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Distinct functions of IRF-3 and IRF-7 in IFN-alpha gene regulation and control of anti-tumor activity in primary macrophages

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ABSTRACT

Type I IFN (IFN-α/β) have important biological functions ranging from immune cell development and activation, to tumor cell killing and most importantly inhibition of virus replication. Following viral infection or activation of Toll-like receptors (TLRs) via distinct ligands, IFN-α/β are produced. Two members of the interferon regulatory factor (IRF) family – IRF-3 and IRF-7 – are the major modulators of IFN gene expression. Activation of IRF-3 and IRF-7 by TBK1/IKKε mediated phosphorylation promotes IFN gene expression and potentiates the production of IFN responsive genes important to the development of an effective antiviral immune response. IFN treatment can augment anti-tumor properties and they are potentially key players in cancer therapy. For example, adoptive transfer of IFN-γ-activated macrophages can mediate tumor cell killing via direct cell–cell contact, as well as release of soluble cytotoxic pro-inflammatory molecules. A recent study investigated whether IRF-3 and IRF-7 could mediate the acquisition of new anti-tumor effector functions in macrophages. Adenovirus mediated transduction of the active form of IRF-7 into primary macrophages resulted in the production of type I IFN, upregulation of target genes including TRAIL and increased tumoricidal activity of macrophages; in contrast, the active form of IRF-3 led to induction of cell death. These studies indicate that IRF-7 transduced macrophages may be an attractive candidate for *in vivo* adoptive therapy of cancer.

1. Introduction

The interferon (IFN) family constitutes an important class of cytokines and is composed of transcriptionally activated and secreted proteins with pleiotropic biological effects on the host. IFNs play a central role in the resistance of mammalian hosts to pathogens, and in the modulation of antiviral and immune responses [30,68]. The IFN proteins group into two classes: type I (IFN-α and -β) and type II (IFN-γ), which bind two distinct cell surface receptors, type I and type II IFN receptors, respectively (for review see [51]). It is important to point out that other antiviral, IFN-like molecules termed IFN-λ, which

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could be classified as type III IFN, have recently emerged [78]. Although, some immunomodulatory effects are shared by IFN-α/β and IFN-γ, type I IFNs exert stronger antiviral, anti-proliferative and antiangiogenic effects than IFN-γ and are widely administered as adjuvant therapy in cancer and viral related diseases.

2. Biological effects of type I interferons

IFN-α/β exerts a vast spectrum of biological functions, the best characterized of which is its role in the effective inhibition of replication of many RNA and DNA containing viruses. After IFN stimulation, hundreds of cellular genes known as interferon-stimulated genes (ISGs) are transcriptionally activated [17]. These genes encode proteins such as RNase L, dsRNA-dependent protein kinase (PKR), and 2′–5′ oligoadenylate synthetase (OAS), that mediate antiviral activities directly or indirectly.

In the establishment of an effective antiviral response, type I IFN upregulates various effector molecules that directly affect protein synthesis, cell proliferation and cell survival. IFN-α and -β are used in clinical settings for the treatment of chronic hepatitis C. In addition, it has been shown that IFN-α treatment reduces the risk for HCC in patients with chronic hepatitis C (for review see [50,71].

IFN-α/β also exerts profound effects on the immune system (Fig. 1). IFN-α/β regulates the homeostatic differentiation of hematopoietic cells such as B cells, T cells, osteoclasts, myeloid dendritic cells (DCs) and natural killer (NK) cells. In the case of immature DCs, activation and maturation of DCs can be induced by IFN-α/β, leading to the upregulation of major histocompatibility complex (MHC) molecules (especially class I MHC), chemokines, chemokine receptors and costimulatory molecules (CD40, CD80, CD86), which in turn leads to efficient homing in secondary lymphoid organs and $CD8^+$ or $CD4^+$ T cell responses [70]. In NK cells, IFN-α/β increases levels of perforin and leads to the induction of cytotoxic activity [6]. T lymphocyte responses are also modulated through IFN-α/β promotion of Th1 differentiation [9,49]. In particular, the inhibition of T cell death is promoted directly by IFN-α [43]. The development and function of B cells are also affected by type I IFNs. B cell receptor (BCR) mature B2 cell responses are enhanced by IFN-α/β [8] and as with T cells, type I IFN can inhibit B cell development and survival by increasing resistance to Fas-mediated apoptosis.

IFN-α/β also possessses potent anti-proliferative and anti-tumor functions [5,64]. In clinical settings, IFN-α/β is administered to patients suffering from various malignancies such as melanoma, hairy cell leukemia, renal cell carcinoma and Kaposi's sarcoma. The immunomodulatory role of type I IFN on DC and T lymphocyte function may explain various aspects of IFN-induced tumor immunity [5] (for review see [67]). IFN-α/β are also known to induce apoptosis; type I IFN exerts a pro-apoptotic effect associated with an increase in cyclin kinase inhibitors and several pro-apoptotic molecules (Fas/FasL, p53, Bax, Bak), as well as activation of pro-caspases 8 and 3 [11].

3. Role of IRFs in virus mediated IFN activation

IFN-β and/or IFN-α are rapidly produced following the sensing of incoming viral particles (via nucleic acid or ribonucleoprotein complexes) in the cytoplasm of cells or after engagement of Toll-like receptors (TLRs) in immune cells [31,73]. Viral entry or engagement of TLR3 or TLR4 induces the activation of latent transcription factors involved in immunomodulation, including IRF-3, NF-κB and ATF-2/c-Jun [40,61]. The production of

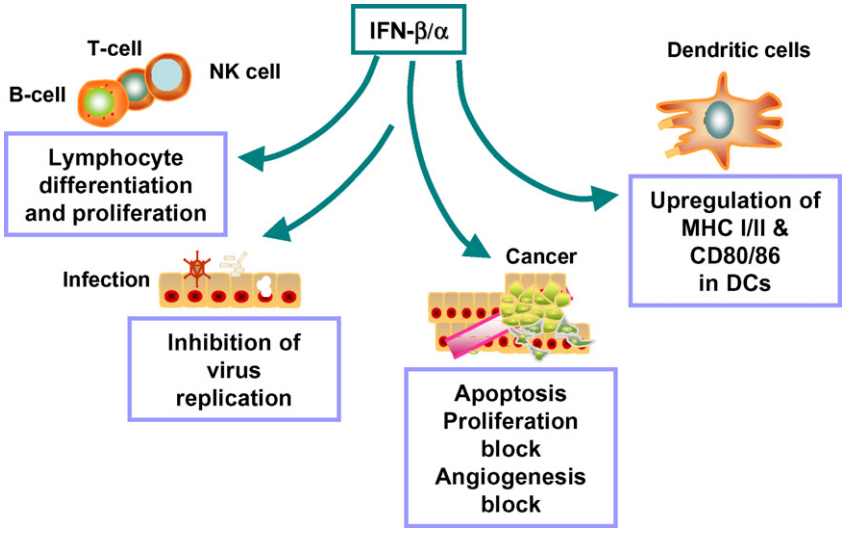

Fig. 1 – Diverse biological effects of type I interferons (IFN-α/β). IFN-α/β plays a central role in modulating host antiviral and immune responses by exerting a wide variety of biological functions. Immune cell development and differentiation are affected by type I IFN expression. These cytokines induce dendritic cell maturation by upregulating MHC class I and II and CD80/86 co-stimulatory molecules, as well as B-cell, T-cell and NK-cell differentiation and proliferation. In addition, IFN-α/β possesses pro-apoptotic, anti-proliferative and anti-angiogenic functions. Because of their potent effects on immune regulatory cell activation, IFN-α/β mounts an effective innate and adaptive immune response necessary for the inhibition of virus replication.

IFN-β relies in part on the C-terminal phosphorylation and activation of the cytoplasmic interferon regulatory factor (IRF)-3 transcription factor [61]. IRF-3 is a constitutively expressed protein of 55 kDa (427 aa) present in most cell types [1]. In addition to an N-terminal DNA binding domain that is conserved amongst all the IRF family members, IRF-3 contains a highly condensed, hydrophobic C-terminal structure involved in protein–protein association and gene activation [46,52,65]. The C-terminal region comprises a cluster of phosphoacceptor sites ^{382}GGASSLENTVDLHISNSHPLSLTSDQYKAYLQD^{414} and virus-induced phosphorylation is thought to realign the C-terminal domain and relieve autoinhibition [22,53]. Once phosphorylated, IRF-3 dimerizes and translocates to the nucleus, associates with CREB binding protein (CBP/p300) co-activators and participates in the transcriptional activation of the IFN-β and human IFN-α1 (or murine IFN-α4) promoters [19,23,35,58]. Binding of the newly secreted IFN-β and IFN-α1 to receptors on adjacent cells leads to the activation of the Jak/STAT signaling pathway. The ISGF3 complex (ISGF3γ/IRF-9-STAT1-STAT2) binds to interferon-stimulated response elements (ISRE) found in numerous IFN-induced gene promoters such as PKR, 2′–5′ oligoadenylate synthase and IRF-7 [33,41,55,58,59,61]. Microarray analysis demonstrated that a constitutively active form of IRF-3 also induces the expression of other ISGs such as ISG56, ISG54 and ISG60 [19].

IRF-7 was first described to bind and repress the Epstein Bar Virus (EBV) Qp promoter, which regulates expression of the EBV nuclear antigen 1 (EBVA1) [77], and shortly after its discovery, the importance of IRF-7 in IFN regulation was recognized [2,56,76]. IRF-7 is an IFN-inducible and virus-inducible protein in most cells with transcriptional activity that depends on C-terminal phosphorylation. Similarly to IRF-3, IRF-7 is activated by virus-induced phosphorylation at its C-terminus [10,34]. Active IRF-7 homodimers or heterodimers with IRF-3, bind to promoters of all IFN-α genes, and are thus ultimately responsible for the induction of delayed type I IFNs and amplification of the interferon response [41,58,60]. The serine rich C-terminal region between aa471–487, is the target of virus-induced phosphorylation [34,42,56] by TBK1 and IKKε [62,69]. Serines 477 and 479 appear to be critical targets for TBK1 and IKKε, since their substitution to alanine resulted in an IRF-7 that was unable to respond to virus challenge [36,69]. Interestingly removal of the aa247–467 region also creates a highly active form of IRF-7 (known as IRF-7 Δ247–467) [36]. IRF-7 protein has a short half-life of approximately 30 min, which may represent a mechanism that ensures transient IFN induction [57].

Using IRF-7−/− mice, Honda et al. demonstrated that IRF-7 is essential for the induction of type I IFN via virus-mediated, MyD88-independent and -dependent TLR signaling pathways. Although IRF-7−/− mice developed normally with no overt differences in the hematopoietic cell populations, they were more susceptible than MyD88−/− mice to viral infection [25]. This observation correlates with a complete inhibition of IFN-α mRNA induction and a significant reduction in IFN-β levels in IRF-7−/− cells. Also, serum IFN levels were significantly lower in IRF-7−/− MEFs [25]. In the double IRF-3/IRF-7 knockout, IFN-β levels were completely abrogated. In contrast, MyD88−/− MEFs induced IFN-α/β mRNA to similar levels as wild-type MEFs in response to virus, suggesting that IFN induction, was MyD88-independent but IRF-7-dependent [25].

4. IFN regulation in plasmacytoid dendritic cells

Plasmacytoid dendritic cells (pDC) are major IFN producing cells and stand out amongst other cells in their ability to produce high amounts of IFN-α/β following engagement of TLR9 by unmethylated DNA or stimulation of hTLR7/mTLR8 by single stranded viral RNA [14,18,20,21,74]. pDC utilize a MyD88-dependent pathway of IFN-α/β induction, which is also dependent on IRF-7 [25]. Coccia et al. hypothesize that the regulation of IFN-α production in pDCs may not be mediated by the positive feedback effect of IFN-β due to constitutive expression of IRF-7 [13] and the ability of MyD88 to recruit IRF-7, but not IRF-3, through a molecular complex including TRAF6 and IRAK4 [24]. Furthermore, comparison between IRF-3−/− and IRF-7−/− pDCs revealed that IFN induction through TLR7/8, or 9 was normal in IRF-3−/− cells, but completely ablated in IRF-7−/− cells, thus demonstrating that IRF-7 is essential for the MyD88-dependent induction of IFN-α/β genes via the nucleic acid recognizing TLR subfamily [25]. IκB kinase-α (IKK-α) is also involved in TLR7/9 induced IFN-α production in that IFN production was severely impaired in IKK-α deficient pDCs, whereas a decrease in the induction of inflammatory cytokines was observed. In addition, IKK-α associated with and phosphorylated IRF-7, thus identifying a role for IKK-α in the TLR7/9 signaling pathway [26].

5. Transcription of IFN-α gene promoters mediated by IRF-3 and IRF-7

The activation of the human IFN-α multigenic family is an important example of differential gene regulation mediated by IRF-3 and IRF-7. Among different members of human IFN-α multigenic family, IFN-α1 gene is the only immediate-early gene expressed following virus infection in different cell lines. IFN-α1 exhibits higher expression levels compared to other IFN-α genes following IFN-α/β gene amplification, depending on the cell type and virus species [3,15,76]. Analysis of IFN-α gene expression patterns in Sendai virus-infected pDCs, monocytes and conventional dendritic cells showed that each population expressed IFN-α1 as the major subtype, but that the range of IFN-α subtypes expressed in pDCs was broader [28]. In contrast, all IFN-α subtypes were expressed at the same level in influenza virus-infected monocyte-derived or plasmacytoid dendritic cells [13]. Cell type and virus specific patterns of IFN-α subtype expression are not well characterized, although differential IFN-α expression is essentially based on the differential response of the corresponding IFN-α gene promoters to IRF-3 and/or IRF-7.

In mice, maximal virus-induced transcription of IFN-α4 gene requires the presence of three IRF-elements located in the virus-responsive element, VRE-A4, delimited to the [−120 to −40] region of IFN-A4 gene promoter [4,7]. The first element corresponds to the [−98 to −87] GAAAGTGAAAAG sequence (B module), where the GT residues determine the specificity

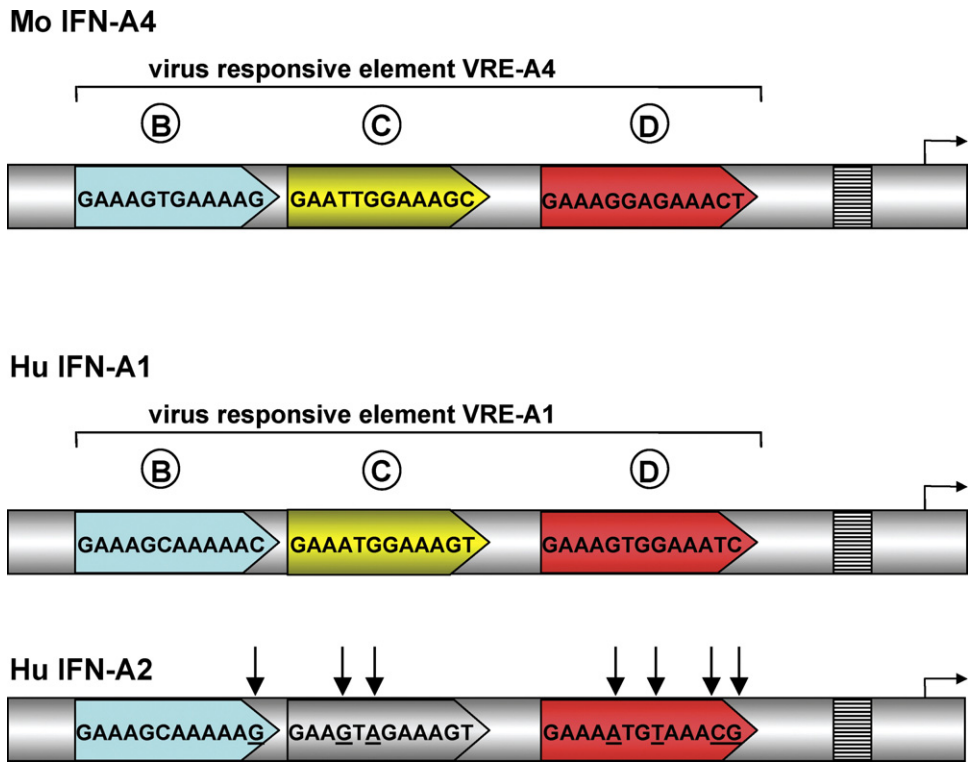

Fig. 2 – Nucleotide substitutions potentially affecting IRF-mediated transcription of mouse IFN-A4 and human IFN-A1 and IFN-A2 gene promoters. The virus responsive element VRE-A4 located in the proximal [−119 to −26] region of the mouse IFN-α4 gene promoter is presented schematically [12]. Nucleotide sequence of IRF-elements (modules B to D) and substitutions in C and D modules that potentially affect the IRF-mediated transcription of human IFN-αl and IFN-α2 gene promoters are indicated. The sequences (accession number AL 353732) were retrieved from Genbank database.

for IRF-7 and IRF-3 (Fig. 2). The [−86 to −75] GAATTGGAAAGC sequence corresponding to the C module is also a selective target for IRF-3 binding activity. The [−57 to −45] GAAAGGA-GAAACT sequence (D module) is weakly recognized both by IRF-3 and IRF-7 [45]. These IRF-binding sites differ in their individual affinity for IRF-3 and IRF-7, contribute differently to the overall recruitment of each factor to the promoter and determine the IFN-α4 gene expression levels in different cell types during virus infection [12]. Among the three modules required for IRF-3-mediated transcription, the C module is critical for IRF-3 recruitment to the IFN-α4 promoter, but is dispensable for transactivation by IRF-7, which is essentially mediated by B and D modules.

In vivo recruitment of IRF-3 and CBP to the IFN-α4 gene promoter following virus infection is dictated by the precise arrangement of the IRF-elements; recruitment is dramatically reduced by reversal of B or C module in IFN-α4, resulting in impaired IRF-3-mediated transcription. In contrast, reversal of the B or C module does not affect IRF-7-dependent transactivation, suggesting that cooperative transcription of IFN-α4 promoter by IRF-7 homodimers bound to B and D modules is distinct from its IRF-3-dependent transcription. The absence of one or two IRF-elements in the promoter destabilizes the ordered assembly required for IRF-3-mediated cooperative transcription and CBP recruitment, as in the case of IFN-α11 gene [12]. Naturally occurring nucleotide substitutions in both the C and D modules abrogate IRF-3-mediated transcription of IFN-α11 gene promoter, but affect IRF-7 mediated transcription to a lesser extent. These data suggest that IFN-α promoters lacking one or two IRF-elements may nevertheless respond to virus infection in cells expressing IRF-7.

A similar promoter organization is present in human IFN-α1 gene: three IRF-binding elements are located at similar positions and in similar orientations within the promoter proximal −120 bp region (Fig. 2). This analysis also reveals that the sequences corresponding to potential B and D elements are more conserved in all human IFN-α gene promoters than those corresponding to module C. Sequence homology between the C modules of human IFN-α1 and mouse IFN-α4 suggests that this module plays a pivotal role for IRF-3-mediated transactivation of human IFN-α1 gene promoter. IRF-3-mediated expression of other IFN-α genes may be selectively attenuated, since nucleotide substitutions considerably alter the GAAATG motif of the C module in their promoter (GAAGTA in IFN-α2). Based on the model proposed for murine IFN-α4, the B, C and D modules may participate in IRF-3-mediated transcription of human IFN-α1, whereas activation by IRF-7 is essentially mediated by B and D. This model predicts that IRF-3, but not IRF-7 mediated transcription must be attenuated in other human IFN-α gene promoters lacking the C module. Constitutive expression of IRF-7 and IKKε in lymphoid cells or mature dendritic cells may thus reduce differential expression of IFN-α rather leading to massive IFN-α expression. Since the expression of IRF-7 and

IKKε are both virus-inducible, differential expression of IFN-α may predominate early after virus infection, but may be balanced in other cell types such as fibroblasts and macrophages during late phase induction [38,57,69].

6. Macrophages and their anti-tumoral properties

Macrophages are present in a resting state in many tissues, where they perform diverse functions including tissue remodeling, inflammation, immunity, clearance of apoptotic cells, healing and angiogenesis. However, macrophages can also play a dual role in host defense. They can trigger both an innate immune response and contribute to the development of the adaptive immune response. For example, macrophages are able to phagocytose microbial particles through the use of Fcγ receptors (FcγR), leading to the secretion of pro-inflammatory chemo- and cytokines and the release of oxygen radicals and tumor necrosis factor (TNF) that will directly harm the microorganism [32]. Furthermore, upon pathogen recognition, macrophages also promote antigen processing and presentation through the binding of the antigen-MHC II complex to the T cell receptor (TCR) of $CD4^+$ T cells, resulting in the polarization of T cells to a T helper 1 (Th1) response [39].

Macrophages are capable of recognizing and killing tumor cells. When properly activated, macrophages exert tumoricidal activities via direct cell-to-cell interaction. This results in membrane disruption and lysis of target tumor cells through phagocytosis and antibody-dependent cell cytotoxicity (ADCC). Soluble cytotoxic pro-inflammatory macrophage-derived products such as IL-1, TNF, TRAIL, FasL, nitric oxide (NO) and oxygen radicals (ROS) may also lyse tumor cells (for review see [32]). Furthermore, macrophages primed with interferon (IFN)-γ increases the expression of FcγR, inducible nitric oxide synthase (iNOS), TRAIL (TNF-related apoptosis inducing ligand), cell adhesion molecules, and production of IL-1, resulting in enhanced tumor cell killing [39].

Adoptive transfer of activated macrophages is an attractive complement to conventional cancer therapies. Phase I and II studies have demonstrated the feasibility and safety of IFN-γ-activated macrophages (MAK® cells, developed by IDM, Paris, France) transfer in several malignancies, including mesothelioma, ovarian, and bladder cancer [16,44,48,72].

7. Expression of active IRF-3 and IRF-7 in primary human macrophages

As type I IFN and activated macrophages are currently used in the clinical setting for the treatment of various cancers, we tested the hypothesis that IRF-3 and IRF-7 contribute to the acquisition of new anti-tumor effector functions of macrophages. Recombinant adenoviruses (Ad) were generated expressing constitutively active forms of IRF-3 (IRF-3 5D) and IRF-7 (IRF-7 Δ247–467) known as Ad-IRF-3 and Ad-IRF-7, respectively. IRF-3 5D induces strong activation of the *IFNB* promoter in the absence of virus induction [35,37] and IRF-7 Δ247–467 activates *IFNA* gene transcription more than 1000-fold compared to wild-type IRF-7 [36]. Ad-IRF-3 transduced macrophages induced low levels of IFN-β and IFN-α2 mRNA, but no detectable IFN-α1 mRNA. For Ad-IRF-7 on the other hand, the mRNA levels for IFN-β, IFN-α2, IFN-αl were considerably higher. Expression of active IRF-3 5D by primary human macrophages resulted in rapid cell death, while expression of IRF-7 Δ247–467 was not pro-apoptotic. As IFN-β is known to upregulate p53 expression and thereby the apoptotic cellular response, it can be argued that cell death induced by Ad-IRF-3 could be mediated by IFN-α/β [66]. However, cell death was not solely associated with IFN-α/β expression, since Ad-IRF-7 was a more potent inducer of *IFNA* and *IFNB* transcription compared to Ad-IRF-3. IRF-3 and IRF-7 harbor distinct DNA binding recognition sequences and other transcriptional properties such as the recruitment of transcriptional co-activators and may therefore differentially regulate expression of apoptotic genes (for review [61]). Studies are underway to investigate IRF-3 or IRF-7 target genes in the apoptotic response in primary macrophages or DC.

Ad-IRF transduced macrophages produced type I IFNs and displayed increased expression of genes encoding TRAIL, IL-12, IL-15, ISG-56 and CD80. mRNA levels, with the exception of IL-12, are higher for Ad-IRF-7 relative to Ad-IRF-3 transduced macrophages. Macrophages and tumor cells are known to release pro-tumorigenic factors that constitute a major drawback for macrophage adoptive transfer in cancer therapy. Recombinant IFN-α inhibits expression of IL-8, VEGF, MMP-2 and MMP-9 by tumor cells *in vitro* and *in vivo* [27,29,54,63,75]. Molecular mechanisms underlying these effects are not well characterized, although IFN signaling may create competition for transcriptional modulators that bind preferentially to ISG promoters and decrease the expression of other genes [47]. Interestingly, we observed that the transcription of the pro-angiogenic gene VEGF was down-regulated by expression of active IRF-7 in macrophages, whereas downregulation of the metastatic gene MMP-2, was seen in Ad-IRF-3 transduced macrophages, suggesting that IRFs may increase macrophage anti-tumor properties while reducing their pro-tumorigenic effects. Furthermore, Ad-IRF-7-transduced macrophages exerted a cytostatic activity on different cancer cells lines, including SK-BR-3, MCF-7 and COLO-205, even though the latter cells were previously shown to be insensitive to MAK cells.

In vivo, primary macrophages transduced with Ad-IRF-7 may mediate their anti-tumor effects by four distinct mechanisms (1) directly, via secretion of type I IFN (in the case of type I IFN-sensitive tumors); (2) after activation of macrophages by either IRF-7 or type I IFN, enhancing their effector functions; (3) indirectly, via recruitment and polarization of other immune cells by type I IFN or other macrophage-derived factors, including chemokines; and (4) via down-regulation of expression of genes known to promote metastasis and angiogenesis.

8. Conclusion

Transduction of active IRF-7 into primary macrophages of MAK cells is an attractive strategy for *in vivo* cancer therapy via adoptive transfer. The present studies illustrate that IRF-3 and IRF-7 activate distinct gene expression programs in macrophages and demonstrate that IRF-7 modulates the anti-tumor properties of primary macrophages (Fig. 3). While IRF-3

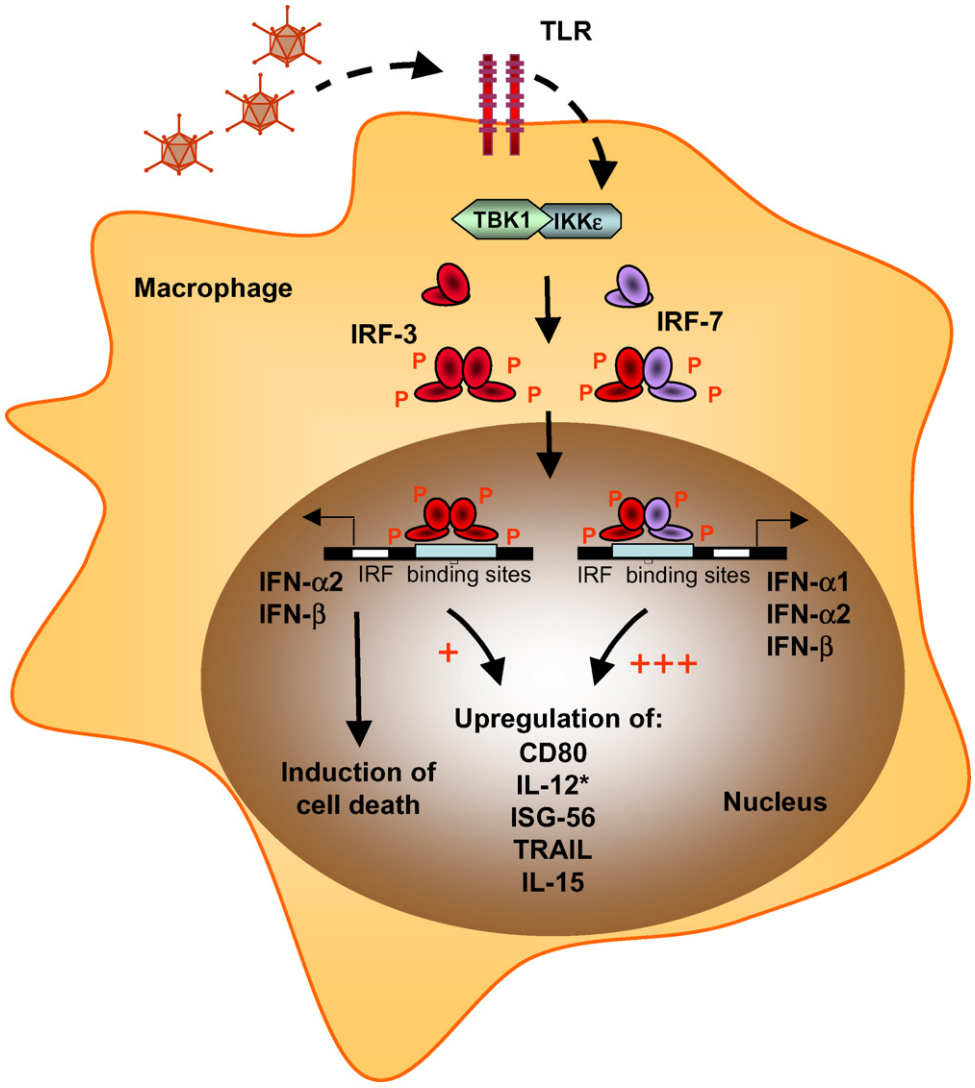

Fig. 3 – Distinct roles of IRF-3 and IRF-7 in macrophage function. Following viral entry into macrophages via TLRs, various downstream pathways are activated, including the non-canonical IKK-related kinases TBK1 and IKKε. These kinases are responsible for the C-terminal phosphorylation and activation of IRF-3 and IRF-7 transcription factors. Following dimerization and translocation to the nucleus, IRF-3 and IRF-7 participate in the transcriptional activation of IFN promoters. While active IRF-3 induces low levels of transcriptional activation of IFN-α and IFN-β, the activated form of IRF-7 triggers higher expression of these genes. Phosphorylated forms of IRF-3 and IRF-7 enhance the transcription of CD80, ISG-56, TRAIL, IL-15 and IL-12. Activated IRF-7 induces a higher expression of these genes (indicated as +++) relative to IRF-3 activation (indicated as +) with the exception of IL-12 (*) in primary human macrophages. In contrast, activated IRF-3 induces significant apoptosis in primary macrophages.

appears to be involved in the early induction of apoptosis, IRF-7 induces the higher expression of a distinct set of genes such as TRAIL, IL-15, ISG-56 and CD80. Harnessing the immunomodulatory and anti-tumor properties of an IRF-7 genetic program may ultimately improve the utility of adoptive transfer of macrophages in cancer therapy.

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