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Identification of IRF-8 and IRF-1 target genes in activated macrophages.
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Identification of IRF-8 and IRF-1 Target Genes in Activated Macrophages

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Abstract

Interferon Regulatory Factor 1 (IRF-1) and IRF-8, also known as Interferon Consensus Sequence Binding Protein (ICSBP), are important regulators of macrophage differentiation and function. These factors exert their activities through the formation of heterocomplexes. As such, they are coactivators of various interferon-inducible genes in macrophages.

To gain better insights into the involvement of these two transcription factors in the onset of the innate immune response and to identify their regulatory network in activated macrophages, DNA microarray was employed. Changes in the expression profile were analyzed in peritoneal macrophages from wild type mice and compared to IRF-1 and IRF-8 null mice, before and following 4hrs exposure to IFN- γ and LPS.

The expression pattern of 265 genes was significantly changed (up/down) in peritoneal macrophages extracted from wild type mice following treatment with IFN- γ and LPS, while no changes in the expression levels of these genes were observed in samples of the same cell-type from both IRF-1 and IRF-8 null mice. Among these putative target genes, numerous genes are involved in macrophage activity during inflammation. The expression profile of 10 of them was further examined by quantitative RT-PCR. In addition, the promoter regions of three of the identified genes were analyzed by reporter gene assay for the ability to respond to IRF-1 and IRF-8. Together, our results suggest that both IRF-1 and IRF-8 are involved in the transcriptional regulation of these genes. We therefore suggest a broader role for IRF-1 and IRF-8 in macrophages differentiation and maturation, being important inflammatory mediators.

Key words

Interferon Regulatory Factors (IRFs); IRF-8; IRF-1; ICSBP; Macrophage activation; Transcriptional regulation; CXCL16; H28; LIF; MAP4K4; MMP9; MYC; PCDH7; PML; SOCS7;

Abbreviations

CML, Chronic Myelogenous Leukemia; CXCL16, Cxc Chemokine Ligand 16; ICSBP, Interferon Consensus Sequence Binding Protein; IFN, Interferon; IL-17R, Interleukin-17 Receptor; IRF, IFN Regulatory Factor; KO, Knockout; LIF, Leukemia Inhibitory Factor; MAP4K4, Mitogen Activated Protein Kinase 4; MiHC, Minor Histocompatibility Complex 28 (H28); MMP9, Matrix Metalloproteinase 9; MYC, Myelocytomatosis oncogene; PCDH7, Protocadherin 7; PML, Promyelocytic Leukemia; WT, Wild Type;

1. Introduction

Interferon (IFN) Regulatory Factor-8 (IRF-8), also known as Interferon Consensus Sequence Binding Protein (ICSBP), and IRF-1 are members of the IRF family of transcription factors, which include 9 cellular members (Mamane et al. 1999). In general, these factors are important immunomodulators and are essential components of the IFN signaling cascade. Using in-vitro assays, these two transcription factors were identified in association on the promoter region of numerous macrophage essential genes (Marecki and Fenton 2000; Xiong et al. 2003). IRF-8 is a key element for the differentiation of myeloid progenitor cells towards macrophages and for mature macrophage activity. Accordingly, IRF-8^{-/-} mice exhibit clinical manifestation that resembles the human Chronic Myelogenous Leukemia (CML). Comparable to CML patients, these mice experience a rapid and systemic expansion of granulocytes that eventually results in a fatal blast crisis (Holtschke et al. 1996). Consistent with this, lack of IRF-8 was reported in human myeloid leukemias, implicating its pivotal role as a tumor suppressor gene (Schmidt et al. 1998). Thus, IRF-8 drives bipotential myeloid progenitor cells towards mature macrophages while inhibiting the differentiation pathway towards granulocytes (Tamura et al. 2000). In addition, it was shown that IRF-8 is an essential factor for proper functioning of mature macrophages. For example, IRF-8 null mice fail to mount Th1-mediated immune response, since they fail to produce the IL-12 p40 subunit (Wang et al. 2000). Similarly, IRF-1^{-/-} are also devoid of Th1 responses due to deficiency of the same IL-12 subunit (Taki et al. 1997; Lohoff et al. 1997). Further, clinical studies have assigned important role for IRF-1 in the onset of myelodysplastic syndromes, which are clonal myeloid disorders (Willman et al. 1993). These studies indicate that IRF-1, like IRF-8, is a tumor suppressor gene with essential functions in the maturation of myeloid cells to macrophages.

Unlike other IRF members, IRF-8 is capable of binding to its target DNA sequence only following association with either IRF-1 and IRF-2 or non-IRF transcription factors, such as

PU.1, an essential factor for hematopoiesis (Sharf et al. 1997; Wang et al. 2000; Dahl and Simon 2003) The domain essential for these protein-protein associations, termed IRF Association Domain (IAD), is conserved among all other IRF members excluding IRF-1 and IRF-2. The association modules of IRF-1 and IRF-2 with IRF-8 were identified as PEST domains, enriched with proline, glutamic acid, serine, and threonine, originally shown in PU.1 (Levi et al. 2002). The specific partner that interacts with IRF-8 dictates not only the DNA binding site but also the transcriptional activity e.g. activation or repression. The stoichiometry between the interacting partners was not determined and it is possible that several PEST domains interact with an IAD, as observed on promoters of several macrophage specific genes, such as IL-12, IL-1 β , and ISG15 (Meraro et al. 2002; Marecki et al. 2001b).

As mentioned above, IRF-1 is one of the partners associated with IRF-8. Together, they play critical role in the regulation of several pro-inflammatory genes that function during macrophage activation; These two factors synergistically regulate the transcription of iNOS (Xiong et al. 2003), the two components of the phagocyte respiratory burst oxidase: p67 and gp91 (Eklund and Kakar 1999), the transcription of IL-12 (Masumi et al. 2002) , IL-18 (Kim et al. 1999), IL-1 β (Marecki et al. 2001b), and ISG15 (Meraro et al. 2002) . Taken together, IRF-8 and IRF-1 are essential components of macrophage functioning and innate immune response.

To get better insight into the role of these transcription factors in the onset of the innate immune response and to identify their regulatory network in macrophages, we performed DNA microarray analysis. We have used mRNA retrieved from macrophages extracted from IRF-8 Knockout (KO) mice, IRF-1 KO mice, and C57BL/6J, the counterpart wild type (WT) strain. Changes in the expression profiles were analyzed before and after four hours of exposure to both IFN- γ and LPS. The levels of mRNA transcripts from treated and untreated macrophages were compiled and compared. The expression of 265 genes was

significantly altered (fold of change ≥ 2.0 $p < 0.001$) in WT mice while no changes were observed in the transcripts of these genes in both KO mice. Thus, our results suggest that both IRF-1 and IRF-8 are involved in the transcriptional regulation of these differentially-expressed genes. Among these putative target genes, numerous are involved in macrophage activity during inflammation pointing to the pivotal regulatory role of both IRF-1 and IRF-8 in the inflammatory process.

2. Materials and methods

2.1 Animals

The mouse strains; C57BL/6J (Harlan Biotech, Israel), IRF-1 deficient (Kindly obtained from Dr. Rubinstein, The Weizmann Institute, Israel, originally from The Jackson Laboratory), and IRF-8 deficient (Holtschke et al. 1996), were maintained in microisolator cages in a viral pathogen-free facility.

2.2 Isolation of peritoneal macrophages

4 days prior to experiment, mice were injected i.p. with 3 ml 3% sterile Thioglycolate medium. Mice were sacrificed by CO₂ inhalation, 10ml sterile PBS was injected to the peritoneal cavity, peritoneal lavage enriched in macrophages were extracted. Peritoneal macrophages were washed in 15ml of pre-warmed RPMI 1640 supplemented with 2%FCS, 2mM glutamine, 30mM HEPES, 0.4% sodium bicarbonate, and penicillin and streptomycin (100IU/ml and 100 µg/ml, respectively). ~ 2.5X10⁶ cells were plated in tissue culture Petri dishes (100mm) at 37°C and 6% CO₂. 4 hours later, non-adherent cells were removed by two successive washes and 24 hours later, adherent cells were either not treated or treated for 4 hours with 100U/ml IFN-γ (CytoLab, Rehovot, Israel) and 50 ng/ml LPS (Sigma). All animal work conformed to the guidelines of the animal care and use committee of Technion – Israel Institute of Technology.

2.3 Cell lines

Murine bone marrow derived RAW264.7 macrophage cell line was obtained from ATCC (Manassas, VA). CL-2 macrophage cells, established from IRF-8 KO mice, were previously reported (Wang et al. 2000). Cells were maintained in RPMI 1640, supplemented with 40µM β-mercaptoethanol, 10% FCS and antibiotics. 5ng/ml M-CSF and CSF (R&D systems, Minneapolis, MN) were added to the growth medium of CL2

cells. Treatment with IFN- γ and LPS was performed as described for peritoneal macrophages.

2.4 Plasmids

Mammalian expression vectors encoding for IRF-8, IRF-1, and PU.1 were all described previously (Alter-Koltunoff et al. 2003). The reporter construct pGL3-MMP9, containing the -634 bp to +30 bp of the MMP9 promoter, driving the expression of the luciferase gene, was kindly obtained from Dr. Douglas D. Boyd (The University of Texas MD Anderson Cancer Center, Houston, Texas). The pGL3-PML containing the 1.44 kb promoter region conjugated to luciferase was a kind gift from Dr. Hugues de Thé (Hopital St. Louis, Paris, France). Genomic DNA fragment corresponding to the promoter region of pCDH7 (from position -1, upstream to the translation initiation site, up to position -1000) was PCR amplified (5' primer CCGCTAGCCCTCACTTCAGCTCTTAC; 3' primer GAGATCTTGTTGGAGCAGTGGGATC) and cloned between the SacI and XhoI restriction sites of the basic reporter plasmid pGL3 (Promega).

2.5 RNA extraction, Sample preparation, hybridization, and Array processing

Plates were washed once with 10 ml of ice cold PBS, then lysed with 1 ml/plate of Tri-Reagent (Sigma) and RNA was prepared according to the manufacturer instructions.

Total RNA from each sample was used to prepare biotinylated target RNA, with minor modifications from the manufacturer's recommendations. Briefly, 10 μ g of total RNA was used to generate first-strand cDNA by using a T7-linked oligo (dT) primer. After second-strand synthesis, in vitro transcription was performed with biotinylated UTP and CTP (Enzo Diagnostics), resulting in approximately 100-fold amplification of RNA, which was then processed as per manufacturer's recommendation using an Affymetrix GeneChip Instrument System. Briefly, spike controls were added to 10 μ g fragmented cRNA before

overnight hybridization. Arrays were then washed and stained with streptavidin-phycoerythrin, before being scanned on an Affymetrix GeneChip scanner.

Quality and amount of starting RNA were confirmed using an agarose gel. Following scanning, array images were assessed by eye to confirm scanner alignment and the absence of significant bubbles or scratches. 3'/5' ratios for Glyceraldehyde-3'-Phosphate Dehydrogenase (GAPDH) and β -actin were confirmed to be within acceptable limits range from QC report here, and BioB spike controls were found to be present on 100%, with BioC, BioD and CreX also present in increasing intensity. When scaled to a target intensity of 150 (using Affymetrix MAS 5.0 array analysis software), scaling factors for all arrays were within acceptable limits as were background, Q values and mean intensities.

All experiments were performed using Affymetrix Mouse 430A 2.0 oligonucleotide arrays, as described at <http://www.affymetrix.Com/products/arrays/specific/mgu74.affx>.

Genes were filtered using Mas 5 algorithm results. A list of 14025 probe sets, "valid genes", representing probe sets with signals higher than 20 and detected as "present" in at least one sample was obtained.

2.6 DNA transfections and reporter gene analyses

RAW 264.7 and CL-2 cells were diluted to 10^6 cells/ml 16-24 hrs prior to transfection. At the time of transfection, cells were washed twice in phosphate buffered saline (PBS) and suspended at 4×10^7 cells/ml in RPMI medium lacking fetal calf serum and antibiotics. 0.25 ml of the cell suspension was placed into 0.4 cm electroporation cuvette (Bio-Rad, Richmond, CA, USA) with up to 30 μ g of plasmids DNA suspended in no more than 20 μ l of distilled water, including 8 μ g of the various reporter gene constructs and 1 μ g of IRF-8 and/or IRF-1 expression plasmids (as indicated in the text). Cells and plasmids DNA were incubated for 5 min at room temperature prior to electroporation at 960 μ F and 300V for RAW264.7 cells and 250V for CL-2 (Bio-Rad gene pulser). Following the electric shock, cells were left at room temperature for additional 15min and then diluted into 10 ml of

RPMI containing 10% FCS. 24 hrs following electroporation, cells were harvested, lysed using the lysis buffer of the Dual Luciferase assay kit (Promega), and luciferase activities were determined according to the manufacturer's instructions using a TD-20/20 luminometer (Turner Design, Promega). Reporter gene activities were normalized for protein concentration and transfection efficiencies as described (41). Each set of transfection experiments was repeated at least three times generating similar results.

2.7 Quantitative RT-PCR analysis

A quantitative Real-Time-PCR analysis of mRNA expression was performed to confirm the differential expression of the selected genes. 100ng total RNA from each sample was reverse-transcribed to cDNA using Reverse-iTTM 1st strand synthesis kit (ABgene, Surrey, UK) according to manufacturer's instructions. cDNA was amplified with two primers for each gene (Table I) using ABsolute SYBER Green ROX Mix (ABgene, Surrey, UK) and Rotor-Gene 3000TM Real-Time thermal cycler (Corbett Research, Australia) according to the manufacturer's instructions. Briefly, 4 µl of cDNA template (diluted 1:10) was mixed with 5 pmole of each primer (Sigma), 12.5µl *ABsolute* reaction mix and H₂O to reach 25µl per reaction. The amplification conditions for all reactions were 1 x 95°C for 15 minutes followed by 40 x 95°C for 10 seconds, 60°C for 20 seconds, 72°C for 15 seconds. Melting reaction (72-99°C) for 5 seconds/1°C verified that the reactions contained a unique product. Transcript levels were normalized against the expression levels of GAPDH. Standard curves were constructed for each run by using serial dilutions of the corresponding cDNA from RAW 264.7 cells. Each standard and sample was measured in triplicates. Quantitative results of Real-Time PCR were assessed by determining the relative Calculated Concentration (Copies) value. A calibration curve was derived by plotting the Calculated Concentration values obtained for each dilution. The calibration curves showed a correlation coefficient (R^2) between 0.98 and 1.01. The estimated amount of transcripts was normalized to GAPDH mRNA expression to compensate for variations in quantity or

quality of starting mRNA and for differences in reverse transcriptase efficiency. The gene of interest/GAPDH ratio is expressed in percentages.

2.8 Bioinformatics analysis of promoter regions

Analysis of the promoter sequences was carried out with GEMS Launcher (Genomatix Software GmbH, Munich, Germany) using the FrameWorker tool which is based on the algorithms described (Frech et al. 1997; Frech and Werner 1997; Quandt et al. 1995; Quandt et al. 1996).

3. Results

3.1 Stimulation of peritoneal macrophage with IFN- γ and LPS leads to differential expression of genes involved in various aspects of macrophage activity

To look for changes in peritoneal macrophages expression profile following stimulation, mice were injected intraperitoneally with Thioglycolate and cells were extracted by peritoneal lavage 4 days later. The isolated macrophages were either treated or not treated with IFN- γ and LPS to mimic bacterial infection. Four hours later, total RNA was extracted and utilized for DNA microarray analysis. We focused on early response transcripts whose expression level changed significantly following stimulation in macrophages from WT mice.

12,713 probe sets indicated valid signals from which data could be analyzed. Among these, the expression level of 1009 genes was significantly changed (up or down) in macrophages from WT mice treated with IFN- γ and LPS (Fig. 1). This list includes numerous transcripts that have been reported to be altered following macrophage activation, such as IL-1 β , IL-6, iNOS, TNF receptor 1, GBP and more (for review see (Ricciardi-Castagnoli and Granucci 2002)). Altogether, these data demonstrate the validity of the results obtained by the array analysis.

3.2 Microarray analysis revealed a group of genes regulated by both IRF-1 and IRF-8

Our main goal in this study was to identify genes that are potentially regulated by both IRF-1 and IRF-8 using DNA microarray with mRNA extracted from macrophages of the corresponding KO mice. We focused on transcripts whose expression level changed significantly following stimulation in WT macrophages, but were unchanged in macrophages from both IRF KOs. Out of the 1009 genes that were either up- or down-regulated in activated macrophages extracted from WT mice, 387 and 360 genes did not exhibit any significant change in the IRF-1 and IRF-8 KO mice respectively (Fig. 1). Among these unaffected genes in the KO mice, 265 transcripts did not exhibit any change

in expression pattern in both KO strains following macrophage activation. Thus, our results suggest that both IRF-1 and IRF-8 are essential transcriptional regulators of these 265 genes in activated macrophages.

3.3 Quantitative RT-PCR analysis confirms the role of both IRF-1 and IRF-8 in the regulation of genes involved in innate immune response

Many genes essential for innate immune response were among those that their expression was not changed in macrophages from KO mice compared to macrophages from the WT mice. This group included ten genes of interest; Cxc Chemokine Ligand 16 (CXCL16), Minor Histocompatibility Complex (MiHC) 28 (H28), Interleukin-17 Receptor (IL-17R), Leukemia Inhibitory Factor (LIF), Mitogen Activated Protein Kinase 4 (MAP4K4), Matrix Metalloproteinase 9 (MMP9), Myelocytomatosis oncogene (MYC), Protocadherin 7 (PCDH7), Promyelocytic Leukemia (PML), and Suppressor Of Cytokine Signaling 7 (SOCS7). To further validate the microarray results observed for these genes, quantitative RT-PCR studies were performed (Fig. 2.). As can be seen in Fig. 2A, the expression levels of CXCL16, H28, IL-17R, LIF, MAP4K4, MMP9, MYC, PCDH7, PML, and SOCS7 were significantly increased in macrophages extracted from WT mice (black columns) following activation for 4hrs with IFN- γ and LPS. However, no changes in the expression of these genes were observed in cells extracted from IRF-8, as well as from IRF-1 null mice (white and gray columns, respectively). The only exceptional gene was LIF whose expression level in IRF-1^{-/-} was similar to that of WT. The expression level of IL-6 is not dependent on IRF-1 and IRF-8 and therefore served as a control.

3.4 Promoter analysis reveals the participation of both IRF-1 and IRF-8 in the activation of target promoters

In silico analysis of the putative promoter region (~ 1kb upstream to the predicted transcription start site) revealed that all ten genes harbor common DNA binding motifs like Interferon Simulated Response Elements (ISREs) to which IRFs can bind, as well as sites

for the putative binding of NF κ B, PU.1, AP-1, SP1 and E-box binding proteins. This data implies that their signalosome context might share similar characteristics.

To verify the ability of some of the promoters of the tested genes to respond to IRF-1 and IRF-8, reporter gene assays were performed. The macrophage cell line RAW264.7 was transfected with the reporter constructs driven by either the MMP9, or the PCDH7 or the PML promoter. Cells were cotransfected with expression constructs for IRF-1 IRF-8 or both, and either not treated or treated with IFN- γ and LPS for 24 hours. The level of the luciferase reporter gene expression was determined. As seen in Fig. 3A (black columns), treatment of transfected cells with IFN- γ and LPS led to the activation of the MMP9 reporter construct. Further, cotransfection of IRF-8 expression construct alone elicited stronger activation of this reporter than IRF-1 alone. Cotransfection of both expression constructs for IRF-1 and IRF-8 led to an additive activation of the reporter construct. This further supports the role of both factors as immediate regulators of MMP9 in macrophages. The same type of experiments were performed with the reporter constructs harboring the promoters of PCDH7 (Fig. 3B) and PML (Fig. 3C). In principal, similar results were observed. Both promoters were activated in the transfected cells by IFN- γ and LPS and by each of the transfected factors or combination. The same type of experiments was also performed in CL-2 cells, a macrophage cell line originated from IRF-8^{-/-} mice (Fig. 3, white columns). In these cells, stimulation with IFN- γ and LPS was not sufficient to activate all three reporter constructs, indicating the pivotal role of IRF-8 in the regulated expression of these three tested promoters. Reintroduction of IRF-8 to these cells was sufficient to activate all three promoters, and maximal activation was observed when both IRF-8 and IRF-1 were cotransfected. However, transfection of IRF-1 alone was not sufficient to activate both MMP-9 and PCDH7 promoters, indicating that IRF-8 and IRF-1 are essential co-regulators. Taken together, these results point to the key role of IRF-8 and IRF-1 in the expression of MMP9, PCDH7 and PML in activated macrophages.

4. Discussion

Both IRF-1 and IRF-8 have important roles in the maturation and the function of macrophages. Accordingly, mice with null mutations for these two genes exhibit severe defects in both innate and adaptive immunity. These mice manifest abnormalities in the early phases of myelopoiesis, are unable to mount Th1 mediated immune response, and are sensitive to various pathogens (Tamura and Ozato 2002; Taniguchi et al. 1901; Testa et al. 1995). These two IRF members associate through characterized domains to create transcriptional heterocomplexes that bind and regulate various promoters of macrophage essential genes (Levi et al. 2002; Marecki et al. 2001a; Tamura et al. 2000). To search for yet unidentified target genes for these two transcription factors in macrophages, DNA microarray analysis was employed. We compared the expression profile of peritoneal macrophages from WT mice following stimulation with IFN- γ and LPS to that of treated macrophages from IRF-1 and IRF-8 KO mice. As shown in Fig.1, numerous genes (1009) exhibited significant changes in their expression pattern in WT macrophages in accordance to previously published data (Ricciardi-Castagnoli and Granucci 2002). To our surprise, the expression of about a third of these genes was significantly un-induced in IRF-1 (387) and IRF-8 (360) defective mice. This data points to the pivotal role of each IRF member in the regulation of gene expression in activated macrophages. The list of genes is not restricted only to those essential for macrophage function but also to genes with more general activities.

Among the genes whose expression was changed in WT following activation, we looked for those whose expression was not changed both in IRF-1^{-/-} and IRF-8^{-/-} macrophages. We assumed that the skewed expression observed in the KO mice after activation, may result from the absence of the respective transcription factors, implying that these non induced genes are potentially regulated by both IRF-1 and IRF-8. The expression level of 265 transcripts was changed in WT macrophages and not in both KOs following activation. We

chose to further validate the microarray results for 10 genes that may take part in the innate immune response. *in-silico* analysis of the putative promoters of these 10 genes (1kb upstream to the predicted transcription start site) revealed common putative DNA motifs for the binding of IRFs, PU.1, NFkB and AP1. This suggests that these genes might respond to similar signaling cues in macrophages and therefore their enhanceosomes are organized in a similar architecture. The promoters of three of these genes were further analyzed by reporter gene assays demonstrating the role of both IRF-8 and IRF-1 in their coordinated regulation in activated macrophages.

As mentioned, we have looked at only ten of the many putative genes that are regulated by both IRF-1 and IRF-8 in activated macrophages. These genes are involved in key processes taking part in the onset of innate response. Based on our data we suggest that in addition to the already described activities of these two transcription factors in mature macrophages, they also play significant roles in adhesion, chemotaxis, ECM degradation, antigen presentation, cytokine signaling, and apoptosis. The partial putative regulatory network for IRF-1 and IRF-8 in activated macrophages through the regulation of PCDH7, H28, LIF, PML, CXCL16, IL-17R, MYC, MMP9, MAP4K4, SOCS7, is illustrated in Fig. 4.

Cells congregation and cell-cell interactions of macrophages is a fundamental event in the inflammatory cascade following infection. To get an efficient response, macrophages enhance their mobility and aggregate in the infected site (Peters et al. 2000). This accumulation of macrophages in the infected site may be accomplished through the expression of cell surface adhesion molecules, such as PCDH7, which belongs to the protocadherins family of cadherins. These proteins play an important role in cell-cell adhesion of specific neurons in the central nervous system (Yoshida 2003). Of the reported PCDHs, relatively little is known about the function of PCDH7. However, its putative involvement in the inflammatory process through IKK/NFkB signaling was reported (Li et

al. 2002). We suggest that PCDH7 adhesion molecule, regulated by both IRF-1 and IRF-8, contributes to macrophages cell-cell adhesion and their interaction with target cells.

Two other important processes taking place during innate immune response; chemotaxis and the recruitment of immune cells to the infected site. Being involved in the initial steps in the onset of inflammation upon infection, macrophages signal to other professional immune cells. Through chemotaxis and antigen presentation, neutrophils, T-cells, and NK cells are recruited to the inflamed site. We suggest that IRF-1 and IRF-8 are important factors in this event through the regulation of CXCL16, IL-17R, and MiHC H28. CXCL16 is a chemokine expressed by macrophages, Dendritic Cells (DCs), and endothelial cells (Matloubian et al. 2000;Shimaoka et al. 2003;Wilbanks et al. 2001). When expressed at the cell surface, CXCL16 functions as a scavenger receptor, binding and internalizing oxidized low-density lipoprotein and bacteria. As a soluble form, CXCL16 is a chemoattractant for activated CD4⁺ and CD8⁺ T cells through binding its receptor, CXCR6. CXCL16 is synthesized as an intracellular precursor that is rapidly transported to the cell surface where it undergoes metalloproteinase-dependent (ADAM10) cleavage, causing the release of a soluble chemoattractant (Matloubian et al. 2000;Wilbanks et al. 2001). IL-17 is a cytokine secreted exclusively by activated T cells. Its receptor, IL-17R, is expressed in all tissues examined to date (Moseley et al. 2003) and signals through Extracellular signal-Regulated protein Kinase (ERK), c-jun N-terminal kinase (JNK), and p38 MAP kinase pathways resulting in the induction of pro-inflammatory cytokines (Shalom-Barak et al. 1998;Martel-Pelletier et al. 1999;Subramaniam et al. 1999a;Subramaniam et al. 1999b). H28 belongs to the MiHC (H locus) and is being presented by antigen presenting cells following induction with inflammatory cytokines (Malarkannan et al. 2000).

The accumulation of macrophages at the infected site is an important part of innate immunity and the inflammatory response. The tissue destruction that accompanies infection

is facilitated by the matrix metalloproteinases (MMPs). This family of proteinases is expressed in macrophages in response to inflammatory stimuli, such as LPS or IFN- γ and subsequently degrades the ECM. MMP9, also known as Gelatinase B, can degrade extracellular matrix components such as collagens and elastins. Like most MMPs, it is made as a proenzyme that requires proteolysis that is the limiting step in the degradation of extracellular matrix. (Min et al. 2002; Pendas et al. 1997). Interestingly, Koenders *et al.* reported a critical role of IL-17R signaling in the expression of proinflammatory and catabolic mediators, such as IL-1 and MMP-9 during chronic T cell-mediated synovitis, which is macrophage-driven joint inflammation (Koenders et al. 2005). Like other immune cells, macrophages are produced in large amounts in response to a stimulus and eventually undergo apoptosis (Xaus et al. 2001). These two processes may be achieved by the simultaneous expression of both proliferative and pro-apoptotic antagonistic genes. Therefore, it is not surprising to find both c-Myc and PML upregulated in activated macrophages. c-Myc regulates diverse cellular processes integral to cell growth, survival, and development, and its deregulation leads to cancer (Nesbit et al. 1999). Conversely, increased PML protein levels inhibit proliferation (Koken et al. 1995; Liu et al. 1995), mainly through gene expression control by sequestering and modulating the post-translational status of several transcription factors (Salomoni and Pandolfi 2002). It was recently suggested that PML is involved in the surveillance of c-Myc activity participating in c-Myc post-translational modifications (Cairo et al. 2005). In addition, PML and c-Myc may be involved in apoptosis through the regulation of p27(kip1). The latter is a target of transcriptional repression by c-Myc (Yang et al. 2001) and is recruited by PML along with other apoptotic factors to nuclear bodies (Quignon et al. 1998). Interestingly, the Acute Promyelocytic Leukaemia (APL) PML/RAR α oncoprotein delocalizes them from these bodies (Quignon et al. 1998). PML is a direct gene regulated by IRF-8 in activated macrophages, and IRF-8^{-/-} mice are defective in induced expression of PML in activated

macrophages that is accompanied by lack of nuclear bodies (Dror et al, in preparation). Interestingly, three of the target genes identified, i.e. MAP4K4, IL-17R, and SOCS7 are involved in different stages of the NF κ B signaling pathway that eventually leads to the transcriptional regulation of a wide array of pro-inflammatory genes in macrophages (Karin and Greten 2005)(Fig. 4). Therefore, our results suggest an important role for IRF-1 and IRF-8 in the tight control of this pathway. Furthermore, an interesting aspect of this regulatory network may be the link between IRFs, SOCS7, and LIF (Fig. 4). Following treatment with IFN- γ and LPS the induced expression of IRF-1 and IRF-8 is regulated by STAT-1. Subsequently, the induced IRF-1 and IRF-8 regulate the expression of SOCS7 and in addition, IRF-8 regulates the expression of LIF. LIF has potent proinflammatory properties, being the inducer of the acute phase protein synthesis and affecting the cell recruitment into the area of damage or inflammation (Verfaillie and McGlave 1991;Chodorowska et al. 2004). LIF also regulates the expression of SOCS1 (and possibly of SOCS7 as well) leading to the downregulation loop of SATA1 signaling.

Taken together, we propose a pivotal role for IRF-1 and IRF-8 in regulating the expression levels of genes that participate in significant biological processes during macrophage activation while functioning as part of the innate immune response.

At this stage of our research, the exact nature of the regulatory role of both IRF-1 and IRF-8 on these identified genes is still elusive. One possible scenario is that both proteins regulate these target genes in concert, either directly through the association to the promoters of these genes, or indirectly, via regulating other transcription elements that eventually lead to the regulation of these genes. Collectively, our results suggest that IRF-1 and IRF-8 are key elements in major processes taking place during macrophage activation and are crucial to the macrophage functioning in inflammation.

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Fig. 1: Venn diagram illustrating transcripts distribution.

The expression level of 1009 transcripts was changed in WT macrophages activated by IFN- γ and LPS, in comparison to untreated cells. Similarly, the level of 387/360 transcripts was changed in WT activated macrophages and not in comparable samples from IRF-1^{-/-} and from IRF-8^{-/-} mice, respectively. Among these genes, the RNA level of only 265 genes was changed in samples from WT macrophages and not in samples from both IRF-1^{-/-} and IRF-8^{-/-} mice.

Fig. 2: Quantitative Real-Time RT-PCR analysis of IRF-8 and IRF-1 target genes.

mRNA was extracted from peritoneal macrophages of WT (black columns) as well as IRF-8 (gray columns) and IRF-1 (white columns) KO mice before and following 4hrs exposure to IFN- γ and LPS and quantitative RT-PCR was performed as detailed in Material and Methods. The results are presented as fold of expression calculated as the values of the PML/GAPDH ratio observed in activated cells, divided by the ratio observed in resting cells. Statistical significance (p value less than 0.05) was determined by Students T-test.

Fig.3: The promoters of MMP9, PCDH7 and PML are activated by IRF-1 and IRF-8.

RAW264.7 cells (black columns) and CL-2 cells (white columns) were transiently transfected with luciferase reporter constructs driven by either the MMP9 (Panel A), PCDH7 (Panel B) or PML (Panel C) promoters. The cells were either treated with IFN- γ and LPS for 24hrs or cotransfected with expression constructs for IRF-1 and IRF-8 as indicated. 24hrs later, the cells were harvested and luciferase levels were measured. The luciferase activities were normalized for transfection efficiency and cell number. Data are given as means and STD of three independent experiments.

Fig.4: Schematic illustration of IRF-1 and IRF-8 regulatory network in activated macrophages. Target genes indicated by gray boxes. Biological processes indicated by

white boxes. Direct regulatory pathways by IRF-8 and IRF-1 are indicated by black arrows, while general regulatory pathways are indicated by dashed arrows.

Table 1: Primers used for Real-Time PCR

Transcript	5' Primer ^a	3' Primer ^a	Amplicon size (bp)
CXCL16	CCTTGCTCTCTTGCGTTCTTCC	TCCAAAGTACCCTGCGGTATC	139
H28	TTCAACTCAGTGGAAGTCTGCT	GGAGTGTTTCCCCGCTTTTTC	134
IL-17R	AGTGTTTCCTCTACCCAGCAC	GAAAACCGCCACCGCTTATTAC	194
LIF	GCTATGTGCGCCTAACATGAC	CGCTCAGGTATGCGACCAT	101
MAP4K4	TCTGGCTTGTTATGGAGTTT	CTTTGAGAGTGTTCCCTTTG	78
MMP9	CGATTTGAAGTCTCAGAAGG	TACTGGAAGATGTCGTGTGA	96
MYC	TCTCCATCCTATGTTGCGGTC	TCCAAGTAACTCGGTCATCATCT	114
PCDH7	GTTAATGCCAAAGACAAAGG	TTGCATAAACTTAGGGTCGT	99
PML	CAGGCCCTAGAGCTGTCTAAG	ATACACTGGTACAGGGTGTGC	78
SOCS7	TCCTCCAAGAAGAAGCCTCA	GGAGGTAGGGGGAAAGACTG	108
GAPDH	AGGTCGGTGTGAACGGATTTG	TGTAGACCATGTAGTTGAGGTCA	123

^aprimers were designed using Primer3 software: <http://www-genome.wi.mit.edu/cgi-bin/primer/primer3>

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