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A Role for Triggering Receptor Expressed on Myeloid Cells-1 in Host Defense During the Early-Induced and Adaptive Phases of the Immune Response[1]

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Triggering receptor expressed on myeloid cells (TREM)-1 is a cell surface molecule expressed on neutrophils and monocytes implicated in the propagation of the inflammatory response. To further characterize the function of this molecule in different phases of the immune response, we examined TREM-1 in the context of host defense against microbial pathogens. In primary human monocytes TREM-1 activation did not trigger innate antimicrobial pathways directed against intracellular *Mycobacterium tuberculosis*, and only minimally improved phagocytosis. However, activation of TREM-1 on monocytes did drive robust production of proinflammatory chemokines such as macrophage inflammatory protein-1α and IL-8. Engagement of TREM-1 in combination with microbial ligands that activate Toll-like receptors also synergistically increased production of the proinflammatory cytokines TNF-α and GM-CSF, while inhibiting production of IL-10, an anti-inflammatory cytokine. Expression of TREM-1 was up-regulated in response to TLR activation, an effect further enhanced by GM-CSF and TNF-α but inhibited by IL-10. Functionally, primary monocytes differentiated into immature dendritic cells following activation through TREM-1, evidenced by higher expression of CD1a, CD86, and MHC class II molecules. These cells had an improved ability to elicit T cell proliferation and production of IFN-γ. Our data suggest that activation of TREM-1 on monocytes participates during the early-induced and adaptive immune responses involved in host defense against microbial challenges. *The Journal of Immunology,* **2003, 170: 3812–3818.**

Following an infection the immune response develops in phases that build in specificity and complexity, resulting ultimately in clearance of the infection. The innate response serves as the first line of defense and is initiated following activation of pattern recognition receptors, such as the Toll-like receptors (TLRs)[3], by various pathogen-associated microbial patterns (1). Mechanisms of innate immunity include activation of direct antimicrobial pathways (2, 3), induction of nitric oxide (4), and phagocytosis (5), which require relatively little time to initiate and lead to the rapid clearance of foreign organisms. Activation of TLRs during the innate response also triggers the release of cytokines and chemokines (6–8) that mark the beginning of the early-induced response. This response follows within hours of the innate response, allowing for additional inflammatory cells to be activated and recruited to the site of infection. The adaptive immune response is the final phase of an immune response, relying primarily on T and B cells. This arm of the immune response takes several days to fully develop, largely due to the selection and amplification of lymphocyte populations with the most optimal Ag receptors for recognition of pathogen-derived epitopes and subsequent destruction of infected host cells. This process is enhanced by the differentiation of monocytes into highly efficient APCs called dendritic cells (DC), which facilitate the activation of effector T and B cells (9). Unlike the innate and early-induced responses, the adaptive response is unique in its ability to generate memory, which reduces the time required to mount a response against future challenges by the same pathogen.

Triggering receptor expressed on myeloid cells (TREM)-1 is a recently discovered cell surface molecule that has been identified on neutrophils and a subset of monocytes (10). It belongs to a growing family of receptors related to NK cell receptors that activate downstream signaling events with the help of an adapter protein called DAP12 (10–12). Expression of TREM-1 on these cells is induced by LPS, a ligand for TLR4, as well as lipoteichoic acid (10, 13). Although its natural ligand has not been identified, engagement of TREM-1 on monocytes with agonist mAbs results in production of the proinflammatory cytokines TNF-α and IL-1β, as well as chemokines such as IL-8 and monocyte chemoattractant protein (MCP)-1. These effects were dramatically enhanced when LPS was used as a costimulus. In an animal model of LPS-induced septic shock, blockade of TREM-1 signaling inhibited hyperresponsiveness and death (13), further highlighting the crucial role of this molecule in inflammation.

Although there is evidence suggesting that TREM-1 helps to establish proinflammatory conditions, little is known regarding its specific involvement in the different phases of host defense. To address this, we examined the role of TREM-1 in mechanisms of

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[3] Abbreviations used in this paper: TLR, Toll-like receptor; DC, dendritic cell; TREM, triggering receptor expressed on myeloid cell; MCP, monocyte chemoattractant protein; iDC, immature DC; *M. tb, M. tuberculosis*; MIP, macrophage inflammatory protein; MFI, mean fluorescence intensity; ΔMFI, change in MFI.

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innate, as well as early-induced and adaptive, immunity used to combat microbial infection.

Materials and Methods

Preparation of primary monocytes and immature DC (iDC) from peripheral blood

Peripheral blood taken from healthy donors was centrifuged over Ficoll gradients (Amersham Pharmacia, Uppsala, Sweden) to isolate PBMC. Resulting cells were counted, and then passed over Percoll gradients (Amersham Pharmacia) at a concentration of 5×10^7/ml to further deplete lymphocytes. The resulting primary monocytes were routinely between 80 and 90% $CD14^+$, as determined by flow cytometry. Cells were cultured in a 5% CO_2 incubator at 37°C in complete medium (RPMI 1640, 0.1 mM sodium pyruvate, 2 mM penicillin, 50 μg/ml streptomycin; Life Technologies, Grand Island, NY) supplemented with 10% FCS (Omega Scientific, Tarzana, CA). To generate iDC, primary monocytes were cultured in complete medium supplemented with 10% FCS containing 800 U/ml GM-CSF (Immunex, Seattle, WA) and 1000 U/ml IL-4 (Peprotech, Rocky Hill, NJ) for 5 days, as previously described (14).

In vitro stimulation of monocytes

For activation of cells via TLRs, primary monocytes were cultured in 24- and 96-well flat-bottom tissue culture plates (Corning, Corning, NY) in the presence of: purified *Salmonella minnesota* LPS (10 ng/ml), 19 kDa lipopeptide (10 μg/ml), or poly(I:C) (20 μg/ml). In some experiments, these stimuli were given in combination with 2.5 U/ml rTNF-α, 500 U/ml rIL-10 (Endogen, Woburn, MA) or 500 U/ml rGM-CSF (Immunex).

To activate monocytes through TREM-1, an agonist anti-TREM-1 mAb (clone 9E2, mouse IgG1) was generated and purified as described elsewhere (10, 15). Flat-bottom plates (24- or 96-well) were precoated with 10 μg/ml anti-TREM-1 or an isotype-matched control (mouse IgG1; Sigma-Aldrich, St. Louis, MO) for 4 h at 37°C. After thorough washing with 1× PBS, monocytes were added to wells containing the appropriate stimuli, and briefly spun in a centrifuge at 1200 rpm to engage TREM-1. In some experiments, cell-free supernatants were analyzed for production of TNF-α, GM-CSF, and IL-10 by ELISA. All Abs used for tissue culture were azide-free and contained <1 pg/ml endotoxin contamination, as determined by *Limulus*-Amebocyte Assay (BioWhittaker, Walkersville, MD).

Growth of Mycobacterium tuberculosis

M. tuberculosis (*M. tb* virulent strain H37Rv) was grown in suspension with constant, gentle rotation in roller bottles containing Middlebrook 7H9 broth (BD Biosciences, Heidelberg, Germany) supplemented with 1% glycerol (Roth, Karlsruhe, Germany), 0.05% Tween 80 (Sigma-Aldrich), and 10% Middlebrook OADC enrichment (BD Biosciences). Aliquots from logarithmically growing cultures were frozen in PBS containing 10% glycerol, and representative vials were thawed and enumerated for viable CFUs on Middlebrook 7H11 plates. Staining of bacterial suspensions with fluorochromic substrates differentiating between live and dead bacteria (BacLight; Molecular Probes, Leiden, The Netherlands) revealed a viability of the bacteria above 90%.

Antimicrobial assays and quantification of bacterial growth

Adherent PBMC obtained from healthy donors were infected with single cell suspensions of *M. tb* (multiplicity of infection of 1). After 4 h, nonphagocytosed bacteria were removed by extensive washing with PBS. The efficiency of infection was 25 ± 5%, as quantified by staining of control cultures on Permanox chamber slides (Nunc, Naperville, IL) with an auramine-rhodamine stain. Viability of infected cells was determined by trypan blue exclusion and was >96% in all experiments. Infected cells were then harvested and 3×10^5 cells were plated in 24-well plates coated with anti-TREM-1 or isotype control mAbs. Some wells received 2 μg/ml 19 kDa to induce antimicrobial activity, as previously described (2). Plates were quickspun to simultaneously engage TREM-1, and incubated for 48 h.

To measure bacterial growth, CFUs were assessed. Infected monocytes were lysed with 0.3% saponin and sonicated in a 37°C waterbath sonicator (Elma, Singen, Germany) for 5 min. Aliquots of sonicate were diluted 10-fold in 7H9 medium and plated in duplicate on 7H11 agar plates. These plates were then incubated for 21 days at 37°C and 5% CO_2, and CFU were enumerated.

Isolation of RNA, cDNA synthesis, and RT-PCR

Total RNA was extracted from primary monocytes cultured in the presence of various stimuli using a TRIzol reagent (Life Technologies), and reverse transcribed using Superscript RT II (Life Technologies) to generate cDNA for use in RT-PCR. Conditions used for all reactions were 94°C, 30 s/65°C, 30 s/68°C, 1 min for 30 cycles. Reactions typically contained 2.5 mM $MgCl_2$, 0.2 mM dNTP, 2.0 U *Taq* polymerase, and 20 pM 5′ and 3′ oligonucleotide primers (Life Technologies). The sequences of the primer pairs used, 5′ and 3′, were the following: TREM-1, TTGTCTCAGAACTCCGAGCTGC and GAGACATCGGCAGTTGACTTGG; IL-8, ACAA GCTTCTAGGACAAGAGCC and AAACTTCTCCACAACCCTCTGC; and β-actin, GGACGACATGGAGAAGATCTGG and ATAGTAATGT CACGCACGATTTCC. PCRs were run on agarose gels and visualized by ethidium bromide staining.

Flow cytometry and FITC-dextran uptake

Cells cultured in the presence of various stimuli were harvested and blocked with 20% human serum in 1× PBS for 1 h on ice to reduce nonspecific Ab binding by FcRs. Cells were then stained with unconjugated mAb recognizing TREM-1 (9E2, IgG1), or CD1a (OKT6, mIgG1), CD14 (3C10, $IgG2_b$), HLA-DR (L243, $IgG2_a$) prepared from hybridomas purchased from American Type Culture Collection (Manassas, VA). A PE-conjugated anti-CD86 (IT2.2, $IgG2_b$) was also used, purchased from BD PharMingen (San Diego, CA). In some cases, cells were double-stained with anti-TREM-1 and a FITC-conjugated anti-CD14 mAb (Caltag Laboratories, South San Francisco, CA) to analyze expression of TREM-1 on gated $CD14^{high}$ monocytes. Appropriate unconjugated (Sigma-Aldrich) or FITC- and PE-conjugated isotype control mAbs (Caltag Laboratories) were used in all experiments. After staining, cells were washed and fixed in 1% paraformaldehyde, and analyzed on a FACSCalibur flow cytometer (BD Biosciences). All data analysis was conducted using WinMDI 2.8 (J. Trotter, Scripps Research Institute, San Diego, CA).

For intracellular staining, cells were cultured in the presence of monensin (2 μg/ml) for 3 h before harvesting. Cells were harvested, washed, and fixed in 4% paraformaldehyde for 20 min. Cells were then permeabilized in saponin buffer (0.5% saponin, 5% FCS) for 20 min, washed, and stained with PE- or FITC-conjugated mAbs against MCP-1, MCP-3, macrophage inflammatory protein (MIP)-1α, and IL-8 (all from BD PharMingen) or the appropriate controls. Cells were then fixed, and acquired as above.

For analysis of FITC-dextran uptake, monocytes and iDC were harvested and incubated in complete medium containing 10% FCS on ice for 30 min. After blocking, 10 μl of a 10 mg/ml solution of FITC-dextran (Molecular Probes) was added to the cells, and incubated for an additional 30 min at either 4°C or 37°C. After incubation, cells were washed three times, fixed with 1% paraformaldehyde and acquired as above.

MLR and T cell assays

MLRs were performed as previously described (16). Briefly, primary monocytes were isolated, irradiated (3000 rad from a ^{137}Cs source), and cultured (1×10^4/well) in 96-well plates coated with isotype control or anti-TREM-1 mAbs, as described above. On day 2, T cells were purified from an unrelated donor using a T cell enrichment kit (Stem Cell Technologies, Vancouver, British Columbia, Canada) followed by adherence in RPMI 1640 containing 1% FCS for 2 h to eliminate contaminating monocytes. The resulting T cells were determined to be 95% pure by flow cytometry. The T cells were added to wells containing monocytes at a ratio ranging from 1:10–1:100 monocytes/T cell and cultured for an additional 5 days. On day 5, cultures were pulsed with 1 μCi/well [^{3}H]thymidine, incubated for an additional 4 h, and T cell proliferation was measured.

For T cell assays, primary monocytes (1×10^4/well) were stimulated in 96-well plates with isotype control or agonist anti-TREM-1 mAbs for 2 days, as described above. These activated monocytes were then pulsed with serial dilutions of the 10 kDa Ag derived from *Mycobacterium leprae* or total *M. leprae* sonicate for 30 min, and used to stimulate the MHC class II-restricted T cells lines D103.5 or C10E, as previously described (17, 18). After 2 days, supernatants were harvested and assayed for production of IFN-γ and IL-4 by ELISA. IFN-γ and IL-4 ELISA kits were purchased from BD PharMingen and BioSource International (Camarillo, CA), respectively.

Results

TREM-1 does not enhance direct innate antimicrobial activity against M. tb

We have identified a novel antimicrobial pathway in human monocytes that is rapidly induced through TLR2 ligation, and is therefore part of the innate immune response (2). To determine whether TREM-1 can induce and/or augment this antimicrobial activity, we tested its ability to reduce the viability of intracellular bacteria.

Primary human monocytes were infected with *M. tb* and then cultured in the presence of isotype control or activating anti-TREM-1 mAbs. A portion of these cells was also grown in the presence of *M. tb* 19-kDa lipopeptide (19 kDa), a TLR2 ligand used to trigger the antimicrobial pathway (2, 4). After 2 days, cells were harvested and CFU were measured. As shown in Fig. 1*A*, addition of the 19-kDa lipopeptide dramatically inhibited CFU by ~75%, as expected. However, this effect was completely blocked by the agonist TREM-1 mAb, restoring CFU to control levels. Interestingly, the addition of TREM-1 alone increased CFU by ~50% relative to untreated controls. This data suggests that TREM-1 does not enhance TLR-induced antimicrobial activity in monocytes, and may actually inhibit it instead.

To determine whether activation through TREM-1 can improve phagocytosis, another innate immune mechanism, we cultured primary monocytes in the presence or absence of TREM-1 mAbs for 2 days and measured uptake of FITC-dextran particles by flow cytometry. Fig. 1*B* shows there was only a very modest increase in phagocytic activity following TREM-1 activation, ~15% higher than the control. This result suggests that TREM-1 does not significantly enhance phagocytosis. Together these data suggest that TREM-1 is unlikely to play a prominent role in direct mechanisms of innate immunity.

TREM-1 triggers release of proinflammatory chemokines

Based on data suggesting that TREM-1 does not positively regulate mechanisms of innate immunity, we hypothesized that it may instead influence the early-induced and/or the adaptive phases of the immune response that follows. During the early-induced phase of the response, lymphocytes are recruited to the site of infection by chemokines (19), which help determine which cells will convene to mediate the ensuing adaptive response. To determine whether TREM-1 activation could induce chemokine production, monocytes were stimulated with isotype control or anti-TREM-1 mAbs for 20 h and assayed for production of various chemokines by intracellular flow cytometry. As shown in Fig. 2, cells activated with anti-TREM-1 mAbs strongly up-regulated expression of MCP-1, MCP-3, MIP-1α, and IL-8. Production of IL-8 was most dramatically enhanced, with 91% of the cells staining positive. Interestingly, the up-regulation of MCP-1 appeared to be linked to induction of MCP-3, as the percentage of MCP-1 single-positive cells did not change following TREM-1 activation, whereas the percentage of MCP-1/MCP-3 double-positive cells increased 10-fold. This data suggests that ligation of TREM-1 on monocytes drives robust production of proinflammatory chemokines involved in the recruitment of immune cells.

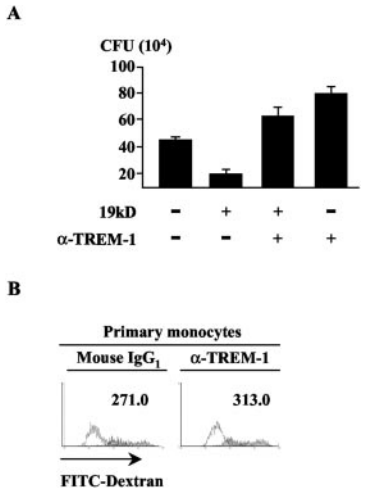

FIGURE 1. TREM-1 does not enhance the efficacy of innate immune mechanisms. *A*, Primary monocytes infected with *M. tb* were stimulated in the presence or absence of 19 kDa and either isotype control (mouse IgG1) or anti-TREM-1 mAbs to assess their effect on the viability of intracellular bacteria. After 48 h, cell lysates were plated and CFU were counted. Error bars represent SEM for the replicates. Results shown are representative of three independent experiments. *B*, To measure phagocytosis, primary monocytes were cultured with control or anti-TREM-1 mAbs for 48 h, and then incubated with FITC-dextran particles either on ice (open histograms) or at 37°C (filled histograms) to initiate particle uptake. Numbers in the corner of each histogram correspond to the change in MFI (ΔMFI) between the signal intensity at these two temperature conditions. Results shown are representative of three experiments.

TREM-1 and TLR synergize in the production of proinflammatory cytokines

As effector cells are recruited by chemokines, cytokines are responsible for their activation and commitment toward the Th1 or Th2 lineage (20). Given the ability of TREM-1 to induce production of proinflammatory chemokines in monocytes, we wanted to determine its ability to mediate production of Th1-inducing, proinflammatory cytokines. In our initial experiments to address this issue, the levels of TNF-α induced by the anti-TREM-1 mAb were significantly lower than we had expected; roughly 10-fold lower than published results. Therefore, we performed a similar series of experiments in which cells were treated with anti-TREM-1 mAbs in combination with various TLR ligands, because it had been shown that LPS, a ligand for TLR4, dramatically enhanced TNF-α production triggered by TREM-1 (10). For this, monocytes were cultured in the presence of isotype control or anti-TREM-1 mAbs costimulated with various TLR ligands, and were assayed for cytokine production by ELISA. As shown in Fig. 3, we observed between a 5- and 20-fold increase in TNF-α production from cells cultured in both anti-TREM-1 mAbs and TLR ligands as compared with those cultured with the Ab alone. We also detected a significant increase in GM-CSF production when anti-TREM-1 was administered in combination with a TLR ligand (~30 pg/ml vs 150–225 pg/ml). Interestingly, the low levels of anti-inflammatory IL-10 produced by LPS and poly(I:C) were inhibited by 80–90%

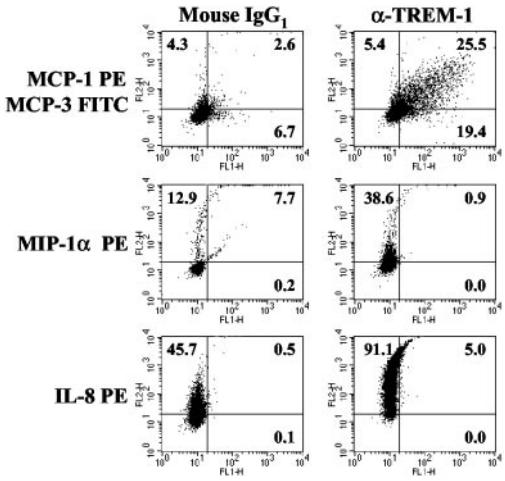

FIGURE 2. TREM-1 triggers the expression of proinflammatory chemokines. Primary monocytes were stimulated with isotype control (mouse IgG_1) or anti-TREM-1 mAbs for 20 h and assayed for intracellular expression of various proinflammatory chemokines by flow cytometry. The numbers in each quadrant represent the percentage of cells staining positive for the Ab indicated. The results shown are representative of at least three independent experiments.

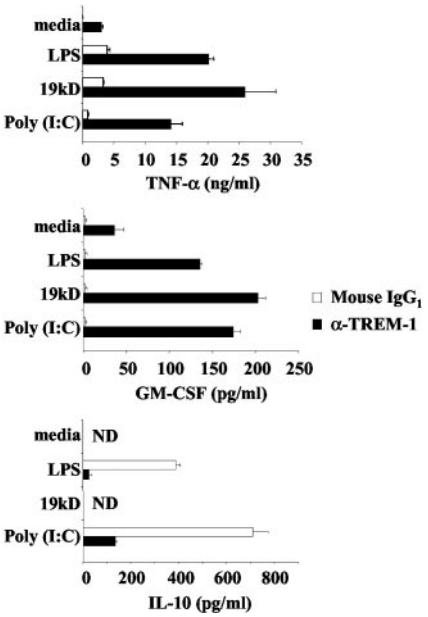

FIGURE 3. TREM-1 synergizes with TLRs in proinflammatory cytokine production. Primary monocytes (5×10^4/well) were cultured in a 96-well plate with an isotype control (mouse IgG_1) or agonist anti-TREM-1 mAb in the presence of various stimuli to elicit cytokine production. After 24 h, cell-free supernatants were harvested and analyzed by ELISA. Levels of detection in the ELISA were the following: TNF-α, 30 pg/ml; GM-CSF, 15 pg/ml; and IL-10, 30 pg/ml. Error bars represent the SEM for each condition. Results shown are representative of data obtained from four experiments.

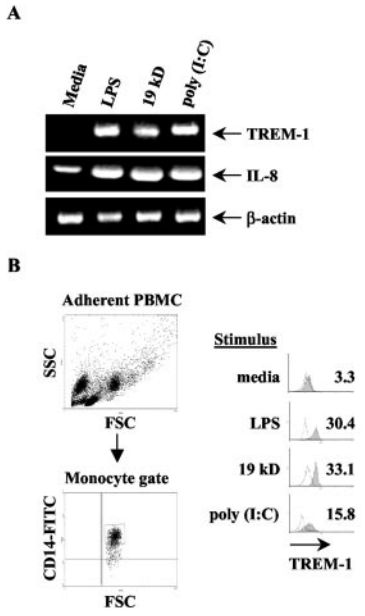

FIGURE 4. TLR ligands up-regulate TREM-1 expression on primary monocytes. *A*, cDNA derived from primary monocytes cultured for 24 h in the presence of LPS, 19 kDa, or poly(I:C) was analyzed for the presence TREM-1 mRNA by RT-PCR. Responsiveness of the monocytes to each stimuli is demonstrated by IL-8 mRNA induction. All reactions were normalized to β-actin levels to ensure equivalent amounts of template were used. *B*, Primary monocytes were cultured with various TLR agonists, as above, and analyzed for TREM-1 protein expression by flow cytometry. Cells were stained with mouse IgG1 (open histograms) or anti-TREM-1 mAbs (filled histograms). For data analysis, gating was set on the $CD14^{high}$ monocyte population. Numbers on each histogram represent ΔMFI. Actual ΔMFI for TREM-1 in response to each stimulus varied, but the data shown are representative of a typical result.

following TREM-1 engagement. Other proinflammatory cytokines normally produced by TLR ligation, such as IL-12, were induced to equivalent levels but were not affected by anti-TREM-1 (data not shown). These data suggest that TLRs synergize with TREM-1 to up-regulate proinflammatory cytokines and down-regulate anti-inflammatory cytokines produced during the early-induced immune response.

TREM-1 is up-regulated on monocytes in response to TLR ligands

We reasoned that the ability of TLR ligands to synergize with TREM-1 in the production of proinflammatory cytokines might be due to the up-regulation of TREM-1 expression resulting from activation of TLRs. To test this, primary monocytes were cultured in the presence of medium, LPS (TLR4), 19 kDa (TLR2), or poly(I:C) (TLR3) for 24 h and cDNA was generated for use in RT-PCR. As shown in Fig. 4*A*, TREM-1 mRNA was induced to similar levels by all the TLR ligands we tested. As a control for cellular activation, we determined that mRNA encoding IL-8, a chemokine produced by activated monocytes (21), was up-regulated in response to all TLR stimuli tested. Reactions were normalized to β-actin mRNA levels to ensure equal amounts of starting material were used.

To determine whether TREM-1 protein could also be induced, monocytes were cultured for 24 h in the presence of the same TLR agonists as above, and analyzed for TREM-1 expression by flow cytometry. As depicted in Fig. 4*B*, we gated on the $CD14^{high}$ monocyte subset previously shown to express the highest levels of TREM-1 protein (10). Cell surface expression of TREM-1 was induced to comparable levels following stimulation with LPS and the 19-kDa lipopeptide, and to a lesser extent in cells stimulated with poly(I:C). These data suggest that TREM-1 is up-regulated by innate cues that activate TLRs.

Pro- and anti-inflammatory cytokines differentially regulate TREM-1 expression

One mechanism to quickly amplify the intensity of a particular signaling pathway is through the use of autocrine loops, in which products derived from the pathway act to sustain the expression of receptors that respond to the signal. The ability of TLR ligands to up-regulate TREM-1 expression, and their synergy with TREM-1 in cytokine production led us to suspect that proinflammatory cytokines produced by this combination of receptors may further up-regulate TREM-1 expression. To address this possibility, monocytes were cultured for 24 h in medium, LPS, or 19 kDa in the presence of TNF-α, GM-CSF, or IL-10 and analyzed for cell surface expression of TREM-1 by flow cytometry. As shown in Fig. 5, when monocytes cultured with LPS or 19kDa were co-stimulated with GM-CSF or TNF-α, the level of TREM-1 expression increased roughly 2-fold relative to LPS or 19 kDa alone, and 5- to 10-fold above resting levels, as judged by the change in mean fluorescence intensity (MFI). In contrast, IL-10 completely negated up-regulation of TREM-1 by TLRs. In the absence of TLR ligation, GM-CSF and TNF-α alone were able to induce a modest up-regulation of TREM-1, whereas IL-10 alone had effect. Other cytokines we tested, IL-1β, IL-4, IL-12, and IL-18, had no effect on TREM-1 expression alone or in combination with LPS or 19 kDa (data not shown). This data indicates that the same proinflammatory cytokines produced by TREM-1 and TLR coactivation further up-regulate expression of TREM-1, whereas the opposing anti-inflammatory cytokines have an inhibitory effect.

TREM-1 triggers differentiation of primary monocytes into iDC

Our data point to a potential role for TREM-1 in the production of proinflammatory cytokines and chemokines produced during the early-induced phase of an immune response. This links TREM-1 indirectly to the string of events that influences adaptive immunity, however, we wanted to ascertain whether TREM-1 had a more direct impact on this branch of the immune response. Our data, combined with other previous reports, led us to reason that TREM-1 might play a role in monocyte differentiation into DC. First, monocytes up-regulate the DC marker CD83 following activation through TREM-1 (10, 22). Second, our data has highlighted the ability of TREM-1 to induce robust production of TNF-α and GM-CSF, two cytokines that have been used to derive iDC from monocytes in vitro (14, 23). Finally, another TREM family member, TREM-2, has been shown to participate in the maturation and survival of DC (11), raising the possibility that this family of receptors regulates different stages of maturation in myeloid cells.

To test this possibility, primary monocytes were cultured in medium, or GM-CSF and IL-4 in the presence of an isotype control or anti-TREM-1 mAb. Some cells were stained immediately (day 0, column 1) to assess baseline expression of monocyte and DC markers. The remaining cells were cultured for 5 days to allow for differentiation, and analyzed for expression of monocyte and DC markers by flow cytometry. As shown in Fig. 6, there was remarkable similarity in CD1a and CD14 expression patterns between monocytes cultured in medium with anti-TREM-1 mAbs alone (column 3) and iDC derived in the conventional manner with GM-CSF and IL-4 (column 4). Both groups showed increased CD1a expression concomitant with a down-regulation of CD14, a characteristic phenotype of monocyte-derived iDC. Interestingly, iDC derived with GM-CSF and IL-4 in the presence of TREM-1 mAbs (column 5) demonstrated markedly higher CD1a expression, as well as a complete loss of CD14 expression, compared with those derived in the presence of control mAb. In addition to its influence on monocyte and iDC markers, TREM-1 was also found to increase expression of molecules related to T cell activation and Ag presentation, namely CD86 and MHC class II (HLA-DR). Expression of CD80, another related costimulatory molecule, was unaffected (data not shown). This data suggests that TREM-1 promotes the differentiation of monocytes into cells phenotypically resembling iDC.

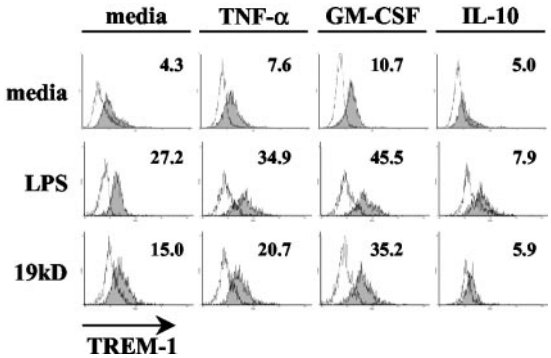

FIGURE 5. Cytokines differentially affect expression of TREM-1 induced through TLRs. Primary monocytes (3×10^5/ml) cultured in the presence of medium, LPS, or 19 kDa were cocultured with various pro- and anti-inflammatory cytokines over a 24-h period, and analyzed for cell surface expression of TREM-1 by flow cytometry. Numbers in *upper right corner* of each histogram indicate ΔMFI of the TREM-1 signal. These results are representative of three independent experiments.

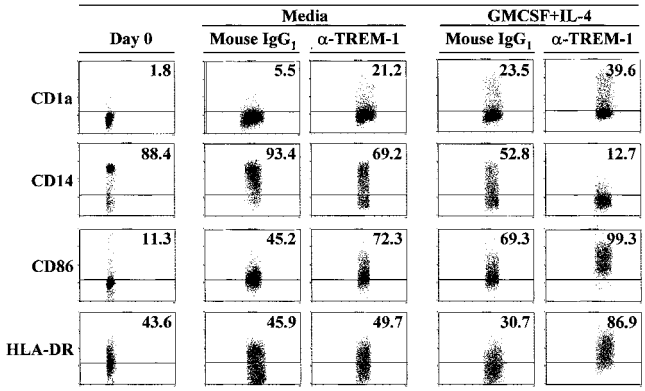

FIGURE 6. Engagement of TREM-1 induces monocyte differentiation into iDC. Primary monocytes (3×10^5/ml) were cultured in 24-well plates coated with isotype control (mouse IgG_1) or anti-TREM-1 mAbs. Portions of these cells were also cultured in GM-CSF and IL-4 to derive iDC for comparison to TREM-1-matured cells. On days 0 and 5, cells were harvested and stained with control or mAbs directed against various monocyte and DC markers and analyzed by flow cytometry. Each plot represents the percentage of cells that stain positive for the marker indicated relative to the intensity of isotype control staining (horizontal line). Data are representative of three experiments.

TREM-1-derived DCs more efficiently stimulate T cells

To determine whether the changes caused by the engagement of TREM-1 endowed monocytes with a superior ability to stimulate T cells, we tested their ability to function as APC in an MLR. Primary monocytes were cultured for 2 days in the presence of isotype control, or anti-TREM-1 mAbs, to begin inducing the iDC phenotype. Purified T cells from an unrelated donor were then added at various ratios and T cell proliferation was measured by

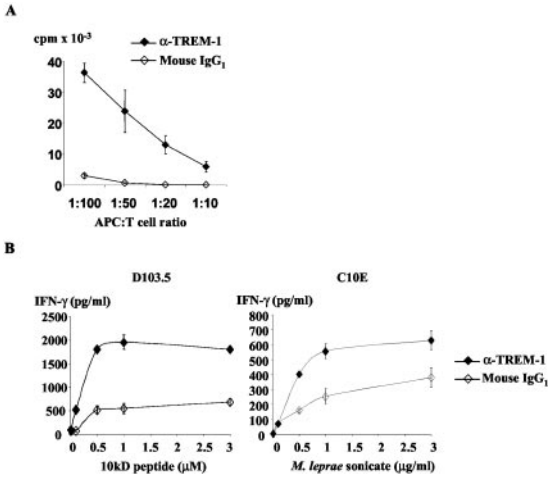

FIGURE 7. TREM-1-derived DCs more efficiently stimulate T cells. *A*, Irradiated primary monocytes (1×10^4/well) were cultured in the presence of isotype control (◇) or anti-TREM-1 mAbs (◆) for 2 days, and then cocultured with varying ratios of purified T cells from an unrelated donor for 5 days in a MLR. After 5 days, T cell proliferation was measured by [^{3}H]thymidine incorporation. Error bars represent SEM for triplicate samples. *B*, Primary monocytes (1×10^4/well) were cultured in 96-well plates for 2 days in the presence of isotype control (◇) or anti-TREM-1 mAb (◆). Cells were then pulsed with dilutions of Ag and incubated for 30 min to allow uptake. D103.5 or C10E T cells (1×10^4/well) were added to the monocytes, and cultured for an additional 24 h. Supernatants were then harvested and assayed for IFN-γ production by ELISA. Error bars at each Ag concentration represent the SEM for triplicate samples. Results shown are representative of three independent experiments.

[^{3}H]thymidine incorporation after an additional 5 days in culture. As shown in Fig. 7*A*, TREM-1-activated monocytes promoted a dramatic increase in T cell proliferation compared with cells activated with isotype control mAbs. The effect was true across all APC-T cell ratios we tested. Robust proliferation was observed in all samples in response to IL-2 (data not shown).

We also tested the ability of monocytes activated through TREM-1 to stimulate production of IFN-γ in two MHC class II-restricted T cell lines, D103.5 and C10E, which are reactive against a 15-mer peptide epitope of the *M. leprae* 10-kDa Ag and whole *M. leprae* sonicate, respectively (17, 18). Primary monocytes were activated for 2 days in the presence of isotype control, or anti-TREM-1 mAbs as above, and then pulsed with various concentrations of the 10-kDa peptide or *M. leprae* sonicate. The monocytes were then used as APC to activate D103.5 and C10E in a routine T cell assay. After 24 h, supernatants were harvested and assayed for production of IFN-γ and IL-4 by ELISA. As shown in Fig. 7*B*, both D103.5 and C10E demonstrated a large increase in IFN-γ production when stimulated by TREM-1-activated monocytes. This trend was consistent over the range of Ag concentrations we tested, suggesting it was a dose-dependent effect and not due to the particular conditions used. Furthermore, the lack of IFN-γ production in the absence of Ag suggests the enhancement of T cell activation is Ag-dependent, and not the result of other nonspecific effects mediated by TREM-1. No production of IL-4 was detected in the same supernatants (data not shown). This data highlights a potential role for TREM-1 in shaping the maturation of monocytes into iDC with a superior ability to activate T cells.

Discussion

The immune response is a highly evolved machine designed to offer protection against microbial pathogens. The combined use of pre-existing innate and inducible adaptive immune mechanisms ensures that the host will be able to mount an appropriate immune response against a wide range of pathogens. Based on its previously described relevance in inflammation, we wanted to more clearly define the role of TREM-1 in innate and acquired mechanisms of host defense. In this study, we show that engagement of TREM-1 on primary monocytes did not enhance antimicrobial activity or phagocytosis, two direct pathways of the innate response. However, we did detect increased production of proinflammatory chemokines and cytokines and strong inhibition of the anti-inflammatory cytokine IL-10 when monocytes were coactivated with anti-TREM-1 and TLR ligands. TLR ligands also up-regulated TREM-1 expression, an effect that was enhanced by TNF-α and GM-CSF and completely repressed by IL-10. Finally, engagement of TREM-1 caused a subset of primary monocytes to differentiate into iDC based on expression of CD1a and CD14 markers. These cells also expressed higher levels of CD86 and MHC class II molecules, which was consistent with their superior ability to drive T cell proliferation and IFN-γ production. Collectively, our data suggests that activation of TREM-1 by itself, and in combination with TLRs, enhances the early-induced and adaptive immune responses which combine to provide host defense against microbial infection.

We believe the importance of TREM-1 during the early-induced response manifests primarily in its ability to up-regulate monocyte production of proinflammatory cytokines and chemokines that mediate the activation, maturation, and recruitment of lymphocytes involved in combating an infection. The chemokines we tested, MCP-1, MCP-3, MIP-1α, and IL-8, were all significantly up-regulated in the majority of cells following TREM-1 ligation. It is likely that the previously described ability of TREM-1 to drive inflammation may be partially attributable to the induction of one or several of these chemokines. In fact, all have been shown to mediate the recruitment of inflammatory Th1 cells (24–27), however conflicting data suggest that MCP-1 may preferentially support Th2 cytokine-producing cells (28). In addition to chemokines, ligation of TREM-1 led to an up-regulation of GM-CSF and TNF-α, two proinflammatory cytokines also part of the early-induced response. Cells coactivated with various TLR ligands further enhanced production of these cytokines, demonstrating the synergy between TREM-1 and TLR signaling pathways. In stark contrast, the IL-10 produced in response to some TLR ligands was almost completely inhibited by TREM-1, further confirming its specificity in potentiating proinflammatory, Th1-inducing conditions. Interestingly, the same pro- and anti-inflammatory cytokines affected by TREM-1 also modulated its cell surface expression, suggesting the possible existence of an autocrine loop designed to sustain TREM-1 signaling.

Perhaps the most intriguing finding to surface in this study was the ability of TREM-1 to cause primary monocytes to differentiate into cells that phenotypically resemble iDC. More importantly, these phenotypic changes correlated with the superior ability of the cells to function as APC, stimulating T cell proliferation in a MLR and production of IFN-γ by two MHC class II-restricted T cell lines. The selective up-regulation of CD86 and not CD80 may be noteworthy in light of earlier reports suggesting that T cells activated through these coreceptors preferentially express the Th1 and Th2 cytokines IFN-γ and IL-4, respectively (29), although this issue remains controversial. It would also be of interest to determine whether TREM-1-activated monocytes could differentially stimulate Th1 and Th2 T cell responses. These findings provide evidence for a more direct link between TREM-1 and mechanisms of adaptive immunity.

Based on the close homology of TREM-1 to other DAP12-associated NK cell receptors, many of which are involved during the innate immune response (30), we were surprised to find that TLR-induced antimicrobial activity against *M. tb* was not enhanced, and in fact was inhibited, by TREM-1 activation. One possible explanation for this result comes from a report demonstrating that DCs are inferior to macrophages in containing mycobacterial growth (31, 32). The authors suggest that this discrepancy may involve one of many functional differences between these cells induced by IL-10. It is tempting to speculate that the inability of TREM-1 to elicit antimicrobial activity may result from its repression of IL-10, as well as its role in driving the maturation of monocytes into iDC. Future studies that more thoroughly characterize the genes modulated by TREM-1 will undoubtedly shed light on the mechanisms behind these and other functional properties of this molecule.

In summary, our data suggest that TREM-1 participates during early-induced and adaptive responses that cooperate to provide host protection against microbial challenges. Future studies directed at identifying natural ligands for TREM-1 will contribute to our understanding of its physiological significance during an immune response. They may also lead to the development of new clinical manipulations of this molecule, in which targeted activation or blockade of TREM-1 may help to maximize the efficacy of existing treatments.

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References

1. Aderem, A., and R. J. Ulevitch. 2000. Toll-like receptors in the induction of the innate immune response. *Nature 406:782.*

2. Thoma-Uszynski, S., S. Stenger, O. Takeuchi, M. T. Ochoa, M. Engele, P. A. Sieling, P. F. Barnes, M. Rollinghoff, P. L. Bolcskei, M. Wagner, et al. 2001. Induction of direct antimicrobial activity through mammalian Toll-like receptors. *Science 291:1544.*
3. Kaiser, V., and G. Diamond. 2000. Expression of mammalian defensin genes. *J. Leukocyte Biol. 68:779.*
4. Brightbill, H. D., D. H. Libraty, S. R. Krutzik, R. B. Yang, J. T. Belisle, J. R. Bleharski, M. Maitland, M. V. Norgard, S. E. Plevy, S. T. Smale, et al. 1999. Host defense mechanisms triggered by microbial lipoproteins through Toll-like receptors. *Science 285:732.*
5. Aderem, A., and D. M. Underhill. 1999. Mechanisms of phagocytosis in macrophages. *Annu. Rev. Immunol. 17:593.*
6. Medzhitov, R., P. Preston-Hurlburt, and C. A. J. Janeway. 1997. A human homologue of the Drosophila Toll protein signals activation of adaptive immunity. *Nature 388:394.*
7. Akira, S., K. Takeda, and T. Kaisho. 2001. Toll-like receptors: critical proteins linking innate and acquired immunity. *Nat. Immunol 2:675.*
8. Luster, A. D. 2002. The role of chemokines in linking innate and adaptive immunity. *Curr. Opin. Immunol. 14:129.*
9. Banchereau, J., and R. M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature 392:245.*
10. Bouchon, A., J. Dietrich, and M. Colonna. 2000. Cutting edge: inflammatory responses can be triggered by TREM-1, a novel receptor expressed on neutrophils and monocytes. *J. Immunol. 164:4991.*
11. Bouchon, A., C. Hernandez-Munain, M. Cella, and M. Colonna. 2001. A DAP12-mediated pathway regulates expression of CC chemokine receptor 7 and maturation of human dendritic cells. *J. Exp. Med. 194:1111.*
12. Chung, D. H., W. E. Seaman, and M. R. Daws. 2002. Characterization of TREM-3, an activating receptor on mouse macrophages: definition of a family of single Ig domain receptors on mouse chromosome 17. *Eur. J. Immunol. 32:59.*
13. Bouchon, A., F. Facchetti, M. A. Weigand, and M. Colonna. 2001. TREM-1 amplifies inflammation and is a crucial mediator of septic shock. *Nature 410: 1103.*
14. Sallusto, F., and A. Lanzavecchia. 1994. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor α. *J. Exp. Med. 179:1109.*
15. Cella, M., C. Dohring, J. Samaridis, M. Dessing, M. Brockhaus, A. Lanzavecchia, and M. Colonna. 1997. A novel inhibitory receptor (ILT3) expressed on monocytes, macrophages, and dendritic cells involved in antigen processing. *J. Exp. Med. 185:1743.*
16. Hertz, C. J., S. M. Kiertscher, P. J. Godowski, D. A. Bouis, M. V. Norgard, M. D. Roth, and R. L. Modlin. 2000. Microbial lipopeptides stimulate dendritic cell maturation via TLR2. *J. Immunol. 166:2444.*
17. Kim, J., A. Sette, S. Rodda, S. Southwood, P. A. Sieling, V. Mehra, J. D. Ohmen, J. L. Oliveros, E. Appella, Y. Higashimoto, et al. 1997. Determinants of T cell reactivity to the *Mycobacterium leprae* GroES homologue. *J. Immunol. 159:335.*
18. Sieling, P. A., M. T. Ochoa, D. Jullien, D. S. Leslie, S. Sabet, J. P. Rosat, A. E. Burdick, T. H. Rea, M. B. Brenner, S. A. Porcelli, and R. L. Modlin. 2000. Evidence for human $CD4^+$ T cells in the CD1-restricted repertoire: derivation of mycobacteria-reactive T cells from leprosy lesions. *J. Immunol. 164:4790.*
19. Zlotnik, A., and O. Yoshie. 2000. Chemokines: a new classification system and their role in immunity. *Immunity 12:121.*
20. O'Garra, A. 1998. Cytokines induce the development of functionally heterogeneous T helper cell subsets. *Immunity 8:275.*
21. Caux, C., C. Massacrier, B. Vanbervliet, B. Dubois, C. Van Kooten, I. Durand, and J. Banchereau. 1994. Activation of human dendritic cells through CD40 cross-linking. *J. Exp. Med. 180:1263.*
22. Zhou, L. J., and T. F. Tedder. 1995. Human blood dendritic cells selectively express CD83, a member of the immunoglobulin superfamily. *J. Immunol. 154: 3821.*
23. Zhou, L. J., and T. F. Tedder. 1996. $CD14^+$ blood monocytes can differentiate into functionally mature $CD83^+$ dendritic cells. *Proc. Natl. Acad. Sci. USA 93: 2588.*
24. Huang, D. R., J. Wang, P. Kivisakk, B. J. Rollins, and R. M. Ransohoff. 2001. Absence of monocyte chemoattractant protein 1 in mice leads to decreased local macrophage recruitment and antigen-specific T helper cell type 1 immune response in experimental autoimmune encephalomyelitis. *J. Exp. Med. 193:713.*
25. Siveke, J. T., and A. Hamann. 1998. T helper 1 and T helper 2 cells respond differentially to chemokines. *J. Immunol. 160:550.*
26. Fujisawa, T., Y. Kato, J. Atsuta, A. Terada, K. Iguchi, H. Kamiya, H. Yamada, T. Nakajima, M. Miyamasu, and K. Hirai. 2000. Chemokine production by the BEAS-2B human bronchial epithelial cells: differential regulation of eotaxin, IL-8, and RANTES by TH2- and TH1-derived cytokines. *J. Allergy Clin. Immunol. 105:126.*
27. Bonecchi, R., N. Polentarutti, W. Luini, A. Borsatti, S. Bernasconi, M. Locati, C. Power, A. Proudfoot, T. N. Wells, C. Mackay, et al. 1999. Up-regulation of CCR1 and CCR3 and induction of chemotaxis to CC chemokines by IFN-γ in human neutrophils. *J. Immunol. 162:474.*
28. Chensue, S. W., K. S. Warmington, J. H. Ruth, P. S. Sanghi, P. Lincoln, and S. L. Kunkel. 1996. Role of monocyte chemoattractant protein-1 (MCP-1) in Th1 (mycobacterial) and Th2 (schistosomal) antigen-induced granuloma formation: relationship to local inflammation, Th cell expression, and IL-12 production. *J. Immunol. 157:4602.*
29. Kuchroo, V. K., M. P. Das, J. A. Brown, A. M. Ranger, S. S. Zamvil, R. A. Sobel, H. L. Weiner, N. Nabavi, and L. H. Glimcher. 1995. B7-1 and B7-2 costimulatory molecules activate differentially the Th1/Th2 developmental pathways: application to autoimmune disease therapy. *Cell 80:707.*
30. Moretta, A., C. Bottino, M. Vitale, D. Pende, C. Cantoni, M. C. Mingari, R. Biassoni, and L. Moretta. 2001. Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis. *Annu. Rev. Immunol. 19:197.*
31. Fortsch, D., M. Rollinghoff, and S. Stenger. 2000. IL-10 converts human dendritic cells into macrophage-like cells with increased antibacterial activity against virulent *Mycobacterium tuberculosis. J. Immunol. 165:978.*
32. 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.*