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Interleukin-4 induced interferon regulatory factor (Irf) 4 participates in the regulation of alternative macrophage priming

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ABSTRACT

Interleukin (IL)-4 is a central regulator of T helper 2 (Th2) immune responses, and also has a major impact on innate immune cells. This cytokine primes macrophages for immune responses to parasites and induces a distinct macrophage phenotype that may also promote tissue repair. IL-4 signaling in macrophages is primarily mediated by the transcription factor signal transducer and activator of transcription 6 (Stat6), which in turn regulates a number of secondary DNA binding proteins that may participate in shaping the resulting phenotype. The impact of secondary transcription factors on IL-4-treated macrophages, however, is largely unknown. Here we show that interferon regulatory factor 4 (Irf4) is strongly induced on RNA and protein level in bone marrow-derived macrophages upon priming with IL-4. Using microarray-based whole genome expression analysis, we also demonstrate that a subset of IL-4 regulated genes, including several MHC-II genes, *Ciita*, *Cyp1b1*, and *Il1rn*, are dysregulated in *Irf4*-deficient macrophages. The presented data suggests a non-redundant role for *Irf4* in shaping the phenotype of alternatively primed macrophages.

Introduction

In the presence of the two T helper 2 (Th2) cytokines interleukin (IL)-4 and IL-13, which both signal through the common IL-4 receptor α chain, macrophages develop a phenotype consistent with a role in humoral immunity and tissue repair (Gordon, 2003; Stein et al., 1992). This type of macrophage polarization is generally referred to as "alternatively activated" or "alternatively primed" to contrast macrophages that encountered the classical priming stimulus interferon-γ (IFNγ) in a Th1 environment (Gordon and Taylor, 2005; Martinez et al., 2009).

The main signaling pathway initiated through the common IL-4 receptor α chain on macrophages includes the phosphorylation and dimerization of the DNA binding factor signal transducer and activator of transcription (Stat)6, which activates down-stream target genes including typical murine alternative macrophage markers like arginase I (*Arg1*) (Gray et al., 2005), chitinase 3-like 3 (*Chi3l3*, also called Ym1) (Welch et al., 2002), or resistin like alpha (*Retnla*, also called FIZZ1) (Stutz et al., 2003). In contrast to IL-13, IL-4 also activates the insulin receptor substrate-2 (IRS2) signaling cascade through the γc chain, but its impact on macrophage gene expression is less well defined (Heller et al., 2008; Wills-Karp and Finkelman, 2008). We have recently noted that IL-4 treatment of bone marrow-derived macrophages (BMM) induces the expression of a number of secondary transcription factors, including early growth response 2 (*Egr2*), basic helix–loop–helix domain containing, class B2 (*Bhlhb2*), kruppel-like factor 4 (*Klf4*), transcription factor EC (*Tcfec*), as well as interferon regulatory factor 4 (*Irf4*) (Rehli et al., 2005; Schilling et al., 2009). It is currently unknown, to which extent these factors contribute to both the steady state phenotype and the activation program of alternatively primed macrophages.

The transcription factor Irf4 is essential for the homeostasis and function of mature T and B lymphocytes (Mittrucker et al., 1997). In B cells, it is involved in plasma cell differentiation and class–switch recombination (Klein et al., 2006). In the T cell compartment, Irf4 is required for the IL-4 induced differentiation of Th2 cells (Lohoff et al., 2002), the survival of Th cells after T cell receptor (TCR) activation (Lohoff et al., 2004) and the induction of Th17 cells (Huber et al., 2008; Brustle et al., 2007).

In contrast to the lymphoid lineage, the function of Irf4 is less well studied in the myeloid cell compartment. Anecdotal evidence for regulated and functionally important Irf4 expression in both human and murine mononuclear phagocytes exists (Lehtonen et al., 2005; Pedchenko et al., 2005; Marecki et al., 1999; Gauzzi et al., 2005; Tamura et al., 2005; Suzuki et al., 2004), but we know relatively little about its target genes in macrophages and dendritic cells.

Abbreviations: BMM, bone marrow-derived macrophage; CSF, colony-stimulating factor; IL, interleukin; TCR, T cell receptor; Th, T helper

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Assuming that its marked IL-4-dependent upregulation indicates a functional role of Irf4 in alternatively primed macrophages, we studied global IL-4 dependent gene expression changes in *Irf4*-deficient bone marrow-derived macrophages. Our data provide novel insights into the function of Irf4 in alternatively primed macrophages.

Materials and methods

Chemicals

All chemical reagents were purchased from Sigma-Aldrich (Berlin, Germany) unless otherwise noted. Protease inhibitors were obtained from Roche Applied Science unless otherwise noted. IL-4 was obtained from PeproTech Inc. (Rocky Hill, NJ, USA) and LPS from Sigma-Aldrich. Oligonucleotides were synthesized by Metabion (Martinsried, Germany). Oligonucleotide primers were designed using PerlPrimer software (Marshall, 2004) and controlled using UCSC In-Silico PCR and BLAT (http://genome.ucsc.edu/). All primer sequences are listed in Table 1.

Mice

The mutant mouse strain deficient in *Irf4* was described previously (Mittrucker et al., 1997). To obtain a pure C57BL6 background *Irf4*-deficient mice were backcrossed with C57BL6 mice for ten generations.

Cells and cell culture

To generate bone marrow-derived macrophages (BMM), bone marrow cells were flushed from femurs and tibias of 8–12 weeks old $Irf4^{-/-}$ or $Irf4^{+/-}$ mice using cold PBS. Cells from several animals were pooled and plated on bacteriological 100 mm^2 plastic plates (Bibby Sterilin, Staffordshire, UK Ltd.) at 5×10^5 cells/ml in 20 ml endotoxin-free RPMI 1640 medium (Biochrom KG, Berlin, Germany) supplemented with glutamine, vitamins, pyruvate, nonessential amino acids, β-mercaptoethanol (all purchased from Invitrogen, Germany), 10% FCS (PAA Laboratories GmbH, Austria), and 200 ng/ml human rCSF1 (Cetus) per plate for 5 days. Complete medium was replaced on day 5, cells were harvested on day 6 and seeded with rCSF1 at a density of 10×10^6 cells/10 ml medium on 10 cm tissue culture dishes (Falcon). On day 7, cells were incubated for 4 or 18 h with or without IL-4 (10 ng/ml). Cells were collected, pelleted and directly used for RNA preparation.

Total RNA preparation and quantitative (q) RT-PCR

Total RNA was isolated using the RNeasy midi kit (Qiagen). RNA (1 μg) was reverse transcribed using Superscript II MMLV-RT (Promega). Reverse transcribed products were diluted 1:4 in RNase free water and analysed by qRT-PCR on a Mastercycler Ep Realplex (Eppendorf) using the the QuantiFast SYBR Green PCR Kit (Qiagen) according to the manufacturer's instructions. Primers are given in Table 1. Melting curves were analysed to control for specificity of the PCR reactions. Expression data for all genes were normalized to the housekeeping gene *Hprt1*. The relative units were calculated from a standard curve plotting three different concentrations of log dilutions against the PCR cycle number (CP) at which the measured fluorescence intensity reached a fixed value. The amplification efficiency E was calculated from the slope of the standard curve by the formula: $E = 10^{-1/\text{slope}}$. For each sample, data of at least three independent experiments were averaged.

Microarray handling and data analysis

RNA preparations from at least three independent BMM cultures were analysed using Whole Mouse Genome Oligo Microarrays (Agilent). Labelling and hybridization were performed using the Agilent Gene Expression system according to the manufacturer's instructions. In brief, 200–1000 ng of high-quality RNA were amplified and Cyanine 3-CTP labelled with the One Color Low RNA Input Linear Amplification Kit (Agilent). Labelling efficiency was controlled using the NanoDrop spectrophotometer, and 1.65 μg labelled cRNA were fragmented and hybridized on the Whole Mouse Genome Expression Array (4×44K, Agilent). Images were scanned using a DNA microarray scanner (Agilent), and processed with Feature Extraction Software 9.5.1 (Agilent) using default parameters. Extracted data were further processed with GeneSpring GX 11. Data were normalized to the 75th percentile and to the untreated $Irf4^{+/-}$ samples. Microarray data have been submitted and are available from the NCBI/GEO repository (accession number GSE21853).

Western blot analysis

Western blot analysis was performed as described (Rehli et al., 2005) using anti-Irf4 (sc6059, Santa Cruz) and anti-β-actin (Sigma-Aldrich) antibodies.

Results

Regulation of Irf4 in IL-4-treated bone marrow-derived macrophages

It is known that some effects of IL-4 on macrophage functions are delayed and require new protein synthesis. These may include a second wave of regulator proteins that are induced by the initial Stat6-dependent response (Major et al., 2002). Previous profiling of the IL-4 induced transcriptome indeed identified a number of transcription factors that were induced in alternatively primed macrophages (Schilling et al., 2009; Rehli et al., 2005). One of

Table 1
Oligonucleotide primers used for qRT-PCR.

Gene symbol	Sense primer	Antisense primer
Hprt1	5′-CTCATGGACTGATTATGGACAGGAC-3′	5′-GCAGGTCAGCAAAGAACTTATAGCC-3′
Irf4	5′-GACCAGTCACACCCAGAAATCCC-3′	5′-GTTCCTGTCACCTGGCAACC-3′
Tlr4	5′-TTTCAGAACTTCAGTGGCTGG-3′	5′-AGTTTGAGAGGTGGTGTAAGCC-3′
Bhlhb2	5′-CAGCTGAAGGATCTCCTACCCG-3′	5′-CTTGTAAACCGCTCTGCAGGG-3′
Ciita	5′-TCTAGGACCTCACTGAACTATTTGG-3′	5′-GTTGACCTGGGGCATCTCAC-3′
Cyp1b1	5′-ACTATTACGGACATCTTCGGAGCCA-3′	5′-TGGTCCAACTCAGCCTGCAC-3′
Ccl24	5′-CTTGCTGCACGTCCTTTATTTCCA-3′	5′-GGTCAGTACAGATCTTATGGCCCT-3′
Mpp6	5′-CAAGGTGTGGGCAGAAGGAG-3′	5′-TTCGTCTTCTCTTGGCTTCCGT-3′
Socs2	5′-CAAGATCCCTTGTGCCCGGA-3′	5′-GCAGAATGGTGTGGCAAAGTCTC-3′
Il1rn	5′-TGTGTTCTTGGGCATCCACGG-3′	5′-CTTGCTCAGATCAGTGATGTTAACTTCC-3′