CD16 Regulates TRIF-Dependent TLR4 Response in Human Monocytes and Their Subsets

Irina N. Shalova, Tasneem Kajiji, Jyue Yuan Lim, Vanessa Gómez-Piña, Irene Fernández-Ruíz, Francisco Arnalich, Philip Tsau Choong Iau, Eduardo López-Collazo, Siew-Cheng Wong and Subhra K. Biswas

J Immunol 2012; 188:3584-3593; Prepublished online 16 March 2012; doi: 10.4049/jimmunol.1100244
http://www.jimmunol.org/content/188/8/3584

References This article cites 69 articles, 18 of which you can access for free at: http://www.jimmunol.org/content/188/8/3584.full#ref-list-1

Why The JI? Submit online.
• Rapid Reviews! 30 days* from submission to initial decision
• No Triage! Every submission reviewed by practicing scientists
• Fast Publication! 4 weeks from acceptance to publication

Subscription Information about subscribing to The Journal of Immunology is online at: http://jimmunol.org/subscription

Permissions Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
CD16 Regulates TRIF-Dependent TLR4 Response in Human Monocytes and Their Subsets

Irina N. Shalova,* Tasneem Kajiji,* Jyue Yuan Lim,* Vanesa Gómez-Piña,† Irene Fernández-Ruiz,‡ Francisco Arnalich,‡ Philip Tsau Choong Iau,‡ Eduardo López-Collazo,† Siew-Cheng Wong,* and Subhra K. Biswas*

Blood monocytes recognize Gram-negative bacteria through the TLR4, which signal via MyD88- and TRIF-dependent pathway to trigger an immune-inflammatory response. However, a dysregulated inflammatory response by these cells often leads to severe pathologies such as sepsis. We investigated the role of CD16 in the regulation of human monocyte response to Gram-negative endotoxin and sepsis. Blood monocytes from sepsis patients demonstrated an upregulation of several TRIF-dependent genes as well as a selective expansion of CD16-expressing (CD16⁺) monocytes. Gene expression and biochemical studies revealed CD16 to regulate the TRIF-dependent TLR4 pathway in monocytes by activating Syk, IFN regulatory factor 3, and STAT1, which resulted in enhanced expression of IFNB, CCL5, and CXCL10. CD16 also upregulated the expression of IL-1R–associated kinase M and IL-1 receptor antagonist, which are negative regulators of the MyD88-dependent pathway. CD16 overexpression or small interfering RNA knockdown in monocytes confirmed the above findings. Interestingly, these results were mirrored in the CD16⁺ monocyte subset isolated from sepsis patients, providing an in vivo confirmation to our findings. Collectively, the results from the current study demonstrate CD16 as a key regulator of the TRIF-dependent TLR4 pathway in human monocytes and their CD16-expressing subset, with implications in sepsis. The Journal of Immunology, 2012, 188: 3584–3593.

Sepsis is an extremely complex pathology and an outcome of a dysregulated inflammatory response to persistent bacterial infection (1). It accounts for one of the highest mortalities in intensive care units worldwide (2). Yet, the molecular mechanism of sepsis is still poorly understood. Monocytes/macrophages are one of the key immune cells that play a crucial role in mediating the dysregulated inflammatory response during sepsis (3). During the initial phase of sepsis, these cells contribute to the overt inflammatory response and the “cytokine storm,” whereas during the later phase of the disease, these cells elaborate anti-inflammatory cytokines and become refractory to further endotoxin challenge, a phenomenon referred to as endotoxin tolerance (3–8). The polarization of monocytes/macrophages to an anti-inflammatory and endotoxin tolerant state is believed to be one of the important reasons for sepsis-induced immunosuppression and mortality (1, 9). Therefore, studying the functional phenotype of these cells in course of sepsis as well as the molecular mechanisms(s) that shape their dysregulated phenotype is crucial in understanding the molecular basis of sepsis.

At the molecular level, monocytes/macrophages respond to Gram-negative bacteria (and their endotoxins) through the TLR4-signaling pathway (10–12). TLR4 signaling can be segregated into two distinct branches, depending on the usage of the adaptor molecules MyD88 or TRIF (13, 14). Both these cascades lead to distinct outcomes: the MyD88-dependent pathway induces the expression of proinflammatory cytokine genes such as IL-6, CXCL1, CCL3, and TNF through activation of NF-kB (15, 16), whereas the TRIF-dependent or the MyD88-independent pathway leads to IFN regulatory factor 3 (IRF3)/STAT1-induced expression of type I IFNs (IFN-α and IFN-β) and IFN-inducible chemokines such as CXCL10, CCL5, and CCL2 (17). Indeed, TRIF knockout mice show drastic downregulation of IFNB, CXCL10, CCL5, and CCL2 genes, qualifying them as TRIF-dependent genes (17). Dissection of TLR4 signaling in endotoxin tolerant human monocytes and murine macrophages have reported multiple alterations along the MyD88-dependent signaling pathway, leading to inhibition of inflammatory gene transcription in these cells (3, 18–21). In contrast, the role of the TLR4/TRIF-dependent pathway in endotoxin tolerance and sepsis has not been investigated to a great detail. We have reported a nonredundant role for the TRIF/IFN-β pathway in mediating lipid A (LPA)-induced endotoxin tolerance in mouse embryonal fibroblasts (22). Other observations on the defective expression of classical proinflammatory cytokines such as TNF and IL-1β in TRIF-deficient mice and the fact that the TRIF pathway induces ~74.4% of the LPS-induced transcriptome in murine macrophages argue for an important role of this pathway in regulating sepsis response at least in the mice (16, 17). In line, a more recent study shows the differentiation of monocytes to dendritic cells and their TRIF-dependent mobilization to the lymph nodes during gram-negative bacterial infection in mice (23).
Another level of complexity in monocyte response is their cellular heterogeneity (24, 25). Two distinct monocyte subsets have been defined in mice and humans based on the expression of chemokine receptors CCR2 and CX3CR1 (25–31). In mice, the two subsets are phenotypically defined as Gr1+CCR2+CX3CR1+ monocytes and the Gr1+CX3CR1+CCR2+ monocytes. The "inflammatory" Gr1+ monocytes, which form the major subset, are found mostly at the sites of inflammation and specialize in the production of inflammatory mediators like TNF, reactive oxygen species, and NO, in response to infection with bacteria or parasites (26, 32). In contrast, the "resident" Gr1− monocytes patrol the endothelial linings of blood vessels, can differentiate into macrophages upon extravasation, and are associated with tissue repair (32, 33). In humans, gene expression and surface marker studies showed the CD14+CD16− monocytes (also called CD14+ monocytes) to resemble the mouse Gr1+ monocytes, whereas the CD16+ monocytes resemble the Gr1− mouse monocytes (26, 27, 29, 30). Although the CD14+ monocytes were reported to produce IL-10 and weakly induce TNF, the CD16+ monocytes showed an inflammatory phenotype, characterized by increased expression of TNF, IL-1β, reactive oxygen species, MHC class II molecules, efficient Ag presentation, and higher FcγR-induced phagocytosis (28, 34–36). Further studies have now shown heterogeneity among the CD16+ monocyte population indicating a further division into the CD14+CD16+ monocytes and the CD16+CD14− (or CD14−) monocytes (37–39). Although CD16+ monocytes constitute a minor fraction of the blood monocytes, they expand greatly under different pathological states, including sepsis (40–44). Because each monocyte subset possess a distinct functional phenotype, how the selective expansion of a particular monocyte subset may affect the overall mononuclear response in sepsis is an important question. Similarly, whether the expansion of specific monocyte subsets could explain the differential regulation of intracellular signaling pathways and the functional plasticity of monocytes in course of sepsis remains to be studied (3).

One of the distinguishing features of the human monocyte subsets is the differential expression of CD16 or FcγRIII. The FcγRIII receptor belongs to the family of activatory Fc receptors, which are characterized by an ITAM that triggers activatory functions in phagocytes (45). Indeed, Ab-mediated Fc receptor activation on phagocytes plays a crucial role in modulating their response in autoimmunity, cancer, and infections. However, it remains to be investigated whether the differential expression of CD16 on human monocytes (and their subsets) regulates their response to Gram-negative endotoxins and sepsis.

Addressing some of the above issues, we report in this paper the crucial role of CD16 in orchestrating the response of human monocytes and their subsets during Gram-negative sepsis and endotoxin challenge. To our knowledge, we demonstrate for the first time CD16 as a key regulator of the TRIF-dependent TLR4 signaling in monocytes and the preferential activation of this pathway in the CD16+ monocyte subset. In addition, CD16 was implicated in driving the expression of negative regulators of the MyD88-dependent TLR4 pathway in monocytes that shape their endotoxin tolerant phenotype in sepsis.

Materials and Methods

Cell lines, reagents, and cell culture

All murine cells were cultured in DMEM (Life Technologies) containing 4500 mg/l D-glucose, 1-glutamine without sodium pyruvate and sodium bicarbonate. Medium was supplemented with containing 10% FBS (HyClone) and 100 U/ml penicillin–streptomycin. The following reagents were used for cell treatment: lipid A, Escherichia coli, serotype R515(Re) (Alexis Biochemicals, Lausen, Switzerland), CD16 mAb (clone 3G8, catalog number 555403; BD Pharmingen), CpG (Roche Diagnostics, Singapore), flagellin, and Pam3Cys (InvivoGen). CD16 plasmid viz. cMyc-DDK-tagged open reading frame clone of human FCGR3A transcript variant 1 was obtained from OriGene. Control plasmid, viz. pmaxGFP plasmid encoding the GFP from Pontellina p., was obtained from Amaxa Biosystems (Lonzza, Switzerland). Syk 1 inhibitor (spleen tyrosine kinase inhibitor, 3-(1-methyl-[1H]-indol-3-yl)-methylene)-2-oxo-2,3-dihydro-[1H]-indole-5-sulfonamide) and Syk II Inhibitor (2-(2-aminoethylamino)-4-(3-trithiomethylamino)-pyrimidine-5-carboxamide, Dihydrochloride, Dihydrate) were bought from Calbiochem.

Sepsis patient selection

We studied patients (age, 51 ± 12 y; mean ± SD) consecutively admitted to the Department of Internal Medicine at the Hospital “La Paz” with microbiologically confirmed Gram-negative bacteremia (positive blood cultures for E. coli) secondary to urinary tract infection, who met the diagnostic criteria for sepsis (46). Blood samples were taken within 48 h after blood culture collection when they met the sepsis criteria for the first time. The following exclusion criteria were imposed: malignancy and chronic inflammatory diseases, treatments with steroids or immunosuppressive drugs during the last month, hepatic failure (serum aspartate aminotransferase and/or alanine aminotransferase level > 100 IU/l; prothrombin time < 60%, total bilirubin level > 60 μmol/l), renal insufficiency (plasma creatinine level > 200 μmol/l), AIDS, virus B or C hepatitis, gestation, and age over 70 y. The control group consisted of healthy volunteers (age, 49 ± 12 y; mean ± SD). An additional source of blood samples from sepsis patients (with the same exclusion criteria described above) was obtained from National University Health System (Singapore). Written informed consent was obtained from all subjects. The studies were approved by the local ethics committee. Monocytes were isolated from the healthy volunteers and the patients as described in the following sections.

Mice

Wild-type C57BL/6 mice were maintained in our animal facility and used for the isolation of blood monocytes. All mice were used between 6 and 8 wk of age and in accordance with institutional guidelines proposed by the Institutional Animal Care and Use Committee of the Bio Resource Center, A*STAR (Singapore). Isolation of peripheral blood monocytes was done as follows: Peripheral blood collected from the mice by cardiac puncture, followed by RBC lysis, isolation of PBMCs, and flow sorting of Gr1+ and Gr1− monocyte subsets on a BD FACSaria cell sorter (BD Biosciences).

Isolation and culture of human blood monocytes

PBMCs were isolated from buffy coats obtained from the Blood Bank of Health Sciences Authority (Singapore), using Ficoll-Hypaque density gradient centrifugation. Monocyte isolation was performed using the CD14+ monocyte isolation kit from Miltenyi Biotec (Bergisch Gladbach, Germany), according to the manufacturer’s instruction. Aliquots of the isolated monocytes were then stained with anti-CD14 (61D3) and CD16 (3G8) (BioLegend, San Diego, CA) and tested by flow cytometry to determine their purity. Monocytes were plated in 6-well tissue culture plates (Nunclon) at 2 × 10⁶ cells/well in incomplete IMDM (IMDM-modified HyClone medium containing HEPES and 4 mM l-glutamine; Thermo Scientific) for 1 h at 37°C in the tissue culture incubator. Thereafter, the adherent monocytes were cultured in IMDM containing 5% human serum (v/v). These cells were then treated as indicated in Results. Isolation of the CD14+ and CD16+ human monocyte subsets from peripheral blood was done either by flow sorting using BD FACSaria cell sorter (BD Biosciences) or the CD16 and CD14 monocyte isolation kits (Miltenyi Biotec), as per the manufacturer’s instructions. Aliquots were stained with CD14 and CD16 Abs (as mentioned above) and analyzed by flow cytometry to determine the purity. The cell culture conditions of monocyte subsets were the same as indicated for total monocytes.

Nucleoporation of monocytes and CD16 overexpression

Monocytes were transfected using Human Monocyte Nucleofector Kit (AMAXA Biosystems). Cells were washed twice with MACS buffer, 1 × 10⁶ monocytes were resuspended in 100 μl human monocyte nucleofector solution, mixed with 0.5 μg purified CD16-plasmid or with control plasmid pmaxGFP (Amaza), and electroporated in Amaza-certified cuvettes using an Amaza Nucleofector apparatus with optimal program Y-001. After electroporation, cells were immediately mixed with 500 μl fresh Amaza human monocyte nucleofector medium and transferred into 6-well plates containing 1 ml of the same medium. Cells were incubated at 37°C for 24 h. Following incubation, the medium was changed to fresh complete
IMDM, monocytes were rested for 12 h, and then, treatments were given. Transfection was verified by fluorescence microscopy in pmaxGFP-transfected cells and CD16 overexpression by flow cytometry as well as immunoblotting.

Small interfering RNA knockdown of monocytes

RNA interference was performed using small interfering RNAs (siRNAs) targets against human FCγRIII and a negative control siRNA (Invitrogen). Lipofectamine 2000 (Invitrogen Life Technologies) transfection reagent and siRNA were diluted in Opti-MEM medium with reduced serum (Invitrogen Life Technologies), and mixed together to form liposomes containing siRNA with final concentration 10 nM. Monocytes were washed twice with PBS and incubated with liposome/siRNA complexes for 5 h. After incubation, the transfection reagents were removed, and the cells were placed in into complete IMDM (described above) for 48 h; treatments were given thereafter. CD16 knockdown was assessed by flow cytometry and immunoblotting.

Quantitative PCR

Cells were lysed with TRIzol (Invitrogen Life Technologies), and total RNA was prepared using the RNAeasy kit (Qiagen), according to the manufacturer’s instructions. Total RNA (0.5 μg) was reverse transcribed, the cDNA used for quantitative PCR on an iCycler iQ5 Real-Time PCR detection system (Bio-Rad) and analyzed as described earlier (47).

Immunoblotting

Monocytes were treated as indicated in Results. Protein extracts were prepared and processed for immunoblot as described earlier (47). The following Abs were used for the immunoblots: anti–phospho-Syk (Tyr525/526), anti–phospho-STAT1(Tyr701), anti–phospho-IRAK1 (Ser376), anti-Myc, anti-MyD88, and anti–β-actin from Santa Cruz Biotechnology. For immunoprecipitation, equal amount (0.25 mg) of protein lysates were preclear with protein A/G-agarose (Santa Cruz Biotechnology) for 1 h and then incubated with Myc–Tag Ab (Cell Signaling Technology) overnight at 4°C on a rotor. Thereafter, protein A/G beads were added and incubated for 3 h at 4°C on the rotor. IgG–protein A/G complexes were collected by centrifugation, washed, and boiled in Laemmli Sample buffer (Bio-Rad) for 5 min before loading onto a 10% polyacrylamide gel. Further immunoblotting was performed as reference above. Densitometric analysis of immunoblots was done using the ImageJ software.

Immunofluorescence and confocal microscopy

Monocytes were cultured overnight on the pyrogen-free glass coverslips, and then, the treatment was given for 60 min. After incubation, cells were washed with PBS, fixed with 2% paraformaldehyde (s/w) for 10 min, permeabilized with 0.03% Triton X-100 for 10 min, and blocked with 2% BSA for 1 h at room temperature. Thereafter, samples were incubated with anti–phospho-STAT1 overnight at a dilution 1:50 at 4°C. Then, samples were washed and subsequently incubated with anti-rabbit IgG conjugated with Alexa 488 (Invitrogen) at a dilution 1:500 for 1 h at room temperature. Samples were extensively washed, mounted with Vectashield mounting medium with DAPI (Vector Laboratories), and analyzes using Zeiss LSM 5 Duo confocal microscope (Carl Zeiss MicroImaging). Images were captured and processed using Zeiss LSM Image Browser (Carl Zeiss MicroImaging), and image analysis was done with ImageJ software. DAPI was pseudocolored as red.

Statistical analysis

Statistical significance of observed data was performed using the Student t test where p < 0.05 was considered as statistically significant.

Results

Expression of TRIF-dependent genes in monocytes from Gram-negative sepsis

The expression of TRIF-dependent genes in human monocytes during Gram-negative sepsis has not been investigated. We checked the expression of the TRIF-pathway specific genes IFNB, CCL5, and CXCL10 in monocytes from sepsis patients (17). Fig. 1A shows elevated expression of these genes in the sepsis monocytes, as compared with normal monocytes from healthy donors. Because human monocytes consist of two distinct subsets namely, the CD14+ and the CD16+ monocytes, a selective expansion of any of these subsets could contribute to the elevated expression of the TRIF-dependent genes. Therefore, further investigations were undertaken.

Flow cytometric analysis of sepsis monocytes showed a significantly increased percentage of CD16+ monocytes in the patients, as compared with monocytes from healthy donors (Fig. 1B, left panel). Fig. 1B, right panel, shows a representative FACS plot indicating the relative distribution of CD16+ and CD14+ monocytes in healthy donor and sepsis patient. Note the increased proportion of CD16+ monocytes in the latter.

We examined the expression of TRIF-dependent genes in the CD16+ and CD14+ monocyte subsets isolated from the peripheral blood of sepsis patients. Fig. 1C shows that CD16+ monocytes from sepsis patients display a markedly higher expression of the TRIF-dependent genes IFNB, CCL5, and CXCL10 than the CD14+ monocytes from these patients. To check the specificity of this response, we assessed in parallel the expression of the MyD88-dependent genes IL6, CCL3, and CXCL1. CD16+ sepsis monocytes showed downregulated expression of IL6, CCL3, and CXCL1 as compared with the CD14+ sepsis monocytes (Fig. 1C). Taken together, these results not only indicated the expansion of CD16+ monocytes in sepsis patients but also suggested the higher expression of TRIF-dependent genes in this monocyte subset as compared with the CD14+ monocytes in sepsis.

Stimulation of CD16 and TLR4 synergize to enhance the expression of TRIF-dependent genes

Because our experiments with sepsis monocytes showed enhanced expression of TRIF-dependent genes in the CD16+ monocyte subset, we wondered whether signaling through the CD16 itself might play a role in the modulation of these genes in response to TLR4 stimulation. This is a possibility because Gram-negative pathogens besides stimulating TLR4 also bind to CD16 (48).

We first determined whether stimulation of CD16 in conjunction with TLR4 stimulation could selectively enhance the expression of TRIF-dependent genes in the CD16+ monocyte subset, as compared with the CD14+ monocyte subset. CD14+ and CD16+ monocyte subsets were isolated from healthy donor blood and treated as indicated below. TLR4 stimulation was performed by using LPA (100 ng/ml), whereas stimulation of CD16 was elicited by using an agonistic Ab, clone 3G8, described in earlier studies (49–51). CD16+ monocytes treated simultaneously with 3G8 and LPA (indicated by “Ab+LPA”) showed a significantly higher expression of IFNB, CCL5, and CXCL10, as compared with the CD14+ monocytes, which received the same treatments (Fig. 2A, left panel). However, the expression of the MyD88-dependent genes IL6, CCL3, and CXCL1 in the CD16+ monocytes in response to Ab+LPA treatment showed either a downregulation or no significant change compared with CD14+ monocytes receiving the same treatments (Fig. 2A, right panel). We also performed the same experiment in whole monocytes. Monocytes treated with Ab+LPA significantly enhanced IFNB, CCL5, and CXCL10 expression (but not the expression of IL-6, CCL3, or CXCL1) as compared with monocytes treated with an isotype-matched Ab plus LPA (indicated by “Iso-Ab+LPA”) (Fig. 2B). In all cases, when monocytes were treated with Ab or Iso-Ab alone, no significant modulation of any of the above genes was noted. To further demonstrate the specificity of the observations described above, we performed additional experiments, where monocytes were stimulated with Ab or Iso-Ab in the presence or absence of TLR ligands that exclusively signal through the MyD88-dependent pathway (e.g., PAM3Cys5 for TLR2, flagellin for TLR5, and CpG for TLR9). As shown in Fig. 2C, treatment of monocytes with Ab and PAM3Cys4 (Ab+PAM), flagellin (Ab+Flag), or CpG (Ab+CpG) failed to show any significant modulation of the TRIF-dependent genes as compared with Ab or Iso-Ab alone.
with Iso-Ab+PAM, Iso-Ab+Flag, or Iso-Ab+CpG), indicating the specificity of CD16 stimulation in enhancing TRIF-dependent responses.

Taken together, the above results suggest that CD16 stimulation acts in concert with TLR4 to preferentially enhance the expression of TRIF-dependent genes IFNB, CCL5, and CXCL10.

**CD16 overexpression or knockdown specifically affects the expression of TRIF-dependent genes**

To further establish the regulatory role of CD16 in the expression of TRIF-dependent genes, we performed CD16 overexpression and knockdown studies in monocytes. As shown in Fig. 3A, upper panel, control- or CD16-transfected monocytes were stimulated with Ab+LPA and checked for the expression of TRIF-dependent genes IFNB, CCL5, and CXCL10. The results demonstrate CD16-transfected monocytes to show significantly higher expression of IFNB, CCL5, and CXCL10 than the control transfected monocytes, upon Ab+LPA treatment. However, no such significant modulation was seen for IL6, CCL3, and CXCL1 upon stimulation with Ab+LPA treatment. Using a converse approach, CD16 siRNA knockdown in monocytes caused a significant attenuation in the expression of the three TRIF-dependent genes, as compared with the control siRNA-treated monocytes. CD16 knockdown was confirmed by its downregulated expression using flow cytometry (Fig. 3B, lower panel).

**CD16 stimulation modulates TRIF-dependent TLR4 signaling pathway in monocytes**

We investigated the mechanism by which CD16 regulates the preferential expression of TRIF-dependent genes in monocytes. For this purpose, TRIF-dependent signaling in monocytes was studied following stimulation through CD16 (using the agonistic Ab, Ab), TLR4 (using LPA), or both together (Ab+LPA). Incubation with an isotype control Ab alone (Iso-Ab) or together with LPA (Iso-Ab+LPA) was used as the control. Triggering of the TLR4/TRIF pathway leads to the activation of IRF3 and STAT1, which drives the transcription of genes such as IFNB, CCL5, and CXCL10 (13, 17). We studied activation of IRF3 and STAT1 by measuring their phosphorylation in monocytes stimulated by Iso-Ab+LPA versus Ab+LPA for the indicated time points. Fig. 4A shows that monocytes stimulated with Ab+LPA expressed higher levels of phospho-IRF3 and phospho-STAT1 at 15- to 60-min post-stimulation as compared with monocytes stimulated with Iso-Ab+LPA. Densitometric analysis indicated besides each blot show...
a quantitative confirmation of the above results. In addition, we also repeated the same experiment and visualized the cells using confocal microscopy for STAT1 activation. As demonstrated in Fig. 4B, Ab+LPA-treated monocytes showed an increased phospho-STAT1 expression (indicated by increased Alexa 488 staining intensity in the densitometry graph; Fig. 4B, right panel) as well as more nuclear translocation (indicated by more yellow staining in the merge panel) than the Iso-Ab+LPA–treated monocytes (Fig. 4B). Collectively, the above results indicated the ability of CD16 to trigger the activation of key components of the TRIF-dependent TLR4 signaling cascade such as IRF3 and STAT1.

To confirm the activation of TRIF-dependent signaling by CD16 and TLR4 stimulation in vivo, we determined the status of this signaling pathway in monocyte subsets isolated from Gram-negative sepsis patients. Because Gram-negative bacteria bind to TLR4 as well as CD16 (48), we hypothesized that signaling through these receptors should yield enhanced activation of the TRIF-dependent pathway in the CD16+ monocytes than CD14+ monocytes in sepsis. Indeed, our immunoblotting data showed a marked increase in phospho-STAT1 expression but not phospho-IRAK1 expression (a key molecule in the MyD88-dependent TLR4 pathway) in the CD16+ but not CD14+ monocytes from sepsis patients (Fig. 4C).

To further demonstrate how CD16 triggered TRIF-dependent TLR4 signaling, we performed other biochemical studies. Monocytes were transfected with a CD16-Myc expressing plasmid to allow the overexpression of CD16. These monocytes were stimulated or not with Ab+LPA or LPA for 1 h and used to identify signaling molecules that coprecipitated with CD16. Immunoprecipitation with Myc followed by immunoblotting revealed significantly higher levels of phospho-STAT1 for Ab+LPA-treated monocytes than those treated with LPA alone (Fig. 4D, first panel). However, STAT1 expression was comparable for both treatments (Fig. 4D, second panel), emphasizing the fact that the enhanced phospho-STAT1 expression (i.e., activation of STAT1) was specific for Ab-induced CD16 signaling. Because activation of STAT1 requires upstream tyrosine kinases and the tyrosine kinase, Syk is activated by CD16 signaling (52), we investigated whether activated Syk (i.e., phospho-Syk) could be detected among the CD16 coprecipitates. Indeed, immunoblotting revealed phospho-Syk as a coprecipitate with CD16 upon Ab+LPA stimulation but not LPA stimulation alone (Fig. 4D, third panel). In addition, in these immunoprecipitation experiments with CD16-overexpressing monocytes, we also found TRIF as a coprecipitant with CD16 in the LPA and Ab+LPA monocytes (Fig. 4D, fourth panel), confirming its role in the above pathway. However, immunoblotting for an unrelated molecule, MyD88 did not yield any coprecipitation, serving as a negative control in the above experiment.

To further confirm the role of Syk, we performed stimulated monocytes with Ab+LPA in presence or absence of Syk inhibitors. Fig. 4E shows 2-fold or more suppression in the expression of certain cytokine genes. These results indicate the role of Syk in the CD16-mediated TRIF signaling pathway.

**FIGURE 2.** Stimulation of CD16 augments TLR4-induced expression of TRIF-dependent cytokine genes. (A) CD14+ and CD16+ monocyte subsets isolated from normal donors were subjected to treatment with CD16 agonist Ab, 3G8 (5 μg/ml) together with LPA (100 ng/ml) (= Ab+LPA) for 4 h. Thereafter, RNA was extracted, and quantitative PCR performed for the indicated genes. *p < 0.05, CD16+ Mo versus CD14+ Mo. (B) Total monocytes from normal donors were stimulated either with isotype control Ab (= Iso-Ab) or CD16 agonistic Ab (= Ab) in the absence or presence of LPA for 4 h. Thereafter, quantitative PCR was carried out on the extracted RNA for the indicated genes. *p < 0.03, Iso-Ab+LPA versus Ab+LPA. (C) Total monocytes from normal donors were stimulated either with isotype control Ab (= Iso-Ab) or CD16 agonistic Ab (= Ab) in the absence or presence of 100 ng/ml PAM3Cys4 (PAM), 3 μg/ml CpG or 100 ng/ml flagellin (Flag) for 4 h. Thereafter, quantitative PCR was carried out on the extracted RNA for the indicated genes. Values in (A) are mean ± SEM representative of three independent experiments. Values in (B) and (C) are mean ± SEM of three independent experiments.
TRIF-dependent genes CCL5 and CXCL10 in the Ab+LPA-stimulated monocytes that were pretreated with Syk inhibitors as compared with their vehicle controls. Vehicle controls consisted of monocytes treated with the vehicle (i.e., DMSO) and subsequently stimulated with LPA+Ab.

The above results collectively demonstrate that signaling through CD16 promoted the TRIF-dependent TLR4 pathway by interacting and activating Syk, STAT1 and IRF3.

CD16 induces the expression of negative regulators of MyD88-dependent pathway

IRAK-M and IL-1R antagonist (IL-1RA) are negative regulators of the MyD88-dependent TLR4/IL-1R pathway and have been reported to be upregulated in sepsis (8, 53). Overexpression of CD16 in monocytes caused a significant increase in the expression of IRAKM and IL1RA as compared with the control-transfected cells, following Ab+LPA stimulation (Fig. 5A). Conversely, knocking
down CD16 using an siRNA inhibited the expression of these negative regulator genes in Ab+LPA-treated monocytes (Fig. 5B). In line with this observation, we could also demonstrate that CD16+ monocytes from sepsis patients showed significantly increased expression of IRAKM and IL1RA than the CD14+ monocytes (Fig. 5C).

Taken together, the above observations demonstrate the role of CD16 in driving the elevated expression of negative regulators IRAKM and IL1RA, which may possibly result in a concomitant decrease in MyD88-dependent signaling.

**TRIF-dependent genes are differentially modulated in mouse Gr12 monocyte subset in response to endotoxin shock**

Recent transcriptomal data have shown the CD16+ human monocytes to correspond to the Gr12 mouse monocytes (both express CD16), whereas CD14+ human monocytes correspond to the Gr1+ mouse monocytes (29). On the basis of our above findings on the differential regulation of TRIF-dependent genes in human blood monocytes and their subset in sepsis, we wanted to ascertain whether this was also true in their murine counterparts. For this purpose, mice were exposed to an endotoxin shock by i.p. injection of LPA, and blood monocytes were collected after 4 h of injection. Gr1+ and Gr12 blood monocytes were FACS sorted, and the expression of TRIF-dependent genes was compared between the monocyte subsets. As shown in Fig. 6, Gr12 monocytes showed a significantly higher expression of the TRIF-dependent genes IFNB, CCL5, and STAT1 as compared with their Gr1+ counterparts. This result is in support with our data from the human monocytes and their subsets. However, further studies are needed to clarify whether the molecular mechanism also remains the same as shown for the humans.

**Discussion**

Monocytes play a key role in host immune response during sepsis (3, 4). However, not much has been studied with regards to their molecular characterization and the mechanistic basis of their dysregulated inflammatory response in course of this disease. Our study on human monocytes from Gram-negative sepsis patients...
CD16 siRNA (10 nM) in response to Ab+LPA for 4 h. and IL1RA expression in total monocytes treated with control siRNA or (CD64) or Fc of MyD88-dependent cytokines such as TNF, IL-6, and IL-1 receptor on monocytes has been reported to trigger the expression phenotypes (56, 57). In line with this idea, cross-linking of CD16 whereas binding to activatory Fc receptors such as FcγRI (CD64) or FcγRII (CD16) can lead to inflammatory macrophage phenotypes (56, 57). In line with this idea, cross-linking of CD16 receptor on monocytes has been reported to trigger the expression of MyD88-dependent cytokines such as TNF, IL-6, and IL-1β (51, 56). However, modulation of TRIF-dependent genes by signaling through CD16 has not been demonstrated. Our investigation in sepsis monocytes revealed that signaling through CD16 contributed to enhanced expression of the TLR4/TRIF-dependent genes. This was evident from three lines of data, namely: 1) preferential upregulation of TRIF-dependent genes in monocytes stimulated through TLR4 and CD16, as compared with only TLR4-stimulated monocytes; 2) inhibition or upregulation of TRIF-dependent genes in CD16 knockdown or overexpressing monocytes following stimulation through CD16 and TLR4; and 3) preferential upregulation of TRIF-dependent signaling and its target genes in the CD16+ monocyte subset in sepsis. Mechanistically, we demonstrated that stimulation of monocytes through CD16 and TLR4 synergistically enhanced phosphorylation of Syk, IRF3, and STAT1 as compared with monocytes stimulated through TLR4 only. Further immunoprecipitation studies confirmed the interaction of CD16 with each of these signaling molecules as well as TRIF. Supporting our observations, an earlier study shows that the selective blockage of the inhibitory FcγRIIB receptor in human dendritic cells and monocytes induced the expression of IFN-inducible genes through STAT1 activation (58). A balance of activating/inhibitory Fc receptors is possibly important for the regulation of IFN signaling in these myeloid cells. In an in vivo setting like sepsis there are multiple signals such as immune complexes, TLR ligands, and bacteria, which simultaneously trigger TLR and Fcγ receptor pathways. In fact, many Gram-negative bacteria such as E. coli bind to multiple receptors such as CD16, scavenging receptor, in addition to TLR4 (48). Furthermore, our mechanistic findings on the involvement of Syk in TRIF-dependent signaling, in response to CD16 and TLR4, is supported by a very recent study. Although activation of Syk by the activatory Fc receptors is well-known, this recent study suggests a Syk-dependent TLR4/TRIF response in mouse APCs (59). However, in this case, CD14 was suggested to mediate this cascade. In light of our data, it is possible that CD16 may act as an important pathway to activate a Syk-dependent TRIF pathway, especially under conditions when CD14 is downregulated as has been reported for sepsis monocytes (60).

Supporting the findings on the role of CD16 in regulating monocyte response in human sepsis, we and others have observed an expansion of the CD16+ monocyte subset in the peripheral blood of Gram-negative sepsis patients (40, 44). Our ex vivo studies on these sepsis monocytes revealed that the CD16+ monocyte subset expressed markedly higher levels of TRIF-dependent genes (INFβ, CCL5, and CXCL10), compared with the CD14+ monocyte subset. Mechanistically, the elevated expression of TRIF-dependent genes in the CD16+ sepsis monocytes was linked to elevated phospho-STAT1 (but not the phospho-IRAK1, a mediator of MyD88 pathway) expression, indicating
the preferential activation of TRIF-dependent pathway in this monocyte subset. The expression of IFN-inducible genes such as CCL5 or CXLCL10 through STAT1 activation is also associated with TLR7/8 signaling in response to viruses and nucleic acid (13). In this context, the CD14+CD16+ monocytes have been recently shown to be strong sensors of viral nucleic acid through the TLR7/8 pathway (37). This finding in conjunction to our present results suggests a dominant IFN-inducible response in the CD16+ monocytes (albeit through the TLR4/TRIF pathway, in our case). We also observed the elevated expression of TNF (data not shown) with the TRIF-dependent genes in the CD16+ sepsis monocytes, which can be explained by recent studies showing the regulation of TNF by a TRIF-dependent IRF3 pathway (61). As with TNF, there are other inflammatory genes that are induced either by both TRIF and MyD88 pathway for their maximal expression or switch between these two pathways, depending on the time of activation. However, investigating the differential modulation of the full-range of inflammatory genes in the human monocyte subsets in sepsis would warrant a future transcriptome study. In mice macrophages, the LPS-induced transcriptome was shown to be largely mediated by the MyD88-independent pathway (16), but the lack of TRIF mutants in humans (as yet) make it difficult to dissect the role of this pathway in human monocytes.

What could be the functional significance of an elevated production of IFN-inducible chemokines such as CCL5 and CXCL10 by the CD16+ monocytes during bacterial infection? Chemokines such as CCL5 and CXCL10 are potent chemotactic factors for Th1 cells and monocytes/macrophages, respectively (62). Thus, elevated expression of such chemokines by the CD16+ monocytes may serve to amplify the recruitment of inflammatory cells, as is the case with the mouse Gr1− monocytes during early time points of Listeria monocytogenes infection (28). Indeed, in this model, the Gr1− monocytes showed elevated expression of TRIF-dependent genes CXCL9 and CXCL10 during the early time points of infection (28). Our data from LPA-injected mice demonstrated Gr1− monocytes to preferentially upregulate TRIF-dependent genes such as IFNB, CCL5, and STAT1 than the Gr1+ monocytes. In an E. coli infection model in mice, CD16 has been implicated in promoting sepsis by preventing phagocytotic killing of bacteria and facilitating inflammation (48). However, whether CD16 modulated the response of blood monocytes and their subsets during Gram-negative sepsis infection in mice and whether such a mechanism was similar or disparate with the humans, remains to be investigated.

One of the common features of monocytes from sepsis patients is their endotoxin tolerant phenotype (3, 63). Although we showed CD16 to regulate expression of TRIF-dependent genes, this molecule was also responsible for regulating the expression of IRAK-M and IL1RA, which are well-known negative regulators of TLR4/MyD88-dependent pathway and hallmarks of sepsis-related endotoxin tolerance (3). CD16 overexpression and knockdown experiments clearly demonstrate its role in the regulation of IRAK-M and IL1RA in monocytes. Ex vivo study of sepsis monocytes also showed the CD16+ monocytes to upregulate expression of IRAK-M and IL1RA. The upregulation of these negative regulators is in line with the downregulation of MyD88-dependent genes such as IL6, CXCL1, and CCL3, as seen in our various assays. In addition, CD16+ monocytes from sepsis patients also show higher expression of the anti-inflammatory gene IL10 as compared with their CD14+ monocytes (data not shown). The coexpression of IL10 with TRIF-dependent genes is plausible based on the evidence supporting its regulation by a TRIF-dependent type I IFN pathway (64, 65). Collectively, the above data suggests the CD16 molecule (and the monocyte subset expressing it) may be a key regulator of the endotoxin tolerant phenotype in human sepsis. Because TNF is a major inducer of endotoxin tolerance in monocytes, it would be interesting to investigate the proposition as to whether the CD16+ monocytes through the elevated production of this proinflammatory cytokine (36) in the early phase of sepsis contributes to the induction of endotoxin tolerance in the later phase of the disease. This is possible in view of the plasticity of the Gr1− monocytes that switch from an inflammatory phenotype to an alternative-activated phenotype during L. monocytogenes infection (32).

In conclusion, the present investigation identifies a key role for CD16 in regulating the TRIF-dependent TLR4 response in human monocytes and their subset. Besides its role in the differential regulation of the TRIF pathway, CD16 was also implicated in the induction of negative regulators such as IL1RA and IRAKM, suggesting its contribution to endotoxin tolerance. Taken together, our results also suggest CD16 in monocytes and their subset as important players in human Gram-negative sepsis. Incidentally, a role for CD16+ monocytes and the CD16-mediated signaling pathway has also been suggested in other pathologies such as autoimmune diseases and some cancers (66–69). Thus, targeting CD16 may serve as means to regulate monocyte/macrophage response in a variety of diseases.

Acknowledgments

We thank Drs. Mickey Koh and Garnett Sucks (Health Sciences Authorities, Singapore) for help and support with the blood supply.

Disclosures

The authors have no financial conflicts of interest.

References


