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IL-10 Enhances MD-2 and CD14 Expression in Monocytes and the Proteins Are Increased and Correlated in HIV-Infected Patients

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Soluble proteins that bind LPS, like myeloid differentiation-2 (MD-2) and CD14, have essential roles in regulating LPS signaling through TLR4. During a Gram-negative bacterial infection, the host may control the response by adjusting the levels of soluble MD-2 and CD14. To address the surface expression of MD-2 on human leukocytes, we developed a mAb, IIC1, that recognized MD-2 both free and when bound to TLR4. MD-2 was found on the surface of freshly isolated monocytes, on a subpopulation of CD19+ B-cells and on CD15+ neutrophils. LPS transiently reduced the MD-2 levels on monocytes, which is most likely due to endocytosis of the LPS receptor complex since MD-2 colocalized with TLR4 in early endosomes after LPS stimulation. In the absence of LPS, MD-2 partly colocalized with TLR4 in Golgi trans and medial compartments. Cultivating monocytes for 18–20 h resulted in loss of MD-2 expression on the surface, which was reversed either by LPS or IL-10. Furthermore, addition of IL-10, but not LPS, resulted in a considerable increase in mRNA for both MD-2 and CD14. Using ELISA, we demonstrated that IL-10 had a profound dose- and time-related effect on the release of soluble MD-2 and soluble CD14 from monocytes. In HIV-infected patients, the amounts of MD-2, CD14, and IL-10 increased significantly in the patient group with AIDS. Of interest, we found that IL-10, CD14, and MD-2 levels were positively correlated, suggesting that IL-10 may be a driving force for increased release of MD-2 and CD14 during systemic inflammation. The Journal of Immunology, 2009, 182: 588–595.
highly susceptible to *Salmonella typhimurium* infections (11). Elevated levels of sCD14 are associated with various systemic inflammatory diseases (3, 12, 13). Plasma from patients with sepsis also contains increased levels of MD-2 (14). MD-2 is secreted by various cell types, and a recent report has shown that IL-6 is a potent inducer of MD-2 secretion from human hepatocytes (15). One would think that the regulation of sMD-2 and sCD14 may serve important functions in host defense against Gram-negative bacteria. However, little is known about the regulation of MD-2 and CD14 secretion from cells.

To address the question on MD-2 protein expression, we developed a novel mAb against human MD-2 that is suitable for the analysis of cell surface bound MD-2. By using this mAb, we describe analyses of MD-2 surface expression on various leukocyte populations. Surprisingly, we found that IL-10 was a very potent stimulator of MD-2 and CD14 mRNA and protein in monocytes. Using ELISAs, we found that both CD14, and in particular MD-2, were markedly elevated in sera from HIV-infected patients and notably, both proteins were significantly correlated with serum levels of IL-10 in these patients. These data provide new information about the role of IL-10 in the regulation of sMD-2 and sCD14.

**Materials and Methods**

**Reagents**

LPS (0111:B4) from *Escherichia coli* was purchased from Invivogen. Pam3Cys was purchased from Boehringer Mannheim Biochemica. Poly(I:C) was from Amersham Pharmacia Biotech. IL-1β, IL-10, and a neutralizing IL-10 mAb were purchased from R&D Systems. TNF-α was obtained from Genentech. Anti-TLR4 (HTA125) was provided by Dr. K. Miyake (Saga Medical School, Japan), anti-CD14 was either 5C5 (3) or 3C10 (American Type Culture Collection). Anti-Flag (M2) was purchased from Sigma-Aldrich. Abs against EEA-1 (EEA1:FITC), Golgin-84, CD19, CD27, CD15, RIPI05, and isotype-matched controls were obtained from Becton Dickinson. Rabbit anti-human Giantin conjugated with Alexa Fluor 488 was purchased from Covance Research Products. Abs were conjugated with Alexa fluorochromes as indicated according to the manufacturer’s protocol (Invitrogen).

**Cells and cell stimulation**

Monocytes were isolated from A− buffy coats (The Blood Bank, St. Olavs Hospital) by Lymphoprep (Axis-Shift) and plastic adherence (16), and grown in RPMI 1640 with 5% A− serum (The Blood Bank). LPS was sonicated for 5 min and preincubated in RPMI 1640/5% A− serum at 37°C for 5 min before addition to cells. For stimulation with LPS, Pam3Cys, TNF-α, IL-1β, and IL-10, a concentration of 20 ng/ml was used. For Poly I:C 5 μg/ml was used. For confocal imaging, the cells were seeded on 35 mm glass-bottom gamma-irradiated dishes (MatTek Corporation). Tonsils were obtained from one patient undergoing routine tonsillectomy at the Department of Otolaryngology, Head and Neck Surgery, St. Olav’s University Hospital. The patient sample was obtained after informed consent and the study was approved by the regional ethics committee. The tonsil was minced, diluted in PBS, and filtered through a 70-μm pore size nylon filter (BD Biosciences) to deplete larger cells. Lymphocytes were isolated by Lymphoprep (Axis-Shift) and enriched for B cells (98% pure CD19 filter (BD Biosciences) to deplete larger cells. Lymphocytes were isolated from PBMCs by Lymphoprep (Axis-Shield) and plastic adherence (16), and preincubated in RPMI 1640/5% A− serum, then detached with a cell scraper (Sarstedt). To address the question on MD-2 protein expression, we developed a novel mAb against human MD-2 that is suitable for the analysis of cell surface bound MD-2. By using this mAb, we describe analyses of MD-2 surface expression on various leukocyte populations. Surprisingly, we found that IL-10 was a very potent stimulator of MD-2 and CD14 mRNA and protein in monocytes. Using ELISAs, we found that both CD14, and in particular MD-2, were markedly elevated in sera from HIV-infected patients and notably, both proteins were significantly correlated with serum levels of IL-10 in these patients. These data provide new information about the role of IL-10 in the regulation of sMD-2 and sCD14.

**Flow cytometry**

Adherent monocytes and B cells were incubated with stimuli as indicated in RPMI 1640/5% A− serum, then detached with a cell scraper (Sarstedt). The cells were washed once with PBS (0–4°C), fixed with 1% FCS/PBS. Non-specific binding was blocked with PBS/20% A− serum for 15 min on ice, washed once with PBS/2% A−, and labeled with 10 μg/ml primary Ab in PBS/2% A− for 30 min on ice. The cells were washed three times with PBS/2% A− before secondary labeling with goat anti-mouse Ig FITC (Becton Dickinson) for 30 min on ice, washed, and analyzed by flow cytometry (Excel, Coulter).

**Confocal imaging**

For intracellular imaging, cells were fixed with 3% paraformaldehyde/PBS on ice for 15 min, washed once with 1% FCS/PBS, permeabilized with 0.1% saponin/20% A−/PBS at 22°C for 20 min, then incubated with the appropriate Abs (5 μg/ml in 2%/A− serum/0.1% saponin/PBS) for 45 min at 22°C. The cells were washed three times with 2%/A− serum/0.1% saponin/PBS before incubation with 2 μl PBS. Images were captured with an Axiovert 100-M microscope housing a Zeiss LSM 510 META scanning unit and a 1.4 × 63 plan apochromat objective.

**RNA isolation and cDNA synthesis**

Monocytes were stimulated as indicated for 6 or 18 h in RPMI 1640/5% A− serum. Cells were washed once with PBS (0–4°C), then detached with a cell scraper (Sarstedt). Total RNA was isolated from monocytes with the RNeasy Mini Kit (Qiagen) according to the manufacturer’s protocol. Total RNA was quantified with ND-1000 Spectrophotomer (NanoDrop). cDNA was made using the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer’s protocol.

**Real-time PCR**

The sequences of primers used were as follows: GAPDH: 5′-GAA-GGT-GAA-GGT-CGG-AGT-C-3′ and 3′-GAA-GAT-GAT-GAT-GGG-ATT-TC-3′; MD-2: 5′-TAC-ACC-TAC-TGT-CAT-AAA-ATG-3′ and 3′-AAA-GAG-TAA-TCG-TCA-TCA-3′; CD14: 5′-GCCATGACGCCACGCGCAGAAGACTCT-3′ and 3′-CGACGCCCCCTGGGACCGACGATCCAA TTGCA-3′; TLR4: 5′-CGT-GCC-CTGC-GGT-GCT-TTA-3′ and 3′-AAA-GGC-TCC-CAG-GGG-TAA-AC-3′. Standard curves for MD-2, CD14, TLR4, and appurtenant GADPH were obtained from cDNA from the MD-2 expressing OH-2 myeloma cell line and PBMC, respectively. cDNA from monocytes was prepared for analyzes according to the manufacturer’s protocol using LightCycler Fast Start DNA MasterPLUS SYBR Green I kit (Roche). CD14, MD-2, TLR4, and GADPH cDNA expressions were measured with a LightCycler (Roche), using the Roche Molecular Biochemical LightCycler Software version 3.5. Measurements obtained were quantified with RelQuant software (Roche). The data are presented as mean fold increase in mRNA expression ± SEM of three independent experiments.
ELISA for sMD-2 and sCD14

sMD-2 was captured on immunoplates coated with a TLR4-Fc fusion protein (18), and detected with digoxygenin labeled MD-2 mAb 5D7 (14) or digoxygenin labeled IIC1 and anti-digoxygenin-HRP (Roche). The MD-2 standard was either purchased from R&D Systems or provided by Dr. R. Jerala (National Institute of Chemistry, Ljubljana, Slovenia). Measurements of sCD14 were performed using a sandwich ELISA with CD14-specific mAbs 3C10 and 5C5 according to a previously published protocol (3).

Blood sampling from HIV-infected patients

Blood samples were collected from 49 HIV-infected patients (30 males and 19 females, 32 ± 7 years) classified according to Centers for Disease Control and Prevention (CDC) in CDC group A (asymptomatic HIV infection, n = 17), CDC group B (symptomatic non-AIDS, n = 20) and AIDS (CDC group C, n = 12). Blood samples were obtained in periods without any acute infection or exacerbation of chronic infection. Blood samples were also taken from 38 patients with ongoing infection with *Mycobacterium avium* complex (MAC) infection (29 males and 9 females, 35 ± 8 years). None of the patients received highly active antiretroviral therapy. HIV RNA levels were measured by quantitative reverse PCR (Amplicor HIV Monitor; Roche Diagnostic Systems). The limit of detection was 40 copies/ml plasma. For comparison, 30 HIV-seronegative sex- and age-matched healthy blood donors were also included in the study. Informed consent for blood sampling was obtained from all subjects. The study was conducted according to the ethical guidelines at our hospital in conformity with the Helsinki declaration and was approved by the local ethical committee. Peripheral venous blood was drawn into sterile blood collection tubes without any additives (Becton Dickinson), immediately immersed in melting ice, and allowed to clot before centrifugation (1000 g for 10 min). Serum samples were stored at −80°C and were thawed less than three times.

Statistical analysis

For comparisons of more than two groups, the Kruskal-Wallis test was used. If a significant difference was found, the Mann-Whitney U test was used to determine the differences between each pair of groups. Coefficients of correlation were calculated by the Spearman rank test. Probabilities are two-sided and considered to be significant when *p* < 0.05.

Results

Generation of a monoclonal anti-human MD-2 Ab

To assess MD-2 expression in human cells, we wanted to generate a mAb that could bind MD-2 either free or in complex with TLR4. Few mAbs against human MD-2 are available (14, 19), and some will only detect MD-2 when bound to TLR4 (14). A hybridoma secreting a mAb, IIC1, was generated and selected for further studies as this mAb bound to recombinant (r) MD-2 and stained HEK293 cells transfected with CD14, TLR4, and Flag-tagged MD-2 (Fig. 1), but not to Flag-tagged TLR2 expressing cells (Fig 1B). The mAb IIC1 also stained CHO cells transfected with MD-2 and TLR4, but not cells transfected with TLR4 alone (data not shown).

IIC1 identified a protein with a mw of around 26 kDa in Western blots (Fig. 1C). A strong MD-2 band was detected in CHO cells transfected with TLR4 and Flag-tagged MD-2 immunoprecipitated

![FIGURE 1. Characterization of a human MD-2 mAb. A, HEK293 cells stably transfected with CD14 and Flag-tagged MD-2 were stained with Abs against CD14 (3C10), TLR2 (TL2.1), MD-2 (IIC1), and Flag (M2) followed by addition of goat anti-mouse Ig-FITC. As a control, the same staining procedure was performed on HEK293 cells stably expressing Flag-tagged TLR2 (B). Gray shadow fill represents the staining obtained with the goat anti-mouse Ig-FITC only. C, Immunoblots with the MD-2 mAb (IIC1). Lysates from CHO-TLR4 cells with and without MD-2 (Flag) were prepared. The mAbs were conjugated to tosyl-activated Dynabeads (30 μg/10^8 beads) for 24 h at 37°C and washed/blocking with 100 mM Tris buffer for 24 h before incubation with the lysates. The eluates were run on 12% BisTris SDS-PAGE gels and immunoblotted with the IIC1 mAb. The MD-2 band is clearly detectable in CHO-TLR4-MD-2 cells with IIC1. Lane 1, CHO cells expressing MD-2 and TLR4 immunoprecipitated with IIC1; lane 2, CHO cells expressing TLR4/Flag-tagged MD-2 immunoprecipitated with anti-Flag; lane 3, CHO cells expressing Flag-tagged TLR4 immunoprecipitated with anti-Flag.](http://www.jimmunol.org/DownloadedFrom.jpg)
with IIC1 (Fig. 1C, lane 1) or anti-Flag (Fig. 1C, lane 2). Respective control was Flag-TLR4 transfected CHO cells with anti-Flag-conjugated beads (Fig. 1C, lane 3). A weak MD-2 band was also detected in monocytes when IIC1 was used for immunoprecipitation and detection (data not shown). These data show that the IIC1 mAb recognizes a protein that is compatible in size with MD-2. This mAb is of the IgM isotype and the TNF inducing abilities of LPS on monocytes is not inhibited by IIC1 (data not shown). Furthermore, IIC1 does not affect the binding of LPS to monocyte membranes (data not shown). In summary, the IIC1 is a nonagonistic mAb that recognizes human MD-2 and which is useful for flow cytometric detection and immunofluorescence microscopy.

Expression of surface MD-2 on leukocytes

Next, we wanted to use the IIC1 mAb to obtain information on the expression levels of the MD-2 protein in leukocytes. Human PBMC were stained with A488-IIC1 in combination with anti-CD14PE and anti-CD19PE. As can be seen from Fig. 2A, >90% of freshly isolated CD14 positive monocytes expressed considerable amounts of surface-bound MD-2. Around 6% of the CD14 negative lymphocytes also had detectable amounts of MD-2. Since B cells are reported to contain mRNA for MD-2 (20), we examined whether CD19 positive B cells expressed MD-2. Around 40% of the CD19+ cells expressed MD-2 on the surface (Fig. 2A). Since MD-2 was expressed in a population of B cells, we also wanted to verify that B cells isolated from tonsils contained detectable levels of MD-2 on the cell surface. In B cells from tonsils, surface bound MD-2 was detected in around 57% of the CD19+ population (Fig. 2B), however, the MD-2 expression was not confined to the whole CD27+ memory population as only ~50% of CD27+ cells expressed MD-2 (Fig. 2B). In support of these findings, B cells from tonsils also contained significant levels of MD-2 mRNA (data not shown). Furthermore, CD15 positive neutrophils contained surface

**FIGURE 2.** Expression of MD-2 on leukocytes. A, PBMC were either stained with anti-CD14PE and anti-MD-2-A488 (Alexa Fluor 488) (IIC1) or anti-CD19PE and anti-MD-2-A488 (IIC1) with corresponding isotype matched controls. Each dot plot represents 30,000 cells analyzed. B, FACS analyses of B cells isolated from human tonsils. B cells were isolated by negative selection using the B cell isolation kit II (Miltenyi Biotec). The cells were labeled with anti-CD19TriColor, anti-CD27PE and anti-MD-2-A488 (IIC1), with corresponding isotype matched controls. In brief, 20,000 cells were analyzed. C, Neutrophils and monocytes were separated both by their CD15 expression and their scatter signal during FACS analysis and the cells were stained with MD-2-A488 mAb IIC1. The gray shadow fill represents staining with an isotype matched control mAb. Dot plots and histograms are representatives from three experiments.

**FIGURE 3.** Confocal microscopy of MD-2 and TLR4 localization in human monocytes. Colocalizations are represented as intensity profiles along the respective arrows in the boxed pictures. A and B, MD-2 and TLR4 located on early endosomes. Monocytes were stimulated with medium only (A) or LPS (20 ng/ml) for 5 min (B), fixed and permeabilized, then labeled with anti-TLR4 (HTA125-Alexa Fluor 546), anti-MD-2 (IIC1-Alexa Fluor 647), and an early endosome marker (anti-EEA-1-FITC). C and D, MD-2 and TLR4 in Golgi media and trans-Golgi, respectively. Nonstimulated monocytes were fixed and permeabilized, then directly labeled with anti-TLR4 (HTA125-Alexa Fluor 546), anti-MD-2 (IIC1-Alexa Fluor 647 or IIC1-Alexa Fluor 488), and a Golgi media or trans-Golgi marker (Giantin-Alexa 488 or anti-Golgin-Alexa Fluor 647), respectively. The images in the figure are representatives from three independent experiments.
bound MD-2, however, the level of expression was lower when compared with monocytes (Fig. 2C).

**MD-2 and TLR4 localization in human monocytes**

Previous studies have indicated that MD-2 is required for trafficking of TLR4 to the plasma membrane (11). Thus, we wanted to visualize by immunofluorescence microscopy the spatial distribution of TLR4 and MD-2 in monocytes. Nonstimulated monocytes and monocytes stimulated with LPS (20 ng/ml) were fixed and permeabilized, then directly labeled with HTA125-Alexa Fluor 546 (anti-TLR4) and IIC1-Alexa Fluor 647 or IIC1-Alexa Fluor 488. Early endosomes, cis and trans-Golgi were identified by direct labeling with anti-EEA-1-Alexa Fluor 488, anti-Giantin-Alexa Fluor 488, and anti-Golgini-Alexa Fluor 647, respectively. MD-2 was found to colocalize with TLR4 in small clusters on the plasma membrane (data not shown) and also, after LPS stimulation for 5–15 min, in early endosomes (Fig. 3, A and B), where TRAM/TRIF signaling is taking place (21, 22). MD-2/TLR4 colocalization was only seen in a smaller part of cis-and trans-Golgi. MD-2 was much less prominent in the Golgi apparatus than compared with TLR4, which was observed in parts of the trans- and cis-Golgi (Fig. 3, B and C). Thus, it is likely that the specific glycosylation essential for cell surface expression of TLR4 (23) occurs at distinct areas of the Golgi.

**Regulation of MD-2 and CD14 surface expression**

Since addition of LPS resulted in localization of MD-2 in early endosomes (Fig. 3B), it is likely that LPS induces receptor-mediated uptake of MD-2 as has been observed for TLR4 (2). As can be seen from Fig. 4A, addition of LPS to freshly isolated monocytes for 2 h resulted in a marked down-regulation of surface MD-2 expression. Cultivating monocytes at 37°C overnight (18–20 h) considerably reduced the amount of surface-bound MD-2 (Fig. 4B). However, adding LPS prevented the loss of MD-2 from the monocyte membrane. Also, by adding 20 ng/ml of IL-10 for 18–20 h the MD-2 levels were retained (Fig. 4B). This IL-10 treatment did not significantly affect the TLR4 or RH105 expression in monocytes (data not shown). The surface expression of CD14 is high on monocytes and LPS and IL-10 only marginally increased the surface expression of CD14 (Fig. 4C). These results suggest that LPS and IL-10 may either stabilize MD-2 on the surface, or activate MD-2 transcription. Monocytes were also stimulated for 18–20 h with cytokines such as IFN-γ, IL-1β, IL-6, or TNF. None of these cytokines had any effect on MD-2 surface expression on monocytes (data not shown). In addition, treatment of PBMC with IL-10 overnight did not increase the MD-2 expression on the CD19+ lymphocytes (data not shown).

**Regulation of MD-2 and CD14 mRNA expression**

Since addition of either LPS or IL-10 resulted in a marked increased in surface expression of MD-2 on monocytes, we next examined whether the mRNA level for MD-2 was affected by these stimuli. Monocytes received either medium or LPS, IL-1β, TNF-α, IL-10, Pam3Cys, or Poly(I:C). Total RNA was harvested after 6 and 18 h of stimulation and cDNA was made with Reverse Transcriptase Superscript III. MD-2 and CD14 gene expression was measured with real time PCR, using GAPDH as the reference gene, and fold change compared with nonstimulated cells was calculated (Fig. 5, A and B). LPS clearly reduced MD-2 mRNA after 18 h, but no effect was seen after 6 h. Pam3Cys and IL-1β gave similar response patterns as LPS, but not as prominent. Poly(I:C) seemed to marginally increase MD-2 mRNA after 18 h, and TNF had little effect. We noticed that LPS, Pam3Cys, and IL-1β had the opposite effect on CD14 compared with MD-2, tending to increase rather than decrease mRNA expression. Surprisingly, IL-10 was a strong inducer of both MD-2 and CD14 mRNA expression already after 6 h, and expression was still high after 18 h. Addition of IL-10 did not increase TLR4 mRNA expression (fold increase in TLR4 mRNA expression was 0.67 ± 0.03 after 6 h and 1.06 ± 0.03 after 18 h stimulation).

**IL-10 induces monocytes to secrete high amounts of sMD-2 and sCD14**

Since IL-10 was such a powerful inducer of MD-2 and CD14 mRNA, we wanted to examine whether IL-10 also induced secretion of sMD-2 and sCD14 from monocytes. Monocytes were given medium alone or stimulated with 0.2, 2, or 20 ng/ml IL-10 and harvested after 18 h. As shown in Fig. 5, C and D, IL-10 stimulated the production of sMD-2 and sCD14 in a dose-dependent manner. The MD-2 levels were almost doubled with 20 ng/ml IL-10 while the release of sCD14 increased 3–4-fold. An increase in sCD14 release was observed already at 0.2 ng/ml IL-10, while a 10-fold higher IL-10 concentration was required to induce an increased release of sMD-2 (Fig. 5, C and D). The release kinetics of both sMD-2 and sCD14 were rather slow and IL-10 had to be present for 10–20 h before an effect was observed (Fig. 5, E and F). The IL-10-induced production of sMD-2 seemed
to proceed more rapidly that sCD14. The IL-10 induced production of sMD-2 and sCD14 was specific since a neutralizing IL-10 Ab inhibited the induction (data not shown).

Having observed that IL-10 is a potent inducer of sMD-2 and sCD14 in human monocytes, we wanted to test whether this effect also could be observed in a clinical situation. We chose to examine serum samples taken from HIV-infected patients. These patients show persistent inflammation and are characterized by raised serum levels of IL-10 that significantly correlate with disease severity (24). As depicted in Fig. 6, HIV-infected patients (n = 49) had raised serum levels of IL-10, sCD14, and in particular of sMD-2 when compared with healthy controls (n = 30) with increasing levels according to disease severity (i.e., CDC classification), significantly correlated with number of HIV RNA copies in plasma (r = 0.38, p < 0.01; r = 0.56, p < 0.001; r = 0.54, p < 0.001; sMD-2, IL-10, and sCD14, respectively). Moreover, we have shown that IL-10 potently induce the release of MD-2 and CD14 in monocytes, and notably, in HIV-infected patients with raised levels of all these parameters, IL-10 was significantly correlated with MD-2 (r = 0.36, p < 0.01) and sCD14 (r = 0.55, p < 0.001).

To strengthen the correlation between CD14, MD-2, and IL-10, an additional population of 38 HIV-infected patients with severe immunodeficiency (CDC group C, CD4+ < 500/L) and ongoing symptomatic MAC infection were analyzed. A similar correlation was also revealed in these patients with a significant correlation between IL-10 and MD-2 (r = 0.41, p < 0.05) and IL-10 with sCD14 (r = 0.49, p < 0.01).

**Discussion**

In this article, we have addressed the expression of MD-2 in leukocytes and surprisingly found that IL-10 has a profound effect on MD-2 and CD14 mRNA and protein levels in monocytes. To characterize the surface expression of MD-2 on human leukocytes in a reliable manner, we developed a new mAb, IIC1 that gives a robust signal in flow cytometry. The IIC1 recognizes MD-2 either as free or bound to TLR4. MD-2 has high affinity for TLR4 (Kd of 12 nM, Ref. 25), and one would suspect that the MD-2 on membrane surfaces represents molecules bound to TLR4. However, MD-2
has also been reported to bind to MD-1 via RP105 (26), which may also contribute to the observed MD-2 expression. Using an Ab that detects MD-2 in complex with TLR4, Elson et al. (27) recently reported that MD-2 is expressed on the surface of monocytes and endothelial cells, however, they found little MD-2 on neutrophils. With our IIC1 mAb, we observed that monocytes had the highest surface expression of MD-2, nevertheless, neutrophils and around 40% of CD19<sup>+</sup> B cells also expressed MD-2. It has previously been reported that memory B cells (CD19<sup>+</sup>, CD27<sup>+</sup>) contain mRNA for MD-2. By analyzing cells from tonsils we found that around 50% of the CD19<sup>+</sup>, CD27<sup>-</sup> memory B cell expressed MD-2. Human B cells express very low levels of TLR4 (20), however, the TLR4-like gene RP-105 is expressed in higher amounts in memory B cells than naive B cells. Thus, the possibility exists that a part of the MD-2 expression detected in B-cells represents binding to RP-105 (26). The functional consequences of MD-2 expression on normal human B-cells are not known. Ruprecht et al. (28) have reported that LPS alone does not affect proliferation of naive human B cells. However, malignant human B cells that express TLR4 and MD-2 respond with high IL-6 production and proliferation when LPS is added (29).

Freshly isolated monocytes expressed considerable amounts of surface bound MD-2 that was progressively diminished over time in culture. This reduction in surface bound MD-2 coincided with an increase in the spontaneous release of MD-2 (Fig. 5E). We have previously reported that LPS mediates a reduction in TLR4 surface expression on monocytes, which is due to ligand-induced endocytosis of TLR4 (2). This effect is transient and TLR4 plasma membrane expression is not affected when LPS is added to monocytes for 24 h (T. Espevik and L. Ryan, unpublished data). Since MD-2 has a strong affinity for TLR4, it is likely that MD-2 follows TLR4 during LPS induced endocytosis that can be detected at 2 h of LPS incubation. It has been reported that monomeric form of human MD-2 is unstable at 37°C (30) and that LPS has a stabilizing effect on MD-2 (31). Thus, it is reasonable to believe that the stabilizing effect of LPS on MD-2 can explain the enhanced surface expression of MD-2 observed in the presence of LPS. Interestingly, IL-10 increased the mRNA for both MD-2 and CD14, and IL-10-stimulated monocytes expressed increased levels of surface MD-2. However, IL-10 did not enhance TLR4 expression. Recently it has been reported that IL-6 is a potent cytokine for the induction of MD-2 in hepatocytic cells and also in the promonocytic cell line THP-1 (15). In human monocytes, IL-6 had no effect on MD-2 mRNA- and protein expression (data not shown). Thus, differences may exist between various cell types regarding susceptibility to stimuli that result in MD-2 secretion.

Because of the marked effect of IL-10 on MD-2 mRNA and cell surface expression in monocytes, we established an ELISA method to quantify MD-2 levels in biological fluids. The MD-2 ELISA is based on a human TLR4-Fc construct as a coat and an MD-2 Ab, SD7, (a subclone of 18H10), which detects MD-2 only when bound to TLR4 (14). The SD7 mAb was chosen as detector since it gave higher sensitivity for MD-2 detection compared with IIC1 (data not shown). Only the monomeric form of MD-2 interacts with TLR4 (25), thus, the values obtained with this ELISA represent the amount of monomeric MD-2. The reported levels of MD-2 in human serum are not consistent. One report has estimated the levels to be around 1.7 μg/ml (25), which is in accordance with the values that we observe in the control serum samples with this ELISA (Fig. 6). However, a recent report describes that the MD-2 level in serum is in the range of 10 ng/ml (18). The reason for these discrepancies is likely to be due to different assay methods and the ratio between monomers and multimers in the recombinant MD-2 standards used in the various assays.

By using the MD-2 ELISA, we found that IL-10 induced a dose-related increase in sMD-2 with a rather slow kinetics that required ~20 h to reach a maximal stimulatory effect. The amount of sCD14 produced by monocytes increased 3–4-fold by addition of IL-10. However, IL-10 only had minimal effect on the amount of membrane CD14 on monocytes. IL-10 plays a predominant role in reducing the production of proinflammatory cytokines (32). Stimuli that induce the production of proinflammatory cytokines also result in production of IL-10, however, the kinetic of induction differs (33). Macrophage recognition of apoptotic cells triggers an anti-inflammatory response that is mediated by IL-10 and TGF-β (34). Previous reports have demonstrated that IL-10 enhances LPS-induced production of IL-1 receptor antagonist (35). Also, IL-10 increases the levels of membrane and soluble p75 TNFR on human monocytes (36). It is not known in detail whether the stimulatory effect of IL-10 on IL-1RA and p75 TNFR has a role in inhibiting systemic inflammation.

The demonstration that IL-10 increases the production of both sMD-2 and sCD14 may have several implications. Soluble MD-2 is elevated during Gram-negative sepsis (14) and sCD14 is elevated in plasma from both septic and HIV-infected patients (3). IL-10 may be a driving force for increased serum levels of sMD-2 and sCD14, which was supported in our studies on HIV-infected patients where the IL-10, sCD14, and sMD-2 levels were significantly correlated (Fig. 6). Soluble MD-2 may have opposing effects on LPS activation as low amounts greatly increases LPS stimulation on cells expressing TLR4, whereas higher amounts inhibit LPS induced activation (37, 38). During the most severe clinical stages of HIV (i.e., AIDS), the sMD-2 levels increased ~5-fold, reaching around 5–5.5 μg/ml (160 nM). Concentrations of MD-2 higher than 110 nM inhibit LPS induced IL-6 production (38), and it is tempting to hypothesize that the high sMD2 levels found in AIDS patients could impair their ability to mount an appropriate inflammatory response against various microbes. In addition, sMD-2 can function as an opsonin that enhances phagocytosis of Gram-negative bacteria (15), but whether this potential anti-microbial function is related to the concentration of sMD2 is at present not clear. Also, sCD14 enhances LPS responses, particularly on cells that do not express membrane CD14 (4). This occurs since CD14 binds LPS and transfers it to MD-2 after which the LPS-MD-2 complex binds and activates TLR4 (37). The role of sCD14 in relation to LPS-induced septic shock is controversial. Treatment with anti-CD14 mAb provides therapeutic benefits after in vivo exposure to endotoxin (39). However, mice overexpressing sCD14 retain LPS in the circulation and are resistant to LPS-induced lethality (40). In contrast to sMD-2, an excess of sCD14 inhibits phagocytosis of Gram-negative bacteria (41), which is likely to be due to the fact that mCD14 is a phagocytosing receptor for Gram-negative bacteria, mycobacteria, and apoptotic cells (42, 43). The combined effects of sMD-2 and sCD14 on regulating LPS responses and phagocytosis of Gram-negative bacteria have not been addressed in clinical situations, however, the possibility exists that the high amounts of sMD-2 and sCD14 that is induced by IL-10 during systemic inflammation inhibit LPS induced responses. While such a down-regulation of LPS-induced responses could be beneficial in patients with fulminant sepsis, it could potentially contribute to immunodeficiency in HIV-infected patients by impairing a protective inflammatory response against invading bacteria such as MAC in HIV-infected patients.

Disclosures

The authors have no financial conflict of interest.