Hypoxia modulates the gene expression profile of immunoregulatory receptors in human mDCs: identification of TREM-1 as a novel hypoxic marker in vitro and in vivo.

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Hypoxia modulates the gene expression profile of immunoregulatory receptors in human mature dendritic cells: identification of TREM-1 as a novel hypoxic marker *in vitro* and *in vivo*

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Running Title: TREM-1 induction by hypoxia on mature DCs

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Abstract

Dendritic cells (DCs) are a heterogeneous group of professional antigen presenting cells functioning as sentinels of the immune system and playing a key role in the initiation and amplification of innate and adaptive immune responses. DC development and functions are acquired during a complex differentiation and maturation process influenced by several factors present in the local milieu. A common feature at pathologic sites is represented by hypoxia, a condition of low pO_2 which creates a unique microenvironment affecting cell phenotype and behavior. Little is known about the impact of hypoxia on the generation of mature (m)DCs. In this study, we identified by gene expression profiling a significant cluster of genes coding for immune-related cell surface receptors strongly upregulated by hypoxia in monocyte-derived mDCs and characterized one of such receptors, TREM-1, as a new hypoxia-inducible gene in mDCs. TREM-1 associated with DAP12 in hypoxic mDCs, and its engagement elicited DAP12-linked signaling, resulting in ERK-1, Akt, and IκBα phosphorylation and pro-inflammatory cytokine and chemokine secretion. Finally, we provided the first evidence that TREM-1 is expressed on mDCs infiltrating the inflamed hypoxic joints of children affected by Juvenile Idiopathic Arthritis, representing a new *in vivo* marker of hypoxic mDCs endowed with pro-inflammatory properties.

Introduction

Dendritic cells (DCs) are a heterogeneous group of professional antigen-presenting cells involved in the initiation and amplification of innate and adaptive immunity, which develop through different haematopoietic pathways[1]. Myeloid DC immunostimulatory properties are acquired during a complex differentiation and maturation process. Their precursors extravasate from the bloodstream to non-lymphoid peripheral tissues, where they reside in an "immature" stage (iDCs) at sites of potential pathogen entry, and are an important component of the leukocyte infiltrate in inflammatory tissues [1,2]. iDCs are specialized for antigen capture and processing, functioning as sentinels of the immune system[3]. Antigen uptake and activation by endogenous factors, such as proinflammatory cytokines and tissue damage-associated molecular patterns (DAMPs), or exogenous factors, such as pathogen-associated molecular patterns (PAMPs), induce iDCs to undergo phenotypic and functional changes that culminate in their maturation into mDCs, which have a higher capacity for antigen presentation[1]. mDCs switch their chemokine receptor repertoire down-regulating inflammatory receptors and upregulating those required for homing to secondary lymphoid organs, where they prime naive T cells triggering specific immune responses[2-4]. The local microenvironment contributes to the regulation of DC development and functions [2,5,6]. A common feature of inflamed tissues is represented by hypoxia, a condition of low partial oxygen pressure (pO_2, 0-20mm Hg) which arises as a result of dysfunctional vascular network and diminished O_2 supply and affects the phenotype and functions of every cell exposed to it [6-9]. Activation of gene transcription is the primary mechanism by which mammalian cells respond to decreased pO_2, and the underlying molecular pathways have been elucidated in detail and extensively reviewed. Briefly, transcriptional activation is mediated primarily by the hypoxia inducible factor-1 (HIF-1), a heterodimer of the constitutive HIF-1β subunit and an oxygen sensitive α subunit (HIF-1α-2α). α subunits are post-translationally stabilized under hypoxia and translocate to the nucleus where they dimerize with HIF-1β transactivating the hypoxia responsive element (HRE) present in the promoter of many O_2-sensitive genes [7,8,10]. HIF-1 expression and activity is tightly regulated by various cofactors and transcription factors, and HIF-independent pathways mediating gene induction by hypoxia have also been described [6,10,11].

Recent studies have investigated hypoxia effects on DC differentiation, maturation, and functions. We reported that monocyte differentiation into iDCs under chronic hypoxia promotes the onset of a unique migratory phenotype by differentially modulating the expression profile of chemokines/receptors and genes involved in cell adhesion and tissue remodeling [12,13]. Other studies indicated that acute iDC exposure to low pO_2 may either impair [14] or promote [5,15] their maturation, by affecting expression of chemokine receptors, costimulatory molecules, and T cell priming ability. However, the impact of chronic hypoxia on mDC development and functional behaviour in inflammatory states is still largely unknown.

In this study, we demonstrate that the gene expression pattern of mDCs generated from human monocytes under chronic hypoxia (H-mDCs) is distinct from that of mDCs developing under normal O_2 levels and characterized by upregulation of genes coding for surface costimulatory and adhesion molecules, immunoregulatory and pattern recognition receptors (PRRs). Among them, we identify the triggering receptor expressed on myeloid cells (TREM)-1, a member of the Ig superfamily of immunoreceptors and a strong amplifier of the immune responses [16,17], as a new hypoxia-inducible gene in mDCs. We provide evidence that this receptor is biologically active and expressed *in vivo* on mDCs recruited to the hypoxic joints of Juvenile Idiopatic Arthritis (JIA) patients, pointing to a pathogenetic role for this molecule in the disease.

Methods

DC generation and culture

Blood monocytes were isolated from healthy volunteers at a purity of >93%, plated into sixwell culture plates (BD-Falcon) in RPMI 1640 (Euroclone) supplemented with 10% heatinactivated FCS (HyClone), and incubated for 4 days under normoxic (20% O_2) or hypoxic (1% O_2) conditions in the presence of GM-CSF and IL-4 (both 100 ng/ml), as detailed [12,13]. A cocktail of proinflammatory mediators containing TNF〈 (50 ng/ml), IL-1β (50 ng/ml), IL-6 (10 ng/ml), and PGE_2 (1mM) was added for the last 48hr to induce DC maturation. Hypoxic conditions were obtained by culturing cells in an anaerobic work-station incubator (CARLI Biotec) flushed with a mixture of 1% O_2, 5% CO_2, and 94% N_2. Medium was allowed to equilibrate in the hypoxic incubator for 2hr before use, and pO_2 was monitored using a portable oxygen analyzer (Oxi 315i/set, WTW).

Synovial fluid mononuclear cell isolation

Synovial fluid (SF) samples were obtained at the time of therapeutic knee arthrocentesis from eight children affected by oligoarticular JIA [18] and collected into sodium-heparin tubes under vacuum. pO_2 levels in SF samples were monitored to confirm hypoxic conditions. Paired PB samples and PB from five age-matched control subjects undergoing venepuncture for minor orthopedic procedures were obtained on the occasion of routine venepuncture and collected as for SF. Informed consent was obtained according to the procedure approved by the Gaslini's Ethical Committee. Specimens were centrifuged to prepare cell-free SF and plasma and separated by Ficoll to isolate mononuclear cells (SFMCs and PBMCs). SF-derived samples were handled in the anaerobic incubator to prevent cell reoxygenation, as detailed [19,20].

Cytokines and antibodies

Human recombinant GM-CSF, IL-4, TNFα, IL-1β, and IL-6 were from PeproTech; PGE_2 was from Sigma.

mAbs used for FACS: anti-CD83-FITC or PE-Cy5 and anti-CD86-FITC (BD-Pharmingen), anti-TREM-1-PE, anti-CXCR4-FITC, and anti-CCR7-FITC (R&D Systems), anti-CD1a-FITC or -APC

(Serotec), anti-CD1-FITC and anti-CD141-APC (Miltenyi-Biotec). Proper isotype-matched control Abs (Dako) were used.

Abs used for western blot: mouse anti-human TREM-1 (R&D Systems), mouse anti-human HIF-1α (BD-Biosciences), rabbit anti-human phospho (p)-ERK)-1, p-Akt, and p-IkBα (Cell Signaling Technology), mouse anti-human DAP12, rabbit anti-human ERK-1, Akt, IkBα, and β-actin (SantaCruz Biotechnology),

Flow cytometry

Flow cytometry was performed as described[12,13]. Cells resuspended with PBS supplemented with 0.2% BSA, 0.01% NaN_3 were incubated with fluorochrome-conjugated mAbs for 30 min at 4°C, after blocking nonspecific sites with rabbit IgG (Sigma). Fluorescence was quantitated on a FACSCalibur flow cytometer equipped with CellQuest software (BD-Biosciences). Cells were gated according to their light-scatter properties to exclude cell debris.

RNA isolation and GeneChip hybridization

Total RNA was purified from different donor-derived mDCs using the Qiagen RNeasy MiniKit and reverse-transcribed into double-stranded cDNA on a GeneAmp PCR System 2700 thermal cycler (Applied Biosystems) using the one-cycle cDNA synthesis kit (Affymetrix). cDNA derived from three donors was purified and biotin labeled with the IVT-expressed kit (Affymetrix), as described[21]. Fragmented cRNA was hybridized to Affymetrix HG-U133 plus 2.0 arrays (Genopolis Corporation) containing 54,000 probe sets coding for 38,500 genes, chips were stained with streptavidin-phycoerythrin (Invitrogen) and scanned using an Affymetrix GeneChip Scanner 3000, as described [21]. Data capturing was conducted with standard Affymetrix analysis software algorithms (Microarray Suite 5.0). Comparative analysis of hypoxic relative to normoxic expression profiles was carried out with GeneSpring Expression Analysis Software Gx9.0 (Silicon Genetics), and expression data were normalized using “per chip normalization” and “per gene normalization” algorithms. Fold-change was calculated as the ratio between the average expression level under hypoxia and normoxia. We selected a modulated gene list of two-fold induction/inhibition with a false discovery rate of 0%. The significance of gene expression differences between the two experimental conditions was calculated using the Mann-

Whitney *U*-test. Only genes that passed the test at a confidence level of 95% ($P<0.05$) were considered significant. Complete data set for each microarray experiment was lodged in the Gene Expression Omnibus public repository at NCBI (www.ncbi.nlm.nih.gov/geo/) [accession Nr.GSE22282]. Gene Ontology (GO) data mining for biological process at level 1 was conducted online using the Database for Annotation, Visualization and Integrated Discovery (DAVID) software (http://david.niaid.nih.gov). HRE consensus elements consisting of a 4nt core (**CGTG**) flanked by degenerated sequences ((T|G|C)(A|G)**(CGTG)(**C|G|A)(G|C|T)(G|T|C)(C|T|G)) were mapped in the promoter regions of genes represented in the chip, as detailed [12].

Real-time RT-PCR

Real time PCR (qRT-PCR) was performed on a 7500 Real Time PCR System (Applied), using SYBRGreen PCR Master Mix and sense/antisense oligonucleotide primers (TIBMolbiol) (listed in Table S1). Expression data were normalized on the values obtained in parallel for three reference genes (indicated in Table S1) selected among those not affected by hypoxia in the Affymetrix analysis using the Bestkeeper software, and relative expression values were calculated using Q-gene software, as detailed [21].

Cross-linking of TREM-1-positive cells

12-well flat-bottom tissue culture plates (Costar) precoated with 10μg/ml of agonist anti-TREM-1 mAb or control IgG1 were incubated ON at 37°C before seeding $8x10^5$ H-mDCs/well/ml of RPMI 1640 w/o cytokines. Plates were briefly spun at 1200 rpm to engage TREM-1. After 24hr stimulation under hypoxia, supernatants were harvested and tested for cytokine/chemokine content by ELISA. In a set of experiments, cells were plated in medium w/o FCS, plates centrifuged and incubated for 20 min at 37°C under hypoxic conditions. ERK1, Akt and IκBα phosphorylation was then assessed by western blot.

Western blot analysis and immunoprecipitation

Total protein extracts were prepared as described [13], subjected to SDS-PAGE, transferred to PVDF membranes (Millipore), and probed with specified Abs. In a few experiments, lysates were subjected to immunoprecipitation with 10μg/ml anti-TREM-1 mAb or control IgG1 ON at 4°C, and protein G-Sepharose 4B (GE-Healthcare) for 45 min at 4°C. Precipitates were separated by SDSPAGE and immunoblotted with anti-DAP12 mAb. Chemiluminescence detection was carried out with peroxidase-conjugated goat anti-rabbit and anti-mouse Abs using an ECL kit (Pierce).

ELISA

Conditioned medium (CM) was replaced on day 3 or 4 of mDCs generation with fresh medium supplemented with cytokines for 24hr, and tested for soluble (s)TREM-1 content by ELISA (R&D Systems) after additional 24hr culture. sTREM-1 was also quantified in SF and plasma samples. TNFα, IL-6, IL-12p70, IL-10, IL-8, CCL4, and CCL5 were measured in CM from mDCs triggered with anti-TREM-1 mAb or control mAb by specific ELISA (R&D Systems). Data were analyzed with the GraphPad Prism-5 Software.

Statistical analysis

Data are the mean ±SE of three independent experiments, unless differently specified. The Student's t-test was used to determine results'significance ($p<0.05$). sTREM-1 concentrations in SF and plasma specimens were evaluated by the Wilcoxon rank test ($p<0.05$ statistically significant).

Results

Gene expression profile of hypoxic mDCs

mDCs were generated by culturing human monocytes under normoxic and hypoxic conditions in the presence of GM-CSF/IL-4 for 4 days and a cocktail of pro-inflammatory stimuli for the last 48hr. As determined by flow cytometry (Fig.1), both mDCs and H-mDCs displayed the mature phenotype, characterized by high surface expression of CD1a differentiation marker, CD83 maturation marker, and CCR7 chemokine receptor, undetectable in fresh monocytes, and by upregulation of CD86 costimulatory molecule and CXCR4 chemokine receptor, in agreement with previous observations [13].

H-mDC transcriptional profile was then assessed by microarray analysis. Pairwise comparison between data sets from normoxic and hypoxic samples revealed differential modulation of a large number of transcripts. The majority of differentially expressed genes were identified as unique and named in the GenBank™, whereas the remaining transcripts were either ESTs or hypothetical. After restricting the profile to sequences exhibiting ≥2-fold expression differences, we identified 563 up- and 402 down-regulated genes by hypoxia (Fig.2), which were selected for further analysis. These results provided the first indication that mDCs and H-mDCs had a different gene expression signature.

To gain insights into the nature of hypoxia-induced changes, selected genes were clustered into various functional categories according to GO data mining. We highlighted 33 interrelated functional pathways, containing a statistically significant portion of hypoxia-modulated genes (Fig.2). H-mDC transcriptional profile was mainly associated to cell growth, differentiation, and/or maturation, and angiogenesis regulation. Several upregulated genes fell into pathways implicated in signal transduction, metabolic processes, and apoptosis. Interestingly, a prominent set of upregulated genes had immunological relevance, coding for proteins involved in immune regulation, inflammatory responses, cell migration and adhesion.

Characterization of hypoxia-inducible immune-related genes

Among immune-related genes, profound differences were observed in the expression of a large cluster (63) of cell surface receptor-encoding genes (Table I). These include several PRRs critical to host defense, such as CD180, various complement receptor components, toll-like receptors (TLRs)-1 and -2,

C-type lectin receptors CLEC-2D,-2B,-7A, and macrophage receptor with collagenous structure (MARCO). Of interest is also the upregulation of scavenger receptors implicated in the regulation of fatty acid and/or cholesterol uptake/transport, such as thrombospondin receptor (CD36) and apolipoprotein B48 receptor (APOB48R). A set of genes coding for costimulatory and adhesion/homing molecules was also upregulated, such as several integrin family members, L1 cell adhesion molecule (L1CAM/CD171), neuropilin-1 (NRP1), ADAM metallopeptidase 8 (ADAM8/CD156), platelet/endothelial cell adhesion molecule (PECAM-1/CD31), and lymphocyte adhesion molecule-1 (LECAM1). Other hypoxia-inducible genes coded for immunoregulatory receptors, the most relevant of which are: Ig-Fc receptors, TREM-1, SLAM family member-9 (SLAMF9), blood dendritic cell antigen (BDCA3/CD141), leukocyte immunoglobulin-like receptors (LILR)-A1,-A2, and semaphorins (SEMA)-4B,-4D.

These data suggest that hypoxia can exert a profound modulatory effect on mDC innate and adaptive immune functions.

The possible relationship between gene inducibility by hypoxia and HRE presence in the promoter was then investigated by mapping HRE sequences in the first 2000 bases upstream the transcription initiation site. Interestingly, we found that ≈56% of upregulated genes contained at least one member of the HRE family in the promoter, whereas the others were HRE- (Table I), indicating that a remarkable portion of the hypoxic transcriptome does not require HIF-1 binding.

To validate microarray results, mRNA levels for a subset of genes selected among those listed in Table I were quantified by qRT-PCR in pooled RNA from mDCs and H-mDCs generated from different donors (Fig.S1). A few known hypoxia target genes were analyzed in parallel as positive controls (indicated in Fig.S1). We found a ≈90% concordance between qRT-PCR and Affymetrix data with respect to the direction of the expression changes, although differences in the extent of modulation were observed for some genes. CD85, CD9, and CLEC2B upregulation by hypoxia was not confirmed by qRT-PCR.

TREM-1 is expressed in hypoxic mDCs

Among validated genes, TREM-1 was that displaying the highest expression difference in H-mDCs *vs* mDCs (Fig.S1). TREM-1 was previously reported to be selectively expressed in blood neutrophils and a

subset of blood monocytes and tissue macrophages and to be completely downregulated during monocyte differentiation into DCs [16,17]. Hence, we were interested in further investigating TREM-1 regulation and functional significance in H-mDCs.

To address the issue of donor-to-donor variability, we evaluated TREM-1 mRNA expression in H-mDCs generated from five donors by qRT-PCR (Fig.3A). Expression of CAXII metalloenzyme was assessed in parallel as an index of response to hypoxia [22]. TREM-1 transcript levels were significantly higher in H-mDCs than in mDCs from all tested samples, paralleling those of CAXII, with differences ranging from 23- to 516-fold among individual donors. In keeping with the mRNA data, marked TREM-1 immunoreactivity was observed by Western blot analysis in HmDCs lysates, whereas TREM-1 was almost undetectable in mDCs (Fig.3B), suggesting that its expression is restricted to cells generated under hypoxia.

TREM-1 surface expression was then measured by cytofluorimetry. The left panel of Figure 3C depicts CD83/TREM-1 staining pattern of mDCs and H-mDCs from a representative donor, and the right panel of Figure 3C shows the percentage of TREM-1+ cells in the CD83-gated population from eight individual samples at day 4 of culture. Fifty to 86% of CD83+ H-mDCs expressed TREM-1. Conversely, no TREM-1+ mDCs were detectable in any of the donors examined, confirming previous evidence [17]. Immunophenotypic analysis of TREM-1+ and TREM-1- cells demonstrated a different expression profile of CD1c (BDCA-1) and CD141 (BDCA-3), two surface markers recently identified in functionally distinct subgroups of myeloid DCs (MDC1 and MDC2) *in vivo* [23], with a significantly ($p<0.05$) higher percentage of TREM-1+ H-mDCs displaying CD141 and a higher CD1c expression detectable on TREM-1- cells (Table S2), suggesting a correlation between TREM-1 and CD141 expression. A soluble form of TREM-1 (sTREM-1) was described in biological fluids during inflammation [16,24]. We evaluated sTREM-1 content in CM from mDCs and H-mDCs. sTREM-1 was released by H-mDCs but not by mDCs, ranging from 79 to 207 pg/10^6 cells/ml in eight different donors (Fig.3D), consistent with the expression pattern of the membrane-bound form.

H-mDCs reoxygenation by exposure to normoxic conditions (Reox) for 24hr resulted in a pronounced downregulation of TREM-1 mRNA levels compared to cells maintained under hypoxia for the same

length of time (Fig.4A). Accordingly, reduction of TREM-1 surface expression was measured by FACS both in terms of mean fluorescence intensity and percentage of positive cells (Fig.4B) and was paralleled by a comparable decrease of sTREM-1 release into the supernatant (Fig.4C), suggesting that hypoxia simulatory effects on TREM-1 were reversible. The role of low pO_2 as a stimulus for TREM-1 in mDCs was further supported by data showing that TREM-1 mRNA (Fig.5A) and surface protein (Fig.5B) were induced in mDCs exposed to acute (24hr) hypoxia, although at levels and in a percentage of cells lower than those detectable in H-mDCs.

DC exposure to hypoxia was previously shown to be associated with HIF-1α protein accumulation and target gene induction [13-15]. Given the presence of HRE in the gene promoter (Table I), we investigated the potential involvement of HIF-1α in TREM-1 hypoxic induction by RNA interference. As depicted in Supplemental Figure 2, high levels of HIF-1α were present in mDCs cultured for 24hr under hypoxia and were associated with TREM-1 expression. HIF-1α silencing decreased TREM-1 protein levels, whereas it did not modify the expression of β–actin used as an internal control. These data suggest that TREM-1 inducibility by hypoxia in mDCs is mediated at least in part by HIF-1α.

TREM-1 cross-linking on H-mDCs promotes DAP12-signaling activation and proinflammatory cytokine and chemokine release

TREM-1 is a transmembrane receptor which lacks signaling motifs in the short cytoplasmic tail and non-covalently associates with the adapter molecole, DAP12, for signal transduction in human monocytes and neutrophils [17,24,25]. To confirm TREM-1 association with DAP12, 4-day HmDCs were subjected to immunoprecipitation with anti TREM-1 mAb, and the precipitates were analyzed by anti-DAP12 immunoblotting. As shown in Figure 6A, TREM-1 paired with DAP12, consistent with a role for this protein in mediating TREM-1 signaling in H-mDCs. To determine whether TREM-1 was functionally competent, H-mDCs were plated on a plastic surface coated with an agonist mAb or control IgG for 20 min under hypoxia, and activation of DAP12-signaling pathway was assessed. TREM-1 cross-linking resulted in increased phosphorylation of DAP12-linked molecules, ERK-1, Akt, and IκBα, indicating that TREM-1 engagement can transduce activating signals in H-mDCs (Fig.6B).

TREM-1 is an amplifier of the inflammatory response [17,24]. Hence, we investigated whether its activation triggered proinflammatory cytokine and chemokine production by H-mDCs.

Supernatants from H-mDCs were collected 24hr after stimulation with agonist anti-TREM-1 or control IgG1 under hypoxic conditions and analyzed for cytokine and chemokine content by ELISA (Fig.6C). TREM-1 cross-linking significantly enhanced TNF-α, IL-6, IL-12p70, CXCL8, CCL4, and CCL5 secretion compared to Ig, whereas production of the anti-inflammatory cytokine IL-10 was not affected. No changes in cytokine secretion were observed upon triggering with an isotype matched anti-MHC-I mAb (data not shown), confirming that H-mDC activation by anti-TREM-1 mAb was not due to aspecific Fc receptor ligation. Overall, these data suggest that TREM-1 is a functional receptor selectively expressed by H-mDCs and not by the normoxic counterpart whose engagement can drive production of cytokines and chemokines involved in innate and adaptive immunity, raising the question of the existence of TREM-1+ mDCs *in vivo*.

TREM-1 is expressed *in vivo* on H-mDCs recruited to the synovial fluid of JIA patients

TREM-1 has been recently implicated in the pathogenesis of non-infectious inflammatory disorders, including rheumatoid arthritis (RA) [26,27]. Hypoxia is a common feature of the inflamed rheumatoid synovium [8,28], and evidences suggest that DCs are enriched in arthritic joints playing a role in the initiation and perpetuation of the inflammatory process [23,29]. To evaluate TREM-1+ mDCs occurrence *in vivo*, we investigated whether TREM-1 was expressed in synovial mDCs from children affected by JIA. We first analyzed plasma and SF samples obtained from JIA patients for sTREM-1 content by ELISA. As shown in Figure 7A, plasma sTREM concentrations in JIA patients (61-394 pg/ml) were significantly higher ($P < 0.01$) than those in control subjects (12-16pg/ml) and were further increased (P value<0,001) in matched SF samples (1120-3800 pg/ml).

These data are in line with previous observations in other forms of inflammatory arthritis, such as RA [26,27], indicating disease activity.

SFMCs were then isolated from a subset of patients and analyzed for TREM-1 surface expression. HIF-1α protein was also evaluated in parallel by western blotting to confirm cell adaptation to the hypoxic synovial environment. SFMCs, but not paired PBMCs, constitutively accumulated high levels of HIF-

1α (Fig 7B), confirming recent observations [19,20]. The presence of TREM-1+ mDCs in SFMCs was then determined by three-color flow cytometric analysis with mAbs to CD1a, CD83, and TREM-1. As shown in Figure 7C, a subset of SFMCs, ranging from 4.6%- to 6.6% in three different patients, expressed CD1a (*left panels*), confirming DC enrichment in JIA SF. A high proportion of DCs in JIA SF expressed CD83 (Fig.7C, *right panels*), comprising 42, 25, and 20% of the total CD1a-gated cells in patient 1, 3, and 6, respectively (upper + lower right quadrants). Noteworthily, 88, 60, and 80% of cells within the CD83+ subset expressed TREM-1, accounting for 37, 15, and 16% of the total CD1a-gated population (upper right quadrants). These findings provide the first evidence of the existence in a human hypoxic tissue *in vivo* of a population of CD83+ mDCs expressing TREM-1, correspondent to mDCs generated *in vitro* under low pO_2, suggesting the potential relevance of this molecule as an *in vivo* marker of hypoxic mDCs.

Discussion

mDC development from monocytic precursors recruited at sites of inflammation and infection occurs in the setting of low pO_2 [5,6]. The impact of the hypoxic microenvironment on DC maturation process is still controversial. This study characterizes the transcriptional profile of mDCs generated from human monocytes under chronic hypoxic conditions similar to those present in diseased tissues, demonstrating that H-mDCs are functionally reprogrammed through the differential expression of a large number of genes involved in innate and adaptive immunity.

Futhermore, we define a new subpopulation of hypoxic mDCs characterized by the CD1a+/CD83+/TREM-1+ phenotype.

Divergent effects of hypoxia on DC maturation were reported in previous studies based onthe expression of maturation markers, costimulatory molecules, chemokine receptors, and T cell priming ability. Mancino et al. showed that acute hypoxia impaired iDC phenotypic and functional maturation in response to LPS [14], whereas Rama et al. and Jantsch et al. demonstrated that it promoted their maturation both by itself[5] and in combination with LPS [15]. Two recent reports addressing the effects of chronic hypoxia suggested inhibition of monocyte-derived DC maturation [30,31]. In contrast, our results indicate that chronic exposure to low pO_2 does not substantially affect mDC phenotypic maturation, because comparable CD1a, CD83, CD86, CCR7 and CXCR4 surface expression was displayed by mDCs and H-mDCs, confirming and extending previous data by our group[13] and by Zhao et al. [32]. A possible explanation for these conflicting results could be the different experimental approaches used with regard to source and purity of DC precursors, differentiation/maturation protocols, degree and duration of the hypoxic stimulus, and difference of species (human *vs* mouse).

Clustering of hypoxia-modulated genes according to GO data mining identified 33 interrelated functional categories, containing a statistically significant portion of genes controlling cell metabolism, differentiation/maturation, and functional properties. Interestingly, a large cluster of genes belonging to immune-related pathways was upregulated in H-mDCs respect to mDCs.

These findings extend to mDCs the characteristic trend of response to hypoxia of other types of mononuclear phagocytes, including primary monocytes, monocyte-derived macrophages and iDCs [12,21,33], emphasizing the critical contribution of reduced oxygenation to the control of immune responses. mDCs are critical for the induction of protective immunity to microbial invasion and the maintenance of self-tolerance [34]. Their functions are tightly regulated by a complex network of inhibitory and activating signals transduced by a defined repertoire of cell surface receptors [4,24,35,36], and dysregulated expression of these molecules may result in an aberrant response characterized by amplification of inflammation and loss of tolerance [2,4,34]. DC maturation under chronic hypoxia resulted in the upregulation of genes encoding various members of these receptor families. Profound changes were observed in genes coding for PRRs critical to host defense, which are endowed with the capability to recognize specific PAMPs on infectious agents and trigger proinflammatory cytokine production [36-38], suggesting enhanced inflammatory functions of mDCs generated in hypoxic than in normoxic areas. Upregulation of mRNA for the scavenger receptors, CD36 and APOB48R, is also of note and consistent with the view that hypoxia may exert a pathogenetic role in atherosclerosis[7], because these molecules function as endocytic transporters for lipoproteins and phospholipids, contributing to lipid-loaded foam cell formation [39,40]. mDC migration is known to be highly sensitive to microenvironmental changes [2-4]. We observed higher expression of mRNA for several adhesion/homing receptors and costimulatory molecules in H-mDCs compared to mDCs. Given the role of the encoded proteins in cell-cell and cell-matrix interactions, transendothelial migration and trafficking, and T cell costimulatory activity [41,42], it is likely that H-mDCs have an increased capacity to migrate to secondary lymphoid organs and activate adaptive immune responses than the normoxic counterparts.

Hypoxia also affected the expression pattern of genes coding for immunoregulatory Ig-like receptor family members involved in the regulation of myeloid cell functions in innate and adaptive immunity. Of relevance, is the increased mRNA for Ig-Fc receptors. These are activating receptors characterized by “immunoreceptor tyrosine-based activating motifs” (ITAM) in their cytoplasmic domain (FcγRIIC, FcαR, FcεRI) or pairing with an ITAM-containing transmembrane adaptor proteins for signaling (FcγRIIIA), which trigger phagocytosis and immune complex clearance, inflammatory cytokine

production, ADCC, and respiratory burst [43]. Hypoxic upregulation of other genes encoding Ig family members, such as BDCA3, LILRA2, and SEMA4D [35,44,45], was also observed. These molecules modulate DC maturation, immunogenicity, cooperation with T cells, and pro-inflammatory cytokine production. Our results suggest that hypoxia can affect mDC functional behaviour in diseased tissues and potentially contribute to the pathogenesis of allergic inflammatory disorders and/or autoimmune diseases, which are associated with overexpression of these receptors [44-46]. The challenge of future studies will be to validate these data *in vivo*.

An intriguing finding of this study is the identification of TREM-1 as a hypoxia-inducible gene in mDCs. Previous reports have shown that TREM-1 is selectively expressed on neutrophils and on a subset of monocytes and tissue macrophages, where it can be markedly upregulated by various stimuli, such as TLR ligands and proinflammatory cytokines, whereas it is completely down-regulated during monocyte differentiation/maturation into DCs [16,17,24,25]. Our results provide the first evidence that TREM-1 can be expressed in mDCs generated under chronic hypoxia.

TREM-1 mRNA was consistently detected in H-mDCs from different donors and paralleled by expression of the membrane-bound receptor and release of its soluble form. Interestingly, TREM-1mRNA and protein expression was also inducible upon mDC exposure to acute hypoxia, although at levels lower than those induced by chronic hypoxia, and was reversible, because cell reoxygenation resulted in TREM-1 downregulation. Consistent with these findings, HIF/HRE system appeared to be involved at least in part in TREM-1 hypoxic induction. These results suggest that TREM-1 expression in mDCs *in vivo* may vary dynamically with the degree of local oxygenation, which is quite heterogeneous and rapidly fluctuating in diseased tissues[9], giving rise to distinct mDC subsets potentially endowed with different functional properties.

TREM-1 expressed on H-mDC paired with DAP12 adapter protein and was biologically active, because its engagement resulted in the activation of DAP12-linked signaling, as shown by increased ERK-1 and Akt kinase phosphorylation. These data are in agreement with previous observations in primary monocytes and neutrophils [17,24,25]. TREM-1 cross-linking on H-mDCs triggered secretion of TNFα, IL-6, CXCL8, CCL4, and CCL5, which are important proinflammatory and chemotactic factors [4,13,21,29],

and of IL-12p70, a Th1-inducing cytokines essential for adaptive immune responses against intracellular pathogens and tumors [4,34]. These data support and extend previous findings implicating TREM-1 as an amplifier of inflammation [17,24] and suggest that the hypoxic environment enhances mDC ability to regulate leukocyte recruitment at inflammatory sites and Th1 priming by stimulating TREM-1 expression. Increased nuclear levels of NF-kBα were observed in response to TREM-1 cross-linking in monocytes and neutrophils [25]. NF-κBα proteins are sequestered in the cytoplasm by inhibitory proteins (IκBα), which become phosphorylated and subsequently degraded after cell activation, allowing NF-κB nuclear translocation and activity[47]. Our data showing IκBα phosphorylation in response to TREM-1 crosslinking support a role for the NF-κB pathway in TREM-1-mediated proinflammatory responses also in H-mDCs.

Increased TREM-1 expression and sTREM-1 release were observed in several infectious and non-infectious inflammatory disorders, suggesting a pathogenetic role for this molecule [16,17,24,26,27,48]. Our data add to the growing list of diseases associated with TREM-1 overexpression, providing the first evidence of elevated sTREM-1 levels in JIA. JIA is the most common chronic pediatric rheumatic disease[18], and sTREM-1 presence in JIA-SF is an index of the underlying inflammatory process whose persistence and amplification is dictated by the functional cooperation among several inflammatory cell populations recruited to the synovium of affected joints [28], including TREM-1+ monocytes/macrophages and neutrophils [26,27,48]. We have recently documented the presence of reduced pO_2 in the JIA synovium and the contribution of the hypoxic environment to the persistence of inflammation by triggering leukocyte recruitment and activation [19,20,28]. The demonstration that SFMCs express HIF-1α confirms the adaptation of synovial inflammatory cells to local hypoxia. Previous studies have demonstrated accumulation of both DC precursors and myeloid DC subsets in the SF and ST from children affected by JIA [23,28].

Consistent with these findings, our immunophenotypical data demonstrated enrichment of cells expressing the DC marker CD1a in JIA synovial effusions, which comprised up to 6.6% of the total SFMC population. Although CD1a+ DCs had a predominantly immature phenotype (CD83-), a high percentage of mature (CD83+) DCs was also present in hypoxic JIA-SF, and the majority of cells in the

CD83+ subset expressed TREM-1. Taken together with *in vitro* findings, the demonstration of TREM-1 expression *in vivo* on mDCs present in hypoxic JIA-SF strongly suggest the potential relevance of this molecule as a marker of mDCs generated under hypoxic conditions.

Furthermore, the observation that also the majority of cells belonging to the CD83- subset in JIASF was TREM-1+, indicates that TREM-1 expression is independent of the DC maturation stage, representing a common *in vivo* marker of hypoxic DCs. Sustained TREM-1 induction on synovial DCs by intra-articular hypoxia may be of pathological relevance, representing a potential mechanism of amplification of synovial inflammation, and future studies will be aimed at assessing the role of TREM-1 putative ligand(s) [49] present in the inflamed arthritic synovium [50] in triggering DC proinflammatory cytokine and chemokine production.

In conclusion, this study provides novel insights into the mechanisms linking low pO_2 to the regulation of immune and inflammatory responses, leading to new perspectives of the role of hypoxia in programming mDC functions within pathological tissues.

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Authorship contributions

M.C.B. designed and supervised research, contributed to data analysis, and wrote the manuscript; D.P. and F.B. contributed to the design and carry out of most of the research and analyzed flow cytometric and microarray data; F.R. contributed to DC culture and gene expression analysis; C.V. performed western blot analysis; M.G. provided synovial and blood samples from JIA patients and discussion; A.E. provided critical discussion; F.N. contributed to data analysis; P.C. analyzed cytokine expression profile; M.G. and L.V. conceived and coordinated research, contributed to data analysis, assisted with manuscript preparation and revision.

Conflicts of Interest Disclosure: the authors declare no competing financial interests

References

[1] Banchereau J, Briere F, Caux C et al. Immunobiology of dendritic cells. *Annu Rev Immunol.*2000;18 (4) :767-811.

[2] Cavanagh LL, Von Andrian UH. Travellers in many guises: the origins and destinations of dendritic cells. *Immunol Cell Biol.* 2002;80 (5) :448-462.

[3] Mellman I, Steinman RM. Dendritic cells: specialized and regulated antigen processing machines. *Cell.* 2001;106 (3) :255-258.

[4] Allavena P, Sica A, Vecchi A et al. The chemokine receptor switch paradigm and dendritic cell migration: its significance in tumor tissues. *Immunol Rev.* 2000;177 (1) :141-149.

[5] Rama I, Brune B, Torras J et al. Hypoxia stimulus: An adaptive immune response during dendritic cell maturation. *Kidney Int.* 2008;73 (7) :816-825.

[6] Bosco MC, Puppo M, Blengio F et al. Monocytes and dendritic cells in a hypoxic environment: Spotlights on chemotaxis and migration. *Immunobiology.* 2008;213 (9-10):733-749.

[7] Semenza GL. Hypoxia-inducible factor 1: oxygen homeostasis and disease pathophysiology. *Trends Mol Med.* 2001;7 (8) :345-350.

[8] Muz B, Khan MN, Kiriakidis S, Paleolog EM. Hypoxia. The role of hypoxia and HIFdependent signalling events in rheumatoid arthritis. *Arthritis Res Ther.* 2009;11 (1) :201-209.

[9] Finger EC, Giaccia AJ. Hypoxia, inflammation, and the tumor microenvironment in metastatic disease. *Cancer Metastasis Rev.* 2010;29 (2) :285-293.

[10] Cummins EP, Taylor CT. Hypoxia-responsive transcription factors. *Pflugers Arch.* 2005; 450 (6) :363-371.

[11] Rius J, Guma M, Schachtrup C et al. NF-kappaB links innate immunity to the hypoxic response through transcriptional regulation of HIF-1alpha. *Nature.* 2008;453 (7196) :807-811.

[12] Ricciardi A, Elia AR, Cappello P et al. Transcriptome of hypoxic immature dendritic cells: modulation of chemokine/receptor expression. *Mol Cancer Res.* 2008;6 (2) :175-185.

[13] Elia AR, Cappello P, Puppo M et al. Human dendritic cells differentiated in hypoxia downmodulate antigen uptake and change their chemokine expression profile. *J Leukoc Biol.*

2008;84 (6) :1472-1482.

[14] Mancino A, Schioppa T, Larghi P et al. Divergent effects of hypoxia on dendritic cell functions. *Blood.* 2008;112 (9) :3723-3734.

[15] Jantsch J, Chakravortty D, Turza N et al. Hypoxia and Hypoxia-Inducible Factor-1{alpha} Modulate Lipopolysaccharide-Induced Dendritic Cell Activation and Function. *J Immunol.* 2008;180 (7) :4697-4705.

[16] Klesney-Tait J, Turnbull IR, Colonna M. The TREM receptor family and signal integration. *Nat Immunol.* 2006;7 (12) :1266-1273.

[17] Bouchon A, Dietrich J, Colonna M. Cutting edge: inflammatory responses can be triggered by TREM-1, a novel receptor expressed on neutrophils and monocytes. *J Immunol.* 2000;164 (10) :4991-4995.

[18] Ravelli A, Martini A. Juvenile idiopathic arthritis. *Lancet* 2007;369 (9563) :767-778. [19] Bosco MC, Delfino S, Ferlito F et al. Hypoxic synovial environment and expression of macrophage inflammatory protein MIP-3⟨/CCL20 in Juvenile Idiopathic Arthritis. *Arthritis Rheum.* 2008;58 (6) :1833-1838.

[20] Bosco MC, Delfino S, Ferlito F et al. The hypoxic synovial environment regulates expression of vascular endothelial growth factor and osteopontin in juvenile idiopathic arthritis. *J Rheumatol.* 2009;36 (6) :1318-1329.

[21] Bosco MC, Puppo M, Santangelo C et al. Hypoxia modifies the transcriptome of primary human monocytes: modulation of novel immune-related genes and identification of CCchemokine ligand 20 as a new hypoxia-inducible gene. *J Immunol.* 2006;177 (3) :1941-1955.

[22] Ivanov S, Liao SY, Ivanova A et al. Expression of hypoxia-inducible cell-surface transmembrane carbonic anhydrases in human cancer. *Am J Pathol.* 2001;158 (3) :905-919.

[23] Smolewska E, Stanczyk J, Brozik H et al. Distribution and clinical significance of blood dendritic cells in children with juvenile idiopathic arthritis. *Ann Rheum Dis.* 2008;67 (6):762-768.

[24] Sharif O, Knapp S. From expression to signaling: roles of TREM-1 and TREM-2 in innate immunity and bacterial infection. *Immunobiology.* 2008;213 (9-10) :701-713.

[25] Tessarz AS, Cerwenka A. The TREM-1/DAP12 pathway. *Immunol Lett.* 2008;116 (2) :111-116.

[26] Collins CE, La DT, Yang HT et al. Elevated synovial expression of triggering receptor expressed on myeloid cells 1 in patients with septic arthritis or rheumatoid arthritis. *Ann Rheum Dis.* 2009;68 (11) :1768-1774.

[27] Kuai J, Gregory B, Hill A et al. TREM-1 expression is increased in the synovium of rheumatoid arthritis patients and induces the expression of pro-inflammatory cytokines. *Rheumatology (Oxford).* 2009;48 (11) :1352-1358.

[28] Bosco M.C., Varesio L. Monocytic Cell Gene Regulation by the Hypoxic Synovial Environment in Juvenile Idiopathic Arthritis: Implications for Disease Pathogenesis. *J Clinical Rheumatol Musculoskel Med.* 2010;1 (1) :47-55.

[29] Lutzky V, Hannawi S, Thomas R. Cells of the synovium in rheumatoid arthritis. Dendritic cells. *Arthritis Res Ther.* 2007;9 (4) :219-231.

[30] Yang M, Ma C, Liu S et al. Hypoxia skews dendritic cells to a T helper type 2-stimulating phenotype and promotes tumour cell migration by dendritic cell-derived osteopontin. *Immunology.* 2009;128 (1) :e237-e249.

[31] Wang Q, Liu C, Zhu F et al. Reoxygenation of hypoxia-differentiated dendritic cells induces Th1 and Th17 cell differentiation. *Mol Immunol.* 2010;47 (4) :922-931.

[32] Zhao W, Darmanin S, Fu Q et al. Hypoxia suppresses the production of matrix metalloproteinases and the migration of human monocyte-derived dendritic cells. *Eur J Immunol.* 2005;35 (12) :3468-3477.

[33] Fang HY, Hughes R, Murdoch C et al. Hypoxia-inducible factors 1 and 2 are important transcriptional effectors in primary macrophages experiencing hypoxia. *Blood.* 2009;114 (4):844-859.

[34] Schakel K. Dendritic cells--why can they help and hurt us. *Exp Dermatol.* 2009;18 (3) :264-273.

[35] Colonna M, Nakajima H, Cella M. A family of inhibitory and activating Ig-like receptorsthat modulate function of lymphoid and myeloid cells. *Semin Immunol.* 2000;12 (2) :121-127.

[36] Kanazawa N. Dendritic cell immunoreceptors: C-type lectin receptors for patternrecognition and signaling on antigen-presenting cells. *J Dermatol Sci.* 2007;45 (2) :77-86.

[37] Kawai T, Akira S. The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nat Immunol.* 2010;11 (5) :373-384.

[38] Bowdish DM, Gordon S. Conserved domains of the class A scavenger receptors: evolution and function. *Immunol Rev.* 2009;227 (1) :19-31.

[39] Chabowski A, Gorski J, Calles-Escandon J, Tandon NN, Bonen A. Hypoxia-induced fatty acid transporter translocation increases fatty acid transport and contributes to lipid accumulation in the heart. *FEBS Lett.* 2006;580 (15) :3617-3623.

[40] Brown ML, Ramprasad MP, Umeda PK et al. A macrophage receptor for apolipoprotein B48: cloning, expression, and atherosclerosis. *Proc Natl Acad Sci U S A.* 2000;97 (13):7488-7493.

[41] Maddaluno L, Verbrugge SE, Martinoli C et al. The adhesion molecule L1 regulates transendothelial migration and trafficking of dendritic cells. *J Exp Med.* 2009;206 (3) :623-635.

[42] Hegde VL, Singh NP, Nagarkatti PS, Nagarkatti M. CD44 mobilization in allogeneic dendritic cell-T cell immunological synapse plays a key role in T cell activation. *J LeukocBiol.* 2008;84 (1) :134-142.

[43] Gerber JS, Mosser DM. Stimulatory and inhibitory signals originating from the macrophage Fcgamma receptors. *Microbes Infect.* 2001;3 (2) :131-139.

[44] Mamegano K, Kuroki K, Miyashita R et al. Association of LILRA2 (ILT1, LIR7) splice site polymorphism with systemic lupus erythematosus and microscopic polyangiitis. *GenesImmun.* 2008;9 (3) :214-223.

[45] Takamatsu H, Okuno T, Kumanogoh A. Regulation of immune cell responses by semaphorins and their receptors. *Cell Mol Immunol.* 2010;7 (2) :83-88.

[46] Yerkovich ST, Roponen M, Smith ME et al. Allergen-enhanced thrombomodulin (blood dendritic cell antigen 3, CD141) expression on dendritic cells is associated with a TH2-skewed immune response. *J Allergy Clin Immunol.* 2009;123 (1) :209-216.

[47] Ghosh S, Karin M. Missing pieces in the NF-kappaB puzzle. *Cell.* 2002;109 (2) :S81-S96.

[48] Cavaillon JM. Monocyte TREM-1 membrane expression in non-infectious inflammation. *Crit Care.* 2009;13 (3) :152-153.

[49] El Mezayen R, El Gazzar M, Seeds MC et al. Endogenous signals released from necrotic cells augment inflammatory responses to bacterial endotoxin. *Immunol Lett.* 2007;111 (1):36-44.

[50] Foell D, Wittkowski H, Roth J. Mechanisms of disease: a 'DAMP' view of inflammatory arthritis. *Nat Clin Pract Rheumatol.* 2007;3 (7) :382-390.

Figure legends

FIGURE 1. Phenotype of monocyte-derived mDCs generated under hypoxic conditions.

Human monocytes were cultured for 48 hr with IL-4 and GM-CSF followed by incubation with the proinflammatory mediators, TNFα, IL-1β, IL-6, and PGE_2 for additional 48 hr under 20% O_2 (mDCs) or 1% O_2 (H-mDCs) conditions, and expression of the indicated cell surface molecules was analyzed by flow cytometry after 4 day culture, as described in the *Methods.* The expression profile of unstimulated monocytes is shown for comparison. Cells were gated according to their light scatter properties to exclude cell debris. Solid histograms represent the fluorescent profile of cells stained with specific FITC-conjugated Abs, whereas open histograms represent the fluorescent profile of cells stained with isotype-matched controls. Data are plotted as fluorescence intensity on a log scale *vs* the number of positive cells. In each histogram, the percentage of positive cells is indicated. Results are from one representative experiment out of ten performed with cells from different donors.

FIGURE 2. Functional classification of hypoxia-responsive genes by GO data mining. The gene expression profile of H-mDCs *vs* mDCs was analyzed using high-density oligonucleotide arrays, as described in the *Methods*. Unique genes showing at least 2-fold change in expression levels between cells generated under hypoxic and normoxic conditions were selected and clustered in different KEGG pathways according to GO data mining for biological process at level 1 using the DAVID software. Based on this classification scheme, genes can be placed in more than one category if more than one function of the encoded protein was established. Bars on the right of the y axis represent upregulated genes; bars on the left of the y axis represent downregulated genes. Immunerelated pathways are highlighted in bold.

FIGURE 3. TREM-1 expression in H-mDCs. mDCs and H-mDCs were generated from different donors, CM was replaced on day 3 of generation with fresh medium supplemented with cytokines, and TREM-1 was analyzed at day 4 of culture. **(A)** TREM-1 mRNA expression. Total RNA was reverse-transcribed and tested for TREM-1 expression by qRT-PCR. CAXII mRNA levels were assayed in parallel as positive control. Expression changes were evaluated as detailed in the *Methods*. Data are expressed as mean normalized gene expression values, calculated on the basis of triplicate

measurements for each experiment/donor, relative to the values obtained for the reference genes. **(B)** TREM-1 protein expression. Total cell lysates were prepared from mDCs generated from three of the donors shown in panel A under normoxic (-) or hypoxic (+) conditions. Proteins (100 (μg) were resolved on a 10% SDS-PAGE, and the blots were hybridized with Abs directed to TREM-1 and β-actin as a loading control. A representative immunoblot is shown. Strong bands are seen at ≈30 kDa, the expected TREM-1 molecular weight. **(C)** TREM-1 surface expression. mDCs and HmDCs were double-stained with anti-CD83-FITC and anti-TREM-1-PE Abs and analyzed by flow cytometry on a FACScan, as specified in the *Methods*. Cells were electronically gated according to their light scatter properties to exclude cell debris. *Left panel*: results from one of eight independent experiments are shown as dot plots. The percentage of single and double-positive cells is indicated: TREM-1/CD83 double-positive cells are contained in the upper right quadrant, whereas CD83 and TREM-1 single-positive cells are contained in the lower right and upper left quadrants, respectively. Cells stained with control Abs were contained the lower left quadrants. *Right panel:* Data are expressed as percentage of TREM-1+ cells within CD83+ mDCs and H-mDCs generated from eight individual donors (dots). Horizontal lines represent median values for each group. *p* value by the Student's t test is indicated. **(D)** sTREM-1 secretion**.** Cell free supernatants were harvested and assayed for sTREM content by ELISA. Data were obtained from the same preparations analyzed in panel C and are expressed as pg/1x10^6 cells/ml (dots). Horizontal lines represent median values for each group. *p* value by the Student's t test is indicated.

FIGURE 4. Effects of reoxygenation on TREM-1 expression. Four day H-mDCs were generated from different donors, CM was replaced with fresh medium supplemented with cytokines, and TREM-1 was analyzed after additional 24 hr culture under 20% O_2 (Reox). **(A)** TREM-1 (*black bars)* and CAXII (*gray bars)* mRNA expression was assessed by qRT-PCR. Data from one representative experiment of three performed are expressed as in the legend to Figure 3A. **(B)** TREM-1 surface expression was evaluated by flow cytometry. Solid histograms represent the fluorescent profile of TREM-1-expressing cells, whereas open histograms represent the fluorescent profile of cells stained with the isotype-matched control Ab. The percentage of TREM-1+ cells and the mean fluorescent intensity (between

brackets) are indicated. Results from one of three independently tested donors are shown. **(C)** sTREM-1 release was measured by ELISA**.** Data from one representative experiment of three performed are expressed as pg/1x10^6 cells/ml.

FIGURE 5 Effects of acute hypoxia on TREM-1 expression. Four day mDCs were exposed to hypoxia for 24 hr and **(A)** TREM-1 (*black bars)* and CAXII (*gray bars)* mRNA expression was assessed by qRT-PCR. Data from one representative experiment of three performed are expressed as in the legend to Figure 3A. **(B)** TREM-1 surface expression was evaluated by flow cytometry. Solid histograms represent the fluorescent profile of TREM-1-expressing cells, whereas open histograms represent the fluorescent profile of cells stained with the isotype-matched control Ab. The percentage of TREM-1+ cells and the mean fluorescent intensity (between brackets) are indicated. Results from one of three independently tested donors are shown.

FIGURE 6. Activation of H-mDCs by TREM-1 cross-linking. **(A)** mDCs were generated under hypoxic conditions from two different donors, and total cell lysates were subjected to immunoprecipitation with anti-TREM-1 mAb or control IgG1. The precipitates were resolved on a 12% SDS-PAGE and immunoblotted with anti-DAP12 mAb, as described in the *Methods*. DAP12 and Ig light chain (IgL) are indicated by arrows. Molecular weight markers are given at the side. **(B,C)** H-mDCs were seeded onto plates pre-coated with agonist anti-TREM-1 mAb or control IgG1 at 10 μg/ml, after extensive washing to remove cytokines, and cultured for 20 min **(B)** or 24 hr **(C)** under hypoxic conditions. **(B)** Protein phosphorylation. Cell lysates (40 μg) were resolved on 10% SDS-PAGE, and immunoblotted with Abs anti-phospho (p)-ERK, Akt, and I*k*Bα. Abs against the non-phosphorylated forms (tot) were used as loading controls. Representative experiments performed with cells from two different donors are shown. T_0 indicates H-mDCs from one of the donors not subjected to Ab cross-linking, used as a negative control of phosphorylation. The figure was arranged by combining the lanes containing not-crosslinked samples (T_0) with those containing samples subjected to cross-linking from differenta parts of the same gel. **(C)** Cytokine production. Conditioned medium was assayed for TNF-α, IL-6, IL-12p70, IL-10, CXCL8, CCL4, and CCL5 content by specific ELISA. Results are expressed as pg or ng/8x10^5 cells/ml and are represented as the mean ± SE of three different experiments. *

P<0.05; **Pp<0.001; ***P<0.0001: values significantly different from those of H-mDCs cross-linked with IgG1.

FIGURE 7. TREM-1 expression i*n vivo* in hypoxic mDCs from JIA-SF. SF was collected from children affected by JIA, and SFMCs were purified as detailed in the *Methods*. **(A)** sTREM-1 concentrations were quantified by ELISA in paired plasma and SF from eight JIA patients and plasma from five age-matched control subjects. Individual samples were run in triplicate. Boxes show the values falling between the 25^{th} and 75^{th} percentiles, whiskers the highest and lowest values for each soubgroup. Bold horizontal lines represent median values. *p* value by the Wilcoxon rank test is indicated: *P<0.01 relative to plasma controls; **P<0.001 relative to plasma from JIA patients. **(B)** HIF-1α expression was assessed by Western Blot analysis on whole protein extracts from three pairs of fresh PBMCs and SFMCs purified from a subset of the JIA patients shown in panel A. Protein (100 ⌈g) were resolved on a 8% SDS-PAGE, and the blots were hybridized with anti-HIF-1α mAb. β-actin was evaluated as a loading control. **(C)** Flow cytometric analysis of TREM-1 expression in SF-mDCs. SFMCs from the three JIA patients shown in panel B were stained with anti-CD1a-APC, CD83-FITC, and-TREM-1-PE Abs and analyzed on a FACScan. Cells were electronically gated according to their light scatter properties to exclude cell debris. The gated population was analyzed for CD1a positivity (*left panels, region R1)*, and CD1a+ cells were then examined for CD83 and TREM-1 expression (*right panels*). Non-specific staining was corrected using isotype-matched Abs. Numbers represent the percentage of single and double-positive cells within the CD1a-gated population and are indicated for each patient: TREM-1/CD83 double positive cells are contained in the upper right quadrant, whereas CD83 and TREM-1 single-positivecells are contained in the lower right and upper left quadrants, respectively. Cells stained with control Abs are in the lower left quadrant. The percentage of TREM-1 positive cells relative to the total number of CD83+ cells (representing TREM-1-expressing mDCs) was 88, 60, and 80% in patient 1, 3, and 6, respectively, whereas the percentage of TREM-1 positive cells relative to the total number of CD83- cells (representing TREM-1-expressing iDCs) was 74, 80, and 80% in patient 1, 3, and 6, respectively.

Table II: Relative expression of genes encoding surface immunoregulatory/pattern-recognition receptors and costimulatory/adhesion molecules in H-mDCs *vs* N-mDCs^

Gene Bank Accession No.	Gene symbol	Full Name	Main function(s) of gene product	Fold Increase§
NM_018690	APOB48R*	apolipoprotein B48 receptor	Expressed on reticuloendothelial cells; binds the apolipoprotein B48 and regulates lipid transport into the cell	2.9
AJ277151	CD134	tumor necrosis factor receptor superfamily, member 4	Regulates T-cell expansion and survival; activate NF-kB through its interaction with adaptor proteins TRAF2 and TRAF5	2.1
AW119113	CD141 (BDCA3)	thrombomodulin, blood dendritic cell Ag	Expressed on DCs, associated with TH2-skewed immune responses, pathogenetic role in atopy and asthma	11.4
D37781	CD148*	protein tyrosine phosphatase, receptor type, J	Receptor PTP expressed in macrophages in response to pro-inflammatory stimuli.; regulates cell spreading, cytoskeletal re-arrangements and chemotaxis; positive regulator of Src family kinases	2.3
NM_001109	CD156a (ADAM8)*	ADAM metallopeptidase domain 8	Membrane-anchored proteins implicated in cell-cell and cell-matrix interactions,	4.5
AI653981	CD171 (L1CAM)*	L1 cell adhesion molecule	Costimulatory adhesion molecule belonging to the immunoglobulin supergene family; plays an important role in nervous system development, including neuronal migration and differentiation; regulates DC transendothelial migration and trafficking; implicated in motility and metastasis stimulation of several cancer cel types.	6.7
NM_005582	CD180 (RP105)	CD180 antigen	Expressed on myeloid and B cells; belongs to the TLR4 subfamily of TLRs, negative regulator of TLR4 signaling and of innate immune responses to pathogens and danger:; activates NF-kappa-B	13.5
NM_001877	CD21 (CR2)	complement component (3d/Epstein Barr virus) receptor 2	Receptor for Epstein-Barr virus (EBV) on T and B cells; overexpressed on B cells and follicular DCs; required for the generation of immune responses to T-dependent antigens.	4.4
AW241983	CD307	Fc receptor-like 5	Differentiation antigen expressed in B-lineage cells, involved in B-cell development and differentiation. May have an immunoregulatory role	5
M37780	CD31 (PECAM-1)*	platelet/endothelial cell adhesion molecule	Cell adhesion molecule expressed on platelets, endothelial and other cells	3
NM_013447	CD312 (MR2)	EGF-like domaincontaining, mucin-like, hormone receptor-like 2	Member of the adhesion-GPCR family differentially expressed during differentiation, maturation, and activation of myeloid cells; plays a role in their migration and adhesion	3.8

U90940	CD32	Fc fragment of IgG, low affinity IIc, receptor for	Expressed on myeloid cells; involved in effector and regulatory functions such as phagocytosis of immune complexes and modulation of antibody production by B-cells	11
NM_001772	CD33	CD33 antigen (gp67)	Myeloid-restricted transmembrane protein of the sialic acid-binding Ig-like lectin (Siglec) family marker of myeloid cell differentiation; negative regulator of myeloid cell activation and DC generation	4.7
NM_000573	CD35 (CR1)	complement component (3b/4b) receptor 1	Member of the receptors of complement activation (RCA) family, expressed on follicular DCs, eritrocytes, and leukocytes; mediates cellular binding of complement-bound opsonins and immune complexes;. enhances adaptive immune responses	2.6
AW299226	CD36*	CD36 antigen (collagen type I receptor, thrombospondin receptor)	Mulri-functional class B scavenger receptor; involved in the uptake of oxidized LDL, collagen, thrombospondin, anionic phospholipids, and apoptotic cells. Adhesion molecule	5
U87967	CD39 (ENTPDase1)	ectonucleoside triphosphate diphosphohydrolase	Surface enzyme that hydrolyzes nucleoside tri- and diphosphates , reduces inflammatory cell adhesion and trafficking and consequent tissue injury.; contributes to vascular homeostasis regulation	2.7
U94903	CD44	CD44 antigen	Receptor for hyaluronic acid, osteopontin, collagens, and matrix metalloproteinases (MMPs); involved in cell-cell interactions, cell adhesion and migration;; participates in lymphocyte activation, recirculation and homing, hematopoiesis, and tumor metastasis.	2.7
NM_001778	CD48*	CD48 antigen (B-cell membrane protein)	Member of the immunoglobulin supergene family, activation-associated glycoprotein expressed on leukocytes, Ligand for CD2, facilitates interaction between activated lymphocytes. Involved in the regulation of NK and T-cell activation	2.7
NM_000560	CD53	CD53 antigen	Member of the tetraspanin family, complexes with integrins, plays a role in the regulation of leukocyte activation, growth and motility, contributes to the transduction of CD2-generated signals in T and NK cells	3.6
BE676623	CD71	Transferrin receptor (p90)	Regulator of cellular uptake of iron and iron metabolism; IgA receptor expressed on DCs	4.5
AI681260	CD85 (LILRB1)*	Leukocyte immunoglobulin-like receptor B1	Inhibitory leukocyte Ig-like receptor, expressed on myeloid lineage cells and up-regulated during DC differentiation; binds major histocompatibility complex class I; contains ITIM domains; inhibitor of immune and inflammatory responses	2.6
X74039	CD87	plasminogen activator, urokinase receptor	Receptor for urokinase plasminogen activator (U-PA); regulates cell-surface plasminogen activation; mediates integrin-independent cell adhesion by binding to vitronectin, modulate the proliferative, adhesive, and migratory cellular phenotype	3.8

U43677	CD89*	Fc fragment of IgA, receptor for	Expressed on cells of myeloid lineage; ITAM-bearing receptor of the Fc region of immunoglobulins alpha; can mediate both pro- and anti-inflammatory functions of IgA; activator of Src family kinases	55
AK025016	CD9*	CD9 antigen (p24)	Member of the tetraspanin family,; facilitates the organization of multimolecular membrane complexes, including integrins; plays a role in leukocyte activation, differentiation, and migration; Involved in platelet activation and aggregation.	8
NM_012072	CD93 (C1qR1)	complement component 1, q subcomponent, receptor 1	Defence collagen receptor for C1q, mannose-binding lectin (MBL) and surfactant protein A; expressed on monocytes, neutrophils, endothelial cells, platelets, glial and stem cells; modulate phagocytosis and clearance of apoptotic cells; mediate s leucocyte-endothelial interactions	8.6
NM_002414	CD99*	CD99 antigen	Mediate leucocyte adhesion amd transendothelial migration	3.4
BC005254	CLEC2B	C-type lectin domain family 2, member B	Member of the natural killer cell C-type lectin-like family of immunoregulatory receptors; platelet activation receptor for snake venom, rhodocytin,, HIV, podoplanin; triggers platelet activation and aggregation; activator of Src and Syk families of tyrosine kinases;involved in tumor growth/metastatization.	4.8
AF285089	CLEC2D*	C-type lectin domain family 2, member D	Member of the natural killer cell C-type lectin receptor family.; pattern recogntion receptor expressed on DC subrypes, monocytes, macrophages, granulocytes, and B cells; regulates the cross-talk between NK cells and APCs; mediates phagocytosis and proinflammatory cytokine production by myeloid cells; activator of Src and Syk families of tyrosine kinases;	4.1
AF313468	CLEC7A*	C-type lectin domain family 7, member A (dectin-1)	Member of the C-type lectin-like family of immunoregulatory receptors; pattern recognition receptor for fungal beta-glucan predominantly expressed on DCs, monocytes, macrophages, neutrophils and microglia, ; activates phagocytosis, ROS, and cytokine production; DC-maturaion and T cell costimulatory capacity	2.9
NM_020377	CYSLT2R	cysteinyl leukotriene receptor 2	Member of the superfamily of G protein-coupled receptors; plays a major role in endocrine and cardiovascular systems; in immune cells expressed in eosinophils and macrophages, stimulates IL-8 secretion; up-regulates early inducible genes; mediates cell trafficking and innate immune responses; involved in the pathogenesis of chronic inflammatory diseases and allergic reactions	2.9
M74921	EDNRB*	endothelin receptor type B	G protein-coupled receptor; binds the vasoactive peptide, endothelin	2.2

AF213460	EPHA3	Ephrin receptor A3	Receptor for members of the ephrin-A family of receptor tyrosine kinases; mediates processes involved in cell-cell contact, cellular adhesion and angiogenesis, developmental remodeling and neuronal mapping	4
M34986	EPOR*	erythropoietin receptor	Member of the cytokine receptor family mainly expressed on the erythroid colony-forming units but also expressed in other hemopoietic cells; binds erythropoietin; immunostimulatory activities	2.1
NM_001432	EREG*	epiregulin	Member of the epidermal growth factor family, binds EGFR and members of ErbB tyrosine-kinase receptor family; mediates cell proliferation; promotes wound healing; the membrane-bound form is involved in proinflammatory cytokine production by macrophages	20.8
NM_004106	FCER1G*	Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide	ITAM-containing subunit of the tetrameric high affinity IgE receptor and of other Fc receptors; expressed on mast cells and APCs; key molecule involved in allergic reactions mediated by IgE; involved in the regulation of several aspects of the immune response	3.6
NM_000570	FCGR3 (CD16a) *	Fc fragment of IgG, low affinity IIIa, receptor	Intermediate affinity type 1 transmembrane glycoprotein of the Ig superfamily; expressed by a T lymphocyte subset, mast cells, NK cells, and phagocytes; activating receptor involved in phagocytosis, inflammatory mediator release, ADCC, and immune complex clearance	9.6
U81501	FPRL1*	formyl peptide receptor-like 1 ; formyl peptide receptor-like 1	High affinity receptor for bacteria-derived and host-derived N-formyl-methionyl peptides; key player in innate immunity and host defense mechanisms against pathogen infection , mediates phagocyte chemotactic migration and IL-1 production	2.6
AI246590	IRAK-2*	interleukin-1 receptor-associated kinase 2	Members of the IRAK family of kinases; key components in the signal transduction pathways utilized by IL-1R and TLRs; critically involved in the regulation of intracellular signaling networks controlling inflammation, such as NF-kappaB activation	9.7
N95414	ITGA2 (CD49B)*	Integrin, alpha 2	Receptor for laminin, collagen, fibronectin, and E-cadherin; involved in cell adhesion and cell-surface mediated signalling, generation and organization of newly synthesized extracellular matrix	6.9
NM_002205	ITGA5 (CD49)*	integrin, alpha 5	Receptor for fibronectin and fibrinogen; involved cell adhesion to fibronectin and cell-surface mediated signalling	3.6
NM_002207	ITGA9	integrin, alpha 9	Receptor for VCAM1, tenascin and osteopontin; participates in cell adhesion and motility	2
NM_000889	ITGB7	integrin, beta 7	Interacts with the cell surface adhesion molecules MADCAM1, expressed on vascular endothelium , with	8.3

			VCAM1 and fibronectin; plays a riole in adhesive interactions	
AW131039	ITGB8*	Integrin, beta 8	Receptor for fibronectin; mediate cell-cell and cell-extracellular matrix interaction	3
NM_000655	LECAM1	selectin L (lymphocyte adhesion molecule 1)	Cell surface adhesion protein, belonging to a family of adhesion/homing receptors. required for binding and rolling of leucocytes on endothelial cells, facilitating their migration into secondary lymphoid organs and inflammation sites	3
AF025529	LILRA1 (LIR6)*	leukocyte immunoglobulin-like receptor A1	Member of the leukocyte Ig-like immunoreceptor family expressed predominantly on monocytes/macrophages and B cells and at lower levels on dendritic cells and natural killer (NK) cells; it associates with Fcgamma R and acts as receptor for class I MHC antigens	3.5
NM_006866	LILRA2 (ILT1)*	leukocyte immunoglobulin-like receptor A2	Member of the leukocyte Ig-like immunoreceptor family, selectively expressed on myeloid lineage cells, including DCs and in inflammatory tissues, such as RA synovium and lesions of lepromatous leprosy; functions as an activating receptor pairing with FcRg for signaling, promoting an inflammatory response	6.4
NM_002346	LY6	lymphocyte antigen 6 complex, locus E	Glycosylphosphatidylinositol-anchored molecule that belongs to the Ly-6 family., also known as stem cell antigen 2 (SCA2); is a type I interferon-inducible gene; highly expressed in a variety of human cancer cells	2.5
NM_006770	MARCO	macrophage receptor with collagenous structure	Member of the class A scavenger receptor family, phagocytic pattern recognition receptor preferably expressed by phagocytes and upregulated in response to TLR agonists and Gram+/- bacteria, implicated in macrophage phagocytic activity and pro-inflammatory cytokine production; critical role in host defense to pathogens; implicated in cell-cell recognition and modified LDL scavenging.	3.7
BE870509	MET (HGFR)*	met proto-oncogene (hepatocyte growth factor receptor)	Proto-oncogene encoding the trosine-kinase receptor for hepatocyte growth factor and scatter factor; expressed in a variety of organs and cell types; plays a role in cell growth and motility in many physiologic and pathologic processes	3.4
AF280547	NRP1*	neuropilin 1	Membrane-bound coreceptor to tyrosine kinase receptors for vascular endothelial growth factor, class 3 semaphorins, and placenta growth factor; involved in the control of cell growth and migration; plays a role in angiogenesis and axon guidance; mediates contacts between DCs and T cells and is essential for the initiation of the primary immune response	5.8
M80436	PAFR	platelet-activating factor receptor	G protein-coupled receptor for platelet-activating factor (PAF); transduces pleiotropic functions including cell motility, smooth muscle contraction, cytokine synthesis and	2.8

			release; implicated in diverse pathologic processes, such as allergy, asthma, septic shock, arterial thrombosis, and inflammatory processes	
S78505	PRLR*	prolactin receptor	Major mediator of prolactin effects on cell proliferation, survival, cytoskeletal modifications and differentiation;expressed in a wide variety of cells and tissues, including PBMC.	2.1
NM_000956	PTGER2	prostaglandin E receptor 2	Receptor for prostaglandin E2, expressed on macrophages and mDCs; activates the expression of multiple genes implicated in the regulation of immunity and inflammation; involved in CCR7-mediated DC migration	3.7
BE908995	MYADM (SB135)*	myeloid-associated differentiation marker	Selectively expressed in myeloid cells, including monocytes, DCs, promyeloid or monocytic leukemia cell lines; marker of myeloid cell differentiation	2.1
AK026133	SEMA4B	Semaphorin 4B	Integral membrane protein with a Ig extracellular domain, widely expressed throughout the nervous system; provides axonal guidance cues during neuronal development and regulates formation or function of synaptic specializations	4.1
NM_006378	SEMA4D (CD100)*	Semaphorin 4D	Integral membrane protein with a Ig extracellular domain; controls proliferation, survival, and migration of cells of the nervous system and positively regulate angiogenesis through Plexin-B1 receptor; plays a role in both humoral and cellular immunity via interactions with CD72; promotes DC maturation and function, B-cell aggregation and Ab production; Ag-specific T-cell activation, Mn migration, tumor-infiltrating Mf tumorigenic activity, T/B and T/DC interactions.	3.1
AJ130712	SIGLEC7	sialic acid binding Ig-like lectin 7	Member of the CD33-related Siglec recceptor family; highly expressed on NK cells and weakly on monocytes and a minor subpopulation of CD8 T lymphocytes; acts as an nhibitory receptor (ITIM motif).	8
NM_033438	SLAMF9*	SLAM family member 9	Member of the signaling lymphocytic activation subfamily of the immunoglobulin superfamily; involved in innate and acquired immuno responses through the regulation of multiple hematopoietic cell functions, including lymphocyte and monocyte/macrophage activation, NK killing,a DC maturaion, andplatelet aggregation	12.2
201666_at	TIMP1*	TIMP metallopeptidase inhibitor 1	inhibitor of most of the known MMPs, can promote proliferation of several cell types and have an anti-apoptotic function	15.1
AL050262	TLR1*	toll-like receptor 1	Member of the Toll-like receptor (TLR) family; expressed on cells of the innate immune system, plays a fundamental role in the recognition of pathogen-associated molecular patterns	3.6

			(PAMPs) expressed on infectious agents and mediates the production of cytokines necessary for the activation of innate immunity	
NM_003264	TLR2*	toll-like receptor 2	Member of the Toll-like receptor (TLR) family; expressed on cells of the innate immune system, plays an essential role in the innate immune recognition of pathogen-associated molecular patterns (PAMPs) expressed on infectious agents leading to the generation of an immune response; implicated in the pathogenesis of several auto-immune and inflamatory diseases	7.7
NM_018643	TREM-1*	triggering receptor expressed on myeloid cells 1	Ig superfamily immunoregulatory receptor expressed on monocytes/macrophages and neutrophils; stimulates their proinflammatory functions and acts as an inflamatory amplifier priming cells to respond to other stimuli via functional interaction with TLRs; regulation of integrin function via Plexin-A1.; involved in inflammatory diseases and septic shock.	39.8
1557444_at	TREML3	triggering receptor expressed on myeloid cells-like 3	Ig superfamily immunoregulatory receptor expressed on myeloid cells, structurally related to the TREM genes and located in the TREM gene cluster on human chromosome 6	5.9

^Gene expression profiling was carried out independently on the RNA purified from three independent N- and H-mDC preparations, and comparative analysis of gene expression diferences between the two experimental conditions was conducted as described in the *Materials and Methods.* Each gene is given a representative GeneBank accession number, a common gene symbol, a brief gene description, and the fold increase value. Underlined genes were validated by qRT-PCR.

§The indicated values are calculated as the ratio of hypoxic/normoxic signals (mean of expression level of three experiments). Genes increased by ≥2 fold are shown.

*Genes whose promoter contain members of the HRE family

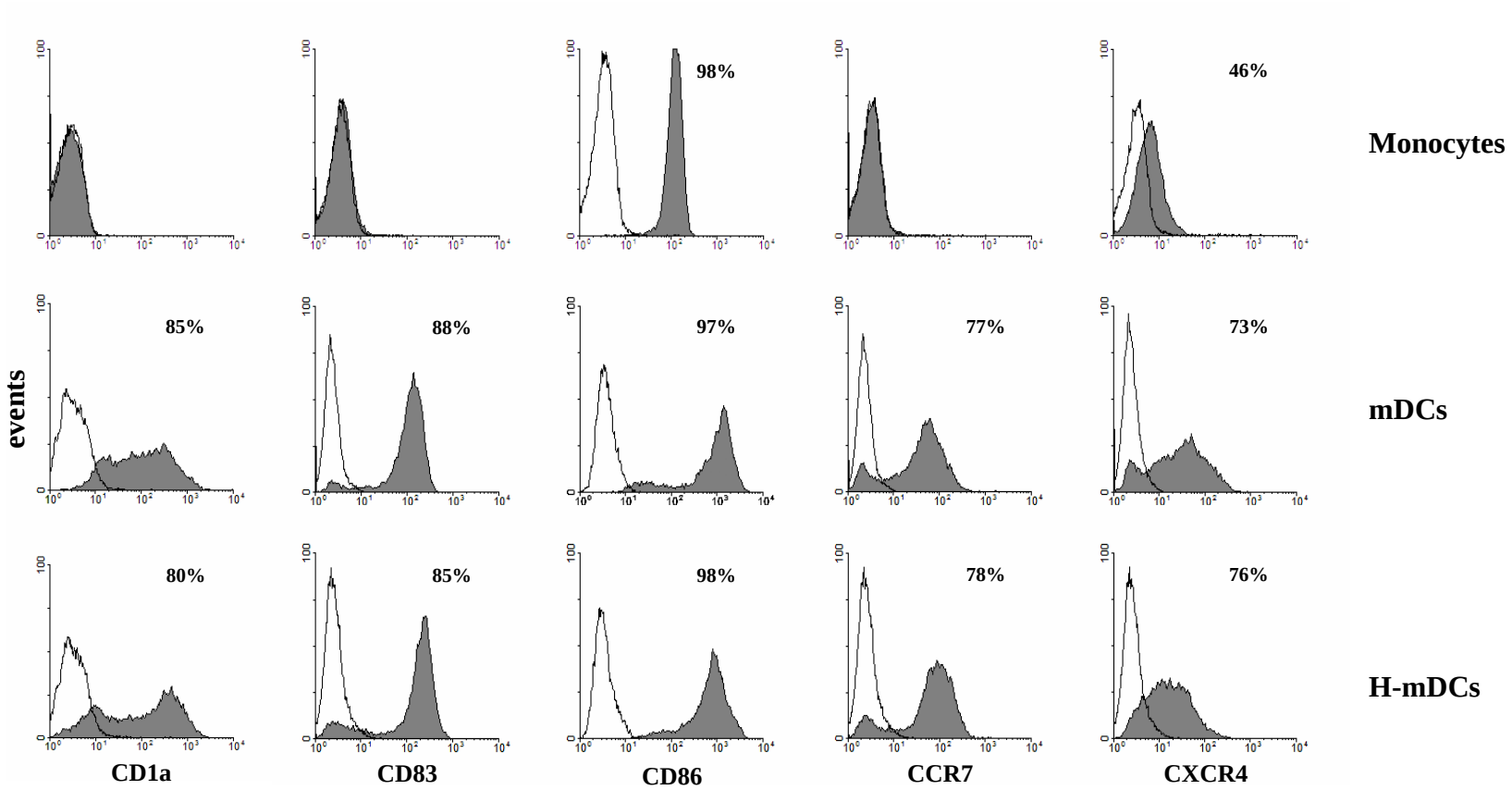

events
98%
46%
Monocytes
85%
88%
97%
77%
73%
mDCs
80%
85%
98%
78%
76%
H-mDCs
CD1a
CD83
CD86
CCR7
CXCR4

Fig.1

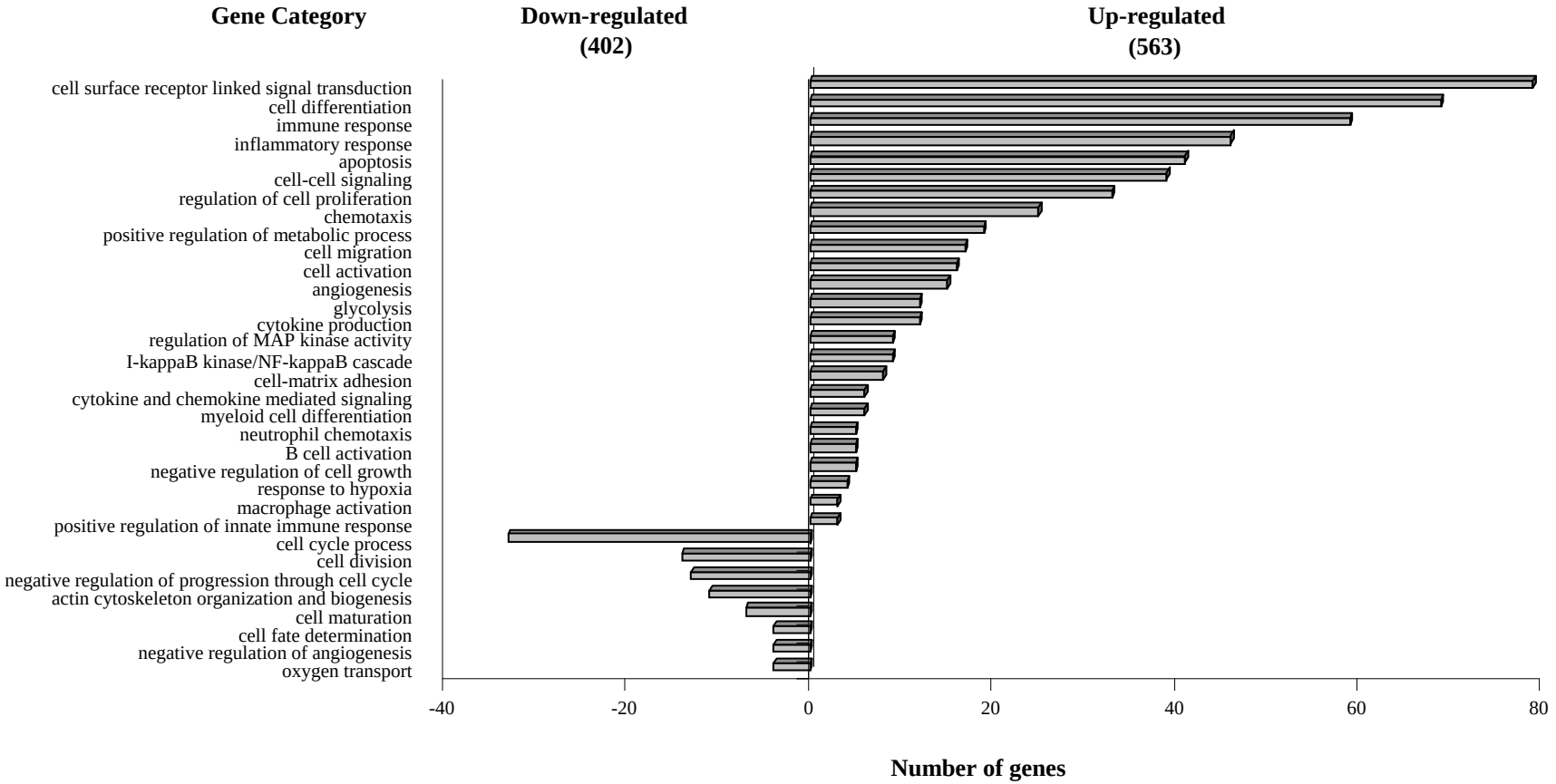
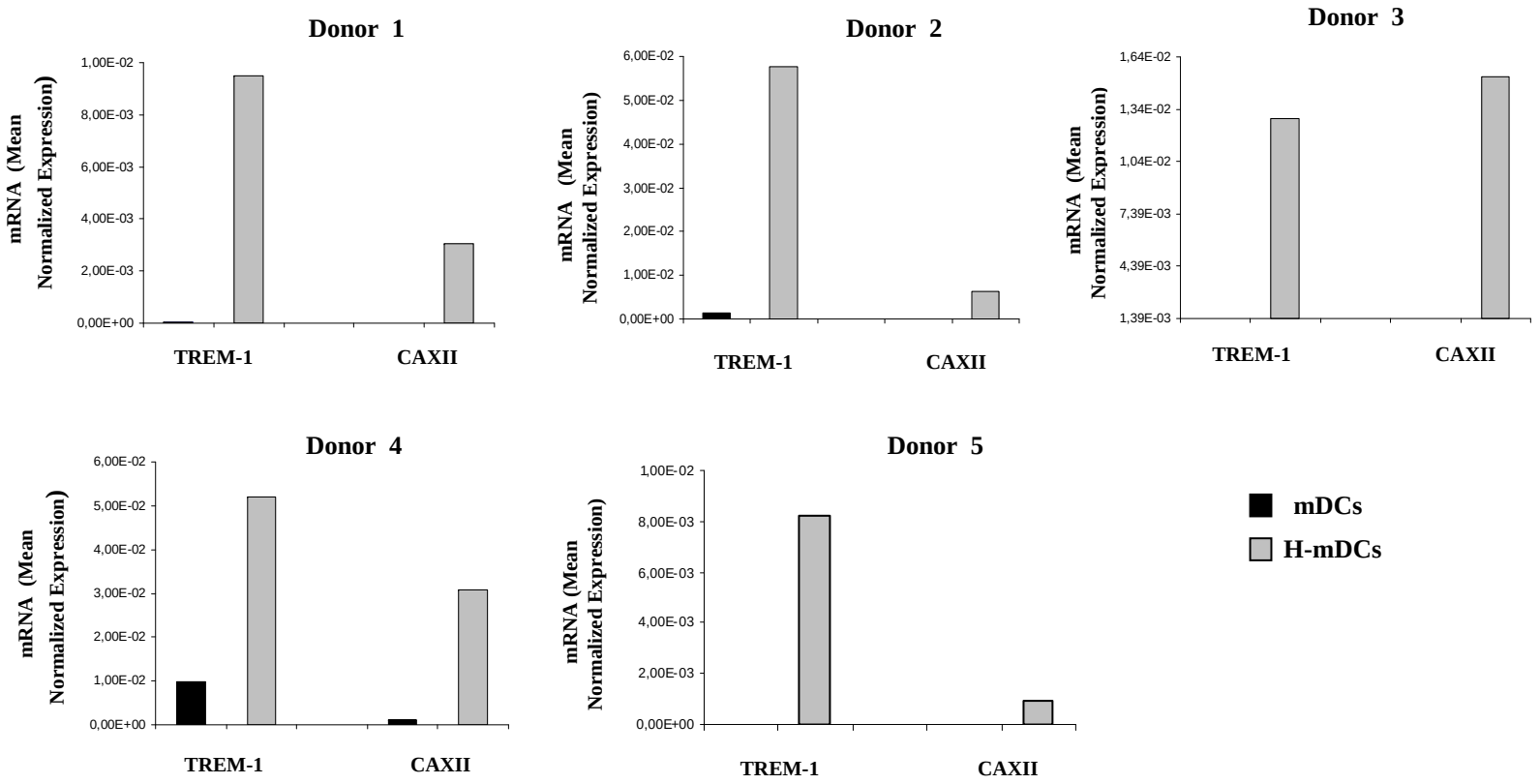

Gene Category
Down-regulated
(402)
Up-regulated
(563)
cell surface receptor linked signal transduction
cell differentiation
immune response
inflammatory response
apoptosis
cell-cell signaling
regulation of cell proliferation
chemotaxis
positive regulation of metabolic process
cell migration
cell activation
angiogenesis
glycolysis
cytokine production
regulation of MAP kinase activity
I-kappaB kinase/NF-kappaB cascade
cell-matrix adhesion
cytokine and chemokine mediated signaling
myeloid cell differentiation
neutrophil chemotaxis
B cell activation
negative regulation of cell growth
response to hypoxia
macrophage activation
positive regulation of innate immune response
cell cycle process
cell division
negative regulation of progression through cell cycle
actin cytoskeleton organization and biogenesis
cell maturation
cell fate determination
negative regulation of angiogenesis
oxygen transport
-40
-20
0
20
40
60
80
Number of genes

Fig.2

A


Donor 1
Donor 2
Donor 3
Donor 4
Donor 5
mRNA (Mean Normalized Expression)
TREM-1
CAXII
mDCs
H-mDCs

B

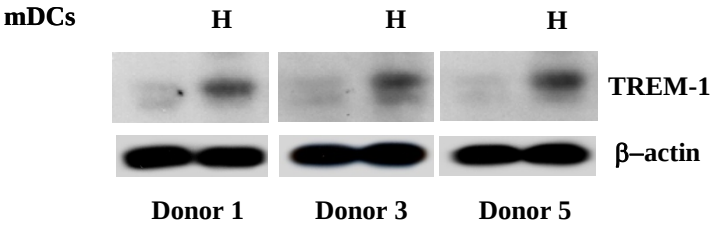
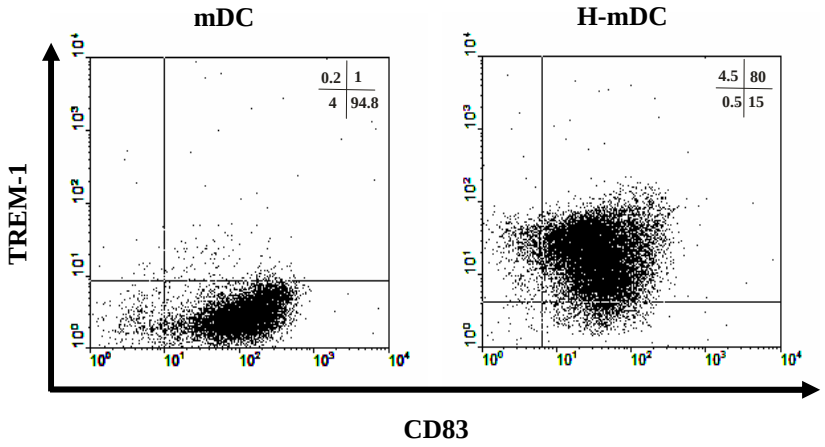
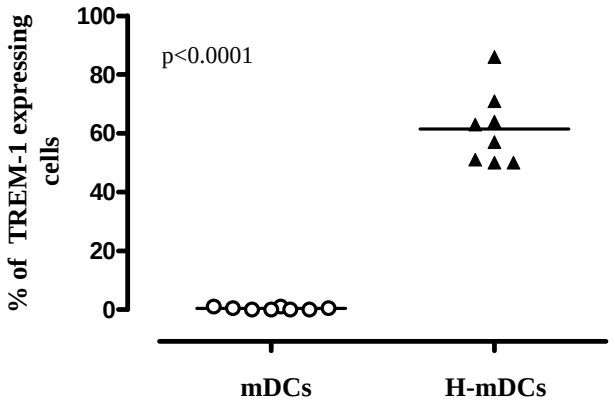

mDCs
H
H
H
TREM-1
β–actin
Donor 1
Donor 3
Donor 5

Fig.3A,B

C


mDC
H-mDC
TREM-1
CD83
0.2
1
4
94.8
4.5
80
0.5
15


% of TREM-1 expressing cells
p<0.0001
mDCs
H-mDCs

D

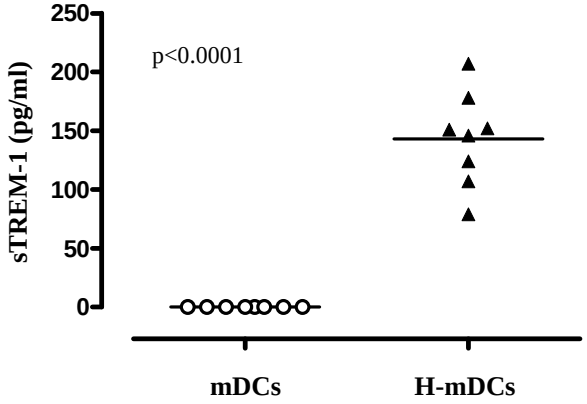
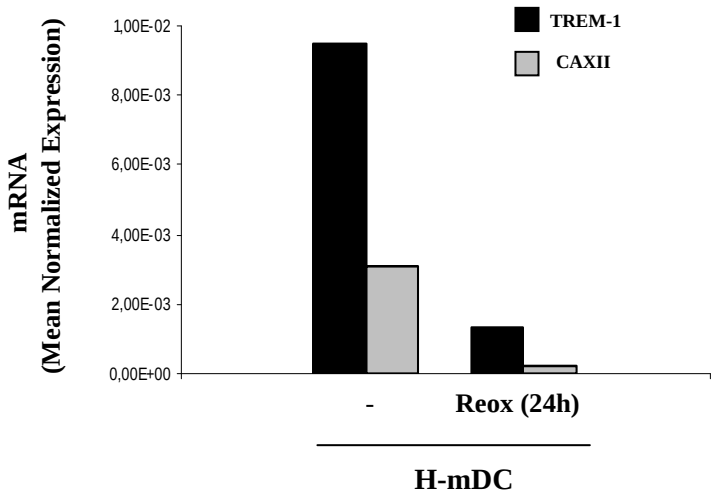
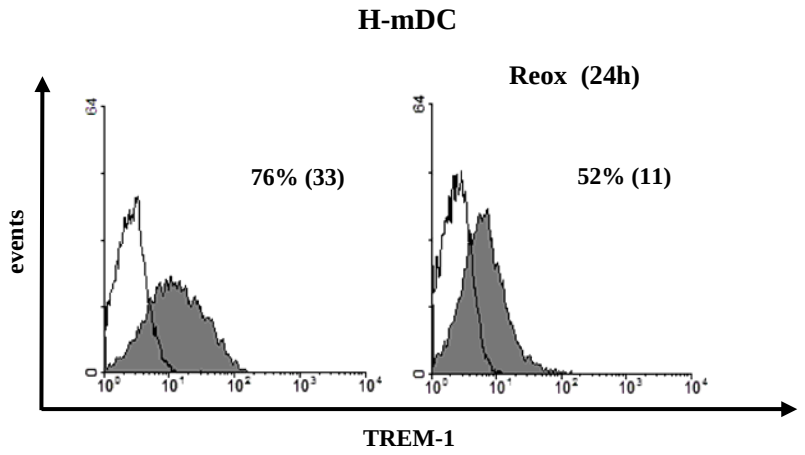

sTREM-1 (pg/ml)
p<0.0001
mDCs
H-mDCs

Fig.3C,D

A


mRNA
(Mean Normalized Expression)
1,00E-02
8,00E-03
6,00E-03
4,00E-03
2,00E-03
0,00E+00
TREM-1
CAXII
-
Reox (24h)
H-mDC

B


H-mDC
Reox (24h)
events
76% (33)
52% (11)
TREM-1

C

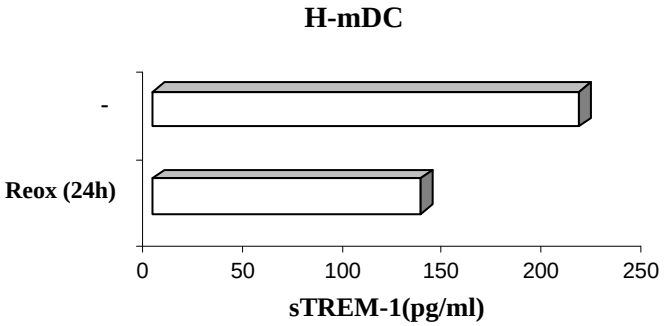

H-mDC
-
Reox (24h)
0
50
100
150
200
250
sTREM-1(pg/ml)

Fig.4

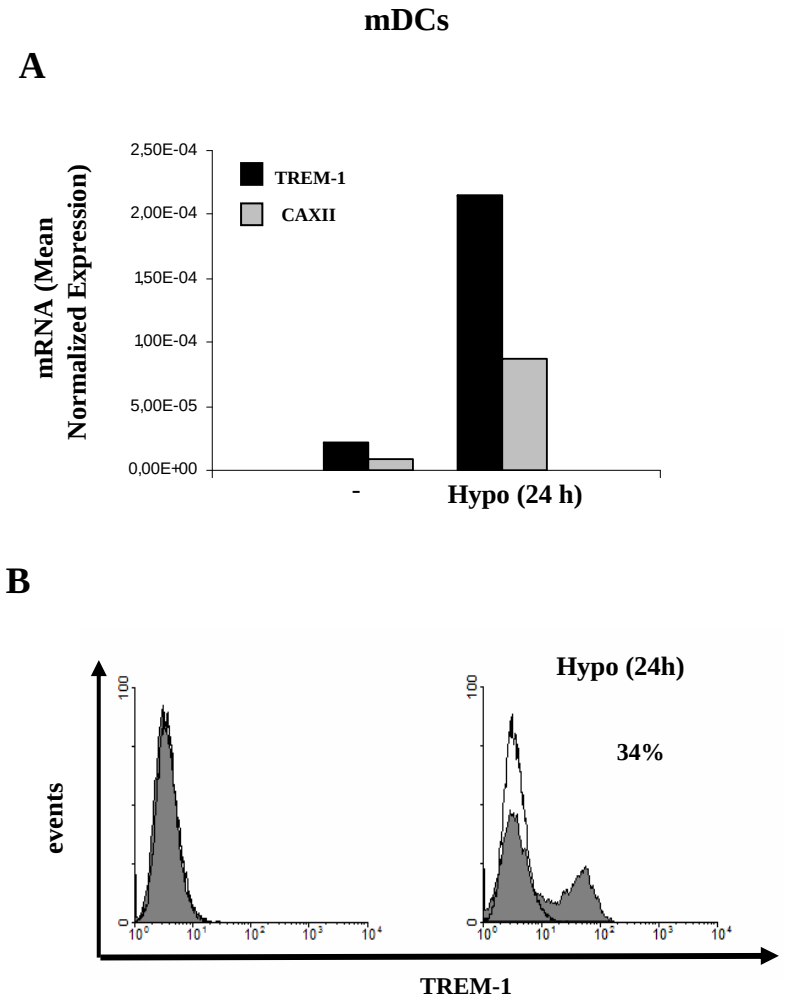

mDCs
A
mRNA (Mean Normalized Expression)
2,50E-04
2,00E-04
1,50E-04
1,00E-04
5,00E-05
0,00E+00
TREM-1
CAXII
-
Hypo (24 h)
B
events
Hypo (24h)
34%
TREM-1

Fig. 5

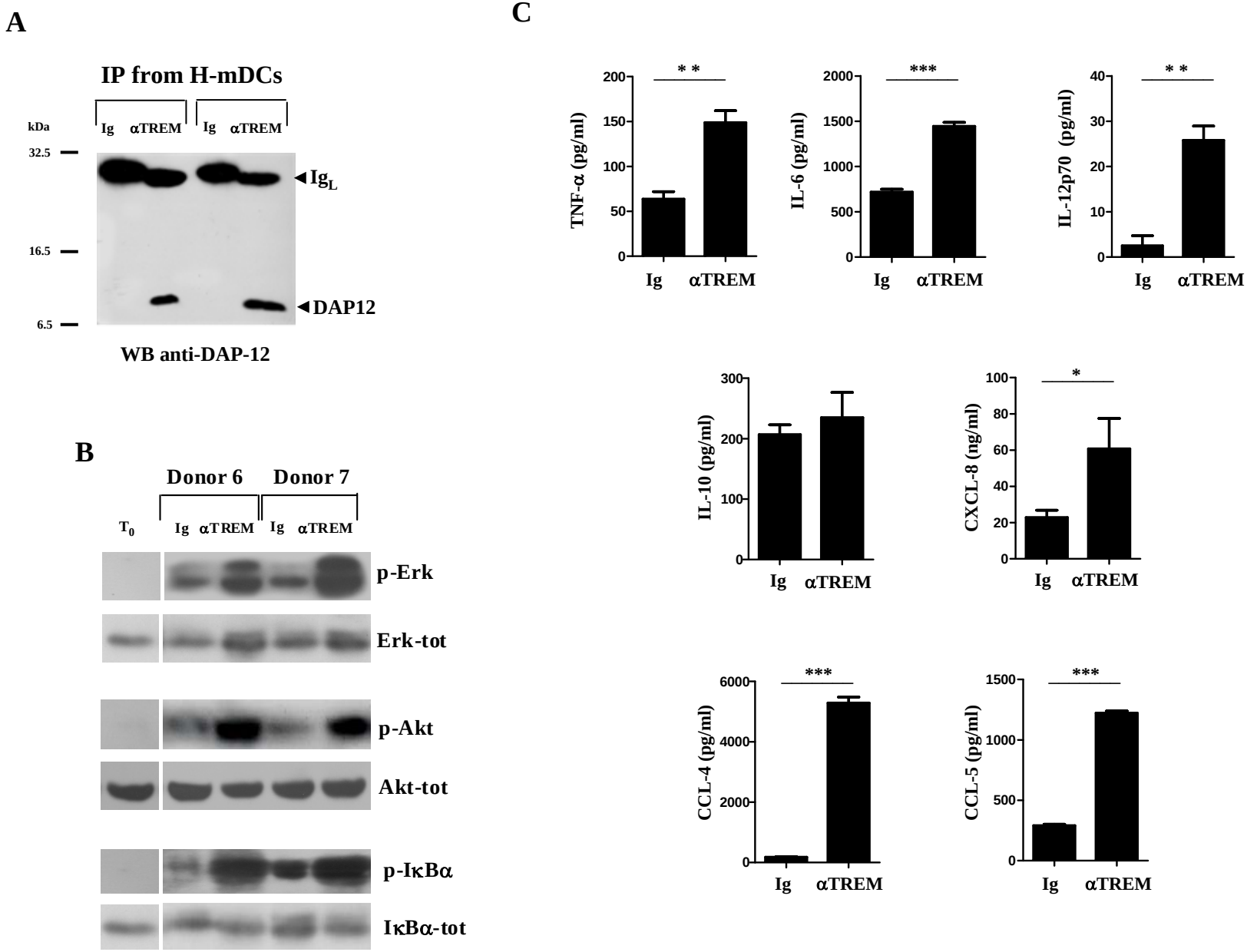

A
IP from H-mDCs
kDa
Ig
αTREM
Ig
αTREM
32.5
16.5
6.5
IgL
DAP12
WB anti-DAP-12
B
Donor 6
Donor 7
T0
Ig
αTREM
Ig
αTREM
p-Erk
Erk-tot
p-Akt
Akt-tot
p-IκBα
IκBα-tot
C
TNF-α (pg/ml)
IL-6 (pg/ml)
IL-12p70 (pg/ml)
IL-10 (pg/ml)
CXCL-8 (ng/ml)
CCL-4 (pg/ml)
CCL-5 (pg/ml)
Ig
αTREM

Fig. 6

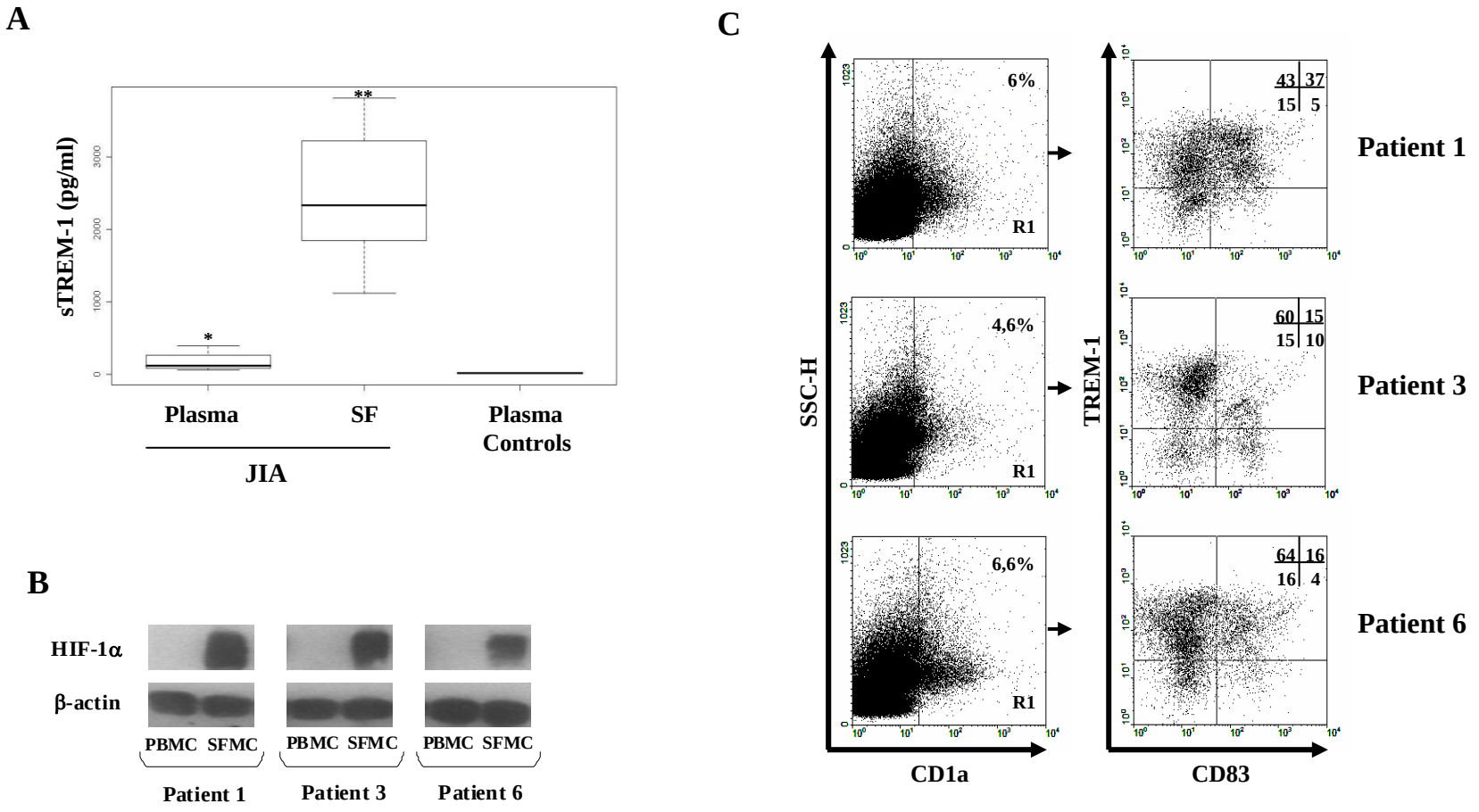

A
sTREM-1 (pg/ml)
0
1000
2000
3000
**
*
Plasma
SF
Plasma Controls
JIA
B
HIF-1α
β-actin
PBMC SFMC
PBMC SFMC
PBMC SFMC
Patient 1
Patient 3
Patient 6
C
SSC-H
CD1a
6%
R1
4,6%
R1
6,6%
R1
TREM-1
CD83
43 37
15 5
60 15
15 10
64 16
16 4
Patient 1
Patient 3
Patient 6

Fig. 7