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# IRF2 Deficient Primary Macrophges Cells Acquire an Inflammatory Programme upon Activation of TLR9 Innate Receptor

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## Abstract

*Activation of Toll-like receptor (TLRs) is known to be critical for innate immune system homeostasis. TLRs downstream signaling transduction pathway induces activation of transcription factors like IFN-regulatory factors (IRFs) and Nuclear Factor-kappa B (NF-kB) which leads to the upregulation or suppression of genes that induces inflammatory genes responses and other genes transcription. Since other IRFs are known to play a critical role in TLRs downstream signaling, in our study we shown that IFN-regulatory factor 2 (IRF2) is critical for the activation of NF-kB/p65, following stimulation of TLR9 by CpG B ligand stimulation and HSV1 infection. In the course of our experiments, the peritoneal macrophages from IRF2-deficient primary macrophages indicated a decrease in the gene induction of inflammatory cytokines Il6, TNFα and a marked increase in Il12p40, Ifnβ ,Cxcl10 and iNos, compared with control macrophages upon CpG B stimulation and HSV-1 infection. Moreover, the NF-kB/p65 showed a delayed phosphorylation in IRF2-deficient primary macrophages. Thus, our research has placed the IRF2 in a new context regarding the complex nature of NF-kB/p65 regulation upon CpG B-TLR9 induction signaling.*

**Keywords:** *IRF2-deficient mouse macrophages; TLR9; NF-kB; Proinflammatory cytokine; Herpes Simplex Virus type-1 (HSV-1).*

## 1. Introduction

Immune homeostasis is essential for the host functioning of the immune system. Exacerbated inflammation is induced manly by primary macrophages trough inflammatory cytokines induction and it is harmful. Thus the immune responses might be modulated at the intracellular level to maintain the homeostasis.

The presence of a pathogen is detected by the innate immune system, which is the main mechanism for immediate response. The main receptor of innate immunity the Toll-like receptors (TLR)-triggered immune response are required for the initiation of an effective host defense [1]. When TLRs are activated, these receptors trigger the downstream signaling transduction pathway trough interferon transcription factors (IRFs) [2] to induce the expression of type-1 interferons (IFNs) genes and transcription factor Nuclear Factor-kappa B (NF-kB) activation and proinflammatory cytokines and chemokines gene expression [1] [3].

An array of IFN-regulatory factors (IRFs), namely, IRF1, IRF3, IRF4, IRF5, IRF7, IRF8, are known to play critical roles in the TLR-mediated immune response [4]. The interferon regulatory factor-2 (IRF2) evidences oncogenic potential and a transcriptional repressor of Interferon-β (*Ifnβ*) gene. Moreover, the IRF2 is expressed in most lymphoid cells while it is constitutionally expressed in all cells and its deletion in mice leads to skin inflammation, splenomegaly, and premature death [5].

Although the Toll-like receptor 9 (TLR9)-mediated recognition of bacterial and viral infection is required for the initiation of an effective host defense. Activation of TLR9 signaling lead to inflammation through activation of interferon regulatory factor 7 (IRF7) and interferon regulatory factor 5 (IRF5), found to play a role in the upregulation of IFNs and the activation of NF-kB target inflammatory cytokines and chemokines [6], [7], [8].

NF-kB is a transcription factor essential for the control of cell proliferation, survival, differentiation, immune response, and inflammation [9] [10] [11]. The role of NF-kB pathway in inducing angiogenesis is well understood [12], but its identification as the factor triggering this signaling pathway in the context of TLRs represents a relatively new approach. Cell homeostasis is mediated by the operation of a complex network of signaling pathways and transcription factors via innate receptors, such as Toll-like receptors (TLRs) [13].

Many members of the IRFs family of transcription factors function distinctly from one another, depending on the nature of the signal emanating from a given innate receptor. IRFs often cooperate/antagonize with other IRFs transcription factors and NF-kB transcription factor [14 [15]. The IRF1 and IRF2 are the first identified to play a role as regulators of the type-1 Interferon *Ifnα/β* genes [16]. Biologically, IRF2 plays an important role in cell growth regulation, and has been shown to be a potential oncogene in NIH 3T3 cells and tumor formation in mice [17]. Thus, until now it has been shown that IRF2 has been suggested to induce oncogenic malignant tumors, but little is known about the mechanism underlying the oncogenic activities of IRF2 [18] Although IRF2 was found in several types of human cancer types, lately it was shown that many cancers are triggered by various pathogens and chronic inflammation [19] [20]. Some of the studies on IRF2-deficient mouse which have focused on the hepatocytes of IRF2-deficient mouse showed a significant inhibition of the expression of *Il12R* and *Ifnγ*, as well as *Il1β* and *Tnfα* level of mRNA are increased significantly in TLR4 ligand stimulation [21].

Lately, there has been a growing interest in the role of the IRFs family of transcription factors in the regulation of immune responses, cytokine signaling, and oncogenesis and TLRs pathway signaling activation. In this context, the present study investigates the role of IRF2 in the TLR9 signaling activation, considering that this aspect has not been studied until now.

Since IRF2 absence resulted in defective host defense, we analyzed the primary peritoneal macrophages *ex vivo* upon CpG B stimulation of TLR9 innate receptor activation. Our results has shown that the NF-kB target genes, respectively proinflammatory cytokines *Tnfα*, *Il6* mRNA were slightly downregulated. The gene expression of *Il12p40*, *Ifnβ*, *Irf7*,*Cxcl10* and *iNos* mRNA were highly upregulated in IRF2-deficient peritoneal macrophages in comparison with control cells upon synthetic oligodeoxynucleotides CpG B stimulation. It has been known that the Herpes Simplex Virus type-1 (HSV-1) infection leads to TLR9 signaling downstream activation [33]. In this context, we investigated the mRNA of the *Tnfα*, *Il6* showed a decreased expression and *Ccl5*, *Cxcl10*, *iNos*, *Irf7* and *Ifnβ* mRNA level which were increased in IRF2-deficient macrophages in comparison with the control

cells. Our finding indicates that IRF2 modify the intensity of TLR9 downstream signaling pathway activation in primary macrophages and maintains intracellular immune homeostasis. Proinflammatory cytokines IL6 and TNFα were usually induced by NF-kB/p65 canonical pathway activation leading us to further investigate phosphorylation of p65. NF-kB also controls expression of the downstream IFN auto-amplification loop through STAT1, IRF1, IRF5, and IRF7 transcription factors. Our results indicate that the absence of IRF2 showed different kinetic of p65 phosphorylation it probably due to IRF2 direct interaction with NF-kB/p65 or an possible cross-regulation with other IRFs, as shown in the recent study [29]. Further, we investigated the *Irf7*, *Irf5* and *Irf1* mRNA level upon CpG B stimulation. Both, *Irf7* and *Irf5* mRNA level were increased and showed their activation as *Ifnβ* inducible molecules as long as the *Irf1* mRNA level was not affected. Our research suggests that IRF2 may contribute to new insights into the molecular mechanisms governing the inflammatory process in macrophages upon CpG B-TLR9 activation.

Thus, our research indicates that IRF2 transcription factor is required to maintain the immune homeostasis upon activation of the above mentioned TLRs and to avoid uncontrolled inflammation induced by infections with pathogens that activate innate immune receptors.

## 2. Materials and Methods

**Mice, cells and reagents**: The generation of IRF2 deficient mice has been described [22]; mice were maintained on the C57BL/6 (B6) genetic background. All of the mice were maintained under specific pathogen-free conditions and used at 8–12 weeks of age. Mice were injected i.p. with 2 ml 3% thioglycolate (DIFCO) and cells were harvested from the peritoneal cavity 3 days day after injection. Peritoneal macrophages cells were prepared or maintained as described [28]. All primary cells used for *ex vivo* experiments were on the B6 background. CpG-B (ODN 1668) was purchased from FASMAC.

**Viral Infections:** HSV-1 was prepared as described. Peritoneal macrophages ($5 \times 10^5$) were infected with viruses (a multiplicity of infection (MOI) of 1 for HSV-1). Quantitative RT-PCR for the expression of *Ifnβ*, *Il6*, *Il12p40*, *Tnfα, Cxcl10* and *Ccl5* mRNA levels were evaluated after normalization to the expression of GAPDH mRNA. The *t* test was used for statistical analysis of triplicate experiments.

**RNA analysis**: RNA was extracted and reverse-transcribed was performed using Rneasy kit from QIAGEN and reverse tanscription (RT) to cDNA were performed using Prime Script 1$^{st}$ strand synthesis kit (TAKARA). Quantitative RT-PCR analysis was done with a Lightcycler 480 and SYBR Green system (Roche Molecular Biochemicals). All data are presented as relative expression units after normalization to the expression of GAPDH mRNA (glyceraldehyde phosphate dehydrogenase). The primer sequences for mouse *Gapdh*, *iNos*, *Il6*, *Tnfα*, *Il12p40*, *Cxcl10*, *Ccl5*, *Irf1*, *Irf5*, *Irf7* and *Ifnβ* mRNA are as follows: *Gapdh*, 5'-CAC ATT GGG GGT AGG AAC AC-3' (forward) and 5'-CTC ATG ACC ACA GTC CAT GC-3' (reverse); *Ifnβ*, 5'-CCA CCA CAG CCC TCT CCA TCA ACT AT-3' (forward) and 5'-CAA GTG GAG AGC AGTTGAGGACAT C-3' (reverse); *Tnfα*, 5'-TCA TAC CAG GAG AAAGTCAACCTC-3'(forward)and5'-GTATAT GGG CTCATACCAGGGTTT-3'(reverse); *Il6*, 5'-ACG ATG ATG CAC TTG CAG AA-3' (forward) and 5'-GTAGCTATGGTA CTCCAGAAGAC-3'(reverse); *IL12p40*, 5'-GACACGCCTGAAGAAGATGAC-3'(forward) and5'-TAGTCCCTTTGGTCCAGTGTG-3'(reverse);*Ccl5*,5'CGTGCCCACGTCAAGGAG TAT-3'(forward)and5'CCCACTTCTTCTCTGGGTTG3'(reverse);*Cxcl10*,CCATCAGCA

CCATGAAGACTTTGGGG3'(forward)and5'CTTCACTCCAGTTAAGGAACC3'(reverse), *iNos*, 5'CACCTTGGAGTTCACCCAGT3'(forward)and5'ACGACTCGTACTTGGGATGC-3'(reverse),*Irf5*,5'TAGAGGCTACCCAGGAGCAA3'(forward)and5'GCCCACTCCAGAA CACCTTA3'(reverse);*Irf1*,5'TTCCAGATTCCATGGAAGCACGC-3'(reverse) and 5' ATTCGCCAATGACAAGACGCTGG-3'(forward),*Irf7*,(forward)5'-GCAAGGGTCACCAC A CACTA-3'and (reverse) 5'-CAAGCACAAGCCGAGACT-3',

**Western blot analysis:** The i.p primary peritoneal macrophages from control and IRF2 deficient mouse were isolated (as described [15]), then stimulated with 3µM CpG-B for the indicated time. Whole cells extract were lysed in lysis buffer (50mM Tris-HCl (pH8), 150 mM NaCl, 1% Nonidet P-40) suplemented with protease inhibitor cocktail (Roche). Proteine concentration were determined by Bradford assay (Bio-Rad), and equivalent amounts of cellular extract concentration were used. Immunoblotting were carried out using anti-phospho NF-kB p65 antibody (Cell Signaling), p65 anti-mouse antibody (Santa Cruz). The enhanced chemiluminescence system (Amersham Pharmacia) was used for revealing the bands, followed by exposure to x-ray film (Biomax MR, Eastman Kodak Co.).

**Statistical analysis**: Differences between control and experimental groups were evaluated with Student's *t*-test. Each experiment was conducted through at least three independent experiments with consistent results. The representative gel or blot from each experiment is presented in this manuscript. In reporter assay, a mean value and standard deviation of the triplicates is used to represent the reporter activity. The data was analyzed using student's T-test with significance $p < 0.05$.

## 3. Results and Discutions

### IRF2-deficient peritoneal macrophages gene expression profile upon activation of TLR9 ligand stimulation

The IRF2 deficient mouse develop psoriatic skin-like disease upon ageing [23] and it is common knowledge that often psoriatic lesions will get infected by environmental pathogens leading to supra-infections and lately to chronic inflammation [24]. In this context, it has been shown that bacterial or viral infection activates TLR9 and downstream transcription factor such as NF-kB and IFNs [25]. Chronic inflammation arises due to the continuous presence of a stimulus or the genetic factors and physiological alterations, disrupting the cellular feedback mechanism that normally reduces the intensity of the immune response [26],[27].

Therefore, our study has proposed to analyze the TLR9 innate receptor activation in IRF2 deficient primary macrophages ($IRF2^{-/-}$) and control macrophages ($IRF2^{+/+}$). In this regard, the NF-kB dependent genes, such as proinflammatory cytokines *Tnfα*, *Il6*, *Il12p40*, chemokine *Cxcl10* and *iNos*, following CpG B stimulation of TLR9 where quantified. Primary macrophages where co-stimulated using 3µM of CpG B for 0, 4 and 8 hour time points. The mRNA of above genes where evaluated by quantitative RT-PCR as relative expression units after normalization to *Gapdh* mRNA.

*Ex vivo* peritoneal macrophages from IRF2-deficient mouse showed a downregulation of the proinflammatory cytokines *Tnfα*, *Il6* mRNA as long as the expression level of *Il12p40* and *iNos* genes were significantly upregulated in comparison with their control macrophages by quantitative RT-PCR assay (Fig1. A, B, C and E). *Il12p40* mRNA level increased almost 35 fold times at 8 hours, *Il6* and *Tnfα* mRNA was observed to be

diminished at 4 hours and respectively 8 hours upon CpG B stimulation in absence of IRF2. *Il12p40* usually is highly expressed in activated macrophages and has anti-angiogenic activity. Modestly decrease of only 2 times was observed in the case of *Tnfα* to maximum mRNA induction level at 4 hours of CpG B stimulation in IRF2-deficient macrophages. The treatment with CpG B resulted in differences in gene expression in activated NF-kB pathway.

Furthermore, the genes induced by IFN pathway, gene encoding the inflammatory cytokine *Ifnβ* was increased 10 fold times in IRF2 deficient cells at 4 hours post-stimulation and chemokines *Cxcl10* mRNA was observed to increase 2 fold time (Fig.1 C and D) in IRF2 deficient cells at 8 hours, maximum induction level comparing with control littermate macrophage cells, suggesting that absence of IRF2 resulted in the upregulation of inflammatory cytokine and chemokine. It has been shown that *Irf7* gene are activated participating in the inflammatory response induced by IFN pathway upon CpG B treatment. Beside this IRF2 is known to function as negative regulator (transcriptional attenuator) of the IFN-ISGF3 pathway and the loss of IRF2 leads to the constitutionally upregulation of the IFN-inducible genes [17]. The promoter region of the *Ifnβ* gene contains at least four regulatory cis-elements: the positive regulatory domains (PRD) I, II III and IV, PRD II and PRD IV which are activated by NF-kB [14]. These results show that IRF2 is an important negative regulator of modulating gene expression in TLR9 signaling pathway and IFN-pathway in macrophages.

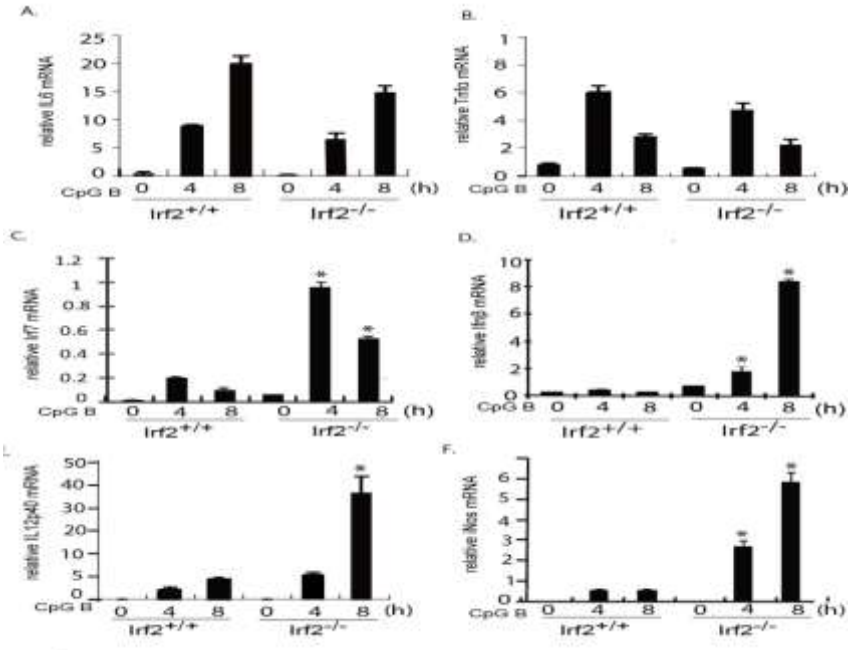


Figure1. The genes expression in IRF2-deficient primary macrophages ($IRF2^{-/-}$) and control macrophages ($IRF2^{+/+}$) upon CpG B treatment. Macrophages were stimulated with CpG B 0.3μM for 0, 4 to 8 hours for activation of TLR9 receptor. Proinflammatory cytokines and chemokines mRNA where evaluated; the results are presented relative to *Gapdh* mRNA expression $*P < 0.05$ and $**P < 0.01$ (Student's *t*-test). Data are representative of three independent experiments (mean and s.d. of triplicates).

As a next step, we sought to determine the induction of inducible nitric oxide synthase *iNos* mRNA level in CpG-TLR9 activation in IRF2-deficient macrophages and the control

primary cells. This gene is known to be involved in host immunity and participate in anti-microbial and anti-tumor activities as part of the oxidative burst of macrophages [28]. The level of *iNos* mRNA level was upregulated in IRF2 deficient macrophages 3 fold time at 4 hours post-stimulation and 11 fold times at 8 hours post-stimulation with CpG B comparing with the control macrophages (Fig.1F).

Proinflammatory cytokines *Il6* and *Tnfα* were usually induced by NF-kB/p65 canonical pathway in TLR 9 receptor activation, leading us to further investigate phosphorylation of p65 in IRF2-deficient and control macrophages.

We next examined whether the loss of IRF2 affects the activation of NF-kB/p65 in response to treatment with 3 µM of CpG B ligand in IRF2 deficient and control peritoneal macrophages.

Activation of TLR9 following CpG B ligand stimulation also results in the degradation of IkBα to release NF-kB subunits p65 and p50, which dimerize and translocate to the nucleus to initiate gene transcription [18].

In order to determine whether IRF2 deficiency could affect NF-kB activation, the IRF2-deficient and control primary macrophages were incubated with 3 µM CpG B for 0, 5, 15, 30 and 60 minutes time points, and the activation of NF-kB/p65 was assessed by immunoblotting with specific antibodies (Fig. 2A).

The control macrophages showed normal activation of p65 upon 3 µM CpG B stimulation, as revealed by the induction of p65 phosphorylation at expected times but, in contrast, IRF2-deficient macrophages NF-kB were activated and actually went up at 60 minutes (see Fig.2A).

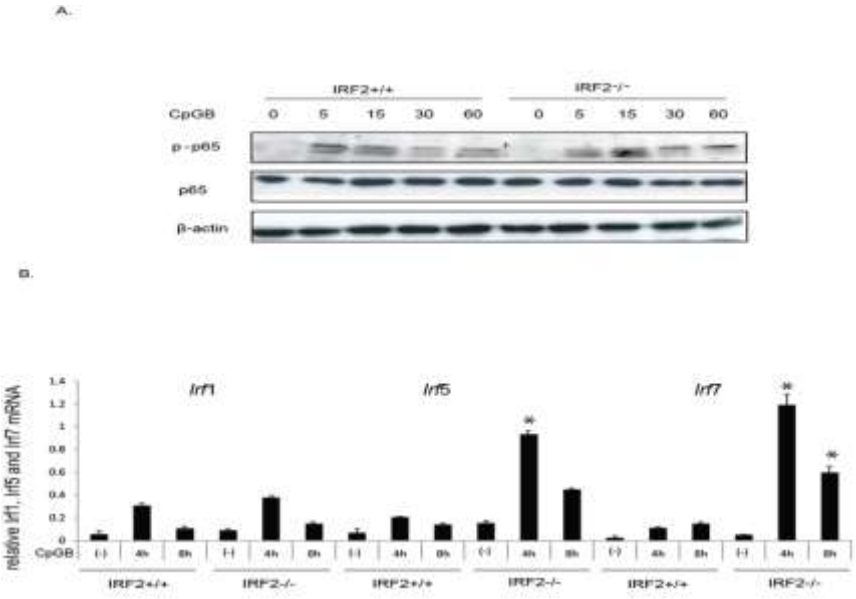


Figure2. **A)**. NF-kB/p65 activation upon 3µM CpG-B treatment of control ($IRF2^{+/+}$) and IRF2-deficient ($IRF2^{-/-}$) macrophages in absence of IRF2. P65 subunit of NF-kB, phosphorylated p65 (p-p65) and the control β-actin where evaluated at 0, 5, 15, 30 and 60 minutes of stimulation with 3µM CpG B ( Fig.2A). **B).** Quantitative RT-PCR analysis of *Irf1*, *Irf5* and *Irf7* mRNA in control and IRF2-deficient peritoneal macrophages stimulated for 0, 4 and 8 hours with 3µM CpG-B; results are presented relative

to *Gapdh* mRNA expression **P* < 0.05 and ***P* < 0.01, ( Student's *t*-test). Data are representative of four experiments (mean and s.d. of triplicates).

At the same time, weak residual activation of p65 could still be detected in CpG B treatment in the macrophages lacking IRF2. These results showed that IRF2 is required either directly for NF-kB/p65 activation or the absence of IRF2 influences the level of other IRFs factor involved directly in NF-kB/p65 pathway.

Recently it was shown that NF-kB and IRFs pathways have cross-regulatory mechanism on target genes promoter level. NF-kB0 also controls expression of the downstream IFN auto-amplification loop through STAT1, IRF1, IRF5, and IRF7 transcription factors [29]. Both the IRF5 [30] and IRF7 proteins cooperate extensively in regulation of target proinflammatory cytokines following TLR9 in macrophages. Other research report showed that IRF2 competes with other DNA-binding proteins, namely IRF5 and IRF7, for the same promoter region [31]. Moreover due to the close relationship between IRF2 and IRF1 we next analyzed the *Irf1*, *Irf5* and *Irf7* genes expression in IRF2 deficient and control macrophages for indicated time points upon stimulation with CpG B ligand (see Fig2. B). The expression of IRF1, IRF5 and IRF7 transcription factors was assessed by quantitative RT-PCR following stimulation with CpG B ligand in absence and in the presence of IRF2 in macrophages.

To test whether other IRF2 or other above mentioned IRFs transcription factor are responsible for NF-kB/p65 activation upon CpG B stimulation, we carried out quantitative RT-PCR assay using different *Irf1*, *Irf5* and *Irf7* genes primers in IRF2 deficient macrophages and the control cells.

In this regard, the gene expression of *Irf1*, *Irf5* and *Irf7* mRNA was determined, following TLR9 ligands stimulation at 4 hours respectively 8 hours post-stimulation with CpG-B in immune cells as mouse primary peritoneal macrophages from $IRF2^{-/-}$ deficient mouse and control $IRF2^{+/+}$ *ex vivo*. *Irf1* mRNA gene expression was induced by TLR9 activation following CpG B ligand stimulation but the level of *Irf1* mRNA was low in the absence of IRF2. IRF1 gene has a good conserved binding site for NF-kB but none for the other IRF family members. We can speculate that IRF2 may not influence NF-kB activation trough some cross-regulation directly with IRF1. *Irf5* and *Irf7* mRNA level significantly increased in the absence of IRF2, upon CpG B stimulation (see Fig.2 B). Interestingly, IRF2 may play a role in attenuating the function of IRF5 or IRF7 gene transcription or other molecular mechanism, which might be further investigated.

**Gene profiles induced expression by TLR9 receptor activation in HSV-1 infection**

We next examined whether IRF2 is involved in host defense against infections by DNA viruses such as HSV-1 in TLR9 signaling pathway. In macrophages HSV-1 infection is recognized by TLR9 receptor which initiate a signaling cascade leading to NF-kB activation [33]. Control and IRF2-deficient macrophages were infected with HSV-1 of 1MOI for 4 hours respectively 8 hours and assayed by quantitative RT-PCR.

The gene expression of *Il6* was downregulated in IRF2 deficient macrophages at 8 hours post-infection (Fig.3 A). The induction of proinflammatory cytokine *Tnfα* mRNA by HSV-1 infection was slightly downregulated after post-infection in IRF2 deficient macrophages in comparison with the control macrophages (Fig.3B). Meanwhile, for the

chemokines *Ccl*5 and *Cxcl*10 genes the mRNA showed a significantly increase of their mRNA at 8 hours post-infection as shown in IRF2 absence (Fig.3E and F). Moreover, CXCL10 is known a critical inhibitor of angiogenesis.

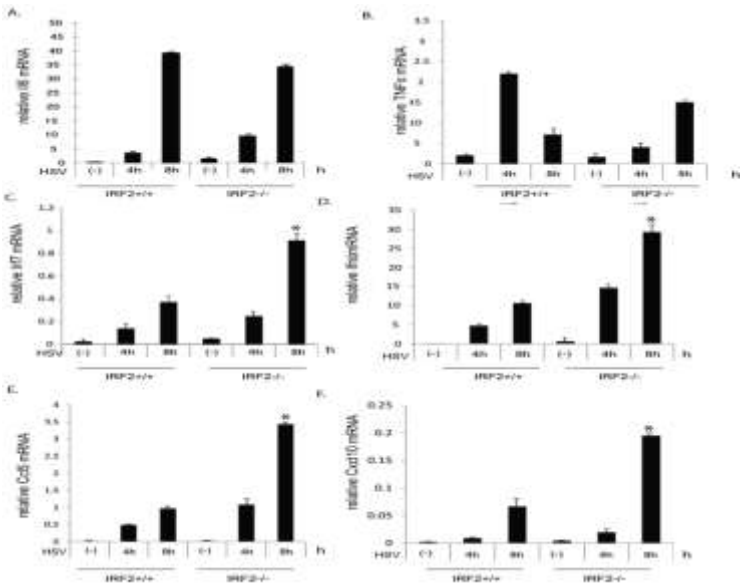

Figure 3. HSV-1 infection induces activation of TLR9 and upregulation of proinflammatory cytokines and chemokine gene expression in IRF2-deficient macrophages ($IRF2^{-/-}$) and control ($IRF2^{+/+}$) cells (Fig.3). The primary macrophages were infected with HSV-1 (MOI-1) for 4 and 8 hours. Proinflammatory cytokines *Il6*, *Tnfα* and chemokines gene *Ccl5*, *Cxcl10* mRNA were measured by quantitative RT-PCR (Fig3. A,B,E,F). Analysis of cytokine *Ifnβ* and *Irf7* mRNA upon infection with HSV-1 (Fig3. C, D); results are presented relative to *Gapdh* mRNA expression **P* < 0.05 and ***P* < 0.01, ( Student's *t*-test). Data is representative of three independent experiments (mean and s.d. of triplicates).

Macrophages as innate immune cells are potently inducers of *Ifnβ* gene expression following TLR9 receptor activation. Next, we analyzed the *Ifnβ* and *Irf7* mRNA level in IRF2-deficient and control macrophages. The *Ifnβ* mRNA level increased upon HSV-1 infection, as expected, on macrophages laking IRF2. Our results confirm IRF2 role as attenuator of IFNα/β gene and induced by IRF7 transcription factor in macrophages following TLR9 activation. The *Irf7* mRNA increased 3.5 fold times 8 hours post infection in the absence of IRF2. Both CpG B ligand and HSV-1 virus infection activate TLR9 downstream signaling pathways IFN and NF-kB in the same regulatory mechanism in absence of IRF2 transcription factor, with some differences on genes expression.

## 4. Conclusions

Our experiments have demonstrated that early kinetics of NF-kB/p65 activation decreased by IRF2 gene knockdown macrophages, suggesting that IRF2 may be a positive regulator of p65 expression. Similar results were observed for known NF-kB-dependent genes, such as *Il6*, expression levels of which were upregulated in response to IRF2 absence. Here we present the IRF2 transcription factor function downstream of TLR9 signaling pathway and is required for activation kinetics of NF-kB directly or indirectly via other IRF

transcription factor and NF-kB. We may speculate that the possible interactions between NF-kB and IRF2 pathways occur at gene promoter level but this should be further investigated.

Our data indicated that IRF2 role in *Il12p40* gene expression is more interesting and could be exploited for the manipulation of exacerbated immune responses and in inflammatory diseases, including some autoimmune diseases. Therefore NF-kB appears to be a crucial mediator involved in the pathogenesis of many autoimmune diseases including tumors. Studies on cytokine production pathways have also pointed towards a key role for NF-kB activation in the immunopathologic conditions.

## 5. Aknowledgements

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