The Journal of Immunology

RESEARCH ARTICLE | APRIL 01 2002

The Proinflammatory $CD14^+CD16^+DR^{++}$ Monocytes Are a Major Source of TNF[1]

FREE

Kai-Uwe Belge; ... et. al

J Immunol (2002) 168 (7): 3536–3542.
https://doi.org/10.4049/jimmunol.168.7.3536

The Proinflammatory $CD14^+CD16^+DR^{++}$ Monocytes Are a Major Source of TNF[1]

Kai-Uwe Belge,* Farshid Dayyani,‡¶ Alexia Horelt,* Maciej Siedlar,[2]* Marion Frankenberger,‡ Bernhard Frankenberger,† Terje Espevik,§ Löms Ziegler-Heitbrock[3]‡¶

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)-Lys[4]-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)-Lys[4]-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/μl 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

*Institute for Immunology, University of Muenchen, and †GSF National Research Center for Environment and Health, Institute of Molecular Immunology, Muenchen, Germany; ‡Clinical Cooperation Group, Aerosols in Medicine, Institute for Inhalationbiology, GSF National Research Center for Environment and Health and Asklepios-Fachkliniken Muenchen-Gauting, Gauting, Germany; §Institute of Cancer Research and Molecular Biology, Medisinsk Teknisk Senter, Trondheim, Norway; and ¶Department for Microbiology and Immunology, University of Leicester, Leicester, United Kingdom

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

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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

[2] M.S. is on leave from the Department of Clinical Immunology, Jagiellonian University Medical College, Krakow, Poland.

[3] Address correspondence and reprint requests Dr. Löms Ziegler-Heitbrock, Department for Microbiology and Immunology, University of Leicester, University Road, Leicester, LE1 9HN, U.K. E-mail address: ziehei@gmx.de

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

0022-1767/02/$02.00

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 Leu^{11}c-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 AAAGTCTTGATTGATTGG-3′, and 3′ primer, 5′-TTGAAGTTCTCCA 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 coexpressing high levels of CD14 and low levels of DR ($CD14^{++}DR^+$ 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 ± 2.6% in five experiments. Among the $CD14^{++}DR^+$ monocytes only 2.8% were positive for CD16 (Fig. 1, *lower panel*) (average 2.1 ± 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.

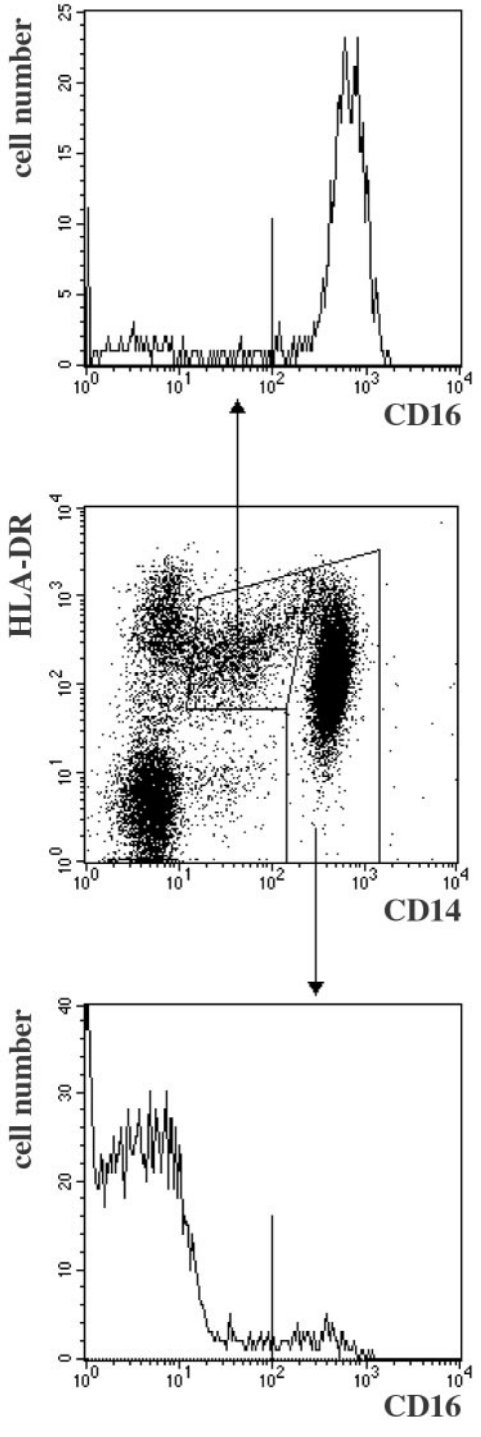

FIGURE 1. The majority of $CD14^+DR^{++}$ cells are CD16 positive. WPB cells were stained in three-color immunofluorescence for CD14, CD16, and HLA-DR. The low CD14 cells coexpressing high levels of HLA-DR were gated as given in the *middle panel* and were analyzed for CD16 expression (*upper* and *lower 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)

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.

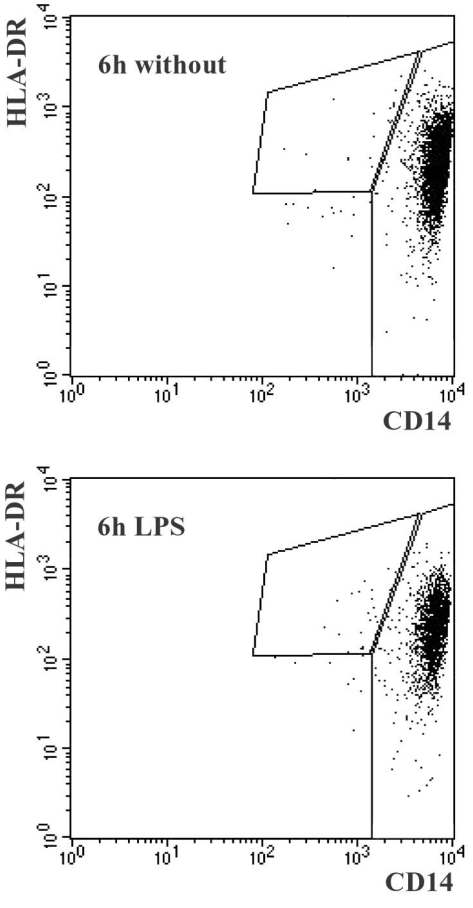

FIGURE 2. Stability of $CD14^{++}$ monocyte phenotype. $CD14^{++}$ monocytes were purified by MACS and were cultured for 6 h in the presence of BFA without (*upper panel*) and with LPS at 100 ng/ml (*lower panel*). Cells were then stained for CD14 and HLA-DR. In the example given, the $CD14^+DR^{++}$ 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 ± 0.6% (not significant).

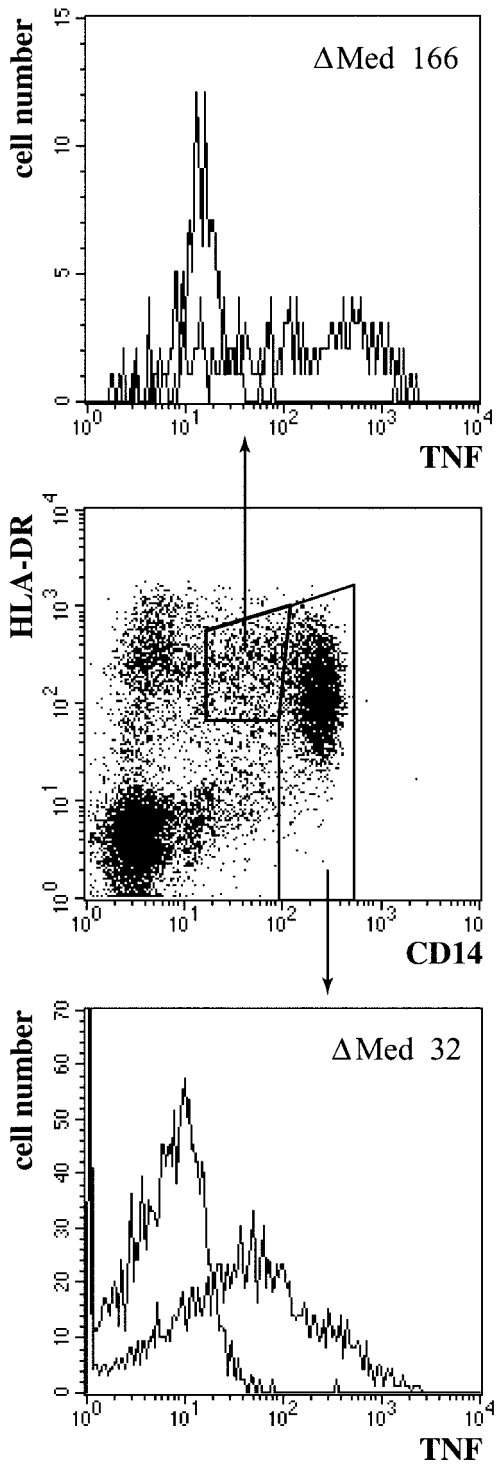

FIGURE 3. TNF expression in monocyte subpopulations. Whole blood samples were stimulated with LPS at 100 ng/ml for 6 h in the presence of BFA and then the cells were stained for CD14, DR (*middle panel*), and TNF. Shown is the anti-TNF staining (*right curve*) vs the control (*left curve*) for the $CD14^{++}$ 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 monocyte 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

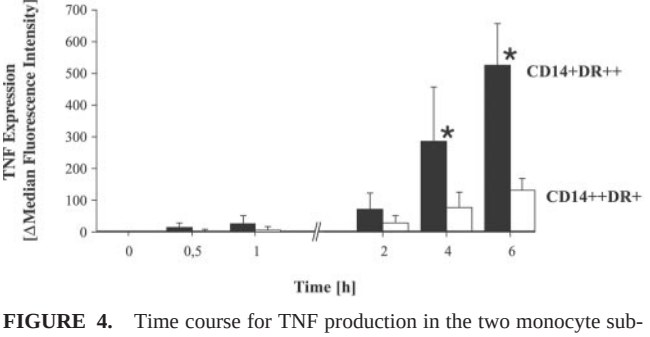

FIGURE 4. Time course for TNF production in the two monocyte subpopulations. WPB samples were stimulated with LPS at 100 ng/ml in the presence of BFA. At various time points cells were stained for CD14, DR, and TNF. Given is the TNF expression as the average ± SD for three donors expressed as specific 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 (*, $p < 0.05$).

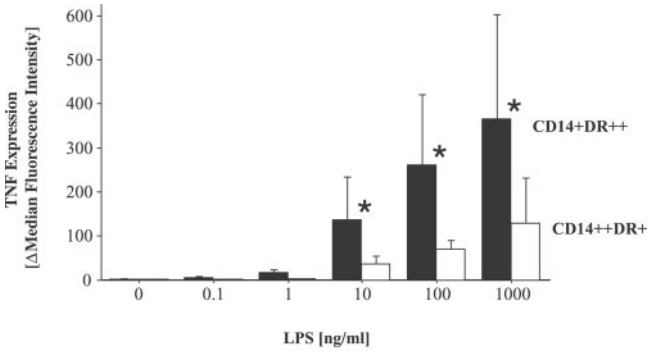

FIGURE 5. Dose response analysis for LPS-induced TNF expression in the two monocyte subpopulations. WPB samples were stimulated with various doses of LPS for 6 h in the presence of BFA. Cells were then stained for CD14, DR, and TNF. Given is the TNF expression as the average ± SD for three donors expressed 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 μg/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.

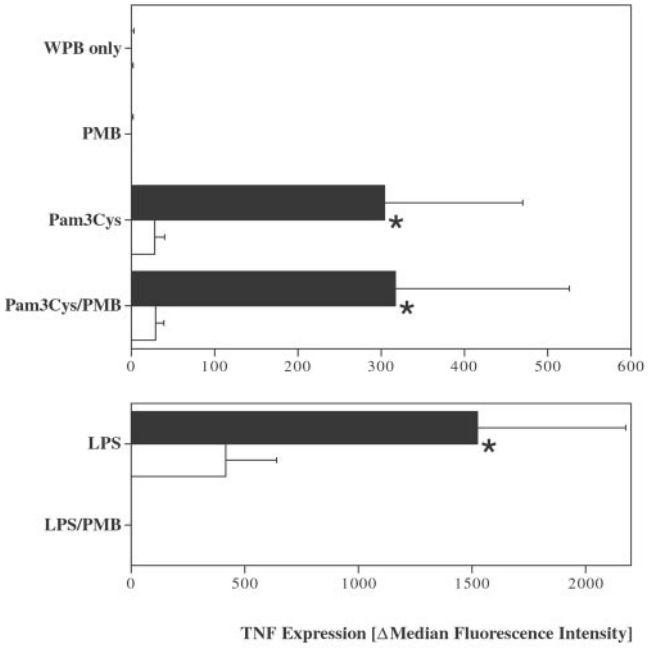

FIGURE 6. Effect of Pam3Cys on TNF production in monocytes subpopulations. WPB samples were stimulated for 6 h in the presence of BFA with LPS (10 ng/ml) or Pam3Cys (100 μg/ml) without and with admixture of PMB. Given is average ± SD of six experiments expressed as specific median fluorescence intensity 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.

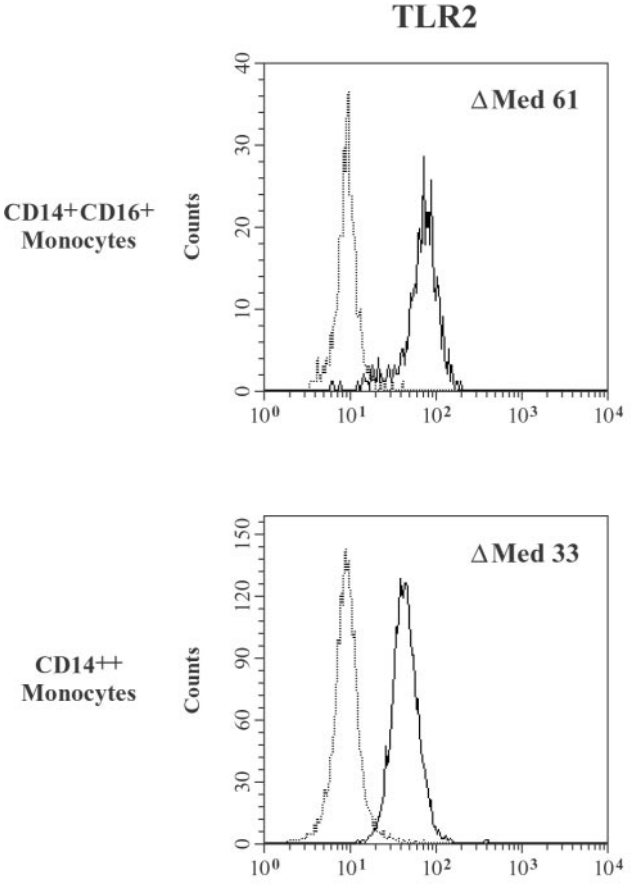

FIGURE 7. Expression of TLR2 by monocyte subpopulations. WPB samples were stained with TLR2 Ab TL2.1 as Alexa 488 conjugate followed by staining with CD14 and CD16 Abs. Shown is the TLR expression relative to isotype staining for both monocyte subpopulations. Given in the *upper right corner* is the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

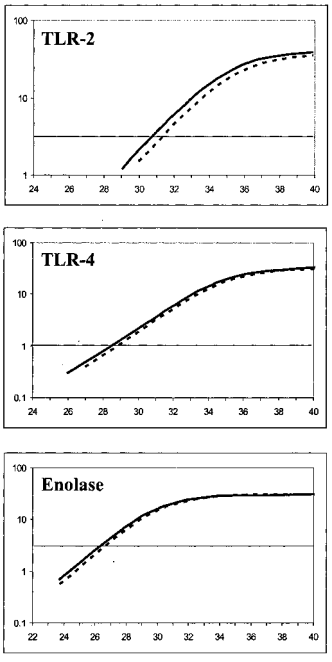

FIGURE 8. Expression of TLR2 and TLR4 mRNA in monocyte subsets. mRNA was prepared from $CD14^+CD16^+$ and $CD14^{++}$ monocytes (purity >90%) and cDNA was amplified in the presence of SYBR green in the LightCycler. Solid line, $CD14^{++}$ monocytes; dashed line, $CD14^+CD16^+$ monocytes. Curves were 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 31.4 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.

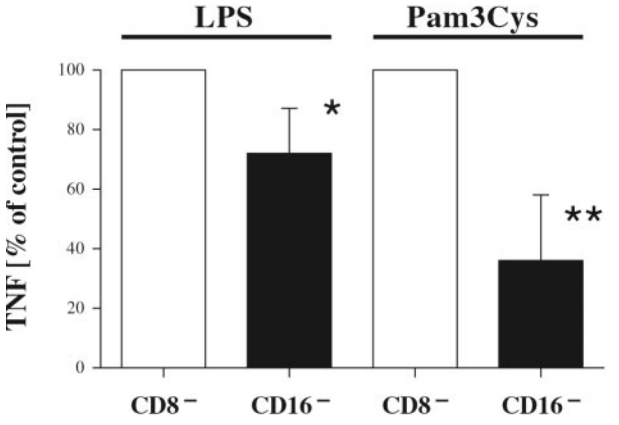

FIGURE 9. Effect of depletion of $CD16^+$ monocytes on LPS- and Pam3Cys-stimulated TNF production. PBMC were depleted of $CD16^+$ cells and for control of CD8-positive cells. After 5 h of stimulation with either LPS or Pam3Cys TNF was measured in supernatants calculated as picograms per 10^6 monocytes 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.

References

1. Poltorak, A., X. He, L. Smirnova, M. Y. Liu, C. V. Huffel, X. Du, D. Birdwell, E. Alejos, M. Silva, C. Galanos, et al. 1998. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in *Tlr4* gene. *Science 282:2085.*
2. Ziegler-Heitbrock, H. W. L. 1996. Heterogeneity of human blood monocytes: the $CD14^+CD16^+$ subpopulation. *Immunol. Today 17:424.*
3. Fingerle, G., A. Pforte, B. Passlick, M. Blumenstein, M. Strobel, and H. W. Ziegler-Heitbrock. 1993. The novel subset of $CD14^+/CD16^+$ blood monocytes is expanded in sepsis patients. *Blood 82:3170.*
4. Blumenstein, M., P. Boekstegers, P. Fraunberger, R. Andreesen, H. W. L Ziegler-Heitbrock, and G. Fingerle-Rowson. 1997. Cytokine production precedes the expansion of $CD14^+CD16^+$ monocytes in human sepsis: a case report of a patient with self-induced septicemia. *Shock 8:73.*
5. Nockher, W. A., and J. E. Scherberich. 1998. Expanded $CD14^+CD16^+$ monocyte subpopulation in patients with acute and chronic infections undergoing hemodialysis. *Infect. Immun. 66:2782.*
6. Steppich, B., F. Dayyani, R. Gruber, R. Lorenz, M. Mack, and H. W. Ziegler-Heitbrock. 2000. Selective mobilization of $CD14^+CD16^+$ monocytes by exercise. *Am. J. Physiol. 279:C578.*
7. Ziegler-Heitbrock, H. W., G. Fingerle, M. Strobel, W. Schraut, F. Stelter, C. Schutt, B. Passlick, and A. Pforte. 1993. The novel subset of $CD14^+/CD16^+$ blood monocytes exhibits features of tissue macrophages. *Eur. J. Immunol. 23:2053.*
8. Frankenberger, M., T. Sternsdorf, H. Pechumer, A. Pforte, and H. W. Ziegler-Heitbrock. 1996. Differential cytokine expression in human blood monocyte subpopulations: a polymerase chain reaction analysis. *Blood 87:373.*
9. Lien, E., T. J. Sellati, A. Yoshimura, T. H. Flo, G. Rawadi, R. W. Finberg, J. D. Carroll, T. Espevik, R. R. Ingalls, J. D. Radolf, and D. T. Golenbock. 1999. Toll-like receptor 2 functions as a pattern recognition receptor for diverse bacterial products. *J. Biol. Chem. 274:33419.*
10. Shimazu, R., S. Akashi, H. Ogata, Y. Nagai, K. Fukudome, K. Miyake, and M. Kimoto. 1999. MD-2, a molecule that confers lipopolysaccharide responsiveness on Toll-like receptor 4. *J. Exp. Med. 189:1777.*
11. Stibenz, D., and C. Buhrer. 1994. Down-regulation of L-selectin surface expression by various leukocyte isolation procedures. *Scand. J. Immunol. 39:59.*
12. De, B. K., J. S. Sampson, E. W. Ades, R. C. Huebner, D. L. Jue, S. E. Johnson, M. Espina, A. R. Stinson, D. E. Briles, and G. M. Carlone. 2000. Purification and characterization of *Streptococcus pneumoniae* palmitoylated pneumococcal surface adhesin A expressed in *Escherichia coli*. *Vaccine 18:1811.*
13. Bessler, W. G., L. Heinevetter, K. H. Wiesmuller, G. Jung, W. Baier, M. Huber, A. R. Lorenz, U. V. Esche, K. Mittenbuhler, and P. Hoffmann. 1997. Bacterial cell wall components as immunomodulators. I. Lipopeptides as adjuvants for parenteral and oral immunization. *Int. J. Immunopharmacol. 19:547.*
14. Schuch, R., and A. T. Maurelli. 1999. The mxi-Spa type III secretory pathway of *Shigella flexneri* requires an outer membrane lipoprotein, MxiM, for invasin translocation. *Infect. Immun. 67:1982.*
15. Tibor, A., B. Decelle, and J. J. Letesson. 1999. Outer membrane proteins Omp10, Omp16, and Omp19 of *Brucella* spp. are lipoproteins. *Infect. Immun. 67:4960.*
16. Brandt, M. E., B. S. Riley, J. D. Radolf, and M. V. Norgard. 1990. Immunogenic integral membrane proteins of *Borrelia burgdorferi* are lipoproteins. *Infect. Immun. 58:983.*
17. Passlick, B., D. Flieger, and H. W. Ziegler-Heitbrock. 1989. Identification and characterization of a novel monocyte subpopulation in human peripheral blood. *Blood 74:2527.*
18. Thoma-Uszynski, S., S. M. Kiertscher, M. T. Ochoa, D. A. Bouis, M. V. Norgard, K. Miyake, P. J. Godowski, M. D. Roth, and R. L. Modlin. 2000. Activation of Toll-like receptor 2 on human dendritic cells triggers induction of IL-12, but not IL-10. *J. Immunol. 165:3804.*
19. Medzhitov, R., P. Preston-Hurlburt, and C. A. Janeway, Jr. 1997. A human homologue of the *Drosophila* Toll protein signals activation of adaptive immunity. *Nature 388:394.*
20. Kirschning, C. J., H. Wesche, T. Merrill-Ayres, and M. Rothe. 1998. Human Toll-like receptor 2 confers responsiveness to bacterial lipopolysaccharide. *J. Exp. Med. 188:2091.*
21. Yang, R. B., M. R. Mark, A. Gray, A. Huang, M. H. Xie, M. Zhang, A. Goddard, W. I. Wood, A. L. Gurney, and P. J. Godowski. 1998. Toll-like receptor-2 mediates lipopolysaccharide-induced cellular signalling. *Nature 395:284.*
22. Krutzik, S. R., P. A. Sieling, and R. L. Modlin. 2001. The role of Toll-like receptors in host defense against microbial infection. *Curr. Opin. Immunol. 13:104.*
23. Yoshimura, A., E. Lien, R. R. Ingalls, E. Tuomanen, R. Dziarski, and D. Golenbock. 1999. Recognition of Gram-positive bacterial cell wall components by the innate immune system occurs via Toll-like receptor 2. *J. Immunol. 163:1.*
24. Hirschfeld, M., Y. Ma, J. H. Weis, S. N. Vogel, and J. J. Weis. 2000. Cutting edge: repurification of lipopolysaccharide eliminates signaling through both human and murine Toll-like receptor 2. *J. Immunol. 165:618.*