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Endotoxin Tolerance Represents a Distinctive State of Alternative Polarization (M2) in Human Mononuclear Cells

Olga M. Pena, Jelena Pistolic, Disha Raj, Christopher D. Fjell, and Robert E. W. Hancock

Classical (M1) and alternative (M2) polarization of mononuclear cells (MNCs) such as monocyte and macrophages is known to occur in response to challenges within a microenvironment, like the encounter of a pathogen. LPS, also known as endotoxin, is a potent inducer of inflammation and M1 polarization. LPS can also generate an effect in MNCs known as endotoxin tolerance, defined as the reduced capacity of a cell to respond to LPS activation after an initial exposure to this stimulus. Using systems biology approaches in PBMCs, monocytes, and monocyte-derived macrophages involving microarrays and advanced bioinformatic analysis, we determined that gene responses during endotoxin tolerance were similar to those found during M2 polarization, featuring gene and protein expression critical for the development of key M2 MNC functions, including reduced production of proinflammatory mediators, expression of genes involved in phagocytosis, as well as tissue remodeling. Moreover, expression of different metallothionein gene isoforms, known for their role in the control of oxidative stress and in immunomodulation, were also found to be consistently upregulated during endotoxin tolerance. These results demonstrate that after an initial inflammatory stimulus, human MNCs undergo an M2 polarization probably to control hyperinflammation and heal the affected tissue. ***The Journal of Immunology*****, 2011, 186: 7243–7254.**

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Inflammation is a complex biological response to harmful stimuli such as microbial infection and tissue injury (1). This rapid process involves a very substantial consumption of metabolic energy and a parallel risk of tissue damage, multiple organ failure, and death (2). Therefore, extremely tight regulation is essential for preventing the deleterious consequences that an excessive response can have on the system. Also called deactivation, adaptation, desensitization, and reprogramming, endotoxin tolerance is defined as the reduced capacity of the host (in vivo) or of cultured immune cells (in vitro) to respond to bacterial signatures, such as LPS, after a first exposure to such a stimulus (3, 4). Because of this characteristic desensitization response, endotoxin tolerance is considered an ancient regulatory mechanism to balance inflammation.

Beeson (5) first reported endotoxin tolerance in 1946 as the abolition of the fever response in rabbits undergoing repeated daily injection of the same dose of typhoid vaccine. In the 1960s, similar results were obtained in humans including reduced fever in response to endotoxin or killed bacteria in secondary infections (6), and later, in 1988, it was demonstrated that macrophages play a central role during endotoxin tolerance (7). Subsequently, it was observed that monocytes isolated from septic patients exhibited a state of cellular hyporesponsiveness, including the absence of proinflammatory cytokine production and low levels of HLA-DR expression (8, 9). Similarly, patients who survive acute septic shock have deficiencies in monocytic cell activation reflecting an endotoxin tolerance state that can persist for up to 2 wk suggesting a stable expression of this phenotype (10).

Several studies have addressed the possible molecular mechanisms that surround endotoxin tolerance. In human systems, this phenomenon has consistently been linked with particular regulatory events including the deficient recruitment of the adapter MyD88 to TLR4 (11), decreased IL-1 receptor-associated kinase (IRAK)4–MyD88 association (12), deficient IRAK1 activation (13), upregulation of negative regulators such as IRAK-M (14), suppressor of cytokine signaling-1 (SOCS-1), TOLLIP and SHIP-1 (15). Additionally, a variation in the composition of NF-κB subunits favoring p50 (16), and RelB (17), as well as the presence of PPARγ (18) have also been found to play an important role in the development of endotoxin tolerance in humans. Notably, it has also been proposed that the responses seen during this process may not be controlled solely at the signaling level. Foster et al. (19) showed that these responses could mainly be attributed to TLR-induced chromatin modifications, and although this has only been demonstrated in mouse models, the presence of strongly conserved methylation patterns between mice and humans suggests that a similar mechanism may also take place in human systems (20). Thus, it is clear that endotoxin tolerance is the consequence of a complex, orchestrated, regulatory response to battle inflammation. However, it is unlikely that this cellular reprogramming occurs merely to reduce inflammation, as the complement of genes differentially expressed during this state appear to direct the cell toward new cellular functions. Therefore, in our attempt to understand endotoxin tolerance from a holistic perspective, rather than from a single-molecule standpoint, we

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The microarray data presented in this article have been submitted to the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE22248.

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Abbreviations used in this article: GO, Gene Ontology; IRAK, IL-1 receptor-associated kinase; MDM, monocyte-derived macrophage; MMP, matrix metalloproteinase; MNC, mononuclear cell; qRT-PCR, quantitative real-time PCR; SOCS-1, suppressor of cytokine signaling-1; TFBS, transcription factor binding site.

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decided to use a systems biology approach to study this cellular reprogramming and compare it with other known cellular programs. Our results led us to conclude that endotoxin tolerance is indeed a distinctive program of alternative polarization.

Classical (M1) and alternative (M2) polarization are very general terms used to classify the responses observed in macrophages toward different stimuli in the microenvironment. Classical macrophage polarization is driven in response to microbial products or Th1 cytokines, such as IFN-γ, and is characterized by an enhanced capacity to kill intracellular microorganisms and produce generous amounts of proinflammatory mediators. Conversely, alternative macrophage polarization can be generated in response to a variety of stimuli such as Th2 cytokines (IL-4, IL-13, and IL-10), glucocorticoids, or a mixture of Ig complexes and TLR ligands, producing different forms of M2 polarization. The functions of alternatively activated macrophages involve the control of inflammatory responses, enhanced phagocytic activity, and tissue repair (21–23). This study illustrates the substantial similarities that exist between M2 polarization and endotoxin tolerance states and proposes that this phenomenon can be considered as another form of alternative activation triggered by bacterial signatures such as LPS.

Materials and Methods

Cells and reagents

For the isolation of blood mononuclear cells (MNCs), venous blood was collected from healthy volunteers into heparin-containing Vacutainer tubes (BD Biosciences, San Jose, CA) in accordance with the ethical approval guidelines of the University of British Columbia Research Ethics Board. PBMCs and monocyte-derived macrophages (MDMs) were isolated as described previously (24, 25) and cultured in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine, 25 mM HEPES, and 1 mM sodium pyruvate (all from Invitrogen, Carlsbad, CA). Human bronchial epithelial cells (16HBE4o$^-$) were obtained as a gift from Dr. D. Gruenert (University of California, San Francisco, San Francisco, CA) and cultured in MEM medium with Earle's salts (Invitrogen) supplemented with 10% (v/v) FBS and 2 mM L-glutamine. All cells were cultivated in a humidified 37°C incubator containing 5% CO_2.

LPS was isolated from *Pseudomonas aeruginosa* (PA-H103), grown overnight in Luria–Bertani broth at 37°C using the Darveau–Hancock method (26). The isolated LPS pellets were extracted with a 2:1 chloroform/methanol solution to remove contaminating lipids. Purified LPS samples were quantitated using an assay for the specific sugar 2-keto-3-deoxyoctosonic acid (KDO assay) and resuspended in endotoxin-free water (Sigma-Aldrich, St. Louis, MO) and used at a concentration of 10 ng/ml.

MDM differentiation

For MDM differentiation, PBMC isolation procedures were performed exclusively in PBS. PBMCs were then resuspended in serum-free RPMI 1640 media and plated at 5×10^6 cells/well in 6-well plates for 30 min. Subsequently, media was changed, and fresh complete media containing M-CSF (10 ng/ml) (Research Diagnostic, Concord, MA) was added. Cells were cultured for 7 d, with media changes every second day. On day 7, cells were subjected to LPS and LPS/LPS treatments.

Endotoxin tolerance induction experiments

Endotoxin tolerance was induced in cells using 10 ng/ml LPS as first and second stimulus (Fig. 1*A*). The experiment consisted of three treatments: control/untreated cells (no LPS), single-dose LPS stimulated cells (LPS), and tolerized cells or cells that had been treated twice with LPS (LPS/LPS).

For LPS/LPS tolerance treatments, cells were initially treated with LPS and incubated for 24 h before the second treatment. Supernatants were then collected from the plates, followed by washing and addition of fresh media. Cells were subsequently treated a second time with LPS. Four hours after the second treatment, supernatants and cell lysates were collected. Supernatants were stored at −20°C until cytokine and chemokine analysis was done, and cell lysates were stored at −80°C in RLT lysis buffer (Qiagen, Valencia, CA) until RNA isolation.

RNA isolation

RNA isolation was carried out as described previously (24). Briefly, RNA was isolated from cell lysates using Qiagen RNA Isolation Kit (RNeasy-Mini Kit; Qiagen), treated with RNase-free DNase (Qiagen) and eluted in RNase-free water (Ambion, Austin, TX) as per the manufacturer's instructions. The RNA concentration was determined using a NanoDrop spectrophotometer, and RNA integrity and purity were assessed with an Agilent 2100 Bioanalyzer using RNA Nano kits (Agilent Technologies).

Microarray experiment and analysis

Microarrays using RNA samples obtained 4 h after treatment of PBMCs obtained from four different healthy donors were performed using the Illumina platform at the Genome BC Microarray Facility Platform (Prostate Centre, Vancouver General Hospital, Vancouver, BC, Canada). Complete

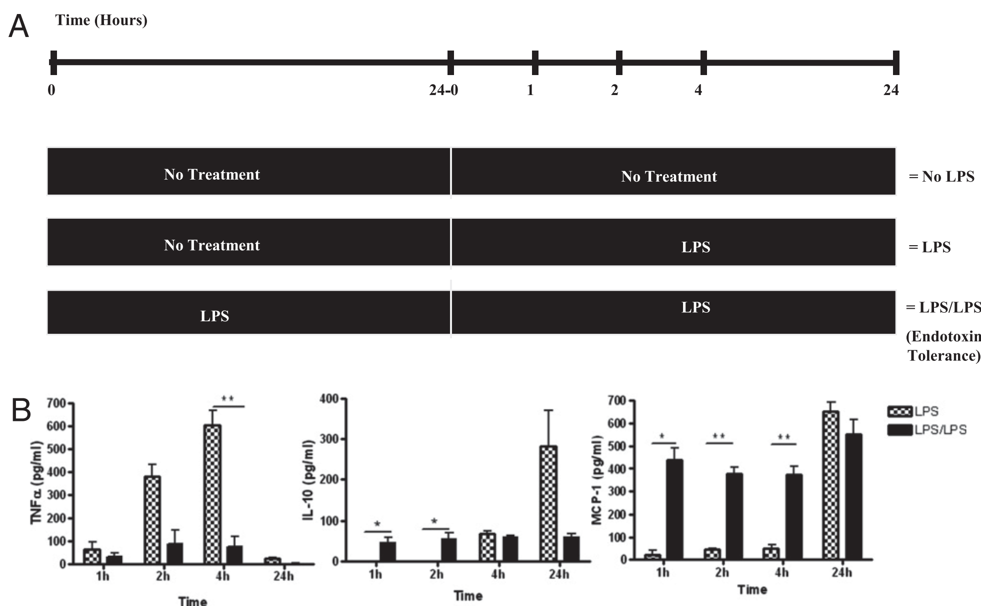

FIGURE 1. Kinetics of cytokine and chemokine secretion in LPS-tolerant cells. *A*, PBMCs were left untreated or challenged with LPS (10 ng/ml) in a single dose (LPS) or two doses at a 24-h interval (LPS/LPS) and incubated for 1, 2, 4, and 24 h as indicated. *B*, Tissue culture supernatants were collected, and TNF-α, IL-10, and MCP-1 production was determined by capture ELISA. Mean values ± SD of four biological replicates are shown. *$p < 0.05$, **$p < 0.01$.

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microarray data have been deposited in the Gene Expression Omnibus public database (accession number GSE22248; http://www.ncbi.nlm.nih.gov/geo/). Differential express genes were selected based on an adjusted p value <0.05 (a table is available from the authors upon request). Transcriptional analysis of the microarray data was performed using system biology tools developed in our laboratory including the InnateDB database (http://www.innatedb.ca) (27), Cerebral, which was used for pathway network visualization, and MetaGEX, which was used to obtain Gene Ontology (GO) terms and transcription factor overrepresentation analysis (C.D. Fjell, unpublished observations) (http://marray.cmdr.ubc.ca/metagex/). Genes with fold change of 2 or more were considered differentially expressed.

Quantitative real-time PCR

Differential gene expression identified through microarray analysis was validated via quantitative real-time PCR (qRT-PCR), which was performed using the SuperScript III Platinum Two-Step qRT-PCR kit with SYBR Green (Invitrogen) as per the manufacturer's instructions, and the ABI Prism 7000 sequence detection system (Applied Biosystems, Carlsbad, CA). Briefly, 500 μg total RNA was reverse transcribed using qScript cDNA Synthesis Kit (Quanta Biosciences, Gaithersburg, MD). PCR was conducted in a 12.5-μl reaction volume containing 2.5 μl 1/5 diluted cDNA template. A melting curve was performed to ensure that any product detected was specific to the desired amplicon. Fold changes for LPS and LPS/LPS samples were calculated compared with the "no LPS" control, after normalizing the change in expression of the gene of interest to the housekeeping gene β_2-microglobulin using the comparative threshold cycle method (28). Sequences of the primers [all from Invitrogen except matrix metalloproteinase (MMP)-9, which was from α-DNA, Montreal, QC, Canada] used for qRT-PCR are available from the authors upon request.

ELISA

ELISA was performed on supernatants collected 4 h posttreatment. These included TNF-α, MCP-1, CCL-22 (R&D Systems), IL-10 (eBioscience), and CCL-3 (Biosource). ELISA assays were performed according to the kit manufacturers' instructions.

In vitro scrape assay

16HBE4o$^-$ cells were grown to confluence in 6-well plates. Confluent cell monolayers were mechanically wounded using a rubber cell scraper (Sarstedt). Wounded 16HBE4o$^-$ cells were washed three times with 1× PBS to remove loose cells and debris and were then incubated for 24 h in a 37°C incubator containing 5% CO_2, with supernatants collected at 4 h from treated PBMCs. Re-epithelialization of the unstained wounds was observed using an IX70 inverted microscope (Olympus, Center Valley, PA) with a camera using UltraView v4 software (PerkinElmer Life Sciences, Wellesley, MA). A magnification of ×10 was used to allow a major surface area to be covered. Photographs taken were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD; http://imagej.nih.gov/ij/) to measure the re-epithelialization area. The entire surface area of each well was photographed (three fields of view per well) for each independent experiment, and analysis of re-epithelialization was done using ImageJ software to measure the average surface area of wound closure. Calculations of mean percentage re-epithelialization represent mean values ± SD of six independent experiments normalized to the RPMI 1640 medium control.

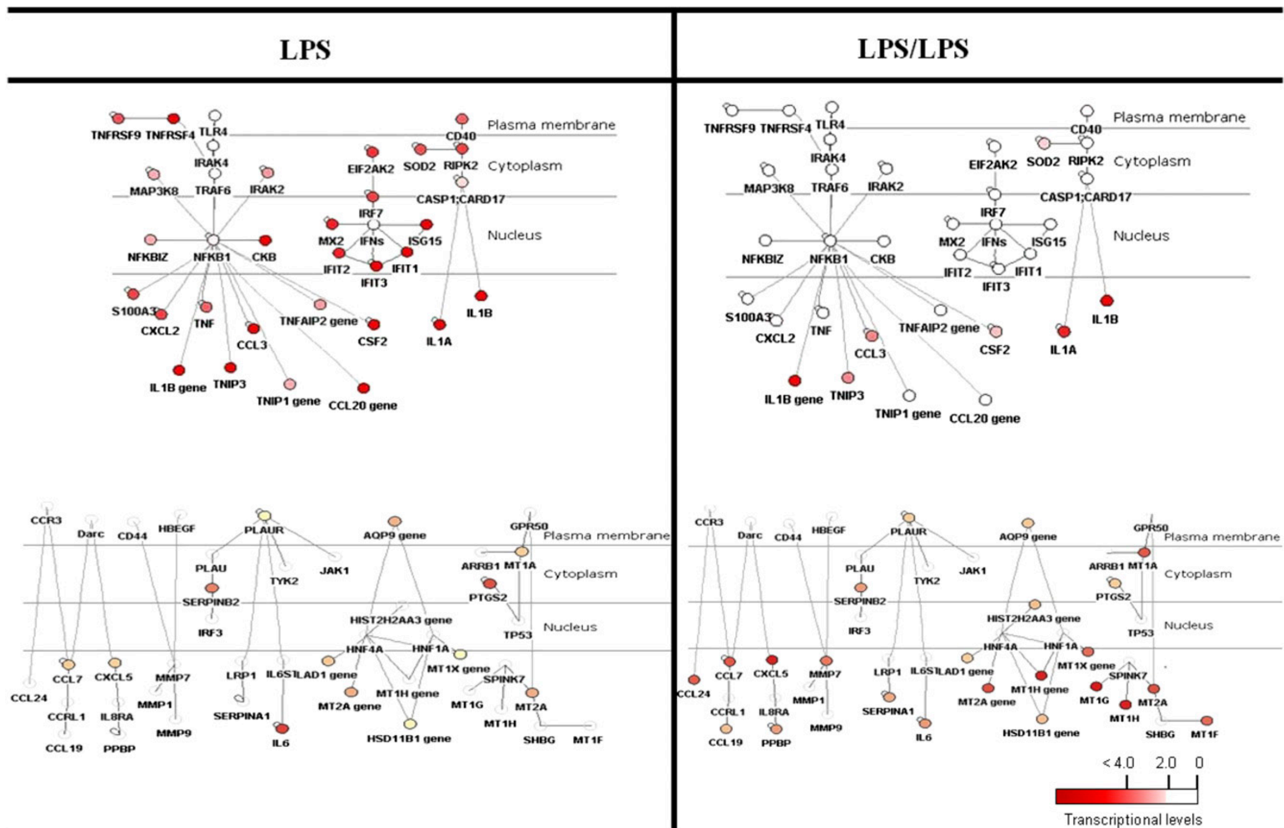

FIGURE 2. Microarray analysis revealed strong differences in gene expression during endotoxin tolerance. The demonstrated network/pathway diagrams are taken from an InnateDB analysis of the microarray data based on upregulated genes and visualized using the visualization tool Cerebral. In this visualization, genes/proteins (nodes) are shown as circles, and interactions between these nodes are shown as lines (edges). Two groups of subnetworks are shown with the *upper subnetworks* being the TLR4 to NF-κB pathway, characteristic of LPS and the *lower subnetworks* including wound-healing proteins such as chemokines, serpins, and metallothioneins, characteristic to LPS/LPS. Nodes are colored according to the degree of dysregulation from highly upregulated (deep red) through baseline (white). Endotoxin tolerance was induced in PBMCs (as shown in Fig. 1*A*, with a second LPS stimulus for 4 h) and RNA isolated and used to perform microarrays using the Illumina platform. The full data set has been deposited in the Gene Expression Omnibus database (accession number GSE22248). Data analysis was performed using the InnateDB database (http://www.innatedb.ca), and pathway network visualization based on cellular interactions was performed using the linked Cytoscape plugin Cerebral.

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Flow cytometry analysis

Whole-blood samples were treated in a similar manner to that described earlier for PBMCs. Briefly, 2 ml whole blood was treated with either a single LPS dose, or LPS/LPS at 10 ng/ml dose (to induce tolerance), or were left untreated. IFN-γ at 20 ng/ml, IL-10 at 10 ng/ml, or IL-4 at 20 ng/ml were used as controls for classical and alternative cell polarization, respectively. Cells were prepared for flow cytometry by staining 100 μl whole blood with fluorescently tagged monoclonal anti-CD163, anti-CD14, and anti-CD206 Abs (eBioscience) for 40 min, followed by RBC lysis with RBC lysis buffer (BD Biosciences) for 10 min at room temperature in the dark. Cells were then washed three times in 1× PBS and resuspended in 0.5% formaldehyde in PBS. Analysis was performed using a FACSCalibur system and FlowJo software (Tree Star), with a $CD14^+$ gate used to select for monocytes.

Statistical analysis

Statistical significance was determined using a two-tailed Student *t* test for paired comparisons and a one-way ANOVA for multiple data sets using the Prism 4.0 software.

Results

Kinetics of cytokine and chemokine production during endotoxin tolerance in PBMCs

To determine the kinetics of cytokine and chemokine production during endotoxin tolerance in human MNCs, PBMCs were treated with either a single LPS dose or two LPS doses to induce tolerance (LPS/LPS) and were incubated for either 1, 2, 4, or 24 h (as shown in Fig. 1*A*). Cytokine/chemokine levels were determined by ELISA from harvested cell-free supernatants. Fig. 1*B* shows that TNF-α was consistently downregulavted during endotoxin tolerance, with the strongest difference occurring at 4 h after the second LPS stimulation ($p < 0.01$). In contrast, the anti-inflammatory cytokine IL-10 was found to be strongly upregulated at 24 h after a single LPS stimulation and then stayed at lower concentrations during endotoxin tolerance (LPS/LPS). The chemokine MCP-1 was either upregulated or present at similar levels of expression during endotoxin tolerance compared with that after a single LPS treatment.

Microarray analysis revealed strong differences in gene expression during endotoxin tolerance

Global transcriptional profiling of PBMCs at 4 h after the second LPS stimulus was done using the Illumina microarray platform. The microarray analysis performed on LPS- and LPS/LPS-treated samples compared with "no LPS" samples consistently confirmed findings obtained previously by ELISA in terms of inflammatory mediators and revealed a large number of differentially expressed genes. An InnateDB analysis based on upregulated genes was performed showing proinflammatory mediators, such as TNF-α, IFN-related genes, and inflammasome-related genes unchanged during endotoxin tolerance (Fig. 2, *upper subnetworks*), whereas a large variety of other genes such as chemokines and, notably, other genes including the scavenger receptor MARCO, metalloproteinases, and a whole family of metallothionein isoforms were shown to stay at a similar level of upregulation or were increased when compared with single LPS stimulations (Fig. 2, *lower subnetworks*). To determine which biological processes were most strongly associated with the dysregulated genes, GO terms overrepresentation analysis was filtered to display those biological processes with the highest *p* values and odds ratios that were related to innate immunity. The highest odds ratios showed the main biological processes that were present under each condition, such as biosynthesis of IL-12, IFN-γ, and their respective activation of signaling pathways during single treatments with LPS. In contrast, negative regulation of certain signaling pathways

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Table I. GO terms overrepresentation analysis

GO Term	LPS		LPS/LPS	
	p Value	Odds Ratio	*p* Value	Odds Ratio
Regulation of cytokine biosynthetic process	6.46E−08	4.94	0.03536	2.74
Positive regulation of I-kB kinase/NF-κB cascade	0.00032	2.68	NS	NS
Leukocyte activation during immune response	0.00034	6.23	0.00658	NS
Positive regulation of innate immune response	0.00041	7.27	NS	NS
Positive regulation of NO biosynthetic process	0.00042	7.27	0.03595	3.77
Cytokine-mediated signaling pathway	0.00067	3.84	0.01366	2.84
Regulation of apoptosis	0.00122	2.58	NS	NS
Positive regulation of NF-κB transcription factor activity	0.00266	3.66	NS	NS
Response to IL-1	0.00450	9.32	NS	NS
Regulation of epidermal cell differentiation	0.00748	7.46	0.03108	5.66
Positive regulation of IL-12 biosynthetic process	0.00786	13.98	NS	NS
JAK–STAT cascade	0.01241	3.74	NS	NS
TNF-mediated signaling pathway	0.01672	5.33	NS	NS
Positive regulation of IFN-γ production	0.02371	6.99	NS	NS
Regulation of autophagy	NS	NS	0.00202	33.97
Positive regulation of VEGF	NS	NS	0.00234	11.33
Positive regulation of pseudopodium assembly	NS	NS	0.00890	11.32
Proteoglycan metabolic process	NS	NS	0.01462	8.50
Negative regulation of I-kB kinase/NF-κB cascade	NS	NS	0.01465	8.49
Positive regulation of actin filament polymerization	NS	NS	0.02203	6.79
Chemotaxis	0.00483	1.94	8.32E−13	4.77
Positive regulation of smooth muscle cell proliferation	NS	NS	0.00955	4.72
Regulation of adaptive immune response	NS	NS	0.00096	4.27
Positive regulation of mononuclear cell proliferation	0.00113	3.55	0.00108	3.79
Regulation of tyrosine phosphorylation of STAT protein	NS	NS	0.01920	3.24
Locomotory behavior	0.02035	1.59	2.66E−9	3.23
Positive regulation of leukocyte activation	0.00069	2.75	0.00048	3.06
Ras protein signal transduction	NS	NS	0.02029	2.44

GO terms with the highest *p* values and odds ratios that were related to innate immunity are presented.

such as the NF-κB cascade and cellular migration and proliferation were characteristic of endotoxin tolerance (Table I). Similarly, we analyzed the upstream regions of dysregulated genes for overrepresentation of transcription factor binding sites; drastic differences were found between the single LPS and endotoxin tolerance treatments. This analysis revealed in keeping with published data (29–31) that the binding sites for NF-κB, IFN regulatory factor-1, and STAT family members were the most significantly associated with inflammatory responses during single LPS treatments, whereas different members of the ETS family of transcription factors were most associated with endotoxin tolerance (Table II). In addition, we performed a parallel bioinformatic analysis with another endotoxin tolerance microarray study reported in the literature recently (32). Our findings (presented as Supplemental Table I) showed substantial similarities in transcription factors including those from the ETS family (ETS1, ETV4, SPI1) and MTF1, which modulate metallothionein responses. We also found matching signaling pathways that are mainly correlated with membrane trafficking and cell motility (ARF1, Ras), endocytosis, wound healing (FGF, VEGF, EGF, PDGF), as well as insulin responses. The molecular basis for this differential expression in endotoxin tolerant cells compared with endotoxin stimulated cells was studied further and is discussed in more detail later.

Proinflammatory mediators and chemokine gene expression profiles during endotoxin tolerance were similar to those found during M2 polarization

To confirm the microarray analysis, qRT-PCR analysis was performed. The pattern of dysregulation of several genes with prominent proinflammatory functions, as well as several chemokines, presented the first evidence that endotoxin tolerance might mediate an M2 polarization phenotype in PBMCs. Similar to the observations for cytokine secretion shown in Fig. 1*B*, and consistent with literature observations (33, 34), TNF-α gene expression was significantly reduced to basal levels ($p < 0.001$) during endotoxin tolerance (4 h after the second treatment) (Fig. 3*A*), as was tissue factor ($p < 0.01$). Cyclooxygenase-2 was also significantly downregulated during tolerance relative to the single LPS treatment. M1 polarization-associated chemokines CCL-3 and CCL-20 (Fig. 3*B*) were both significantly reduced in cells exhibiting endotoxin tolerance ($p < 0.01$). In contrast, during endotoxin tolerance, M2 polarization-associated chemokines CCL-22 and CCL-24 were enhanced by ~8-fold ($p < 0.001$) and 73-fold ($p < 0.01$), respectively, relative to the single LPS treatment (Fig. 3*C*). Some of these findings were then followed up at the protein level by ELISA using PBMCs (Supplemental Fig. 2*A*) and MDMs (Fig. 3*D*), where similar results were obtained, confirming the previous findings.

Table II. Transcription factor binding site overrepresentation analysis

Transcription Factor	LPS *p* Value	LPS/LPS *p* Value
IFN regulatory factor 1 (IRF1)	3.38E−10	NS
Signal transducer and activator of transcription 1 (STAT1, 91 kDa)	1.51E−09	NS
CCAAT/enhancer binding protein (CEBPB [C/EBP], β)	4.80E−08	NS
IFN regulatory factor 2 (IRF2)	7.63E−06	NS
Recombination signal binding protein for Ig k J region (RBPJ)	9.33E−06	NS
Signal transducer and activator of transcription 3 (STAT3)	1.10E−05	NS
v-rel reticuloendotheliosis viral oncogene homolog (REL [avian])	1.89E−05	NS
Signal transducer and activator of transcription 5B (STAT5B)	3.91E−05	NS
Fusion (FUS [involved in t(12;16) in malignant liposarcoma])	0.00034	NS
Signal transducer and activator of transcription 5A (STAT5A)	0.00053	NS
v-rel reticuloendotheliosis viral oncogene homolog A (RELA [avian])	0.00065	NS
IFN regulatory factor 7 (IRF7)	0.00070	NS
Tumor protein p63 (TP63)	0.00070	NS
Heterogeneous nuclear ribonucleoprotein K (HNRNPK)	0.00129	NS
Runt-related transcription factor 1 (RUNX1)	0.00132	NS
v-myc myelocytomatosis viral oncogene homolog (MYC [avian])	0.00132	NS
Nuclear respiratory factor 1 (NRF1)	0.00194	NS
Aryl hydrocarbon receptor nuclear translocator (ARNT)	0.00205	NS
IFN regulatory factor 3 (IRF3)	0.00224	NS
SFFV proviral integration oncogene spi1 (SPI1)	0.01598	2.82E−05
Ets variant 4 (ETV4)	NS	4.73E−05
Sterol regulatory element binding transcription factor 1 (SREBF1)	NS	0.00048
Sp1 transcription factor (SP1)	NS	0.000238
v-ets erythroblastosis virus E26 oncogene homolog 1 (ETS1 [avian])	0.03253	0.00152
GA binding protein transcription factor α subunit (GABPA, 60 kDa)	NS	0.00224
E74-like factor 1 (ELF1 [ets domain transcription factor])	NS	0.00259
FBJ murine osteosarcoma viral oncogene homolog (FOS)	0.01491	0.00282
Forkhead box O3 (FOXO3)	NS	0.00377
Serum response factor (SRF)	NS	0.00391
cAMP responsive element modulator (CREM)	NS	0.00471
metal-regulatory transcription factor 1 (MTF1)	NS	0.00471
CCAAT/enhancer binding protein (CEBPE [C/EBP], ε)	NS	0.00886
GATA binding protein 6 (GATA6)	NS	0.00886
Sp2 transcription factor (SP2)	NS	0.00886
v-rel reticuloendotheliosis viral oncogene homolog B (RELB)	NS	0.01869
Wilms tumor 1 (WT1)	NS	0.01869
Zinc finger protein 76 (ZNF76 [expressed in testis])	NS	0.01869
Zinc finger protein 143 (ZNF143)	NS	0.01869
Aryl hydrocarbon receptor (AHR)	NS	0.02192

Overrepresentation of the binding sites for transcription factors were determined based on evaluation of the promoter regions for dysregulated genes; those with highest significance (*p* values) were selected.
SFFV, spleen focus forming virus.

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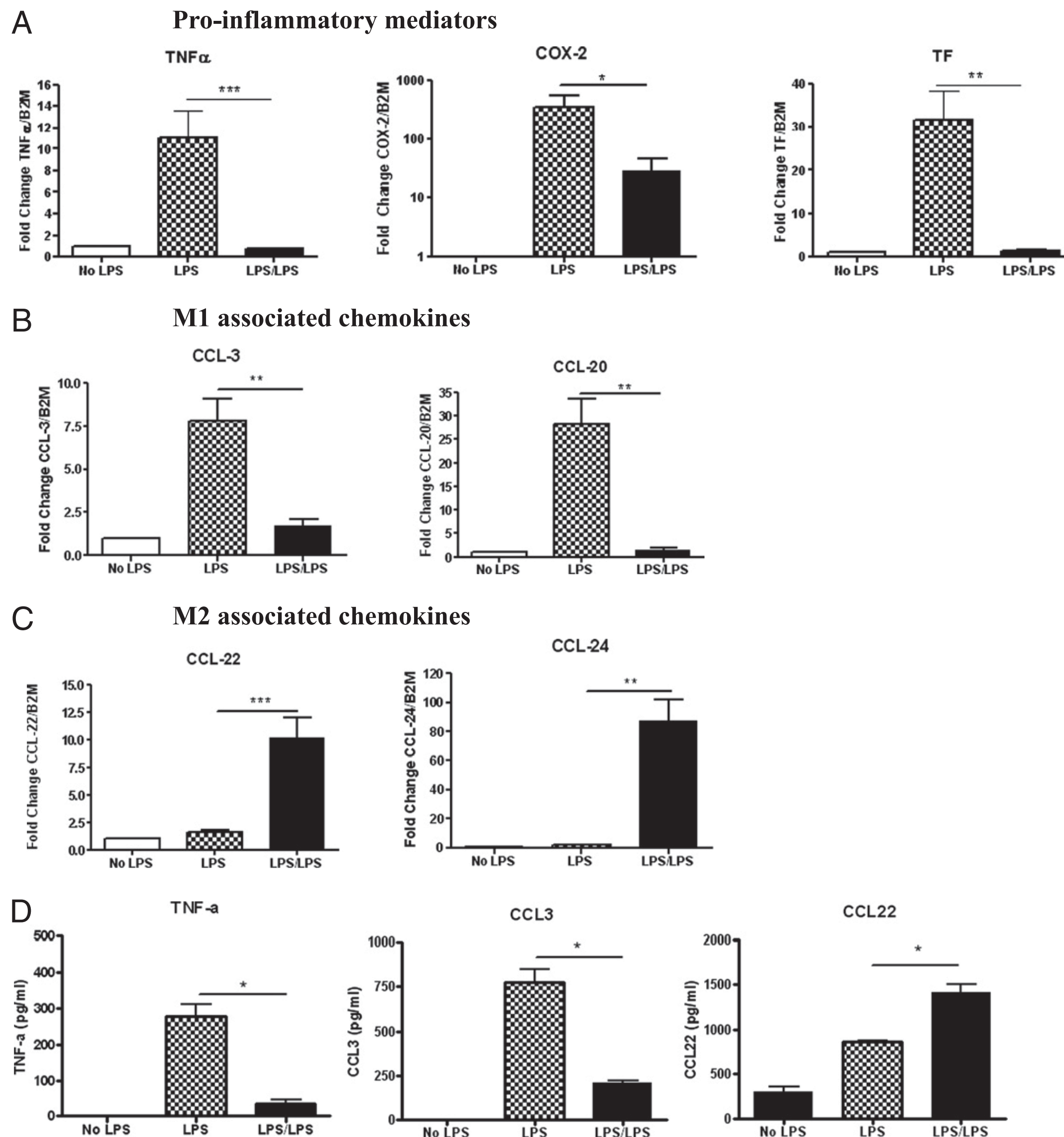

FIGURE 3. Proinflammatory mediators and chemokine profile responses during endotoxin tolerance were similar to those observed during M2 polarization. Endotoxin tolerance was induced as shown in Fig. 1*A*, in PBMCs and MDMs, with a 4-h second LPS stimulus in the case of the LPS/LPS situation. Gene expression of proinflammatory mediators (*A*) and chemokines (*B*, *C*) was analyzed in PBMCs, selected on the basis of the output of the microarray studies, and was assessed using qRT-PCR. Fold changes (*y*-axis) were normalized to β_2-microglobulin (B2M). Protein expression was analyzed by ELISA on supernatants collected from MDMs 4 h poststimulation (*D*). Results are shown as the mean ± SD of at least three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Cell surface marker expression revealed a unique profile during endotoxin tolerance with similarities to an M2 polarization state

CD163 and CD206 (mannose receptor) are cell surface markers that are induced in M2-polarized cells (35). Based on the above data that was consistent with the hypothesis that endotoxin tolerance skews cell polarization into an M2-like phenotype, a cell surface marker analysis was performed in a $CD14^+$ monocyte population to determine expression levels of CD163 and CD206 by flow cytometry using different stimuli for M1 and M2 polarization controls. As shown in Fig. 4, CD163 was significantly induced in tolerized (LPS/LPS) ($p < 0.05$) compared with single LPS treatment or untreated sample. These results were similar to those obtained in the control samples treated with IL-10 (M2 polarization-inducing control) compared with IFN-γ (M1 polarization-inducing control) treated cells ($p < 0.05$). In contrast, CD206 did not present any significant expression among LPS treatments, although significant upregulation of this marker was observed on IL-4–treated cells compared with untreated cells (no LPS) ($p < 0.05$), M1-polarized cells (IFN-γ) ($p < 0.01$), or LPS (single treatment) treated cells ($p < 0.01$).

Key genes related to phagocytosis and wound healing were strongly upregulated during endotoxin tolerance

In addition to extensive differential gene regulation of cytokine and chemokine expression, endotoxin tolerance also induced genes associated with phagocytosis and wound healing. Endotoxin tolerance was induced in PBMCs using a 10 ng/ml LPS dose [as shown in Fig. 1*A*, with a 4-h second LPS (tolerizing) stimulus], and RNA/cDNA from these samples was used in gene expression analysis by qRT-PCR. The scavenger receptors MARCO and CD23 have been previously linked with phagocytosis (36, 37). Both genes were strongly unregulated during endotoxin tolerance compared with expression after a single LPS treatment, with

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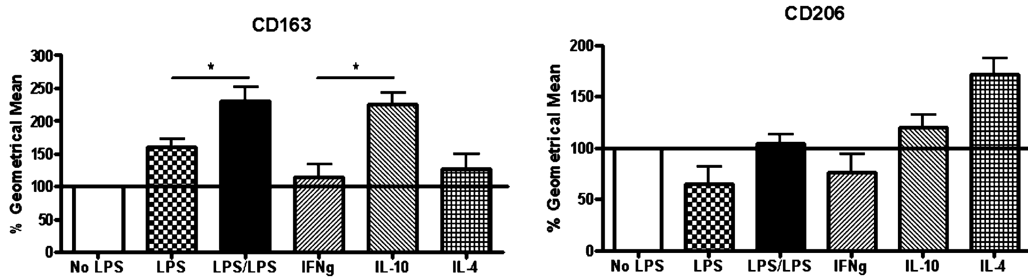

FIGURE 4. Cell surface marker expression revealed a unique profile during endotoxin tolerance with similarities to an alternative polarization state. Whole-blood samples were treated with a single 4-h LPS treatment (LPS) or endotoxin tolerance with a 4-h second LPS stimulus (LPS/LPS) or with IFN-γ at 20 ng/ml, IL-10 at 10 ng/ml, or IL-4 at 20 ng/ml. Upon RBC lysis, the expression of the known alternative polarization markers CD163 and CD206 (mannose receptor) was analyzed by flow cytometry, gating for $CD14^+$ monocytes. Results are shown as mean values ± SD of three independent experiments. *$p < 0.05$.

MARCO increasing by ~20-fold ($p < 0.001$) and CD23 increasing by 5-fold ($p < 0.01$) at 4 h (Fig. 5*A*). Gene expression analysis by qRT-PCR showed that growth factors VEGF and FGF-2 (Fig. 5*B*), previously described to be produced by human monocytes (33, 38), were significantly induced during tolerance conditions ($p < 0.01$ and $p < 0.05$, respectively). Similar results were observed for the metalloproteinases MMP-7 and MMP-9 ($p < 0.05$). In addition, both the proteoglycan versican and formyl-peptide receptor ligand-1, which have been shown to play significant roles in wound healing (34, 39), were strongly upregulated during endotoxin tolerance by ~20-fold ($p < 0.001$) and 70-fold ($p < 0.05$), respectively, relative to expression after a single LPS stimulation. These findings were confirmed using human MDMs (Fig. 6), as well as primary monocytes (Supplemental Fig. 2*B*) with very similar results to the ones found in PBMCs. In addition, MMP-9 expression was also confirmed at protein level during tolerance (data not shown).

Endotoxin tolerance conditions enhanced wound-healing properties of epithelial cells

Given that gene expression analysis revealed upregulation of a variety of genes involved in wound healing, including growth factors and MMPs (Figs. 5*B*, 6), a functional wound-healing assay was performed using bronchial epithelial cells in an in vitro scrape model. A monolayer of $16HBE4o^-$ cells was "wounded" by mechanical removal of cells and supernatants from PBMCs treated with LPS alone, LPS/LPS, or no LPS for 4 h poststimulation (as per Fig. 1*A*) were applied to wounded epithelial monolayers.

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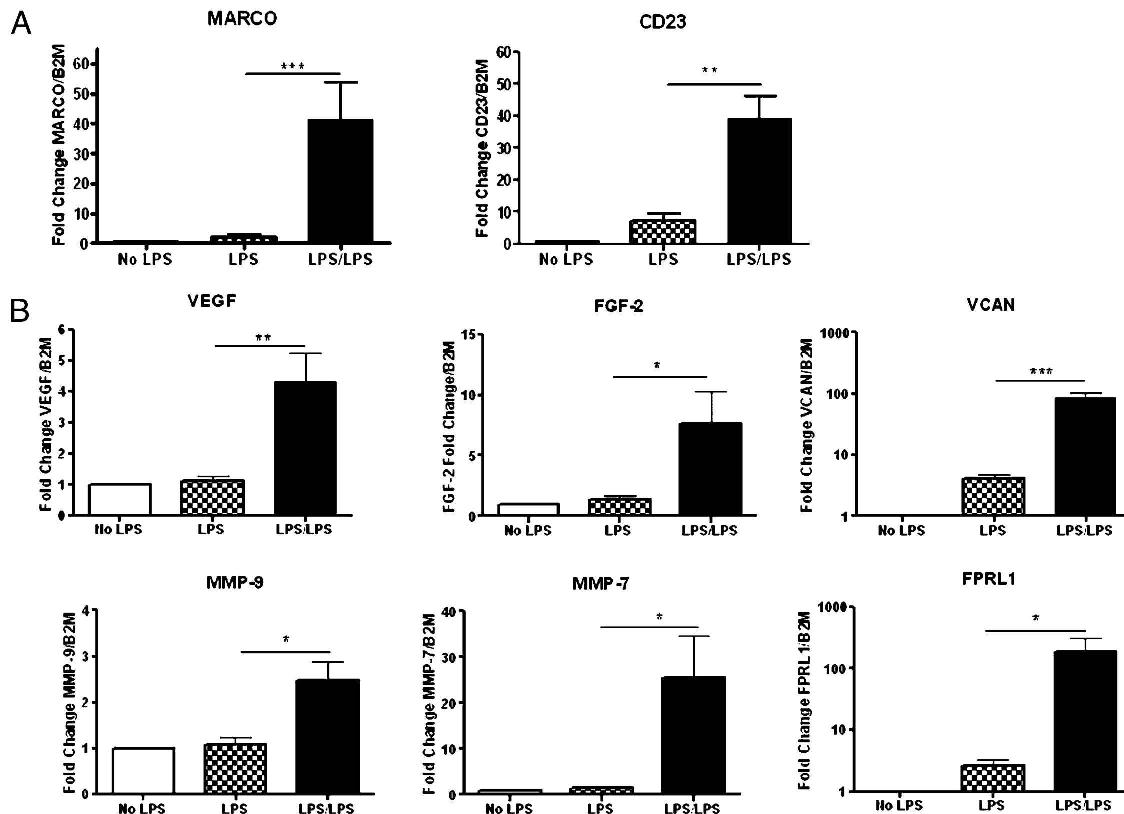

FIGURE 5. Key genes related to phagocytosis and wound healing were consistently upregulated during endotoxin tolerance. Gene expression of phagocytosis-associated (*A*) and wound-healing–associated (*B*) genes was analyzed by qRT-PCR in LPS or LPS/LPS endotoxin tolerant PBMCs (as per Fig. 1*A*, with a 4-h second LPS stimulus). Results shown are mean values ± SD of four independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. FPRL-1, formyl-peptide receptor ligand-1; VCAN, versican.

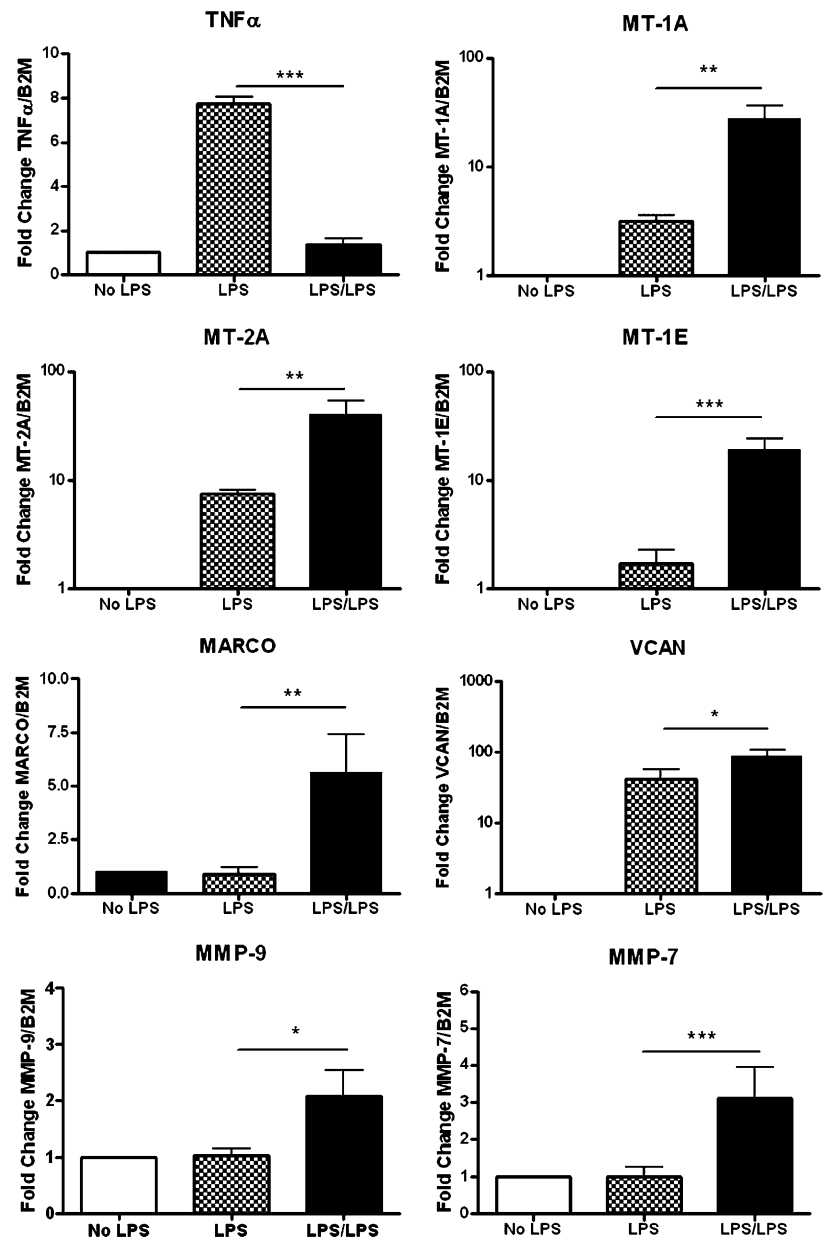

FIGURE 6. Upregulation of key genes related to phagocytosis and wound healing during endotoxin tolerance in human MDMs. Expression of genes involved in phagocytosis and wound healing was analyzed by qRT-PCR in LPS or LPS/LPS endotoxin tolerant human MDMs (as per Fig. 1*A*, with a 4-h second LPS stimulus). Results shown are mean values ± SD of four independent experiments. The responses in human monocytes are found in Supplemental Fig. 2*B*. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. VCAN, versican.

After 24 h of incubation, photographs were taken of the migration of 16HBE4o^{-} cells into the wounded area, relative to the margins of the scrape zone. The surface areas of the re-epithelialization were determined relative to the original boundaries of the scrape zone using ImageJ software, and the percentage mean re-epithelialization (or wound closure) was determined relative to the media-only treatment (fresh RPMI only). As shown in Fig. 7, supernatants from tolerized PBMCs were able to induce significantly higher ($p < 0.05$) re-epithelialization relative to the LPS-alone treatment in six independent experiments. Additionally, to confirm that the levels of re-epithelialization observed were due to factors produced during the tolerance state and not to residual LPS, PBMCs were treated as described previously but with a slight change in the protocol. In this, during the second LPS stimulation, cells were only treated for 1 h, followed by two washes to remove residual LPS, and then incubated for the following 4 h. This conditioned medium was then used during the scrape assays performed on epithelial cells. The data obtained showed that when using conditioned media that did not include trace amounts of LPS, a similar level of re-epithelialization and significant upregulation upon endotoxin tolerance was observed compared with those of the regular method (Fig. 7).

Metallothioneins are strongly upregulated during endotoxin tolerance

Metallothioneins are highly conserved metal-binding proteins. They are characterized by their multiple involvements in metal homeostasis, detoxification, modulation of inflammation, and cell proliferation (40). As shown in Supplemental Fig. 1, metallothioneins as a group were strongly upregulated in endotoxin tolerance. In particular, MT-2A, MT-1E, and MT-1X showed an upregulation during endotoxin tolerance by ~10-, 16-, and 18-fold, respectively. MT-1A and MT-1F furthermore showed an even more enhanced gene expression under tolerance conditions (25- and 35-fold increase over LPS-alone treatment), while MT-1H showed the highest level of induction by >230-fold ($p < 0.01$). Selected metallothioneins (MT-1A, MT-2A, and MT-1E) were analyzed on tolerized MDMs revealing very similar findings to the

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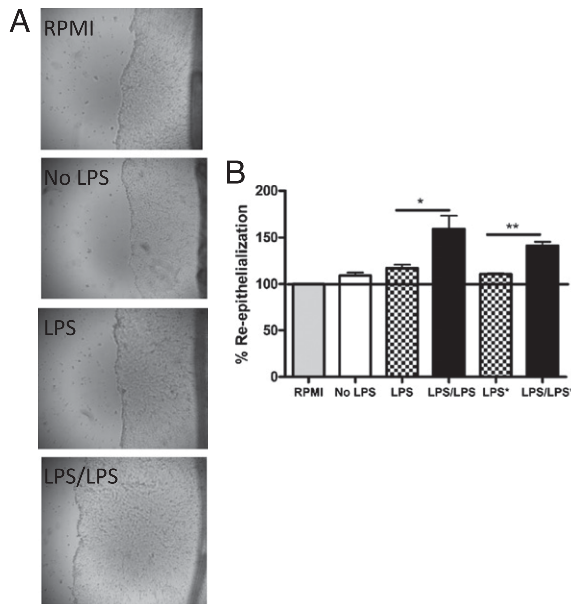

FIGURE 7. Endotoxin tolerance enhanced wound-healing properties in epithelial cells. Supernatants from single LPS treated or endotoxin tolerized PBMCs (as per Fig. 1*A*, with a 4-h second LPS stimulus) or RPMI 1640 medium were applied to a mechanically wounded monolayer of 16HBE4o$^-$ cells. In addition, during the second stimulus, cells were only treated for 1 h, followed by two washes to remove residual LPS, and then fresh medium re-added and incubated for the following 4 h (treatments are labeled with an asterisk [i.e., LPS* and LPS/LPS* in *B*]). After that time, this conditioned medium was removed and used during the scrape assays performed on 16HBE4o$^-$ epithelial cells. *A*, After 24 h of incubation, photographs were taken of the advancing growth front of 16HBE4o$^-$ cells, relative to the scrape point of origin marked on each well. The measurements of surface areas of re-epithelialization were determined using ImageJ software. Images shown are from a single experiment representative of six separate trials. Original magnification ×10. *B*, The quantitation of re-epithelialization represents the mean values ± SD of at least three independent experiments. $*p < 0.05$, $**p < 0.01$.

ones obtained with PBMCs (Fig. 6). However, we were unable to confirm these changes at the protein level because of the lack of suitable reagents.

Discussion

Although endotoxin tolerance has been widely studied, there are still many unknown aspects. In our efforts to obtain a better understanding of this phenomenon from a broader perspective than that of many previous studies, we used a systems biology approach to observe the range of selective changes that occurred during endotoxin tolerance. These changes were similar to those that occur in M2-polarized macrophages, leading us to propose that endotoxin tolerance represents a distinct state of M2 polarization. Our findings revealed the downregulation of a broad variety of proinflammatory mediators. For example, the proinflammatory cytokine TNF-α, an accepted marker for demonstrating endotoxin tolerance, was consistently reduced in our study, consistent with numerous other studies (e.g., Refs. 41, 42). Similar findings were obtained for cyclooxygenase-2 and tissue factor, mediators that play important roles in the activation of inflammation and coagulation, respectively. In addition, chemokines previously associated with classical activation or the M1 phenotype, such as CCL-3 and CCL-20 (43), were downregulated, whereas chemokines that have been associated with the alternative activation or the M2 phenotype were upregulated during endotoxin tolerance in human PBMCs and MDMs. We also observed that while CD206, a major IL-4–induced alternative macrophage marker, was unchanged, the expression of CD163, a key IL-10–induced alternative macrophage marker, was enhanced (23). As there appears to be more than one type of alternative macrophage activation state (21), it is important to note that our data lead to the conclusion that endotoxin tolerance is a form of alternative activation but does not exclude the possibility that there will be distinct and possibly definitive differences from the M2 state induced by other agents.

Furthermore, GO terms overrepresentation analysis normally used to determine the operative biological processes demonstrated that a single (inflammatory) LPS stimulation directs cells toward proinflammatory functions such as positive regulation of mediators like TNF-α, IFN-γ, IL-12, and NO. Conversely, the induction of endotoxin tolerance redirects biological processes turning off the aforementioned functions while activating other immunological functions such as chemotaxis, cell proliferation, and, notably, wound-healing related processes such as the positive regulation of VEGF and proteoglycans. These findings are in agreement with our results from transcription factor binding site (TFBS) overrepresentation analysis, which predict the operative transcription factors by looking for common TFBS in the upstream regions of dysregulated genes. During single LPS stimulation, the analysis showed the presence of key transcription factors like the NF-κB subunit RelA (p65), as well as IFN regulatory factor and STAT family members, which are important for the production of proinflammatory mediators. In contrast, during endotoxin tolerance we found the presence of transcription factors such as the NF-κB family member RelB involved in the modulation of inflammation (44), and which was previously described as an active participant of endotoxin tolerance (17). Additionally, endotoxin tolerance led to the overrepresentation of dysregulated genes with binding sites for the transcription factors important in wound-healing processes. For example, the TFBS for Sp1, a transcription factor involved in angiogenesis through AKT-mediated induction of VEGF expression, was overrepresented (33, 45). The TFBS for members of the ETS family like ETS-1, ELF-1, and SPI1 which are involved in regulation of extracellular matrix (46), vascular development (47), and cellular migration (48), were also significantly overrepresented during endotoxin tolerance. These findings could also be connected to the substantial upregulation of MCP-1 (CCL-2) observed in our time-course experiments during endotoxin tolerance, as MCP-1 has been previously linked with the regulation of angiogenesis through activation of the ETS-1 transcription factor (49).

A characteristic function of an M2 polarization state is the enhancement of wound-healing processes (21). In alternatively activated murine macrophages, this function has been linked to the actions of IL-4, leading to the expression of arginase-1 and the consequent expression of polyamines, which are important for wound repair (50). In humans, this appears not to be the case (51), as arginase-1 is not expressed during alternative activation. Instead, it has been suggested that wound-healing functions are linked to the presence of various MMPs, such as MMP-9, which has a role in attracting blood vessel-associated stem cells (52), as well as MMP-12, which participates in remodeling of the extracellular matrix (53). We have demonstrated for the first time, to our knowledge, that, similar to alternative activation, cells undergoing endotoxin tolerance upregulate not only MMP-9 but also a wide variety of genes that are important in wound repair, including MMP-7, also important in remodeling of the extracellular matrix, and formyl-peptide receptor ligand-1, which has been linked to wound repair through its ligand LL-37 (39). In addition, we observed upregulation of growth factors such as VEGF and FGF-2, important for the growth of endothelial and epithelial

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layers, and proteoglycans such as versican, which is an important component of the extracellular matrix in blood vessels. Using a scrape assay with HBE cells, we confirmed that enhanced wound-healing properties were associated with the cellular reprogramming observed during endotoxin tolerance in vitro. This result is consistent with the enhanced expression of metalloproteinases such as MMP-9, which plays a vital role in airway epithelial wound repair by regulating cellular functions, and matrix-bound growth factors (54, 55). Moreover, the presence of growth factors such as VEGF, which is associated with airway epithelial cell proliferation (56), could also be attributed to the improvement in re-epithelialization observed in our model. These results suggest that cellular reprogramming during endotoxin tolerance also acts to enhance repair of tissue damage after the response to an infectious insult. However, to verify the role of the complete wound-healing program during endotoxin tolerance, further in vivo analysis would be required.

Macrophages undergoing M2 polarization also tend to demonstrate enhanced phagocytic activity (57). We have shown that during endotoxin tolerance, molecules involved in various aspects of phagocytosis, such as MARCO and CD23, are upregulated (36, 58). These findings are supported by previous observations revealing the induction of these molecules in IL-10– and IL-4–induced alternative macrophages, respectively (53, 59, 60). Our results complement previous observations showing enhanced phagocytosis during endotoxin tolerance (32, 61), suggesting an important role for this phenomenon in the clearance of microbes or apoptotic bodies after an initial inflammatory response.

Moreover, we identified for the first time, to our knowledge, the strong and consistent presence during endotoxin tolerance of six different metallothionein isoforms. TFBS overrepresentation analysis also demonstrated the likely importance of the metallothionein-associated transcription factor, MTF1. Metallothioneins are a group of small proteins with a variety of functions, including protection from oxidative damage, as well as influencing zinc homeostasis and angiogenesis, all of which may have important roles during endotoxin tolerance. Metallothionein expression has also been linked with protection from acute lung injury and cardiac dysfunction during endotoxemia mainly by modulating inflammation as well as enhancement of endothelial integrity (62, 63). Moreover, metallothionein expression has also been shown to be increased with age leading to a low bioavailability of zinc, a consequent thymic involution and a basic level of immunosuppression in the elderly, phenomena that become stabilized at a very old age (64). Only the expression of the MT-2A isoform has been described in the IL-10–induced M2 polarization state (65), suggesting that the cellular reprogramming occurring during endotoxin tolerance might be a type of M2 polarization distinct from those described in the literature to date and that metallothioneins could be used as a powerful marker of this state. However, further analyses are needed to confirm this statement and to gain a better understanding of the biological role of these molecules in vivo.

The variety of cellular modifications that occur during human endotoxin tolerance could be linked to the autocrine and paracrine responses mediated by soluble factors such as IL-10, which, as shown here, is normally expressed in response to an initial LPS stimulus, and its expression is maintained at lower levels after a secondary stimulus. This would tend to lead to the generation of a transcriptional profile similar to that observed in M2 polarization by IL-10. In fact, most of the genes shown by William et al. (66) to be upregulated by a combination of LPS and IL-10 were also upregulated here during endotoxin tolerance. Furthermore, when we performed a parallel bioinformatic analysis with other endotoxin tolerance microarray data available in the literature (32), the results (Supplemental Table I) were consistent with the conclusions here, suggesting that important known alternative M2 functions such as phagocytosis (23), insulin responses (67), and wound healing (23) are associated with a tolerant/reprogrammed state. Additionally, different negative regulators have been linked to the transcriptional changes seen during the tolerance state; however, some of these have been identified only in animal models. To confirm the presence of these negative regulators in our model system, we performed qRT-PCR whereby 14 different known negative regulators were screened (Supplemental Fig. 3). The results showed an enhanced expression of eight regulators during the single LPS treatments (ST2L, SOCS-1, IRAK-M, SARM, RelB, A20, NFBIA, and IKBZ). Most of these important regulators still demonstrated increased expression during the tolerance state (LPS/LPS). In two instances, SOCS-1 and RelB, expression was strongly upregulated at a level similar to or higher than that observed during a single LPS treatment. The increased presence of these two essential negative regulators coincided with our microarray findings and our TFBS overrepresentation analysis. These consistent findings may indicate that those negative regulators expressed during a single LPS treatment may be important for the initial termination of TLR responses, whereas those present during double LPS treatments may be responsible for the development and maintenance of a cellular tolerant/reprogrammed state.

It is important to mention that some of the above-discussed data were also confirmed in MDMs (Fig. 6) and human primary monocytes (Supplemental Fig. 2*B*). These findings may suggest that the endotoxin tolerance phenomenon can take place independently of adaptive immunity, as an ancient biological alternative to prevent excessive inflammation in situations where adaptive immunity is impaired or not present. In this context, endotoxin tolerance is an ideal method for the immune system to reach a balance; modifying existing responses through cellular reprogramming to focus on the phagocytosis of microbes and dead cells and the healing of the affected site of infection. In the usual response of mammals to minor or chronic insults, where adaptive immunity is present, the cross-talk between monocytes/macrophages and T cells may be key to regulating classical and alternative activation through the expression of Th1 and Th2 cytokines. However, in a rapid and strong inflammatory response such as during infections leading to sepsis, the role of adaptive immunity might be lost due to the prompt development of events plus the fact that their responses may be impaired due to the profound depletion of T and B cells (68), leaving endotoxin tolerance as the main mechanism to control inflammation. Nevertheless, this suppressed-inflammatory/wound-healing state would likely be dangerous if sustained for a prolonged period, as the strong immunosuppression might lead to susceptibility to secondary infections, increasing the risk of death. Therefore, an understanding of this ancient mechanism from a broad perspective will help with the effort to develop more effective approaches for treating dysregulated inflammatory disorders like sepsis while taking into account the positive aspects of this phenomenon.

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Disclosures

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