INTERFERON REGULATORY FACTOR 6 REGULATES MACROPHAGE POLARIZATION

An Undergraduate Research Scholars Thesis

by

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Submitted to Honors and Undergraduate Research
Texas A&M University
in partial fulfillment of the requirements for the designation as an

UNDERGRADUATE RESEARCH SCHOLAR

Approved by
Research Advisor: Dr. Beiyan Zhou

May 2015

Major: Chemical Engineering

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ABSTRACT

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Under obese stress, adipose tissue macrophages (ATMs) undergo a phenotypic switch from anti-inflammatory status (M2) to proinflammatory (M1) status, a major contributor to the development of chronic tissue inflammation and insulin resistance which are causal factors for type II diabetes. However, the mechanisms underlying the control of macrophage activation statuses have not been fully elucidated. In this study, we demonstrate that interferon regulatory factor 6 (IRF6) exerts a profound impact on macrophage polarization.

Interestingly, we observed that the expression of IRF6 was dramatically suppressed in M2 macrophages upon IL4 stimulation, but not in LPS-activated M1 macrophages, as compared to naive (M0) macrophages. In addition, IRF6 expression differs distinctly between lean and obese ATMs. We further investigated the role of IRF6 using gain and loss of function strategies in a well-defined in vitro system. Knockdown of IRF6 with a gene-specific shRNA successfully suppressed IRF6 expression level in macrophages. Interestingly, significantly enhanced M2 responses were demonstrated by elevated levels of the activation-related cell surface markers CD69 and CD86 and the expression of M2-related genes including IRF4, PPARγ, Arginase1, and IL10. Conversely, bone marrow derived macrophages (BMDMs) with ectopic expression of IRF6 displayed blunted

M2 responses in the presence of IL4, compared to the M2 BMDMs transfected with an empty vector. In addition, the gain or loss of IRF6 expression did not significantly affect M1 responses of BMDMs upon LPS stimulation, suggesting the regulatory effect of IRF6 primarily acts on enhancing macrophage alternative activation.

In summary, our findings identified a novel transcription factor, IRF6, in mediating macrophage alternative activation program. Further analysis of IRF6 in controlling ATM activaton in the obese context will provide crucial information to understand the ATM action and their contribution to adipose tissue function and subsequent obesity-induced chronic inflammation and insulin resistance.

CHAPTER I

INTRODUCTION

Macrophages display a phenotypic spectrum ranging from pro-inflammatory (classically activated) M1 to anti-inflammatory (alternatively activated) M2.[1] M1 activation occurs in response to exposure to T helper type 1 cytokines or stimulation with lipopolysaccharide (LPS) or free fatty acids.[2, 3] In contrast, Th2 cytokines including interleukin 4 (IL4) and IL13 induce M2 expression.[4, 5] M2 macrophages function in tissue remodeling and repair and are phenotypically favored in lean adipose tissue macrophages (ATMs). The ATM composition is extremely important for physiological function. On a high fat diet, both M1 and M2 macrophage abundance increase in adipose tissue.[6] However, the increase is significantly more for the M1 subclass, which leads to chronic inflammation and insulin resistance.[7-9] Despite a significant amount of research, the molecular mechanisms regulating macrophage polarization have yet to be fully elucidated.

Interferon regulatory factors (IRFs) are a family of nuclear transcription factors, many of which regulate immune cell maturation. Each IRF share a homology with their N-terminal DNA-binding domain and recognize a consensus DNA sequence, known as the IFN-stimulated response element.[10] Several members of the IRF family have been demonstrated to play an important role in the determination of macrophage phenotypes.[11] IRF1, IRF3, and IRF4 all favor the M2 anti-inflammatory phenotype. Conversely, IRF5 and IRF8 promote M1 inflammatory macrophage responses. IRF4 in particular is a potent regulator of M2 activation and may also participate in negative-feedback regulation of TLR signaling.[12, 13] Investigation into IRF

expression revealed that IRF4 and IRF6 have differential expression levels between M0, M1, and M2. While the importance of IRF4 has already been established, the role of IRF6 in macrophage polarization has yet to be elucidated. IRF6 mutations have been linked to Van der Woude and popliteal pterygium syndromes but no link with adipose tissue inflammation has been discovered yet.[14] Our study shows the importance of IRF6 in regulating the macrophage polarization process.

CHAPTER II

METHODS

Bone marrow isolation and macrophage differentiation

Bone marrow-derived macrophages (BMDMs) were obtained as described previously. After red blood cell lysis, bone marrow cells were seeded at 2×10^6 cells/mL with Iscove's Modified Dulbecco's Medium (IMDM) medium containing 10% FBS and 15% L929 culture supernatant as a source of granulocyte macrophage colony-stimulating factor (GM-CSF) for differentiation of bone marrow cells to monocytes. After 7 days, the formation of mature monocytes was evaluated by flow cytometry using antibodies against CD11b and F4/80.

Macrophage polarization analysis

BMDMs were stimulated by lipopolysaccharide (LPS; 100 ng/mL) for M1 activation or IL4 (20 ng/mL) for M2 activation. After 48 h of stimulation, BMDMs were examined for activation of expression of associated surface antigens CD69 and CD86 using flow cytometry.

Flow cytometry analysis

Unless specified, antibodies were obtained from eBioscience. BMDMs were stained with fluorescence-conjugated antibodies to detect their activation. Activation of macrophages was detected using antibodies against F4/80 (Cat. No. 53-4801), CD11b (Cat. No. 45-0112), CD86 (Cat. No. 17-0862), and CD69 (Cat. No. 12-0691). Flow cytometry analysis was performed using Accuri C6 (BD Bioscience), and results were analyzed using Flowjo or Accuri C6 software (BD Bioscience).

Western blotting

Total protein was extracted from BMDMs using a Radio-Immunoprecipitation Assay (RIPA) buffer (Cat. No. 9806S; Cell Signaling Technology®), and protein concentrations were determined using the Bradford assay. Proteins were separated on PROTEAN® TGX Stain-Free™ Precast Gel (Cat. No. 456-8081; Bio-Rad) and transferred onto a polyvinylidene fluoride (PVDF) membrane followed by detection using antibodies directed against IRF6.

Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted from BMDMs using the Trizol extraction protocol according to the manufacturer's instructions (Cat. No. R2052; Zymo Research). Gene expression analysis was performed using an iScript One-Step RT-PCR kit with SYBR Green (Cat.No. 170-8893; Bio-Rad) on Bio-Rad CFX384 (Bio-Rad). The data presented correspond to the mean of $2^{-\Delta\Delta Ct}$ from at least three independent experiments after being normalized to β-actin.

Luciferase Reporter Assay

The luciferase reporter assay was carried out as described previously. To verify that IRF6 binds to the upstream region of PPARγ, a 478-bp DNA fragment (-1857 to -1379 relative to the 5' end) within upstream region of PPARγ was inserted into upstream of the firefly luciferase expression cassette of the pGL3 basic vector (Cat. No. E1751; Promega). The luciferase activity was determined by transient transfection of the murine macrophage cell line RAW264.7 with Bright-Glo luciferase reporter system (Cat. No. E2620; Promega) and normalized to the internal control firefly luciferase activity.

Gain and loss of IRF6 assays

To overexpress IRF6, the open reading frame (ORF) sequence of IRF6 was inserted into the downstream portion of the CMV promoter of XZ201 construct. To knockdown the expression of IRF6, the pLKO.1-CMV-TurboGFP^{TM} vector (Sigma-Aldrich) with inserted short hairpin RNA (shRNA; targeting IRF6) was generated. The empty vector was used as the control.

Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed as described previously. Briefly, M2 BMDMs were cross-linked for 10 min with 1% formaldehyde and quenched with 125mM glycine. After nuclei were isolated by centrifugation, the pellet was resuspended in lysis buffer containing 0.1% SDS and sonicated to achieve fragment sizes of 200-500bp. The immunoprecipitation was conducted with ChIP-grade protein G magnetic beads using an antibody against IRF6 (Cat. No. sc-98829; Santa Cruz Biotechnology). IgG protein (Cat. No. ab46540; Abcam) was used as the negative control. To validate the enrichment, quantitative PCR was performed with tiled primers.

Data and statistical analyses

Results are expressed as means ± SEM. Each data point derived from qRT-PCR assays represents an average of two technical replicates, and data were averaged over independently replicated experiments (n = 3-4 independently collected samples) and analyzed using the Student's *t* test. The overall group-effect was analyzed for significance using two-way ANOVA and Bonferroni post-test for each factor at each individual time. Data analyses were performed using Graphpad Prism version 6.0 software. A value of $P<0.05$ was considered statistically significant and is denoted by a *, $P<0.01$ by **, and $P<0.001$ by ***.

CHAPTER III

RESULTS

IRF6 differentially expressed in macrophages

Due to the significance of the IRF family in regards to macrophage phenotype, we performed an analysis of IRF expression levels in M0, M1, and M2. The results showed that only IRF4 and IRF6 were differentially expressed among macrophage phenotypes (Figure 1, 2).

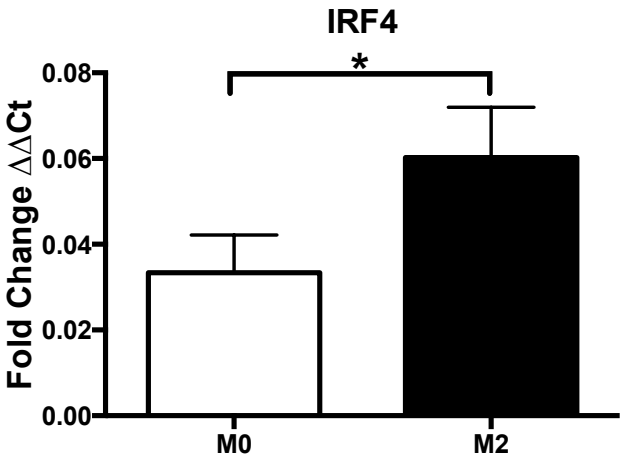


Figure 1: Relative expression of IRF4 is shown from a qRT-PCR. The RNA from the M2 cells was taken 48 hours post activation

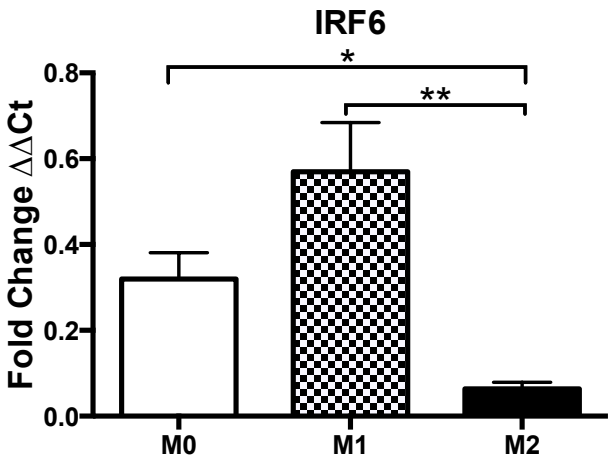


Figure 2: Relative expression of IRF6 is shown from a qRT-PCR. The RNA from the M2 and M1 cells was taken 48 hours post activation

IRF6 expression is diminished in M2 macrophage

To gain initial insights into the role of IRF6 in macrophage activation, we investigated the expression pattern of IRF6 in response to the stimuli using a well-established in vitro model. Bone marrow-derived macrophages (BMDMs) were treated with LPS (100 ng/mL) for M1 activation or IL4 (20 ng/mL) for M2 activation. The macrophage polarization was confirmed by determining the expression level of activation-associated cell surface markers CD69 and CD86

using flow cytometry assays. Interestingly, after 24 hours of IL4 treatment, macrophages exhibited suppressed expression of IRF6, compared to the native macrophages (M0). In addition, we observed that IRF6 expression was dramatically reduced in M2 macrophages as early as 5 h and maintained till 72 h in response to IL4 exposure (Figure 3). Upon LPS stimulation, M1 macrophages maintained similar expression level of IRF6 with M0 macrophages. Thus, these results indicate the suppression on expression of IRF6 during M2 activation but not in M1 macrophages.

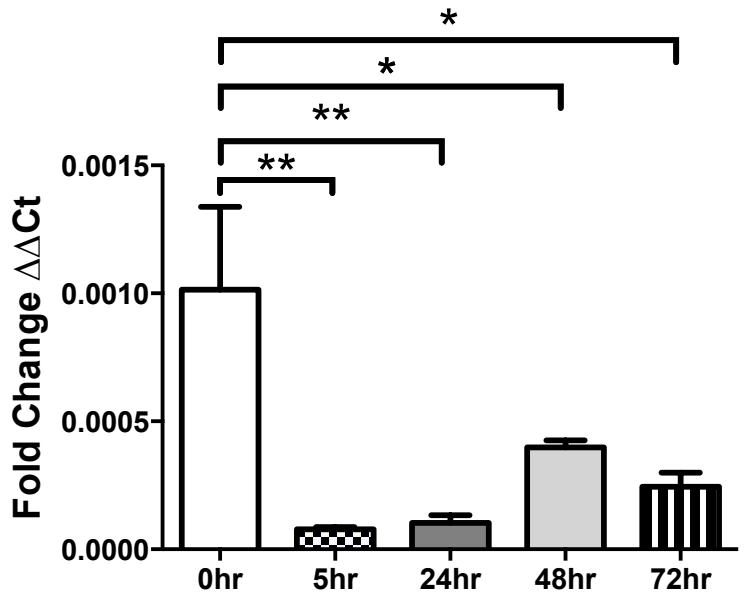


Figure 3: The 0 hr represents M0, naïve macrophages. The 5hr, 24hr, 48hr, and 72hr are all M2 macrophages that have been activated for that period of time by IL4.

IRF6 stalls M2 activation

Next, to further investigate if the differentiated expression pattern of IRF6 is crucial for macrophage polarization, we used both gain and loss of IRF6 assays. To ectopic expression of IRF6, the construct harboring open reading frame (ORF) sequence of IRF6 was transfected into BMDMs. After validated overexpression of this gene in BMDMs (Figure 4), cells were subjected

to activation by LPS or IL4, and activation features were evaluated as previously described. After IL4 stimulation, BMDMs transfected with IRF6 overexpression construct showed suppressed expression of PPARγ that is a key mediator promoting M2 responses, compared to the cells transfected with empty vector (Figure 5). Consistently, these macrophages with ectopic expression of IRF6 exhibited blunted M2 activation in response to IL4 stimulation, as evidenced by reduced expression level of activation-related surface markers CD69 and CD86 (Figure 6). In addition, we observed that overexpression of IRF6 led to less abundance of interleukin 10 (IL10) and arginase 1 (Arg1) that characterize the M2 phenotype, compared to the cells transfected with empty vector (Figure 4). To knockdown the expression of IRF6, we generated gene specific short hairpin RNA (shRNA) construct against IRF6 and transfected it into the BMDMs. After loss of IRF6, M2 macrophages displayed enhanced PPARγ expression upon IL4 stimulation, followed by improved M2 responses including the expression of key genes IL10 and ARG1 and activation-related cell surface markers CD69 and CD86 (Figure 7, 8).

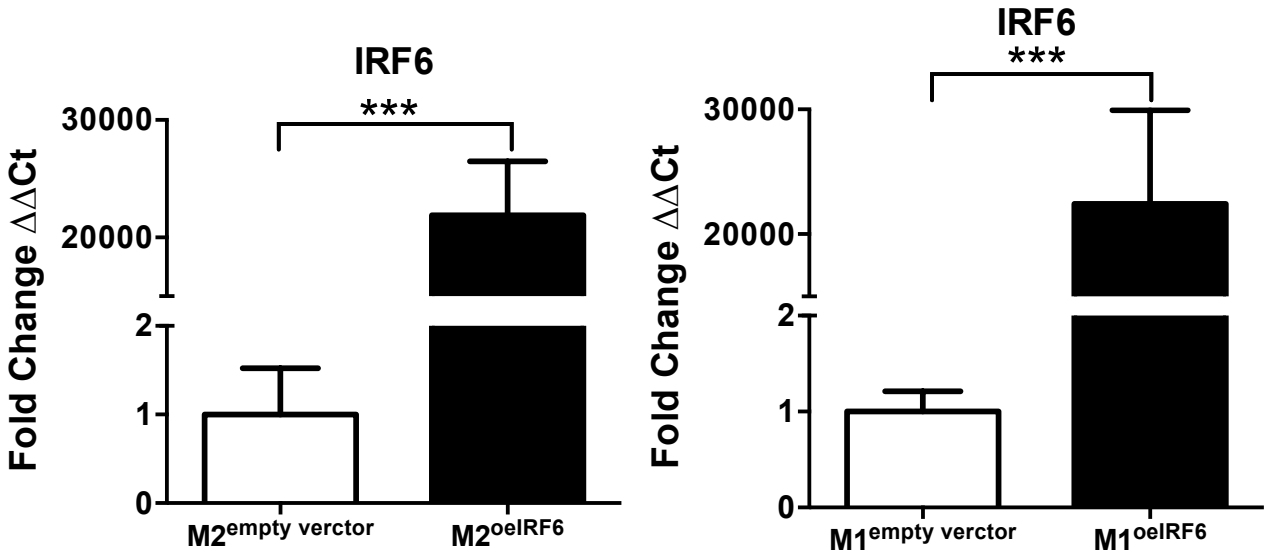


Figure 4: The above data shows the difference in expression for IRF6 when treated with the empty vector as compared to the overexpression construct.

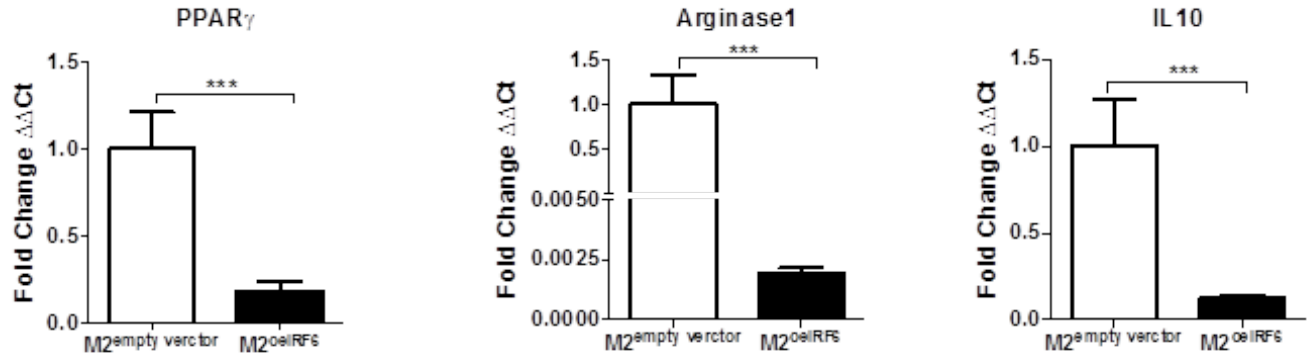


Figure 5: Results from the qRT-PCR show that PPARG expression is significantly lower when IRF6 is overexpressed. The same is true of arginase 1 and interleukin 10, which are anti-inflammatory genes that exhibit increased expression in M2 macrophages.

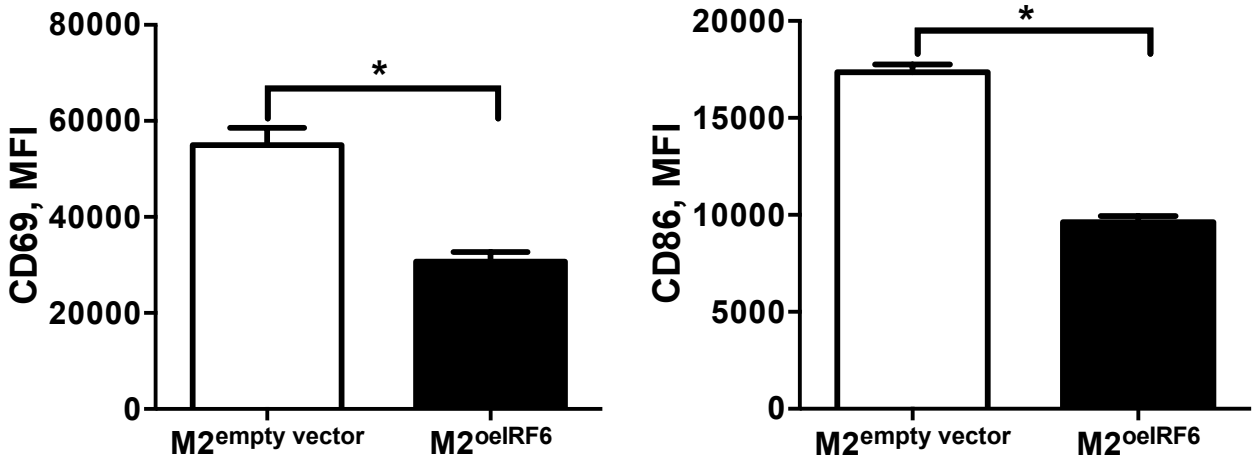
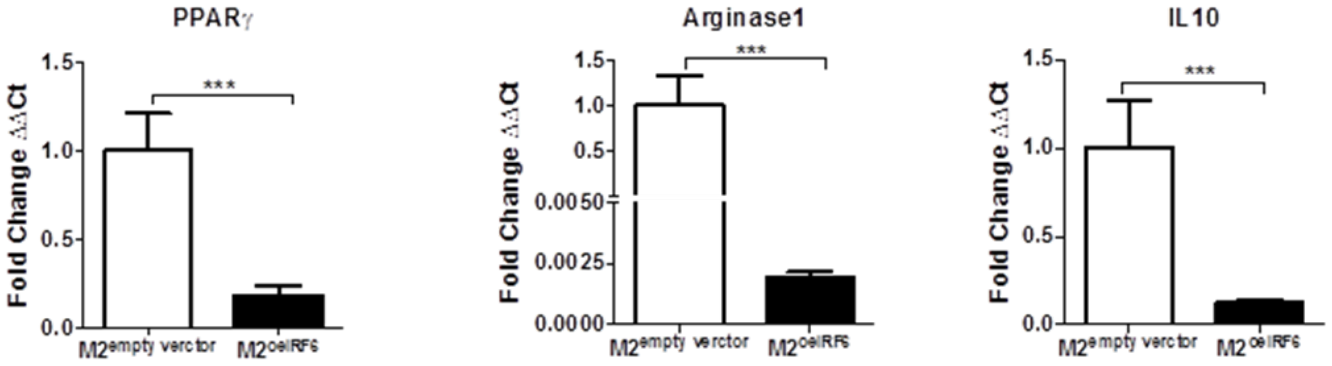


Figure 6: CD69 and CD86 are activation related cell markers for the macrophage. The decreased expression of M2 correlates with the gene expression data.



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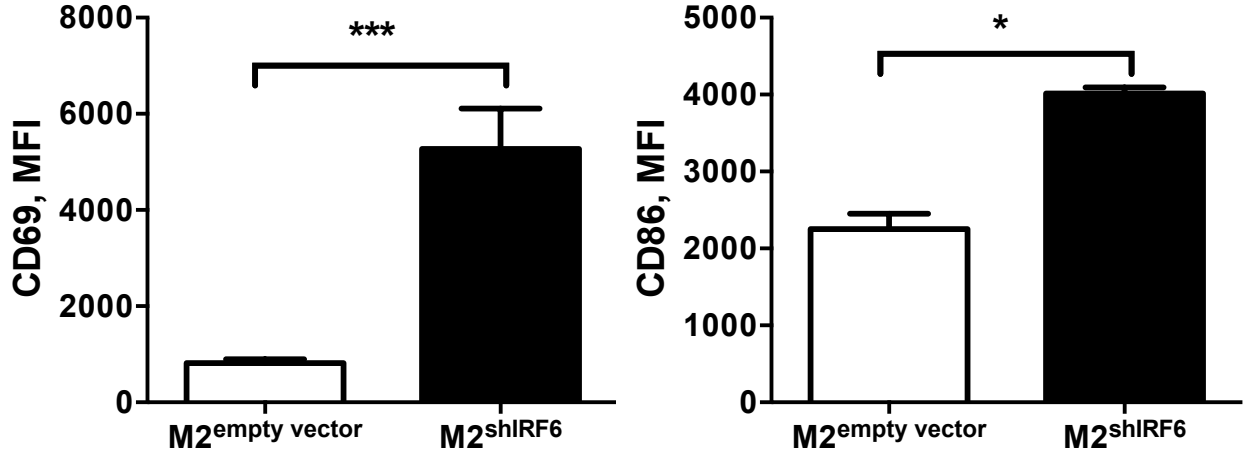

Figure 8: CD69 and CD86 are activation related cell markers for the macrophage. The increased expression of M2 correlates with the gene expression data for the shRNA knockdown.

IRF6 has a similar structural homology with IRF5,[11] which is a critical regulator for M1 macrophage responses. However, after LPS stimulation, ectopic or knockdown of IRF6 expression had minimal impact on M1 macrophage activation, as evidenced by the expression of activation-related cell surface markers and pro-inflammatory cytokines TNFα and IL1β with cells transfected with empty vector (Figure 9, 10).

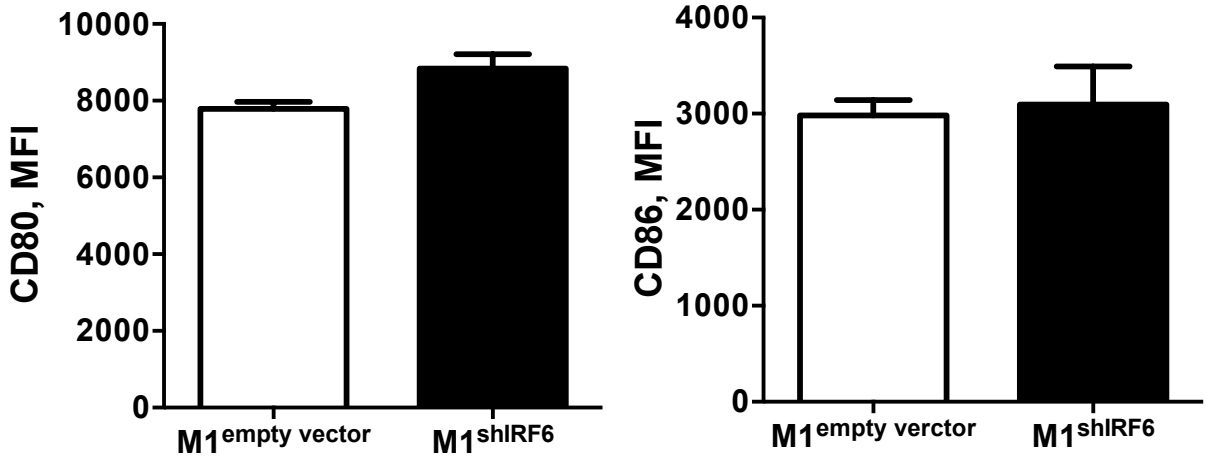

Figure 9: CD80 and CD86 are activation related cell markers for the macrophage. The lack of a difference in the expression of the activation related cell markers mirrors the inflammatory gene expression.

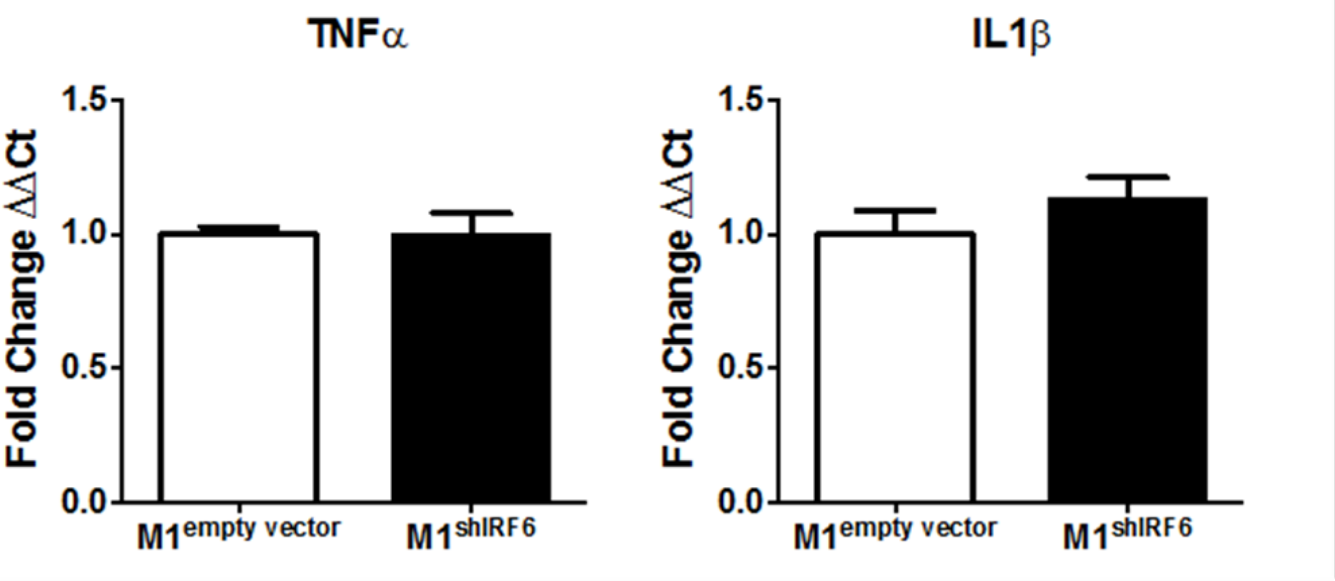

Figure 10: TNFα and IL1β are inflammatory related genes that exhibit higher expression in M1 macrophages. The lack of a difference in expression levels is an indicator that the M1 expression levels are the same between the empty vector and knockdown treated cells.

Taken together, these results demonstrate that IRF6 is a critical mediator for macrophage M2 activation but has no apparent impact on M1 activation.

IRF6 directly inhibits PPARγ expression by binding to its promoter

Given the significant impact on PPARγ expression by IRF6, we surveyed the upstream region of PPARγ for the interferon-stimulated response elements (ISREs). Using the JASPAR algorithms[15], we predicted 4 potential classical ISREs (GAAANNGAAAG/CT/C) within 4kb upstream of PPARγ (Figure 11). To identify the genuine binding sites, we used chromatin immunoprecipitation (ChIP) methodology with antibodies against IRF6 in nucleolus isolated from alternatively activated BMDMs. The protein/DNA complexes were stabilized by formaldehyde crosslinking and isolated from BMDMs stimulated with IL4, and the sonicated DNA fragments (200-500bp) were immunoprecipitated using an antibody against IRF6. The enrichment of ISREs was examined by quantitative PCR with primer pairs flanking each

predicted ISRE. Interestingly, two ISREs (-2061 to -2057, -1788 to -1782, relative to the 5' end of PPARγ coding region) displayed significant enrichment as evidenced by the fold change relative to total DNA input and IgG control (Figure 12).

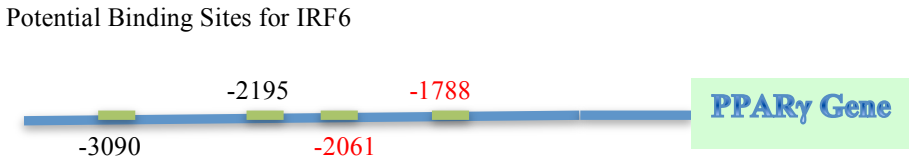

Figure 11: The numbers are negative because the binding sites are in the upstream region of the PPARγ gene. The sites highlighted in red exhibited significant DNA enrichment.

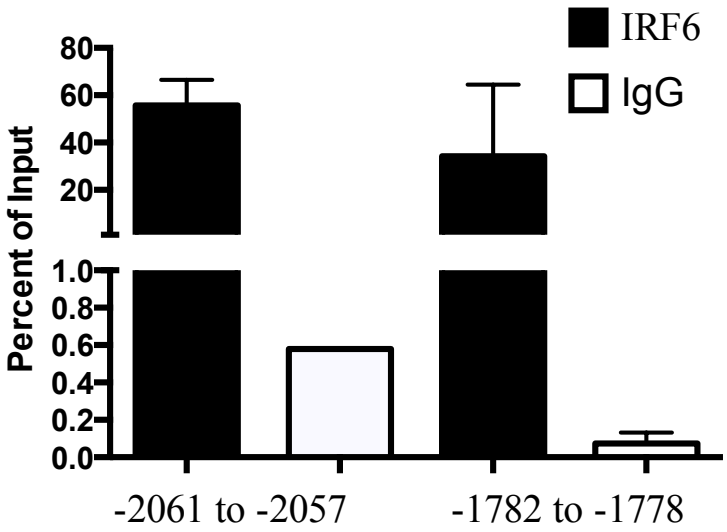

Figure 12: The higher percentage of DNA pulled down with IRF6 as compared to IgG, the control, demonstrates that IRF6 binds effectively to the targeted sites.

CHAPTER IV

CONCLUSIONS

Macrophages serve as a key mediator of immune responses in response to various stimuli. A well-orchestrated network tightly regulates the plasticity and functional polarization of macrophages and IRFs exert critical roles in controlling macrophage activation through modulating the activities TLRs-mediated signaling pathways. However, the impact of IRFs on the action of PPARγ-dependent signaling, which play a central role in promoting macrophage M2 responses, is unclear. In this study, we provided evidence to support the crucial roles of IRF6 in modulating macrophage alternative activation through its regulation on the expression of PPARγ. Our findings reveal that IRF6 can repress PPARγ expression by directly binding to the ISREs located in the PPARγ upstream region, and IL4-induced down-regulation of IRF6 expression is critical to initiate PPARγ-dependent alternative M2 activation.

The rapid change in expression pattern of IRFs is essential for their transcriptional functions that subsequently result in the responses of macrophage encountering distinct stimuli. Indeed, in this study, we observed that IRF6 expression was repressed at the initial period of M2 activation, but this gene was not responsive to LPS stimulation. These results suggest that the expression of IRF6 is controlled by IL4-mediated signaling cascades of M2 macrophages. Similarly, other IRF family members also display rapid alteration in their expression in macrophages in response to stimuli. For example, upon stimulation by GM-CSF or IFNγ, activation of TLR mediated signaling cascade leads to induced IRF5 expression, but its expression is insensitive to IL4 stimulation. In contrast, the axis of IL4-STAT6 can enhance the expression of another IRF

family member IRF4 through binding to the promoter region. In addition, IRF4 is an induced gene of Jmjd3 by controlling demethylation of H3K27me3.

The IRF-dependent pathway is one of main downstream signaling pathways triggered by the activation of TLRs, exerting essential transcriptional control on gene profiling. Previous studies have demonstrated that several IRF family members, including IRF1, IRF5, and IRF7, play critical roles in interacting with MyD88, which subsequently triggers TLR-mediated the expression of proinflammatory genes. In contrast, loss of these IRFs blunts proinflammatory responses of M1 macrophages. Interestingly, IRF4 serves as negative regulator of TLR-mediated cascade by competing with IRF5 for binding to MyD88. Our study is the first to report that IRF6 is critical to control the expression of PPARγ, which is a key regulator in controlling macrophage alternative activation. Previous studies have demonstrated that PPARγ-deficient macrophages exhibit impaired M2 responses. Upon IL4 stimulation, we observed that the abundance of IRF6 was rapidly reduced in macrophages, suggesting that IRF6 acts as a "break" for M2 responses till encountering Th2 cytokines such as IL4 or IL13. Indeed, we identified two ISREs located within 4kb upstream of PPARγ coding region and further validated their interaction with IRF6 to suppress the expression of PPARγ using a luciferase reporter assay. In addition, ectopic expression of IRF6 led to further repression on PPARγ expression, whereas knockdown of IRF6 resulted in enhanced PPARγ abundance in M2 macrophages. The ISREs can be recognized by IRFs by their helix-turn-helix motif. Given that IRF4 is induced in M2 macrophages,[12] it is suggested that IRF4 may enhance PPARγ expression through binding these ISREs located within promoter region of PPARγ after removal of IRF6. Indeed, loss of IRF4 can result in blunted M2 responses.

In summary, our findings identified a novel transcription factor, IRF6, in mediating macrophage alternative activation program. Further analysis of IRF6 will provide crucial information to understand the macrophage action and may provide new gene targets for drug development to mitigate inflammatory diseases.

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