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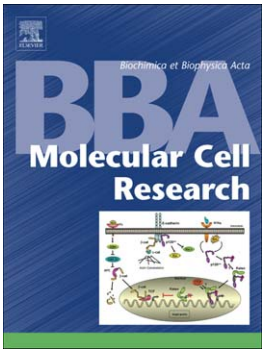

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MafB is a downstream target of the IL-10 / STAT3 signaling pathway, involved in the regulation of macrophage de-activation.

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Running title: MafB role in Macrophage Activation.

ABBREVIATIONS: EMSA, Electrophoretic mobility shift assay; HSC, Hematopoietic Stem Cells; IL-7R, Receptor for Interleukin 7; LPS, Lipopolysaccharides; MARE, MafB Responsive Element; MMP9, Matrix Metalloproteinase 9; PMA, phorbol 12-myristate 13-acetate; QRT-PCR, Quantitative Real Time PCR; RT, Room Temperature; siRNA, small interference RNA; TF, Transcription Factor.

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ABSTRACT

In spite of the numerous reports implicating MafB transcription factor in the molecular control of monocyte - macrophage differentiation, the precise genetic program underlying this activity has been, to date, poorly understood. To clarify this issue, we planned a number of experiments that were mainly conducted on human primary macrophages. In this regard, a preliminary gene function study, based on MafB inactivation and over-expression, indicated *MMP9* and *IL-7R* genes as possible targets of the investigated transcription factor. Bioinformatic analysis of their promoter regions disclosed the presence of several putative MARE elements and a combined approach of EMSA and luciferase assay subsequently demonstrated that expression of both genes is indeed activated by MafB through a direct transcription mechanism. Additional investigation, performed with similar procedures to elucidate the biological relevance of our observation, revealed that MafB is a downstream target of the IL-10 / STAT3 signalling pathway, normally inducing the macrophage de-activation process. Taken together our data support the existence of a signalling cascade by which stimulation of macrophages with the IL-10 cytokine determines a sequential activation of STAT3 and MafB transcription factors, in turn leading to an up-regulated expression of *MMP9* and *IL-7R* genes.

Keywords: MafB, Gene regulation, MMP9, IL7R, Monocyte Activation, IL-10 / STAT3 pathway.

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1. INTRODUCTION

From an evolutionary point of view, macrophages are considered the most primitive cells since related cell types are observed from protozoa up to complex multicellular eukaryotic organisms [1]. Several molecules, such as transcription factors (TFs), growth factors, vitamins, hormones and cytokines, are involved in the regulation of monocyte – macrophage differentiation to assure, across species, the presence of these cells and their functions in innate and adaptive immunity [2]. A large body of evidence highlighted the crucial role played by transcriptional regulation in the monocyte maturation program, demonstrating the combinatorial action and the regulatory interdependence of a considerable number of myeloid TFs, belonging to a limited set of protein families (MAF, Mitf-Tfe, HOX, PPAR, C/EBP, IRF, EGR) [2-8]. Inside this intricate network, MafB may be considered a key player in the genetic control of normal monocytopoiesis. MafB is a leucine zipper DNA binding protein belonging to the Maf family and it shows binding activity to the Maf Responsive Element (MARE) and to the 5'AT-rich half-MARE [9], contained in the promoter regions of target genes. In addition, MafB supports either transcription activation or repression depending on the interacting dimerization partner [10]. This TF is evolutionary conserved [11-13] and several reports indicate that, in human hematopoiesis, its expression is monocyte specific. In fact, data obtained in our laboratory demonstrated that: 1) MafB is highly expressed in monoblasts, monocytes and, accordingly, in monoblastic cell lines (THP1, U937 and Kasumi-1) [5, 14]; 2) virally mediated MafB transduction of human hematopoietic progenitors determines a massive induction of monocyte-macrophage differentiation, coupled to a strong inhibition of erythroid commitment [5]; 3) MafB is a direct target of two monocyte – macrophage related TFs, i.e. Hox-A10 [6] and TFE3 [15]. In addition, data reported in literature underlined that high levels of MafB expression are able to activate the macrophage maturation program at the expense of dendritic cell (DC) differentiation [16, 17] and that its down-regulation is also required for osteoclast differentiation of myeloid cells [18-20].

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Nevertheless, the genetic program controlled by MafB inside the monocyte system is still poorly understood. In this regard, preliminary results were provided by our research group through the analysis of the gene expression profile obtained in hematopoietic stem cells virally transduced with a MafB cDNA [5]. The results of these experiments revealed that MafB is able to control the gene expression of about one hundred genes, involved in monocyte differentiation and macrophage activation. In addition, the analysis of transcriptomes obtained by normal monoblasts [14] and Hox-A10- transduced hematopoietic progenitors [5, 6] disclosed a recurrent co-expression of MafB with several macrophage phenotypic markers that might be considered putative MafB primary response genes. Inside this gene list we focused our attention on two interesting genes: *Matrix Metallo-Proteinases 9* (MMP9) and *Interleukine 7 Receptor* (*IL-7R).* MMP9 is a well-known molecule involved in several aspects of monocyte biology, including monocyte differentiation and polarization [21, 22], and it is also up-regulated in THP1 cells treated with Retinoic Acid, another model of monocyte differentiation characterized by high expression of MafB [23]. In addition, in a previous paper by Montanari et al. [14] we demonstrated that, in the comparison with primary myeloblasts, monoblasts precursors were characterized by a large amount of IL-7R transcript, suggesting it as a novel differentiation marker of human normal monocytopoiesis. In light of these considerations, the rationale of the present study is to demonstrate that *MMP9* and *IL-7R* genes are transcriptionally regulated by MafB through the identification of specific MARE elements within their promoter regions and that this genetic program is involved in the anti-inflammatory process. Interestingly, the results described here allowed us to state that MafB is a downstream target of the IL-10 / STAT3 signaling pathway directly involved in the macrophage de-activating process.

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2.MATERIAL AND METHODS

2.1 Human hematopoietic cells. THP1 monoblastic cell line, obtained from ATCC (Rockwill, MD), was cultured in RPMI 1640 medium (Euroclone, Devon, UK), supplemented with 10 % heat inactivated fetal bovine serum (Biowhittaker, Walkersville, MD; FBS Brazilian origin, Cat. N. DE14-801F, Lot. N. 1SB004) and 1 mM L-Glutamine (Euroclone). THP1 cells were treated with phorbol 12-myristate 13-acetate (PMA; Sigma Chemical Co, St. Louis, MO) at 1.6 nM concentration for 48 hours (h).

Human CD34+ stem-progenitor cells and CD14+ monocytes were purified, respectively, from umbilical cord blood and adult peripheral blood speciments, as already described [5, 14, 24]. Human samples were recruited at the blood transfusion division of Policlinico of Modena, upon a written informed consent of voluntary donors. Cell positive selections were carried out, according to the manufacturer's protocol, by using the immune-magnetic systems "EasySep Human CD34+ Selection Kit" and "EasySep Human CD14 Selection Kit", both provided by Stem Cell Technologies. Primary cells (stem/progenitors and monocytes) were cultured in IMDM (Euroclone) supplemented with 10 % heat inactivated human serum AB (Biowittaker) and 1 mM L-Glutammine (Euroclone). Classical monocyte activation was obtained by a 24 h treatment with a combination of 100 ng/ml LPS (Difco Laboratories) and 20 ng/ml IFNγ (Roche Diagnostics, Mannheim, Germany), alternative polarization was carried out by a 72 h stimulation with 20 ng/ml IL-4 (R&D System); macrophage de-activation was achieved by treatment with a 50 ng/ml concentration of IL-10 cytokine (Miltenyi Biotec, Auburn, CA; USA) for up to 72 h.

2.2 Retroviral vectors construction, cell transduction and purification. Construction of LXIΔN and LMafBIΔN retroviral vectors as well as retroviral transduction of CD34+ progenitor cells were performed as previously reported [5, 6]. Transduced CD34+ cells were NGFR purified at day 7 of liquid culture by using a mouse anti-human p75-NGFR monoclonal antibody (Ab) (BD Biosciences PharMingen, San Diego, CA, USA) and "Easy

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Sep Do It Yourself" selection kit (Stem Cell Technologies). Transduced and purified progenitors were maintained in liquid culture until day 14 in the described conditions and then lysed for RNA extraction and subsequent molecular analysis.

1. *2.3 Gene Silencing.* MafB silencing was achieved in THP1 monoblastic cells and in human normal monocytes using a 100 nM concentration of a mixture of three pre-designed siRNA duplexes provided by Sigma-Aldrich (Sigma Aldrich, St. Louis, MO, USA; Oligo name: SASI_Hs01_00197232; SASI_Hs02_00339624; SASI_Hs02_00339625). Two rounds of nucleofection procedure, performed according to the Amaxa Nucleofector Technology, were carried out on 5 x 10^6 cells every 24 h and, following a 48 h period, a dose of 1,6 nM of PMA or 50 ng/ml of IL-10 was added for additional 24 h, respectively to culture medium of THP1 cells or normal monocytes. Each experiment included a mock and a negative control represented by a non-targeting siRNA synthesized by Sigma-Aldrich in order to exclude non-specific effects of siRNA nucleofection. Quantitative Real Time PCR (QRT-PCR) and Western blot techniques were then used to evaluate MafB silencing upon PMA or IL-10 treatment.

2.4 RNA purification and quantitative RT-PCR. Total cellular RNAs were extracted by means of Qiagen total RNA purification kits (Qiagen, Valencia, CA) and then analyzed by NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) to evaluate RNA integrity and concentration. QRT-PCR was carried out by an ABI PRISM 7900 sequence detection system (Applied Biosystems) on total RNAs (100 ng) reverse transcribed using the High Capacity cDNA Archive Kit (Applied Biosystems), according to the manufacturer's instructions. Each cDNA sample was run in triplicate for targets (MAFB, MMP9, IL-7R, IL-6, TNF-α, IL-1RA, MRC1, CD163) and for GAPDH endogenous control using primers and probes supplied by Applied Biosystems as pre-made solutions and the Faststart Universal Probe Master Mix (Roche Diagnostics). Quantification of QRT-PCR signals was performed using the ($2^{-\Delta\Delta Ct}$) method [25] which calculates relative changes in

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gene expression of the target gene normalized to the GAPDH endogenous control and relative to a calibrator sample. The values obtained were represented in terms of relative quantity of mRNA level variations.

2.5 Protein extract preparation and western blot analysis. Total protein extracts were obtained using a small volume of lysis buffer (50 mM TRIS pH 7.8, 400 mM NaCl, 1 % NP-40, 1mM PMSF, 1X Protease Inhibitor Cocktail), followed by incubation on ice for 30 minutes and centrifugation for additional 30 minutes at high speed. 30 μg of protein extracts were then loaded onto 10 % SDS-polyacrylamide gel electrophoresis and electroblotted to nitrocellulose sheets. Blotted membranes were pre-blocked for 1 h at room temperature (RT) in blocking solution, composed by 5% nonfat milk (Regilat, Saint-Martin-Belle-Roche, France) in 0.05% TBST or by 3% nonfat milk and 2% BSA (Sigma Aldrich, St. Louise, MO, USA) in TBST 0.1%, according to antibodies specificity. The following rabbit anti-human primary antibodies were used at concentrations recommended by the manufacturer's instruction: MafB polyclonal Ab (Sigma-Aldrich), MMP9 monoclonal Ab and polyclonal IL-7R alpha Ab (Abcam, Cambridge, UK). As secondary antibody, conjugated to horse-radish peroxidase, we used a goat anti mouse IgG (Santa Cruz Biotechnology) and goat anti-rabbit IgG (Cell Signaling Technology) at 1: 10000 or 1 : 3 000 dilution, respectively. To normalize analyzed protein samples, a mouse anti-human pan actin monoclonal Ab (Sigma Aldrich) or a rabbit anti-human vinculin polyclonal Ab (Millipore Corporation, Billerica, MA, USA) were used. Detection of Western blot signals was carried out using the Westar EtaC enhanced chemiluminescent substrate (Cyanagen S.r.l., Bologna, Italy).

2.6 Flow cytometry analysis. Surface antigen expression was detected using the following MoAbs: anti-NGFR conjugated to fluorescein isothiocyanate (FITC), phycoerythrin (PE) - conjugated IL-7R, PE – conjugated MRC1 (Miltenyi Biotec), biotin–conjugated CD163 (BMA Biomedicals AG, Augst, Switzerland), TRICOLOR–conjugated streptavidin (Caltag Laboratories, Burlingame, CA). Antibodies incubations were carried out as described

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[24]. Analysis was performed in terms of positivity percentage by using a Coulter Epics XL-MCL (Coulter Electronics, Hialeah, FL) flow cytometer.

2.7 Plasmid expression vectors. A Flag tagged MafB cDNA fragment, obtained as already described [5], was cloned in the *Eco*RI site of pcDNA3.1 plasmid (Invitrogen, Carlsbad, CA, USA) resulting in pcDNA3.1FlagMafB construct. The pCMVSTAT3 expression vector was instead supplied by Origene Technologies (Rockwill, MD). The pT81Luc vectors [26], containing MAFB or STAT3 binding elements, were generated as described below. The following single-strand oligomers and their complements were used:

MMP9 MARE probe: 5'- GATCGACC**CCCTGAGTCAGCA**CTT-3';

IL-7R MARE probe: 5'-GATCGTAGG**GACTGACTCAGCA**CATG-3';

STAT3 ENHANCER 1 probe: 5'- GATCGCTGC**TTC**TCA**GAA**GACC-3';

STAT3 ENHANCER 2 probe: 5'- GATCGACC**TTCC**A**GGAA**ACCC-3'.

The double strand oligomers, obtained by annealing single-strand oligonucleotides modified at the 5' end with the *Bam*H1 and *Sal*I overhang restriction sites, were inserted into the *Bam*HI/*Sal*I digested pT81Luc vector, upstream to a minimal promoter and the luciferase reporter gene. According to these cloning strategies, the following constructs were generated: pT81LucMMP9MARE, pT81LucIL-7RMARE, pT81LucSTAT3Enhancer1 and pT81LucSTAT3Enhancer2. Nucleotide sequence analysis of the plasmids demonstrated that binding sequences had been inserted as single-copy oligomer.

2.8 EMSA and Reporter gene assays. The double strand MARE elements, reported in Table I, were 5' end labeled by γ^{32}P-ATP (6000 Ci/mmol, GE Healthcare Europe GmbH, Freinburg), T4 polynucleotide kinase (New England Biolabs, Ipswich, MA) and purified with Microspin G-25 Columns (GE Healthcare). The mobility shift reactions were carried out as described [6]. 2 μl of "in vitro" transcribed/translated Flag-MafB protein, produced by using the "TnT Quick Coupled Transcription/Translation System" (Promega, Madison, WI) in the presence of pcDNA3.1FlagMafB construct as template, were added to the reaction in a total

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volume of 20 µl. A negative control (Mock), represented by TNT Quick Master Mix incubated with pcDNA3.1 empty vector as template, was also included. Super-shift was obtained by adding, to the gel shift mix, 1 µg of Flag M2 Mouse Monoclonal antibody (Sigma Aldrich). Binding reactions were incubated at RT for 30 minutes and resolved using a non-denaturing 4 % polyacrylamide gel in 0.5 x TBE, pre-run for 1 h at RT. Finally the gel was fixed, vacuum dried, and expose to X-ray films.

2.9 Luciferase Reporter assay. Luciferase transactivation assays were carried out in HEK293 cells as already described [6]. Transient transfection was conducted in 24-well plate with TRANSIT 2020 reagent (Mirus Bio LLC, Madison, Wisconsin), using 200 ng of pT81Luc reporter plasmids, 50 ng of pcDNA3FlagMafB or pCMVSTAT3, 200 ng of pCMVβ-galactosidase (Clontech Laboratories, Inc. Mountain View, CA, USA) to normalize for transfection efficiency, and a carrier plasmid to maintain a total DNA concentration of 800 ng. Following 48 h of incubation, cells were lysed according to standard protocols for β-Galactosidase and Luciferase enzymatic assays.

2.10 Chromatin immune-precipitation. Two different ChIP experiments were developed in the present study in order to verify MafB binding to MMP9 and IL-7R target genes and, subsequently, to evaluate STAT3 binding to MafB promoter region. All the experiments were conducted in human monocytes under normal culture conditions or upon treatment with IL-10 cytokine at 50 ng/ml concentration for 24 h. 3 x 10^7 cells were used for this purpose. Although the aims of ChIPs were different, the experimental settings adopted were similar and are described below. Human monocytes were cross-linked, lysed and washed according to standard protocols. The samples were then sonicated in order to shear DNA to 100-1000 bp fragments and the chromatin was then immune-precipitated by using 5 µg of the following Abs: goat polyclonal anti-MafB Ab (Santa-Cruz), rabbit polyclonal anti-STAT3 Ab (Millipore) or anti-Flag Ab (Sigma Aldrich) used as a control. Subsequently, the enrichment for MAFB- or STAT3- bound sequence was verified by semi-quantitative PCR

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using primers amplifying the genomic region spanning the putative MARE elements or the STAT3 enhancers. In addition, to further validate the specificity of MAFB / STAT3 binding on the respective elements, PCR amplifications were carried out on the microsatellite region localized on chromosome 3, selected as negative control region. PCR reactions were performed using 1.5 μl of immuno-precipitated DNA with 0.3 mM dNTPs, 150 ng of both primers (see Table 1 Supplementary Material), 5x Go-Taq buffer and 2 units of Go Taq (Promega) in a 50 μl volume. The ethidium bromide gel images were analyzed by Image J software (National Institute of Health) and the percentage of enrichment was calculated from the ratio between the amount of PCR-amplified MAFB- or STAT3-bound DNA in ChIP samples and in input chromatin. Inside the same experimental design, three replicates were performed and data were indicated as mean ± s.e.m.

2.11 Statistical Analysis. All experiments were repeated at least three times and results were presented as mean ± s.e.m. values. Pairwise comparisons were carried out using student's *t*-test procedure whereas Multiple comparisons analysis were performed by one-way analysis of variance (ANOVA) followed by Newman-Keuls post-hoc test by using the GraphPad Software [27]. Results of statistical analysis were considered significant when exhibiting p-values ≤ 0.05 and are indicated by asterisks. *, $p < 0.05$; **, $p < 0.01$

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3. RESULTS

3.1 Analysis of MafB, MMP9 and IL-7R gene expression. As described in the Introduction section, the co-expression of MafB with *IL-7R* and *MMP9* genes has been observed in various primary hematopoietic cell contexts analyzed in our laboratory [5, 6, 14]. Nevertheless, to confirm this issue, we performed preliminary experiments in THP1 monoblastic cells treated with phorbol 12-myristate 13-acetate (PMA), a well-recognized in "vitro" model of monocyte-macrophage differentiation. In this cell context, we analyzed the gene expression variations of the investigated genes in two different experimental conditions: PMA treatment and MafB gene silencing. The results achieved by QRT-PCR analysis demonstrated that, upon PMA exposure, THP1 cells exhibited a clear up-regulation of all transcripts (Fig. 1, panel A), whereas MafB inhibition was coupled to a significant reduction of the selected genes expression both at mRNA (Fig. 1, Panel B) and protein levels (Fig. 1, Panel C). The efficiency of gene silencing was verified by QRT-PCR and Western Blot methodologies and showed 70 % reduction of MafB transcript (Fig.1, Panel B) and three fold down-regulation of MafB protein, as compared to the control siRNA (Fig.1, Panel C). Furthermore, we performed a gene expression analysis of the considered genes in a primary cell context over-expressing MafB. This assay was performed in the macrophage progeny obtained from CD34+ hematopoietic stem / progenitor cells retrovirally transduced with MafB cDNA, obtained as already described [5]. A set of experiments, carried out under these conditions, highlighted that MafB over-expression is able to promote, respectively, a 3.5 ± 1.4 and 2.6 ± 0.7 (mean ± s.e.m) fold induction of both *MMP9* and *IL-7R* transcripts, as compared to empty vector transduced cells (Fig.2, Panel A). Accordingly, Western blot and flow cytometry analysis confirmed MAFB over-expression and the consequent MMP9 (Fig. 2, Panel B) and IL-7R (Fig. 2, Panel C) up-regulation in LMafBIΔN - transduced progenitors as compared to the empty vector. Altogether, these preliminary data further supported the

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involvement of MafB in the transcriptional regulation of *MMP9* and *IL-7R* genes, indicating them as putative primary response genes of the considered TF.

3.2 Identification of MARE elements in IL-7R and MMP9 promoter regions. To address this issue, we inspected IL-7R and MMP9 flanking promoter regions in order to identify putative MafB binding sites. This analysis was carried out using the UCSC Genome Browser [28] and considering two important criteria: 1) the possibility to identify elements with nucleotide divergency, since all several known MAREs are different from the canonical consensus sequence [10]; 2) a high level of evolutionary conservation that is frequently associated with relevant gene regulatory elements. Performing this investigation, a putative MARE element was recognized in each analyzed promoter region and is specified in Table I. To evaluate the capacity of MafB to bind the identified motives we performed EMSA assays in a cell-free system, based on the consideration that Maf is a heterogeneous protein family [10], whose members are also expressed in myeloid cells [29, 30], and that the core region of MAREs may be also recognized by the nuclear regulators of AP-1 family [10]. The results reported in Figure 3, panel A, showed that MMP9 MARE and IL-7R MARE sequences were specifically bound by the investigated TF (left panel). In more details, the two autoradiograms highlighted the appearance of a shift (lane 3, lower arrow) and super-shift (lane 4, upper arrow) complex respectively in the presence of Flag-MafB protein and as a result of FLAG M2 antibody incubation. On the contrary, the double point mutated probes, investigated with the same experimental design, did not exhibit such complexes (right panel). These data were further validated by "in-vivo" Chromatin Immuno-precipitation assay. Formaldehyde-cross linked chromatin was extracted from human normal monocytes and then immunoprecipitated with a specific anti-MafB or with an anti- Flag M2 Ab, used as control. The histogram showed in Figure 3, panel B, reported the results (mean ± s.e.m.) obtained from three independent experiments, exhibiting a clear enrichment of MafB-bound chromatin both in MMP9 and in IL-7R promoter regions. Finally, to evaluate the capacity of the isolated MafB

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binding sites to activate transcription, luciferase reporter assays were performed. The obtained data are shown in Fig. 3, panel C. The histograms display a significant increase of luciferase activity in the presence of MafB protein as compared to the control samples, represented by the responsive elements incubated with the empty vector pcDNA3.1. In these experiments, a basal transactivation activity was also detected in the control samples and it was probably ascribed to the capacity of AP-1 family members to recognize and bind the core regions of the analyzed MARE elements. On the basis of this combined approach, it was possible to state that *MMP9* and *IL-7R* are primary response genes of MAFB TF.

3.3 Analysis of MafB expression in activated primary monocytes. Since the *MMP9* gene is a well recognized marker of the anti-inflammatory process regulated by tissue macrophages, we focused our attention on the role exerted by the considered TF in the macrophage polarization process. To this aim, we performed a preliminary analysis of MafB expression in human normal monocytes treated with the following agents: LPS plus IFNγ to achieve a classical activation; IL-4 promoting an alternative activation; IL-10 inducing a macrophage de-activating state [31]. The results of these experiments clearly demonstrated that only IL-10 cytokine was able to induce MafB expression up to a 5.0 ± 2.5 value (mean ± s.e.m; Fig. 4, Panel C) whereas a significant down-regulation of MafB mRNA levels was detected in the alternative activation. On the other hand, any MafB variation was monitored upon LPS plus IFNγ stimulation (Fig. 4, Panel A and B). To validate the adopted experimental model, a similar analysis was extended to well-recognized phenotypic markers, such as IL-6 and TNFα for classical activation, IL-1RA and MRC1 for alternative activation, MRC1 and CD163 for the de-activation process and, as expected, all of them exhibited an up-regulated expression upon treatment with the corresponding agent (Fig. 4, Panel A-C) [32-34]. To further support this observation, we carried out a Western blot analysis aimed to evaluate MAFB protein levels under the experimental settings described above, disclosing a remarkable increment of the studied TF upon exposure of monocytes to IL-10 (Fig. 4, Panel

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D). It is therefore possible to conclude that MafB is involved in the transcriptional control of IL-10-mediated macrophage polarization.

3.4 Analysis of MafB genetic program in IL-10 treated primary monocytes. Based on the data described thus far, we performed a set of time-course experiments in which human primary monocytes were exposed to IL-10 in order to evaluate the expression pattern of MafB and its primary response genes in macrophage de-activation process. The outcome of this investigation, reported as mean ± s.e.m. of three independent stimulations, demonstrated that IL-10 is able to determine a significant induction of all the considered genes underlining, once more, the existence of a clear correlation between MafB and its targets, *MMP9* and *IL-7R* (Fig. 5, panel A; see Table II and Supplementary Material 2 for Student's t-Test, ANOVA and Newman-Keuls Multiple Comparison post hoc tests). To further demonstrate the involvement of the considered TF in IL-10 cellular response, MafB silencing experiments were subsequently carried out on IL-10 treated human monocytes. The histogram reported in Fig. 5, panel B, disclosed a clear MafB down-regulation leading to a significant inhibition of *MMP9* mRNA, that was also confirmed by Western blot analysis (Fig. 5, panel C). Under the same experimental conditions, IL-7R exhibited only a partial down-regulation, detected both at transcriptional (Fig. 5, B) and at translational level (Fig. 5, C). This finding allowed to hypothesize that the genetic control exerted by MafB on this target gene is strong during the up-regulation phase of its expression, while it is marginal during the maintenance of IL-7R basal levels, where other transcription factors are probably involved. Finally, to better characterize the role played by MafB TF in IL-10 anti-inflammatory state, we also analyzed CD163 and MRC1 expression. The data obtained display a clear induction of CD163 and MRC1 upon IL-10 treatment (Fig. 5, Panel D) and a significant down-regulation of both genes following MafB inhibition (Fig. 5, Panel E). Furthermore, by using a protocol for MRC1/CD163 biparametric flow cytometry analysis, we also observed that MafB down-regulation is able to determine a clear reduction of the double positive cell population (Fig. 5,

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Panel F). Taken together, these data allowed us to conclude that MafB is involved in the transcriptional regulation of the macrophage de-activation process mediated by IL-10 enforcing, at the same time, the role that it plays in the transcriptional regulation of *MMP9* and *IL-7R* genes.

3.5 MafB is a downstream target of the IL-10 / STAT3 signaling pathway. To further support our findings, we analyzed the mechanistic correlation between MafB and the intracellular signalling pathway activated by IL-10. This immunosuppressive molecule exerts its effect by binding to its receptor IL-10R, thereby activating the JAK1/STAT3 cascade. [35, 36]. As a consequence, we explored the possibility that the gene coding for MafB could be a STAT3 direct target. In this regard, a preliminary analysis of MafB promoter region, carried out using the UCSC genome browser [28], revealed the presence of two putative canonical STAT3 motives (TTCCnGGAA), respectively localized 9348 bp (STAT3 enhancer 1) and 8805 bp (STAT3 enhancer 2) upstream the transcription start site (TSS) (Fig. 6, panel A). These sites were subsequently validated by ChIP and Luciferase transactivation assays to verify the capacity of the STAT3 TF to bind "in vivo" the MafB promoter region, determining an up-regulation of its transcription and activity. ChIP experiments were carried out in human monocytes exposed to IL-10 cytokine for 24 h and the results achieved demonstrated a specific interaction of STAT3 protein with the Enhancer 1, whereas no binding activity was detected on Enhancer 2 (Fig. 6, Panel B). To evaluate the functional consequences of this finding, we subsequently performed a luciferase transactivation assay using HEK293 cells ectopically transduced with the pCMVSTAT3 vector, driving a constitutive expression of the STAT3 protein, and the pT1Luc construct, in which the considered enhancers had been cloned upstream a luciferase reporter gene. In agreement with ChIP experiments, the data obtained showed that STAT3 Enhancer 1 was able to increase the luciferase activity as compared to control cells whereas the Enhancer 2 appeared devoid of any trans-activation activity (Fig. 6, Panel C). Globally, in light of these results, it is possible to conclude that

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MafB is a STAT3 target gene and it is a downstream effector of the IL-10 / STAT3 signaling pathway involved in the macrophage de-activation process.

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4. **DISCUSSION**

A number of reports have demonstrated the crucial role played by MafB in the transcriptional control of monocyte – macrophage differentiation [5, 6, 14, 15] and the experimental activity developed in our laboratory has significantly contributed to such studies, providing a global view of the genetic program activated by this TF during the mentioned process [5]. In spite of this effort, little is to date known about MafB direct targets that could be responsible for its biological effects. Using a combined approach of gene function studies and DNA – protein interaction analysis, that were mainly conducted in human primary macrophages, we have been able to demonstrate that *IL-7R* and *MMP9* are primary response genes of MafB TF. Although IL-7R has been often associated with T lymphocyte biology in the past [37-39], our results have assigned a novel biological meaning to this receptor and, accordingly, to its ligand. Our previous observations highlighted a strong correlation between MafB and IL-7R, showing that primary human monoblasts express high mRNA levels of both genes [14]. In addition, published studies have recently indicated that IL-7 is able to promote the differentiation of macrophages to dendritic [40] and osteoclast cells [41, 42], i.e. further specialized cell contexts belonging to the same lineage. These considerations put in clear evidence the importance of the molecular mechanism arising by our data and support the idea that IL-7R play an important role in monocyte differentiation and macrophage activation. MMP9 is a well-known protein, implicated in the process of extracellular matrix remodeling, sustained by tissue macrophages. In this regard, a large body of evidence underlined the functional correlation between MMP9 and the "Tumor- Associated Macrophages" (TAMs), the major inflammatory component of stroma in many tumors [21, 22, 43], leading to the hypothesis that MafB might regulate the biological functions exerted by these cells.

The relevance of the results presented in this report also resides in some strictly molecular aspects concerning the nucleotide composition and spatial organization of MARE motives. In fact, the identification of MARE elements has appeared quite complicated for the following

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reasons: 1) binding sites, belonging to this category and isolated so far [44-46], are divergent from the canonical consensus TGCTGAC/GTCAGCA indicated in the scientific literature; 2) a sequence matrix describing virtually all nucleotide combinations in each position of the MARE element has not been defined yet; 3) all members of the AP-1 family recognize the highly conserved TGAC/GTGA core region, but the flanking nucleotides conferring binding specificity to single Maf proteins have been still poorly characterized and may exhibit a significant reciprocal divergence. Based on these consideration, our data will allow a better comprehension of the MARE structure.

Additional experiments described in our paper also disclosed an involvement of MafB in the transcriptional regulation of macrophage polarization, where it is shown to play a role in the de-activation process induced by the IL-10 cytokine. Macrophage activation may be defined as a functional specialization acquired by tissue resident macrophages in response to extracellular or intracellular signals and represents a complex process during which macrophages display a large spectrum of functional phenotypes implying a remarkable plasticity [31, 47, 48]. In this context, macrophage de-activation is an active phenomenon able to switch off both classical and alternative activation. IL-10, together with glucocorticoids and TGFβ, is one of the main regulators of this anti-inflammatory response [33, 49], being able to limit the entity and duration of inflammation and to promote wound healing, tissue remodeling and the restoration of tissue homeostasis. Cellular response to IL-10 stimulation is mediated by the activation of JAK1 kinase, leading to phosphorylation and activation of the STAT3 transcription factor that, in turn, induces the expression of several polarization markers and other TFs [36, 49]. Our data demonstrated, for the first time, that STAT3 is able to up-regulate MafB expression through a direct transcription mechanism and that treatment of macrophages with IL-10 stimulates this effect. This finding allow us to hypothesize the existence of a signaling cascade by which stimulation of macrophages with IL-10 determines a sequential activation of STAT3 and MafB, resulting in the up-regulated expression of

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MMP9 and *IL7R* genes. The final effect of this regulatory pathway would be to resolve the inflammatory condition and to restore tissue integrity.

In conclusion, the data reported here represent, in our opinion, a clear demonstration supporting the involvement of MafB in IL-10 mediated macrophage de-activation. More in general, these results also contribute to shed light on the molecular mechanisms regulating the macrophage polarization process.

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ACKNOWLEDGEMENTS

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FOOTNOTES

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FIGURE LEGENDS

Fig. 1 Analysis of *MAFB, MMP9* and *IL-7R* gene co-expression in PMA treated THP1 cells. PMA stimulation was performed by using a 1.6 nM concentration for 48 h. (**A**) Histogram exhibiting the *MAFB, MMP9* and *IL-7R* transcript variations in PMA treated (grey bars) as compared to untreated cells (white bars). *X-axis*, gene symbol of the considered genes; *y-axis*, Transcript Relative Quantity (RQ). (**B**) Results of MAFB gene silencing leading to a down-regulation of both *MMP9* and *IL-7R* gene expression. Grey bars indicate THP1 cells treated with MAFB siRNA whereas white bars represent the cells treated with a control siRNA and used as calibrator sample. *X-axis*, Gene Symbol of selected genes; *y-axis*, Transcript Relative Quantity (RQ). (**C**) Western blot analysis showing MAFB, MMP9 and IL-7R down-regulation achieved in THP1 cells nucleofected with control siRNA (CONT) or siRNA targeting MafB (MAFB). Normalization of protein extracts was achieved by VINCULIN expression. The protein name and the analyzed samples are indicated, respectively, on the right and at the top of the panel.

Fig. 2 Evaluation of *MAFB, MMP9* and *IL-7R* genes in CD34+ hematopoietic progenitors transduced with empty LXIΔN retroviral vector or with LMafBIΔN construct. (**A**) Histogram displaying the mRNA levels of *MMP9* and *IL-7R* genes (*x-axis*) detected, as Relative Quantity (RQ, *y-axis*) in hematopoietic progenitors over-expressing MafB cDNA (grey bars) as compared to the empty LXIΔN retroviral vector (white bars). The data represent the mean ± s.e.m of six independent experiments. (**B**) Western Blot analysis of MAFB and MMP9 protein expression was performed at day 14 of liquid culture (i.e. 10 days post-infection) on total extracts of transduced/NGFR purified cells. The analyzed protein and samples are respectively indicated on the right and at the top of the panel. Pro, Pro-peptide; Act, active. Expression of ACTIN was also analysed to normalize the amounts of protein extract loaded in the various lanes. (**C**) Results of flow cytometry evaluation, performed to estimate IL-7R positivity in LMAFBIΔN - transduced cells as compared to the control sample infected with

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the empty vector (LXIΔN). Multi-parameter flow-cytometry analysis was used to assess IL-7R and NGFR expression in the indicated samples. The entities of surface antigen expression, measured as positive percentage, are shown inside the blue square (LXIΔN) or red square (LMAFBIΔN).

Fig. 3 EMSA, ChIP and luciferase assays performed to validate MMP9 MARE and IL-7R MARE binding elements. (**A**) Results of gel shift assays to evaluate the MafB *in-vitro* binding to the putative MARE enhancers. As indicated in the table reported below the autoradiograms, lane 1 and 2 represent the negative controls, i.e. the TNT Quick Master Mix incubated, respectively, with pcDNA3.1 empty vector, used as template, and with the anti-FLAG M2 antibody. Lane 3 shows a shift complex (lower arrow) resulting by the incubation with "in vitro" transcribed / translated FlagMafB protein in the presence of [γ-32P]ATP–labeled probes. Lane 4 highlights a supershift complex (upper arrow) generated by the incubation of the same sample with the anti-FLAG M2 antibody. (**B**) Chip experiment performed in human normal monocytes to detect *in-vivo* binding of MafB to the recognized MARE elements. Immuno-precipitation was conducted with an anti - Flag control Ab (CA, white bars) and a specific MafB Ab (MAFB, grey bars). Left panel, histogram showing the ratio between the amount of PCR-amplified MafB-bound DNA in ChIP samples and in input chromatin (% ChIP/INPUT). Right panel, ethidium gel images of semi-quantitative PCR of ChIP experiments. IC, Input Chromatin; CA, anti-Flag control antibody; MAFB, anti-MafB antibody. CTRL. REG., amplification of the microsatellite region localized on chromosome 3 and selected as control. All ChIPs and semi-quantitative PCR were performed in triplicate; a representative experiment is shown. (**C**) Histograms describing the results of luciferase assays performed in HEK293 cells transfected with the pT81Luc reporter plasmids and pcDNA3.1FlagMafB expression vector. *x-axis*, combination of transfected plasmids; *y-axis*, β-Galactosidase normalized Luciferase values. Bars represent the mean luciferase activity ± s.e.m. of at least three independent experiments.

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Fig. 4 MAFB expression in human normal monocytes under polarization treatments. The histograms report the mRNA expression levels of MAFB transcription factor and specific macrophage polarization markers achieved in human monocytes untreated (white bars) or stimulated (grey bars) with a combination of LPS and IFNγ (□□□**A**□), IL-4 (**B**) or IL-10 (**C**). Gene symbols are indicated on *x-axis* whereas mRNA levels, assessed through the QRT-PCR reaction, are reported on *y-axis* as transcript relative quantity (RQ) (mean ± s.e.m) of at least three independent experiments. (**D)** Western Blot analysis of MAFB protein levels under the polarization conditions already explained above. Treatments and proteins are respectively specified on the top and on the right of the panel. To normalize the amounts of protein extract loaded in the various lanes, expression of ACTIN protein was also included in this assay

Fig. 5 Expression of MafB and macrophage polarization markers in response to IL-10 stimulation. The data were achieved in human monocytes treated with a 50 ng/ml concentration of IL-10 cytokine. (**A, B**) QRT-PCR analysis to evaluate MafB, MMP9 and IL-7R transcript levels in IL-10 time course (A) or in MafB silencing experiments (B). Unstimulated monocytes (0H) or samples treated with a non targeting siRNA were used as detector respectively in panel A and B. The results are indicated on *y-axis* as Transcript Relative Quantity (RQ) ± s.e.m. of three independent replicates. (**C**) Western blot examination of MAFB, MMP9, IL-7R proteins carried out in monocytes under MafB inhibition conditions. Combined treatments and protein names are specified at the top and on the right of the panel, respectively. To normalize the amounts of protein extract loaded in the various lanes, expression of ACTIN protein was also included in this assay. (**D**, **E)** Gene expression analysis of *CD163* and *MRC1* genes reported with the same modalities explained in panel A and B. (**F**) Flow cytometry evaluation to estimate CD163 and MRC1 polarization marker expression in monocytes undergoing MafB inhibition followed by IL-10 stimulation. The entities of surface antigen expression, measured as positive percentage, are shown inside the dot plots.

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Fig. 6 Analysis of MafB involvement in IL-10/STAT3 signaling pathway

(**A**) Schematic representation of MafB promoter region showing the position of two putative STAT3 binding sites (STAT3 enhancer 1 and STAT3 enhancer 2) from the Trascriptional Start Site (TSS), indicated by arrow. (**B**) Histogram displaying the results obtained by Chromatin Immuno-precipitation experiments performed in IL-10 treated human monocytes and by using a STAT3 or a Flag antibody as control. On *x-axis*, the combination of IL-10 stimulation and antibodies used are indicated for both the investigated enhancers. On *y-axis*, the chromatin enrichment is reported as the ratio between the amount of PCR-amplified STAT3-bound DNA in ChIP samples and in input chromatin (% ChIP/ IMPUT). (**C**) The histogram shows the data achieved by Luciferase transactivation experiments performed in HEK-293 cells. *X-axis*: combination of transfected plasmids; *y-axis*: β-Galactosidase normalized Luciferase values. The results exhibited in Panels B and C correspond to a mean value ± s.e.m. of at least three independent experiments.

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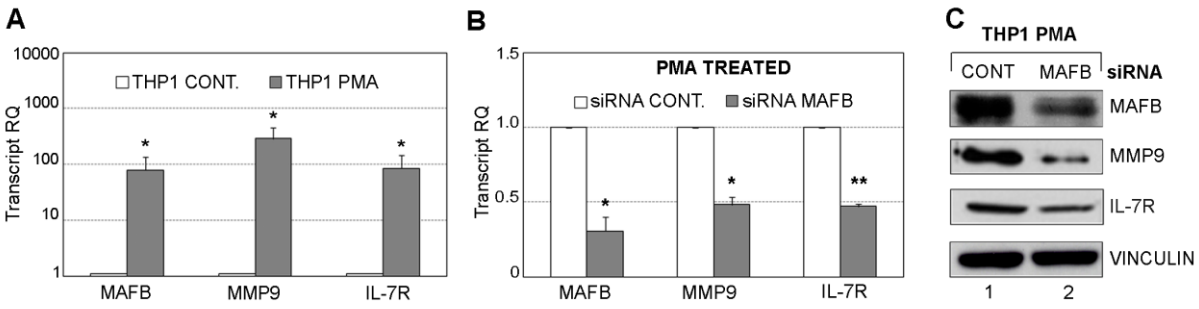

FIGURE 1

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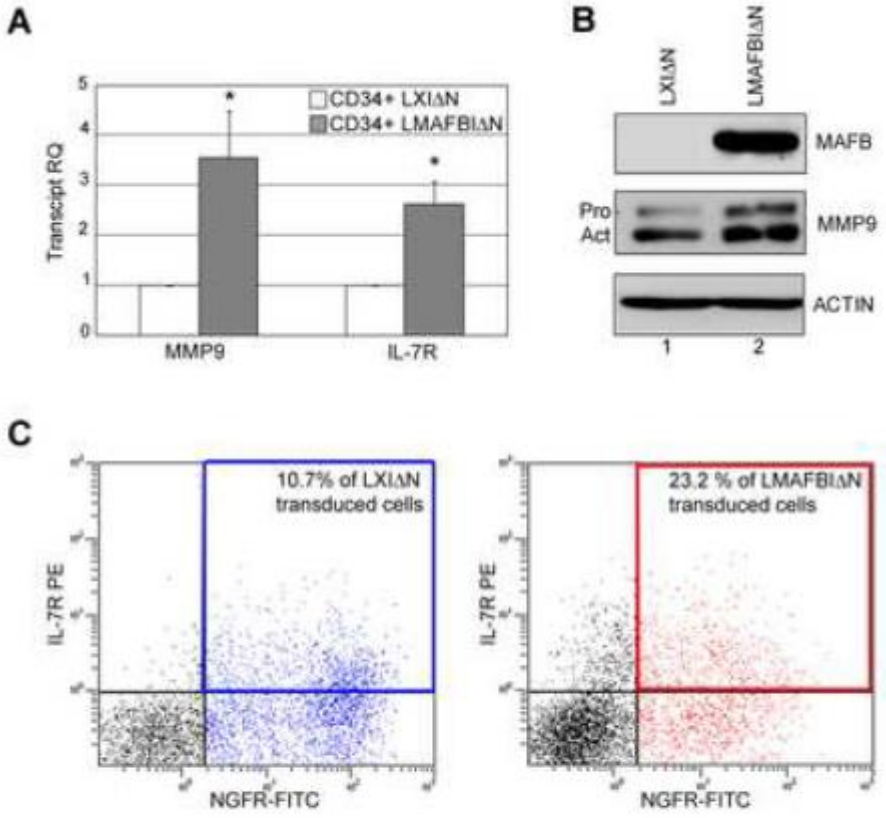
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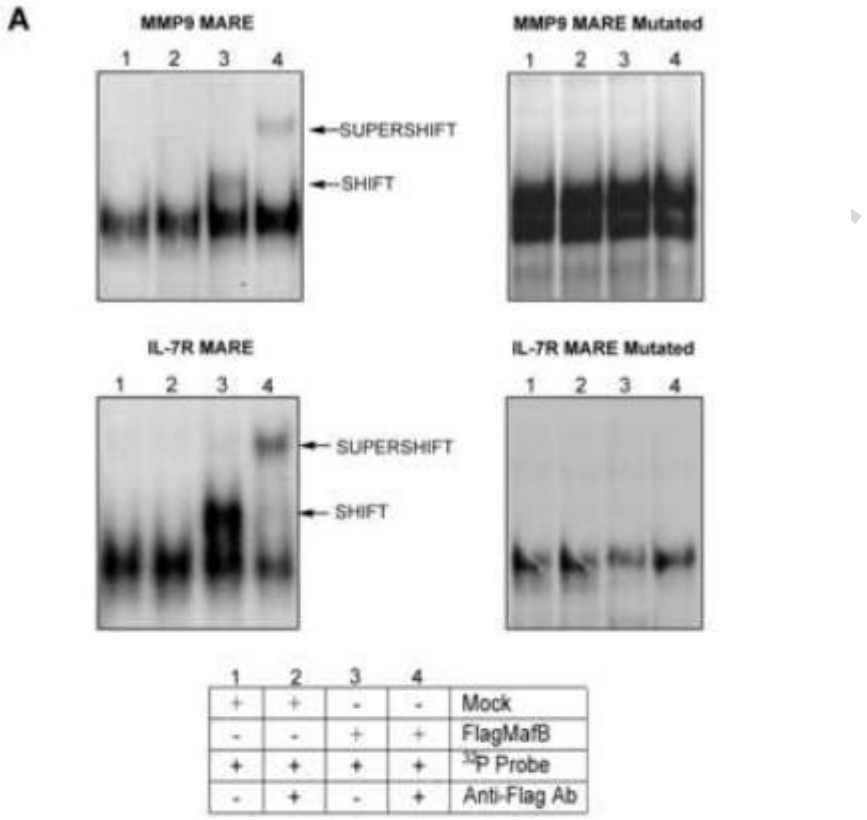
FIGURE 2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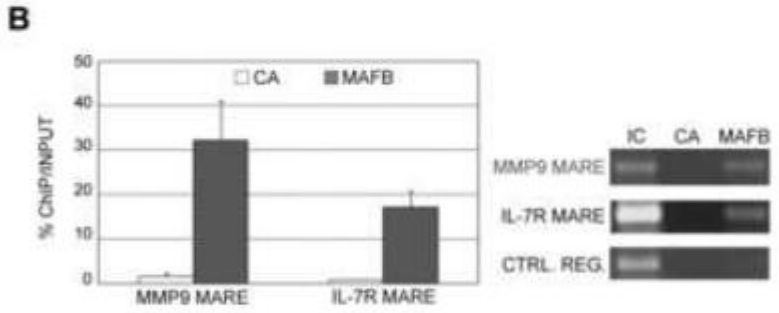
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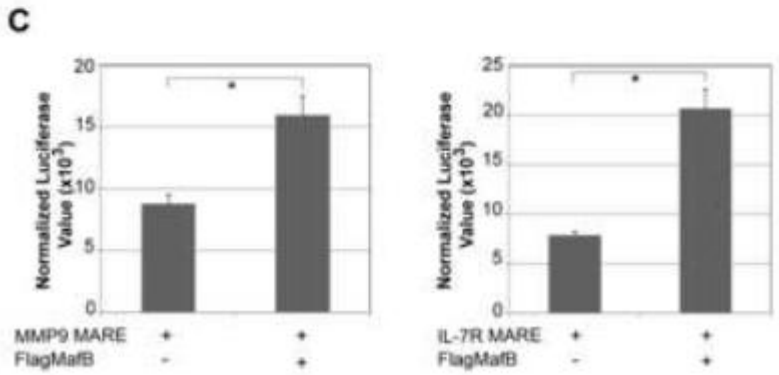
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Normalized Luciferase Value (x10-3)
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FIGURE 3

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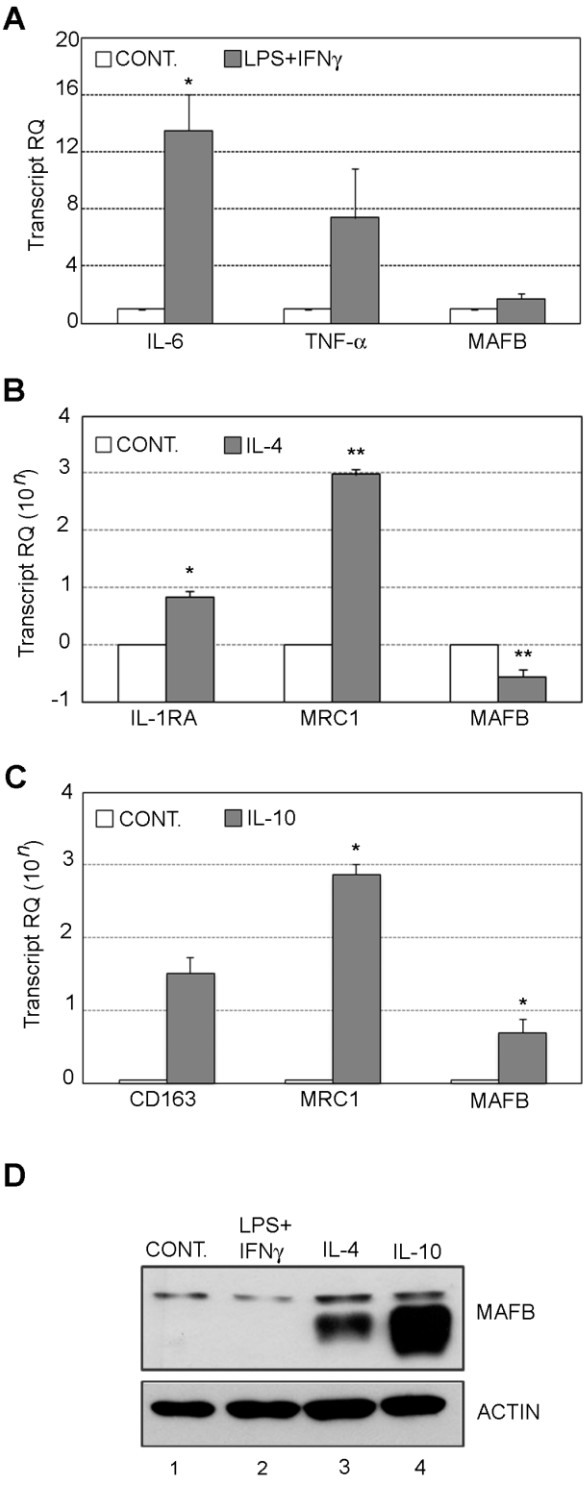

FIGURE 4

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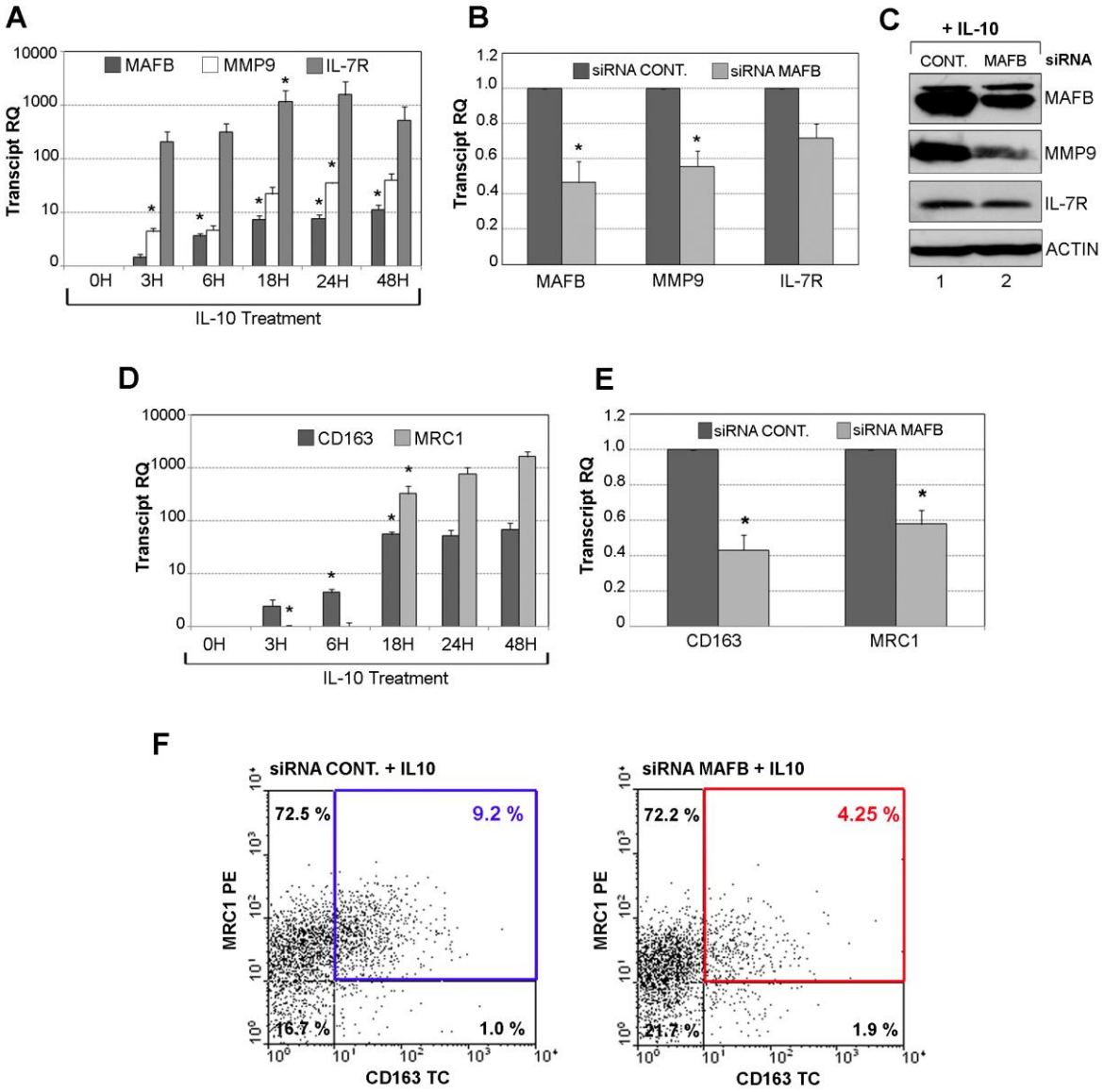

FIGURE 5

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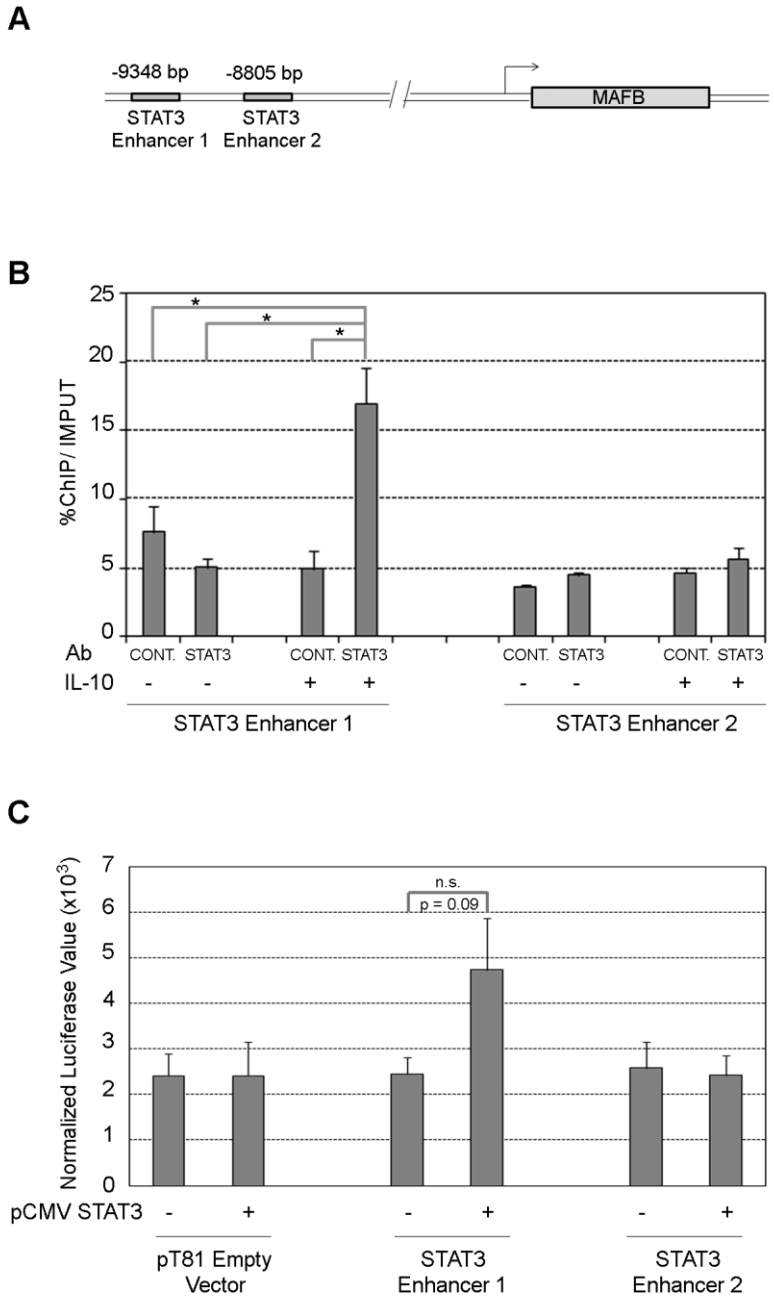

FIGURE 6

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Table I. List of putative MARE elements used as probe in EMSA assays

PROBE NAME	SEQUENCE 5'-3'	POSITION FROM "+1" (bp)
MARE CONSENSUS	5'-TGCTGAC/GTCAGCA**-3'**	
MMP9 MARE	5'-**CC**CTGAGTCAGCA-3'	-84
MMP9 MARE Mutated	5'-**CC**CTGA<u>C</u>TCAGC<u>T</u>-3'	
IL-7R MARE	5'-**GA**CTGACTCAGCA-3'	-4288
IL-7R MARE Mutated	5'-**GA**CTGA<u>G</u>TCAG<u>G</u>A-3'	

Table I reporting the putative MafB binding sites, and its mutated forms, identified in MMP9 and IL-7R promoter regions and used in EMSA experiments. Inside the putative MARE motives, divergent nucleotides are indicated by bold font whereas mutated nucleotides are underlined.

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Table II. Statistical analysis of "time-course experiments" performed in IL-10 treated monocytes.

	p- values				
Treatment Time	**3h**	**6h**	**18h**	**24h**	**48h**
MAFB					
Student's T-Test	0.15	**0.02**	**0.03**	**0.03**	**0.05**
ANOVA Analysis	**0.00042677**				
MMP9					
Student's T-Test	**0.04**	0.07	0.09	**0.001**	0.08
ANOVA Analysis	**0.001044574**				
IL-7R					
Student's T-Test	0.07	0.23	**0.04**	0.11	0.13
ANOVA Analysis	0.443383602				
CD163					
Student's T-Test	0.18	**0.03**	**0.01**	0.06	0.10
ANOVA Analysis	**0.002228024**				
MRC1					
Student's T-Test	**0.05**	0.20	**0.03**	0.09	0.06
ANOVA Analysis	**0.000934677**				

Table II. Statistical analysis applied on gene expression data obtained in the "Time-course experiments" carried out in IL-10 stimulated human monocytes (see Figure 5). Student's T-test analysis was performed by comparing the value obtained in each treatment time versus the untreated cells, used as baseline. Additionally, multiple comparison analysis were carried out by one-way ANOVA and Newman-Keuls post hoc test and the results are reported in the "Supplementary Material 2" file. Statistically significant p-values were reported in bold.

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Highlights

IL-7R and MMP9 are primary response genes of MafB transcription factor
MafB is involved in the macrophage polarization process
MafB is a direct target of the IL-10 / STAT3 signaling pathway

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