Potent Phagocytic Activity with Impaired Antigen Presentation Identifying Lipopolysaccharide-Tolerant Human Monocytes: Demonstration in Isolated Monocytes from Cystic Fibrosis Patients[1]

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Monocyte exposure to LPS induces a transient state in which these cells are refractory to further endotoxin stimulation. This phenomenon, termed endotoxin tolerance (ET), is characterized by a decreased production of cytokines in response to the proinflammatory stimulus. We have established a robust model of ET and have determined the time frame and features of LPS unresponsiveness in cultured human monocytes. A large number of genes transcribed in tolerant monocytes were classified as either "tolerizable" or "nontolerizable" depending on their expression levels during the ET phase. Tolerant monocytes exhibit rapid IL-1R-associated kinase-M (IRAK-M) overexpression, high levels of triggering receptor expressed on myeloid cells-1 (TREM-1) and CD64, and a marked down-regulation of MHC molecules and NF-κB2. These cells combine potent phagocytic activity with impaired capability for Ag presentation. We also show that circulating monocytes isolated from cystic fibrosis patients share all the determinants that characterize cells locked in an ET state. These findings identify a new mechanism that contributes to impaired inflammation in cystic fibrosis patients despite a high frequency of infections. Our results indicate that a tolerant phenotype interferes with timing, efficiency, and outcome of the innate immune responses against bacterial infections. ***The Journal of Immunology,*** **2009, 182: 6494–6507.**

Endotoxin tolerance (ET)[3] is defined as a state of reduced responsiveness to an endotoxin challenge after a primary bacterial insult. Since Paul Beeson first described this phenomenon in 1946 (1), it has been alternatively termed deactivation, adaptation, unresponsiveness, desensitization, refractoriness, or anergy (2). Freudenberg and Galanos demonstrated that macrophages are critical mediators of ET in mice (3); later human cells were also shown to reproduce the ET "phenotype" (2, 4, 5). Furthermore, circulating cells isolated from septic patients possess reduced capacity to produce proinflammatory cytokines when stimulated with endotoxin ex vivo (6, 7). Additionally, ET has been observed in other pathologies such as acute coronary syndrome (8) and cystic fibrosis (CF (9)).

In several studies that have addressed the molecular basis of endotoxin tolerance, LPS unresponsiveness has been associated with decreased expression of NF-κB (10), up-regulation of IL-1R-associated kinase-M (IRAK-M) (7, 11), and SHIP (12), deregulation of TLR4 (13, 14) or the immune signal amplifier triggering receptor expressed on myeloid cells-1 (TREM-1) (9), and inhibition of IRAK-1 phosphorylation (15, 16). The expression of IRAK-M has been pinpointed as a crucial factor in the control of this phenomenon (17), as previously suggested from analysis of IRAK-M$^{-/-}$ mice (18), and later shown by us in humans (7, 11). In these works, the proinflammatory cytokine TNF-α has emerged as a useful marker of tolerant monocytes (7, 19). Other cytokines such as IL-6, IL-10, and TGF-β have also been identified as ET markers in human models or shown to correlate with ET (20). Analysis of the overall down-regulation of proinflammatory factors during ET have revealed a macrophage- and gene-specific epigenetic control mechanism, which appears to circumvent the problem posed by generalized desensitization to repeated LPS signaling (21).

According to their response to LPS, macrophages have been classified as either M1 (if they respond to endotoxin with the production of a proinflammatory repertoire including TNF-α, IL-6, and IL-12) or M2 cells (also termed alternative macrophages),

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[3] Abbreviations used in this paper: ET, endotoxin tolerance; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; FEV_1, forced expiratory volume in 1 s; FVC, forced vital capacity; IRAK, IL-1R-associated kinase; φ, untreated cells; Q-PCR, quantitative PCR; TREM, triggering receptor expressed on myeloid cells; t_{tol}, time of tolerization; t_{rec}, time of recovery; t_{st}, time of stimulation.

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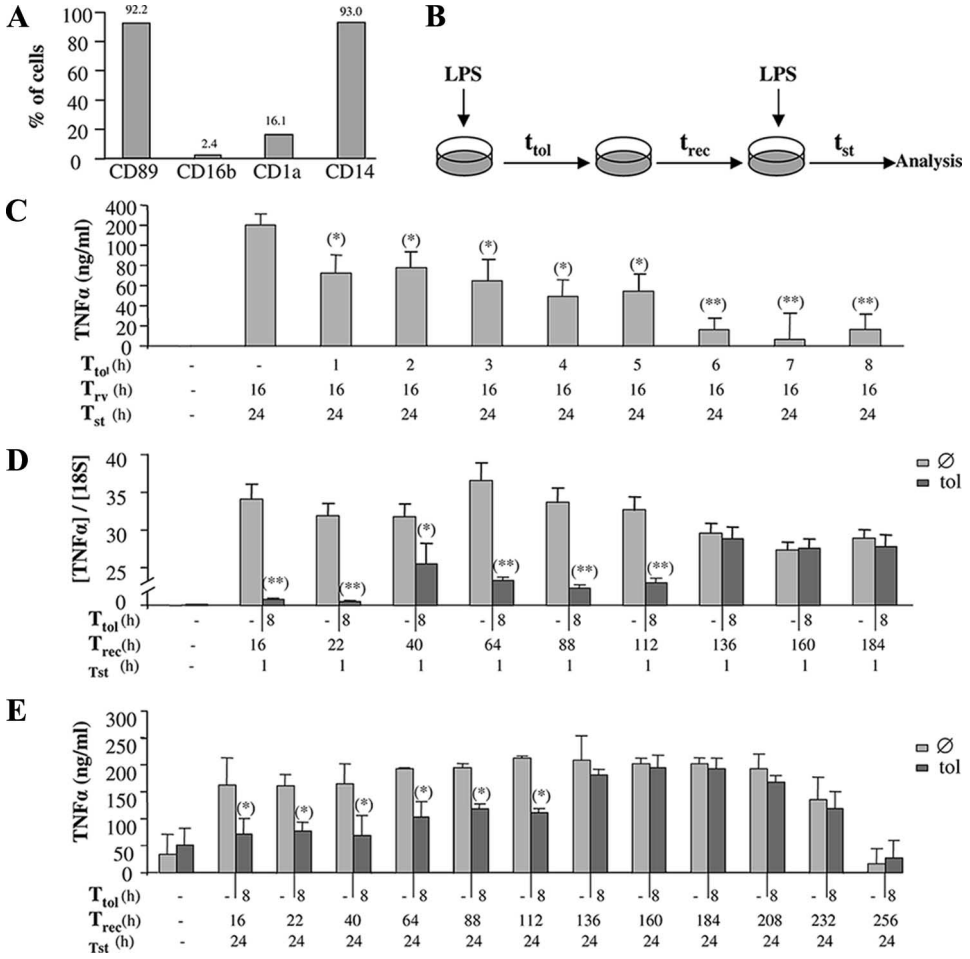


FIGURE 1. Determination of endotoxin tolerance time frame. Circulating human monocytes were isolated from healthy volunteers and cultured. *A*, Purity of the cultures was verified by staining with specific Abs (see *Materials and Methods*) followed by flow cytometric analysis; the fraction of cells stained with each Ab is given ($n = 3$). *B,* Schematic representation of the endotoxin tolerance model used in the present study (t_{tol}, t_{rec} and t_{st} indicate times of tolerance induction, recovery, and restimulation with endotoxin, respectively). *C*, Monocyte cultures were stimulated or not (−) with 10 ng/ml LPS for 1–8 h (t_{tol}). Next, cells were washed twice with PBS and cultured in medium for 16 h (t_{rec}). After this period the cultures were rechallenged or not (−) with 10 ng/ml LPS for 24 h (t_{st}), and TNF-α concentration was evaluated in the supernatant of these cultures using a commercial ELISA ($n = 3$). ∗, $p < 0.05$ and ∗∗, $p < 0.01$ vs non-pretreated cells (−). *D*, Cultures of human monocytes were treated (dark gray bars) or not (light gray bars) with 10 ng/ml LPS for 8 h. Next, cells were washed twice with PBS and cultured in medium for 16–184 h (t_{rec}). Cultures were then restimulated or not (−) with 10 ng/ml LPS for 1 h (t_{st}), and TNF-α mRNA expression was analyzed by Q-PCR at real time ($n = 3$). ∗, $p < 0.05$ and ∗∗, $p < 0.01$ vs control (∅). *E*, The same experiment as performed in *D* but with t_{st} of 24 h; TNF-α concentrations are shown ($n = 3$). ∗, $p < 0.05$ vs control (∅).

which produce high levels of IL-10 and several chemokines instead (22). It has been recently shown that M1 macrophages present an IL-12^{high}IL-23^{high}IL-10^{low} phenotype, while M2 macrophages exhibit the complementary IL-12^{low}IL-23^{low}IL-10^{high} phenotype with a variable signal-dependent capacity to produce inflammatory cytokines (23). Tolerant monocytes seem to exhibit an M2 phenotype (24).

Despite all of these works, a clear picture of the mechanism that governs ET in human monocytes and its (patho)physiological implications is still missing. Here, we present a detailed study of tolerant human monocytes via microarray techniques. First, we established the time frame of endotoxin exposure necessary to induce ET in human monocytes, and conducted an in-depth study of this period. Microarray analysis allowed us to classify several "tolerizable" and "nontolerizable" genes in tolerant monocytes. Tolerant monocytes exhibit rapid IRAK-M overexpression, high levels of TREM-1 and CD64, and a marked down-regulation of MHC molecules and NF-κB2. We also confirmed that they share an IL-12^{low}IL-23^{low}IL-10^{high} phenotype with M2 cells. Furthermore, the evident up-regulation of the high-affinity IgG Fc receptor, CD64/FcRI, and a clear down-regulation of HLA class II molecules in tolerant macrophages suggest both a high phagocytic ability and a poor capability for Ag presentation (25, 26). Phagocytosis of labeled bacteria and lymphocyte proliferation assays corroborated these features of ET monocytes. Finally, we have also tested the phagocytosis capability and Ag presentation in monocytes isolated from CF patients. We show that monocytes isolated from these patients have impaired Ag presentation machinery, while maintaining their high phagocytic ability. Our results provide clear evidence of a specific phenotype in ET cells that is characterized by an increased potential for bacterial phagocytosis, along with an impaired presentation of Ags via MHC molecules that could contribute to immune impairment.

Materials and Methods

Patients and healthy controls

We studied 25 nonsmoker adults, 11 women and 14 men, diagnosed with CF on the basis of established criteria (clinical phenotype, sweat testing, and cystic fibrosis transmembrane conductance regulator (CFTR) genotyping), who had not used corticosteroids within the 3 mo previous to the

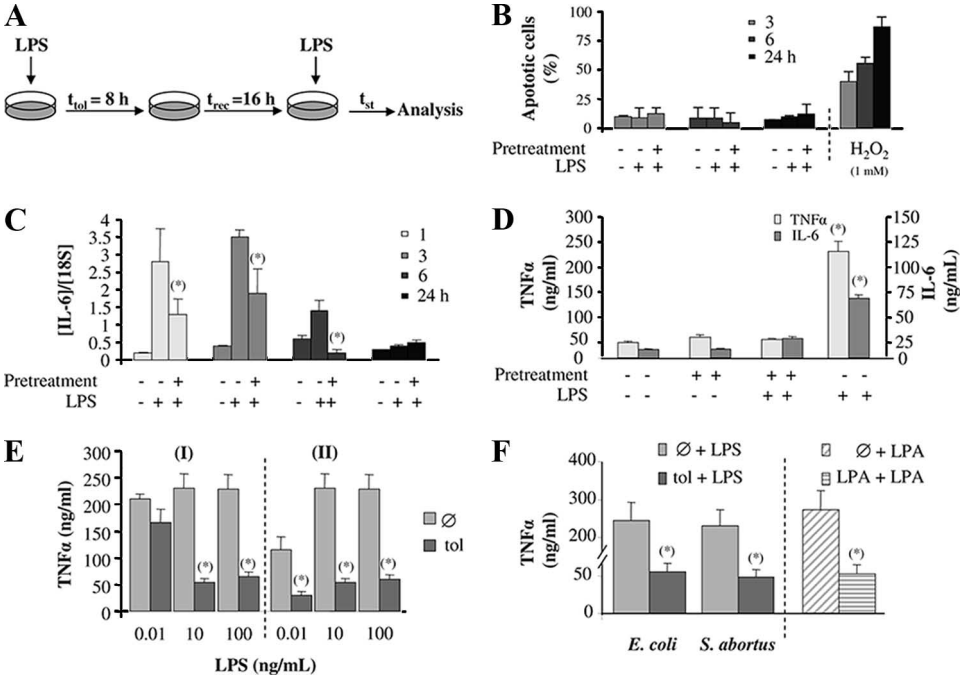


FIGURE 2. Endotoxin tolerance model. *A*, Schematic representation of the employed endotoxin tolerance model. *B* and *C*, Cultures of human monocytes were pretreated (+) or not (−) with 10 ng/ml LPS for 8 h, washed twice with PBS, cultured in medium for 16 h, and finally restimulated with 10 ng/ml LPS for indicated times. *B*, The percentage of apoptotic cells was determined by flow cytometry using annexin V staining; cells treated with 1 mM H_2O_2 were used as positive control. *C*, IL-6 mRNA expression levels evaluated by real-time Q-PCR ($n = 3$). ∗, $p < 0.05$ vs the same condition without pretreatment. *D*, Results of a similar experiment, but with t_{st} of 24 h. TNF-α (light gray bars, left abscissae) and IL-6 concentrations (dark gray bars, right abscissae) in the supernatants of each culture were determined ($n = 3$). ∗, $p < 0.05$ vs the same condition with pretreatment. *E*, Cultures of human monocytes pretreated (tol) or not (∅) with different doses of LPS (0.01, 10, and 100 ng/ml) were rechallenged with 10 ng/ml LPS for 24 h (section I). Alternatively, human monocytes pretreated (tol) or not (∅) with 10 ng/ml LPS were rechallenged with different doses of LPS (0.01, 10, and 100 ng/ml) for 24 h (section II). TNF-α concentrations were measured in the supernatants of each culture ($n = 2$). ∗, $p < 0.05$ vs control (Ø). *F*, *Left panel*, Control (∅) or tolerant (tol) monocytes were restimulated with 10 ng/ml LPS from *E. coli* and *S. abortus* for 24 h. *Right panel*, Human monocytes were stimulated (lipid A (LPA)) or not (∅) with 10 ng/ml LPA for 8 h, and rechallenged with 100 ng/ml LPA for 24 h. TNF-α concentrations were determined in the supernatants of these cultures ($n = 2$). ∗, $p < 0.05$ with respect to control cells (∅).

study. Exclusion criteria included a history of chronic obstructive pulmonary disease, asthma, or other active lung disease, mental or physical handicap, or other significant diseases such as diabetes mellitus, congestive heart failure, ischemic or valvular cardiopathy, or neuromuscular disease. None of the subjects had experienced an exacerbation of respiratory tract infection within the previous 4 wk, and none of them had had oral corticosteroid therapy for at least 3 mo before the study (see also supplemental Fig. 2).[4] Ten sex- and age-matched healthy volunteers without personal history of CF or other significant illness were included as controls.

The following clinical variables were collected on each subject: age, sex, CFTR genotype, exacerbations in the last year, microorganisms in sputum, and usual therapy. Standard, calibrated scales and stadiometers were used to determine height, weight, and body mass index. Spirometry (forced expiratory volume in 1 s (FEV_1) and forced vital capacity (FVC)) was performed with a MasterScope system (VIASYS Healthcare) according to the American Thoracic Society/European Respiratory Society criteria (27). Results were expressed as percentage of normal values, using best postbronchodilator measurements. Predicted values were calculated from the equations for adults of the European Community for Steel and Coal (28). Written informed consent was obtained from all subjects enrolled. This study was approved by the local Ethics Committee ("La Paz" Hospital Ethics Committee).

Abs and reagents

The following Abs were used: anti-CD3-PE, anti-CD64-FITC, anti-MHCII-DM-PE, anti-MHCII-DQ-FITC and anti-MHCII-DR-allophycocyanin (BD Biosciences); anti-CD14-allophycocyanin (Miltenyi Biotec); anti-TREM-1-PE (R&D Systems); and anti-CD1a-FITC, anti-CD16b-FITC, and anti-CD89-FITC (Serotec). The medium used for cell culture was DMEM from Invitrogen. LPS from *Salmonella abortus* was a kind gift from Dr. Galanos (Max-Planck-Insitut für Immunobiologie, Freiburg, Germany), LPS from *Escherichia coli* was acquired from Sigma-Aldrich, and lipid A (*Escherichia coli* R515) was purchased from Alexis Biochemicals. All other reagents were obtained from Sigma-Aldrich unless otherwise stated.

PBMC isolation and cell culture

PBMC were isolated from buffy coats by centrifugation on Ficoll-Hypaque Plus (Amersham Biosciences) as we described before (7–9, 29, 30). The composition of this adherent population of cells was analyzed by FACS. All reagents used for cell culture were endotoxin-free, as assayed with the *Limulus* amebocyte lysate test (Cambrex).

Once seeded, adherent cells were treated or not with 10 ng/ml LPS during the time of tolerization (t_{tol}). After that, cells were washed three times with PBS and kept in complete medium for different times through the time of recovery (t_{rec}) phase. Then, cells were restimulated or not with 10 ng/ml LPS for periods of time ranging from 1 to 48 h (the time of stimulation, or t_{st}). Throughout this work, nontreated cells are denoted "φ". The treatment termed "tol" corresponds to 8 h of t_{tol}, 16 h of t_{rec}, and the indicated t_{st} of restimulation, as illustrated in Fig. 2 (see also "Results"). Experiments using lipid A instead of LPS were also performed.

Starting from peripheral blood of CF patients or healthy volunteers, adherent cells were purified following the same protocol employed for buffy coats. The composition of the adherent population was analyzed by FACS. Once plated, cells were exposed or not to 10 ng/ml LPS for various times according to particular experiments.

RNA isolation and cDNA synthesis

Cells were washed once with PBS and their RNA was isolated using the High Pure RNA Isolation Kit from Roche Diagnostics. cDNA was obtained by reverse transcription of 1 μg of RNA using the High Capacity cDNA Reverse Transcription Kit from Applied Biosystems.

[4] The online version of this article contains supplemental material.

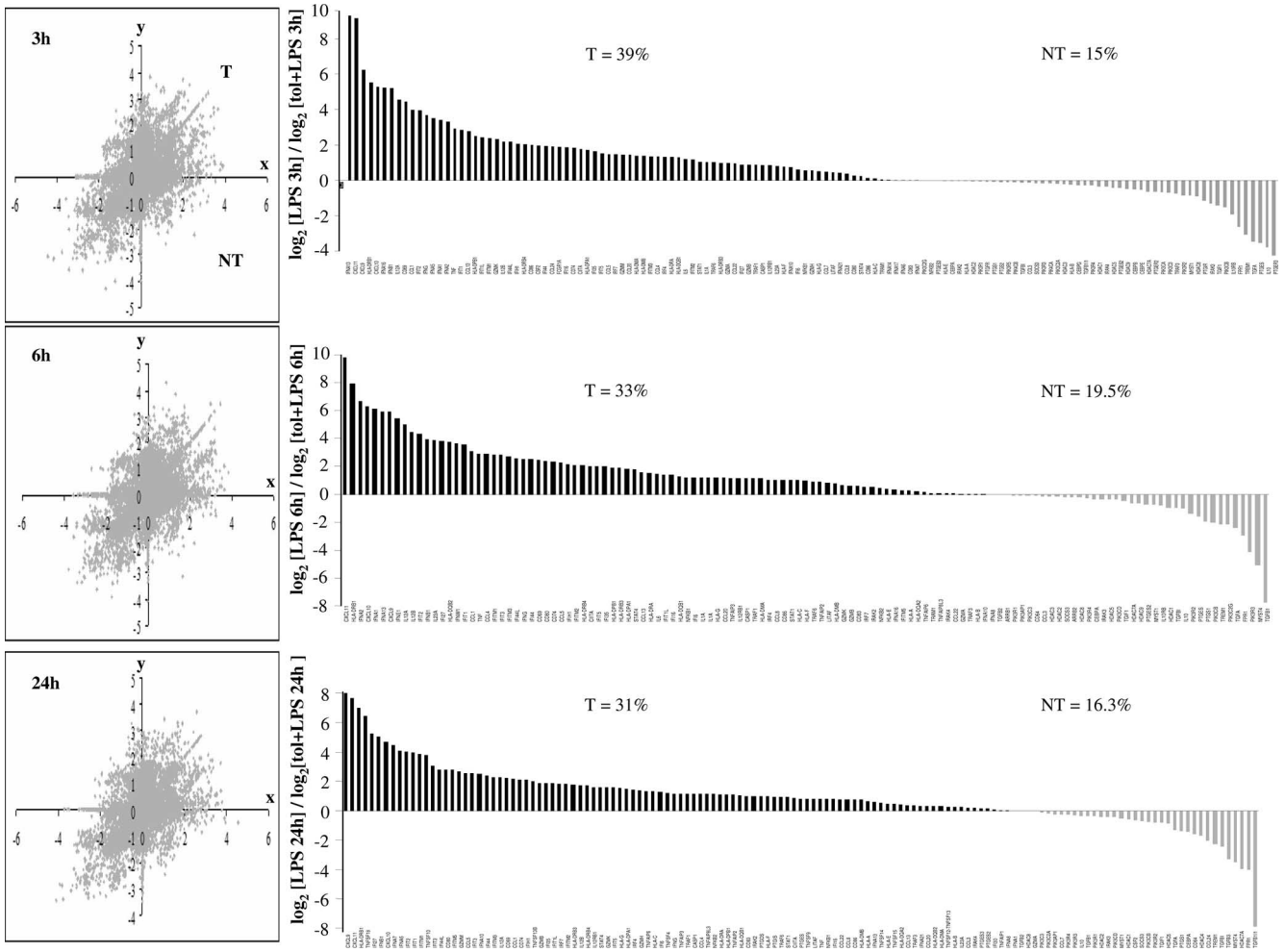


FIGURE 3. Microarray analysis of gene expression in tolerant human monocytes. Control and tolerant human monocytes were treated with 10 ng/ml LPS for 3, 6, or 24 h. Next, mRNA was isolated and cDNA synthesized. A cDNA microarray (22,178 genes) was used to analyze changes associated with the ET phase, as described in *Materials and Methods*. The relative expression levels of expressed genes are represented as points (x, y) in graphics where $x = \log_2([\varnothing + \text{LPS}]/[\varnothing])$, while $y = \log_2([\varnothing + \text{LPS}]/[\text{tol} + \text{LPS}])$. Thus, the first quadrant ($x > 0$, $y > 0$) contains genes stimulated in a first encounter with endotoxin, but down-regulated in tolerant cells, and which have been termed tolerizable (T). Genes in the fourth quadrant ($x > 0$, $y < 0$) are nontolerizable (NT) (*left panels*; $n = 3$). The ratios $\log_2([\varnothing + \text{LPS}]/[\text{tol} + \text{LPS}])$ of major T and NT class genes are also represented next to each (x, y) graphic (*right panels*).

Microarray experiments and analysis

RNA was quantitated with a NanoDrop 1000A spectrophotometer, and sample concentrations were adjusted to 100 ng/μl. cRNA synthesis and hybridization to the Sentrix HumanRef-8_V2 BeadChip Human from Illumina containing 22,178 probes were performed according to the manufacturer's protocols.

The obtained probe-level data were imported into Illumina BeadStudio software for quality control assessment and background correction. Background-corrected data were then normalized using the loess normalization method from BioConductor R packages. Genes with low expression levels across all the samples were filtered out. Differentially expressed genes were determined based on whether the genes showed the same direction of change and their fold changes were all >1.5 across all donors.

mRNA quantitation

Gene expression levels were analyzed by real-time quantitative PCR (Q-PCR) using the LightCycler system from Roche Diagnostics and cDNA obtained as described above. Q-PCR was performed using a QuantiMix Easy SYG kit from Biotools and specific primers. Results were normalized to the expression of the β-actin or 18S genes, and the cDNA copy number of each gene of interest was determined using a 7-point standard curve as we described before (7–9, 29, 30).

Primers

The sequences of oligonucleotides used and their annealing temperatures are shown in supplemental Fig. 3. All primers were synthesized, desalted, and purified by Bonsai Biotech.

ELISA for TNF-α, IL-6, and IL-10

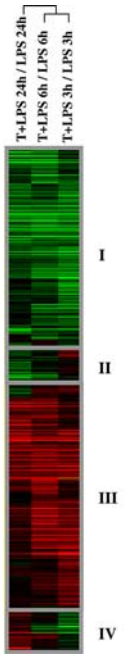
Concentrations of TNF-α in supernatants were determined using the ELISA development kit supplied by PeproTech. IL-6 and IL-10 levels in supernatants were also determined with two commercial ELISAs purchased from Bender MedSystems.

Protein arrays

We measured the concentrations of several cytokines in supernatants of cultured monocytes using the RayBio Human Inflammation Ab Array II from RayBiotech following the manufacturer's instructions. Data were normalized against positive controls. After that, arbitrary units of intensity were measured for each cytokine present in the membranes.

Flow cytometric analysis

Several surface proteins were detected using the specific Abs indicated above, as previously described (30).



Cluster	Representative genes
I	CASP1, CCL1, CCL13, CCL20, CCL22, CCL3, CCL4, CCL5, CCL8, CCL40, CD69, CD74, CD86, CIITA, CXCL10, CXCL11, CXCL9, FAS, FASLG, GZMK, IFI35, IFI44, IFNγ, IFNβ1, IL12p35, IL12p40, IL1α, IL23p19, IL6, IRF4, IRF7, IRF8, LITAF, MHCI-B, MHCI-F, MHCI-G, MHCII-DMβ, MHCII-DPα1, MHCII-DPβ1, MHCII-DQβ1, MHCII-DRα1, MHCII-DRβ1, MHCII-DRβ3 MHCII-DRβ4, SOCS1, STAT1, STAT4, TNFAIP2, TNFAIP3, TNFα, TRAF1, TRAF6
II	CD80, CD83, IRAK2, IRAK4, MHCI-A, MHCI-C, NFκB2/p100, TRAF3,
III	FPR1, IL10, IL19, IL24, IRAK-M, PIK3C3, PIK3CB, PIK3R1, PIK3R2, PTGES2, PTGS1, TGFα, TGFβ, TREM-1
IV	CCL24, CCL7, CD64, CSF2, SOCS3

FIGURE 4. Analysis of expression data identifies four gene clusters according to their behavior upon LPS restimulation. Differentially expressed genes in tolerant human monocytes, as identified by cDNA microarray-based profiling, are represented in form of a heat map (green to red scale; green, [T + LPS]/[LPS]) < 0; red, [T + LPS]/[LPS] > 0). Notice the separation in four distinct clusters, two of which (I and III, respectively) correspond to the previously coined tolerizable (T) and nontolerizable (NT) gene classes. Cluster II groups genes that were classified as NT after a 3-h rechallenge but T afterward, and for which we propose the terms late or slowly tolerizable genes. Finally, genes included in cluster IV were tolerizable at early points, but became nontolerizable later; they might be described as transiently tolerizable factors. Selected genes from each cluster are given next to the heat map; those verified by Q-PCR and/or protein microarrays are underlined.

Phagocytosis and bacteria killing assays

We followed protocols previously described by de las Heras and coworkers (31) or da Silva and coworkers (32).

Proliferation assay

We followed a protocol previously described by Hernandez-Fuentes and coworkers (33) and Adam and coworkers (34).

Data analysis

The number of experiments analyzed is indicated in each figure. Data were collected from a minimum of three experiments to calculate the mean ± SD. The statistical significance was calculated using the unpaired Student's *t* test, and differences were considered significant at p values of <0.05. For analysis of patients' data we used box-plot graphics and performed Mann-Whitney *U* tests using GraphPad Prism 4.0 software (GraphPad Software).

Results

Pretreatment of human monocytes with LPS induces endotoxin tolerance in vitro

During infection, monocytes are one of the primary effectors of innate immunity. However, after a first encounter with LPS, monocytes enter a refractoriness state termed endotoxin tolerance (ET), in which further responses to an endotoxin challenge are severely blunted. To study the mechanisms involved in ET in greater detail, we first set out to establish conditions under which this process can be reliably induced in isolated human cells.

The purity of human monocytes isolated as previously described (7, 8, 29) was verified by FACS analysis (Fig. 1*A*). Following the experimental design schematically depicted in Fig. 1*B*, cultures of human monocytes were pretreated or not with 10 ng/ml LPS for 1–8 h (t_{tol}), washed twice with PBS, and left overnight in the incubator (t_{rec} of 16 h) in the presence of complete medium. These cultures were then challenged again with 10 ng/ml LPS for 1 or 24 h (t_{st}). After each step, TNF-α concentrations in the culture supernatants were determined using a commercial ELISA (Fig. 1*C*). Our data indicate that pretreatment with LPS for 6 h induces an endotoxin tolerance state. Note that 1 h of LPS pretreatment was enough to induce a refractory state to further LPS challenges.

We subsequently performed experiments to determine the appropriate recovery time (t_{rec}, see Fig. 1*B*) and thus identify the "tolerance time frame" in our system. Cells that were pretreated with LPS for 8 h remained in a state of tolerance for at least 112 h (~5 days; Fig. 1, *D* and *E*). After 6 days, the response to LPS stimulation was similar in LPS-pretreated cultures and in controls (Fig. 1, *D* and *E*). Similar results were obtained when levels of IL-6 or IL-12 were followed instead (data not shown). Taking into account all of these data, we have established an in vitro model for further studies of endotoxin tolerance that consists of 8 h of LPS pretreatment (t_{tol}) and 16 h of recovery (t_{rec}) (Fig. 2*A*). In all experiments performed neither apoptosis nor reduction in the number of cells was observed, indicating that the refractory state does not influence cell viability (Fig. 2*B*).

Additionally, to verify the robustness of our model we also determined mRNA and protein levels of IL-6, a cytokine that is also crucially involved in endotoxin shock (Fig. 2, *C* and *D*). In other experiments, we determined that 0.01 ng/ml LPS was a suboptimal dose to induce endotoxin tolerance (Fig. 2*E*, section I). Moreover, we also tested different LPS concentrations (from 0.01 to 100 ng/ml) for monocyte restimulation (Fig. 2*E*, section II). We also verified that the observed phenomenon was unrelated to the particular LPS source (Fig. 2*F*). Additionally, no significant differences were detected between LPS and lipid A (LPA) from *E. coli* in this regard (Fig. 2*F*). Note that our ET model differs from previously reported protocols (7, 21, 35) in that we introduce a time of recovery; this period corresponds to the time frame between first and second infections in a clinical context.

Transcriptional profile of tolerant human monocytes

To study transcriptional changes associated with endotoxin tolerance in human monocytes, we performed a genome-wide microarray analysis to compare virgin (i.e., nonstimulated) monocytes (∅), monocytes stimulated once with LPS (∅ plus LPS), and tolerant monocytes restimulated with LPS (tol plus LPS). To understand the temporal regulation of genes during the ET phase, and to distinguish between early, intermediate, and late events, the last group was analyzed at three different times after a second LPS challenge

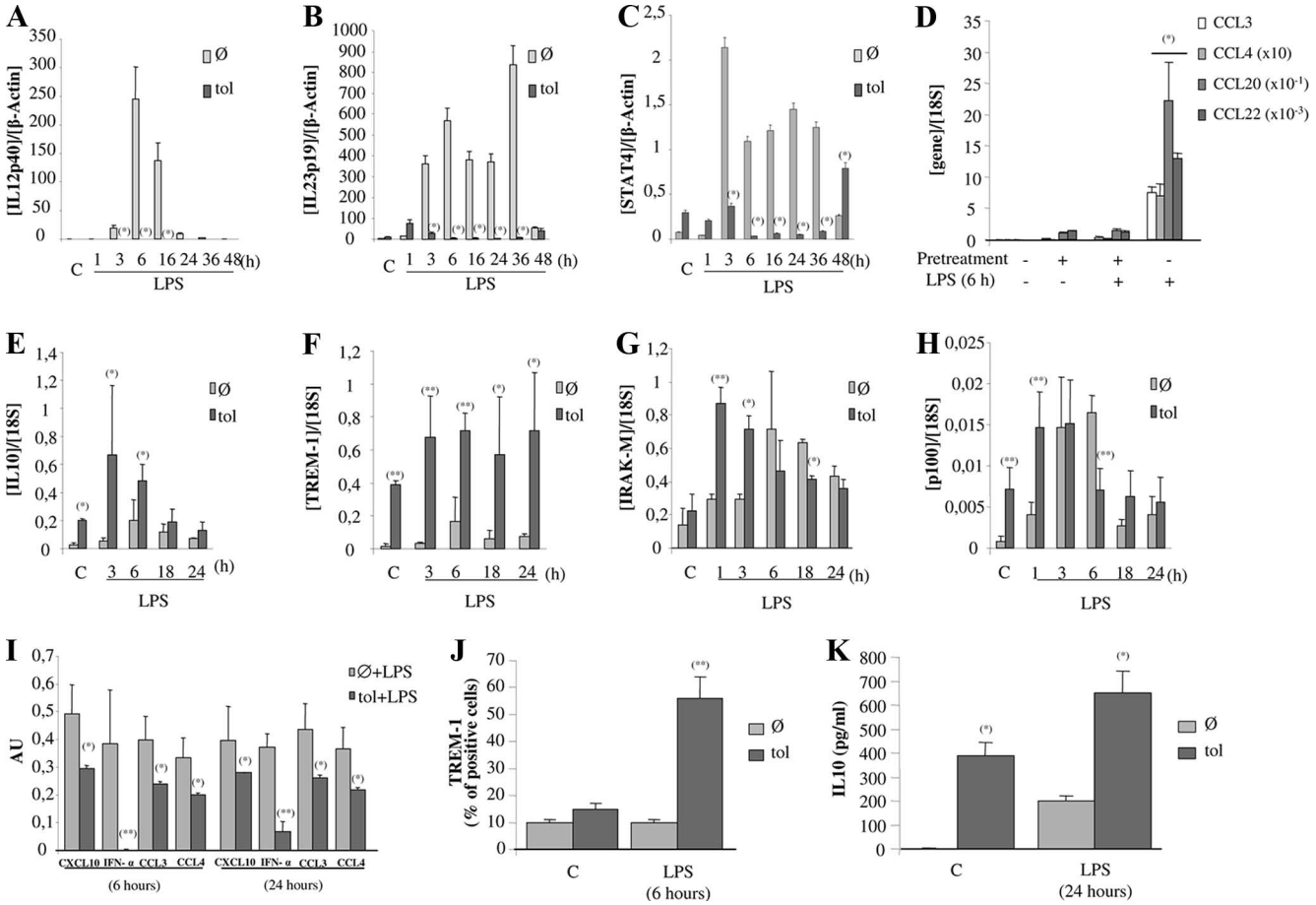


FIGURE 5. Real-time Q-PCR and protein expression analysis confirm major class T and NT genes identified by DNA microarray. *A–C*, Control (light gray bars) and tolerant human monocytes (dark gray bars) were treated or not with 10 ng/ml LPS for 1–48 h. Expression of *IL-12p40*, *IL-23p19*, and *STAT4* was analyzed by real-time Q-PCR. The ratio [gene]/[β-actin] is given ($n = 3$). $*$, $p < 0.01$ vs control (∅). *D*, Monocytes were pretreated (+) or not (−) with 10 ng/ml LPS and then stimulated with 10 ng/ml LPS for 6 h. mRNA levels of selected T class genes were analyzed by real-time Q-PCR; the ratio [gene]/[18S] is shown ($n = 3$). $*$, $p < 0.01$ vs control. *E–H*, Control (light gray bars) and tolerant human monocytes (dark gray bars) were treated or not (C) with 10 ng/ml LPS for 1–24 h. Expression of selected NT genes was analyzed by real-time Q-PCR. The ratio [gene]/[18S] is given ($n = 3$). $*$, $p < 0.05$ and $**$, $p < 0.01$ vs control (∅). *I*, Control (light gray bars) or tolerant human monocytes (dark gray bars) were stimulated with 10 ng/ml LPS for 6 or 24 h ($n = 3$). The concentrations of several proteins in the supernatants of these cultures were determined using a commercial protein array (Antibody Array II; RayBiotech). AU indicates arbitrary units. $*$, $p < 0.05$ and $**$, $p < 0.01$ vs control (∅). *J*, Control and tolerant human monocytes were incubated (LPS) or not (C) with 10 ng/ml LPS for 6 h. Cells were analyzed by flow cytometry; the percentage of TREM-positive cells is given ($n = 5$). $**$, $p < 0.01$ vs control. *K*, The same experiment was performed but LPS stimulation lasted 24 h. IL-10 concentration was evaluated in the supernatants of the cultures. $*$, $p < 0.01$ vs control.

(t_{st} of 3, 6, and 24 h). As expected, a large number of genes induced after a first encounter with the endotoxin were either not reinduced or induced to much lower levels upon a second challenge. Interestingly, a global analysis revealed that expressed genes could be classified into two major classes: nontolerizable (class NT) and tolerizable genes (class T), according to whether they were induced or not in tolerant monocytes upon reexposure to endotoxin. This classification follows the one previously introduced by Foster and coworkers (21).

Data obtained from microarray analyses are given as a two-dimensional plots in Fig. 3, *left panels*, which explicitly shows the regions occupied by NT ($x > 0$, $y < 0$) and T class genes ($x > 0$, $y > 0$). (Note that the abscises are $x = \log_2([\varnothing \text{ plus LPS}]/[\varnothing])$, and thus values of $x > 0$ correspond to genes stimulated in a first encounter with the endotoxin, while the ordinates are $y = \log_2([\varnothing \text{ plus LPS}]/[\text{tol plus LPS}])$; genes for which $y > 0$ are induced at lower levels in tolerant monocytes.) Besides each two-dimensional plot, the right panel in Fig. 3 includes a selected group of genes with $x > 0$, ordered according to their y values; the complete lists of genes are given in supplemental Fig. 1 (Gene Expression Omnibus (GEO) database, Ref. No. 15595513, www.ncbi.nlm.nih.gov/geo/). The percentage of T genes decreases slightly from t_{st} of 3 h (39%) to t_{st} of 24 h (31%), while that of NT genes was similar at the three time points analyzed (between 15% and 19%). Analysis of our findings revealed that class T genes group several factors that either promote inflammation, such as TNF-α, IL-6, IL-12p40, and IFN-γ, or that they regulate Ag presentation (e.g., MCHII-DRβ1, -DQβ1 and -DPα1). In contrast, the NT class cluster is populated with factors that reduce or block inflammation signaling such as IRAK-M. Additionally, this class also includes several antimicrobial effectors such as formyl peptide receptor 1 (FPR1), acyloxyacyl hydrolase (AOAH), and RNase T2 (RNASET2). This last finding is in agreement with previously published data (21).

Nevertheless, it must be stressed that several genes were classified as either T or NT depending on the restimulation time. Indeed, a heat map analysis of expressed genes identifies four different gene clusters (Fig. 4). While clusters I and III clearly

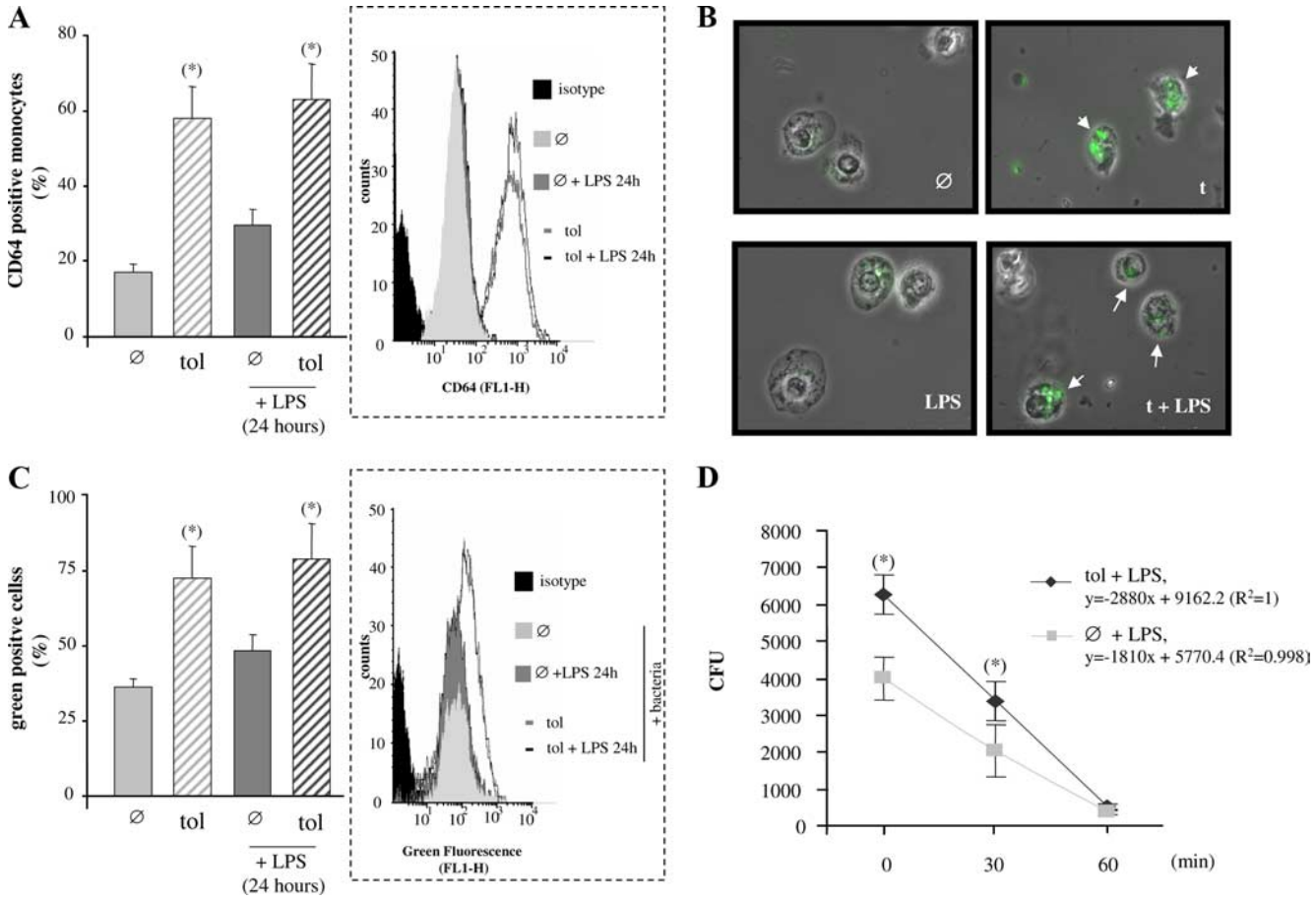


FIGURE 6. Tolerant monocytes overexpress CD64 and exhibit high phagocytic ability. Control (∅) and tolerant human monocytes (tol) were cultured in the presence or absence of 10 ng/ml LPS for 24 h. *A*, The percentage of $CD64^+$ cells is given, as analyzed by flow cytometry; a representative histogram is shown in the inset ($n = 3$). *, $p < 0.05$ vs control (∅). Monocyte cultures were exposed to *E. coli* DH5α bacteria expressing isopropyl β-D-thiogalactoside-inducible GFP ($\sim 10^8$ bacteria/ml) for 1 h. Adherent cells were harvested and analyzed by (*B*) fluorescent microscopy (some monocyte-internalized bacteria are indicated with arrows) and (*C*) flow cytometry; the percentage of green-positive monocytes is given and a representative histogram is shown in the inset ($n = 3$). *, $p < 0.05$ vs control. *D*, *E. coli* cells were incubated with control or tolerant monocytes at 37°C for 30 min. The cells were either lysed by incubation on ice with 0.5% of Triton X-100 for 10 min or incubated at 37°C for up to 60 min before lysis in the same conditions. After monocyte lysis the number of intracellular CFUs was determined after overnight growth on selective medium ($n = 2$). *, $p < 0.05$ vs control.

corresponded to the T and NT gene classes described above, respectively, clusters II and IV grouped genes that could be classified as T or NT depending on the time passed after the second challenge with endotoxin. Cluster II contained genes that were NT at early points and T at a later time, which can be considered as late or slow tolerizable factors (e.g., IRAK2 and IRAK4), while cluster IV grouped genes that could be classified as T at early stages but became NT at later stages (transiently tolerizable genes, such as CCL24 and SOCS3).

Real-time PCR analysis verifies differential regulation of T and NT class genes

To verify our microarray data, we analyzed mRNA expression of a representative set of genes. Three class T genes (*IL-12p40*, *IL-23p19*, and *stat4*) were clearly up-regulated after a first LPS challenge in control human monocytes (∅), but their mRNA levels were significantly reduced in tolerant cells (tol) (Fig. 5*A–C*). Cytokines IL-12 and IL-23 have emerged as classical markers of a proinflammatory state in human monocytes, and low expression levels are characteristic of alternatively activated or M2 monocytes (23, 36). In our model, these two cytokines were down-regulated in tolerant cells for at least 48 h (Fig. 5, *A* and *B*). These data corroborated both our ET model and the results from microarrays analysis.

Additionally, it has been reported that LPS-treated monocytes express high levels of STAT4, while the Th2 cytokine IL-4 and IL-10, specifically down-regulate *Stat4* expression in activated monocytes (37). Our data indicate that this important dual factor is also down-regulated in tolerant cells during the first 36 h of LPS stimulation (Fig. 5*C*). Thus, *Stat4* down-regulation could be considered as a new marker of ET in these cells. We also verified down-regulation of other T class genes in tolerant cells, such as chemokines CCL3, CCL4, CCL20, and CCL22 (Fig. 5*D*). These findings corroborate data obtained from microarray analysis, and they point to a strict control of a specific subset of chemokines and cytokines during the tolerant state. It is particularly noteworthy that the well-defined Th2 chemokine CCL22 is down-regulated in this context, because it is expressed after inflammatory stimulation and is known to act as a bridge to an alternative activation pathway (38).

In a similar manner, we also verified microarray results regarding four NT genes (*IL-10*, *TREM-1*, *IRAK-M*, and *NFκB2/p100*; Fig. 5, *E–H*). Our results concerning the NT character of IL-10 (Fig. 5*E*) reproduce findings previously published by others (39–42). Collectively, our present data confirm that tolerant monocytes exhibit an $IL\text{-}12^{low}IL\text{-}23^{low}IL\text{-}10^{high}$ profile that has been previously linked to M2 cells (23, 36). In this regard, faster expression of IRAK-M in tolerant cultures than in control

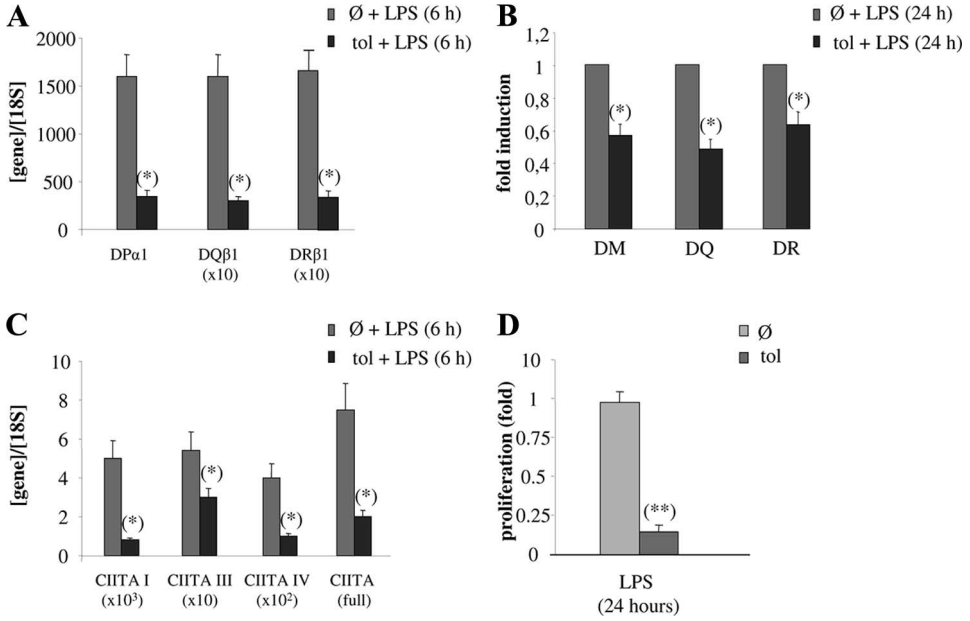


FIGURE 7. MHC class II molecules are down-regulated and Ag presentation is impaired in tolerant monocytes. Control ($\varnothing$) and tolerant (tol) monocytes were treated or not with 10 ng/ml LPS for 6 (*A* and *C*) or 24 h (*B* and *D*). *A* and *C*, Real-time Q-PCR analysis was performed for the following genes: *DPα1*, *DQβ1*, and *DRβ1* (*A*), as well as for *CIITA* and its splice variants, *CIITA I*, *CIITA III*, and *CIITA IV* (*C*) ($n = 4$). *, $p < 0.05$ vs control ($\varnothing$ + LPS). *B*, Cell surface expression of DM, DQ, and DR, as analyzed by flow cytometry ($n = 3$). *, $p < 0.05$ vs control ($\varnothing$ + LPS). *D*, Human heterologous lymphocytes, a nonadherent cell population in the protocol used to obtain monocytes, were labeled with the membrane stain PKH2 green fluorescent cell linker kit (Sigma-Aldrich). Lymphocyte proliferation was measured as loss of green fluorescence intensity in the $CD3^+$ gate; for this analysis, cultures of stained lymphocytes not exposed to monocytes were used as control. The fold induction is shown ($n = 4$). **, $p < 0.01$ vs control ($\varnothing$).

cultures is fully consistent with our previous results (7). Finally, we have confirmed that both TREM-1 and NF-κB2 are up-regulated after LPS stimulation in tolerant monocytes (Fig. 5, *F* and *H*).

Protein expression data also corroborate the results of microarray analysis

In addition to the RT-PCR analysis, we have also studied the translation of a set of selected genes into proteins. This set combines well-established and newly identified ET markers. In particular, we have verified the reduced expression of four class T genes (*IFNα*, *CCL3*, *CCL4*, and *CXCL10*) at two different points (6 and 24 h after LPS restimulation, t_{st}) using a commercial protein microarray (Fig. 5*I*). Moreover, cell surface expression of TREM-1, as well as IL-10 release into the culture supernatant, was also studied by flow cytometry and ELISA, respectively (Fig. 5, *J* and *K*). The data obtained are in perfect agreement with both microarray and RT-PCR analysis.

Phagocytosis is enhanced in tolerant human monocytes

Microarray data indicated that tolerant monocytes up-regulate important genes with a negative role in inflammation or are even responsible for an alternative activation pathway (e.g., *IRAK-M* and *NFκB2/p100*). Additionally, expression of other factors such as CD64 and down-regulation of MHC class II genes was also observed in tolerant cells. Because the cell surface receptor CD64 is a well-known marker for phagocytosis (25), we hypothesized that its increased expression in tolerant monocytes, verified using flow cytometric analysis (Fig. 6*A*), would result in an elevated phagocytic activity by these cells. Interestingly, levels of CD64 expression and phagocytic activity were similar in tolerant cells and in tolerant cells restimulated with LPS. This enhanced phagocytic ability of tolerant monocytes was verified with a standard phagocytosis assay of GFP-labeled bacteria (Fig. 6, *B* and *C*). Additionally, internalized *E. coli* cells were killed at least as efficiently in control monocytes as in tolerant cells stimulated with LPS (Fig. 6*D*; note that the initial differences between tolerant and control cells were due to internalization of higher amounts of bacteria by tolerant macrophages). Our data establish that enhanced phagocytic activity is a physiologically relevant feature of ET monocytes.

Low Ag presentation in tolerant monocytes

In addition to a potent phagocytic function, CD64 is also associated with a low Ag presentation capacity (25, 26). Furthermore, data from our microarray studies indicated that several factors involved in Ag presentation are differentially regulated in tolerant monocytes (Figs. 3 and 4). To study this feature in more depth, we first analyzed the genetic expression of three important MHC class II molecules. Because HLA class II genes are down-regulated in tolerant monocytes (Figs. 3 and 4), and given the presence of three different isotypes of MHC class II molecules (HLA-DP, HLA-DQ, and HLA-DR), we decided to study a representative haplotype of each isotype (DPα1, DQβ1, and DRβ1, respectively) by RT-PCR. Data presented in Fig. 7*A* undoubtedly indicate that these three factors are down-regulated in tolerant cells; restimulation of tolerant monocytes with LPS did not induce mRNA expression of these genes, as observed in control cells challenged with the endotoxin. Furthermore, we analyzed cell surface expression of two of these isotypes (DQ and DR), as well as of the chaperone, DM, by flow cytometry. As Fig. 7*B* illustrates, not only are mRNA levels lower in tolerant cells, but protein expression is also significantly diminished.

These observations prompted us to study the master regulator of MHC class II gene expression, CIITA (43–45). In agreement with previously published data (46), our microarray analysis indicated a

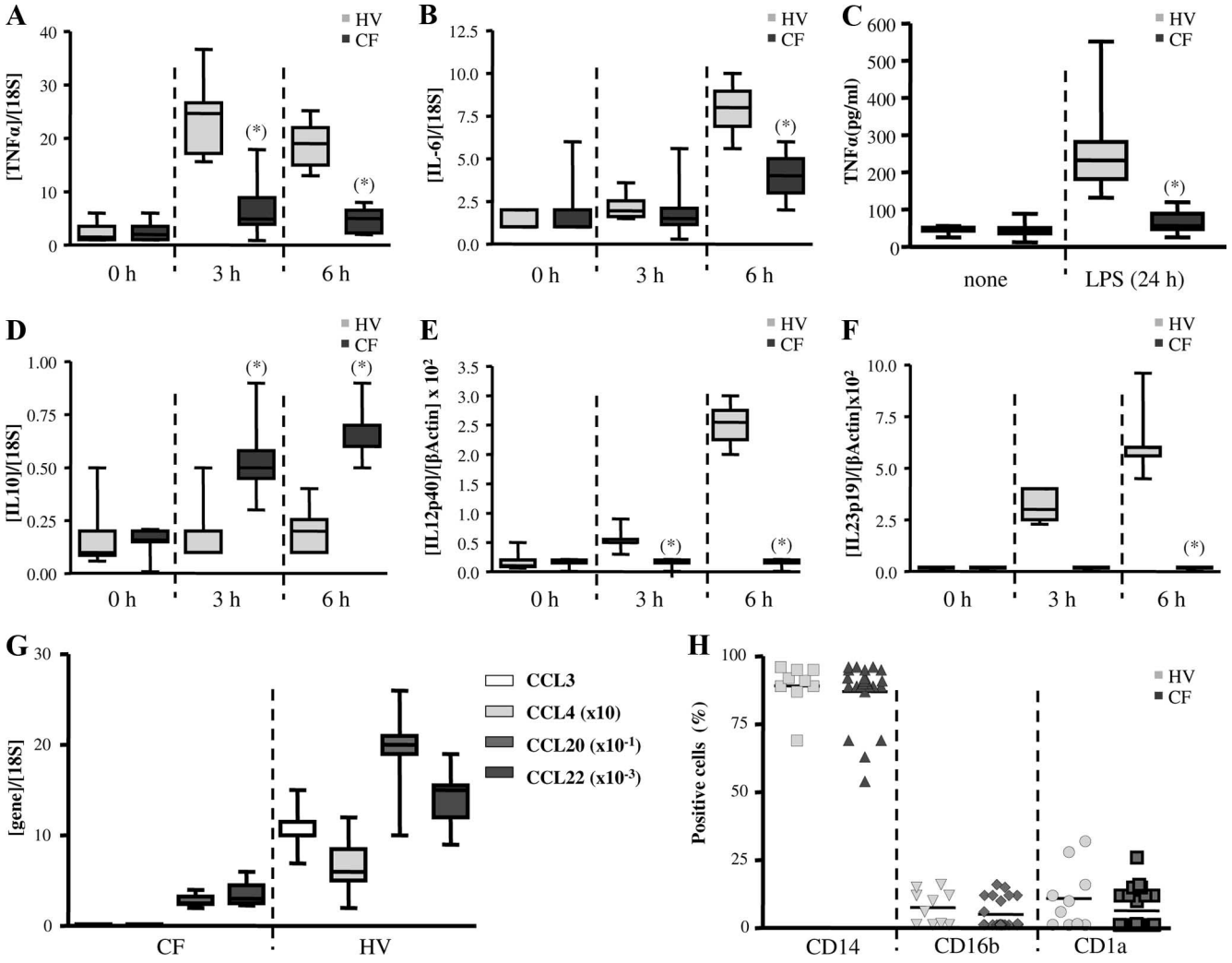


FIGURE 8. Inflammatory responses are impaired in monocytes from CF patients. Monocytes from healthy volunteers (HV, gray boxes, $n = 10$) and from CF patients (CF, black boxes, $n = 25$) were cultured in the presence of 10 ng/ml LPS for indicated times, and mRNA levels of TNF-α (*A*), IL-6 (*B*), IL-10 (*D*), IL12p40 (*E*), and IL23p19 (*F*) were determined by real-time Q-PCR. The ratios [gene]/[18S] are depicted. $*$, $p < 0.01$ vs HV. *C*, Concentrations of TNF-α were determined in supernatants of cultures of human monocytes from healthy volunteers (gray boxes) and CF patients (solid boxes), stimulated or not with 10 ng/ml LPS for 24 h. $*$, $p < 0.05$ vs HV. *G*, Levels of several CCLs were analyzed by real-time Q-PCR in HV and CF monocytes stimulated ex vivo with 10 ng/ml LPS for 6 h. The ratios [gene]/[18S] are depicted. $*$, $p < 0.01$ vs HV. *A–G*, Box plots were used in all cases; horizontal lines represent the median values. Values for p were calculated by the Mann-Whitney U test. *H*, Levels of CD14, CD16b, and CD1a in monocytes isolated from healthy volunteers (gray) and CF patients (dark), as determined by flow cytometry; the fraction of cells stained with specific Abs is given.

down-regulation of CIITA in tolerant monocytes. Given the existence of three splicing variants of CIITA (47, 48), we decided to analyze expression of these three variants (CIITA I, III, and IV), as well as of the full-length form. Our data indicate that all four CIITA forms are down-regulated in tolerant monocytes (Fig. 7*C*). Finally, we have verified the functional impact of CIITA/MHC class II down-regulation via a standard lymphocyte proliferation assay (33, 34). In full agreement with the findings presented above, Ag presentation is significantly impaired in tolerant human monocytes (Fig. 7*D*). Additionally, we are not aware of any direct antiproliferative activity of LPS under these conditions (data not shown).

Circulating monocytes from CF patients exhibit a tolerant phenotype

CF patients suffer from recurrent infections and are prone to bacterial colonization. We have recently reported that circulating monocytes isolated from these patients are "locked" in an ET state (9). To explore the pathophysiological implications of our present findings, we have analyzed the ex vivo effect of LPS stimulation in circulating monocytes isolated from 25 CF patients aged 30 ± 9 years (mean ± SD). Clinical and genetic data of CF patients included in the present study are summarized in supplemental Fig. 2. Their body mass index was 22.2 ± 3.1 kg/m^2, their FEV_1 (percentage predicted) was 41 ± 15%, their FEV_1/FVC ratio was 53 ± 10%, and the mean incidence of pulmonary exacerbations in the previous year was 1.9 episodes (range, 0–5 episodes). We corroborated the tolerant phenotype in the current cohort by showing that the expression of proinflammatory cytokines TNF-α and IL-6 is significantly attenuated in LPS-challenged monocytes isolated from CF patients (Fig. 8, *A–C*). We also confirmed the IL-12^{low}IL-23^{low}IL-10^{high} phenotype and the down-regulation of CCL3, CCL4, CCL20, and CCL22 after LPS challenge (Fig. 8, *D–G*). Note that ~90% of these cells were positive for CD14, while very low levels of CD16b and CD1a were detected (Fig. 8*H*).

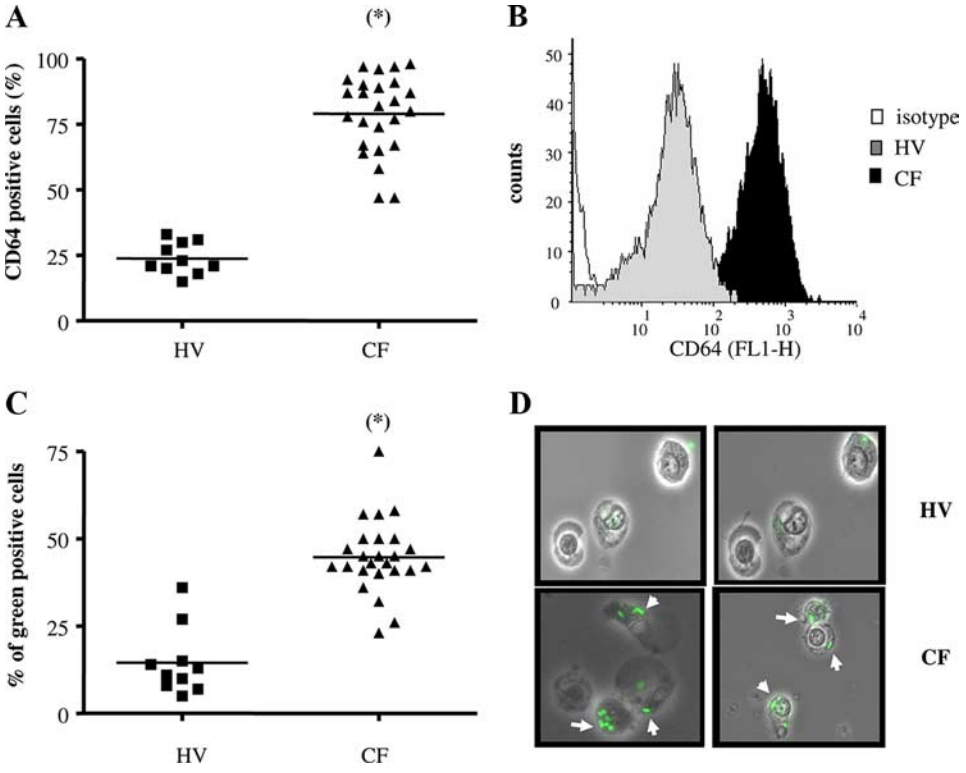


FIGURE 9. CF monocytes overexpress CD64 and exhibit high phagocytic ability. *A*, Percentages of $CD64^+$ cells in monocytes from healthy volunteers (HV, $n = 10$) and CF patients (CF, $n = 25$), as assessed by flow cytometry. Horizontal lines represent the median values. *, $p < 0.01$ vs control group (HV). Values for p were calculated by the Mann-Whitney U test; a representative histogram is shown in *B*. Cultures of CF monocytes and controls were exposed to GFP-labeled *E. coli* DH5α bacteria for 1 h according to the protocol described in *Materials and Methods*. Next, adherent cells were harvested and cell internalization was analyzed by both flow cytometry (*C*, percentage of green-positive cells is given) and fluorescence microscopy (*D*, two representative images from each group are shown; clusters of internalized bacteria in monocytes from CF patients are highlighted by arrows).

Monocytes from CF patients show both high phagocytic activity and low Ag presentation capability

As expected, levels of CD64 were significantly higher in CF monocytes than in healthy controls (Fig. 9, *A* and *B*), thus suggesting an enhanced phagocytic activity in CF. This feature was verified with a phagocytosis assay of GFP-labeled *E. coli* cells (Fig. 9, *C* and *D*). Furthermore, and as observed in monocytes in which the ET state was experimentally induced, MHC class II isotypes (HLA-DP, HLA-DQ, and HLA-DR), as well as CIITA, are clearly down-regulated at the mRNA level in cells isolated from CF patients (Fig. 10*A–D*). Basal levels of these factors were significantly lower in CF patients than in healthy volunteers, and LPS challenge failed to increase their mRNA expression. Additionally, cell surface expression of DM, DQ, and DR was also reduced in CF, when compared with control monocytes (Fig. 10*E*). Finally, the results of a proliferation assay indicated that the Ag presentation machinery is impaired in CF monocytes (Fig. 10*F*).

Discussion

Despite several previous studies that address the mechanisms underlying endotoxin tolerance, a complete description of this phenomenon is still lacking. Here, we have established a robust and reproducible model to study ET in human monocytes. Our data indicate that even a short-term exposition to LPS (1 h) suffices to induce some level of endotoxin tolerance in these cells. The refractory state, however, is more evident when this challenge with LPS lasts for 6–8 h, and this "tolerance memory" is fully kept for up to ~5 days. Only after this period of time do human monocytes "forget" their previous encounter with the endotoxin, but considering their relatively short lifespan they cannot be functionally available for much longer. Importantly, we have observed that the refractory state does not influence cell viability. This finding suggests that monocyte counts could be normal during an infection-induced ET state, but a significant proportion of these cells would not be functional. Along these lines, a refractory state in circulating monocytes isolated from septic patients (confirmed by low levels of TNF-α and IL-6 after ex vivo LPS stimulation) correlates with longer stays in intensive care units, more ventilator days, and a higher incidence of infection (49). Additionally, monocytes isolated from patients who went on to survive their septic episode were found to regain LPS responsiveness, whereas normal reactivity was never restored in nonsurvivors (50). Thus, the phenomenon of endotoxin tolerance is thought to play an important role in the susceptibility to reinfection in patients with severe sepsis (4). We stress that our conclusions are based on data obtained by different and complementary techniques (e.g., real-time Q-PCR, ELISA). It is also noteworthy that our model reproduces the time frame between first and second infections in a clinical context by introducing a definite recovery period between first and second exposure to LPS.

Using our in vitro model, we performed a thorough transcriptional analysis using microarrays to explore the mechanisms involved in ET. A global analysis of the monocyte transcriptome revealed that most of the expressed genes could be grouped into two major classes: NT (nontolerizable) and T (tolerizable), depending on whether they are induced or not in tolerant cells upon a second exposure to LPS, and in complete agreement with recently published data (21). While class T genes are mainly proinflammatory cytokines such as TNF-α, IL-6, and IL-12, class NT is more varied and includes antimicrobial factors (e.g., FPR1, AOAH, and RNASET2), negative regulators of inflammatory signaling such as IRAK-M, a crucial element of the noncanonical NF-κB pathway (NF-κB2/p100), and a well-known marker of alternative activation (IL-10). The up-regulation of class NT genes in tolerant cells points to a profound reprogramming of human monocytes in their tolerance time frame. Thus, the term "immunoparalysis", which has been coined in connection with tolerant monocytes, does not seem to grasp the essence of this phenomenon; "immunomodulation" or "reprogramming" would seem to be more

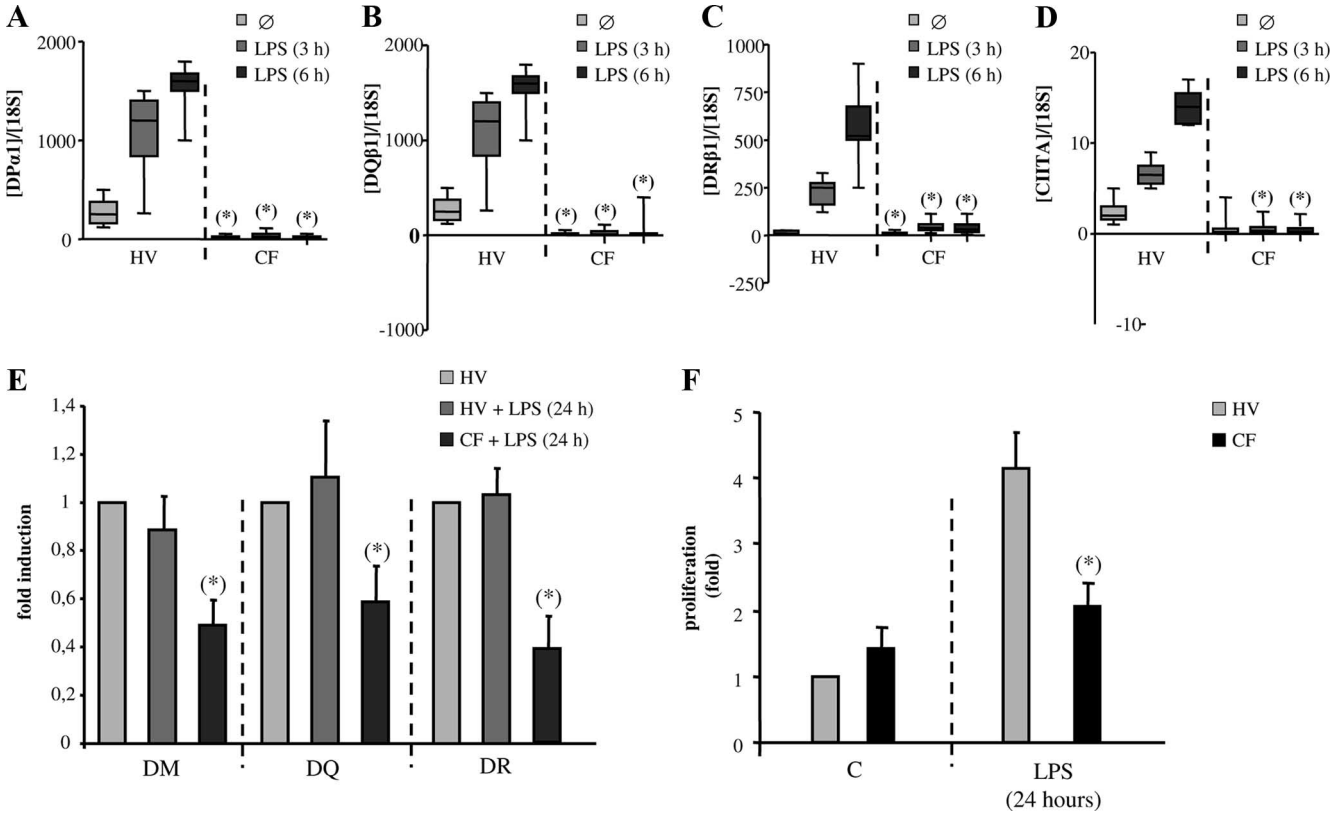


FIGURE 10. MHC class II molecules are down-regulated and Ag presentation is impaired in CF monocytes. Monocytes from healthy volunteers (HV, $n = 10$) and from CF patients (CF, $n = 25$) were cultured in the presence of 10 ng/ml LPS for indicated times. DPα1 (*A*), DQβ1 (*B*), DRβ1 (*C*), and CIITA (*D*) mRNA levels were determined by real-time Q-PCR. The ratios [gene]/[18S] are depicted. *, $p < 0.01$ with respect to HV. Box plots were used to summarize the data; horizontal lines represent the median values. *E*, Cell surface expression levels of DM, DQ, and DR, as analyzed by flow cytometry with appropriate Abs. The fold induction is shown ($n = 3$). *, $p < 0.05$ with respect to control (HV + LPS). *F*, Heterologous human lymphocytes isolated from healthy volunteers were labeled with the membrane stain PKH2 green fluorescent cell linker kit. Following ex vivo LPS stimulation of monocyte cultures for 24 h, stained lymphocytes were added to the plate as responder cells in a relation of 1:5. After three days, nonadherent cells were harvested, and lymphocyte proliferation was assessed by flow cytometry as loss of green fluorescence intensity in the $CD3^+$ gate; for this analysis, cultures of stained lymphocytes not exposed to monocytes along the 3 days of the experiment were used as control. The fold induction is shown ($n = 4$). *, $p < 0.05$ vs control (HV).

appropriate. Additionally, our results suggest that endotoxin tolerance is a dynamic process since an important subset of genes could be classified either as NT or T depending on the time of restimulation (e.g., IRAK2, IRAK4, TRAF3, and SOCS3). Furthermore, and in agreement with previous works, we have also observed an IL-12^{low}IL-23^{low}IL-10^{high} phenotype associated with endotoxin tolerance, which is distinctive of M2 monocytes (51). Our data indicate that tolerant cells down-regulate their inflammatory responses while maintaining an important antimicrobial activity (see below).

We have also observed overexpression of two other important factors in tolerant monocytes: a negative regulator of the TLR pathway, the pseudokinase IRAK-M (7, 11, 18), and the amplifier of innate immune responses, TREM-1 (52). Despite these apparently opposing roles, both genes undergo a significant up-regulation after LPS challenge in tolerant monocytes. We and others have repeatedly found IRAK-M as a negative regulator of inflammation (11, 18, 53), which is in particular overexpressed in ET monocytes (7, 8, 18). The present microarray data confirm these previous results, and the pseudokinase is more rapidly expressed in tolerant cells than in controls after LPS challenge. TREM-1 is as an important proinflammatory factor that strongly enhances leukocyte activation in the presence of microbial products (54), amplifying the innate immune response of both macrophages and neutrophils (54). Despite its well-established role in the amplification of inflammatory responses, TREM-1 was unambiguously identified as a class NT gene in our analysis. The reasons for this apparent contradiction are not known at present. We have recently reported that metalloproteases shed a soluble form of TREM-1 (sTREM-1) via proteolytic cleavage of the membrane anchored form (30). Additionally, the results of several independent lines of research strongly suggest an antiinflammatory role for sTREM-1 (54). We are tempted to speculate that up-regulation of TREM-1 during ET could enhance the source of this negative regulator of inflammation. This putative role of TREM-1 in the control of ET will be addressed in further studies. In this regard, TREM-1 and other NT class genes emerge as putative ET markers, which could be useful for diagnosis of bacterial infections.

Besides overexpression of negative regulators of inflammation and antimicrobial factors, we have observed a clear increase of the well-known marker for phagocytosis, the cell surface receptor CD64, in tolerant human monocytes. CD64 up-regulation correlates with an elevated phagocytic activity of these cells. These results offer a straightforward explanation for recently published data indicating that endotoxin tolerance increases bacterial clearance in mice (55), also in line with human monocytes' enhanced capability to internalize pathogens during the endotoxin tolerance time frame (56). However, both control and tolerant cells exhibited similar ability to kill internalized bacteria.

In addition to a potent phagocytic capacity, CD64 is also associated with impaired Ag presentation function (25, 26). In this

regard, we have observed that several additional factors involved in Ag presentation are down-regulated in tolerant monocytes. Previously, other authors have described a reduced expression of MHC class II molecules during endotoxin tolerance in human monocytes, which has been associated with a diminished capacity for Ag presentation (46, 56). Similar observations have been recently reported for the Ag-presenting molecule, HLA-DR, and the chaperone necessary for loading MHC class II receptors with Ag peptide, HLA-DM (56). Moreover, low HLA-DR expression has been associated with poor outcomes in septic patients and has served as a better predictor of mortality than organ dysfunction scores such as the Sequential Organ Failure Assessment (SOFA ref (57)). Our findings show that not only HLA-DR and HLA-DM are affected during endotoxin tolerance, but that other factors involved in Ag presentation (e.g., HLA-DQ, HLA-DP) are deregulated in tolerant human monocytes as well. In particular, several haplotypes of MHC class II molecules have emerged as class T genes from our microarray analysis (e.g., DRβ1, DRβ3, DQβ1, DPα1, and DMβ).

It has been suggested that down-regulation of MHC class II in tolerant monocytes results from the inhibition of p38 MAPK during the endotoxin tolerance phase (56, 58). Here, we show that all four splicing variants of the master regulator of MHC class II genes, CIITA, are significantly down-regulated in tolerant cells. This finding suggests that, besides impaired p38 activation, MHC class II deregulation during ET could be caused by reduced levels of CIITA. We have also observed a significant down-regulation of some costimulatory molecules (e.g., CD80, CD83, and CD86) in tolerant cells. Taking into account all of these data, we hypothesized that Ag presentation would be diminished in tolerant human monocytes. This was confirmed with a standard alloimmune lymphocyte proliferation assay: while naive human monocytes challenged with LPS were able to induce proliferation of heterologous lymphocytes, tolerant monocytes retreated with LPS exhibited a significantly lower stimulation of lymphocyte proliferation.

Collectively, our results indicate that ET monocytes exhibit a reduced inflammatory response, that is, increased phagocytic ability coupled with a conserved capacity to kill internalized pathogens, but that their Ag presentation function is impaired. On one hand, poor proinflammatory activity together with high phagocytic ability might avoid development of septic shock, while allowing bacterial clearance. On the other hand, failure in Ag presentation inhibits an appropriate switch to adaptive immune response. Thus, a fine balance between refractory and inflammatory states appears to be a compromise solution in clinical situations.

To explore the pathophysiological implications of the tolerant phenotype of human monocytes, we decided to study tolerant monocytes from patients with CF, an autosomal recessive disorder caused by mutations in the CFTR. CF manifests as a multiorgan disease involving the pancreas, salivary glands, genital tubes, and liver canaliculi. Nevertheless, involvement of the respiratory tract, with continuous infectious episodes with *Staphylococcus aureus* and *Pseudomonas aeruginosa*, is the dominant clinical feature, and lung colonization accounts for most of the morbidity and mortality in these patients (59).

We have recently reported that circulating monocytes from CF patients are "locked" in an endotoxin refractory state (9), which was verified in the current cohort. Circulating cells from 25 CF patients were screened for cytokine production, phagocytosis capability, and Ag presentation. We observed a drastic down-regulation of proinflammatory cytokines such as TNF-α, IL-6, IL-12, and IL-23 in ex vivo LPS-stimulated monocytes from these patients in comparison to those from healthy volunteers. Additionally, CF monocytes down-regulated a set of CCLs (CCL3, CCL4, CCL20, and CCL22) after ex vivo challenge with LPS, similar to human monocytes in which the ET state was experimentally induced. Other authors have shown that exposure of primary tracheobronchial epithelial cells to *P. aeruginosa* products also causes selective tolerance via down-regulation of the TLR signaling pathway, likely due to decreased IRAK-1 protein levels and inhibition of its phosphorylation (60).

Furthermore, CF monocytes exhibited high CD64 and low MHC class II expression at both mRNA and protein levels. These findings correlated with a high phagocytic activity and low Ag presentation ability. Our findings are in apparent contradiction with a previous report of significantly reduced phagocytosis observed in lung phagocytes from young CF patients (61), although phagocytosis was normal in peripheral blood phagocytes. Nevertheless, there were important methodological differences between both studies. In contrast to our stable CF adult patients, Alexis and coworkers studied 12 nonstable CF children (6 ± 1 years of age) who underwent clinically indicated bronchoscopies because of worsening respiratory symptoms suggestive of lower airway infection. The authors do not provide information about the use of antibiotics or inhaled corticosteroids, but probably the most serious bias comes from their control group, since 12 control children were not healthy (recurrent pneumonia or bronchitis, upper airway obstruction, hemoptysis, chronic cough, or tracheostomy evaluation). Besides, in five of them pathogenic microorganisms (*Moraxella catarrhalis*, *Streptococcus pneumoniae*, and *Haemophilus influenzae*) were present in bronchoalveolar lavage fluid.

In any case, our results do not allow assessing whether factors of the airway microenvironment might contribute to reduced local phagocytosis. However, in vitro experiments to assess the ability of neutrophils to engulf *P. aeruginosa* revealed a similar efficiency of phagocytosis between neutrophils incubated in normal or CF airway surface liquid (62). In this regard, several observations suggest that CF patients might possess a cell-independent defect in microbial killing due to an intrinsic deficiency in the antimicrobial properties of the airway surface liquid itself (62).

Additionally, there is some previous evidence that is consistent with our finding of a diminished capacity for Ag presentation in CF monocytes. For instance, a reduction of HLA-DR expression has been described in children with CF (61). Moreover, it has been reported that neutrophil elastase-containing secretions from CF patients can disable dendritic cells by reducing their Ag-presenting activity (63).

Endotoxin tolerance might have opposing effects in CF patients. On one hand, defective secretion of Abs and cytokines in response to Ags may underlie a long-suspected, but as yet unproved CFTR-mediated immunological defect underlying pulmonary morbidity and mortality in CF. On the other hand, ET might explain why, despite a high frequency of infection, CF patients do not suffer from a permanent hyperinflammatory state, and the inflammatory response is localized (9, 64). In contrast, patients suffering from another pulmonary disease, chronic obstructive pulmonary disease, who are not in a refractory state (9) exhibit higher levels of inflammatory mediators (65). An impaired Ag presentation ability associated with repeated endotoxin tolerance states might explain the reported Th2 imbalance in the pulmonary immune response to bacteria in CF patients. Besides, underexpression of MHC class II molecules during endotoxin tolerance might contribute to the development of asthma or allergic bronchopulmonary aspergillosis, both of which appear more frequently in CF patients. It has been suggested that HLA-DR molecules such as DR2 and DR5, and possibly also DR4 and/or DR7, contribute to disease susceptibility,

while HLA-DQ2 favors resistance. A combination of these elements determines the outcome of allergic bronchopulmonary aspergillosis in patients with CF (66).

In summary, we demonstrate that tolerant human monocytes are characterized by rapid IRAK-M overexpression, high levels of TREM-1 and CD64, and low HLA expression. We also confirmed the IL-12^{low}IL-23^{low}IL-10^{high} pattern of IL expression. These molecular markers were associated with high phagocytic activity but low Ag presentation ability. Additionally, we demonstrate that the phenotype of CF monocytes mirrors the endotoxin tolerance state in a pathological setting. These findings validate the data obtained with our in vitro model system, and they provide a thorough characterization of CF circulating monocytes. Our results may need to be considered in several clinical situations, and they constitute the basis for the study of ET in other pathological situations. Besides, and given the increasing interest in exploiting the tolerant behavior of human cells as a therapeutic target, they pave the way for developing therapies that better predict and consider the risk of infection in patients with compromised immune responses.

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Disclosures

The authors have no financial conflicts of interest.

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