

The Proinflammatory CD14⁺CD16⁺DR⁺⁺ Monocytes Are a Major Source of TNF¹

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In human blood two monocyte populations can be distinguished, i.e., the CD14⁺CD16⁻DR⁺ classical monocytes and the CD14⁺CD16⁺DR⁺⁺ proinflammatory monocytes that account for only 10% of all monocytes. We have studied TNF production in these two types of cells using three-color immunofluorescence and flow cytometry on whole peripheral blood samples stimulated with either LPS or with the bacterial lipopeptide *S*-(2,3-bis(palmitoyloxy)-(2-RS)-propyl)-*N*-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys⁴-OH, trihydrochloride (Pam3Cys). After stimulation with LPS the median fluorescence intensity for TNF protein was 3-fold higher in the proinflammatory monocytes when compared with the classical monocytes. After stimulation with Pam3Cys they almost exclusively responded showing 10-fold-higher levels of median fluorescence intensity for TNF protein. The median fluorescence intensity for Toll-like receptor 2 cell surface protein was found 2-fold higher on CD14⁺CD16⁺DR⁺⁺ monocytes, which may explain, in part, the higher Pam3Cys-induced TNF production by these cells. When analyzing secretion of TNF protein into the supernatant in PBMCs after depletion of CD16⁺ monocytes we found a reduction of LPS-induced TNF by 28% but Pam3Cys-induced TNF was reduced by 64%. This indicates that the minor population of CD14⁺CD16⁺ monocytes are major producers of TNF in human blood. *The Journal of Immunology*, 2002, 168: 3536–3542.

Cells of the monocyte lineage are important elements of immune defense because these cells can phagocytize foreign material, present Ag to T cells, and produce a host of cytokines, including TNF, IL-1, and IL-6. Cytokine expression by monocytes is efficiently triggered by stimulation with bacterial products like LPS or *S*-(2,3-bis(palmitoyloxy)-(2-RS)-propyl)-*N*-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys⁴-OH, trihydrochloride (Pam3Cys).⁴ Both LPS and Pam3Cys bind to cell surface CD14 with LPS acting via the Toll-like receptor (TLR)4 coreceptor (1) while Pam3Cys acts via the TLR2 coreceptor. Ligation of these receptor complexes leads to activation of a signal transduction cascade, mobilization of transcription factors, and expression of cytokines like TNF. TNF is a master cytokine that regulates the immune response by activating other cells for enhanced proliferation, receptor expression, and migration. In addition, TNF has been shown to regulate the production of other cytokines.

The cells of the monocyte lineage derive from myelomonocytic stem cells in bone marrow. They mature to monocytes and, as such, they go into blood followed by migration into tissue. In tissue these cells, now termed macrophages, differentiate into phenotypically and functionally distinct cell types like alveolar macrophages, osteoclasts, or microglia cells. While the heterogeneity of these tissue macrophages is well established, monocyte heterogeneity has been clearly demonstrated only recently. Using mAbs and flow cytometry we could distinguish the classical CD14⁺ cells and the CD14⁺CD16⁺ monocytes (2). In healthy donors these cells account for ~50 cells/ μ l and ~10% of all monocytes. The CD14⁺CD16⁺ monocytes will increase dramatically in patients with severe infection (3–5), and excessive exercise will also increase the cells by factor 4 due to mobilization from the marginal pool (6). The CD14⁺CD16⁺ monocytes appear to be more mature, they express higher levels of HLA-DR Ags as compared with classical monocytes (7), and they show expression of a distinct pattern of cytokines. Specifically, the CD14⁺CD16⁺ cells have been shown to efficiently produce the proinflammatory cytokine TNF, while they produce no or little of the anti-inflammatory cytokine IL-10 (8). Based on these findings we have termed these cells proinflammatory monocytes.

Cytokine expression by human monocytes previously has been studied after isolation of the cells by density gradient separation, Ab staining, and cell sorting. Because these procedures may alter the function of these cells we have developed in the current study a system that involves stimulation of whole peripheral blood (WPB) followed by staining and analysis of cytokine expression. This system allows for a more meaningful analysis of the regulatory mechanisms involved in cytokine gene expression. Using this method we can demonstrate a higher level of TNF protein expression of the CD14⁺CD16⁺ monocytes as compared with the classical CD14⁺ cells. Also, depletion of these cells from PBMC populations will reduce the amount of Pam3Cys induced TNF in the supernatant by >60%, although the CD14⁺CD16⁺ monocytes account for only 10% of the monocytes. This suggests that in vitro, and even more so in vivo, during bacterial infection the minor

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Received for publication August 6, 2001. Accepted for publication January 25, 2002.

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¹ This work was supported by grants to L.Z.-H. from Deutsche Forschungsgemeinschaft (288/2), from Graduierten-Kolleg Infection and Immunity at the University of Muenchen, and from the Ernst and Berta Grimmke Foundation. M.S. is supported by the Alexander von Humboldt-Foundation.

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⁴ Abbreviations used in this paper: Pam3Cys, *S*-(2,3-bis(palmitoyloxy)-(2-RS)-propyl)-*N*-palmitoyl-(R)-Cys-(S)-Ser-(S)-Lys⁴-OH, trihydrochloride; BFA, brefeldin A; PMB, polymyxin B; TLR, Toll-like receptor; icTNF, intracellular TNF; WPB, whole peripheral blood.

population of CD14⁺CD16⁺ monocytes may be a major source of inflammatory cytokines like TNF.

Materials and Methods

Cells and stimulation

WPB anticoagulated with heparin at 10 U/ml was obtained from apparently healthy donors who were recruited from institute personnel and from students. Six hundred-microliter samples were incubated for 6 h at 37°C in 15-ml polypropylene tubes (catalog no. 188 261; Greiner, Frickenhausen, Germany) in the presence of brefeldin A (BFA) at 10 µg/ml final (catalog no. B-7651; Sigma, Muenchen, Germany). Samples were left untreated or were stimulated with LPS from *Salmonella minnesota* (catalog no. L-6261; Sigma) at 0.1–1000 ng/ml or with Pam3Cys (catalog no. L2000; ECM Microcollections, Tuebingen, Germany) at 1–100 µg/ml. For control of LPS contamination cultures were set up with and without polymyxin B (PMB; Pfizer, Karlsruhe, Germany) at 10 µg/ml. Also, using the Endosafe Limulus Gel Clot assay (catalog no. AE 010; Charles River Breeding Laboratories, Sulzfeld, Germany) (sensitivity: 0.06 EU/ml) for monitoring of our tissue culture reagents, including BFA and heparin, we found no detectable LPS.

Cell surface staining

The stimulated samples were treated with ammonium chloride buffer (0.83% w/v) for ~3 min until erythrocytes were lysed. After washing the leukocytes with staining buffer (PBS/2% FCS), cells were resuspended in 100 µl of staining buffer and Abs to CD14 (My-4-FITC at 18 µg/ml final concentration; catalog no. 6603511; Beckman Coulter, Krefeld, Germany) and HLA-DR (Immu357-PC-5 at 0.06 µg/ml final concentration; catalog no. 2659; Beckman Coulter) were added followed by incubation on ice for 20 min. After washing the samples with staining buffer the cells were fixed with paraformaldehyde at 4% in PBS for 20 min at room temperature followed by two wash steps. The CD16 Ag was detected using Ab Leu¹¹c-PE at 6 µg/ml final concentration (catalog no. 347617; BD Biosciences, Heidelberg, Germany).

Staining for TLRs

For staining of TLR2 the Ab TL2.1 (IgG 2a) (9) was used as Alexa 488 conjugate along with an Alexa 488 isotype control both at 10 µg/ml final concentration.

Intracellular staining

Paraformaldehyde-fixed samples were permeabilized with Perm/Wash solution (catalog no. 2097 KZ; BD Biosciences) for 5 min at room temperature. For detection of intracellular TNF (icTNF) we added PE-conjugated anti-TNF Ab (MP9–20A4, catalog no. RHTNFA04; Caltag Laboratories via Medac, Hamburg, Germany) or as isotype control PE-conjugated rat IgG1 (catalog no. R104; Caltag Laboratories) both at 10 µg/ml for 20 min on ice. Samples were then washed twice and were resuspended in PBS/0.5% paraformaldehyde to be analyzed within 24 h.

Specificity control

The specificity of TNF staining was determined by incubating the anti-TNF-PE conjugate for 10 min at room temperature with a 9-fold molar excess of recombinant human TNF (kindly provided by BASF-Knoll, Ludwigshafen, Germany). This mixture of Ab and cytokine was the added to the permeabilized cells. During our studies we noted that the intensity of icTNF staining in monocytes shows variability between individuals and over time. Therefore, data were interpreted always within a given experimental series.

Flow cytometry analysis

Analysis was done on a FACScan flow cytometer (BD Biosciences) and linearity of the instrument was shown by using Immuno-Brite standard beads (catalog no. PN 6603473; Beckman Coulter). Cells were analyzed by setting scatter gates around monocytes and a portion of the adjacent lymphocytes (see www.monocytes.de for further details). At least 500 CD14⁺DR⁺⁺CD16⁺ monocytes were analyzed per sample and fluorescence intensities were determined by subtracting the median fluorescence intensity of the isotype control from the median fluorescence intensity of the specific Ab. The resultant channels of specific median fluorescence intensity were used as a semiquantitative measure of receptor expression. For icTNF staining (see below) the specific (or $\delta = \Delta$) median fluorescence intensity was calculated by subtracting the signal for anti-TNF staining in the presence of excess rTNF from the specific staining.

Quantitative PCR

Quantitative PCR was performed using the LightCycler system (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions by using primer pairs as noted below. In brief, mRNA was isolated and reverse-transcribed as for conventional RT-PCR. A total of 3 µl of cDNA were used for amplification in the SYBR green format using the LightCycler FastStart DNA Master SYBR Green I kit from Roche Diagnostics (catalog no. 2 239 264). For quantitative PCR, the LightCycler system offers the advantage of speed and real-time measurement of fluorescent signal during amplification. The SYBR green dye binds specifically to the minor groove of dsDNA. Fluorescence intensity is measured after each amplification cycle. During PCR, a doubling of template molecules occurs in each cycle only during the log-linear phase. Although the LightCycler displays signals from every cycle, the instrument uses fluorescent signals only generated during this informative log-linear phase to calculate the relative amount of template DNA.

The following primer pairs were used: TLR2 5' primer, 5'-GCC AAAGTCTTGATGATTGG-3', and 3' primer, 5'-TTGAAGTCTCCA GCTCCTG-3' (product length: 394 bp); TLR4 5' primer, 5'-GAAATGGA GGCACCCCTTC-3', and 3' primer, 5'-TGGATACGTTTCCTTATAAG-3' (product length: 510 bp). As an internal control the housekeeping gene α -enolase was amplified.

Depletion of CD16⁺ monocytes

For depletion of CD14⁺CD16⁺ monocytes from PBMC were isolated by Ficoll-Hypaque (Pharmacia, Freiburg, Germany) density gradient separation. A total of 10×10^6 cells each were resuspended in 100 µl of PBS containing 5 µl of CD16 microbeads (catalog no. 130-045-701; Miltenyi Biotec, Bergisch-Gladbach, Germany) or for control CD8 microbeads (catalog no. 130-045-201; Miltenyi Biotec). After incubation for 30 min at 4°C cells were washed and resuspended in 0.5 ml PBS and this was loaded onto a RS column (catalog no. 130-042-201; Miltenyi Biotec) that was positioned in a MiniMACS magnet (catalog no. 130-042-102; Miltenyi Biotec). Nonadherent cells were recovered and total number of CD14-positive monocytes and percentage of CD14⁺CD16⁺ monocytes were determined by FACS. Cells were then incubated for 5 h at 37°C without or with stimulation by either Pam3Cys (2 µg/ml) or LPS (2 ng/ml). Supernatant TNF was determined by ELISA (PeliKine, catalog no. M1923; Hiss Diagnostics, Freiburg, Germany) and was expressed in picograms per 10^6 monocytes. Average depletion of CD14⁺CD16⁺ monocytes with this procedure was 94%.

Culture of isolated CD14⁺⁺ monocytes for demonstration of the stability of phenotype

For culture of classical monocytes, PBMC were isolated by Ficoll-Hypaque density gradient separation and were depleted of CD16-positive cells using CD16 microbead Abs and MACS as given in the preceding paragraph. Depleted cells were then enriched for classical monocytes using CD14 microbead Ab (catalog no. 130-050-201; Miltenyi Biotec). Purified cells were then cultured in the presence of BFA (10 µg/ml) for 6 h without and with LPS at 100 ng/ml. The monocytes were then stained for CD14-PE (catalog no. 6603262; Beckman Coulter) and HLA-DR-PE-Cyan5 conjugate (catalog no. 2659; Beckman Coulter) and analyzed by FACS.

Statistics

For statistical analysis Student's *t* test was used.

Results

Identification of monocyte populations

Monocyte subpopulations can be readily defined as CD14⁺CD16⁺ and CD14⁺⁺CD16 cells using CD14 and CD16 Abs in two-color immunofluorescence. However, when WPB cells are stimulated with LPS for several hours, then the CD16 cell surface molecule is down-regulated such that the CD14⁺CD16⁺ monocytes cannot be clearly identified anymore (data not shown). Under the same conditions cells coexpressing low levels of CD14 plus high levels of HLA-DR retained their staining pattern. To demonstrate that these

CD14⁺DR⁺⁺ cells are identical to the CD14⁺CD16⁺ monocytes we performed three-color immunofluorescence analyses. In the two-color plot for CD14 and DR (Fig. 1, *middle panel*) a population that is DR-only (DR-positive lymphocytes), a population co-expressing high levels of CD14 and low levels of DR

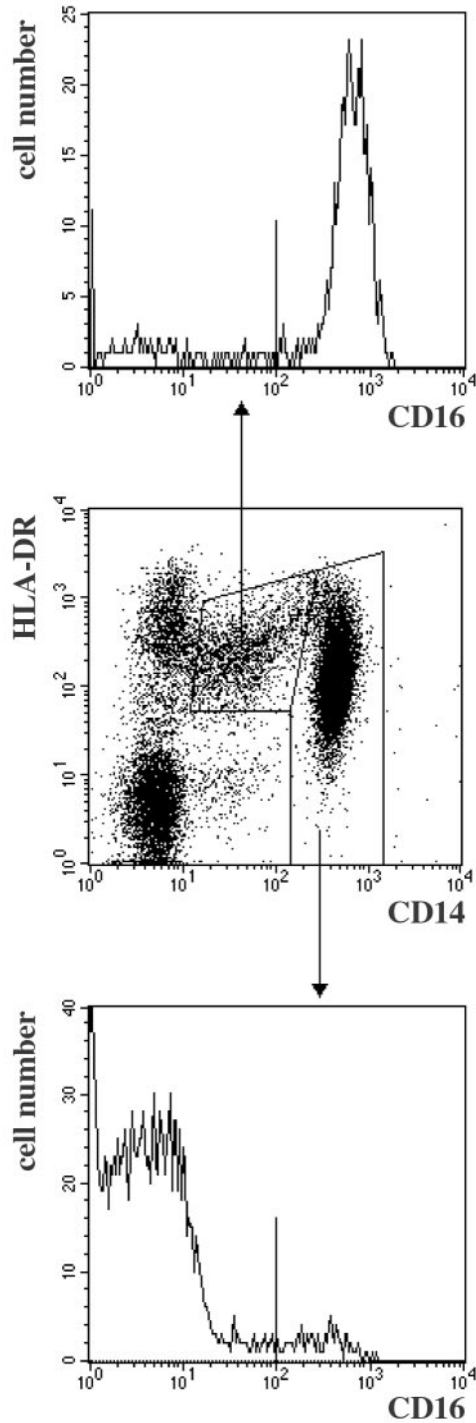


FIGURE 1. The majority of CD14⁺DR⁺⁺ cells are CD16 positive. WPB cells were stained in three-color immunofluorescence for CD14, CD16, and HLA-DR. The low CD14 cells coexpressing high levels of HLA-DR were gated as given in the *middle panel* and were analyzed for CD16 expression (*upper and lower panels*). In this example 93.7% of the CD14⁺DR⁺⁺ cells are CD16 positive. Among the CD14⁺DR⁺ monocytes 2.8% were positive for CD16. The minor population of cells with low CD14 and no HLA-DR showed 9.9% CD16-positive cells (no histogram shown)

(CD14⁺DR⁺ classical monocytes), and a population with the CD14⁺DR⁺⁺ phenotype are visible. When the CD14⁺DR⁺⁺ cells are gated and analyzed for CD16, the majority of cells were found CD16 positive (Fig. 1, *upper panel*).

In the example given, 94% of the CD14⁺DR⁺⁺ cells were CD16 positive with an average of $93.1 \pm 2.6\%$ in five experiments. Among the CD14⁺DR⁺ monocytes only 2.8% were positive for CD16 (Fig. 1, *lower panel*) (average $2.1 \pm 0.5\%$). Thus gating on the CD14⁺DR⁺⁺ population can be used as an alternative approach for the analysis of the CD14⁺CD16⁺ monocytes. To further substantiate this point we have isolated CD14⁺DR⁺ monocytes and have cultured them in the presence of BFA without and with LPS for 6 h. The data in Fig. 2 show that there is no relevant generation of CD14⁺DR⁺⁺ cells from CD14⁺DR⁺ monocytes under these conditions.

TNF expression in LPS-stimulated monocyte subpopulations

We then stimulated whole blood samples with LPS at 100 ng/ml for 6 h in the presence of BFA and stained the cells for CD14, DR, and TNF. A typical example, shown in Fig. 3, gives the TNF expression for the two monocyte populations as depicted in Fig. 3, *middle panel*. The single parameter histograms (Fig. 3, *upper and lower panels*) give the signal obtained with the anti-TNF-Ab (Fig.

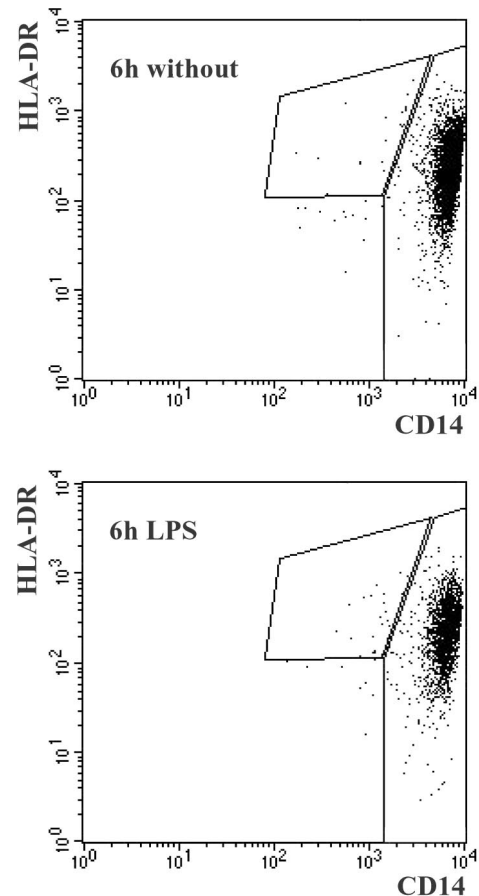


FIGURE 2. Stability of CD14⁺⁺ monocyte phenotype. CD14⁺⁺ monocytes were purified by MACS and were cultured for 6 h in the presence of BFA without (*upper panel*) and with LPS at 100 ng/ml (*lower panel*). Cells were then stained for CD14 and HLA-DR. In the example given, the CD14⁺DR⁺⁺ monocytes were 0.5% without and 0.8% with LPS stimulation. In three experiments the average respective numbers were 0.5 ± 0.1 and $1.1 \pm 0.6\%$ (not significant).

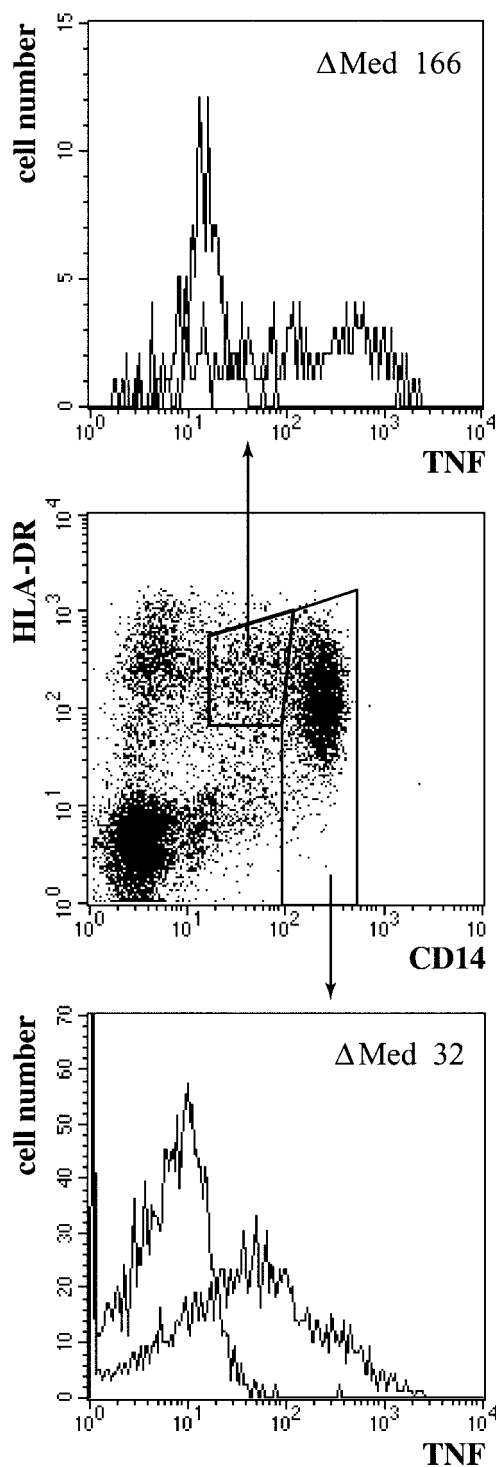


FIGURE 3. TNF expression in monocyte subpopulations. Whole blood samples were stimulated with LPS at 100 ng/ml for 6 h in the presence of BFA and then the cells were stained for CD14, DR (middle panel), and TNF. Shown is the anti-TNF staining (right curve) vs the control (left curve) for the CD14⁺ cells (lower panel) as compared with the CD14⁺DR⁺⁺ monocytes (upper panel). The specific median fluorescence intensity is given in channels (ch) in the upper right corner of each graph. Data are from one of five experiments.

3, upper and lower panels, right curve) as compared with the negative control, which is the anti-TNF staining in the presence of a 9-fold molar excess of rTNF (Fig. 3, upper and lower panels, left curve). It demonstrates a strong expression of TNF by both mono-

cyte populations, but the CD14⁺DR⁺⁺ monocytes exhibit a clearly higher level of expression. In five donors, the average median fluorescence intensity was 37 ± 18.8 in the CD14⁺ monocytes while the CD14⁺DR⁺⁺ monocytes expressed TNF with a median fluorescence intensity of 125.4 ± 55.1 channels ($p < 0.05$); i.e., the signal for TNF protein was ~ 3 -fold higher in these cells.

We then analyzed TNF expression in monocyte subpopulations over time and noted a weak expression already at 0.5 h with an average median fluorescence intensity of 12 channels for the CD14⁺DR⁺⁺ monocytes. Expression increased over time and reached an average of 525 channels at 6 h (Fig. 4). While at all time points the CD14⁺DR⁺⁺ monocytes exhibited a higher immunofluorescence signal for TNF, this difference was only significant for the 4- and 6-h time points.

Given the lower level of CD14 LPS receptor in the CD14⁺DR⁺⁺ monocytes as compared with the classical monocytes, we reasoned that the former cells might exhibit a lower sensitivity to LPS in dose response analysis. As shown in Fig. 5, these cells did, however, show a first response at the 1 ng/ml dose similar to the classical CD14⁺DR⁺ cells. At this dose the cells produced TNF with an average median of 17 channels, expression increased to 261 channels at 100 ng/ml and reached 365 channels at the highest dose of 1000 ng/ml. Again, TNF expression was clearly higher in the CD14⁺DR⁺⁺ monocytes as compared with the classical monocytes at all doses (Fig. 5). Hence, when comparing the two monocyte populations, the CD14⁺DR⁺⁺ monocytes respond at the same low dose of LPS and the response was even higher despite the low-level expression of the CD14 LPS receptor.

TNF expression in Pam3Cys-stimulated monocyte subpopulations

We next asked whether the higher expression of TNF in the CD14⁺DR⁺⁺ monocytes is also seen after stimulation with other microbial products like Pam3Cys. To exclude the possibility that a LPS contamination of the Pam3Cys preparation causes monocyte stimulation we have used PMB for LPS neutralization. PMB, in fact, is very active in that it completely abrogates the LPS-induced TNF production (Fig. 6, lower panel). When looking at TNF production by the two monocyte subsets we observed that Pam3Cys almost exclusively stimulated the CD14⁺DR⁺⁺ proinflammatory monocytes. While the classical CD14⁺DR⁺ monocytes responded minimally with an average median immunofluorescence of 28 ± 12 channels, the proinflammatory monocytes exhibited an

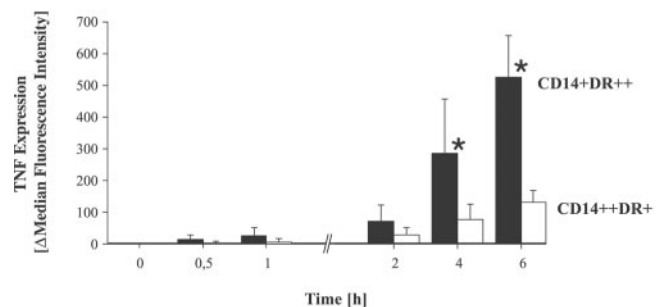


FIGURE 4. Time course for TNF production in the two monocyte subpopulations. WPB samples were stimulated with LPS at 100 ng/ml in the presence of BFA. At various time points cells were stained for CD14, DR, and TNF. Given is the TNF expression as the average \pm SD for three donors expressed as specific median fluorescence intensity in the CD14⁺DR⁺⁺ monocytes (filled bars) and in the CD14⁺DR⁺ cells (open bars). TNF expression in the CD14⁺DR⁺⁺ monocytes was significantly higher at time points 4 and 6 h as compared with the values in the CD14⁺DR⁺ monocytes (*, $p < 0.05$).

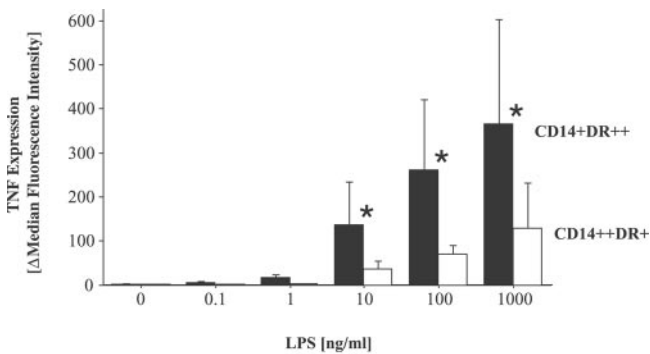


FIGURE 5. Dose response analysis for LPS-induced TNF expression in the two monocyte subpopulations. WPB samples were stimulated with various doses of LPS for 6 h in the presence of BFA. Cells were then stained for CD14, DR, and TNF. Given is the TNF expression as the average \pm SD for three donors expressed as specific median fluorescence intensity in the CD14⁺DR⁺⁺ monocytes (filled bars) and in the CD14⁺⁺DR⁺ cells (open bars). Also, TNF expression in the CD14⁺DR⁺⁺ monocytes was significantly higher at doses 1–1000 ng/ml as compared with the values in the CD14⁺⁺DR⁺ monocytes (*, $p < 0.05$).

average median immunofluorescence intensity of 304 ± 166 channels (Fig. 6, upper panel). Admixture of PMB to the Pam3Cys did not appreciably affect the activity, thereby excluding a significant LPS contamination. Hence, the proinflammatory monocytes show a 10-fold signal for TNF protein after stimulation with Pam3Cys. In dose response analysis Pam3Cys efficiently stimulated TNF expression down to a dose of 1 μ g/ml. Even at this low dose the CD14⁺DR⁺⁺ cells gave a median specific immunofluorescence signal for TNF at 109 ± 68 channels compared with 13 ± 6 channels for the classical CD14⁺⁺DR⁺ monocytes.

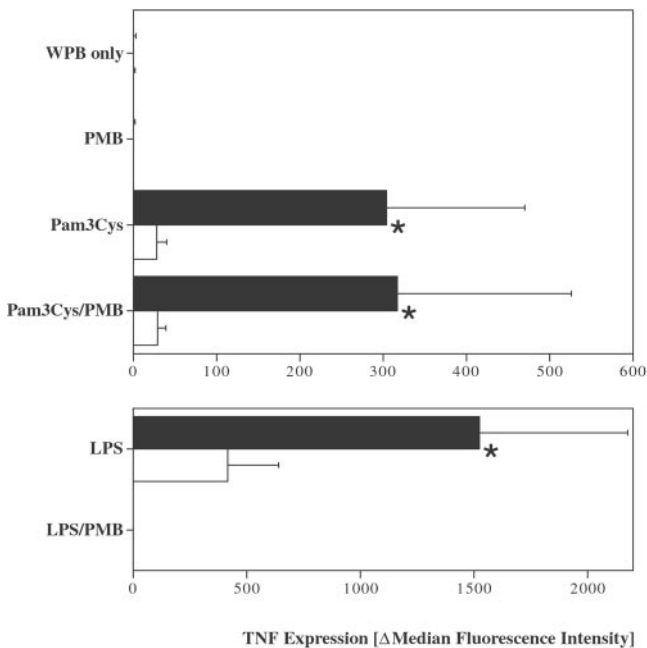


FIGURE 6. Effect of Pam3Cys on TNF production in monocytes subpopulations. WPB samples were stimulated for 6 h in the presence of BFA with LPS (10 ng/ml) or Pam3Cys (100 μ g/ml) without and with admixture of PMB. Given is average \pm SD of six experiments expressed as specific median fluorescence intensity in the CD14⁺DR⁺⁺ monocytes (filled bars) and in the CD14⁺⁺DR⁺ cells (open bars). The higher level of TNF in the CD14⁺DR⁺⁺ monocytes was significant for both stimuli ($p < 0.05$).

Expression of TLRs

Staining for TLR4 using Ab HTA-125 (10) in three-color immunofluorescence in our hands revealed no signal both on the CD14⁺⁺ monocytes and the CD14⁺CD16⁺ monocytes (data not shown). By contrast, TLR2 was clearly detectable and gave a 2-fold-higher specific median fluorescence intensity level in the CD14⁺CD16⁺ monocytes as compared with the classical CD14⁺⁺ cells (Fig. 7). In five donors the average signal for TLR2 in CD14⁺CD16⁺ monocytes was 58.7 ± 9.1 channels as compared with 33.4 ± 9.8 channels for the classical CD14⁺⁺ monocytes ($p < 0.05$), reflecting a 2-fold level of specific median immunofluorescence intensity. A similar pattern was seen when the monocyte subsets were identified as CD14⁺DR⁺⁺ and CD14⁺⁺DR⁺ cells (data not shown).

To detect TLR mRNA in the monocyte subsets we used mRNA analysis by RT-PCR using the LightCycler technology. As shown in the example in Fig. 8 the CD14⁺CD16⁺ monocytes exhibited slightly lower levels of mRNA for TLR2 and almost identical levels for TLR4. In four donors the average mRNA level was calculated to be 1.7-fold lower for TLR2 ($p < 0.05$) and 1.2-fold lower for TLR4 (not significant). Thus CD14⁺CD16⁺ proinflammatory monocytes do not express higher amounts of TLR mRNA; rather, they tend to show slightly lower expression as compared with the classical monocytes.

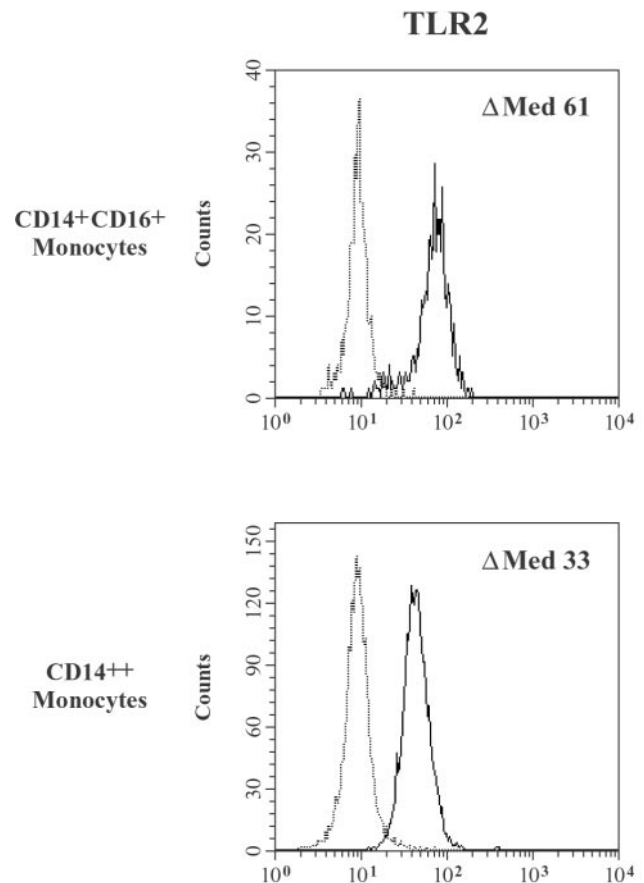


FIGURE 7. Expression of TLR2 by monocyte subpopulations. WPB samples were stained with TLR2 Ab TL2.1 as Alexa 488 conjugate followed by staining with CD14 and CD16 Abs. Shown is the TLR expression relative to isotype staining for both monocyte subpopulations. Given in the upper right corner is the specific median fluorescence intensity. Shown are data from one of five experiments.

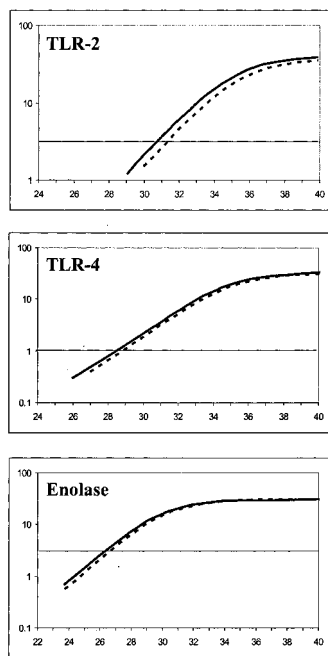


FIGURE 8. Expression of TLR2 and TLR4 mRNA in monocyte subsets. mRNA was prepared from CD14⁺CD16⁺ and CD14⁺ monocytes (purity >90%) and cDNA was amplified in the presence of SYBR green in the Light-Cycler. Solid line, CD14⁺ monocytes; dashed line, CD14⁺CD16⁺ monocytes. Curves were analyzed by determining the cycle number at which the amplification curves intersect with the horizontal line. In this example, in the case of TLR2 the CD14⁺ curve intersects at 30.7 cycles while the CD14⁺CD16⁺ curve intersects at 28.7 cycles. For TLR4 the respective cycle numbers were 28.7 and 29. Shown are data from one of four experiments.

Effect of depletion of CD16⁺ monocytes on levels of secreted TNF

Because the CD14⁺CD16⁺DR⁺ monocytes are much more efficient producers of TNF as detected by intracellular staining we asked whether these cells also contributed a major portion to supernatant TNF secreted by stimulated PBMC. For this we reacted the PBMC with a microbead-conjugated CD16 Ab and depleted these cells using MACS. Upon LPS stimulation this led to a reduction of supernatant TNF by 28% ($p < 0.05$) as compared with control depletion with a CD8 Ab (Fig. 9). When stimulating the same cell preparations with Pam3Cys the CD16 depletion resulted in a much stronger reduction of secreted TNF, i.e., by 64% ($p < 0.005$). These data show that the minor population of CD14⁺CD16⁺ monocytes, which form ~10% of all monocytes in blood, account for the major proportion of the TNF protein that is secreted after stimulation via TLR2.

Discussion

Monocytes produce a host of cytokines (e.g., TNF, IL-1, IL-6) that regulate the immune response and inflammation. Many studies have looked at signals, receptors, and transcription factors involved in production of these cytokines by blood monocytes, but these studies have been performed with cells that have passed one or more steps of purification. Purification of monocytes may, however, alter these cells, as exemplified by the shedding of L-selectin that occurs during the standard density gradient separation of PBMC (11).

Analysis of monocytes directly in WPB can avoid these problems. We therefore have developed a system that employs stimulation of WPB followed by identification of monocytes with Abs to cell surface markers and by detection of cytokines with intracellular staining. The direct addition of stimuli and inhibitors to the freshly drawn blood then allows for the study of cytokine expression unbiased by any manipulation.

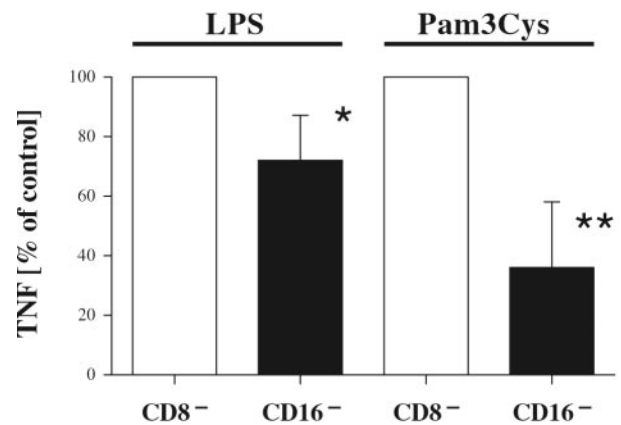


FIGURE 9. Effect of depletion of CD16⁺ monocytes on LPS- and Pam3Cys-stimulated TNF production. PBMC were depleted of CD16⁺ cells and for control of CD8-positive cells. After 5 h of stimulation with either LPS or Pam3Cys TNF was measured in supernatants calculated as picograms per 10⁶ monocytes and expressed as percentage of the CD8 control. Solid bars, CD16 depleted; open bars, CD8 control depletion. Shown is the average of four experiments (*, $p < 0.05$; **, $p < 0.005$).

With appropriate cell surface markers monocytes can be subdivided into two populations, i.e., the CD14⁺CD16⁺ and the CD14⁺ monocytes (2). These cells show a differential pattern of cytokine expression in that the CD14⁺CD16⁺ monocytes fail to produce significant amounts of the anti-inflammatory cytokine IL-10 while they are good producers of the proinflammatory TNF (8). This has led us to term these cells proinflammatory monocytes.

The earlier studies on cytokine expression by monocyte subpopulations were done with cells that were first stained for cell surface markers, then sorted, then stimulated with LPS. The current protocol involves initial stimulation followed by staining. However, LPS stimulation leads to the disappearance of the CD16 molecule from the monocyte cell surface such that the CD14⁺CD16⁺ monocytes cannot be readily identified anymore. The CD14⁺CD16⁺ monocytes are also characterized by a high level of HLA-DR and expression of this molecule is maintained with LPS stimulation. When purified CD14⁺DR⁺ cells with 0.71% remaining CD14⁺DR⁺ cells were cultured for 6 h in the presence of LPS, there was only a slight increase of the CD14⁺DR⁺ cells to 1.59% ($n = 3$). This indicates that the ~10% CD14⁺DR⁺ cells that we analyze for TNF production in whole blood after 6 h of stimulation with LPS are not derived from the CD14⁺DR⁺. Hence, cytokine expression of the CD14⁺CD16⁺ monocytes can be analyzed by looking at the CD14⁺DR⁺ cells, and this can be compared with the CD14⁺DR⁺ cells that are identical with the classical CD14⁺ monocytes.

Previous studies with isolation of monocytes followed by stimulation have demonstrated comparable levels of TNF mRNA and secreted protein for the two monocyte populations after stimulation by LPS (8). Using the WPB stimulation and staining strategy we can demonstrate a 3-fold higher level of LPS-induced TNF protein in the CD14⁺DR⁺ monocytes as compared with the classical monocytes. The detection of higher levels of TNF protein in the current study might be explained by advantages of this system, like the absence of interfering signals due to purification, or it could be due to the higher precision of the flow cytometry method.

Time course analysis revealed a very rapid induction of TNF protein at 0.5 h post-LPS stimulation, and this is most obvious for the CD14⁺DR⁺ monocytes. A maximum TNF production is observed at 6 h, and when prolonging the incubation to 12 h there is still some increase (data not shown). However, for practical reasons we have restricted our analysis to the 6-h time point. When

looking at dose response at this point in time we were surprised to see a pronounced TNF production already at 1 ng/ml LPS in the CD14⁺DR⁺⁺ monocytes while the CD14⁺⁺DR⁺ cells still were close to background (see Fig. 5).

In addition to LPS a dramatically higher response with respect to TNF was seen with Pam3Cys, a product of *Borrelia* and of other types of bacteria (12–16). Also, Zymosan derived from yeast and mycobacterial lipoarabinomannan both induced 2-fold higher levels of specific median immunofluorescence intensity for TNF in the proinflammatory monocytes (data not shown). The higher expression of TNF by the CD14⁺CD16⁺ monocytes might be due to a higher expression of cell surface receptors for these different microbial products. However, the pattern recognition receptor CD14 is lower in the CD14⁺CD16⁺ subset (17) (see Fig. 1). Hence, we have looked for the TLR coreceptors (1, 18–23). LPS responses are mediated by the TLR4 coreceptor. The Ab HTA-125 (10) in our hands unfortunately gave no staining on blood monocytes. Because LPS responses are mediated by TLR4 and because the blood monocytes showed high levels of TNF expression after stimulation with *S. minnesota* LPS in our experiments we expect that with an appropriate reagent TLR4 protein will be detectable on both monocyte subpopulations.

By contrast, levels of TLR2, the coreceptor used by Pam3Cys (18), were readily detected and were 2-fold higher in the immunofluorescence signal in the CD14⁺CD16⁺ monocytes. When we assume that the level of TLR2 is crucial in determining the strength of cell activation, then the higher TLR2 level may, in fact, be responsible in part for the higher response of the CD14⁺CD16⁺DR⁺⁺ monocytes to Pam3Cys.

Of note, the LPS preparation used in this study has not been repurified (24) and therefore it may also partially act via TLR2 due to contaminant lipoproteins.

Based on the higher expression of TNF protein in the CD14⁺CD16⁺DR⁺⁺ monocytes as detected by intracellular staining we may assume that these cells, with their proportion of 10% among all monocytes, could account for a large part of the TNF secreted by stimulated PBMC. Our studies, in fact, demonstrate that depletion of these cells by MACS will reduce secreted TNF by ~30%, consistent with a 3-fold-higher icTNF level in the CD14⁺DR⁺⁺ cells as compared with the CD14⁺⁺DR⁺ monocytes. When stimulated by Pam3Cys the reduction by depletion of CD16⁺ cells was more pronounced (~60%), again consistent with the 10-fold-higher levels of icTNF seen with Pam3Cys. Hence, for Pam3Cys stimulation the minor population of CD14⁺CD16⁺DR⁺⁺ monocytes is responsible for the major part of secreted TNF.

The blood drawn from donors at rest does not cover all leukocytes in circulation because it is restricted to the central pool. A fair amount of additional cells resides in the marginal pool, which comprises cells that are loosely attached to endothelium. These cells can be mobilized, for instance, by stress or excessive exercise. We reported earlier that compared with the CD14⁺CD16⁺ monocytes in the central pool there are three times the number of these cells found in the marginal pool (6). Hence, when looking at total monocytes in circulation (marginal plus central pool), the CD14⁺CD16⁺ monocytes account for 30% of all monocytes. When bacteria or their products seed into blood they will stimulate all CD14⁺CD16⁺ monocytes, including those in the central and in the marginal pool. Hence, in vivo the importance of the proinflammatory monocytes is even higher than demonstrated herein for cell preparations derived from the central pool of blood.

Our data suggest that the CD14⁺CD16⁺ monocytes contribute significantly to LPS-stimulated cytokine production that occurs in Gram-negative infection. The contribution is more pronounced when it comes to Pam3Cys, a compound that represents lipopeptides that are expressed by both Gram-positive and Gram-negative bacteria (12–

16). Hence, lipopeptide-induced cytokines may contribute to any type of bacterial infection. However, while in Gram-negative infection the effects of LPS may predominate, the lipopeptides may be dominant in TLR2-mediated monocyte activation in Gram-positive infection. Based on the present findings, which demonstrate the CD14⁺CD16⁺ monocytes to be the main source or Pam3Cys-stimulated TNF production, we suggest that these proinflammatory monocytes are major players in Gram-positive infection.

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