Bacterial reprogramming of PBMCs impairs monocyte phagocytosis and modulates adaptive T cell responses

Maya C. André,*,†,1 *Christian Gille,** *Philip Glemser,** *Jeanette Woiterski,*† *Hsin-Yun Hsu,*†,§
Baerbel Spring,* *Hildegard Keppeler,*‖ *Boris W. Kramer,*¶ *Rupert Handgretinger,*†
Christian F. Poets,* *Kirsten Lauber,*‖,#,2 *and Thorsten W. Orlikowsky**,**,2

Departments of *Neonatology and †Pediatric Hematology and Oncology, University Children's Hospital, Tuebingen, and
**Department of Neonatology, University Children's Hospital, Aachen, Germany; ‡NMI Natural and Medical Sciences Institute and
‖Department of Internal Medicine I, University of Tuebingen, Germany; §Department of Applied Chemistry, National Chiao Tung
University, Hsinchu, Taiwan; ¶Department of Pediatrics, Maastricht University Medical Center, Maastricht, The Netherlands; and
#Department of Radiation Oncology, Molecular Oncology, Ludwig-Maximilians-University of Munich, Germany

ABSTRACT

Septic diseases are characterized by an initial systemic, proinflammatory phase, followed by a period of anti-inflammation. In the context of the latter, monocytes have been described to display altered functions, including reduced TNF secretion and T cell-stimulating capacities in response to recall antigens. This hyporesponsiveness is supposed to be detrimental for coping with secondary infections. We here characterize bacterially reprogrammed PBMC-derived monocytes with special focus on their phagocytic activity. Hence, we have implemented a surrogate model of the early, postinflammatory period by exposing PBMCs to *Escherichia coli* on d0 and rechallenging them with bacteria on d2. This induced the emergence of a distinct monocytic phenotype with profound phagocytic impairments but a preserved ability for naïve T cell stimulation. The compromising effects on phagocytosis required the presence of bacteria and were not mimicked by TLR4 ligation or exposure to isolated cytokines alone. Moreover, the impairments were specific for the engulfment of bacteria and were coupled to a selective down-regulation of FcγR and SR expression. Intriguingly, this monocytic phenotype contributed to the stimulation of a T_H17-polarized adaptive immune response in the context of secondary infection. Our findings extend the current knowledge of monocytic reprogramming and identify the phagocytic capacity of monocytes as a putative sepsis biomarker. *J. Leukoc. Biol.* **91: 977–989; 2012.**

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

Introduction

Septic diseases induce a massive activation of the immune system with the abundant release of proinflammatory cytokines. Following this initial systemic, proinflammatory response syndrome, many patients show signs of a temporary immunodeficiency in the early postinflammatory period, a phase that has been described with a variety of terms, including anti-inflammatory response syndrome, immune paralysis, LPS-hyporesponsiveness, or endotoxin tolerance [1–3]. The term "endotoxin tolerance" summarizes a complex reprogramming of monocytes as a result of prior exposure to minute amounts of LPS [4]. Pathophysiologically, this process has been associated with alterations in the cytokine milieu with reduced serum levels of proinflammatory cytokines, such as IL-1α and TNF [1, 2], increased concentrations of the anti-inflammatory cytokine IL-10 [5, 6], and an impaired antigen-presenting capacity as a result of low HLA-DR expression [1, 3, 5], together with a reduced T cell stimulatory capacity in response to various recall antigens [3]. So far, the "hyporesponsiveness" of innate immune cells experimentally has mainly been approached by exposing monocytes to LPS but not to whole living bacteria, e.g., *E. coli*. Moreover, the "hyporesponsive" state has been documented primarily by determining the capacity of monocytes to induce cytokine secretion or to induce T cell stimulation upon pathogen-specific antigen presentation, whereas functional data on the phagocytic capacity of bacterially reprogrammed monocytes are missing. In contrast to various studies reporting on the presumed hyporesponsiveness of innate immune cells, one single publication described the up-regulation of CD64 surface expression upon LPS prestimulation, paralleled by an enhanced ability of LPS-tolerant monocytes to phagocytose *E. coli* [7]. Nevertheless, the detailed mechanisms underlying

these bacterially induced alterations in phagocytic processes of CD14$^+$ monocytes remain elusive.

The period of the anti-inflammatory response syndrome can be life-threatening, and a subgroup of septic patients with impaired monocytic functions exhibited an exceptionally poor outcome [8]. Moreover, monocytic function is crucially controlled by bystander cells of the adaptive immune system. Hence, we sought to characterize the precise nature and extent of the functional disability of bacterially reprogrammed monocytes in PBMC cultures. By briefly exposing PBMCs to low doses of whole, live *E. coli* and subsequently culturing them in gentamicin-containing medium for 48 h, we implemented a model system of the early postinflammatory period to study phenotypical properties and functional alterations of bacterially "primed" monocytes, as described earlier by our group [9, 10]. We here demonstrate that prestimulated CD14$^+$ monocytes cultured in the presence of bystander cells have a distinct phenotype and display a severely compromised phagocytic activity specifically conveyed by FcγR (CD16 and CD64) and SR (CD36) down-regulation, but are still capable of inducing T cell proliferation and contributing to the induction of a T$_H$17-polarized adaptive immune response in the context of secondary infection.

MATERIALS AND METHODS

PBMC donors

Randomly selected adults donated the required blood. The study protocol was approved by the Ethics Committee of the University of Tuebingen (Germany).

Cell culture and reagents

PBMCs were isolated from whole blood using Ficoll-Paque (Biochrom, Berlin, Germany) density gradient centrifugation. Washed cells were resuspended in RPMI medium containing 10% FCS (Biochrom) without the addition of antibiotics. Cells were adjusted to 2×10^6 cells/ml and cultured in flat-bottom, 12-well, cell-culture plates (Costar, Bodenheim, Germany) at 37°C and 5% CO$_2$.

Bacterial culture

Prestimulation of PBMCs on d0 was performed using the *E. coli* strain K-12 DH5α. For the determination of the phagocytic capacity on d2, *E. coli* DH5α transformed with pCD353, a plasmid encoding a prokaryotic variant of gfp (gfp-mut2) [10, 11], was used. Single colonies were grown in Lennox L broth medium (Invitrogen, Karlsruhe, Germany) until early logarithmic growth phase (OD$_{600}$=0.55–0.8). For the induction of gfp expression, the growth medium was supplemented with 50 μg/ml kanamycin and 1 mM isopropyl-β-D-1-thiogalactopyranoside (Sigma-Aldrich, Taufkirchen, Germany) for 3 h. Subsequently, bacteria were washed, resuspended in sterile PBS, and used immediately.

Phagocytosis assays

PBMCs were incubated with *E. coli* K-12 DH5α at a MOI of 1:10 (calculated on total PBMCs) for 60 min. PBMCs were then washed, centrifuged on a FCS cushion, resuspended in RPMI medium with 10% FCS and gentamicin (2 μg/ml), and cultured for 2 days. On d2, cells were resuspended in antibiotic-free RPMI medium containing 10% FCS. To determine the phagocytic capacity, cells were exposed to gfp-expressing *E. coli* at a MOI of 1:50 for 60 min, followed by passing them through a FCS cushion, washing, and staining for subsequent flow cytometric analysis. To determine the number of apoptotic PBMCs in our coculture system, we quantified hypodiploid nuclei as described before [9].

Heat attenuation of bacteria

In some experiments, freshly grown *E. coli* K-12 bacteria were heated to 50°C for 2 h prior to coincubation with PBMCs on d0.

Supernatant experiment

Supernatants of d2-prestimulated PBMCs were stored at −20°C. At the time of the experiment, freshly isolated, autologous PBMCs were cultured for 2 days in the presence of supernatant at the indicated concentration and were subsequently exposed to gfp-expressing *E. coli* (MOI of 1:50). In selected experiments, PBMCs were cultured in the presence of the donor-specific supernatant (ratio supernatant:fresh medium, 1:1), together with IL-1Ra (Kineret, 10 μg/ml, Amgen, Munich, Germany) or functional grade-purified anti-IL-10 antibody (2.5 μg/ml; clone JES3-9D7, eBioscience, Frankfurt, Germany). On d2, medium was changed, and cells were subjected to a phagocytosis assay.

Cocultures with apoptotic PBMCs

PBMCs were irradiated with 10 mJ/cm^2 UV-C to induce apoptosis (d-1). Twenty-four hours afterward, apoptosis induction was monitored using Annexin V staining. On d0, apoptotic PBMCs (~70% Annexin V-positive and PI-negative) were then added to 2×10^6 autologous PBMCs at the indicated cell numbers (d0), and coculture was performed for 2 days. On d2, cocultured PBMCs and unstimulated control PBMCs were exposed to gfp-*E. coli*, and the phagocytic capacity was determined.

FCS modification

Phagocytosis on d2 was assessed in RPMI medium without FCS or with the addition of 10% heat-inactivated or IgG-depleted FCS. For heat inactivation, FCS was heated to 56°C for 20 min. IgG depletion was performed using a Protein G sepharose column (GE Healthcare, Freiburg, Germany), and removal of IgG was confirmed by SDS-PAGE analysis and Coomassie staining.

Exposure to various cytokines

PBMCs were exposed to 100 ng/ml rIL-10 and IL-1α (R&D Systems, Wiesbaden-Nordenstadt, Germany) or to TNF (10 ng/ml; R&D Systems) on d0. On d2, cells were resuspended in RPMI medium containing 10% FCS and subjected to a phagocytosis assay.

SR-blocking experiments

Prestimulated or control PBMCs were suspended in FCS-free RPMI medium on d2. Immediately after resuspension, oxLDL particles (Kalen Biomedicals, Montgomery Village, MD, USA) were added (100 μg/ml). Thirty minutes later, phagocytosis of gfp-expressing *E. coli* was measured (MOI of 1:50).

Specific TLR4 activation

In selected experiments, PBMCs were prechallenged on d0 with 100 ng/ml LPS (*E. coli* 055:B5, L6529, Sigma-Aldrich) for 1 h and were then washed and cultivated in RPMI with 10% FSC. On d2, prestimulated and control PBMCs were exposed to gfp-*E. coli* (MOI 1:50) to quantify the extent of phagocytosis.

Phagocytosis of apoptotic cells

Allogeneic granulocytes or Jurkat cells were labeled with PKH26 (Sigma-Aldrich) and irradiated with 10 mJ/cm^2 UV-C to induce apoptosis. Phagocytosis of apoptotic cells was assessed after 24 h (granulocytes) or 4 h (Jurkat cells), respectively. Prestimulated and control PBMCs were resuspended on d2 in serum-free RPMI medium and cocultured with PKH26-labeled

apoptotic prey cells at a phagocyte:prey cell ratio of 1:10. One hour later, cells were washed and stained for flow cytometric analysis.

Flow cytometric analysis of surface receptor expression and determination of phagocytic capacity

For the determination of surface receptor expression, cells were stained with the indicated FITC- or PE-labeled mAb or the corresponding isotype controls (BD Biosciences, Heidelberg, Germany), followed by flow cytometric analysis (FACSCalibur and LSRII, BD Biosciences). The phagocytic capacity was calculated as the percentage of gfp-positive $CD14^+$ monocytes of all $CD14^+$ monocytes. When apoptotic cells were used as prey, phagocytosis was measured as the percentage of PKH26-positive $CD14^+$ monocytes of all $CD14^+$ monocytes.

Cytokine capture and T cell proliferation assay

Stimulation of PBMCs was performed on d0 and d2, as described above. On d2, the percentage of $CD14^+$ monocytes was determined by flow cytometry. PBMCs of identical donors were again isolated on d2, followed by $CD14^+$ monocyte depletion (CD14 Microbeads, Miltenyi Biotec, Bergisch, Gladbach, Germany) and determination of $CD3^+$ T cell frequencies. These PBMCs were then added to the autologous-prestimulated and control PBMCs at a CD14:CD3 ratio of 1:10. Supernatants of these cocultures were collected after 18 or 40 h, respectively, and cytokine concentrations were determined using the human $T_H1/T_H2/T_H17$ CBA kit (BD Biosciences). For the T cell proliferation assay, $CD14^+$-depleted PBMCs were labeled with 2 μM CFSE (Molecular Probes, Eugene, OR, USA), according to the manufacturer's instructions and were then added to autologous-prestimulated or control PBMCs (ratio CD14:CD3 cells, 1:10; calculation based on the number of live $CD14^+$ monocytes). Five days later, cells were stained with anti-CD3 and subjected to flow cytometry. Analysis of T cell proliferation was performed in a histogram blot calculating all peaks with reduced CFSE-fluorescence (M1) as filial cells.

Characterization of monocytic cytokine profile

Stimulation of PBMCs was performed on d0 and d2 as described. Following stimulation on d2, monocytes were isolated using the human Monocyte Isolation Kit II (Miltenyi Biotec), cultured in a gentamicin-containing RPMI medium for 24 h, and supernatants were then stored at $-80°C$ for later analysis. For generation of M1 and M2 macrophages, isolated monocytes were cultured for 7 days in the presence of 1000 U/mL GM-CSF (M1) or 10 ng/mL M-CSF (M2; ImmunoTools, Friesoythe, Germany) or without the addition of exogenous cytokines (M0), as described before [12]. Cytokines were added every 2 days. After 7 days, medium was changed, and 24 h later, the supernatants were collected and stored at $-80°C$, and the monocytes were subjected to flow cytometry. For quantification of the cytokine concentration in the supernatant, a FACS-based ELISA was performed using CBA-Flex sets (BD Biosciences). Measurements were performed using an LSRII (BD Biosciences) with a four-parametric fitting curve to convert MFIs into concentration values.

Multiplex bead assay

For multiplex bead assays, control PBMCs were cultured for 2 days in RPMI medium with 10% FCS and gentamicin as described above. On d2, cells were resuspended in RPMI medium containing 10% FCS without additional antibiotics and exposed to gfp-expressing *E. coli* at a MOI of 1:50 for 1 h. Immediately after phagocytosis, cell-free supernatants were stored at $-70°C$ until further use. A multiplex bead-based assay of these supernatants was performed as described elsewhere [13, 14]. Measurements were performed using a Luminex100 analyzer (Luminex, Austen, TX, USA) with a five-parametric fitting curve to convert MFIs into concentration values.

Purification of monocytes and performance of real-time qPCR

d0-prestimulated and control PBMCs were exposed to *E. coli* on d2 for 1 h (MOI 1:50). Subsequently, cells were washed, and $CD14^+$ monocytes

were purified by MACS selection using the human Monocyte Isolation Kit II (Miltenyi Biotec; purity >92% $CD14^+$ cells, as detected by flow cytometry). Immediately following purification, cells were frozen at $-70°C$ until further use. For real-time qPCR, total RNA was isolated, and cDNA was synthesized as described previously [15]. The resulting cDNA (100 ng) was applied to the following qRT-PCR analyses using an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA) and qPCR Mastermix Plus (Eurogentec, Seraing, Belgium; 20 μl final volume) with 300 nM exon-exon boundary-spanning primers. Relative quantification was performed using the standard curve method. The results were normalized on 18S rRNA, and the mean value of the untreated monocyte population of all donors was used as calibrator.

Statistics

If not stated otherwise, results are shown as pooled data of different experiments with mean values ± SD. For statistics of qRT-PCR analyses, a two-tailed heteroskedastic unpaired Student's *t* test was used (see Fig. 4). All other data were compared by nonparametric Mann Whitney U testing.

RESULTS

Prestimulation with *E. coli* impairs the ability of monocytes for subsequent phagocytosis

To address the question of which phenotypical and functional changes may be induced in monocytes by bacterial prestimulation, we isolated PBMCs from healthy donors, incubated them with low doses of viable *E. coli* (d0), and subsequently, cultured them in the presence of gentamicin, using unstimulated PBMCs as controls. On d2, prestimulated and control PBMCs were exposed to live, gfp-expressing *E. coli*, and the phagocytic competence of monocytes was determined (analysis gate set on $CD14^+$ cells). Interestingly, bacterial exposure on d0 induced a strong impairment in the capacity of PBMC-derived monocytes to phagocytose bacteria efficiently on d2 (**Fig. 1A** and **B**), whereas the phagocytic capacity of isolated, bacterially prestimulated monocytes remained unchanged (Fig. 1C). Preincubation of PBMCs with cytochalasin D greatly reduced the number of gfp^+ monocytes (data not shown). This indicates that gfp-*E. coli* bacteria had been taken up by an active process involving actin polymerization and were not merely passively adhering to the surface of the monocytes. Following this procedure of bacterial priming, only a small subpopulation of cells in the PBMC gate underwent apoptosis (mean/prestimulated PBMCs: 11.4±2.1; mean control: 0.45±0.14%; Fig. 1D), confirming earlier results [16]. Kinetic experiments excluded that an accelerated intracellular digestion of gfp-expressing *E. coli* was responsible for the observed reduction in the percentage of gfp^+ $CD14^+$ monocytes (Fig. 1E). Our data suggest that previous exposure to live *E. coli* induces a severe reduction in the phagocytosis competence of $CD14^+$ monocytes. As the surrogate model described above allowed the physiologic interaction between various cell types under the influence of their respective cell type-specific cytokines, we subsequently performed most of the further experiments with PBMC suspension cultures.
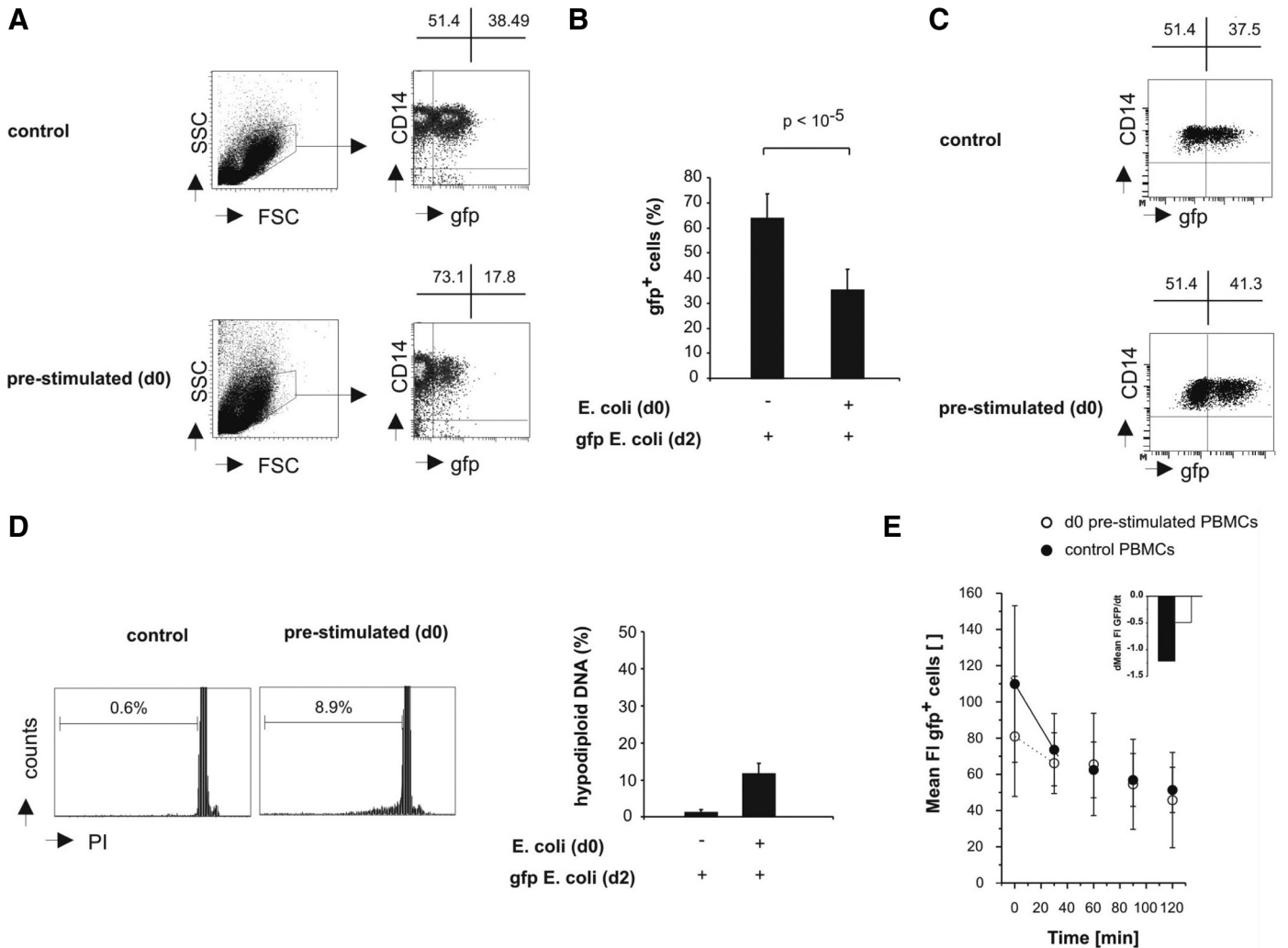
Figure 1. Bacterial prestimulation of PBMCs with *E. coli* induces a strong impairment in the capacity of cultured monocytes to phagocytose bacteria. (A) On d0, PBMCs were incubated with live *E. coli*. Following washing, PBMCs were cultured for 2 days in a medium containing 10% FCS and gentamicin. On d2, phagocytosis of live gfp-expressing *E. coli* was measured in prestimulated and control PBMCs in medium containing 10% FCS without additional antibiotics. SSC, Side-scatter; FSC, forward-scatter. (B) Phagocytosis is presented as the percentage of CD14/gfp double-positive cells of all CD14-positive cells. Data represent pooled data of three different experiments with a total of nine donors. (C) In contrast to monocytes derived from PBMC cultures, isolated, d0-prestimulated and control CD14^{+} monocytes exhibit a comparable ability for phagocytosis on d2. Given is one original dot plot of one experiment performed with three different donors. (D) A small proportion of PBMCs undergoes apoptosis after phagocytosis. PBMCs were exposed to *E. coli* on d0, and the percentage of apoptotic cells was determined 24 h after phagocytosis. The percentage of hypodiploid nuclei in the PBMC gate of pooled data from two experiments with a total of four donors is shown. (E) The reduced gfp signal detected in prestimulated PBMC-derived CD14^{+} monocytes is not a result of an accelerated intracellular digestion of bacteria. Kinetic experiments were performed by feeding prestimulated (○) or control (●) PBMCs on d2 with gfp-*E. coli*. Following washing, aliquots were analyzed for gfp and anti-CD14 fluorescence at the indicated time-points. Given are the MFI (Mean FI) values of gfp/CD14 double-positive cells of all CD14-positive cells. The inset depicts the rate of bacterial digestion for prestimulated and control PBMCs as a linear slope of the initial degradation phase (see lines). Experiments are representative of pooled data of three experiments with seven healthy donors.

Phagocytic impairments require exposure to whole bacteria and cannot be induced by autologous apoptotic PBMCs, LPS treatment, or exposure to isolated cytokines alone

As the presence of apoptotic cells may well induce functional alterations of immune cells, including a reduction in the proliferative capacity or a modulation of cytokine secretion [1, 17], and as a small amount of cells consistently underwent apoptosis in our cell culture system (Fig. 1C), we next tested whether the presence of autologous apoptotic PBMCs itself might impair functional properties of d2-cultured, PBMC-derived monocytes. Interestingly, monocytes cocultured in the presence of apoptotic PBMCs did not exhibit any impairments in their phagocytic capacity on d2 (**Fig. 2A**), thus indicating that under the culture conditions described here, the presence of apoptotic cells alone does not account for the observed
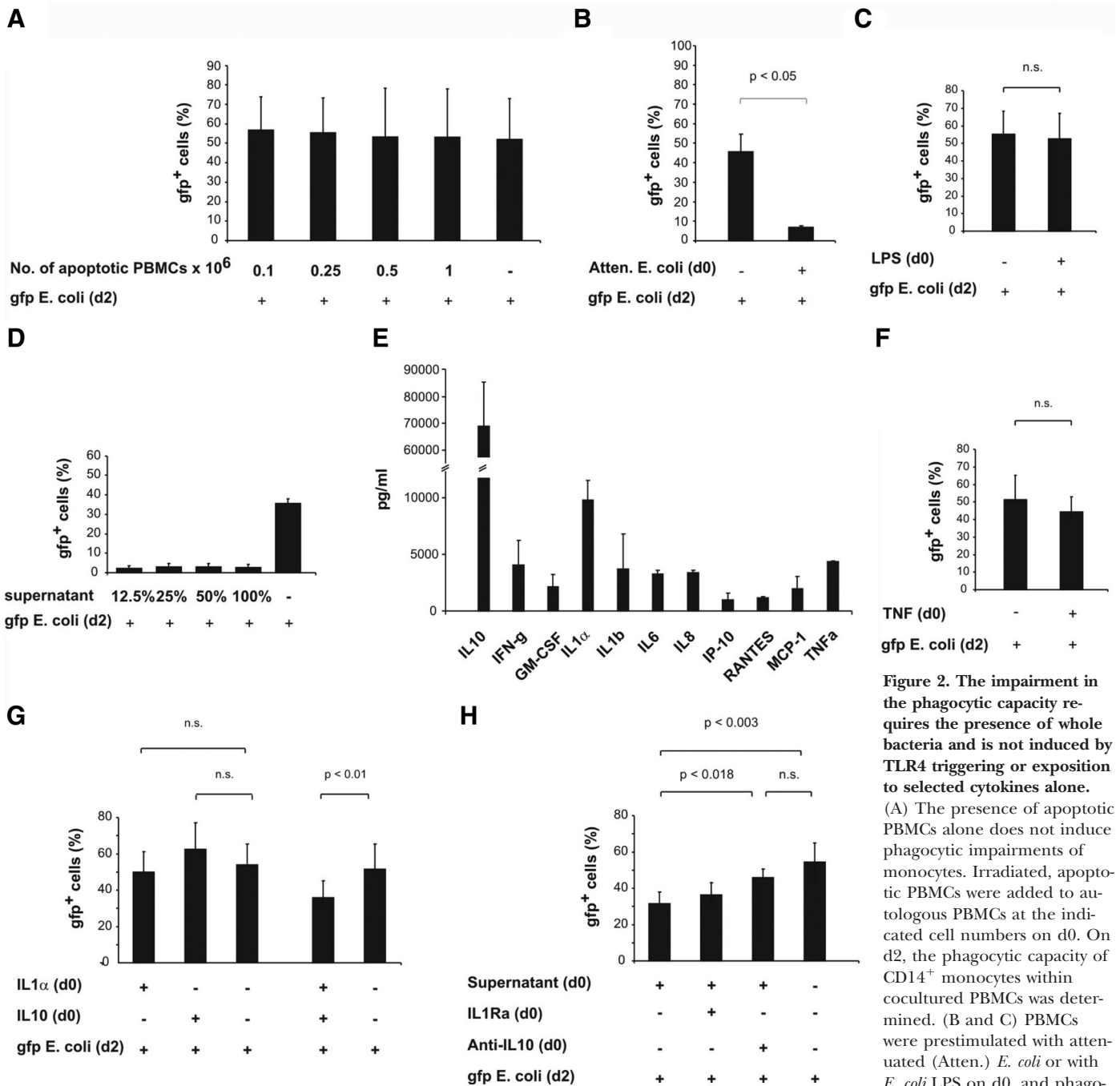
Figure 2. The impairment in the phagocytic capacity requires the presence of whole bacteria and is not induced by TLR4 triggering or exposition to selected cytokines alone. (A) The presence of apoptotic PBMCs alone does not induce phagocytic impairments of monocytes. Irradiated, apoptotic PBMCs were added to autologous PBMCs at the indicated cell numbers on d0. On d2, the phagocytic capacity of CD14^{+} monocytes within cocultured PBMCs was determined. (B and C) PBMCs were prestimulated with attenuated (Atten.) *E. coli* or with *E. coli* LPS on d0, and phagocytosis of gfp-*E. coli* was determined on d2. (D) Culture in the presence of supernatants of bacterially prestimulated PBMCs significantly impairs the ability of naïve PBMCs for phagocytosis on d2. Supernatants of bacterially prestimulated PBMCs were added to freshly isolated PBMCs at the indicated concentration, and 2 days later, the ability of phagocytosis was assessed. (E) d2-cultured PBMCs secrete excessive amounts of IL-10, large amounts of IL-1α, and moderate amounts of TNF upon exposure to *E. coli*. PBMC supernatants were collected immediately after phagocytosis on d2 and were subjected to multiplex bead assay. IP-10, IFN-inducible protein 10. (F and G) Exposure of freshly isolated PBMCs to TNF, IL-10, or IL-1α alone does not impair phagocytosis of d2-cultured PBMC-derived monocytes, whereas the combined exposure to IL-10 and IL-1α induces relevant impairments in the ability for subsequent phagocytosis on d2. PBMCs were cultured until d2 in the presence of TNF, IL-10, or IL-1α or the combination of the latter, respectively. On d2, phagocytosis was assessed. (H) Anti-IL-10 but not IL-1Ra is able to partially restore the phagocytic capacity of PBMCs that are exposed to culture supernatants of bacterially stimulated PBMCs, which were exposed to supernatants and anti-IL-10 or IL-1Ra on d0. On d2, the culture medium was replaced, and phagocytosis was assessed. A represents pooled data of two independent experiments with four donors; B, two experiments with six donors; C–E, one experiment with three healthy donors; F–H, pooled data of two experiments with a total of eight donors.

functional impairments of monocytes. We, therefore, sought to further dissect the requirements for the described inhibition of phagocytosis and prestimulated PBMCs with heat-inactivated *E. coli*. Exposition to attenuated bacteria induced even greater impairments in the phagocytic capacity of d2-cultured monocytes than exposure to live *E. coli* had done (Fig. 2B). Thus, a direct and mutual interaction between the living pathogen and the monocyte is obviously not required for later inhibition of phagocytic processes. To exclude that the mere interaction of *E. coli* surface structures with the TLR4 might be sufficient to compromise phagocytosis, we prestimulated PBMCs with LPS and determined phagocytosis on d2 (Fig. 2C). However, the phagocytic competence of CD14$^+$ monocytes in response to LPS prestimulation was not significantly different from that of the untreated control population, indicating that processes more complex than TLR4 ligation alone are apparently responsible for the observed phenomenon. Notably, culturing PBMCs in the presence of supernatants obtained from bacterially prestimulated PBMCs significantly impaired the subsequent ability of monocytes for phagocytosis (Fig. 2D). Therefore, we next analyzed the cytokine profile released by bacterially stimulated PBMCs and detected a strong production of diverse cytokines, with IL-10, IL-1α, and to a lesser extent, TNF as the most abundant ones (Fig. 2E). However, in contrast to recently published data on tolerance induction by TNF pretreatment of isolated CD14$^+$ monocytes [18], we did not observe functional impairments in TNF-pretreated, PBMC-derived monocytes (Fig. 2F). Additionally, exposure of PBMCs to isolated IL-10 or IL-1α alone was not able to reproduce the observed induction of functional impairments in monocyte phagocytosis (Fig. 2G). However, the combined exposure to IL-10 and IL-1α significantly impaired the phagocytic ability of d2-cultured, PBMC-derived monocytes, and the addition of anti-IL-10 antibody did, to a substantial extent, preserve the phagocytic capacity of monocytes during bacterial reprogramming (Fig. 2H). Collectively, these data indicate that the phagocytic impairments of reprogrammed monocytes are apparently orchestrated by multifaceted mechanisms that require exposure to intact bacteria and include the presence of a specific cytokine milieu, including IL-10 and IL-1α.

Bacterially prestimulated, PBMC-derived monocytes maintain their ability to phagocytose apoptotic cells

To further elucidate whether the reduction in the phagocytic competence was selective for the engulfment of bacteria or rather a result of a more general effect on the process of phagocytosis, bacterially prestimulated PBMCs were exposed to apoptotic allogeneic granulocytes or Jurkat cells on d2, and target cell internalization by monocytes was assessed (**Fig. 3A and B**). Intriguingly, no significant differences in phagocytosis of these apoptotic cells were observed, indicating that mechanisms specifically related to recognition and/or internalization of bacteria were supposedly responsible for the described impairments in *E. coli* phagocytosis. The results presented so far reveal that prestimulation with bacteria, viable or attenuated ones, but not LPS alone, is necessary and sufficient to interfere specifically with the phagocytic competence of monocytes in the context of bacterial rechallenge of PBMC cultures.

Bacterial exposure induces FcγR and SR down-regulation in CD14$^+$ monocytes

The distinct impairment in bacterial phagocytosis suggested that mechanisms selectively controlling the recognition and internalization of bacteria were affected by prestimulation of PBMCs with *E. coli*. To elucidate this in greater depth, we analyzed the mRNA expression pattern of different gene families involved in phagocytosis using qRT-PCR. Compared with the untreated controls, monocytes exposed to bacteria in the context of PBMC cultures exhibited significantly reduced mRNA levels of FcγRs, such as CD16 (FcγRIII); SRs, such as CD36 and the Lox-1; and to a lesser extent, CRs, such as CD11b/CD18 (ITGαM/ITGβ2; **Fig. 4A–C**). Importantly, mRNA levels of the PS receptors specifically involved in apoptotic cell recognition (TIM1 and -4 and BAI1), TLR4, and the small GTPases mediating phagocytic signal transduction (Rho1, RhoG, Rac1, and Cdc42) remained virtually unaltered (Fig. 4D and E). Flow cytometric analysis revealed that for FcγR (CD16 and CD64) and the SR CD36, but not for Lox-1 or the CR subunits CD11b and CD18, the mRNA down-regulation was paralleled by a reduced surface expression of the respective proteins (Fig. 4F). Thus, bacterial exposure on d0 strongly and selectively results in down-regulation of FcγR and SR expres-

Figure 3. Bacterial prestimulation does not affect the ability for subsequent phagocytosis of apoptotic granulocytes or Jurkat cells by monocytes. (A and B) Prestimulated or control PBMCs were exposed to PKH26-labeled apoptotic allogeneic granulocytes (A) or Jurkat cells (B) at a phagocyte:prey ratio of 1:10 in FCS-free medium on d2. Phagocytosis is given as the percentage of CD14/PKH26 double-positive cells of all CD14-positive cells. Data represent pooled data of two independent experiments with a total of six donors.
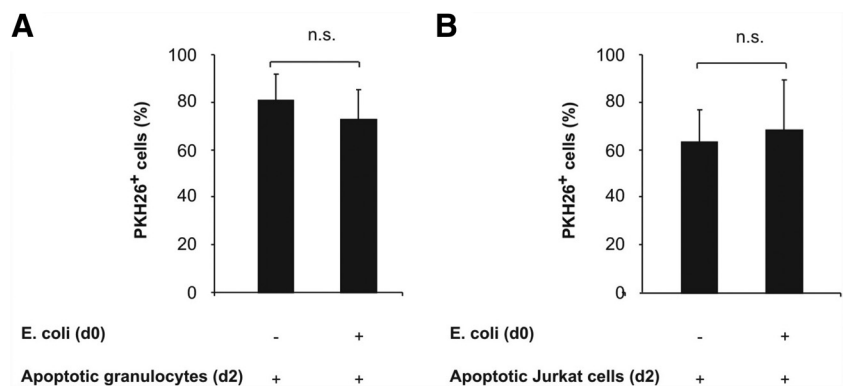
sion, reflecting the specific impairment of monocytes to phagocytose bacteria on d2.

FcγR and SR down-regulation contributes to functional impairments of bacterially reprogrammed monocytes

Phagocytosis of bacteria is commonly accepted to be mediated by the combined action of SRs, FcγRs, and CRs, with the latter two relying on the presence of opsonizing serum-derived IgG or complement proteins, respectively. To dissect the relative contribution of these receptor families to the engulfment of bacteria, we first examined the influence of serum modifications on monocyte-mediated phagocytosis of bacteria. In the absence of serum, bacteria can be internalized by monocytes, supposedly as a result of SR-dependent recognition. Yet, as to be expected, addition of serum strongly enhanced bacterial engulfment (**Fig. 5A**). Apparently, this was largely mediated by opsonizing IgG and only to a lesser extent by complement factors, as depletion of IgG resulted in an inhibition of phagocytosis, whereas complement inactivation had virtually no effect (Fig. 5B). We, therefore, conclude that in our system, FcγRs (CD16 and CD64) and SRs are the key players contributing to bacterial phagocytosis of resting PBMC-derived monocytes. Together with our observation that bacterial exposure robustly suppressed the expression of FcγRs (CD16 and CD64) and CD36, this finding argues for a causal relationship between the impaired bacterial phagocytic capacity and FcγR/SR down-regulation. To finally test this hypothesis, we compared the inhibition of phagocytosis in bacterially reprogrammed monocytes in the presence of control or IgG-depleted serum (Fig. 5C). Interestingly, depletion of IgG did not further reduce phagocytosis, suggesting that bacterially prechallenged, PBMC-derived monocytes do not use FcγR for phagocytosis anymore, supposedly as a result of the down-regulation of FcγR expression. Then, we analyzed the influence of d0 bacterial challenge on phagocytosis in the presence or absence of oxLDL particles, well-known, competing ligands of the SR family (Fig. 5D). Notably, addition of oxLDL did not further impair phagocytosis, implying that also, the SR family is dispensable for the remaining phagocytic activity of bacterially prestimulated PBMC-derived monocytes. In contrast, decomplementation of FCS did profoundly impair the residual phagocytic capacity of bacterially reprogrammed PBMC-derived monocytes (Fig. 5E). Collectively, these experiments demonstrate that FcγRs and SRs are the predominant receptor systems engaged in bacterial phagocytosis by resting monocytes, whereas bacterially reprogrammed, PBMC-derived monocytes importantly rely on CRs to exert their remaining phagocytic functions.
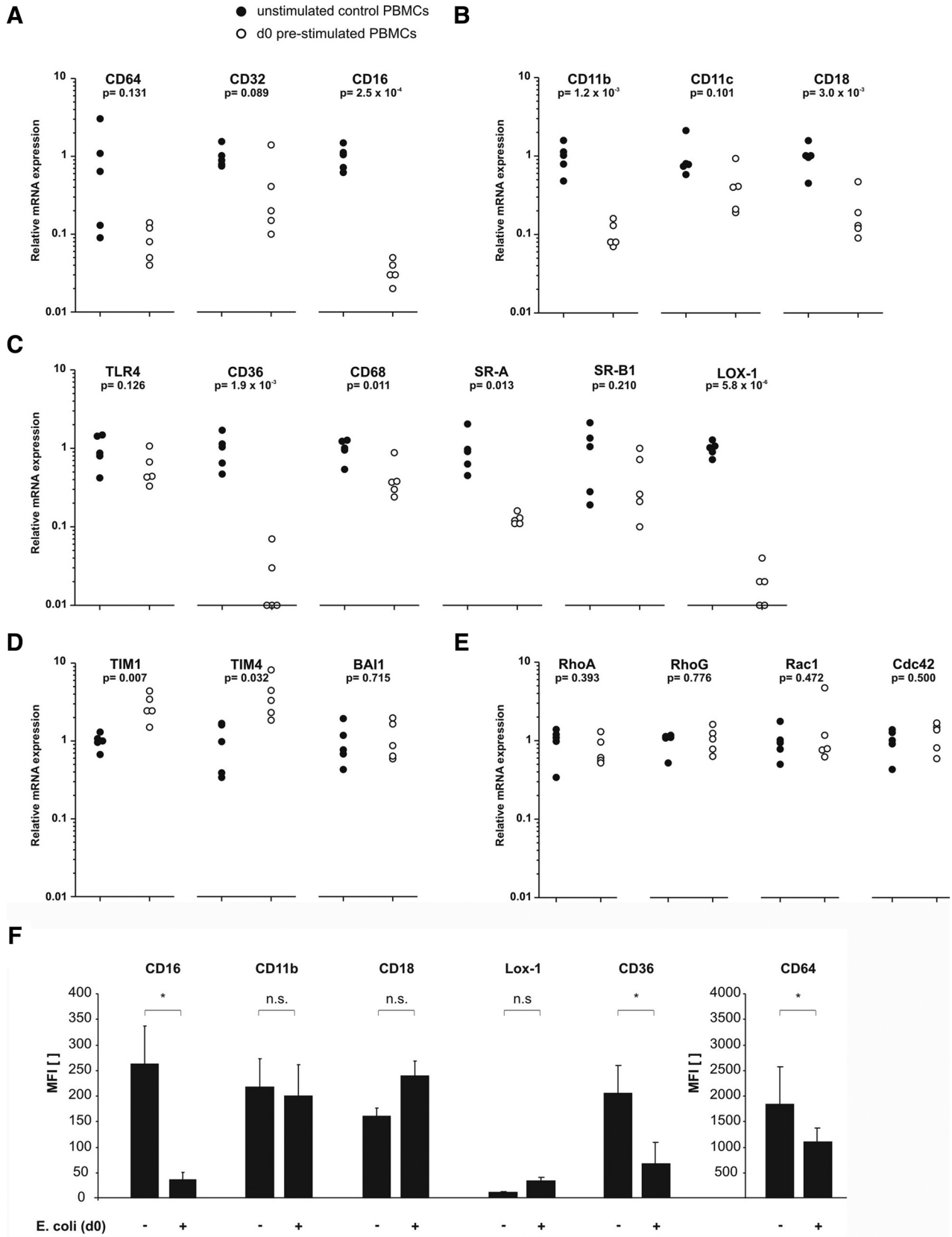
Prestimulated monocytes exhibit a distinct phenotype with preserved T cell-stimulating capacity and altered cytokine responses

To elucidate the functional consequences of bacterial challenge on the induction of subsequent adaptive immune responses by monocytes, we studied the surface expression of costimulatory receptors, known to be important for the interaction of CD14$^+$ monocytes with T cells, and characterized the cytokine milieu generated in a coculture system of bacterially reprogrammed PBMCs with freshly isolated, CD14-depleted PBMCs. Exposure to *E. coli* induced a down-regulation of HLA-DR and to a lesser, statistically not significant, extent, HLA-DQ (data not shown), together with an up-regulation of CD80/CD86 surface expression on d2-cultured CD14$^+$ monocytes (**Fig. 6A** and **B**), indicative of an altered monocytic phenotype with reduced antigen-presenting capacity but preserved T cell-activating potential. In line with the observed up-regulation of costimulatory molecules, prestimulated PBMC suspensions were able to induce proliferation of autologous, naïve T cells to a greater extent than control PBMCs (Fig. 6C). As the expression of costimulatory molecules on PBMC-derived monocytes cultured in the presence of apoptotic cells did not vary (data not shown), we assume that the presence of apoptotic bystander cells alone was not the cause of these increased rates of T cell proliferation. However, the cytokine milieu generated by the naïve coculture system differed fundamentally from that of the bacterially prechallenged one. Whereas in the former, TNF was the dominating cytokine in the early response phase (after 18 h) and T$_H$1-typical IFN-γ as well as T$_R$1-characteristic IL-10 were accumulating in later phases (after 40 h), the production of these cytokines was reduced profoundly in the bacterially prechallenged coculture system. Instead, here, we detected IL-6 and later on (after 40 h), T$_H$17-characteristic IL-17A at levels significantly higher than in the coculture system with naïve PBMCs. Of note, coculture of bacterially prestimulated, isolated monocytes with freshly isolated CD3$^+$ T cells did not result in the generation of T$_H$17 cells (data not shown). Thus, we assume that the cytokines derived from bacterially reprogrammed PBMCs are responsible for the induction of T$_H$17 cells in our model system. Interestingly, bacterially prestimulated and subsequently isolated CD14$^+$ monocytes displayed an IL-6^{hi}IL-10^{lo}IL-12/23p40^{lo}TGF-β^{int} phenotype (**Fig. 7**), which represents an intermediate between the GM-CSF-induced IL-10^{lo}IL-12/23^{hi} M1-macrophage phenotype and the M-CSF-induced IL-10^{hi}IL-12/23^{lo} M2-macrophage phenotype. Regarding surface receptor expression, bacterially reprogrammed monocytes revealed a unique phenotype, which differed significantly from prototypical M1 and M2 macrophages (Supplemental Fig. 1A and B). Hence, bacterially reprogrammed, PBMC-derived monocytes do not persist in an immune-paralytic state but apparently shift their phenotypical and functional orientation toward converting the adaptive immune response in improving T cell proliferation and contributing to a T$_H$17-biased cytokine milieu.

DISCUSSION

To date, it is well acknowledged that sepsis represents an uncontrolled inflammatory response syndrome, and most therapies aim at attenuating the initial proinflammatory response [19]. However, substantial data have accumulated, revealing that many patients do not die as a result of an abundant, proinflammatory cytokine release but as a result of the progressive development of immunosuppression [5, 19, 20]. So far, little is known about varying elements contributing to this second

A

unstimulated control PBMCs

○ d0 pre-stimulated PBMCs

B

CD64 p= 0.131   CD32 p= 0.089   CD16 p= 2.5 x 10⁻⁴

CD11b p= 1.2 x 10⁻³   CD11c p= 0.101   CD18 p= 3.0 x 10⁻³

C

TLR4 p= 0.126   CD36 p= 1.9 x 10⁻³   CD68 p= 0.011   SR-A p= 0.013   SR-B1 p= 0.210   LOX-1 p= 5.8 x 10⁻⁶

D

TIM1 p= 0.007   TIM4 p= 0.032   BAI1 p= 0.715

E

RhoA p= 0.393   RhoG p= 0.776   Rac1 p= 0.472   Cdc42 p= 0.500

F

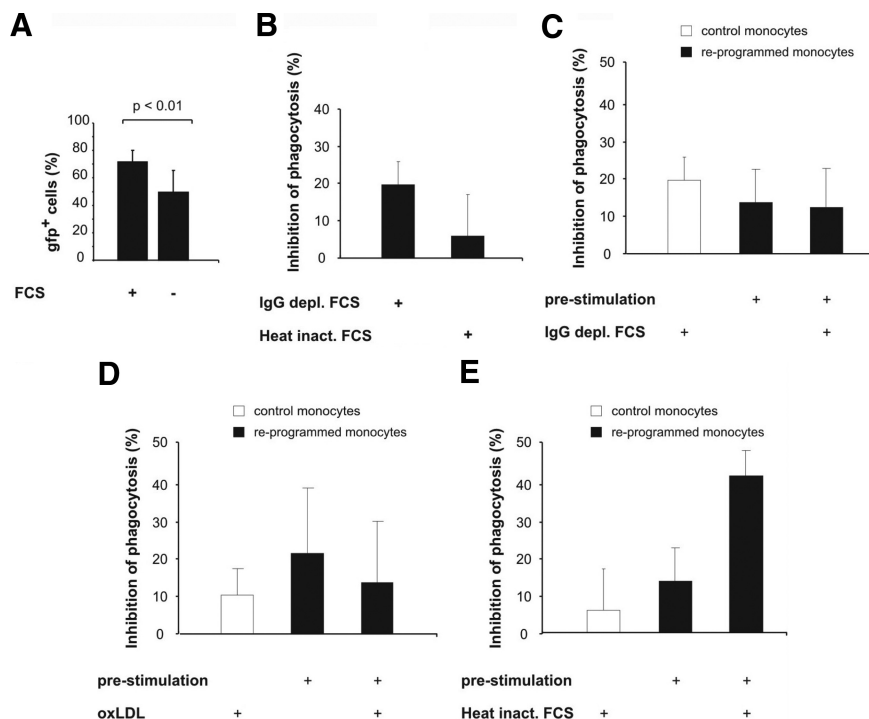CD16   CD11b   CD18   Lox-1   CD36   CD64

Figure 5. Resting monocytes depend on FcγRs and SRs to exert their phagocytic function, whereas reprogrammed monocytes predominantly rely on the use of CRs. (A and B) Freshly isolated PBMCs were cultured in a medium containing 10% FCS. On d2, phagocytosis of gfp-*E. coli* by these resting monocytes was determined in the presence or absence of native FCS (A) and IgG-depleted (depl.) or heat-inactivated (inact.) FCS (B), respectively. Data represent pooled data of four independent experiments with three donors per experiment. (C–E) Reprogrammed monocytes rely on CRs to exert their remaining phagocytic capacity. Freshly isolated PBMCs were bacterially prestimulated as described in Fig. 1. On d2, these reprogrammed PBMCs were washed and resuspended in medium containing 10% native or IgG-depleted FCS (C), in FCS-free medium ± oxLDL (D), or in medium containing 10% heat-inactivated FCS (E). Thirty minutes later, phagocytosis of gfp-*E. coli* was induced and assessed. Given is the percentage of inhibition of phagocytosis by CD14^{+} monocytes cultured in the respective FCS preparations in relation to control incubations performed in a medium containing 10% native FCS (C and E) or in a serum-free medium (D). Data represent collective data of three independent experiments with a total of seven donors.

phase of septic diseases. As monocytes represent key players of the innate immune system, which crucially contribute to the induction of subsequent adaptive immune responses, we sought to characterize the nature of phenotypical and functional alterations of monocytes in the early postinflammatory period. Hence, we used a model system of whole blood-derived PBMCs, which were bacterially prestimulated on d0 and cultured and rechallenged with bacteria on d2 and characterized these reprogrammed PBMC cultures in various readout systems. We did deliberately resign from studying purified monocytes or from inducing endotoxin tolerance by exposing monocytes to LPS, as these approaches do not allow the complex interactions among live pathogens, monocytes, and bystander cells, including CD4^{+} T cells. Our data clearly demonstrate that reprogrammed CD14^{+} monocytes display a distinct IL-6^{hi}IL-10^{lo}IL-12/23^{lo}TGF-β^{int} phenotype with profoundly compromised phagocytic activity (Fig. 1) as a result of FcγR and SR down-regulation but simultaneously contribute to T cell proliferation and the induction of a T_H17-polarized adaptive immune response.

So far, the sepsis-related reprogramming has largely been attributed to increased concentrations of IL-10 [5, 6, 21–23],

and earlier reports have suggested that various alterations in the cytokine milieu associated with the initial hyperinflammatory state are responsible for the functional impairment of monocytes [2, 24]. Indeed, exposure to bacteria induced the secretion of high amounts of IL-1α and excessive amounts of IL-10 in our model system (Fig. 2). Although exposure to supernatants of bacterially prestimulated PBMCs did potently inhibit phagocytosis, the addition of isolated cytokines to the cell culture medium did not significantly affect the phagocytic capacity. Of note, only the combined exposure to IL-10 and IL-1α did induce functional impairments of thus cultured PBMC-derived monocytes comparable with the ones observed with bacterial prestimulation. This is in accordance with the existing literature reporting that IL-10 by itself does not have the capacity to induce an immune-paralysis in (psoriatic) patients [25]. Additionally, in vitro studies using coincubation of PBMCs with *Salmonella typhi flagella* indicated that neutralizing antibodies to IL-10 or IL-1β alone were not able to restore functional alterations of monocytes and T cells [26]. Interestingly, anti-IL-10 but not IL-1Ra was, to a certain extent, able to neutralize the supernatant-mediated, functional

Figure 4. Bacterial prestimulation induces down-regulation of mRNA and surface receptor expression levels of FcγR and SRs in monocytes. (A–E) Exposure to *E. coli* induces down-regulation of mRNA levels of selected engulfment receptors. PBMCs were prestimulated and cultured as described in Fig. 1. On d2, phagocytosis was induced in prestimulated (○) and control (●) PBMCs by adding *E. coli.* after phagocytosis, CD14^{+} monocytes were purified and subjected to qRT-PCR. Transcript levels of important phagocytic receptors, such as FcγRs (A), CRs (B), TLR4 and SRs (C), PS-binding phagocytic receptors (D), and small GTPases (E) were normalized to 18S rRNA and were then calibrated on the mean value of the untreated controls. *P* values indicate the level of statistical significance as determined by Student's *t*-test for unpaired samples. Data represent one experiment with five healthy donors. (F) Surface expression levels of FcγRs (CD16, CD64) and the SR CD36 are reduced in bacterially prestimulated CD14^{+} monocytes. Prestimulated and control PBMCs were cultured as described in Fig. 1. On d2, prior to the induction of phagocytosis, PBMCs were subjected to flow cytometric analysis determining the MFI expression of the respective phagocytic receptors gated on CD14^{+} monocytes. Data on Lox-1 and CD36 expression represent pooled data of three experiments with a total of seven donors, and data on CD16, CD11b, CD18, and CD64 represent pooled data from two experiments with four donors.
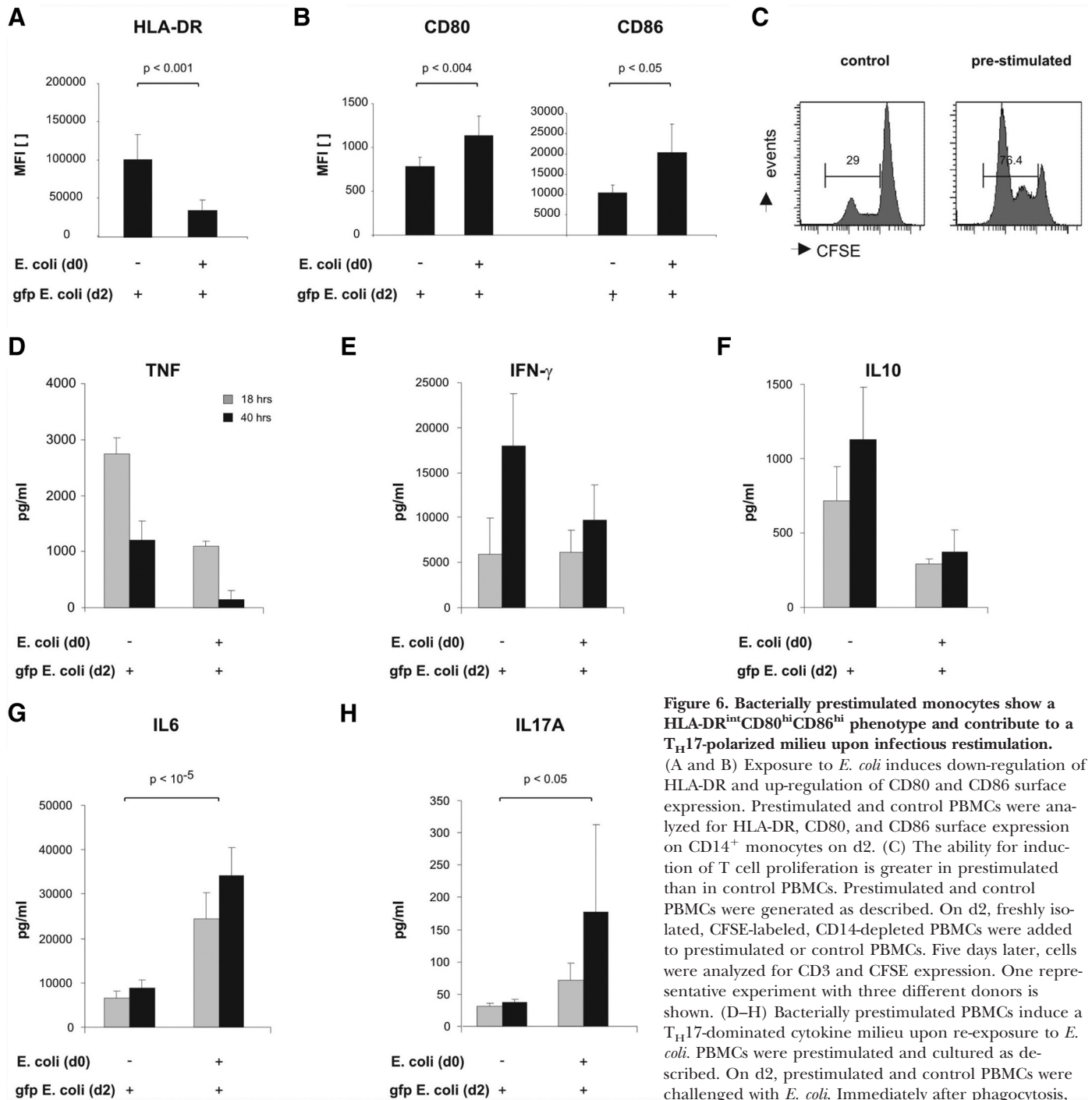
Figure 6. Bacterially prestimulated monocytes show a HLA-DR^{int}CD80^{hi}CD86^{hi} phenotype and contribute to a T$_H$17-polarized milieu upon infectious restimulation. (A and B) Exposure to *E. coli* induces down-regulation of HLA-DR and up-regulation of CD80 and CD86 surface expression. Prestimulated and control PBMCs were analyzed for HLA-DR, CD80, and CD86 surface expression on CD14$^+$ monocytes on d2. (C) The ability for induction of T cell proliferation is greater in prestimulated than in control PBMCs. Prestimulated and control PBMCs were generated as described. On d2, freshly isolated, CFSE-labeled, CD14-depleted PBMCs were added to prestimulated or control PBMCs. Five days later, cells were analyzed for CD3 and CFSE expression. One representative experiment with three different donors is shown. (D–H) Bacterially prestimulated PBMCs induce a T$_H$17-dominated cytokine milieu upon re-exposure to *E. coli*. PBMCs were prestimulated and cultured as described. On d2, prestimulated and control PBMCs were challenged with *E. coli*. Immediately after phagocytosis, freshly isolated, autologous, CD14-depleted PBMCs were added to the cell culture at a CD14:CD3 ratio of 1:10. Supernatants of these cocultures were collected after 18 or 40 h, respectively, and subjected to flow cytometric determination of cytokine concentrations. Data represent pooled data of six donors studied in two independent experiments.

inhibition of monocyte-mediated phagocytosis in our system, again underlining the importance of IL-10 in anti-inflammation.

A number of earlier reports have highlighted the role of apoptotic cells in mediating and perpetuating immune dysfunction in sepsis. However, the functional properties of monocytes exposed to large numbers of apoptotic cells have,

so far, not been addressed. Interestingly, the addition of apoptotic PBMCs to freshly isolated, autologous PBMCs neither impaired the ability for phagocytosis of d2-cultured monocytes nor altered the expression of APC- or T cell-costimulating molecules (Fig. 2, and data not shown). Thus, although our cell culture system did contain low but relevant numbers of apoptotic bystander cells, the presence of these cells cannot suffi-
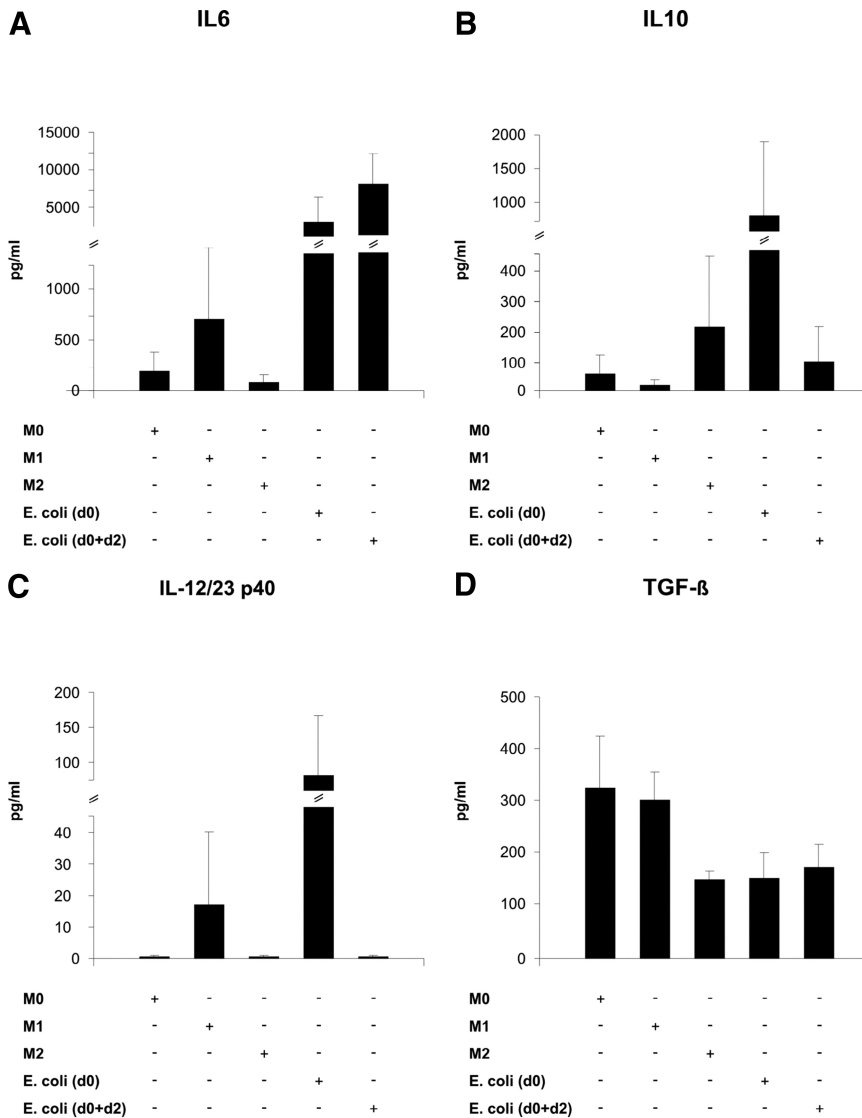
Figure 7. The cytokine profile of bacterially prestimulated monocytes represents an IL-6^{hi}IL-10^{lo}IL-12/23p40^{lo}TGF-β^{int} intermediate phenotype between the IL-10^{lo}IL-12/23^{hi} M1 and the IL-10^{hi}IL-12/23^{lo} M2 macrophage phenotype. (A–D) Prestimulated and control PBMCs were generated as described. After phagocytosis on d2, CD14$^+$ monocytes were isolated and cultured for additional 24 h before analysis of the cytokine profile. M0, M1, and M2 macrophages were generated as described in Materials and Methods. Data represent pooled data of two independent experiments with two donors per experiment.

ciently explain the functional conversion of bacterially reprogrammed monocytes.

Gene expression profiling has shown that most of the monocyte's immediate transcriptional responses to bacterial infection is induced by TLR signaling [27, 28]. However, monocytes express a large repertoire of PRRs, forming a complex that mediates recognition of bacteria [29]. As bacterial prestimulation induced a selective impairment in the phagocytic capacity of d2-cultured monocytes for bacteria but not for apoptotic cells (Fig. 3), we determined the mRNA and surface expression levels of various receptors known to be involved in the process of bacterial cell engulfment. Indeed, exposure to *E. coli* ultimately resulted in a down-regulation of three important phagocytic receptor families in CD14$^+$ monocytes, the FcγRs, CRs, and SRs (Fig. 4). Functional experiments performed in the presence of IgG-depleted serum or the SR-ligand oxLDL corroborated the relative importance of FcγR and SR down-regulation for the induction of phagocytosis-associated monocytic reprogramming (Fig. 5). Interestingly, in line

with our own observations, it has been shown that down-regulation of the SR macrophage receptor with collagenous structure (MARCO), on alveolar macrophages contributed to an enhanced susceptibility for secondary pneumococcal infection in the postinfectious period of a viral disease [30]. Thus, distinct phagocytic receptor families obviously mediate the engulfment of bacteria and the release of proinflammatory cytokines in the early phase of septic diseases, and their silencing, at least in part, accounts for the reprogramming of innate immune cells in the subsequent anti-inflammatory phase.

At first glance, down-regulation of essential phagocytic surface receptor expression in response to bacterial exposure seems paradoxical. However, it is conceivable that this corresponds to a functional conversion that fosters the induction of adaptive immune responses at the transient expense of impaired innate immune responses. Earlier reports have attributed the endotoxin tolerance of monocytes to a reduced HLA-DR expression and a loss of antigen-presenting capacity

[3]. Our phenotypical analyses of monocytes after bacterial prestimulation of PBMCs confirmed those previous reports. However, these monocytes displayed up-regulated T cell-co-stimulating factors, such as CD80 and CD86 (Fig. 6). In the same line, bacterially prechallenged PBMCs revealed improved T cell-stimulating capacity and contributed to a T_H17-biased adaptive immune response by secreting IL-6 and TGF-β (Fig. 7). Interestingly, it has been shown that one relevant physiological stimulus for triggering the differentiation of naive CD4$^+$ T cells into T_H17 cells is the recognition and phagocytosis of infected apoptotic cells by DCs [31]. However, as relevant numbers of DCs were absent in our PBMC culture suspensions (data not shown) and as it has been demonstrated that an induction of T_H17 cells can also be achieved by coculturing CD45RO$^+$ memory CD4$^+$ T cells with LPS-prestimulated monocytes [32], we assume that the observed IL-17 secretion was triggered by cytokines derived from reprogrammed PBMCs. Apart from the induction of inflammatory mediators, T_H17 cells are involved in the activation of neutrophils [33, 34]. With respect to *E. coli* infections, it was demonstrated that neutralization of IL-17 resulted in a reduced infiltration of neutrophils and an impaired bacterial clearance in the early phase of a murine model of i.p. *E. coli* infection [35]. However, data on the role of IL-17 in the pathogenesis of monocytic reprogramming and potential clinical implications are lacking to date. In line with our observations, it has been shown recently that T_H17-dominated immune responses may also contribute to the immunosuppressive phase in postburn injuries [36].

Our findings extend the current knowledge of the functional conversion of monocytes in the status of reprogramming and identify the phagocytic capacity of monocytes as one putative biomarker for the diagnosis of persistent immunosuppression. Severe impairments in the phagocytic capacity of monocytes should be regarded as a strong indicator of a persistent inflammatory hyporesponsiveness, which might result in challenges of envisaged treatment regimens, such as secondary look operations or supportive steroidal therapies.

AUTHORSHIP

M.C.A. and K.L. designed experiments, performed research, analyzed and interpreted the data, and wrote the paper. P.G., J.W., and B.S. performed research. H-Y.H. performed the multiplex bead assay. H.K. performed the qRT-PCR. T.W.O., B.W.K., C.G., C.F.P., and R.H. analyzed and interpreted the data.

ACKNOWLEDGMENTS

REFERENCES

1. Docke, W. D., Randow, F., Syrbe, U., Krausch, D., Asadullah, K., Reinke, P., Volk, H. D., Kox, W. (1997) Monocyte deactivation in septic patients: restoration by IFN-γ treatment. *Nat. Med.* **3**, 678–681.
2. Randow, F., Syrbe, U., Meisel, C., Krausch, D., Zuckermann, H., Platzer, C., Volk, H. D. (1995) Mechanism of endotoxin desensitization: involvement of interleukin 10 and transforming growth factor β. *J. Exp. Med.* **181**, 1887–1892.
3. Wolk, K., Döcke, W. D., von Baehr, V., Volk, H. D., Sabat, R. (2000) Impaired antigen presentation by human monocytes during endotoxin tolerance. *Blood* **96**, 218–223.
4. Kramer, B. W., Ikegami, M., Moss, T. J., Nitsos, I., Newnham, J. P., Jobe, A. H. (2005) Endotoxin-induced chorioamnionitis modulates innate immunity of monocytes in preterm sheep. *Am. J. Respir. Crit. Care Med.* **171**, 73–77.
5. Monneret, G., Finck, M. E., Venet, F., Debard, A. L., Bohe, J., Bienvenu, J., Lepape, A. (2004) The anti-inflammatory response dominates after septic shock: association of low monocyte HLA-DR expression and high interleukin-10 concentration. *Immunol. Lett.* **95**, 193–198.
6. Schroder, M., Meisel, C., Buhl, K., Profanter, N., Sievert, N., Volk, H. D., Grutz, G. (2003) Different modes of IL-10 and TGF-β to inhibit cytokine-dependent IFN-γ production: consequences for reversal of lipopolysaccharide desensitization. *J. Immunol.* **170**, 5260–5267.
7. Del Fresno, C., Garcia-Rio, F., Gomez-Pina, V., Soares-Schanoski, A., Fernandez-Ruiz, I., Jurado, T., Kajiji, T., Shu, C., Marin, E., Gutierrez, del Arroyo, A., Prados, C., Arnalich, F., Fuentes-Prior, P., Biswas, S. K., Lopez-Collazo, E. (2009) Potent phagocytic activity with impaired antigen presentation identifying lipopolysaccharide-tolerant human monocytes: demonstration in isolated monocytes from cystic fibrosis patients. *J. Immunol.* **182**, 6494–6507.
8. Munoz, C., Carlet, J., Fitting, C., Misset, B., Bleriot, J. P., Cavaillon, J. M. (1991) Dysregulation of in vitro cytokine production by monocytes during sepsis. *J. Clin. Invest.* **88**, 1747–1754.
9. Gille, C., Leiber, A., Mundle, I., Spring, B., Abele, H., Spellerberg, B., Hartmann, H., Poets, C. F., Orlikowsky, T. W. (2009) Phagocytosis and postphagocytic reaction of cord blood and adult blood monocyte after infection with green fluorescent protein-labeled *Escherichia coli* and group B Streptococci. *Cytometry B Clin. Cytom.* **76**, 271–284.
10. Gille, C., Spring, B., Tewes, L., Poets, C. F., Orlikowsky, T. (2006) A new method to quantify phagocytosis and intracellular degradation using green fluorescent protein-labeled *Escherichia coli*: comparison of cord blood macrophages and peripheral blood macrophages of healthy adults. *Cytometry A* **69**, 152–154.
11. Dehio, M., Knorre, A., Lanz, C., Dehio, C. (1998) Construction of versatile high-level expression vectors for *Bartonella henselae* and the use of green fluorescent protein as a new expression marker. *Gene* **215**, 223–229.
12. Sierra-Filardi, E. , Puig-Kröger, A., Blanco, F. J., Nieto, C., Bragado, R., Palomero, M. I., Bernabéu, C., Vega, M. A., Corbí, A. L. (2011) Activin A skews macrophage polarization by promoting a proinflammatory phenotype and inhibiting the acquisition of anti-inflammatory macrophage markers. *Blood* **117**, 5092–5101.
13. Hsu, H. Y., Wittemann, S., Joos, T. O. (2008) Miniaturized parallelized sandwich immunoassays. *Methods Mol. Biol.* **428**, 247–261.
14. Hsu, H. Y., Wittemann, S., Schneider, E. M., Weiss, M., Joos, T. O. (2008) Suspension microarrays for the identification of the response patterns in hyperinflammatory diseases. *Med. Eng. Phys.* **30**, 976–983.
15. Peter, C., Waibel, M., Radu, C. G., Yang, L. V., Witte, O. N., Schulze-Osthoff, K., Wesselborg, S., Lauber, K. (2008) Migration to apoptotic "find-me" signals is mediated via the phagocyte receptor G2A. *J. Biol. Chem.* **283**, 5296–5305.
16. Gille, C., Leiber, A., Spring, B., Kempf, V. A., Loeffler, J., Poets, C. F., Orlikowsky, T. W. (2008) Diminished phagocytosis-induced cell death (PICD) in neonatal monocytes upon infection with *Escherichia coli*. *Pediatr. Res.* **63**, 33–38.
17. Hotchkiss, R. S., Swanson, P. E., Cobb, J. P., Jacobson, A., Buchman, T. G., Karl, I. E. (1997) Apoptosis in lymphoid and parenchymal cells during sepsis: findings in normal and T- and B-cell-deficient mice. *Crit. Care Med.* **25**, 1298–1307.
18. Park, S. H., Park-Min, K. H., Chen, J., Hu, X., Ivashkiv, L. B. (2011) Tumor necrosis factor induces GSK3 kinase-mediated cross-tolerance to endotoxin in macrophages. *Nat. Immunol.* **12**, 607–615.
19. Hotchkiss, R. S., Karl, I. E. (2003) The pathophysiology and treatment of sepsis. *N. Engl. J. Med.* **348**, 138–150.
20. Osuchowski, M. F., Welch, K., Siddiqui, J., Remick, D. G. (2006) Circulating cytokine/inhibitor profiles reshape the understanding of the SIRS/CARS continuum in sepsis and predict mortality. *J. Immunol.* **177**, 1967–1974.
21. Kox, W. J., Volk, T., Kox, S. N., Volk, H. D. (2000) Immunomodulatory therapies in sepsis. *Intensive Care Med.* **26** (Suppl. 1), S124–S128.

22. Van der Sluijs, K. F., Nijhuis, M., Levels, J. H., Florquin, S., Mellor, A. L., Jansen, H. M., van der Poll, T., Lutter, R. (2006) Influenza-induced expression of indoleamine 2,3-dioxygenase enhances interleukin-10 production and bacterial outgrowth during secondary pneumococcal pneumonia. *J. Infect. Dis.* **193,** 214–222.

23. Van der Sluijs, K. F., van Elden, L. J., Nijhuis, M., Schuurman, R., Pater, J. M., Florquin, S., Goldman, M., Jansen, H. M., Lutter, R., van der Poll, T. (2004) IL-10 is an important mediator of the enhanced susceptibility to pneumococcal pneumonia after influenza infection. *J. Immunol.* **172,** 7603–7609.

24. Cavaillon, J. M., Pitton, C., Fitting, C. (1994) Endotoxin tolerance is not a LPS-specific phenomenon: partial momicry with IL-1, IL-10 and TGF-β. *J. Endotoxin Res.* **1,** 21–29.

25. Asadullah, K., Sterry, W., Stephanek, K., Jasulaitis, D., Leupold, M., Audring, H., Volk, H. D., Docke, W. D. (1998) IL-10 is a key cytokine in psoriasis. Proof of principle by IL-10 therapy: a new therapeutic approach. *J. Clin. Invest.* **101,** 783–794.

26. Wyant, T. L., Tanner, M. K., Sztein, M. B. (1999) Potent immunoregulatory effects of *Salmonella typhi flagella* on antigenic stimulation of human peripheral blood mononuclear cells. *Infect. Immun.* **67,** 1338–1346.

27. Boldrick, J. C., Alizadeh, A. A., Diehn, M., Dudoit, S., Liu, C. L., Belcher, C. E., Botstein, D., Staudt, L. M., Brown, P. O., Relman, D. A. (2002) Stereotyped and specific gene expression programs in human innate immune responses to bacteria. *Proc. Natl. Acad. Sci. USA* **99,** 972–977.

28. Nau, G. J., Richmond, J. F., Schlesinger, A., Jennings, E. G., Lander, E. S., Young, R. A. (2002) Human macrophage activation programs induced by bacterial pathogens. *Proc. Natl. Acad. Sci. USA* **99,** 1503–1508.

29. Pfeiffer, A., Bottcher, A., Orso, E., Kapinsky, M., Nagy, P., Bodnar, A., Spreitzer, I., Liebisch, G., Drobnik, W., Gempel, K., Horn, M., Holmer, S., Hartung, T., Multhoff, G., Schutz, G., Schindler, H., Ulmer, A. J., Heine, H., Stelter, F., Schutt, C., Rothe, G., Szollosi, J., Damjanovich, S., Schmitz, G. (2001) Lipopolysaccharide and ceramide docking to CD14 provokes ligand-specific receptor clustering in rafts. *Eur. J. Immunol.* **31,** 3153–3164.

30. Sun, K., Metzger, D. W. (2008) Inhibition of pulmonary antibacterial defense by interferon-γ during recovery from influenza infection. *Nat. Med.* **14,** 558–564.

31. Torchinsky, M. B., Garaude, J., Martin, A. P., Blander, J. M. (2009) Innate immune recognition of infected apoptotic cells directs T(H)17 cell differentiation. *Nature* **458,** 78–82.

32. Evans, H. G., Suddason, T., Jackson, I., Taams, L. S., Lord, G. M. (2007) Optimal induction of T helper 17 cells in humans requires T cell receptor ligation in the context of Toll-like receptor-activated monocytes. *Proc. Natl. Acad. Sci. USA* **104,** 17034–17039.

33. Kolls, J. K., Linden, A. (2004) Interleukin-17 family members and inflammation. *Immunity* **21,** 467–476.

34. Ouyang, W., Kolls, J. K., Zheng, Y. (2008) The biological functions of T helper 17 cell effector cytokines in inflammation. *Immunity* **28,** 454–467.

35. Shibata, K., Yamada, H., Hara, H., Kishihara, K., Yoshikai, Y. (2007) Resident Vδ1+ γδ T cells control early infiltration of neutrophils after *Escherichia coli* infection via IL-17 production. *J. Immunol.* **178,** 4466–4472.

36. Neely, C. J., Maile, R., Wang, M. J., Vadlamudi, S., Meyer, A. A., Cairns, B. A. 2011. Th17 (IFNγ-IL-17+) CD4+ T cells generated after burn injury may be a novel cellular mechanism for postburn injuries. *J. Trauma* **70,** 681–690.