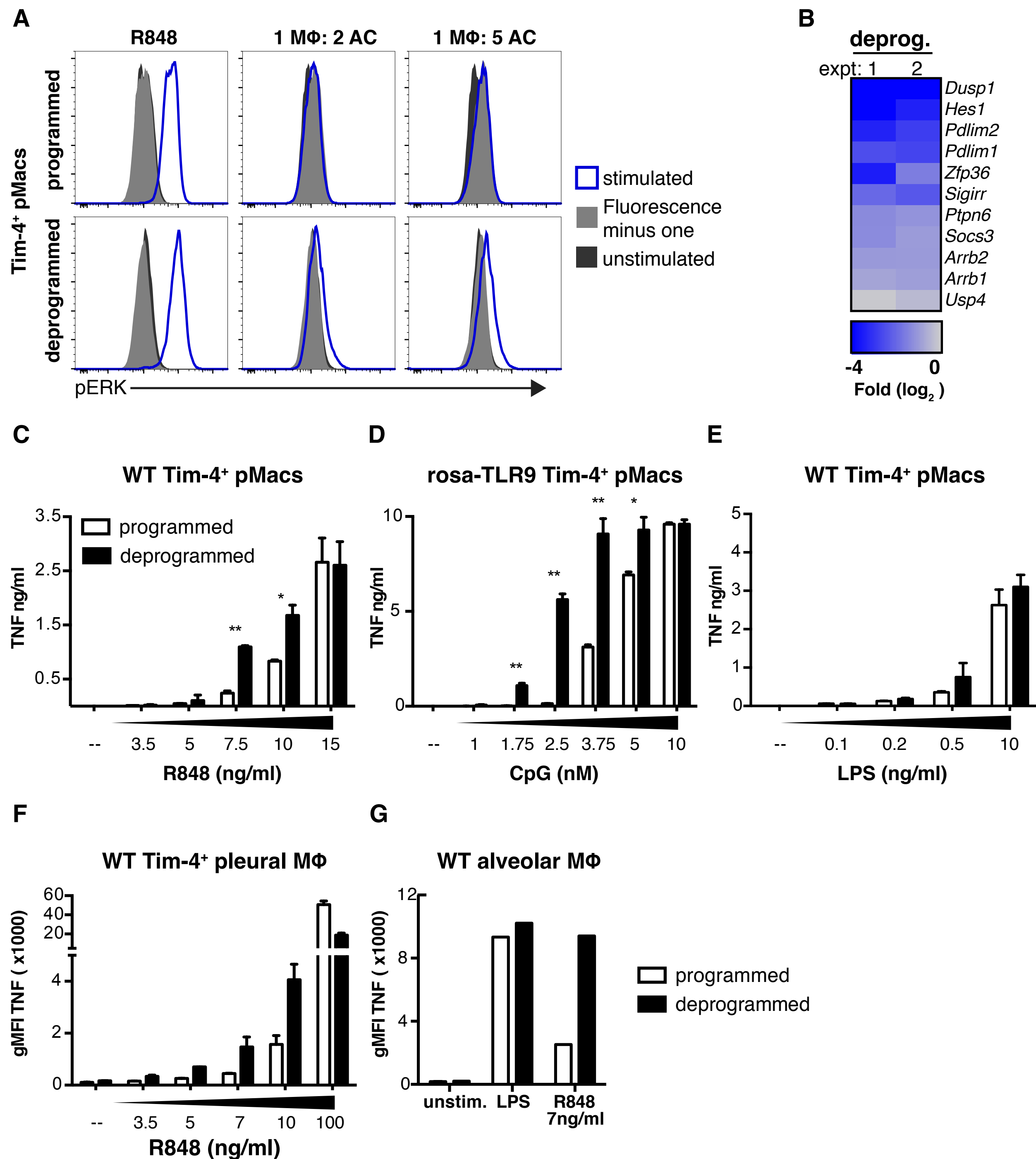


Figure 4.5 Programmed macrophages have a higher activation threshold for TLR7 and TLR9 responses. (A) Deprogrammed macrophages signal in response to ACs while programmed macrophages do not. Programmed and deprogrammed Tim-4+ pMacs were stimulated with R848 (1μg/ml) or ACs for 30min, and phospho-ERK1/2 signal was measured by flow cytometry. Data are representative of three independent experiments. **(B)** Deprogrammed Tim-4+ pMacs downregulate inhibitors of TLR signaling. RNA was isolated from programmed and deprogrammed WT Tim-4+ pMacs and analyzed by RNA sequencing. The heat map indicates genes previously shown to be negative regulators of TLR signaling that are significantly ($p < 0.05$) downregulated in deprogrammed Tim-4+ pMacs relative to programmed Tim-4+ pMacs. Results of two independent experiments are shown.

Replicates have been averaged so only one value per experiment is shown. **(C)** Deprogrammed Tim-4$^+$ pMacs respond to lower doses of TLR7 ligand. Programmed and deprogrammed WT Tim-4$^+$ pMacs were stimulated with increasing doses of R848. TNF was measured by CBA. Data are representative of at least three independent experiments. **(D)** Deprogrammed Tim-4$^+$ pMacs from rosa-TLR9 mice respond to lower doses of TLR9 ligand. Programmed and deprogrammed Tim-4$^+$ pMacs from rosa-TLR9 mice were stimulated with increasing doses of CpG ODN. TNF was measured by CBA. Data are representative of at least three independent experiments. **(E)** Programmed and deprogrammed WT Tim-4$^+$ pMacs were stimulated with increasing doses of LPS. TNF was measured by CBA. Data are representative of at least three independent experiments. **(F and G)** Deprogrammed Tim-4$^+$ pleural and alveolar macrophages more readily respond to low dose TLR7 ligand. Pleural (F) or lung (G) cells from WT mice were harvested and incubated overnight or for 60 hours before stimulation. TNF production was measured by ICS. Data are representative of two independent experiments.

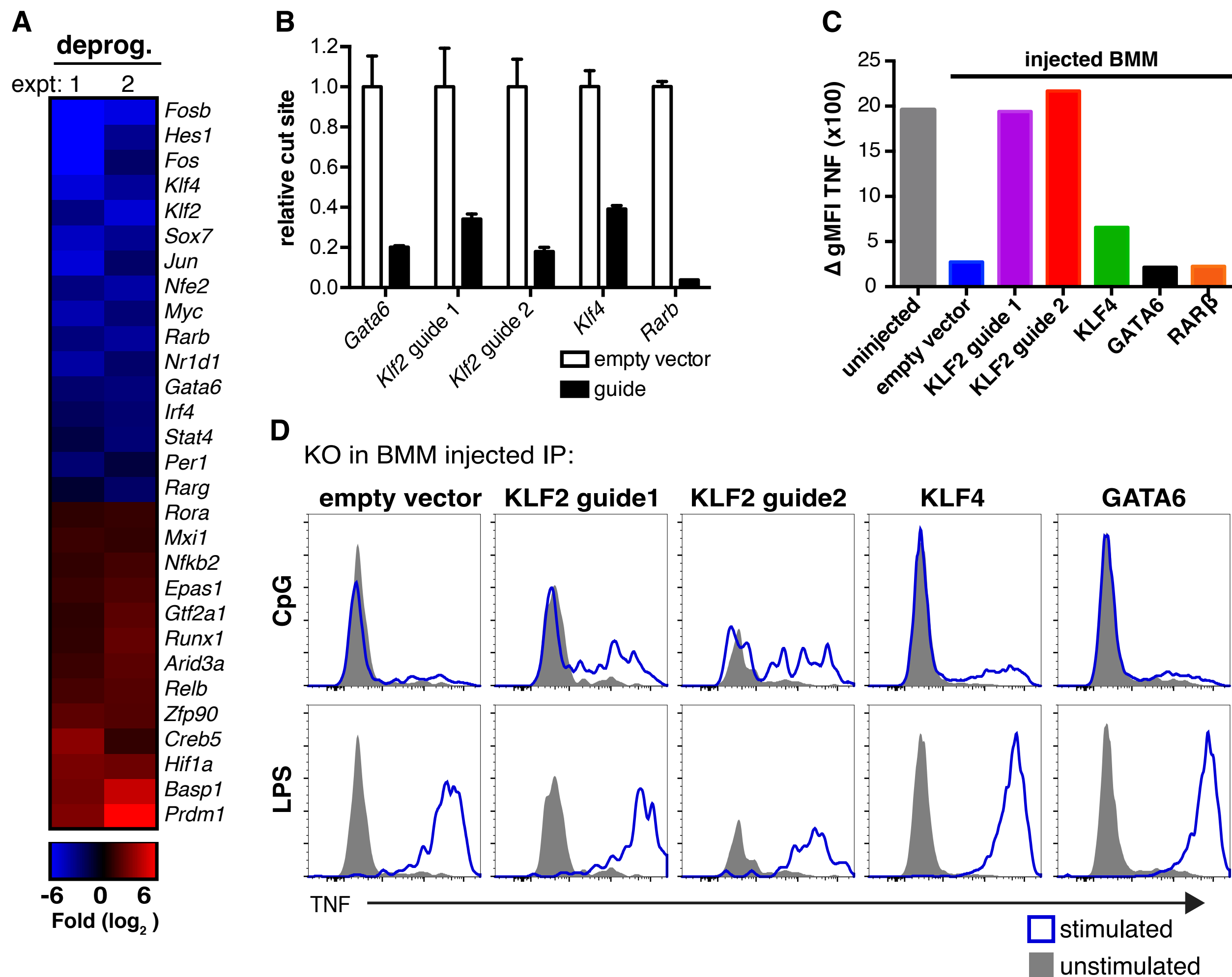
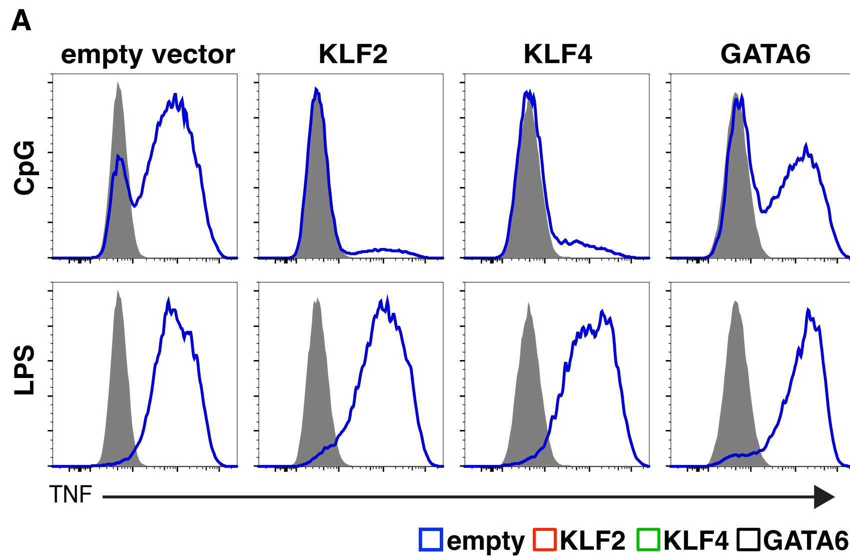
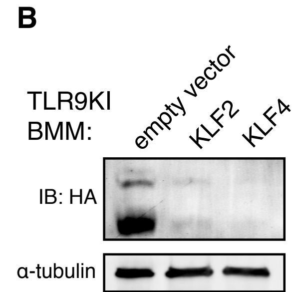
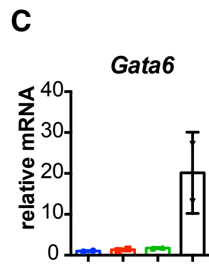


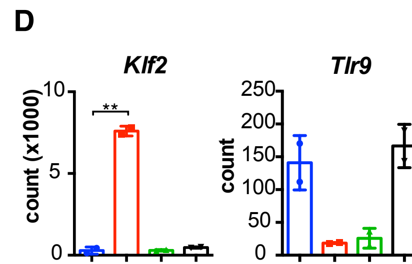
Figure 4.6 BMMs lacking KLF2 retain TLR9 responses in the peritoneal environment. (A) Changes in transcription factor expression associated with deprogramming. RNA was isolated from programmed and deprogrammed WT Tim-4$^+$ pMacs and analyzed by RNA sequencing. The heat map indicates transcription factors that are upregulated or downregulated at least 2-fold ($p < 0.05$) in deprogrammed Tim-4$^+$ pMacs relative to programmed Tim-4$^+$ pMacs. Results of two independent experiments are shown. Replicates have been averaged so only one value per experiment is shown. **(B)** qPCR was performed using primers that bind cas9 cut site to determine efficiency of knock out in BMMs from cas9 overexpressing mice that were transduced with vectors encoding guide RNAs targeting the indicated transcription factors. Genomic DNA from BMMs was isolated and qPCR was performed. Data was normalized to an unrelated site in genomic DNA **(C and D)** In the peritoneal environment BMMs lacking KLF2 maintain responses to TLR9 stimulation. BMMs from cas9 overexpressing mice were transduced with vectors encoding guide RNAs targeting the indicated transcription factors or with empty vector. After differentiation *in vitro*, BMMs were injected intraperitoneally into congenically marked mice. 14 days after injection peritoneal cells were harvested and stimulated with TLR ligands. Uninjected BMMs transduced with empty vector were stimulated as a control. (D) TNF production was measured by ICS. Data are representative of at least two independent experiments. (C) Quantification of IP injected BMMs responses to CpG (ΔgMFI TNF = gMFI TNF in response to CpG – gMFI TNF unstimulated).

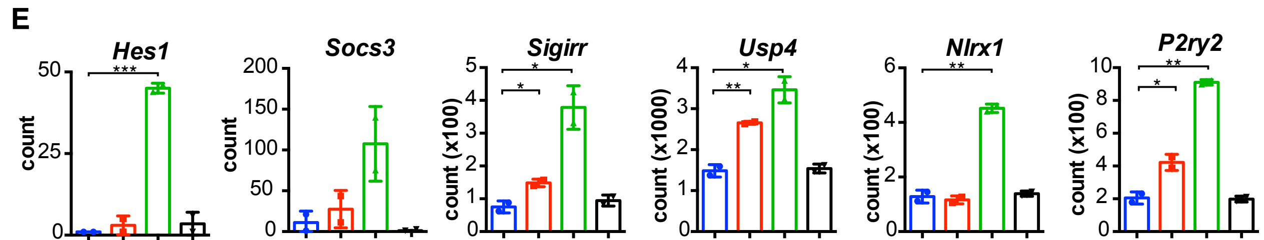


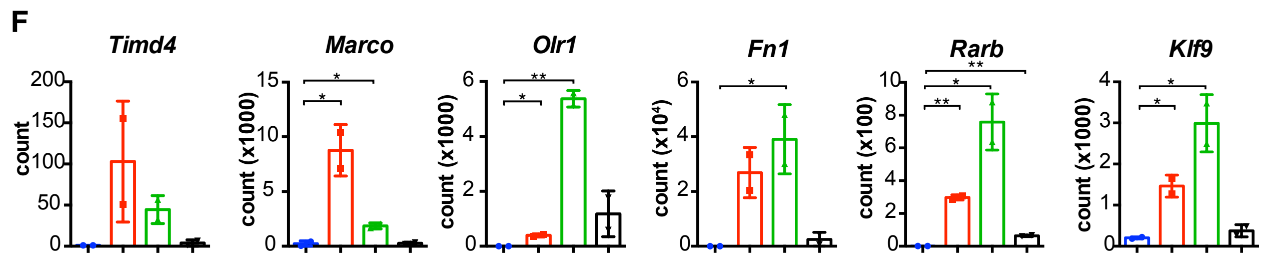


empty KLF2 KLF4 GATA6









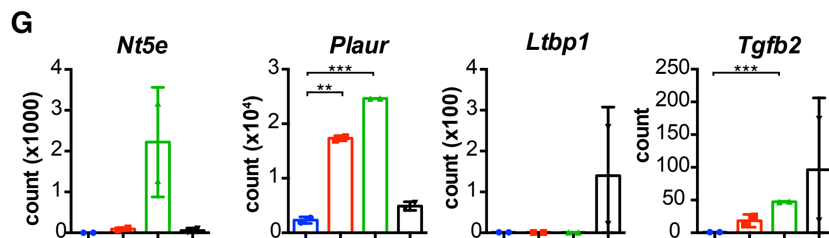


Figure 4.7 Overexpression of KLF2 or KLF4 in BMMs induces a gene expression profile consistent with AC-clearance. (A) Overexpression of KLF2 or KLF4 in BMMs dampens responses to CpG. WT BMMs were transduced with retroviral vectors encoding KLF2, KLF4, GATA6 or with empty vector. Cells were stimulated with TLR ligands and TNF production was measured by ICS. Data are representative of at least two independent experiments. **(B)** Overexpression of KLF2 and KLF4 in BMMs prevents expression of TLR9. TLR9KI BMMs were transduced with retroviral vectors encoding KLF2, KLF4, or empty vector as in (A). Lysates were subjected to immunoprecipitation with anti-HA resin and eluted TLR9 protein was visualized by anti-HA immunoblot. An anti-tubulin immunoblot on the same lysates was performed as a reference. Data are representative of at least two independent experiments. **(C)** BMMs were transduced with retrovirus encoding KLF2, KLF4, GATA6 or empty vector. The expression of GATA6 and KLF4 was determined by qPCR and is expressed as a relative value to *Actb* mRNA. Data are the combined results of two independent experiments. **(D-G)** Overexpression of KLF2 and KLF4 in BMMs induces expression of AC recognition receptors and negative regulators of TLR signaling. BMMs were transduced with retroviral vectors encoding KLF2, KLF4, GATA6 or with empty vector. The levels of mRNA for the indicated genes were quantified using a Nanostring nCounter. Data are the combined results of two independent experiments.

Chapter 5: Influence of inflammatory signals on endosomal TLR responses in AC-engulfing macrophages

Background:

As discussed in chapters three and four, we identified three populations of macrophages that were adept at AC clearance and lack expression of TLR9, the TLR that recognizes DNA. We also demonstrated that these macrophage populations are programmed by signals from their environment to silently engulf ACs. These signals inhibit expression of TLR9 and induce a higher threshold of activation for endosomal TLRs. Although this strict regulation of endosomal TLR responses can be beneficial to the host by avoiding autoinflammatory responses, they may also leave the host vulnerable to infection.

Macrophages can be infected by a variety of microbes. For instance, after being engulfed by a macrophage *Legionella pneumophila* can coopt the phagosome to generate a *Legionella*-containing vacuole where it can replicate [184]. *Salmonella typhimurium* can also survive within macrophages to establish chronic infection and can be reactivated if the host becomes immunocompromised [185], [186].

As discussed in chapter 1, TLR9 is vital for protection from a variety of pathogens including the viruses Ectromelia virus and murine cytomegalovirus, and the bacteria *Klebsiella pneumonia* [31-34]. Interestingly, the number of *K. pneumonia* in the extracellular lung environment as well as within alveolar macrophages was increased in mice lacking TLR9 [36]. This result could suggest a role for alveolar macrophage expression of TLR9 during *K. pneumonia* infection, raising the possibility that infection may induce TLR9 expression.

AC-engulfing macrophage populations that lack expression of TLR9 could be particularly susceptible to microbes that infect macrophages. Infected AC-engulfing macrophages may then become reservoir for latent infection or further spreading of the pathogen. We therefore wondered if these macrophages could adapt to an inflammatory environment to begin to express TLR9 and potentially limit infection.

In this chapter we explore the ability of AC-engulfing macrophages to adapt TLR9 expression to inflammatory conditions. We find that type I and type II IFN can induce expression of TLR9 in these macrophage populations. However, these macrophages still maintain a higher threshold for activation of endosomal TLR signaling, and do not generate responses to ACs. These results suggest that in an inflammatory environment AC-engulfing macrophages can respond to strong TLR9 ligands derived from microbes, without generating responses to self-derived DNA, which contains few unmethylated CpG dinucleotides.

Results:

Inflammatory cues induce TLR9 expression without enabling responses to ACs

As a first step to determine if inflammatory signals affect the AC-clearance program in tissue resident macrophages, we incubated purified Tim-4$^+$ pMacs overnight with different cytokines followed by stimulation with TLR ligands or ACs. These cytokines included IFNγ, which is vital for Th1 responses, IFNβ, which is involved in antiviral immunity, and the pro-inflammatory cytokines TNF and IL-6. Treatment with TNF or IL-6 had no effect, but Tim-4$^+$ pMacs pretreated with IFNγ or IFNβ gained responsiveness to TLR9 stimulation (Figure 5.1 A). Using macrophages from TLR9KI mice, we demonstrated that both IFNγ and IFNβ induced expression of TLR9 protein in Tim-4$^+$ pMacs as well as BMMs (Figure 5.1 B). IFNγ pretreatment also induced TLR9 responses in Tim-4$^+$ pleural and alveolar macrophages (Figure 5.1 C).

We next wanted to determine if IFNγ or IFNβ treatment reversed the entire AC-clearance program. To test this we examined cytokine treated macrophages responses to ACs. Intriguingly, IFNγ or IFNβ did not enable responses to ACs, indicating that while these cytokines induced expression of TLR9 they did not reverse the entire AC-clearance program (Figure 5.2 A). This lack of response was not due to an inability to engulf ACs (Figure 5.2 B).

The lack of response to ACs, despite upregulation of TLR9, in macrophages exposed to inflammatory signals suggested that these cells maintain a high threshold for endosomal TLR signaling. To test this possibility, we compared programmed Tim-4$^+$ pMacs that had been pretreated with IFNβ to deprogrammed macrophages. Although IFNβ treated Tim-4$^+$ pMacs generated responses to higher doses of CpG ODN and R848, their responses to low doses of these ligands were much lower than those of deprogrammed macrophages (Figure 5.2 C and D). These results suggest that IFN treated macrophages may avoid responses to ACs even when expressing TLR9 by maintaining a high activation threshold for endosomal TLR signaling.

Discussion:

We have demonstrated that expression of TLR9 in AC-engulfing macrophages can be modulated by inflammatory signals. IFNβ and IFNγ are able to induce expression of TLR9 in AC-engulfing macrophage populations while maintaining a high threshold for activation of endosomal TLR responses. In this way AC-engulfing macrophages can respond to strong TLR9 ligands, such as those derived from microbes, without generating responses to self-derived DNA, which contains few unmethylated CpG dinucleotides. The ability to respond to microbial DNA could prevent infection of AC-engulfing macrophages. Although prolonged expression of TLR9 in AC-engulfing macrophages may be detrimental, as seen in LysMcre$^{+/-}$Rosa$^{stop\text{-}flox\text{-}TLR9}$ mice in the pristane-induced lupus model, the inability of these macrophages to respond to low-level nucleic acid stimulation may prevent responses to self-derived nucleic acids during acute inflammation. Such responses could prolong inflammation and damage the host. After the inflammation has cleared we imagine AC-engulfing macrophages once again downregulate TLR9 expression.

While TLR9 expression may be beneficial during infections, the observation that forced upregulation of TLR9 subtly exacerbates inflammation during a chronic inflammatory disease brings to mind autoimmune disorders that involve inappropriate IFN responses. For instance, most human lupus patients have a signature of type I IFN exposure [187]. This signature may induce expression of nucleic acid sensing TLRs in cell types that typically lack this expression. It has previously been demonstrated in human peripheral blood monocyte-derived macrophages and DCs that type I IFN can upregulate expression of TLR7 and TLR3 [188], [189]. Perhaps in these patients IFN exposure induces upregulation of nucleic acid sensing TLRs in macrophages that clear ACs, and this continual expression leads to further inflammation. Understanding the regulation of endosomal TLR expression in human AC-engulfing macrophages could reveal potential therapeutic targets. For instance if human AC-engulfing macrophages also utilize KLF2 and KLF4 to control endosomal TLR expression, and this regulation is altered by IFN, increasing KLF2 or KLF4 expression may decrease expression of aberrantly expressed TLRs. In this regard, it is notable that statin treatment can upregulate expression of KLF2 in human peripheral blood monocyte-derived macrophages [190]. Defining the gene expression programs and unique functional characteristics of distinct tissue macrophage populations will undoubtedly open new therapeutic avenues.

Materials and Methods:

Mice

Mice were housed under specific pathogen-free conditions at the University of California, Berkeley. All mouse experiments were performed in accordance with the guidelines of the Animal Care and Use Committee at UC Berkeley. Unless noted mice were analyzed at 6-12 weeks of age. C57BL/6, C57BL/6 CD45.1^{+} (stock #002014), rosa26 $^{stop\text{-}flox\text{-}tdTomato}$ (stock #007909) mice were obtained from Jackson laboratories. A previous graduate student in the lab generated the TLR9 reporter mice. TLR9KI mice were generated using a construct encoded an HA tag on the 3' end of the TLR9 gene followed by an IRES-EGFP sequence.

Tissue harvest

Cells from the peritoneal and pleural cavities were recovered by lavage with ice cold PBS. Perfused lungs were digested with collagenase XI with DNAse I for 45min, single cell suspensions were generated by mechanical disruption through a 100um filter, cells were resuspended in 44% isotonic percoll (GE healthcare #17-0891-01), underlayed with 67% percoll, and spun at 1550xg without brake. Cells from the interface were collected for analysis.

Isolation of Tim-4^{+} peritoneal Macrophages

Peritoneal cells were recovered by lavage with 5ml of ice cold PBS. For RNAseq and western blot experiments B cells were first depleted using anti-CD19-biotin antibody and biotin binder dynabeads (ThermoFisher #11047). Tim-4^{+} cells were isolated using anti-TIM-4 antibody (clone RMT4-54, BioLegend) and anti-rat IgG microbeads (Miltenyi #130-048-501).

Cell culture

BMMs were differentiated for seven days in RPMI complete media (RPMI-1640 supplemented with 10% (vol/vol) fetal calf serum, L-glutamine, penicillin-streptomycin, sodium pyruvate, and HEPES pH 7.2) supplemented with M-CSF containing supernatant from 3T3-CSF cells.

Unless noted peritoneal macrophages *ex vivo* for 60 hours, as well as overnight controls, were cultured in 25% (v/v) omentum supernatant in RPMI complete media. Omentum supernatant was generated by harvesting omenta from C57BL/6 mice and culturing the omenta in RPMI complete media at 1ml/omenta overnight. Supernatant was collected, filtered through a 0.22um filter, and frozen at -80°C for future use. Lung and pleural cells were harvested as described above and plated on non-tissue culture treated plates in RPMI complete media.

For overnight cytokine treatment, cytokines were added to RPMI complete media containing 25% omentum culture supernatant and incubated with macrophages for 16 hours (IFNγ – Tonbo #21-8311-U020, IFNβ – Pbl interferon source #12401-1, TNF – aa84-235 R&D #410-TRNC, IL-6 R&D #406-ML-025, GM-CSF Tonbo #21-8331-U020).

Apoptotic cell generation and engulfment
Thymi were harvest from WT or CD45.1$^+$ rosa-tdTomato mice and single cell suspensions were generated by mechanical disruption through a 70um filter. Cells were irradiated with 600rad and incubated in RPMI complete media at 37°C for 4 hours. Apoptotic cells were then incubated with macrophages at the indicated ratios. For experiments examining AC engulfment capabilities macrophages were allowed to engulf ACs for 60min.

Stimulations
Cells were plated in RPMI complete media. Tissue macrophages were stimulated directly *ex vivo*, after overnight culture, or after 60 hours in culture. Cells were stimulated with TLR ligands (LPS, CpG-B ODN 1668, R848 all from Invivogen), or ACs. For analysis of secreted cytokines, supernatant was collected 7 hours after stimulation. For intracellular cytokine staining, 30 minutes after stimulation brefeldin A (GolgiPlug, BD Biosciences) was added to cells before incubation for another 4 hours.

Flow cytometry and Antibodies
Dead cells were excluded using a fixable live/dead stain (Life technologies) or DAPI (Life Technologies) and all stains were carried out in PBS containing 2% FBS (v/v) and 2mM EDTA including anti-CD16/32 Fc blocking antibody (2.4G2, UCSF monoclonal antibody core) and normal mouse serum (Sigma). Cells were stained for 20min at 4°C with antibodies (see table 3.1). For intracellular TNF staining cells were permeabilized with Fix/Perm buffer (BD) for 20min at 4°C. Cells were then stained with antibodies. All cells were analyzed on an LSRII or LSR Fortessa (BD Biosciences), and data was analyzed with FlowJo (TreeStar).

Western Blot Analysis
Cells were lysed in RIPA buffer (50mM Tris pH 7.4, 150mM NaCl, 1mM EDTA, 0.5mM EGTA, 1% NP-40, 1% DOC, 0.1% SDS) containing protease inhibitors (Roche #05 892 791 001). Cell lysates were immunoprecipitated with anti-HA matrix (Roche #11573000). After washing in RIPA buffer, matrix beads were boiled in SDS-PAGE loading buffer. Protein was run on a 4-15% gel (Biorad #4561083) and transferred to Immobilon-FL membrane (Millipore #IPFL00010). After blocking, membranes were probed with anti-HA antibody, followed by anti-rat-680 secondary (Life Technologies #A21096) or probed with anti-tubulin, followed by anti-mouse-800 secondary (Li-Cor #926-32210). Images were scanned using a Licor Odyssey.

Statistical Analysis
Statistical analysis was performed with the Prism software (GraphPad software). P-values were determined using unpaired two-tailed Students's *t*-test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Figures:

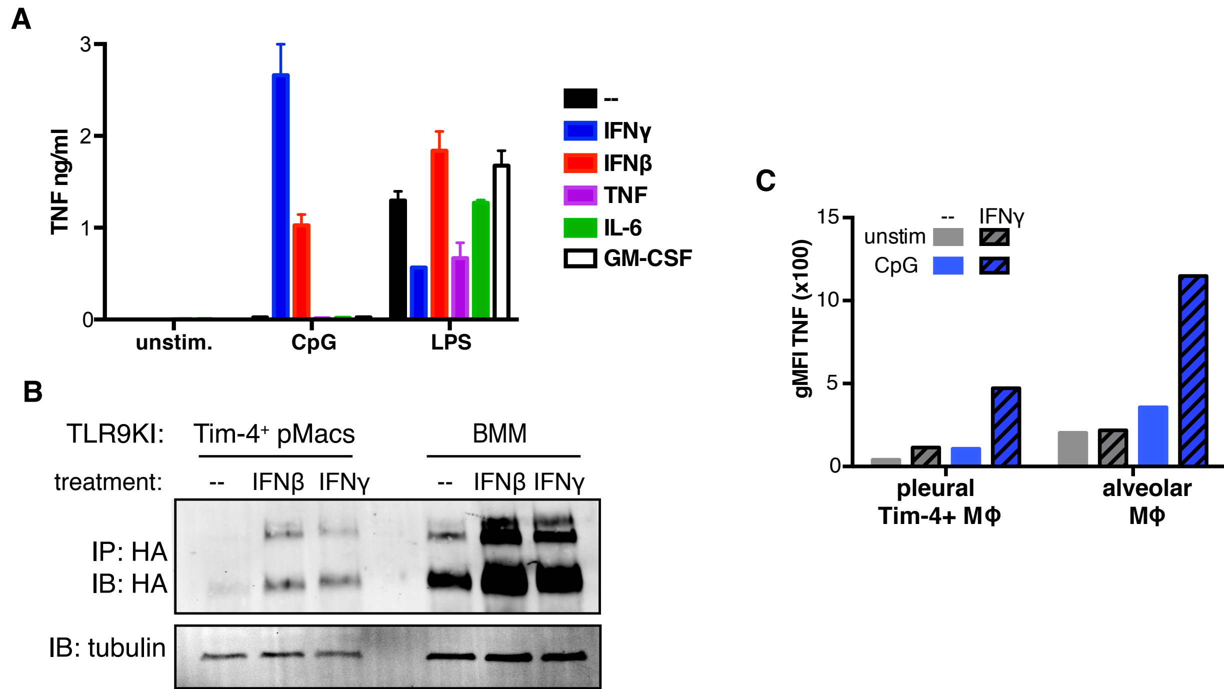


Fig 5.1 Inflammatory cues induce TLR9 expression in AC-engulfing macrophages. (A) IFNγ and IFNβ induce responses to TLR9 ligands in Tim-4$^+$ pMacs. Isolated Tim-4$^+$ pMacs were treated overnight with the indicated cytokines before stimulation with TLR ligands. Concentrations of cytokines used were 10ng/ml IFNγ, 20U/ml IFNβ, 5ng/ml TNF, 50ng/ml IL-6, or 5ng/ml GM-CSF. Cytokine responses were measured by CBA. Data are representative of at least three independent experiments. **(B)** IFNγ and IFNβ induce TLR9 expression in Tim-4$^+$ pMacs. Tim-4$^+$ pMacs and BMMS from TLR9KI mice were cultured overnight ± 10ng/ml IFNγ or 20U/ml IFNβ, and TLR9 levels in lysates were measured by anti-HA immunoprecipitation and immunoblot. An anti-tubulin immunoblot was performed on each lysate as a reference. Wells of Tim-4$^+$ pMacs correspond to lysate of 1.6e6 cell equivalents, and wells of BMMs correspond to lysate of 0.4e6 cell equivalents. Data are representative of at least three independent experiments. **(C)** IFNγ induces Tim-4$^+$ pleural macrophage and alveolar macrophage responses to TLR9 ligands. Lung and pleural cells from WT mice were harvested and incubated overnight ± 100ng/ml IFNγ before stimulation with CpG ODN. TNF production was measured by ICS. Data are representative of two independent experiments

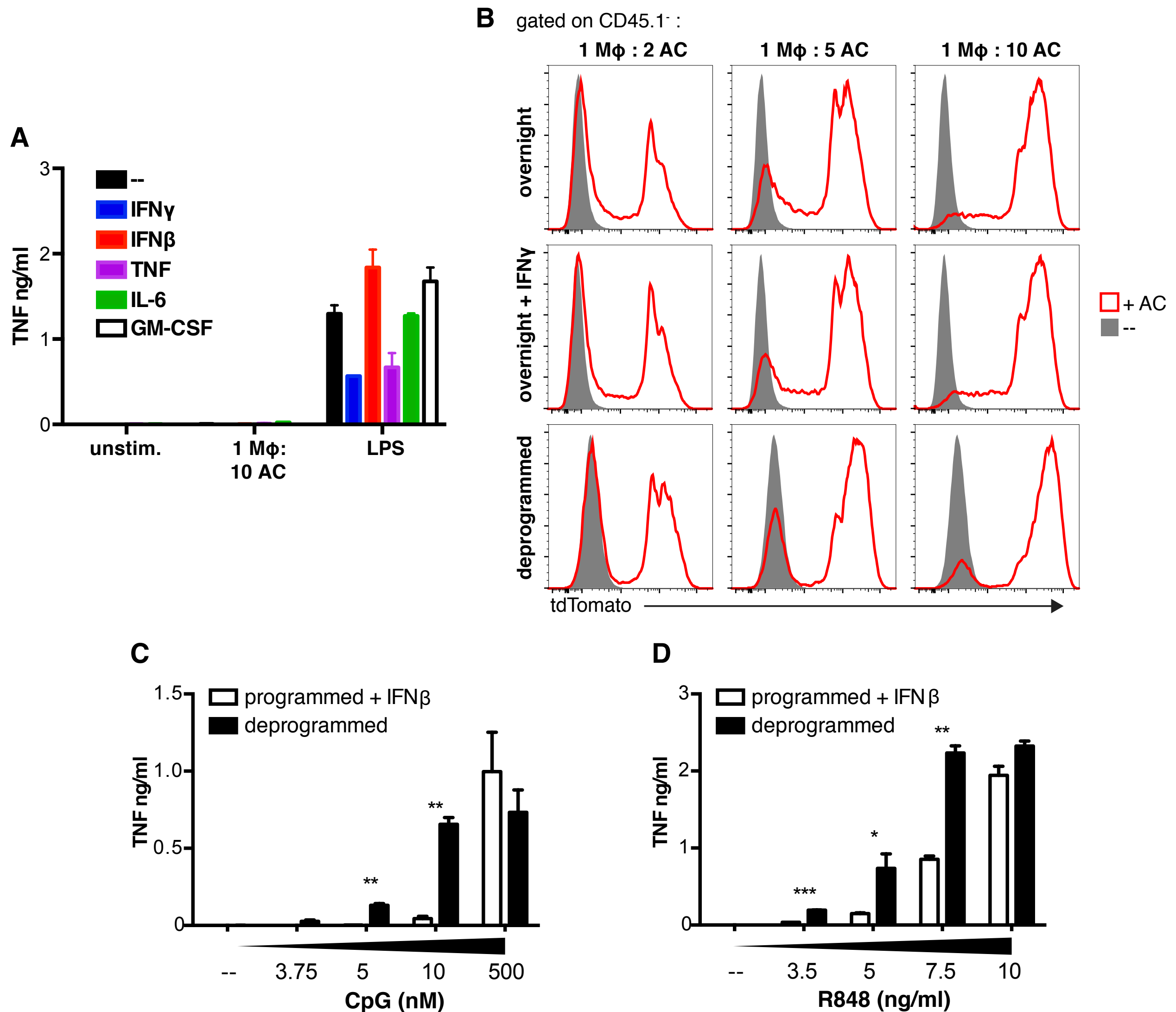


Fig 5.2 Inflammatory cues do not induce responses to ACs by AC-engulfing macrophages. (A) IFNγ and IFNβ induce responses to TLR9 ligands in Tim-4$^+$ pMacs. Isolated Tim-4$^+$ pMacs were treated overnight with the indicated cytokines before stimulation with TLR ligands or ACs. Concentrations of cytokines used were 10ng/ml IFNγ, 20U/ml IFNβ, 5ng/ml TNF, 50ng/ml IL-6, or 5ng/ml GM-CSF. Cytokine responses were measured by CBA. Data are representative of at least three independent experiments. **(B)** Tim-4$^+$ pMacs were isolated from WT mice and cultured overnight ± IFNγ or for 60 hours then incubated with CD45.1$^+$tdTomato$^+$ ACs at indicated ratios and analyzed by flow cytometry. **(C)** IFNβ treated Tim-4$^+$ pMacs maintain a high threshold for TLR9 responses. Isolated programmed IFNβ treated and deprogrammed untreated Tim-4$^+$ pMacs were stimulated with increasing doses of CpG. TNF was measured by CBA. Data are representative of two independent experiments. **(D)** IFNβ treated Tim-4$^+$ pMacs maintain a high threshold for TLR7 responses. Isolated programmed IFNβ treated and deprogrammed untreated Tim-4$^+$ pMacs were stimulated with increasing doses of R848. TNF was measured by CBA. Data are representative of two independent experiments.

Conclusions

In this dissertation I have examined the intersection of nucleic acid sensing TLRs and the clearance of apoptotic cells. As discussed in chapters one and two the ability of the innate immune system to recognize nucleic acid ligands is vital for host survival, as is the ability to maintain tissue homeostasis by efficiently clearing dead cells. However, effectively performing both of these processes without generating inflammatory responses is challenging. The purpose of my work was to illuminate the regulation of this intersection in a physiological setting.

Although it is often proposed that tissue-resident macrophages engulf ACs, whether these cells possess key characteristics that enable immunologically silent clearance of ACs had not been examined. Recent studies demonstrating the heterogeneity of tissue-resident macrophages highlight the functional differences between populations of macrophages. Here I have identified three macrophage populations that engulf ACs *in vivo* and have investigated how they regulate responses to self nucleic acids. I report that these AC-engulfing macrophages are programmed by their tissue environments to clear ACs in an immunologically silent manner. This programming includes inhibition of TLR9 expression as well as upregulation of AC recognition receptors and negative regulators of TLR signaling. I hypothesize that the resulting increased threshold of activation in these cells enables immunologically silent clearance of large numbers of ACs.

All macrophages seem to have some potential to engulf ACs; in fact, the AC recognition receptor MerTK is expressed in all macrophage populations [151], [159]. However, our work identifies distinct tissue macrophage populations as the primary cells involved in AC clearance under homeostatic conditions, at least in certain tissues. Previous studies have suggested that to avoid responses to self-derived nucleic acids recognition of ACs induces expression of anti-inflammatory cytokines [119], [191]. While this could be a mechanism used by phagocytes that engulf limited numbers of ACs, I show that pMacs do not upregulate anti-inflammatory cytokines in response to ACs. Instead, these specialized AC-engulfing macrophages are preprogrammed to strictly regulate endosomal TLR responses in a cell-intrinsic manner. AC clearance is a continual process and perhaps AC-engulfing macrophages cannot constantly secrete anti-inflammatory cytokines without influencing potential responses to pathogens. The Tyro3, Axl, and (TAM) receptor tyrosine kinase family has also been suggested to regulate responses to ACs. In addition to recognizing ACs, TAM receptors have been demonstrated to inhibit TLR signaling by upregulating expression of SOCS genes [138]. However, expression of TAM receptors did not decrease in deprogrammed macrophages, and SOCS genes were not upregulated in programmed macrophages in response to ACs (data not shown). Therefore, although I cannot rule out a role for TAM receptor signaling *in vivo*, I do not believe the AC-clearance program involves this receptor family.

Rather than inducing anti-inflammatory cytokine production or relying on TAM receptor signaling I found that the identified AC-engulfing macrophages likely avoid

responses to AC-derived nucleic acids by strictly controlling the initiation of endosomal TLR signaling pathways. My data suggest that this control is mediated by the transcription factors KLF2 and KLF4. KLF2 and KLF4 expression is induced by still unidentified cues in the tissue environment. Expression of these transcription factors can inhibit expression of TLR9 and induce expression of negative regulators of TLR signaling. In addition to regulating endosomal TLR responses in this manner, KLF2 and KLF4 can induce expression of receptors that mediate AC clearance. I suggest that this coordinated expression of AC receptors and genes that limit innate responses to endogenous nucleic acids helps maintain self-tolerance.

Although this regulation addresses how responses to endogenous ligands can be prevented, I wondered if these mechanisms leave the host susceptible to microbes that may take advantage of the reduced responses to nucleic acid ligands. I found that type I and type II IFN could induce expression of TLR9 in these macrophages, however responses to weak nucleic acid ligands were still controlled. I image that in this way these macrophages are able to respond to stronger DNA ligands derived from microbes, but continue to avoid responses to weaker self-derived ligands such as DNA that contains fewer unmethylated CpG diucleotides.

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