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

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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 naive 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 Th17-polarized adaptive immune response in the context of secondary infection. Our findings extend the current knowledge of monocyctic reprogramming and identify the phagocytic capacity of monocytes as a putative sepsis biomarker. J. Leukoc. Biol. 91: 977–989; 2012.

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 is 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 monocyctic functions exhibited an exceptionally poor outcome [8]. Moreover, monocyctic 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 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 phagocytizing 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 Th17-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) density gradient centrifugation. Washed cells were resuspended in RPMI medium containing 10% FCS and gentamicin 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 phagocytizing 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 Th17-polarized adaptive immune response in the context of secondary infection.

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 (OD₆₀₀ =0.55–0.8). For the induction of gfp expression, the growth medium was supplemented with 50 μg/ml kanamycin and 1 mM isoprropyl-β-D-thiogalactopyranoside (Sigma-Aldrich, Taufkirchen, Germany) for 5 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 JES5-9D7, 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/cm² 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/cm² 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 phagocytose 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-pretreated 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 CBA Flex sets (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-pretreated 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 monocyctic 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 10 ng/mL M-CSF (M2; ImmunoTools, Friesoythe, Germany) and determination of CD3⁺ T cell frequencies. These PBMCs were then added to the autologous-pretreated and control PBMCs (ratio CD14:CD3 cells, 1:10; calculation based on the number of live CD14⁺ monocytes). Supernatants of these cocultures were collected after 18 or 40 h, respectively, and cytokine concentrations were determined using the CBA Flex sets (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-pretreated 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 bacterially reprogrammed monocytes**

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 ± s.d. 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.
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/H11001 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 apparentlyorchestrated. These data indicate that the phagocytic impairments of reprogrammed monocytes are apparently orches-
trated 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 sub-units 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.
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, whereas complement inactivation had virtually no effect on phagocytosis, whereas complement inactivation had virtually no effect (Fig. 5B). 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, whereas complement inactivation had virtually no effect.
A

- **CD64**: p = 0.131
- **CD32**: p = 0.089
- **CD16**: p = 2.3 x 10^-9

B

- **CD11b**: p = 1.2 x 10^-9
- **CD11c**: p = 0.101
- **CD18**: p = 3.0 x 10^-9

C

- **TLR4**: p = 0.126
- **CD36**: p = 1.9 x 10^-9
- **CD68**: p = 0.011
- **SR-A**: p = 0.013
- **SR-B1**: p = 0.210
- **LOX-1**: p = 5.8 x 10^-9

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**: n.s.
- **CD11b**: n.s.
- **CD18**: n.s.
- **Lox-1**: n.s.
- **CD36**: n.s.
- **CD64**: n.s.

E. coli (d0) - - - - - -

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984 Journal of Leukocyte Biology Volume 91, June 2012 www.jleukbio.org
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-6hiIL-10loIL-12/23loTGF-beta 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 Th17-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 potentely 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 coinubcation 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 FcγRs 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 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.
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, 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.

Figure 6. Bacterially prestimulated monocytes show a HLA-DR\textsuperscript{low}CD80\textsuperscript{hi}CD86\textsuperscript{hi} phenotype and contribute to a T\textsubscript{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\textsuperscript{+} 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\textsubscript{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,
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...
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 Th17-biased adaptive immune response by secreting IL-6 and TGF-B (Fig. 7). Interestingly, it has been shown that one relevant physiological stimulus for triggering the differentiation of naive CD4+ T cells into Th17 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 Th17 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, Th17 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. infections, it was demonstrated that CD4+ T cells into Th17 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 Th17 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, Th17 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 monocyte cytophilic reprogramming and potential clinical implications are lacking to date. In line with our observations, it has been shown recently that Th17-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.

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The authors have no financial conflict of interest.

REFERENCES


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*E. coli* · anti-inflammatory response · TLR · T<sub>H</sub>17