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List of contents

Summary

Proper regulation of chromatin structure is necessary for the maintenance of cell type-specific gene expression patterns. Diverse macrophage expression patterns control their polarization and activation/inactivation status. During macrophage activation, interleukin-1 receptor-associated kinase (IRAK)-M acts as a functional decoy preventing uncontrolled toll-like receptor / interleukin-1-mediated responses. Despite its important role, the transcriptional and epigenetic regulation of IRAK-M expression is not fully understood. Our study investigates the role of chromatin regulators and transcription factors in controlling IRAK-M expression upon activation of macrophages by gram (-) bacterial lipopolysaccharide (LPS). Here, we present an RNAi screen in mouse RAW264.7 macrophages of over 60 genes encoding chromatin proteins and transcription factors. We identified 6 proteins that exhibit diverse phenotypes and activation potential upon knockdown following LPS treatment. We focused on two chromatin regulators EZH2 (Histone-lysine N-methyltransferase) and UTX (Ubiquitously transcribed tetratricopeptide repeat, X chromosome) with antagonizing actions. We propose that they are localized on the promoter of IRAK-M to mediate histone H3 lysine 27 trimethylation (H3K27me3). Furthermore, we found that the transcription factor CEBPb directly binds on the promoter of IRAK-M regulating its transcription. Depletion of CEBPb reduced both protein and mRNA IRAK-M expression. These data indicate that EZH2, UTX and CEBPb are key regulators of IRAK-M expression in macrophages activated through TLR4.

Keywords: Epigenetics, IRAK-M regulation, macrophage activation, chromatin regulators, EZH2, UTX.

A. Introduction

1. Inflammation and Endotoxin Tolerance

Immune responses are crucial for the protection of an organism against exogenous or endogenous threats, such as pathogens and cancer respectively. Through evolution vertebrates have developed a complicated recognition system in order to effectively guard themselves, which involves both fast acting innate and slowly activated adaptive immune responses. Innate immune cells such as monocytes/macrophages are able to detect specific molecules through their pattern recognition receptors (PRRs) and thus trigger the initiation events of inflammation (figure 1) [1]. A well studied example is Toll Like Receptor 4 (TLR4), that is known to bind the Lipopolysaccharide (LPS) which is present on the Gram negative bacteria cell wall [1,2].

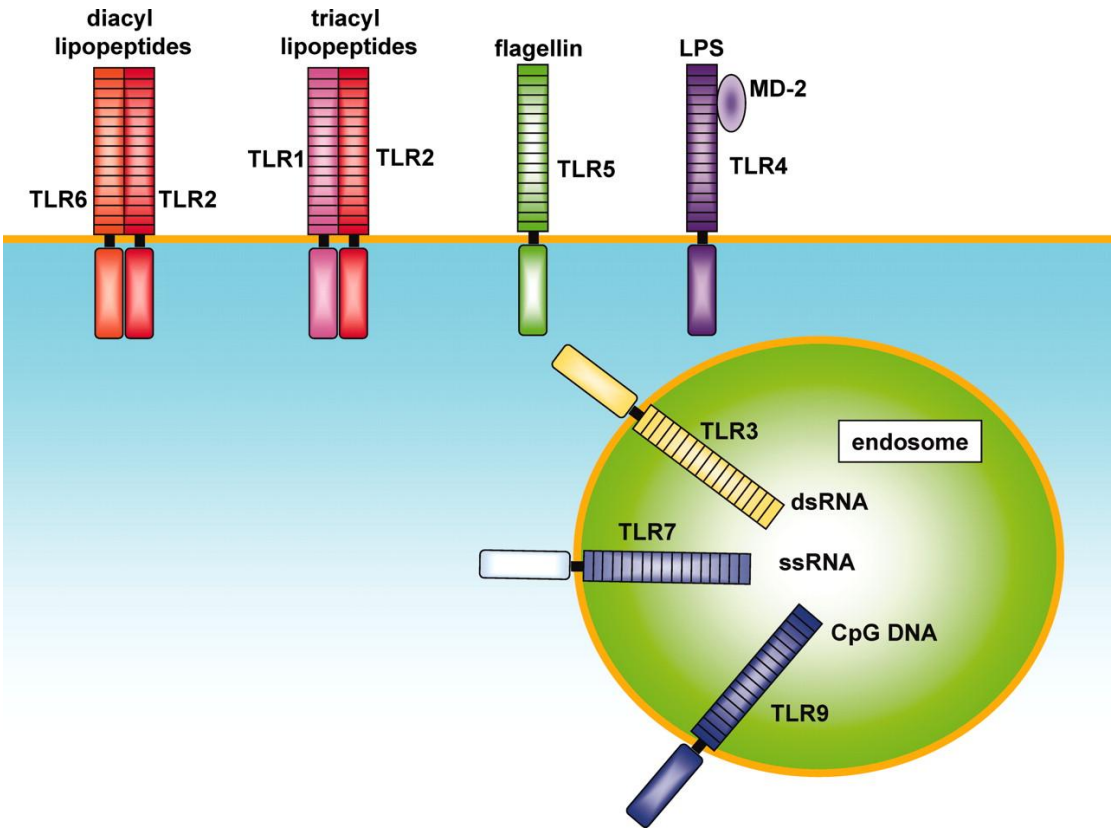


Figure 1. TLRs and their ligands. TLR2 is essential in the recognition of microbial lipopeptides. TLR1 and TLR6 cooperate with TLR2 to discriminate subtle differences between triacyl and diacyl lipopeptides, respectively. TLR4 is the receptor for LPS. TLR9 is essential in CpG DNA recognition. TLR3 is implicated in the recognition of viral dsRNA, whereas TLR7 and TLR8 are implicated in viral-derived ssRNA recognition. TLR5 recognizes flagellin. Thus, the TLR family members recognize specific patterns of microbial components.

Whereas sustained inflammation is essential to battling pathogens, excessive inflammation on the other hand can lead to severe tissue damage. Therefore, immune responses, physiologically, are tightly regulated and in many cases self-restricted via negative feedback signaling loops. For instance, in the case of TLR4, continual stimulation with endotoxins shifts responsive cells into a transient anergic and tolerant state, under which restimulation with LPS is unable to transduce the early pro-inflammatory features, a phenomenon termed Endotoxin Tolerance [3]. This altered status is believed to be the net result of an accordingly altered transcriptional profile. Thus, tolerant macrophages are characterized by the downregulation of pro-inflammatory cytokines, the upregulation of anti-inflammatory cytokines (and cytokine antagonists such as IL-1Ra) and decreased antigen presentation capability, as conferred by the downregulation of MHC-II molecules and CIITA [4].

In view of the fact that the stimulus required for the induction of the tolerant state is an inflammatory one (like LPS in the case of endotoxin tolerance), it is likely that this phenomenon works in negative feedback loops. In other words activation of TLR signaling pathways leads essentially to their own dampening. This involves either the downregulation of key effector molecules in the TLR signaling pathway, such as the TLR4 receptor[5], or the upregulation of negative regulators such as SOCS-1 [6],A20[7] and IRAK-M (also known as IRAK-3) [8]. Regulation is performed at a post transcriptional level also, with the modulation of miRNA expression [9].

- **Inflammation and alternative activation of macrophages**

Macrophages are cells that are characterized by extended flexibility and plasticity. Under various signals such microbial products or damaged cells macrophage undergo either classical M1 activation or alternative M2 activation [115]. M1 polarized macrophages are activated by various TLR ligands and IFN-g while M2 polarized macrophages are activated by IL-4/IL-13. In general M1 macrophages express high levels of pro-inflammatory cytokines and reactive nitrogen intermediates (iNOS) and thus strong activity against microbes and tumor cells. On the other hand, the M2 phenotype is characterized by anti-parasitic activity, tissue remodeling, wound healing and tumor sustained progression through its immunoregulatory activity (Tumor Associated Macrophages). In general M2 macrophages have high expression of scavenging molecules, result in the production of L-ornithine through the arginase pathway and are distinguished by low levels of IL-12 and high levels of IL-10 [115]. Moreover M1 versus M2 macrophages have different chemokine expression profiles.

A key concept is that macrophage polarization and thus its activation status has been linked to pathology. M1 polarized macrophages have been implicated in initiating and at the same time sustain inflammation while the M2 polarized phenotype has been associated with the resolution of chronic inflammation [116]. The general idea is that under conditions such as allergy, cancers and parasite infections the functional macrophages phenotypes in vivo are similar to those of canonical M1-M2 polarized states as defined in vitro [114].

Transcription factors, posttranscriptional regulators, signaling molecules and in general epigenetic mechanisms are implicated in controlling the outcome of

macrophage activation and function. For example the balance between activation of STAT1 and STAT3/STAT6 finely regulates macrophage polarization and activity [117]. Moreover, NF-κB and STAT1 activation promotes M1 macrophage polarization, resulting in cytotoxic and inflammatory functions. In contrast, STAT3 and STAT6 activation results in M2 macrophage polarization, associated with immune suppression and tumor progression [117]. Additionally the nuclear receptors PPARγ and PPARδ control distinct aspects of M2 macrophage activation and oxidative metabolism [118, 119]. Krüppel-like factors such as KLF4 and KLF2 participate in the promotion of M2 macrophage functions by cooperating with STAT6 and suppressing the NF-κB/HIF-1α–dependent transcription, respectively [120]. In addition, IL-4–induced c-Myc activity controls a subset of M2-associated genes such as Scarb1, Alox15, and Mrc1 [121] as well as STAT6 and PPARg activation [121]. At an epigenetic level IL-4 also induces the M2-polarizing Jmjd3-IRF4 axis to inhibit IRF5-mediated M1 polarization [92]. Noncoding RNAs such as mir155 have also been shown to regulate macrophage polarization [122] although their role in macrophage polarization need to be further defined.

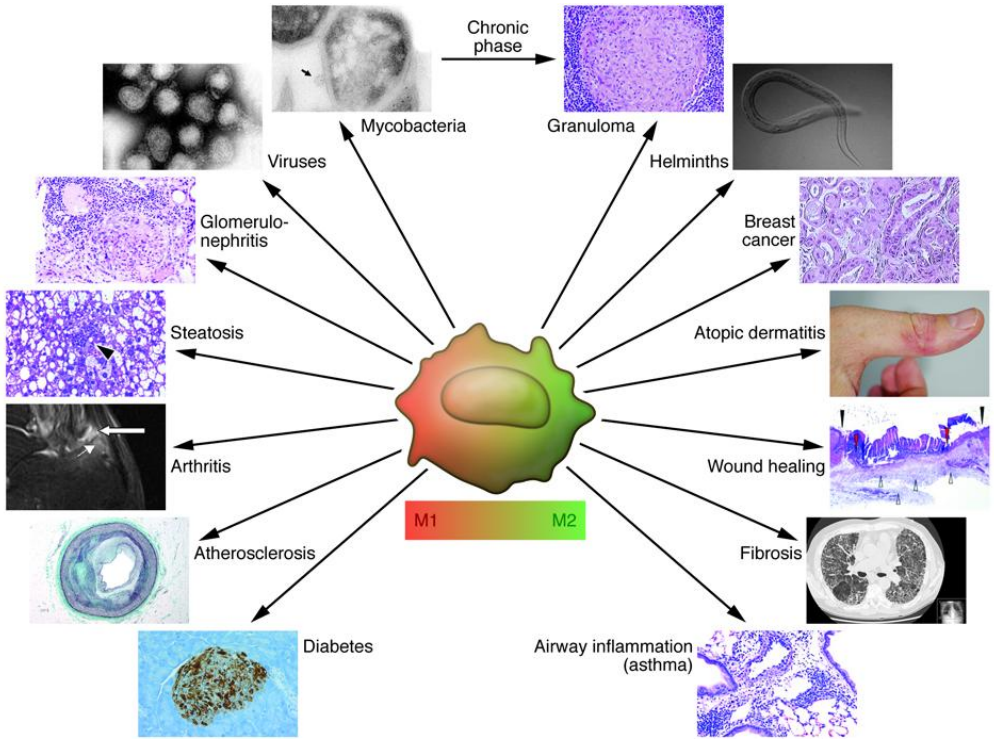


Figure 2. Schematic representation of macrophage plasticity and polarization in pathology. Dynamic changes occur over time with evolution of pathology: for instance, a switch from M1 to M2 macrophage polarization characterizes the transition from early to chronic phases of infection. Images adapted from reference [114].

- **IRAK-M: a central regulator of endotoxin tolerance**

Screening of Expressed Sequence Tags (ESTs) for sequences sharing significant homology with the human Irak gene led to the discovery of *Irak-m* in 1999. Wesche et al [10] were able to clone the cDNA encoding for this novel protein and classify it as a member of the Irak family of kinases that serve as intracellular

signal transducers of various pro-inflammatory stimuli such as LPS or IL-1. The name IRAK-M was appointed because of the restriction in this molecule's expression in cells of monocytic origin. The discovery of the murine counterpart to human *Irak-m* was reported in 2002 by two distinct publications. The first one, by Rosati and Martin [11], identified murine IRAK-M as a 68kDa protein of 609 amino acids, sharing 71% homology with human IRAK-M and displaying the same pattern of minimal autophosphorylation activity (in vitro assays). The second one, by Kobayashi et al [8] is probably the most influential publication regarding this protein. It is the first publication to ever attribute an inhibitory, rather than an effector role for IRAK-M in pro-inflammatory signal transduction and to stably associate it with the induction of endotoxin tolerance in murine macrophages. Moreover it is the first publication to report the generation of IRAK-M deficient mice.

Ever since 2002 IRAK-M is considered as one of the most important inducers of macrophage tolerance. Its role in attenuating TLR signaling in innate immunity has been elucidated by utilizing both ex vivo and in vitro studies and research performed in IRAK-M deficient animals. Moreover, newer data provide further insight in its cellular distribution (table 1) and function, while linking it with adaptive immune responses too. Additionally, beside the basic molecular data, the significance of this protein is strengthened even further by rapidly accumulating evidence from epidemiological studies associating IRAK-M with human diseases.

Table 1: Tissue and cellular distribution of IRAK-M.

Cell or tissue expressing IRAK-M	Species	Source	Method of Detection*
Immune Cells			
Monocytes/Macrophages	Human and Mouse	Wesche et al. 1999	NB
		Rosati et al.2002	WB
Neutrophils	Mouse	Hubbard et al. 2010	WB, RNA
Fibroblasts	Mouse	Rosati et al. 2002	WB
B-cells	Mouse	Meyer-Bahlburg 2007	FACS
Epithelial Cells			
Lung	Human and Mouse	Balaci et al.2007	IHC
		Seki et al.2010	WB, RNA
Intestine	Mouse	Oshima et al. 2010	RNA
Intrahepatic bilary duct	Mouse	Harada et al. 2007	WB,RNA
Tissues (Thymus, Liver, Heart, Brain, Spleen, Kidney)	Mouse	Rosati et al. 2002	WB

*WB: Western Blot, NB: Northern Blot, FACS: Fluorescence-activated cell sorting, IHC: Immunohistochemistry

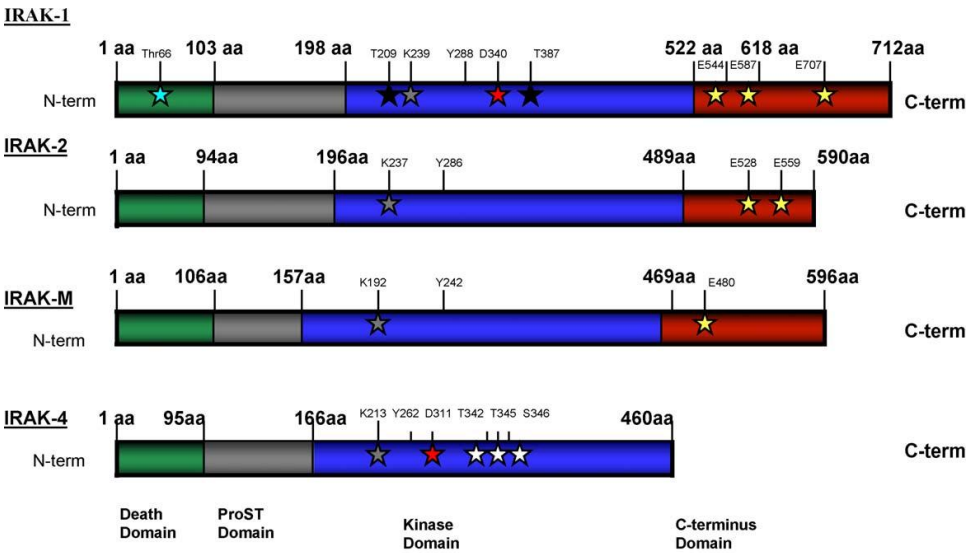
2. IRAK-M gene and protein

The human *irak3* gene is mapped at chromosome 12 spanning over 65 kb. It is comprised of 12 exons. An alternative splice variant of 11 exons (skipping the second exon) of unknown functionality also exists. Northern blot analysis has shown that IRAK-M is expressed predominantly in peripheral blood leukocytes. It is also

expressed in the U937 and THP1 cell lines [10]. Polarization of the THP1 cell line towards macrophages further increases its expression. Human *irak-m* gene encodes for a 68kDa protein of 596 amino acids. The protein shares a 30- 40% homology with other members of the IRAK family [10]. In vitro proteomic analysis has shown that human IRAK-M can be precipitated along with Traf6, MyD88 and members of the IRAK family [10].

Murine IRAK-M is mapped in chromosome 10, spans over 60 kb and is comprised of 12 exons. Northern blot analysis revealed that murine *irak-m* is expressed in all murine tissues and most predominantly in the liver and thymus. It is also expressed in NIH3T3 fibroblasts and RAW 264.7 mouse macrophages [11].It encodes for a 68.7kDa protein of 593 amino acids sharing a 73% homology with murine IRAK-1 [8].

Each member of the IRAK family has a death domain, a proST domain, a conserved kinase domain, and C terminal domain (figure 2). The only exception is IRAK-4 which lacks a C-terminal domain [10, 12]. The death domain was recently shown to facilitate the interaction of IRAK-M with IRAK-1 [38]. The kinase domain can be subdivided into 12 serine / threonine subdomains. Whereas IRAK-M contains a functional ATP binding pocket in subdomain II, the kinase catalytic domain in subdomain IVB is inactive [8, 10, 12]. Namely a critical aspartate residue is replaced by a serine residue. Thus both murine and human IRAK-M has weak autophosphorylation activity [10]. In the case of IRAK-1, it has been shown that each carboxyl terminal subdomain confers the ability for TRAF6 association via the conserved Pro-X-Glu-X-X-(aromatic/acidic residue) motif [13]. Such motifs are not present in IRAK-M.



★	Important for dimerisation
☆	Important for kinase activity
★	Invariant lysine residue
★	Phosphorylated by IRAK-4
★	Critical asparate residue
★	TRAF6 binding motif

Figure 2. Functional domains of the human interleukin receptor-associated kinases (IRAKs). Each member has a death domain, a proST domain, a conserved kinase domain, and C terminal domain. The only exception is IRAK-4 which lacks a C-terminal domain. For human IRAK-1, the death domain contains a critical residue at Thr66 which is important for signaling. The proST domain has been shown to be vital for autophosphorylation. The kinase domain contains Thr209 and Thr387, located in the activation loop, which are potential phosphorylation sites for IRAK-4. The invariant lysine residue in the ATP binding pocket of IRAK-1 is located at K239 and there is a critical aspartate residue at D340, both of which are critical for IRAK kinase function. The tyrosine gatekeeper is located at Y288. The C-terminus contains three TRAF6 binding motifs (E544, E587 and E707). The kinase domain of human IRAK-2 contains an invariant lysine residue in the ATP binding pocket at K237, which is said to be important for its kinase activity. The tyrosine gatekeeper is located at Y286. Its C-terminal contains two TRAF6 binding motifs (E528 and E559). E528 is critical for IRAK-2 function. The invariant lysine residue of IRAK-M is located at K192. The tyrosine gatekeeper is located at Y242. IRAK-M also has a TRAF6 binding motif in its C-terminus (E480). Lastly IRAK-4 contains a death domain, a proST region and a kinase domain. The invariant lysine residue of IRAK-4 is located at K213. There is a critical aspartate residue at D311 which is essential for IRAK-4 kinase function. Other important residues in IRAK-4 kinase domain include T342, T345, and T346. The tyrosine gatekeeper is located at residue Y262.

3. Function of IRAK-M

Members of the Irak family of kinases are thought to regulate signal transduction from several TLRs and cytokine receptors such as IL-1. In brief, these receptors do not possess intrinsic kinase activity. Instead, upon ligand engagement through their LRR (Leucine rich repeats) extracellular domains, they are able to attract intracellular adaptor molecules such as MyD88. MyD88 in particular, has two domains used for protein to protein interactions: a TIR domain for binding onto the respective TIR domain of the TLR and a death domain (DD), which facilitates interactions with downstream molecules carrying similar death domains [1].

All the members of the IRAK family of kinases carry such motifs. Irak1 and Irak4 have been shown to physically interact with MyD88 [14]. This interaction in the receptor complex leads to their auto or cross phosphorylation. Phosphorylated Irak-1 and Irak-4 demonstrate reduced affinity for MyD88 but increased affinity for Traf6 [14, 15]. Thus they escape the receptor complex and form a heterodimer that is able to interact with Tak1 at the cell membrane. Tak1 along with TAB1, TAB2 and TRAF6 are released together into the cytosol without the participation of IRAK and form an active kinase complex with the participation of other proteins [16]. IRAK-1, remains in the cell membrane and is subsequently ubiquitinated and targeted for proteasomal degradation [16].

The most notable substrates of the TAK1/TRAF6/TAB1/TAB2 complex include IKKa/b leading to the activation of the canonical NF-kB pathway, MAP2K3/4/6 phosphorylation also leading to NF-kB induction, MAPK p38 which regulates ATF and Ets mediated transcription (among others) and JNK which leads to AP-1 coupled transcription [17]. While IRAK-M can inhibit the classical pathway in a TLR-4 and -9 dependent manner [8], there is evidence that IRAK-M can selectively inhibit the alternative NF-κB pathway in a TLR-2 dependent manner [23]. It was demonstrated that IRAK-M−/− BMDM display increased RelB (alternative pathway) nuclear translocation relative to WT BMDM. Overall, these data suggest that IRAK-M can negatively regulate both the classical and alternative NF-κB pathway depending on the TLR stimulus. IRAK-M may also negatively regulate activation of the transcription factor AP-1 by inhibiting TLR-mediated MAP kinase activation [22]. Additionally IRAK-M may bind and regulate activation of the co-stimulatory molecule CD80. In response to CD28 engagement, CD80 and CD86 expressed on APCs signal

downstream activation of NF-κB/AP-1 [109]. As determined by confocal microscopy, IRAK-M seems to interact with CD80 and disassociates in response to stimulation with CD28-containing neutrophil lipid rafts which leads to decreased IRAK-M interaction with TRAF6 which may contribute to induction of lethal cytokine storm and pathological inflammation [110], suggesting that IRAK-M may negatively regulate activation of NF-κB/AP-1 via CD80 signaling in addition to TLR/IL-1R signaling (table 2).

As previously mentioned, IRAK-M is one of the major negative regulators of the NF-kB pathway (figure 3). Being a member of the IRAK family it has all the structural characteristics of the other members, but a minimally active kinase catalytical centre [8, 10]. Also proteomic analysis has shown that human IRAK-m can be precipitated along with MyD88, TRAF6 and other family members of the Irak family in transiently transfected HEK cells [10]. IRAK-M can also physically interact with TIRP, another adaptor protein containing a TIR domain [37]. Additionally murine IRAK-M is able to inhibit IRAK phosphorylation but at the same time increase the ratio of phosphorylated IRAK bound to MyD88, despite the reduction in the affinity between these two molecules following IRAK phosphorylation [8]. Thus, placement of this molecule in the TLR/IL-1 signaling cascades should be dictated by these characteristics.

Kobayashi et al proposed two hypothetical mechanisms that could explain these data: IRAK-M negatively regulates intracellular TLR signaling by either antagonizing for effector IRAK phosphorylation, or by stabilizing the TLR4/MyD88/IRAK1-4 complex [8]. Thus functioning as a physiological dominant negative member of the IRAK family, IRAK-M inhibits the TLR signaling pathway, at an early stage. If this is true and IRAK-M should be able to inhibit this pathway at its early transduction stages, then a complete attenuation of branches is expected. Accordingly, bone derived macrophages from IRAK-M knockout mice upon CpG or LPS stimulation, exhibit increased phosphorylation of IkBa, JNK, p38 and ERK1/2 [8]. However discrepancies in this theory do exist: Wesche et al demonstrated that transient transfection of IRAK-M in IRAK-1 deficient HEK 293 cells is able to reconstitute NF-κB activation [10]. Su et al [22] demonstrated that Pam3CSK4 (TLR2 agonist) stimulated IRAK-M deficient macrophages and exhibited increased phosphorylation of p38 MAPK, but not ERK or JNK indicating that IRAK-M exerts its inhibitory effect through an IRAK1 independent pathway. IRAK-M is shown in THP1 cells to exert attenuation of p38 activation through decreasing proteasomal degradation of MKP1, a known inhibitor of p38 MAPK.

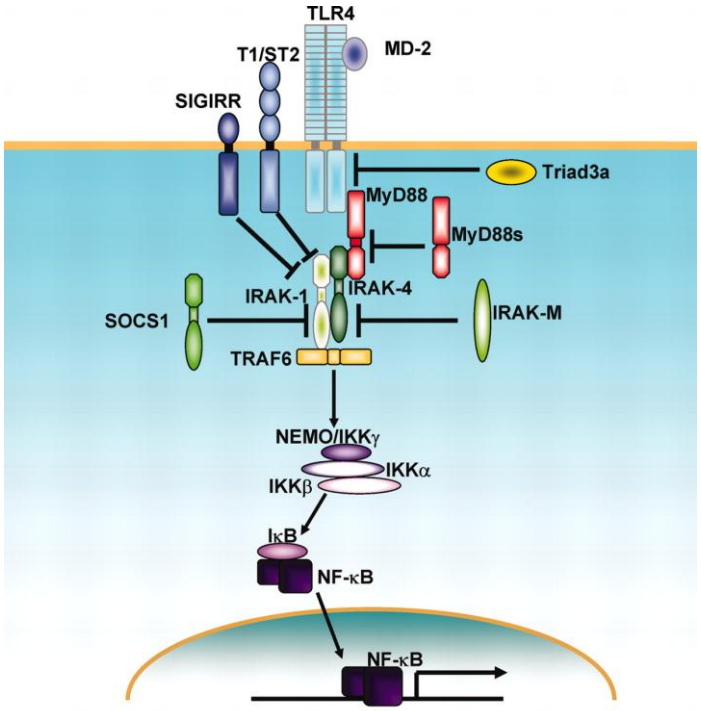


Figure 3. Negative regulation of TLR signaling pathways. TLR signaling pathways are negatively regulated by several molecules. IRAK-M inhibits dissociation of IRAK-1/IRAK-4 complex from the receptor. MyD88s blocks association of IRAK-4 with MyD88. SOCS1 is likely to associate with IRAK-1 and inhibits its activity. TRIAD3A induces ubiquitination-mediated degradation of TLR4 and TLR9. TIR domain-containing receptors SIGIRR and T1/ST2 are also shown to negatively modulate TLR signaling. [figure adapted from Takeda K., Akira S. (2005) Int. Immunol.]

Table 2: a list of all signaling cascades known to be affected by IRAK-M.

Signaling Cascade	IRAK-M contribution	Source
classical NF-kB pathway	↓	Kobayashi K. et al. 2002
alternative NF-kB pathway	↓	Su J. et al. 2009
MAP kinase pathway	↓	Su J. et al. 2007
CD80 signaling	↑	Nolan A. et al. 2009

4. Regulation of IRAK-M expression

- **TLRs**

Probably the most convincing piece of evidence linking IRAK-M to TLR induced tolerance in innate immunity is observations done in IRAK-M knockout mice. They were first generated by Kobayashi et al in 2002[8].These animals appear normal in their gross anatomy, but distinctly display a pro-inflammatory phenotype: Primary macrophages from these animals have an intrinsic inability to become tolerant up until a 24 hour stimulation with TLR agonists: upon challenging with various TLR ligands (or IL-1) or infection with Gram (+) or Gram (-) bacteria, IRAK-M deficient macrophages produce reduced levels of IL-12p40, IL-6 and TNFa. Accordingly, upon TLR stimulation, they also exhibit a substantial increase in cytokine secretion and activation of effector molecules downstream of TLR signaling, such as

NF-κB, JNK, p38 and ERK1/2 [8]. IRAK-M -/- mice infected orally with Salmonella typhimurium, develop more severe enteritis and an increase of both the size and the number of Payer's patches when compared to wild type counterparts [8].Eventually, these animals develop severe osteoporosis, evident in just 4 months after birth due to a substantial increase in the osteoclast number [24]. As shown in ex vivo cell cultures this increase is the net result of accelerated osteoclast differentiation and increased osteoclast survival. Moreover IRAK-M -/- osteoclasts also exhibits a similar intrinsic pro-inflammatory phenotype to their IRAK-M -/- monocytic progenitors: both IL-1 and LPS stimulation hyperactivate the canonical NF-κB pathway and MAPK signaling [24].

IRAK-M deficient mice exhibit higher survival rates when competing in an experimental model for sepsis [30]. Knocking down IRAK-M has also been shown to confer similar effects: siRNA inhibition of IRAK-M is able to reconstitute the secretion of pro-inflammatory cytokines in otherwise TLR-tolerant macrophages [25, 29, 32].
Another indication is the common observation of increased IRAK-M mRNA and protein levels upon TLR ligand stimulation (Table 3). A list of known regulators of IRAK-M expression can be found in Table 1. Whereas, a basal level of IRAK-M expression is present in unstimulated human and murine monocytes/macrophages [8,10,36], transcription of IRAK-M is exponentially induced shortly after TLR ligand stimulation and progresses in fashion analogous to stimulus strength: higher concentrations of TLR ligands and longer incubation periods confer increased IRAK-M mRNA levels [4, 8, 35, 36]. In all listed publications, increased IRAK-M mRNA and protein levels effectively correlate with the consolidation of tolerance in effector cells of innate immunity. These characteristics of IRAK-M expression have been validated in murine and human models for sepsis [30, 39].

Table 3: a list of all TLRs known to affect IRAK-M expression.

Cell surface receptors	IRAK-M expression	Source
TREM-1	↓	Lagler et al., 2009
TLR2	↑	Nakayama et al., 2004
		Kim et al., 2008
TLR4	↑	Kobayashi et al., 2002
		Mages et al., 2007
TLR5	↑	Oshima et al., 2010
TLR7	↑	Hassan et al., 2009
		Hayashi et al., 2009
TLR9	↑	Kim et al., 2008
		Tazi et al., 2009

- **Intracellular signaling, cell surface molecules and soluble factors**

Despite the firm association of IRAK-M to endotoxin tolerance and the relative good understanding of its mechanism of function, too few are known about the actual transcriptional regulation of this gene: whereas cytokines (such as IL1[24], and TNFa [27]) and many TLR ligands - such as LPS (TLR4 agonist) [8], hyaluronan [28] and Pam3CSK4 [34] (TLR2 agonists), have all been shown to promote IRAK-M expression, the mechanisms enabling these inductions are still well hidden. A list of all molecules shown to regulate IRAK-M is summarized at table 4. IRAK-M promoter

is not yet fully characterized and the only firm association between specialized transcription factors and IRAK-M expression was shown only recently. Using chromatin immunoprecipitation and EMSA it was found recently that the up-regulation of Irak-m expression by TLR9 is controlled at the transcriptional level through multiple transcription factors, including NF-kB, AP-1, and CREB [86]. Unpublished ChIP data from our lab also implicates other transcription factors in IRAK-M regulation such as CEBPb, SMAD4 and STATs. Moreover, PU.1 has been shown to promote IRAK-M transcription: IRAK-M is not transcribed in the mAM (mouse Alveolar Macrophage cell line derived from GM-CSF deficient animals), in which PU.1 coupled transcription is not present. Adenoviral reconstitution of PU.1 salvages IRAK-M transcription [44]. However PU.1 responsive sequences in the IRAK-M promoter are not characterized and direct binding of PU.1 is not exhibited, suggesting that the contribution of PU.1 may be primarily towards lineage commitment of the cells to the monocytic/macrophage type, where IRAK-M is predominantly expressed.

The elucidation of the molecular pathways that enable IRAK-M transcription could help in identifying putative key transcription factors. Since the main stimulus for IRAK-M induction is signaling through the TLR cascade of events, transcription factors that are activated by this process should have an impact in IRAK-M transcription. In this way TRL stimulation is self restricted and IRAK-M could help in forming a negative feedback loop that inhibits excess monocyte/macrophage activation. Moreover, other molecules with immunomodulatory properties such as hormones or growth factors could also have an effect in IRAK-M transcription. In this sense, 6-Methylprednisone (6-MP) is able to suppress IRAK-M transcription in in vitro differentiated osteoclasts [45]. 6-MP incubated osteoclasts exhibit an activated phenotype coupled with ERK activation [45].

On the other hand, globular adiponectin (gAd), has been shown to increase IRAK-M expression and promote tolerance in mouse macrophages [36]. gAd however cannot repeat this phenomenon in the presence of MEK and/or Akt1 inhibitors (UO126 and wortmannin respectively) or in the background of akt1 and tpl2 deficient mouse macrophages. Thus, adiponectin could upregulate IRAK-M transcription through Akt1 and tpl2-ERK1/2 mediated processes [36]. These two signaling pathways have been repeatedly shown to regulate transcription factors involved in inflammatory processes. Lastly, despite the lack in experimental data, a role for TGF-β signaling and other anti-inflammatory cytokines in IRAK-M induction should not be excluded. TGF-β has been shown to induce endotoxin tolerance [46]. Since tolerant macrophages do secrete TGFβ, paracrine or autocrine mechanisms could be involved in IRAK-M upregulation upon tolerance. Moreover, a recent report attributes to SMAD4 a direct effect in IRAK-M transcription [58].

Synthetic gangliosides GM1 and GD1a have also been shown to inhibit TLR signaling and promote rapid and reversible tolerance in human peripheral blood monocytes. While the mechanisms governing this phenomenon are largely unknown, enrichment of cell membrane with gangliosides is reported to upregulate IRAK-M [47].

Lastly recent reports attribute another level to IRAK-M regulation: cell trafficking and compartmentalization. In the enterocytes TLR signaling takes places in the Golgi apparatus[48].In an experimental model of necrotic enterocolitis, TLR9

signaling is shown to attenuate the pro-inflammatory, pro-apoptotic TLR4 signaling, thus exerting an anti-inflammatory and protective effect in the intestinal epithelial[49]. In cultured IEC-6 cells this phenomenon is exerted via IRAK-M. In just 30 minutes upon CpG (TLR9 ligand) stimulation, IRAK-M displays increased co-localization with TLR4 in the Golgi apparatus, whereas co-localization of IRAk-1 and TRAF6 is severely reduced. Moreover knocking down IRAK-M reinstates proinflammatory features observed upon TLR4 signaling. Importantly CpG induction to IRAK-M, cannot be attributed to transcriptional activation since this phenomenon has rapid kinetics. Thus TLR9 signaling in intestinal epithelia could facilitate the redistribution of pre-formed IRAK-M [49].

Utilizing cell fragmentation assays in THP1 cells and in situ immunofluorescence in murine bone marrow derived macrophages, Su et al [22] illustrated that IRAK-M distribution involves both the cytoplasmic and the nuclear compartment. Upon Pam3CSK4 stimulation, IRAK-M becomes exclusively cytoplasmic whereas incubation with leptomycin, a nuclear export inhibitor retains it inside the nucleus. The biological significance of this finding requires further clarification.

Table 4: a list of all molecules shown to regulate IRAK-M.

Soluble factors	IRAK-M expression	Source
Adiponectin	↑	Zacharioudaki et al. 2005
α-melanocyte-stimulating hormone	↑	Taylor AW, 2009
PGE2	↑	Hubbard et al., 2010
Gangliosides	↑	Shen et al., 2008
Hyaluronan	↑	Yatabe et al., 2009
GM-CSF	↑	Berclaz et al., 2007
IL-13	↓	Scotton et al., 2005
TGF-β1	↑	Pan et al., 2010
Man-LAMs	↑	Pathak et al., 2005
GNSO	↑	Gonzalez-Leon et al. del Fresno et al., 2004
Intracellular signaling molecules		
PI3K	Unclear	Fukao et al., 2003 Taylor AW, 2005

5. IRAK-M clinical significance

IRAK-M has various roles in immunopathology depending on the disease setting. For example, in the setting of chronic inflammatory diseases, IRAK-M expression is beneficial because it can limit excessive immune responses. In contrast, IRAK-M expression may prevent proper innate immune clearance of pathogens in the context of immunodeficiency. Thus, from a clinical point of view, it is useful to understand situations in which modulation of IRAK-M expression may offer therapeutic benefit.

Collectively it is clear that IRAK-M has potent immunomodulatory properties and is able to promote tolerance under various circumstances. For example prolonged secretion of endotoxins by persistent infection or gut damage can result in septic shock and multi-organ damage. However, upregulation of IRAK-M is of extreme importance in the induction of endotoxin tolerance. This function also

reflects the fact that the host establishes tolerance against normal gastrointestinal tract flora [81]. In a skin allograft model the co-stimulatory blockade procedure used to prolong graft survival is impaired in the absence of IRAK-M and a subsequent *influenza* infection suggests that IRAK-M expression may limit immunopathology without decreasing viral clearance [84]. While IRAK-M may serve an important role in limiting improper immune activation, there are cases in which upregulation of IRAK-M prevents appropriate host defense. For example, in a periodontal tissue infection, an insufficient immune response is observed and thus IRAK- M induction is probably the mean of escape from immune surveillance [83]. Furthermore deficiency in IRAK-M can improve the ability of septic mice to resolve secondary lung infection [30].

Moreover, the ability of IRAK-M to induce endotoxin tolerance correlates with numerous clinical and epidemiological data: IRAK-M deficiency predispose individuals to inflammatory bowel disease (IBD), as IRAK-M is on the genetic susceptibility locus for IBD [82] and monocytes derived from 5 patients suffering from moderate sepsis, upon restimulation with LPS revealed a tolerant phenotype as a result of IRAK-M upregulation [25]. As it is well known, the tumor microenvironment is immunosuppressive, and this may involve the induction of IRAK-M in tumor-associated macrophages (TAMs). Monocytes from patients suffering from chronic myeloid leukemia exhibit high levels of IRAK-M expression [28]. In addition, Xie et al. showed that IRAK-M deficient mice are resistant to melanoma and fibrosarcoma tumor growth following tumor immunization [50]. This is likely attributed to the enhanced anti-tumor effector function of T and B cells in the absence of IRAK-M, given that they display elevated proliferation and activation [50]. Also, monocytes from 30 children suffering from Multi Organ Dysfunction Syndrome (MODS) exhibited increased levels of IRAK-M mRNA [40]. Similar observations were made in a study of 34 ACS (Acute Coronary Syndrome) patients [41]. High IRAK-M levels were shown to correlate with mortality in Gram (-) sepsis [42]. Moreover, mutations in coding sections of the irak3 gene (asthma susceptibility region 12q13-14) have been linked with early onset persistent asthma in Italian populations [43]. Lastly, it has been recently shown that IRAK-M is downregulated in monocytes of obese persons and thus it has been suggested that low IRAK3 in combination with high SOD2 expression and subsequent increase in mitochondrial oxidative stress is a marker of Type 2 diabetes [111].

6. Epigenetic control of macrophage activation/inactivation

The term epigenetics refers to modifications that do not modify the genetic code but instead control how information encoded in DNA is expressed in a tissue and context-specific manner [74]. Epigenetic components and mechanisms are normally interceded by posttranslational modifications (such as phosphorylation, acetylation, methylation etc) of histones and other chromatin proteins that bind DNA, by methylation and hydroxyl methylation of CpG DNA motifs, and by non-coding RNA [75–79]. These epigenetic marks have been considered to be stable, possibly transmissible to offspring, and to underlie steady differentiation into various tissues and cell types that express distinctly different patterns of gene expression,

despite containing identical DNA sequences and genomes. Lately it has become clear that epigenetic chromatin marks are dynamically regulated in response to environmental stimuli. This has resulted in a shift in the utilization of epigenetics to incorporate transient changes in chromatin state or DNA methylation in response to external stimuli that control gene expression [74]. Despite the fact that epigenetic marks are dynamically regulated, they are normally more stable than the quickly fluctuating post-translational modifications of upstream 'traditional' signaling proteins. Subsequently, epigenetic changes that continue to carry on after the original stimulus has ended, provide a mechanism for extending temporary short-lived signals into a more stable and constant cellular response lasting quite a few hours or days (or longer).

A model that has been developed is that the 'epigenetic landscape' of a cell which includes the total patterns of DNA methylation, chromatin modifications, and proteins pre-bound to gene regulatory regions (promoters and enhancers) determines the accessibility for binding and therefore the genomic localization of signaling transcription factors that are activated by acute signals [74, 80]. Consequently, the pattern of gene expression in accordance to an external stimulus is formed by the developmental history of a cell and previous environmental exposures that have shaped the epigenetic landscape. The epigenetic landscape, in turn, could be reformed in response to acute stimulation and polarizing stimuli. Such remodeling of the epigenetic landscape helps to eventually integrate signals and guides reprogramming of cells to change their gene expression pattern in response to subsequent stimuli.

Analysis of the epigenetics of macrophage polarization until now has primarily focused on post-translational modification of histones, with partial analysis of ATP-dependent nucleosome remodeling. There is a huge amount of histone modifications, which can be generally divided into positive and negative marks that promote or suppress transcription, respectively [75, 79, 80]. Table 5 shows the most broadly studied marks relevant for macrophage activation / inactivation (polarization). Whereas trimethylation of histone 3 lysine 4 (H3K4) is associated with active gene transcription, trimethylation of H3K9, H3K27 and H3K79 are linked to silencing of gene expression. These histone marks are 'written' and 'erased' by enzymes called chromatin regulators. The pattern of histone marks forms a sort of 'code' that is 'read' by supplementary chromatin regulators and transcriptional co-activators / co-repressors to determine the rates of transcription initiation and elongation. Therefore, the equilibrium of positive and negative histone marks at gene promoters and enhancers determines transcription rates. The current chromatin state, defined by well-established combinations of histone marks, determines basal transcription level, the extent and kinetics by which a gene locus responds to extracellular stimulus [75, 79, 80]. A key concept is that gene loci relevant for polarized macrophage phenotypes exist in three broad states [71–73]. First, there is a repressed state characterized by the presence of negative marks such as histone 3 lysine 9 trimethylation (H3K9me3) and H3K27me3, absence of positive marks, and a closed chromatin conformation. These genes are refractory to fast induction by activating stimuli. Second, there is a poised state characterized by the existence of activating histone marks (H3K4me3, H3K9, 14-Ac), chromatin conformation that is partially open, and in some cases, a pre-bound RNA polymerase

II that is paused near the transcription start site. Transcription at poised genes is controlled by concurrent presence of the repressive histone marks such as H3K9me3 and H3K27me3, co-repressor complexes, and partially closed chromatin that requires additional positive histone marks and ATP-dependent nucleosome remodeling to be fully accessible to transcription factors. Lastly, there is a third active state that is characterized by active histone marks, an open chromatin conformation, and ongoing transcription.

Table 5: Histone marks that promote or suppress transcription

	Function	Location	Writer	Eraser
H3K4me3	+	P	MLL	KDM5B
H3K9,14-Ac	+	P,E	HAT (CBP/p300)	HDAC3
H4K5,8,12, 16-Ac	+	P,E	HAT (CBP, MOF)	HDAC
H3K27-Ac	+	E,P	HAT (CBP)	HDAC1,2
H3K4me1	+	E	MLL	LSD1/KDM1A
H3K9me3	–	E,P	G9a	JMJD2
H3K27me3	–	P,E	EZH2 (PRC2)	JMJD3, UTX

[a]Ac, acetylation; CBP, CREB binding protein; E, enhancer; H, histone; K, lysine; KDM, lysine demethylase; me, methylation; MLL, mixed lineage leukemia; P, promoter; PRC2, polycomb related complex 2.

- **Histone modifications in M1/M2 macrophage polarization**

Histone acetylation is induced in response to TLR stimulation in macrophages, and is involved in the expression of multiple pro-inflammatory cytokine genes. Acetylated histones are recognized by the bromodomain and extra terminal domain (BET) family of proteins. A small compound (I-BET) interacting with the bromodomain has been identified and this compound was shown to suppress inflammatory gene expression in TLR-stimulated macrophages by disrupting chromatin complexes [85]. Treatment with I-BET rendered mice resistant to endotoxin shock and bacteria-induced sepsis, suggesting that inflammatory responses can be controlled by regulating epigenetic changes on pro-inflammatory gene promoters. Furthermore, trimethylation of H3K4 on cytokine gene promoters was also shown to be induced in M1 macrophages in response to TLR stimulation, indicating that a change in histone modification is induced in the course of M1 macrophage activation leading to chromatin remodeling and inflammatory gene expression [71].

Polycomb repressive complex 2 (PRC2) composed of Ezh2, Suz12 and Eed mediate the methylation of H3K27 [87]. On the other hand demethylases that are known to act on H3K27 by catalyzing trimethylation of H3K27me3 to monomethylation H3K27me1 and harbor a Jumonji-C (JmjC) domain are Jmjd3 (also known as Kdm6b), UTX and UTY, [88–90]. Importantly, the expression of Jmjd3 is induced by TLRs in macrophages via an NF-kB-dependent pathway. In view of the fact that H3K27 trimethylation is implicated in the silencing of gene expression, it has been postulated that Jmjd3 is involved in the fine-tuning of macrophage activation

toward M1 by regulating a set of genes such as Bmp2 and Hox [90, 91]. On the other hand, production of proinflammatory cytokines in response to TLR ligand stimulation was not deregulated in Jmjd3 -/- macrophages, and cytokine production in response to *Listeria monocytogenes* infection was unaffected by Jmjd3 deficiency [92]. Therefore, Jmjd3 is not essential for M1 macrophage polarization. On the contrary, Jmjd3 is crucial for M2 macrophage polarization to helminth infection and chitin administration in mice. Chitin (a polymerized sugar and a structural component of helminths, arthropods and fungi [93]) administration recruits M2 macrophages to the administration site that is important for the following recruitment of eosinophils [93, 94]. Jmdj3 -/- bone marrow chimeric mice were defective in the expression of M2 macrophage markers in $F4/80^{+}CD11b^{+}$ macrophages and eosinophil recruitment in response to administration of chitin. Additionally, activation of M2 macrophages following *Nippostrongylus brasiliensis* infection was severely affected in the absence of Jmjd3. More specifically when GM-CSF was used to induce M1 macrophages from the bone marrow, the production of TNFa and IL-6 in response to TLR ligands was comparable between wild-type and Jmjd3 deficient chimeras [92]. In contrast, expression of the M2 marker genes like Arg1, Ym1, Fizz1, MR and IL-13 was severely impaired in Jmjd3 deficient bone marrow macrophages cultivated in the presence of M-CSF which induces M2 polarization. This observation indicates that Jmjd3 is crucial for the expression of M2 marker genes in bone marrow macrophages. Even though M-CSF-induced or chitin-induced bone marrow macrophages revealed severe defects in M2 marker expression in the absence of Jmjd3, Jmjd3 -/- macrophages were capable of upregulating the expression of M2 genes following IL-4 stimulation. Such data suggest that Jmjd3-mediated H3K27 demethylation is not necessary for the M2 polarization in response to IL-4, and that M2 macrophages should be further subcategorized depending on their requirement for Jmjd3. The expression of the C-terminal part of Jmjd3 containing the JmjC domain, but not its demethylase-defective mutant, was adequate to salvage M2 marker expression in Jmjd3 deficient bone marrow macrophages [88–90]. Conversely, HDAC3 acts as a brake on IL-4-induced M2 polarization by deacetylating putative enhancers of IL-4-induced M2 genes [98]. Consequently, Jmjd3 acts as a demethylase to induce M2 macrophage polarization, although recent studies show a demethylase-independent role in controlling chromatin remodeling [96]. Thus, both histone methylation and acetylation are important for M2 polarization.

In general chromatin immunoprecipitation sequencing analysis shows that trimethylation of H3K27 is enriched in the promoter regions close to the transcription start sites in bone marrow macrophages. M2 marker genes, such as Ym1, Mrc1 and Arg1, were not trimethylated at H3K27 either in the presence or in the absence of Jmjd3, suggesting that these genes are not directly controlled by Jmjd3 through histone modification. Conversely, H3K27 trimethylation of transcription factors such as Irf4 and CEBPb was differentially regulated between wild-type and Jmjd3 deficient macrophages. Irf4 expression was reduced in Jmjd3 deficient macrophages, and its expression was restored in a Jmjd3 demethylase-dependent manner. Indeed, Irf4 deficient mice showed severe defects in M2 macrophage polarization in the presence of M-CSF or in response to chitin administration. While Jmjd3 also controls a set of transcription factors, Irf4 and possibly CEBPb are critical target genes responsible for controlling M2 macrophage

polarization. Differential involvement of IRF and other transcription factors can be important for M1 and M2 macrophage polarization. For example it has been shown that IRF5 is associated with the M1 polarizing phenotype, even though it is unclear whether Irf5 is epigenetically controlled by histone modifications [97].

7. Purpose of this study

Knowledge on IRAK-M transcriptional and epigenetic regulation as stated before is limited. This study aims to elucidate the mechanisms governing IRAK-M expression at an epigenetic level in the initiating events that confer tolerance to LPS. We aimed to identify key chromatin regulators and transcription factors that enable this process and decipher their own regulation in the context of inflammation and endotoxin tolerance, events that are timely and functionally discriminated.

B. Materials and Methods

1. Protocols

Cells used

RAW264.7 macrophages (mus musculus): Abelson murine leukemia virus transformed.

Immunofluorescence

Five thousand per well sample RAW 264.7 mouse macrophages were plated in 384 well plates containing 30 ul DMEM (10% FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin). Cells were starved for 10-12 hours, transfected with 30nM of the specified siRNA using Invitrogen Lipofectamine RNAi Max according to manufacturer's instructions for 24 hours and then incubated in the presence or absence of 100 ng/ml LPS for the indicated time periods. Upon endpoints cells were fixed with 4% PFA, blocked with blocking buffer (5% FBS, 0.1% Triton X-100, 0.01% Saponin, 1x PBS) for 30 minutes, incubated with primary antibody O/N at 4°C and with secondary antibody (including counter stain with DAPI-Hoescht) for 2 hours at RT (wash 5x with 1x PBS between each step with microplate washer). Images acquired with Autoscope (Metamorph software) and signal intensities (sum of all positive pixels divided by the number of cells) were measured and analyzed using Definiens Developer XD. Finally scatter plots were generated using Spotfire.

Primary antibodies used:
Abcam: IRAK-M (ab8116): dilution 1/100.
Arginase1 (ab60176): dilution 1/50
iNOS (ab15323): dilution 1/50

Cell Signaling: GAPDH (D16H11): dilution 1/100.

Secondary antibodies and counterstains used:
Invitrogen: Alexa fluor 647 goat-antirabbit IgG 2mg/ml : dilution 1/500.
Alexa fluor 488 donkey anti-goat IgG 2mg/ml : dilution 1/500.

DAPI-Hoescht : dilution 1/10000.

Quantitative RT-PCR

One million per sample RAW 264.7 mouse macrophages were plated in 24 well plates containing 0.5 ml DMEM (10% FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin). Cells were starved for 10-12 hours, transfected with 30nM of a specified siRNA using Invitrogen Lipofectamine RNAi Max according to manufacturer's instructions for 24 hours and then incubated in the presence or absence of 100 ng/ml of LPS for the indicated time periods. Upon endpoints wells

were washed with ice cold PBS and RNA was isolated with the InviTrap® RNA Cell HTS 96 Kit according to manufacturer's instructions.

The reverse transcription was performed with Applied Biosystems High Capacity cDNA Reverse Transcription Kit (using 1 μg isolated RNA as template) according to manufacturer's instructions.

Real time PCR reaction was performed with the Bioline SensiMix SYBR Hi-ROX Kit according to manufacturer's instructions. Amplification was performed in an ABI PRISM 7900 HT Real- Time PCR 384 well-plate apparatus for a maximum of 45 cycles, as follows: Start steps: 30min at 50°C, 15min at 95°C, Repeat steps: 15 s at 95°C, 30 s at 60°C, and 30 s at 72°C. No by-products were present in the reaction, as indicated by the dissociation pattern provided at the end of the reaction. The amplification efficiency of the mouse IRAK-M product was the same as the one of beta-actin, as indicated by the standard curves of amplification, allowing us to use the formula: fold difference = $2^{-(CtA - CtB)}$, where Ct is the cycle threshold (qBase plus software). Reactions were performed with biological duplicates and technical triplicates, to allow for statistical evaluation (Graphpad Prism software).

Primers used in Real Time-PCR:

Gene	Forward primer 5'	Reverse primer 3'
EZH2	GTGCAGTTATTCCTTCCATGC	ACGCTCAGCAGTAAGAGCAG
EZH2 (BOTH VARIANTS)	CTTCCTACATCCCTTCCATGC	TGGGCGTTTAGGTGGTGTC
MLL2	CAGCAGCTGGCGAATAGTTTC	ACTTGCTGCCTGTTCATACCA
DOT1L	CTGGTGGCCCAGATGATTGA	GTCCATGGTCTCTGCGTACT
ASH1	TGTGATCGAGGCTGTGGTTC	AGGCGTTTCAAGAGGACTGG
NSD1	ATCTGACAAAGCGACCAGCA	TACAAGACAGACGCCCATCG
UTX	GCACCACCTCCAGTAGAACAA	GTCTCATTTGGTGTTGCTGCAT
KDM2A	AAGCAATCCTGTGTCCTCCG	ATTCCTCGTTCAGCAACCCC
PHF8	GAAAGAAGCTTTGCCAGACCA	GCTGGTACCACTCATGTTGA
LSD1 (KDM1A)	CAGACATCATCAGTGGGCCT	GCACAGTATCGCTGTTGTAAGG
CEBPB	GACAAGCTGAGCGACGAGTA	GCTTGAACAAGTTCCGCAGG
SLC9A9	TGTTTGGAGGAGGAACGACC	TATTTGCTTCCTGCTGTGAGGA
SLC9A9	ATGATGCGGTGGCCATAGTC	TGGTCAACAGTGCGGTAACAA
b-Actin	GTCATCACTATTGGCAACGAGC	GCACTGTGTTGGCATAGAGGTC

siRNA sequences:

siRNA Target gene	Structure representation 1	Structure representation 2
Akt2 p1	GAGUCUACAUGGAAGGUCCTC	GGACCUUCCAUGUAGACUCTT
Akt2 p2	GGUAGCUGUCAACAAGGCATT	UGCCUUGUUGACAGCUACCTC
SIRT6	GACGUACUGCGUCUUACACTT	GUGUAAGACGCAGUACGUCTT
PHF8	AAUCUCGACCCAAGAAAAATT	UUUUUCUUGGGUCGAGAUUTC
IRAK-M p1	CUCUUGUUCCACUUUUACCTT	GGUAAAAGUGGAACAAGAGTT
IRAK-M p2	UUCAAUAUGACGAACAUCCAG	GGAUGUUCGUCAUAUUGAATT
SP1 p1	GCAGAAUUGAGUCACCCAATT	UUGGGUGACUCAAUUCUGCTG

SP1 p2	UUCAUAAUUCCCAUGUUGCTG	GCAACAUGGGAAUUAUGAATT
EZH2 p1	AUGUGCACAGGCUGUAUCCTT	GGAUACAGCCUGUGCACAUTT
EZH2 p2	UGACUCUAAACUCAUACACCT	GUGUAUGAGUUUAGAGUCATT
UTX p1	GGUUUACUAAGUUCAGACATT	UGUCUGAACUUAGUAAACCTG
UTX p2	CCAACUAUCUAACUCCACUTT	AGUGGAGUUAGAUAGUUGGTT
LSD1	GCAAACAAGUAAAUAUGGATT	UCCAUAUUUACUUGUUUGCTG
KDM5A	CGCGGCGGAGUUCGUGCCATT	UGGCACGAACUCCGCCGCGTA
CEBPb p1	CUCGAAACGGAAAAGGUUCTC	GAACCUUUUCCGUUUCGAGTT
CEBPb p2	ACGUUUGAUCCGGAUUGCATC	UGCAAUCCGGAUCAAACGUTT
NCoR1	GGAUAAAGCAGAAAAAACATT	UGUUUUUUCUGCUUUAUCCTC
NCoR2	AUAUUUACCCAUGAGUGCCTT	GGCACUCAUGGGUAAAUAUTT
SMAD4	AGAUCACAUGAGGAAAUCCTT	GGAUUUCCUCAUGUGAUCUTT
STAT1 p1	CCUAACCAUUGCUUAUAUATT	UAUAUAAGCAAUGGUUAGGTC
STAT1 p2	GCUGAACUAUAACUUGAAATT	UUUCAAGUUAUAGUUCAGCTC
STAT3 p1	AUUGGCUUCUCAAGAUACCTG	GGUAUCUUGAGAAGCCAAUTT
STAT3 p2	CAUUUUCUGUUCUAGAUCCTG	GGAUCUAGAACAGAAAAUGTT
STAT6	UGAGCGAAUGGACAGGUCUTT	AGACCUGUCCAUUCGCUCATT
MLL1	GCUGUGUUUCCUGAUGACATT	UGUCAUCAGGAAACACAGCTC
HDAC1	UUUUCGGUAGAGACCAUAGTT	CUAUGGUCUCUACCGAAAATT
KDM2A	GGGAAUUUAAGCUUAUCCCTT	GGGAUAAGCUUAAAUUCCCTT
KDM3B	UUUCGUUUCCAGAAGAGGCAC	GCCUCUUCUGGAAACGAAATT
PRDM1	CCACACAAGAAGUUCCUGGTT	CCAGGAACUUCUUGUGUGGTT
IRF4	ACAGGAACCUUUUAUGCUUTT	AAGCAUAAAAGGUUCCUGUCA
MLL2 p1	GCUGCUUACCAAGAAUAACTT	GUUAUUCUUGGUAAGCAGCTG
MLL2 p2	CCUAUGACUAUCAGUUUGATT	UCAAACUGAUAGUCAUAGGTT
MLL3 p1	AGAAAUAUCAGCUAAUGGGTC	CCCAUUAGCUGAUAUUUCUTT
MLL3 p2	CUUUUUGCACAUAUGGUGCTC	GCACCAUAUGUGCAAAAAGTT
NSD1/KMT3B	GAACCUUAUGCCUAUAUCCTT	GGAUAUAGGCAUAAGGUUCTT
DOT1L/KMT4	CCAUACUUGAAAACUAUUUTT	AAAUAGUUUUCAAGUAUGGTG
BMI1	GGGUACUUCAUUGAUGCCATT	UGGCAUCAAUGAAGUACCCTC
SUZ12 p1	GACGUGCUCCAUUUUCGGCTT	GCCGAAAAUGGAGCACGUCTT
SUZ12 p2	GGAUGUAAGUUGUCCAAUATT	UAUUGGACAACUUACAUCCTT
CARM1/PRMT4	GGAUAGAAAUCCCAUUCAATT	UUGAAUGGGAUUUCUAUCCTG
SETD7	UAAUCCGUCAUCGUCCAGGTG	CCUGGACGAUGACGGAUUATT
ASH1/KMT2H	CCACUUUUAAGUGCUUUCCTT	GGAAAGCACUUAAAAGUGGTT
ARID5B	CCUUUGGACUAUGUUUCAATT	UUGAAACAUAGUCCAAAGGTT
PU.1 / SFPI1	UGGUAGGUCAUCUUCUUGCGG	GCAAGAAGAUGACCUACCATT
CREB1	GCCACAGAUUGCCACAUUATT	UAAUGUGGCAAUCUGUGGCTG
SLC9A9	GGCUAGGUCUGGACCAGAATT	UUCUGGUCCAGACCUAGCCTT
NegF	ACGUGACACGUUCGGAGAATT	UUCUCCGAACGUGUCACGUTT
Ambion NegF	AUACGCGAACCUUACGCGATT	UCGCGUAAGGUUCGCGUAUTT
KIF11	AUUGUCUUCAGGUCUUCAGTT	CUGAAGACCUGAAGACAAUTT

2. Instruments

Model	Type	Manufacturer
Discovery 1	Autoscope	MDC
Casy TT	Cell counter	Casy
CyBi®-Well vario	Pipetting robot	CyBio
7900 HT	RT-PCR	Applied Biosystems PRISM
Hydrospeed	Microplate washer	TECAN
Multidrop 384 Reagent Dispenser	Multidrop dispenser	Thermo Scientific
NanoDrop ND-1000	Nucleic acid quantification	Thermo Scientific

3. Data analysis Software

Software	Type	Developer
CyBio® Composer software	CyBio's platform for controlling CyBio's instruments and system integrations (script editor).	CyBio
Detection System (SDS) Software v2.4.1	High-throughput gene expression and genotyping analysis software.	Applied Biosystems
qBASE plus	mRNA gene expression analysis.	Biogazelle
Definiens Developer XD	It enables the development of image analysis solutions for biomedical images.	Definiens AG
MetaMorph Microscopy Automation & Image Analysis Software	Automated microscope acquisition, device control, and image analysis.	Molecular Devices
Spotfire	High throughput data visualization and graphical representation.	TIBCO
GraphPad Prism v5.0	Statistical Analysis and Graphical representation.	GraphPad Software
NCBI Primer-BLAST	A tool for finding specific primers (using Primer3 and BLAST).	NCBI

C. Results

- **In silico analysis of the IRAK-M promoter for TFBS**.

Four search engines, Genomatix, TFsearch, TRANSFAC professional V10.2 library and rVISTA portal 2.0, all available online, were used for in silico analysis of upstream sequences of the IRAK-M gene. Special focus was attributed to transcription factors following bibliographical evidence putatively linking them to IRAK-M transcription. For example NF-κB (the classical pathway for NF-κB activation is induced by TLR signaling), AP-1 (is known to be induced by various MAPKs among which ERK and JNK, downstream effectors of TLR signaling), PU.1 (stably expressed in cells of monocytic origin, phosphorylated by Akt [54] and thus Imperative for IRAK-M transcription), C/EBPβ (master transcription factor able to induce chromatin remodeling), SMADs (downstream effectors of TGFβ signaling), STATs (implicated in macrophage polarization through their association with SOCS). The results of the searches by these four databases are briefly portrayed in figure 4. Numbering is relative to the translation start site. Analysis showed that irak3 is a TATA-less gene [86]. However, a number of putative transcription factor binding sites were revealed and served as the basis for further programming. It also revealed that significant clustering of putative binding sites is exhibited proximally to the 1st exon. Additionally three NF-kB, three C/EBPb, two CREB, two SP1 and five STAT sites were identified in total.

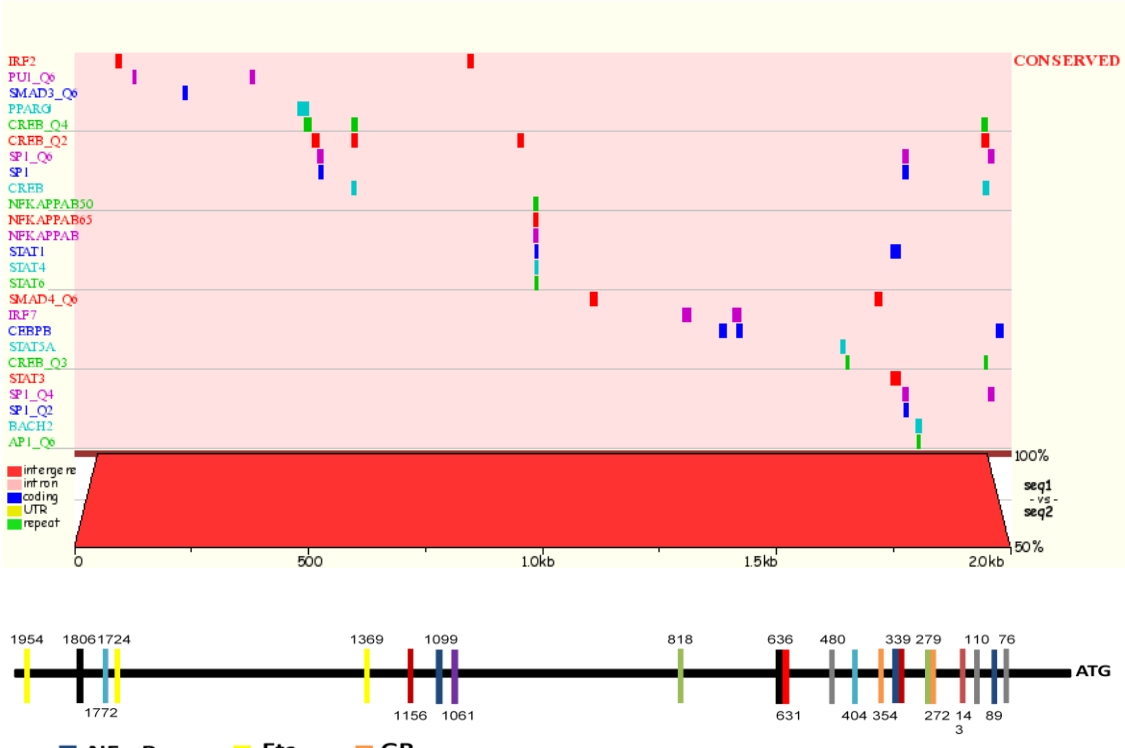


Figure 4: In silico analysis of the promoter (2000 bp upstream of TSS) and identification of binding elements using TRANSFAC professional V10.2 library, rVISTA portal 2.0 (upper panel), Genomatix and TFsearch (lower panel) online prediction engines.

- **Identification of transcriptional and epigenetic regulators of IRAK-M using a small scale RNAi screening**.

Here, we describe a small-scale RNAi screen in RAW264.7 macrophages, in which we examine the roles of 51 loci encoding chromatin proteins and transcription factors. We report more than 20 genes with KD phenotypes, most of which were not previously shown to control IRAK-M expression under LPS stimulation. In depth analysis of a group of hits including genes encoding the PRC2 complex reveals that macrophages lacking this complex over express IRAK-M. We found that UTX, MLL2 and CEBPb are required for the activation of IRAK-M expressed during LPS stimulation.

First, we needed to establish the readout experiment. To do that we treated RAW264.7 macrophages with different LPS concentrations at various time points. Next we stained for IRAK-M and measured the fluorescence intensity of the signal at each condition (sum of all positive pixels divided by the number of nuclei). Treatment of macrophages with 100ng/ml LPS at 48 LPS was sufficient to observe and measure approximately 6 fold difference in IRAK-M intensity (figure 5A).

To identify chromatin proteins important in IRAK-M regulation, we carried out an RNAi screen for chromatin regulators and transcription factors. We employed siRNAs and we typically observed siRNA-mediated KD in > 80% of RAW264.7. When KIF11, a molecular motor protein that is essential in mitosis, knocked down it resulted in apparent proliferation inhibition and extended cell death of nearly all cells (figure 5B). Therefore, siRNAs and transfection conditions are highly effective in RNAi-mediated gene silencing in macrophages.

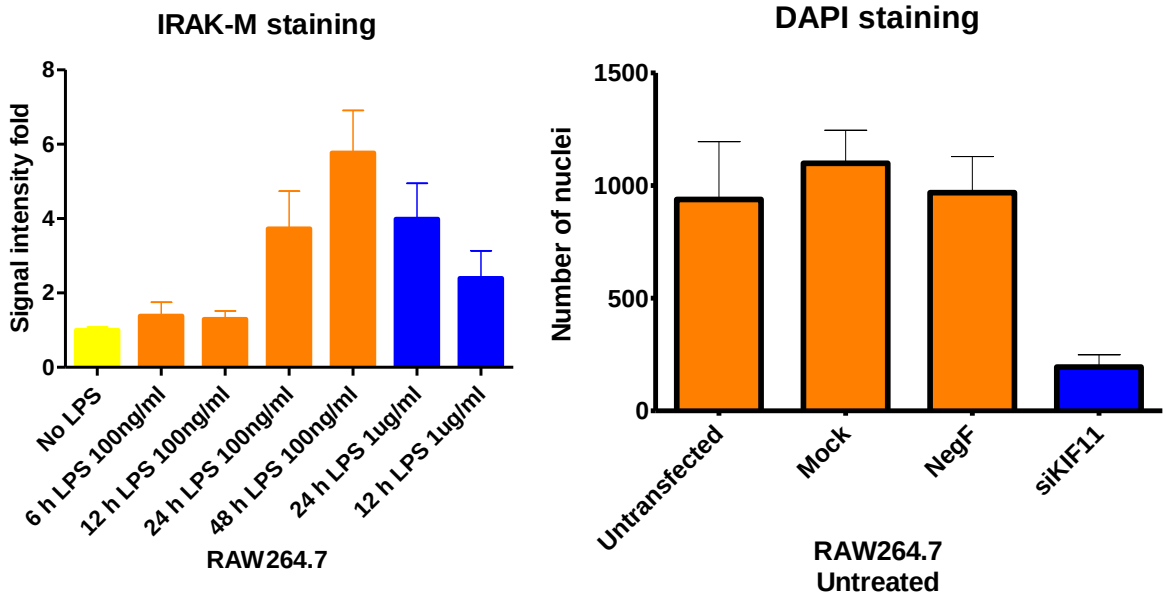
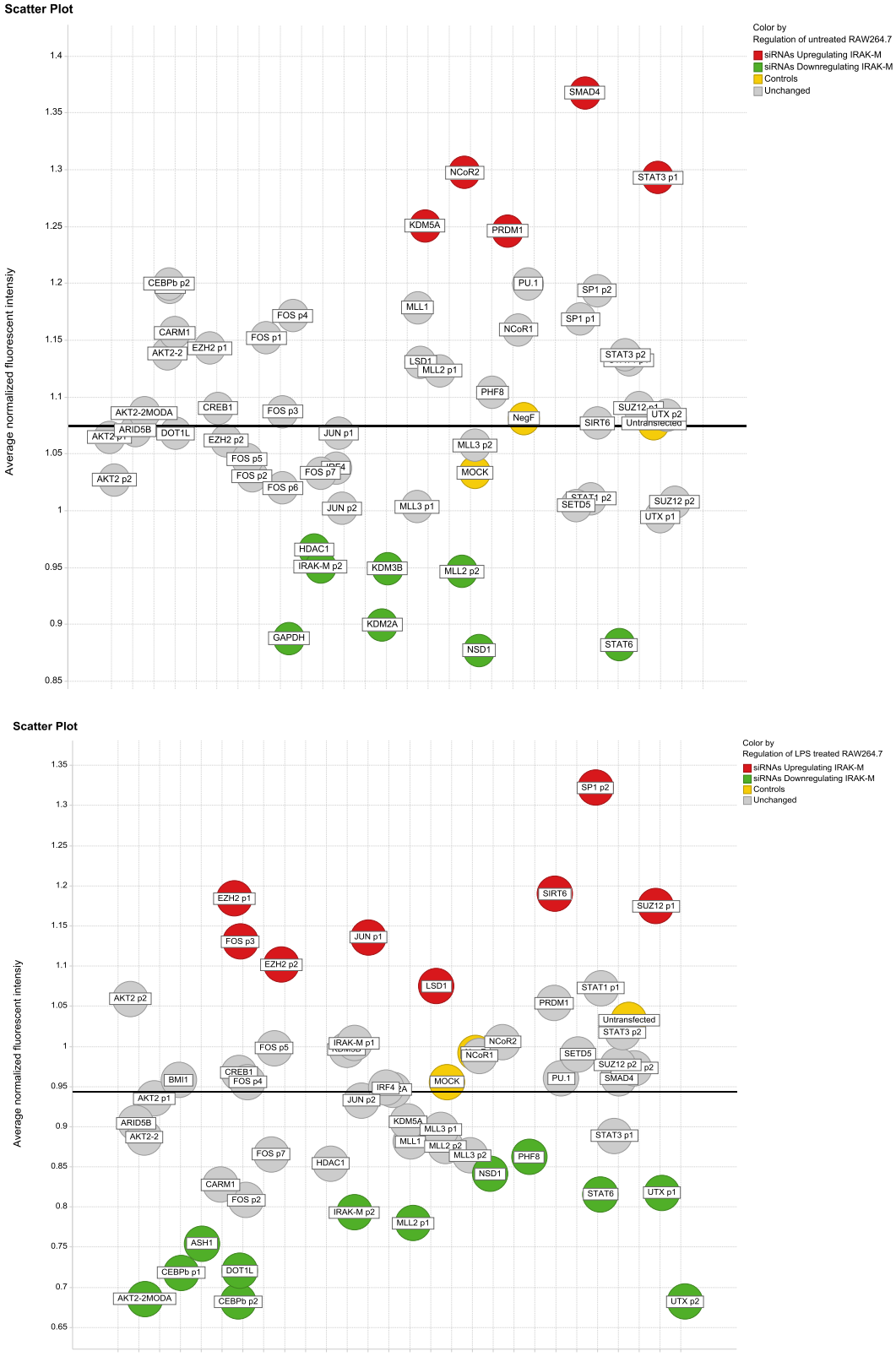


Figure 5. A (left). Treatment of RAW265.7 macrophages with the indicated LPS concentrations and various time points. B (right). Cell viability after transfection with siRNA targeting KIF11.

Next, we selected siRNAs directed against most known or predicted chromatin structural and regulatory proteins as well as transcription factors and transfected them individually into mouse macrophages. Each KD was scored for

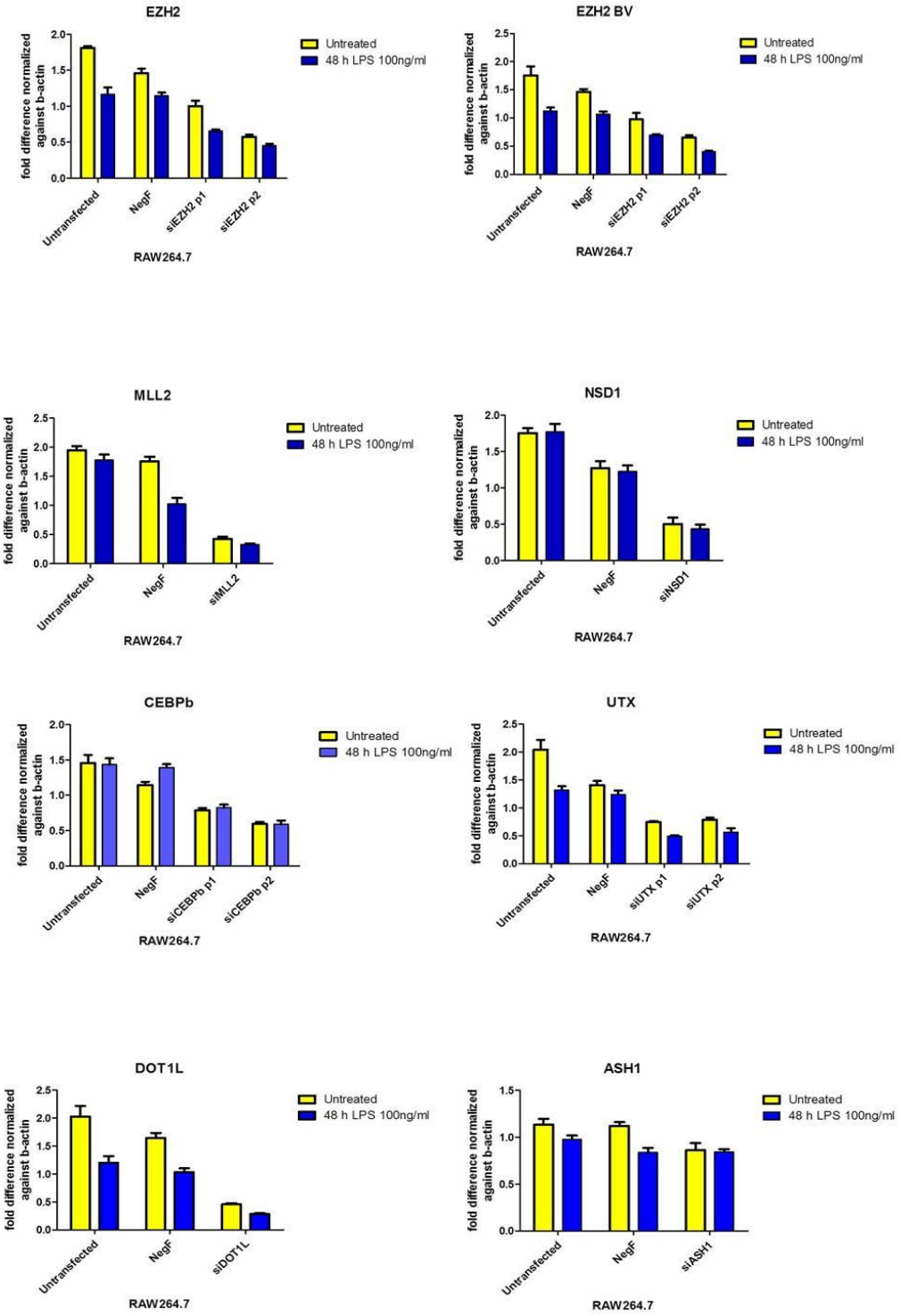
alterations in IRAK-M expression, viability and alterations in cell morphology, each tested twice (figure 6).

Figure 6. Scatter plots showing (Upper plot) IRAK-M IF signal intensity in untreated macrophages following transfection with indicated siRNAs. (Bottom plot) IRAK-M IF signal intensity in LPS (100 ng/ml) treated macrophages for 48 hours following transfection with indicated siRNAs. With red siRNAs upregulating IRAK-M, with green siRNAs downregulating IRAK-M, with grey siRNAs not affecting IRAK-M, and with yellow the controls (untransfected, mock, Negf).



To validate the hits we retested 11 of the top hits with Real Time qPCR both for their direct target gene (figure 7) and the effect on IRAK-M expression. Seven out of eleven (EZH2, CEBPb, MLL2, PHF8, UTX, NSD1, SLC9a9) reproduced the IF results (figure 8). Furthermore, these data strongly suggest that we identified a few false positives probably due to off target effects.

Figure 7. Real Time qPCR of the expression of each selected group of genes (EZH2, MLL2, NSD1, CEBPb, UTX, DOT1L, ASH1, KDM2A, PHF8, LSD1, SLC9A9) to be validated for direct targeting by the siRNAs upon treatment with LPS 100ng/ml for 48 hours.



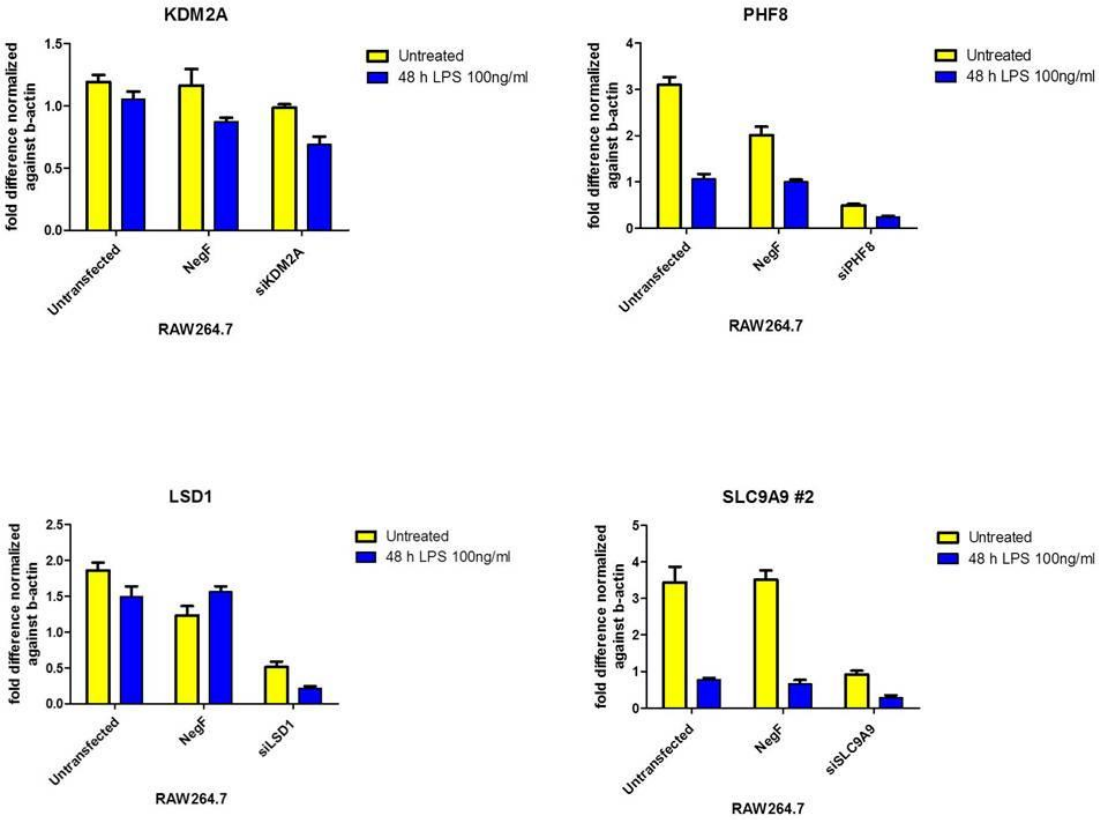
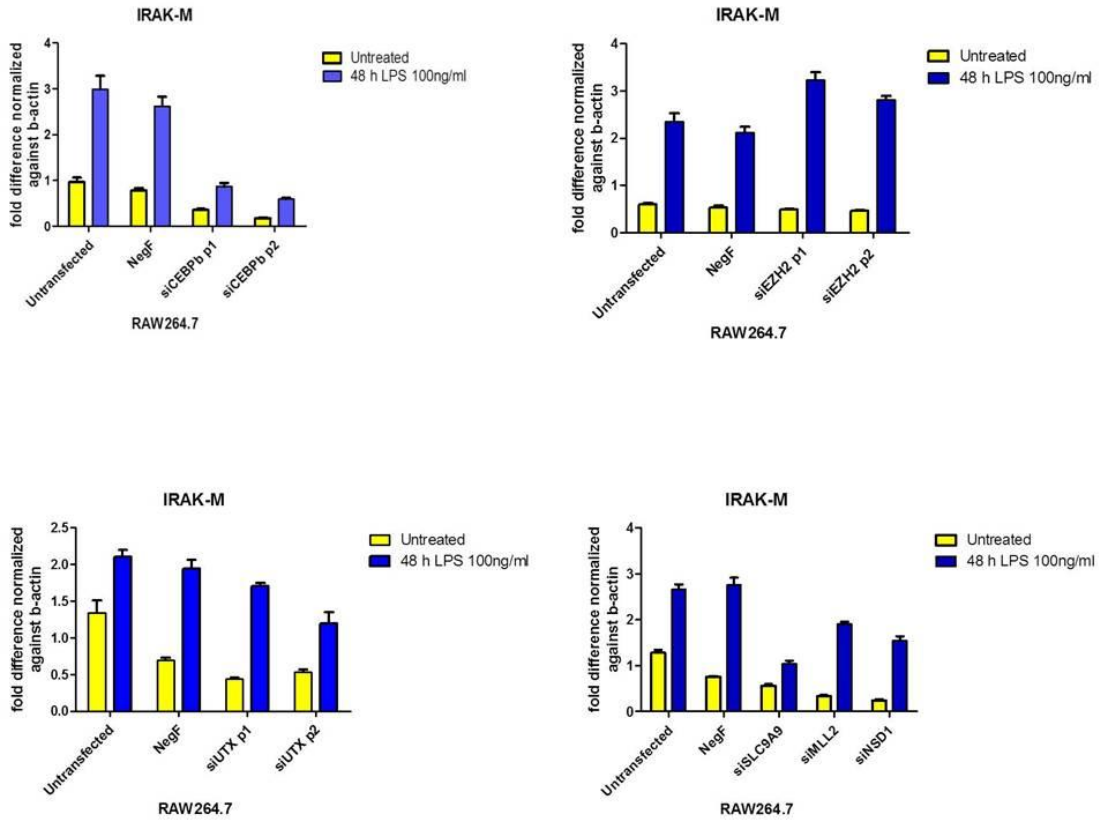
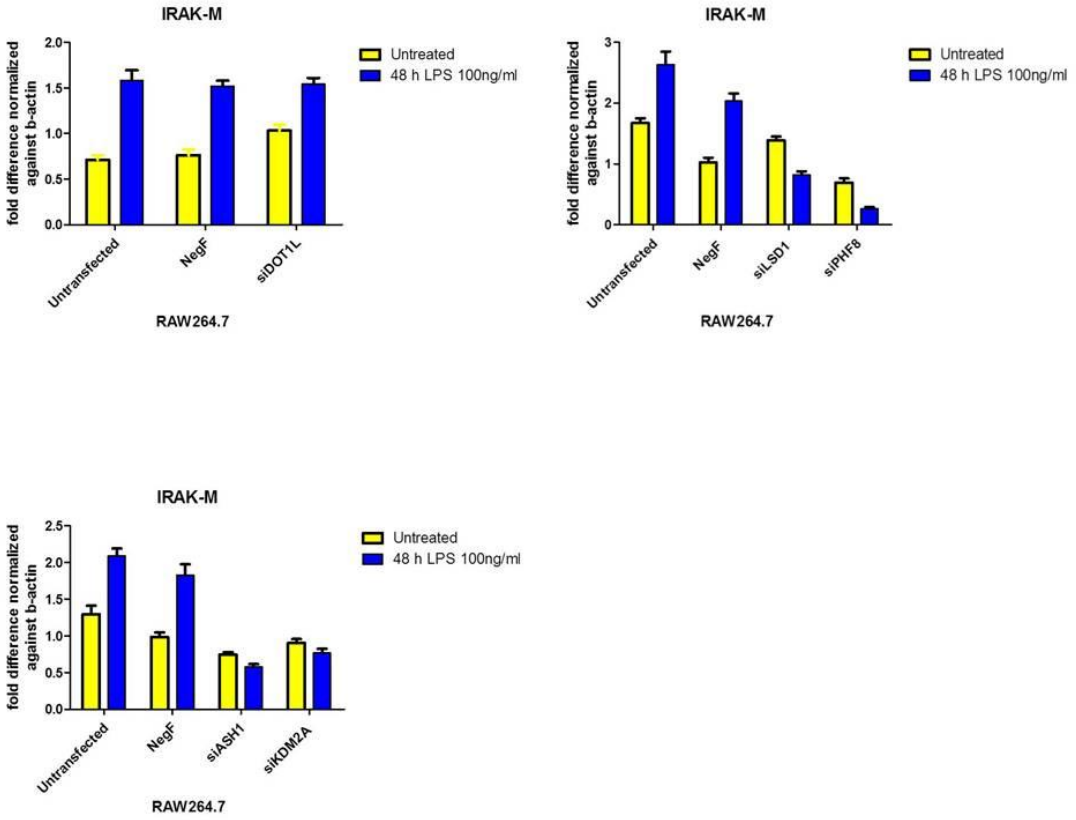


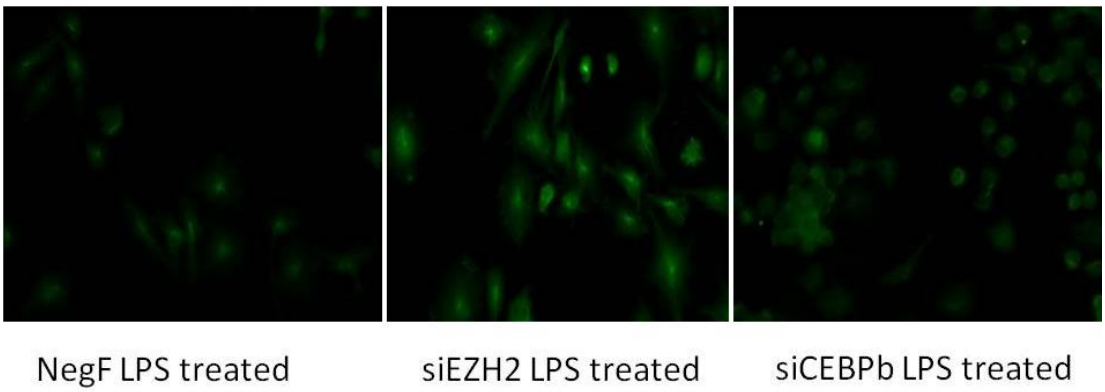
Figure 8. Real Time qPCR of IRAK-M expression on selected group of genes (EZH2, MLL2, NSD1, CEBPb, UTX, DOT1L, ASH1, KDM2A, PHF8, LSD1, SLC9A9) targeted by the siRNAs upon treatment with LPS 100ng/ml for 48 hours.





We observed two classes of KD phenotypes: death or alterations in macrophages morphology. The most prevalent KD phenotype was reduced viability, common among targets like PHF8 that are important for fundamental cellular processes. In addition, KD of some genes caused alterations in macrophage morphology. For example, depletion of the transcription factor CEBPb, resulted in inactivated cells following stimulation with LPS or depletion of EZH2 resulted in hyperactivation of macrophages (figure 9).

Figure 9. Fluorescent images of RAW264.7 transfected with scramble siRNA (Negf), siEZH2 and siCEBPb following treatment with LPS 100ng/ml for 48 hours.



- **Identification of transcriptional and epigenetic regulators of Arginase 1 and iNOS using a small scale RNAi screening**.

To further examine the ability of the chromatin regulators and transcription factors identified to influence IRAK-M expression and thus the outcome of inflammation we checked whether they also regulate hallmarks of macrophage polarization such as iNOS and Arginase1. To do that we first needed to establish the readout experiments. To this end, we treated RAW264.7 macrophages with LPS (100ng/ml) for 48 hours. Next we doubled-stained for Arginase 1 and iNOS and measured the fluorescence intensity of the signal at each condition (sum of all positive pixels divided by the number of nuclei). Treatment of macrophages with 100ng/ml LPS at 48 LPS was sufficient to observe significant fold difference both in Arginase1 and iNOS intensity (figure 10).

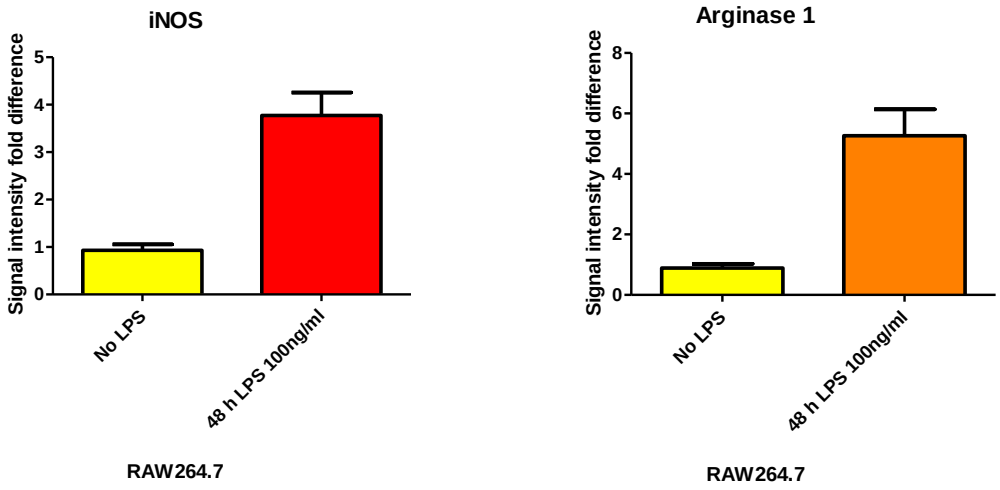
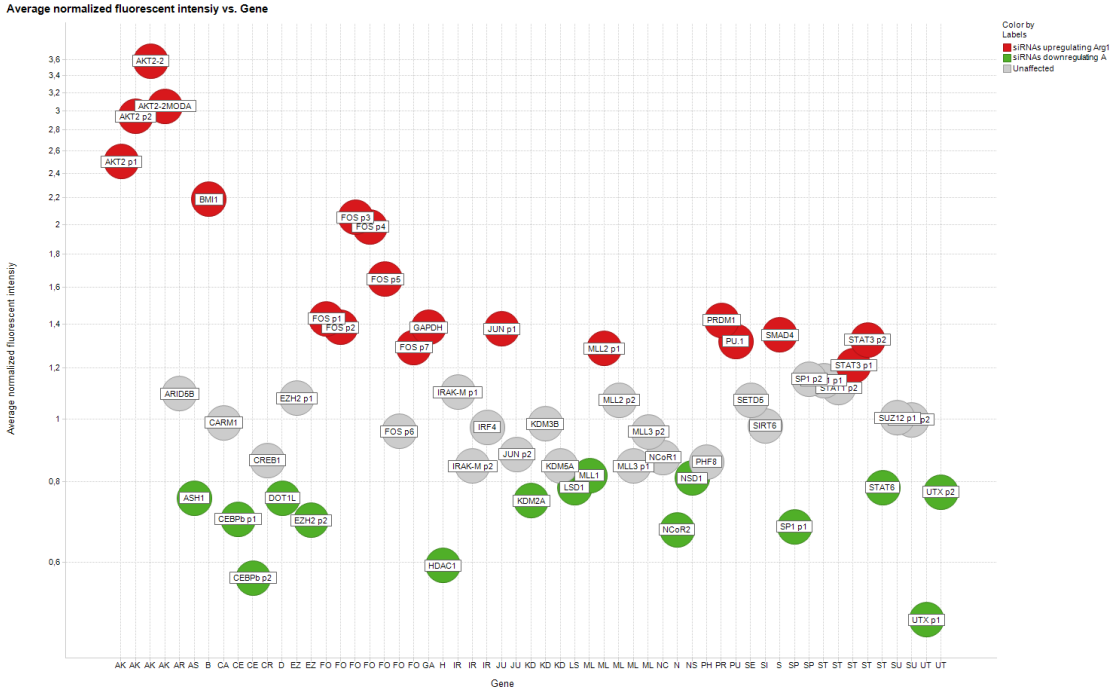
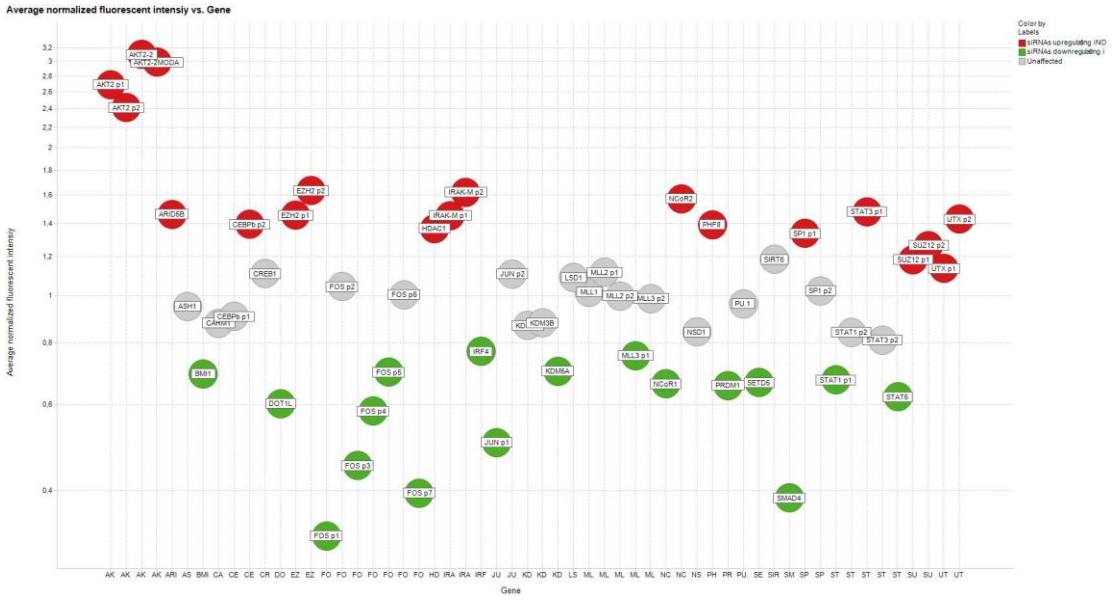


Figure 10. A (left). Treatment of RAW265.7 macrophages with the indicated LPS concentrations and various time points. B (right). Cell viability after transfection with siRNA targeting KIF11.

Next, we selected the same siRNAs directed against the same chromatin structural and regulatory proteins, as well as transcription factors used in the IRAK-M screening analysis, and transfected them individually into mouse macrophages. Each KD was scored for alterations in Arginase 1 and iNOS protein expression, each tested twice (figure 11). Among the most prominent hits regulating Arginase 1 it was CEBPb [112], UTX, NCoR2, HDAC1, BMI1, and of course Akt2 [113]. On the other hand members of the AP-1 transcription factor complex (c-jun, c-fos), EZH2 and ARID5B seem to regulate iNOS expression.

Figure 11. Scatter plots showing (Upper plot) Arginase1 IF signal intensity in LPS (100 ng/ml) treated macrophages for 48 hours following transfection with indicated siRNAs. (Bottom plot) iNOS IF signal intensity in LPS (100 ng/ml) treated macrophages for 48 hours following transfection with indicated siRNAs. With red siRNAs upregulating Arg1 or iNOS, with green siRNAs downregulating Arg1 or iNOS, and with grey siRNAs not affecting them.





D. Discussion

IRAK-M is considered to be closely linked with the adaptation of endotoxin tolerance in cells of the innate immunity. Functioning as a physiological dominant negative isoform in the IRAK family of kinases, it abrogates signal transduction downstream of TLR4 receptor and limits excess inflammatory responses. However, whereas there is a relatively good understanding of its function, its regulation still remains elusive. It has also become well understood that LPS (among other TLR ligands), is a strong inducer of IRAK-M expression. The kinetics of this phenomenon are complex: IRAK-M protein levels increase sharply upon LPS induction, peak at 12 hours and remains high even after 24 hours of continued LPS stimulation [36]. Such a phenomenon can only partly be attributed to fast acting transcriptional mediators, such as NF-κB. Under normal conditions, activation of histone deacetylases (HDACs) inhibits acetylation (Ac) of histones, which results in chromatin packaging, restriction of DNA accessibility for transcription, and controlled target gene expression. During endotoxin tolerance and exposure to LPS stimulation, histone modifications mediated by histone acetyltransferases (HATs) influence elevated transcription of target genes, due to chromatin accessibility, which leads to increased DNA occupancy of transcription factors such as nuclear factor-κB (p50/p65), co-activators and transcriptional complexes, and result in elevated target gene expression (figure 13).

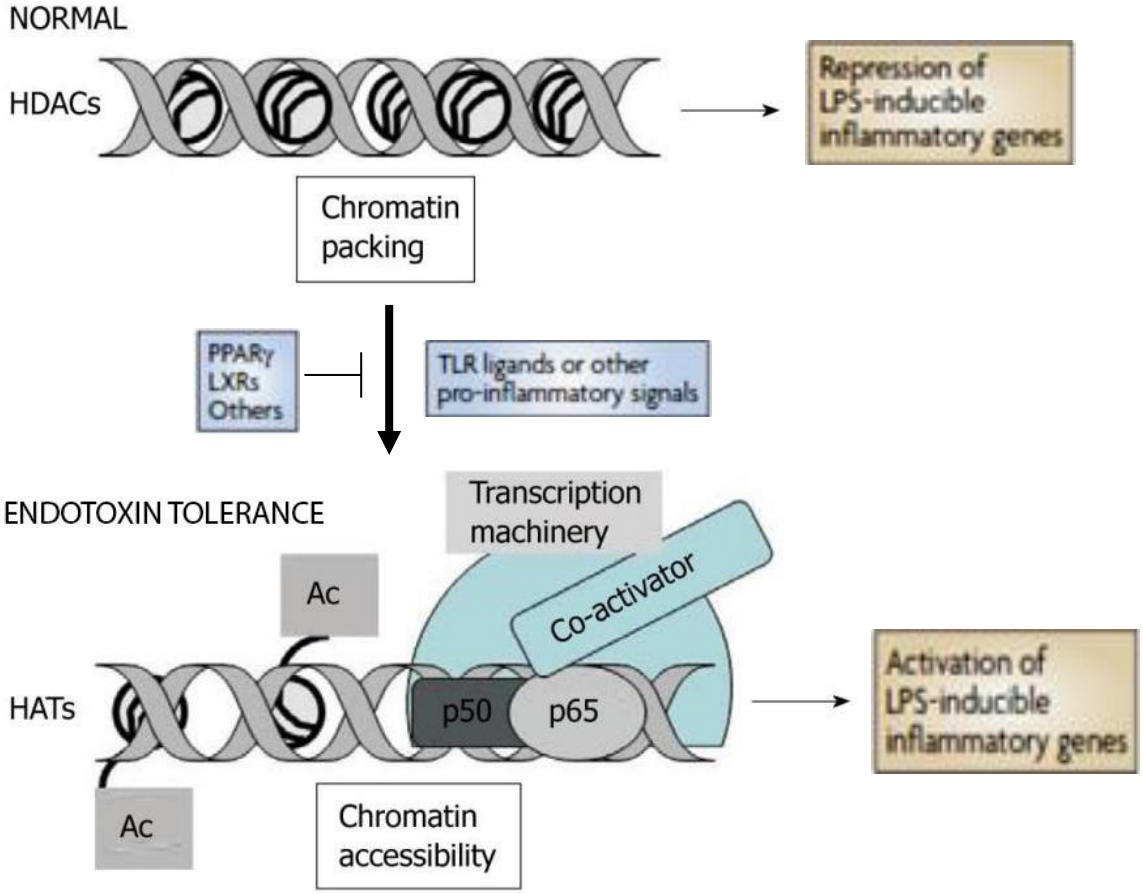


Figure 13. Epigenetic regulation in the context of macrophage activation and endotoxin tolerance.

In this study, we have reviewed available online data concerning *irak3* transcription regulation. Here we show that IRAK-M transcription is CEBPβ regulated. CEBPβ is a member of the CCAAT-enhancer binding proteins, containing a leucine zipper motif. It is inducible by various inflammatory stimuli such as LPS [63] and is also known to cooperate with NF-kB in the regulation of many genes [64, 65, 66]. Moreover, CEBPβ function has been repeatedly shown to be PI3K/Akt regulated [55]. Unpublished data from our lab show that CEBPβ can bind at sites directly upstream of irak3. This is an effect that upon tolerogenic conditions is still strong and putatively drives IRAK-M expression. In conclusion, data accumulated so far point towards a basic concept in gene regulation: signaling pathways lead their own inactivation. In the case of LPS, signal transduction creates a self inhibitory loop: TLR4 signaling promotes CEBPβ activation which in turn leads to the intracellular accumulation of a potent negative regulator, IRAK-M.

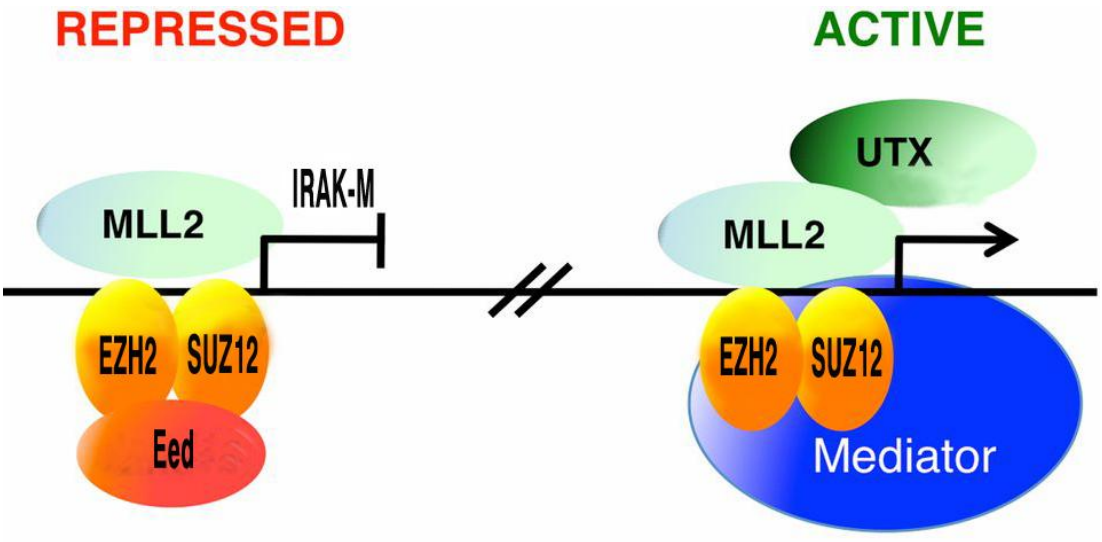
As already stated IRAK-M has a well-established role in reducing immune responsiveness to continuous pathogen exposure. By negatively regulating TLR signaling, IRAK-M inhibits production of pro-inflammatory mediators and contributes to the induction of endotoxin tolerance. Furthermore, it is now appreciated that IRAK-M is expressed in multiple immune and epithelial cells types, and IRAK-M can be induced by a variety of stimuli. This suggests that IRAK-M may play a role in regulating a wider range of inflammatory conditions than previously thought. Despite these recent studies regarding IRAK-M expression and function, little is known about its transcriptional or epigenetic regulation. In order to identify important regulators of IRAK-M expression we performed a small scale siRNA screening analysis of chromatin regulators and transcription factors, in order to identify regulators which induce or inhibit IRAK-M expression (figure 6).

Gene activation encompasses the coordinated removal of histone marks that are refractory to the transcriptional process and introduction of histone marks that are permissive to transcription (103). Coordination of this process is permitted by several mechanisms of cross-talk between histone modifications, including the association of different histone-modifying enzymes within the same protein complex (104, 105). The prime example of cross-talk facilitating gene activation is the association between UTX and MLL3/4, which work together within the same protein complex to remove the repressive mark H3K27me3 and introduce the active mark H3K4me3, respectively (106-108). It will be interesting to check if the MLL2-dependent H3K4me3 mark is detected on the IRAK-M gene. At this point, it is not clear whether MLL2 is enzymatically inactive on the IRAK-M gene or whether the H3K4me3 mark is dynamically removed. In contrast, this gene is probably bound by another demethylase (i.e., UTX), which actively removes the EZH2-dependent repressive mark H3K27me3, thereby allowing activation of the IRAK-M gene. The presence of both MLL2 and UTX on the IRAK-M gene suggests that cross-talk between these histone-modifying enzymes is mediating activation of this gene. Taken together, these results reveal that “active” and “repressive” cross-talk of histone-modifying enzymes coexists on the same locus and plays a crucial role in the precise control of IRAK-M regulated gene expression (figure 12). In the basal state where chromatin is “bivalent” the gene is poised for activation. This form is mainly found in developmental genes and thus it would be interesting to see if it is also found in LPS-activated genes in macrophages.

Mammals have two related H3K27me3 demethylases: UTX and JMJD3. The human UTX has been shown to be associated with components of the H3K4 methyltransferase MLL complex [99-101]. This difference suggests that UTX and JMJD3 may regulate different steps of the transcriptional process and thus different genes, although they both demethylate H3K27me3. Another important difference is that while UTX is abundantly and ubiquitously expressed in many cell types and tissues, JMJD3 is usually lowly expressed, and its expression is elevated in response to development and environmental stimuli [90, 102]. An important question that arises is whether chromatin regulators impart specificity to polarization by gene-specific regulation, or whether they broadly open chromatin to facilitate the function of 'master transcription factors' like CEBPβ that instruct polarization in response to specific signals. The function of HDAC3 in promoting M1-like IFN responses while suppressing IL-4-induced M2 polarization indicates a role for this chromatin regulator in polarization specificity. By contrast, JMJD3 is important for M2 polarization, but also promotes M-CSF and RANKL-induced osteoclast differentiation and, together with related UTX, is required for effective induction of multiple M1 genes by LPS (and possibly IRAK-M). These roles of JMJD3 and UTX are not necessarily paradoxical, but may reflect the locus- and cofactor-specific function of these chromatin modifiers. Transcription factors that bind to promoters and enhancers play a key regulatory role, but binding of many signaling transcription factors is more dynamic than turnover of chromatin marks, which thus can extend and stabilize signals. Cooperation and reciprocal interactions between transcription factors that bind gene loci in a DNA sequence-specific manner and various co-activators, co-repressors, and chromatin regulators that are recruited to regulatory sites to modify chromatin will determine gene expression patterns and macrophage activation status.

IRAK-M remains an interesting target to consider for therapeutic manipulation. Induction of IRAK-M expression in certain settings may serve to limit pathologies associated with excessive cytokine production and innate immune activation. However, specific targeting and limited duration of IRAK-M induction must be employed to minimize host susceptibility to infection. In addition, clinical scenarios associated with increased susceptibility to infection may be improved by inhibiting IRAK-M expression. However, it is not known what degree of suppression of IRAK-M may provide therapeutic benefit. This study by employing RNA silencing technology can help to determine what degree of IRAK-M silencing would restore appropriate immune responsiveness. Moreover, IRAK-M is expressed in multiple cell types and induction or silencing of IRAK-M may need to be cell specific. Overall, the pleiotropic effects of IRAK-M make it an attractive target for therapeutic manipulation to improve the clinical management of patients with a variety of immune-related disorders.

Figure 12. Proposed model of the regulation of transcription on the IRAK-M locus through cross-talk between histone methyltransferases and demethylases. In macrophages, IRAK-M locus is bound by the histone methyltransferases EZH2, which introduce the repressive mark H3K27me3, and MLL2, which introduces the active mark H3K4me3. Upon LPS, the demethylase UTX actively removes the repressive mark H3K27me3, whereas the methyltransferase MLL2 trimethylates H3K4, allowing full activation of IRAK-M.



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