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Soluble Forms of Toll-Like Receptor (TLR)2 Capable of Modulating TLR2 Signaling Are Present in Human Plasma and Breast Milk

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Dysregulation of the initial, innate immune response to bacterial infection may lead to septic shock and death. Toll-like receptors (TLRs) play a crucial role in this innate immune response, and yet the regulatory mechanisms controlling microbial-induced TLR triggering are still to be fully understood. We have therefore sought specific regulatory mechanisms that may modulate TLR signaling. In this study, we tested for the possible existence of a functionally active soluble form of TLR2. We demonstrated the existence of natural soluble forms of TLR2 (sTLR2), which we show to be capable of modulating cell activation. We found that blood monocytes released sTLR2 constitutively and that the kinetics of sTLR2 release increased upon cell activation. Analysis of cells expressing the human TLR2 cDNA or its c-*myc*-tagged version indicated that sTLR2 resulted from the posttranslational modification of the TLR2 protein in an intracellular compartment. Moreover, an intracellular pool of sTLR2 is maintained. sTLR2 was found naturally expressed in breast milk and plasma. Milk sTLR2 levels mirrored those of the TLR coreceptor soluble CD14. Depletion of sTLR2 from serum resulted in an increased cellular response to bacterial lipopeptide. Notably, serum sTLR2 was lower in tuberculosis patients. Coimmunoprecipitation experiments and computational molecular docking studies showed an interaction between sTLR2 and soluble CD14 in plasma and milk. These findings suggest the existence of a novel and specific innate immune mechanism regulating microbial-induced TLR triggering, and may lead to new therapeutics for the prevention and/or treatment of severe infectious diseases. *The Journal of Immunology,* 2003, 171: 6680–6689.

S evere bacterial infection may lead to profound pathophysiological effects, such as myocardial dysfunction, acute respiratory failure, and renal as well as multiple organ failure. These pathological conditions result from the dysregulation of the initial, appropriate host immune response to infection (1–3).

The phylogenetically ancient defense mechanism known as the innate immune system constitutes the initial line of defense against microbial infections through its ability to recognize and respond to a large variety of microorganisms in an immediate manner by using a repertoire of invariant receptors (4). It has become clear that the role of the evolutionarily conserved mammalian Toll-like receptor (TLR)[3] (2) family is critical to the activity of the innate immune system (4, 5). These type I transmembrane receptors are capable of discriminating between different pathogens by recognizing defined molecular structures present in a variety of microorganisms. Ten mammalian TLRs have been reported to date. It is

well established that TLR4, in association with the extracellular accessory molecule MD-2, is the main signaling receptor for most bacterial LPS, the main component of the outer membrane of Gram-negative bacteria. TLR4 also acts as the signal-transducing receptor for whole Gram-negative bacteria and for the fusion protein from respiratory syncytial virus. By contrast, TLR2 mediates the recognition of Gram-positive bacteria, mycobacteria, the hemagglutinin protein of wild-type measles virus, and a broad range of microbial components. The unusual broad-range ligand recognition by TLR2 is achieved at least in part by heterodimerization with TLR6 or TLR1. TLR3, TLR5, and TLR9 were found to recognize dsRNA from virus, bacterial flagellin, and unmethylated CpG motifs of bacterial DNA, respectively (6–8).

In most cases, fully efficient microbial recognition by TLR2 and TLR4 requires the critical activity of a coreceptor, CD14. A typical example is the recognition of LPS by sensitive cells (7, 9). Initially, LPS monomers are shuttled to CD14 by the activity of the catalytic transfer protein, LPS-binding protein. Subsequently, CD14-LPS complexes are believed to directly trigger the activation of TLR4. In this way, CD14 dramatically enhances cell sensitivity to LPS. In fact, in the absence of CD14, cellular responses to most microbial components activating via TLR2 or TLR4 are extremely low (10–12). This coreceptor is expressed as a GPI-anchored molecule mainly in myeloid cells and also in serum as a soluble bacterial coreceptor (sCD14) (13–15). Notably, extremely high levels of functionally active sCD14 in human breast milk have been reported (16, 17).

Engagement of TLRs leads to the production of a variety of proinflammatory and immunoregulatory cytokines, chemokines, and costimulatory molecules (18). This process, initiated by the engagement of TLRs, results in an immediate and efficient response to the microbial challenge. The excessive release of some

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[3] Abbreviations used in this paper: TLR, Toll-like receptor; CHO, Chinese hamster ovary; HEK, human embryonic kidney; LRR, leucine-rich repeat; PSSM, position-specific score matrix; s, soluble.

TLR-mediated proinflammatory molecules may, however, lead to profound deleterious effects, including septic shock and death. Therefore, it would be expected that the triggering of TLRs would be tightly regulated. We have thus sought specific regulatory mechanisms that may modulate signaling via TLR2 in particular, because of this receptor's central role in the innate recognition of a broad range of microbial components. In view of the considerable number of soluble receptors with well-documented regulatory activity that have been reported (19, 20), we have tested for the possible existence of sTLR2, for its potential to modulate TLR signaling, and for its presence in biological fluids.

Materials and Methods

Reagents and Abs

Protein G-Sepharose, PMA, ionomycin, monensin, Nonidet P-40 and Triton X-114 detergents, and LPS (055:B5 strain) were from Sigma-Aldrich. (Dorset, U.K.). Hygromycin B was from Calbiochem (San Diego, CA). The synthetic bacterial lipopeptide Pam$_3$-Cys-Ser-(Lys)$_4$ HCl (Pam$_3$Cys) was from EMC Microcollections GmbH (Tübingen, Germany). All other chemicals were reagent grade. The N terminus 17-mer TLR2 peptide and the polyclonal Ab (sc8689, purified goat IgG) generated by immunization with this peptide, anti-plexin-C1 Ab (goat), normal goat IgG, and anti-goat Igs-HRP conjugate were from Santa Cruz Biotechnology (Santa Cruz, CA). Two anti-TLR2 polyclonal Abs (rabbit) were generated in our laboratories by immunization with an N terminus 20-mer TLR2 peptide (SKEESSNGASLSGDRNGIGK), referred to as TLR2p, or with Chinese hamster ovary (CHO) cells expressing the human TLR2 cDNA (TLR2PM). The specificity of both Abs was confirmed by FACS and Western blot analyses using freshly isolated monocytes and CHO-TLR2 transfectants. The anti-TLR2-specific mAb TL2.1 (mouse IgG2a) and the TL2.1-PE conjugate were from eBiosciences (San Diego, CA). The anti-TLR2 mAb IMG-319 (mouse IgG1) and the anti-TLR2 peptide (180–196, 353–370, 473–489) Ab IMG-410 (rabbit) were from Imgenex (San Diego, CA). The rabbit anti-CD14 Ab was previously described (16). The isotype-matched controls mouse IgG1, IgG2a, and IgG2a-PE conjugate were from Diaclone (Besançon, France). The anti-mouse Igs-PE conjugate, rabbit F(ab')$_2$, was from Dako (Glostrup, Denmark), and the anti-c-Myc epitope mAb clone 9E10 (IgG1) was from Sigma-Aldrich.

Human milk and plasma, cells, cell culture supernatants, and cell activation

Human breast milk and plasma samples were obtained from healthy donors after written consent. Serum from control subjects and newly diagnosed pulmonary tuberculosis patients (HIV negative) was collected at the Chest Clinic, Komfo Anokye Teaching Hospital (Kumasi, Ghana), after local Ethical Committee approval and informed consent from all donors (21). All samples were kept at $-80°C$ until use. Milk and serum sCD14 concentrations were determined by ELISA (IBL, Hamburg, Germany). The human monocytic cell line Mono Mac-6 (kindly provided by H. Ziegler-Heitbrock, Department of Immunology, Leicester University, Leicester, U.K.) was cultured in RPMI 1640 medium (Life Technologies-BRL, Paisley, U.K.) supplemented with 10% FCS (HyClone, Logan, UT; <0.06 U/ml endotoxin), 2 mM glutamine, 1 mM pyruvate, 1% nonessential amino acids, and 10 μg/ml insulin (all from Life Technologies-BRL). Human monocyte preparations were obtained following Ficoll density-gradient centrifugation of buffy coats from heparinized blood of healthy donors and adherence (2 h, 37°C). The purity of the monocyte preparations was always >95%, as evaluated by FACS with anti-CD14, -CD19, and -CD3 mAbs. To test for sTLR2 in cell culture supernatants, cells were washed with phenol red-free RPMI 1640 medium and cultured (1 × 10^6 cells/ml) for the times indicated in *Results* in serum-free and phenol red-free RPMI 1640 medium supplemented with 2 mM glutamine. At the indicated time points, the supernatants were centrifuged and filtered (0.22-μm filters); 0.1% (v/v) Nonidet P-40, 1 μg/ml leupeptin, and pepstatin A were added; and the supernatants were concentrated 10 or 20 times (Centricon YM-10 concentrators; Millipore, Billerica, MA) before immunoprecipitation or Western blotting, respectively. For cell activation experiments (Fig. 1), cell aliquots (5 × 10^6) were cultured in serum-free medium, as described previously, in the absence or presence of 50 ng/ml PMA + 500 ng/ml ionomycin or 10 μg/ml Pam$_3$Cys for the times indicated in *Results*. For functional experiments (Fig. 5, A–C), Mono Mac-6 cells were cultured in 96-well plates (5 × 10^4 cells/well, 50 μl) in the presence of sTLR2 or Fc-sTLR2 containing serum-free culture supernatants, or those from mock-transfected cells (100 μl, collected after 10 h of culture), or the Fc-CD46 control fusion protein (150 ng/ml). The cells were

stimulated with the indicated concentrations of Pam$_3$Cys (50 μl) or LPS for 5 h. Subsequently, the supernatants were tested for IL-8 and TNF-α by ELISA (Duoset; R&D Systems, Minneapolis, MN).

AB serum (100 μl diluted 1/4 with PBS) depleted of sTLR2 used in experiments shown in Fig. 5D was prepared by performing four sequential immunoprecipitations (see below) with the anti-TLR2 Ab sc8689 (6.4 μg) or the irrelevant anti-plexin-C1 Ab, mock depletion. The extent of the sTLR2 depletion was between 85 and 92%, depending on the experiment, as judged by Western blotting, followed by densitometric analysis of the gels (Fig. 5D, right inset). PBMC (2 × 10^5 cells) were stimulated for 15 h with the indicated concentrations of Pam$_3$Cys in the absence or presence of 2% sTLR2 or mock-depleted serum. Cell culture supernatants were tested for IL-8 by ELISA. In control experiments (data not shown), addition of sc8689 (10 μg/ml) to PBMC cultured in the presence of sTLR2-depleted serum did not affect the Pam$_3$Cys-induced release of IL-8. This excluded the possibility that the results described in Fig. 5D were affected by any residual amount of sc8689 present in the depleted serum used.

Immunoprecipitations, Western blot analysis, cell biotinylation, and cell lysate preparations

The immunoprecipitation technique was as previously described (14). In this study, to immunoprecipitate sTLR2 from serum-free culture supernatants, the anti-TLR2 Ab sc8689 (5 μg) or normal goat IgG were used. Immunoprecipitates were washed with Dulbecco's PBS supplemented with 5 mM EDTA, 0.1% (v/v) Nonidet P-40, and 0.02% NaN$_3$ (washing buffer). Immunocomplexes were eluted and loaded onto 10% SDS-PAGE. For sTLR2-sCD14 coimmunoprecipitation experiments using milk, 1-ml samples were diluted 1/10 with Dulbecco's PBS and precleared by incubation (1 h, 4°C, orbital rotation) with normal goat IgG (30 μg), followed by two 1-h rounds, and a further overnight incubation, with protein G-Sepharose (250 μl/round, 50% suspension). The precleared sample was divided into two and incubated (1 h, 4°C, orbital rotation) with the sc8689 anti-TLR2 Ab or normal goat IgG (15 μg). The immunocomplexes were precipitated (90 μl protein G-Sepharose), washed, and boiled in Laemmli reducing sample buffer. For coimmunoprecipitations using plasma, 100 μl samples diluted 1/5 were processed as described for milk samples. In this study, 10 μg of normal goat IgG and 100 μl of protein G-Sepharose were used for preclearing, and 5 μg of sc8689 or goat IgG was used for immunoprecipitations. Immunocomplexes were boiled in Laemmli nonreducing sample buffer. The Western blot technique was previously described (14). To test for sTLR2 in human milk or plasma, samples were diluted 1/150 or 1/300, respectively, with Laemmli reducing sample buffer before 10% SDS-PAGE. Membranes were blocked with a 5% BSA (milk samples) or nonfat dry milk (plasma samples)/PBS, 0.1% Tween 20 (PBS-T) solution and probed with the anti-TLR2 Abs sc8689, TLR2p, TLR2PM (1/2000), IMG-319, or IMG-410 (1 μg/ml) diluted in 2% blocking agent/PBS-T. Detection was conducted by incubation with the species-specific HRP-conjugate secondary Abs, followed by ECL (Amersham Pharmacia, Little Chalfont, Bucks, U.K.). For the analysis of biotinylated sTLR2 in cell culture supernatants, highly viable (≥99%) Mono Mac-6 cells were biotinylated at room temperature for 30 min with EZ-link-sulfo-N-hydroxysuccinimide-biotin (Pierce, Rockford, IL) following the manufacturer's instructions. The cells (10 × 10^6) were washed six times with Dulbecco's PBS, resuspended in PBS (1.7 × 10^6 cells/ml), and biotinylated at a ratio of 80 μg biotin per 1 × 10^6 cells. Subsequently, the cells were washed and recultured in serum-free medium, as described previously. The preparation of Mono Mac-6 cell lysates by using 1% (v/v) Nonidet P-40 detergent was previously described (22). Detergent partitioning of the total cellular proteins was performed as described (23). Typically, 4 × 10^6 Mono Mac-6 cells resuspended in 267 μl of PBS were mixed with 53 μl of precondensed Triton X-114. Following incubation (15 min on ice) and centrifugation (10,000 × g × 10 min, 4°C), the supernatant was collected and warmed at 37°C, and the cloudy solution was centrifuged at 1000 × g × 10 min at room temperature. The resulting upper (aqueous) and lower (detergent) phases were separated and analyzed by Western blotting.

Immunofluorescence, FACS analysis, and intracellular staining

Detection of cell surface TLR2 or Myc-tagged TLR2 with the PE-conjugated anti-TLR2-specific mAb TL2.1 or the anti-c-Myc epitope mAb 9E10, respectively, was performed by immunofluorescence, followed by FACS analysis, as previously described (24). In this study, monocytic cells were preincubated for 10 min with 20% normal rabbit serum before washing and staining. Intracellular detection of TLR2 was conducted following fixation and permeabilization with 0.1% saponin (BDH, Poole, U.K.).

Cloning of the human TLR2 cDNA

Total RNA was extracted from the Mono Mac-6 cell line using the TRIzol reagent (Invitrogen, Paisley, U.K.) following the manufacturer's instructions. RNA (3 μg) was reverse transcribed using either random hexamers (pdN$_6$, 150 pmol) or 40 pmol of a poly(dT) adaptor primer (25). All PCR were conducted in an OmniGene thermal cycler (Hybaid, Middlesex, U.K.) using a mixture of pdN$_6$ (2 μl) and poly(dT) (2 μl)-primed reverse-transcribed RNA or plasmid DNA (100 ng), as previously described (25). The primers used to amplify the full-size TLR2 cDNA were: T2 77, 5′-AGGTAC CTGTGGGGCTCATT-3′ (sense, −77 to −58), and T2 2374, 5′-GCCG GATCCCAAAGATCCCAACTAGACAAAGACT-3′ (antisense, 2374–2397; *Bam*HI site is underlined). Following PCR, the purified DNA was ligated into the pCR II-TOPO cloning vector (TOPO TA cloning kit; Invitrogen). An aliquot was used to electroporetically transform DH5 bacteria. Positive bacterial colonies were expanded, and the plasmid was purified using the QIAprep spin plasmid kit (Qiagen, Valencia, CA). The presence and fidelity of the human TLR2 cDNA were confirmed by in-house DNA sequencing of 12 separate clones (ABI Prism Big Dye sequencing kit, PerkinElmer, Beaconsfield, U.K; ABI 377 DNA sequencer, Applied Biosystems, Warrington, U.K.). For Southern blot analysis of PCR products, a probe was generated by using a fragment encompassing bases 1–1257 of the TLR2 cDNA, which was isolated, [α-^{32}P]dCTP labeled (*Redi*prime II random primer labeling system; Amersham Pharmacia), and purified, as described (25). For constructing the eukaryotic expression vector, the human TLR2 open reading frame cDNA cloned into pCR II-TOPO vector was cloned in pDR2ΔEF1α, as described (25). Plasmids from positive bacterial colonies were amplified in preparation for transfection in CHO or human embryonic kidney (HEK)-293 cells.

Construction of a c-myc-tagged TLR2 cDNA and a Fc-sTLR2 fusion protein

Two oligonucleotides were designed encoding part of the TLR2 protein (aa 19–24, KEESSN) in addition to the c-Myc epitope EQKLISEEDL: sense, 5′-CCAAGGAAGAATCCTCCAATGAACAAAAACTCATCTCAGAA GAGGATCTGT-3′, and antisense, 5′-GACAGATCCTCTTCTGAGAT GAGTTTTTGTTCATTGGAGGATTCTTCCTTG-3′ (c-*myc* epitope is underlined). The oligonucleotides were phosphorylated (T4 polynucleotide kinase; Amersham) and purified (Strataclean resin; Stratagene, La Jolla, CA). Equimolar amounts (300 pmol) of each oligonucleotide were mixed, denatured (5 min, 95°C), warmed (80°C), and annealed. The pCRII-TOPO vector containing the full-length TLR2 cDNA was digested with *Eco*NI (New England Biolabs, Hitchin, U.K.), dephosphorylated, and purified (Geneclean III DNA purification kit; Anachem, Luton, U.K.). The phosphorylated c-*myc*-TLR2 double-stranded oligonucleotide was ligated into the pCRII-TOPO-TLR2 vector. Positive colonies were amplified in preparation for transfection into CHO cells.

A Fc-sTLR2 fusion protein was constructed to allow expression of a soluble chimeric human TLR2 encompassing the full extracellular domain (aa residues 1–586) with a C-terminal Fc tail (26). The L6 expression vector construct consisting of the human IgG4 Fc inserted in pDR2ΔEF1α and the control fusion protein Fc-CD46 were kindly provided by C. Harris (Department of Medical Biochemistry, University of Wales, College of Medicine, Cardiff, U.K.). The plasmid pCRII-TOPO-TLR2 cDNA was used as a template to generate a PCR fragment containing the TLR2 sequence encompassing bases 1–1758, corresponding to aa 1–586, by using the plasmid-derived primer 5′-GTAATACGACTCACTATAGGGCGAA-3′ and primer 5′-**CGCGGATCCGAGGGGGCCTTGAAACAGAACTTC TAA**GTGACATTCCGACACCGAGAGG-3′ (underlined and bold type are the *Bam*HI and PreScission-Amersham-protease cleavage sites, respectively). The PCR product was cloned in the L6 vector. Positive colonies were amplified before transfection into HEK-293 cells.

Cell transfections

Chinese hamster ovary (CHO) and HEK-293 cells, maintained in complete medium (RPMI 1640, 10% FCS, 2 mM glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin), were transfected with the plasmid DNA expression vector constructs (6 μg) using the LipofectAMINE PLUS reagent (Invitrogen) and following the manufacturer's instructions. TLR2 cell transfectants were selected 24 h posttransfection by supplementing the medium with 400 μg/ml hygromycin B. Three weeks posttransfection, TLR2 cell surface expression was tested by FACS, and the transfectants were immunomagnetically sorted to ≥96% purity (Dynal, Oslo, Norway) using the TL2.1 mAb. Expression of the Fc-sTLR2 fusion protein by HEK-293 cells was confirmed by Western blotting of culture supernatants using an anti-TLR2-specific Ab. A major ~110-kDa polypeptide band consistent with the expected electrophoretic mobility of the Fc-sTLR2 under reducing conditions was detected. In preliminary transfection experiments (data not

shown), we confirmed that HEK-293 cells are capable of expressing recombinant cell surface TLR2. However, we did not detect sTLR2 production by the 293-TLR2 transfectants, indicating that posttranslational processing of the full-length protein does not take place in HEK-293 cells.

Molecular modeling

Three-dimensional position-specific score matrix (PSSM) fold recognition method was used to deduce the three-dimensional structure of the CD14 and TLR2 leucine-rich repeat (LRR) domains (27). By this method, several three-dimensional structures of LRR-containing proteins, solved by x-ray crystallography, were identified as a diverse set of templates for modeling LRR-containing proteins (28). The best structural template found for both the CD14 and TLR2 LRR was the LRRs of *Yersinia pestis* outer membrane protein M (29) (CD14 LRR: PSSM E-value, 0.0688; 90% certainty; protein database (PDB) entry 1jl5, chain A. TLR2 LRR: PSSM E-value, 0.0135; 90% certainty; PDB entry 1jl5, chain A). Accordingly, the possible interaction between the LRRs of TLR2 and CD14 was assessed by performing computational molecular docking experiments based on shape complementarity and nonbonded interaction (Van der Walls) terms (30). The nonbonded interactions were computed by using optimized potentials for liquid simulation force-field parameters (31). Molecular docking experiments were also performed to study the interaction of the synthetic lipopeptide Pam$_3$Cys with the supramolecular structure predicted by the CD14 LRR-TLR2 LRR interaction studies.

Results

Detection of sTLR2 polypeptides in normal human plasma

Western blot analysis of normal human plasma samples ($n = 8$) using four different TLR2-specific Abs (IMG 410, sc8689, TLR2p, TLR2PM) showed in each case a major polypeptide band of ~66 kDa and additional polypeptides of 83, 40, 38, and 25 kDa (Fig. 1A, sc8689 shown). The specificity of the detection was confirmed by performing peptide competition experiments by immunoblotting. Preincubation of the anti-TLR2 Ab sc8689 with the peptide used for immunization abrogated, or substantially reduced, the detection of most of the polypeptides (Fig. 1A).

Cellular origin of plasma sTLR2

Because blood monocytes express the highest levels of TLR2 (32, 33), we speculated that they may be the source of plasma sTLR2. To address this issue, we tested for sTLR2 in the culture supernatant of freshly isolated monocytes. Immunoprecipitation followed by Western blotting showed a sTLR2 pattern similar to that in plasma (Fig. 1B). The monocyte-derived sTLR2 pattern showed, however, an additional 70-kDa polypeptide band. Experiments conducted with tonsillar B cells, B cell lines, and peripheral blood T cells failed to demonstrate the release of sTLR2 by these cells (data not shown). These findings thus suggested that blood monocytes may be the main source of plasma sTLR2.

Modulation of sTLR2 release

We asked whether cell activation modulates the release of sTLR2. Analysis of culture supernatants of nonstimulated monocytes showed constitutive release of sTLR2 (Fig. 1C). The amount of sTLR2 accumulated over time, but all the sTLR2 polypeptides were detectable only after 18 h of culture. PMA + ionomycin treatment, however, increased the kinetics of sTLR2 release, because all the sTLR2 polypeptides were detectable after just 30 min of culture (Fig. 1C). The effect of a TLR2-specific ligand, the synthetic bacterial lipopeptide Pam$_3$Cys, was also tested. In this study, we used an anti-TLR2 mAb (IMG-319) that preferentially recognized the three higher M_r sTLR2 polypeptides and found that just 1 h of treatment of Mono Mac-6 monocytes with Pam$_3$Cys induced the release of even higher levels of the major sTLR2 polypeptides than those released by PMA + ionomycin (Fig. 1D). To evaluate the effect of an in vivo exposure to TLR2 as well as TLR4 ligands on sTLR2 release, we tested for sTLR2 in serum samples from pulmonary tuberculosis patients. Notably, sTLR2
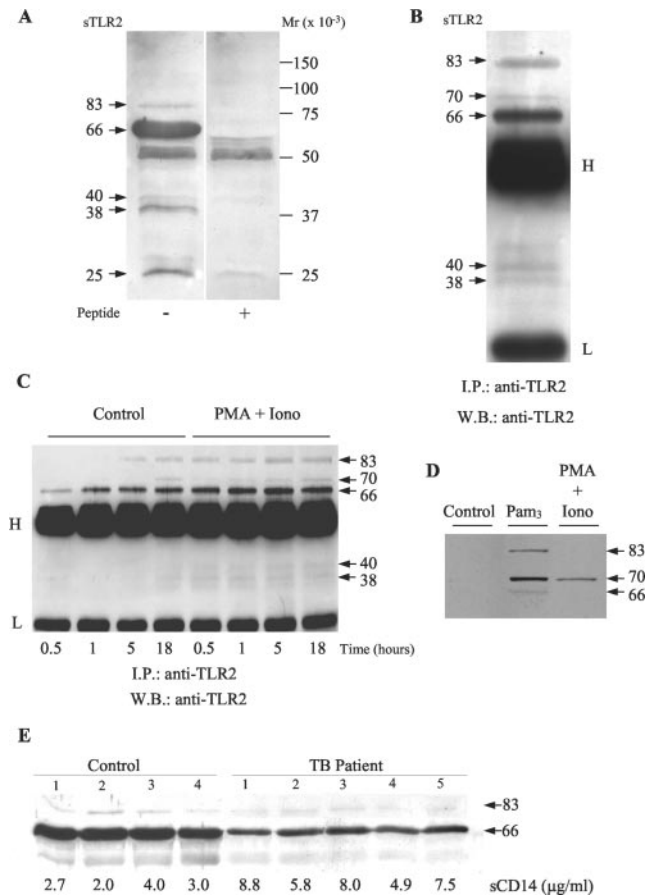
FIGURE 1. sTLR2 detection in normal human plasma and release by human monocytes. *A*, sTLR2 polypeptide pattern in normal human plasma (1/300 dilution) by Western blotting. The specificity of the detection was confirmed by performing peptide competition by immunoblotting. The anti-TLR2 Ab sc8689 was preincubated (+) or not (−) with 5× mass excess of the peptide used for immunization. Shown is a result representative of plasma from eight donors. *B*, Detection of sTLR2 polypeptides in 18-h culture supernatants of freshly isolated monocytes. Immunoprecipitations followed by Western blotting were performed with the sc8689 Ab. H and L: Ig H and L chains. The molecular mass (kDa) of the sTLR2 polypeptides is indicated. *C*, Kinetics of release of sTLR2 polypeptides by normal human monocytes nonactivated (control) or activated with 50 ng/ml PMA + 500 ng/ml ionomycin for the times indicated in the figure. The sc8689 Ab was used for immunoprecipitations and Western blots. *D*, Western blot analysis of sTLR2 released by Mono Mac-6 monocytes stimulated for 60 min with 10 μg/ml of the synthetic bacterial lipopeptide Pam$_3$Cys or 50 ng/ml PMA + 500 ng/ml ionomycin. sTLR2 detection was with the IMG-319 mAb. *E*, Down-modulation of serum sTLR2, evaluated by Western blot (anti-TLR2 peptide Ab TLR2p), and increased levels of sCD14 in pulmonary tuberculosis (TB) patients as compared with healthy controls.

levels were found reduced as compared with those in healthy controls (Fig. 1*E*). This was in contrast to the serum sCD14 concentrations, which were higher in the tuberculosis patients than in controls, as we previously reported (21).

Molecular origin of sTLR2

We then asked how sTLR2 originates. We tested the possibility of a posttranslational modification of the TLR2 molecule, i.e., conversion of cell membrane-bound TLR2 into sTLR2. To this aim, cell aliquots from the experiment on the kinetics of release of sTLR2 described above and in Fig. 1*C* were taken at 15, 30, and 60 min, and tested for TLR2 cell surface expression by FACS (Fig.

2*A*). PMA + ionomycin treatment induced a progressive down-modulation of cell surface TLR2 over the 60-min period. This relatively fast down-modulation correlated with the activation-induced faster kinetics of sTLR2 release described in Fig. 1*C*.

To further explore the possible conversion of cell surface TLR2 into sTLR2, Mono Mac-6 cells or freshly isolated monocytes (data not shown) were surface biotinylated, and the culture supernatants were tested for sTLR2 release by immunoprecipitation, followed by Western blotting with peroxidase-conjugated streptavidin (Fig. 2*B*). The three major sTLR2 polypeptides, 83, 70, and 66 kDa, were found biotinylated in the cell culture supernatants. In addition, a ~48-kDa biotinylated protein, whose identity remains to be confirmed, was consistently coimmunoprecipitated with sTLR2 (see below). Taken together, these results strongly suggested that sTLR2 originates by conversion of cell surface TLR2.
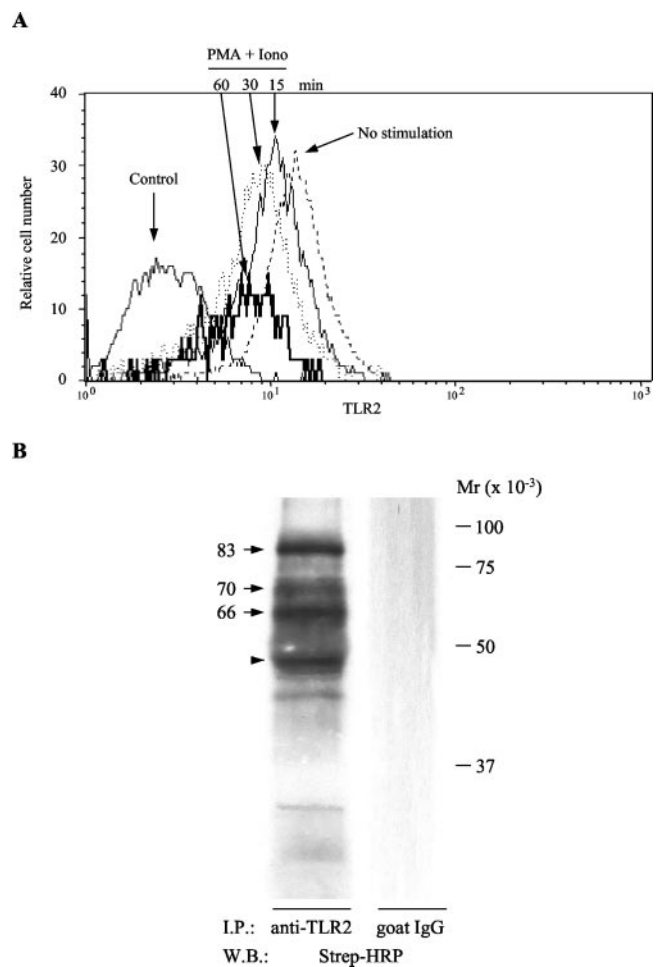

FIGURE 2. Activation-induced down-modulation of cell surface TLR2 and detection of biotinylated sTLR2. *A*, Cell aliquots of the experiment described in Fig. 1*C* were taken at the indicated time points and tested for TLR2 cell surface expression by FACS using the TL2.1 mAb. TLR2 expression in nonstimulated cells was tested after 60 min of culture. Control profile corresponds to the staining with the isotype-matched control Ab. Similar results were obtained from three monocyte preparations tested. *B*, Detection of biotinylated sTLR2 polypeptides in 18-h culture supernatants of cell surface-biotinylated Mono Mac-6 cells (10×10^6 cells) following immunoprecipitation with the anti-TLR2 peptide Ab sc8689 (goat) and detection by Western blotting with HRP-conjugated streptavidin. The arrowhead points at a ~48-kDa polypeptide band that remains to be identified (see text).

sTLR2 can be detected following expression of the human TLR2 cDNA

To confirm that processing of the full-length TLR2 molecule, most likely by proteolytic cleavage, is the mechanism of sTLR2 production, we RT-PCR amplified the human TLR2 cDNA obtained following RNA extraction from Mono Mac-6 monocytes (Fig. 3*A*) as well as from freshly isolated monocytes (data not shown). Analysis of the RT-PCR products and the corresponding Southern blots indicated the presence of a single TLR2 cDNA of ∼2.5 kb. Cloning and sequencing confirmed that this cDNA corresponded to that coding for the full transmembrane TLR2 protein (EMBL U88878; Swissprot 060603). No indication of splice variants, deletions, or mutations of the TLR2 gene was apparent. Subsequently, CHO cells were stably transfected with the cloned 2.5-kb TLR2 cDNA or an N-terminal *myc* epitope-tagged version of the 2.5-kb TLR2 cDNA, and their culture supernatants were tested for sTLR2 or Myc-sTLR2, respectively (Fig. 3*B*). FACS analysis confirmed the cell surface expression of TLR2 and Myc-TLR2 in the cell transfectants (Fig. 3*B*, *upper panel*). The Western blot analysis of the CHO-TLR2/Myc culture supernatants (Fig. 3*B*, *lower panel*) re-vealed a Myc-tagged polypeptide pattern of overall similarity to that observed when human plasma and monocyte cell culture supernatants were tested with anti-TLR2 Abs (Fig. 1). However, some of the Myc-TLR2 polypeptide bands showed slightly different electrophoretic mobility from, or were more or less intense than their plasma- or monocyte-derived counterparts, most likely reflecting the influence of the Myc epitope in the processing as well as a slightly different processing of TLR2 in the hamster cells. CHO-TLR2 culture supernatants also showed sTLR2 polypeptides (Fig. 3*B*). In this study, the 40- and 83-kDa sTLR2 were of a lower intensity. In experiments not shown in this study, we found that PMA + ionomycin modulated the release of sTLR2 by CHO-TLR2 cells in a similar manner to that described in Fig. 1*C* for the monocyte-derived sTLR2.

Overall, the data in Figs. 1–3 confirmed not only that the soluble polypeptides detected in plasma and the monocyte culture supernatants were bona fide TLR2 derived, but also that sTLR2 results from the posttranslational modification of the transmembrane receptor.

FIGURE 3. sTLR2 results from the posttranslational modification of the TLR2 protein. *A*, RT-PCR (3 μg RNA), followed by Southern blot analysis of human TLR2 transcripts obtained following total RNA extraction from Mono Mac-6 monocytes. The primers used to amplify the full-size human TLR2 cDNA were T2 77 (sense, −77 to −58) and T2 2374 (antisense, 2374 to 2397), and are described in *Materials and Methods*. Single primer PCR controls are shown. For Southern blotting, a [α-³²P]dCTP-labeled 1257-bp DNA probe encompassing bases 1–1257 of the TLR2 cDNA was generated and used as described in *Materials and Methods*. Arrow points at the single 2.5-kb cDNA band detected, corresponding to the full-size human TLR2 cDNA (GenBank accession U88878). *B*, *Upper panel*, Cell surface expression level of Myc-TLR2 and TLR2 in the CHO transfectants used for Western blots. *Lower panel*, TLR2 pattern by Western blot of serum-free culture supernatants collected after a 10-h culture of 5 × 10⁶ CHO cells expressing the N-terminal *myc*-tagged human TLR2 cDNA (Myc-sTLR2), the nontagged TLR2 cDNA (sTLR2), or mock-transfected CHO cells. Membranes were probed with the anti-c-Myc epitope mAb clone 9E10 or the anti-TLR2 Ab TLR2p, as indicated.
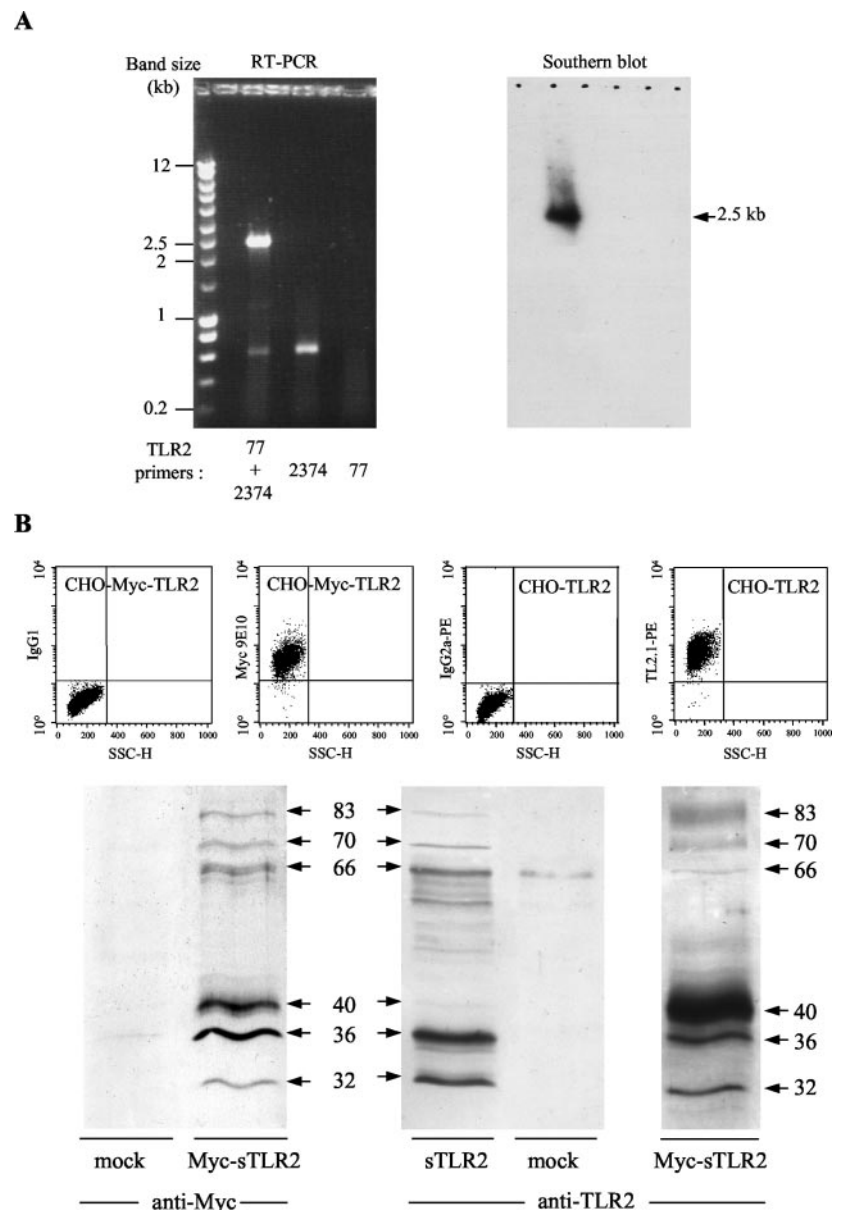
Monensin affects the release of sTLR2

To address the question of whether the processing of the full-size transmembrane TLR2 is a cell surface or an intracellular event, the antibiotic monensin was used. It is known that monensin blocks processing events that take place in internal, acidic compartments, i.e., Golgi apparatus, cisternae, lysosomes, and endosomes, without affecting endocytosis of cell surface molecules or activities that take place at the cell surface (34). Analysis of sTLR2 released by CHO-TLR2 transfectants in the presence of monensin showed accumulation of the 66-kDa sTLR2 polypeptide after 90 min, as compared with control cultures (Fig. 4A). Even after 18 h of chase in the presence of monensin, complete processing of sTLR2, i.e., a substantial accumulation of the lower M_r sTLR2 polypeptides, was not observed. By contrast, monensin treatment induced a detectable increase in the level of the 83- and 70-kDa molecular species over the 18-h period. These results indicated that monensin affects TLR2 processing, thus suggesting that the latter is an intracellular event.

sTLR2 is present in an intracellular pool

To obtain further insight into the origin of sTLR2, we evaluated TLR2 staining in permeabilized and nonpermeabilized Mono Mac-6 monocytes. Following permeabilization, a substantial increase in TLR2 staining was observed (Fig. 4B), suggesting that a significant proportion of the TLR2 protein resides inside the cell, as was previously reported (32, 35). We asked whether sTLR2 contributes to this internal pool. We compared the expression pattern of TLR2 in the total cell lysate of Mono Mac-6 monocytes with that of the higher M_r sTLR2 polypeptides in the corresponding culture supernatant by using Western blotting with the IMG-319 mAb. Three polypeptides showing electrophoretic mobilities consistent with those of 83-, 70-, and 66-kDa sTLR2 were detected in the monocyte cell lysate (Fig. 4C). In addition, the cell lysate showed a strong ~110-kDa TLR2 band consistent with the M_r previously reported for the full-size TLR2 glycoprotein (33). Similar results were obtained by using freshly isolated monocytes
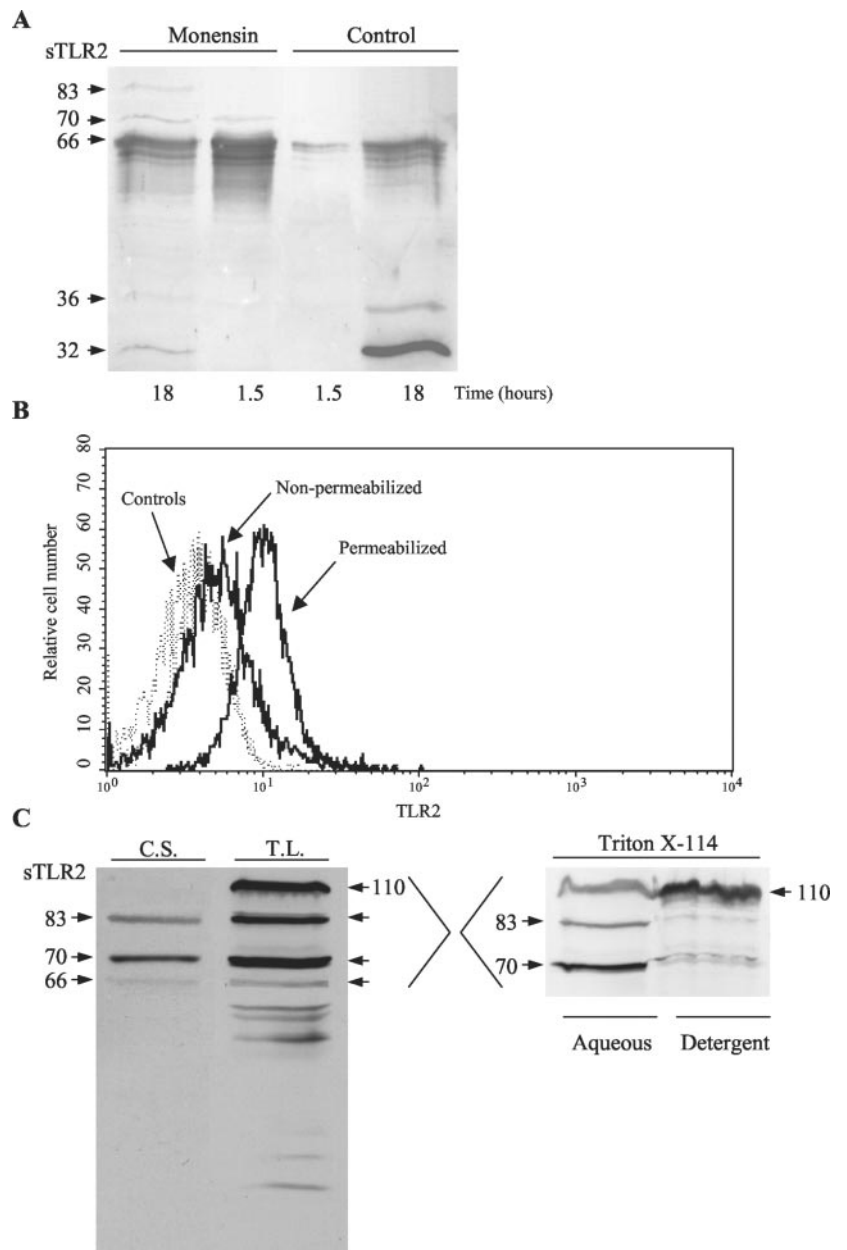

FIGURE 4. Processing and intracellular storage of sTLR2. *A*, Stable CHO transfectants expressing the human TLR2 cDNA were serum free cultured (1.2 × 10⁶ cells/1.5 ml) in the absence or presence of 10 μM monensin. At the indicated time points, the culture supernatants were tested for sTLR2 by Western blotting using the anti-TLR2 peptide Ab TLR2p. *B*, Fluorescence profiles of TLR2 in Mono Mac-6 cells stained with the PE-conjugated TL2.1 mAb before and after cell permeabilization. Dashed and dotted profiles correspond to the staining with the isotype-matched control Ab in nonpermeabilized and permeabilized cells, respectively. *C*, Comparison of the pattern of TLR2 in Mono Mac-6 total cell lysate (T.L.) with that of sTLR2 in the corresponding culture supernatant (C.S.) by Western blotting using the IMG-319 mAb. Following Triton X-114 detergent partitioning of the total cellular proteins (*right inset*), the 83- and 70-kDa polypeptides were found enriched in the aqueous phase, whereas 110-kDa TLR2 was concentrated in the detergent phase.
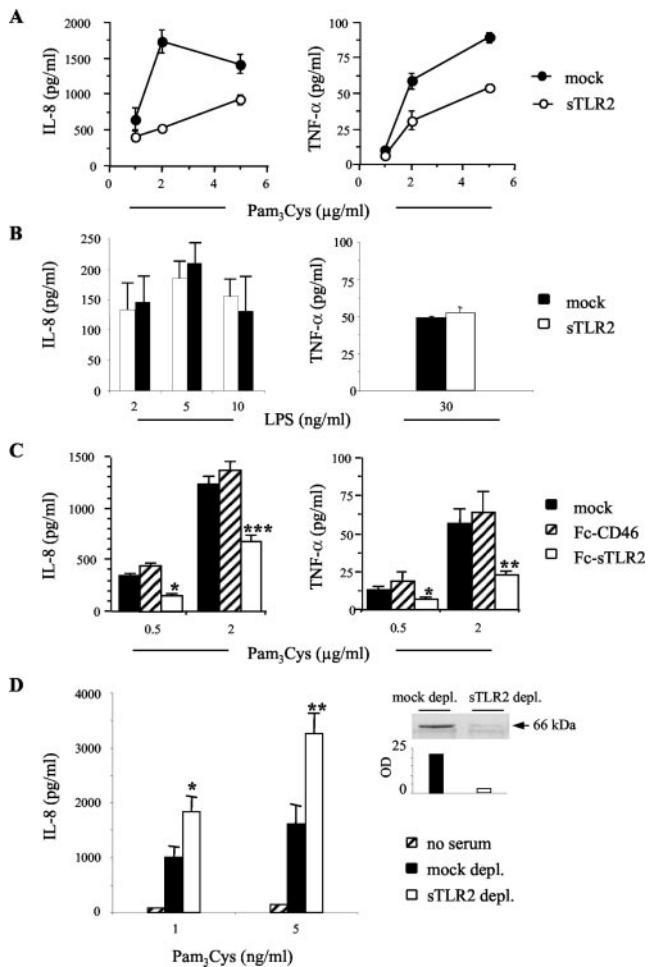
FIGURE 5. sTLR2 inhibits cell activation. IL-8 and TNF-α production by Mono Mac-6 cells (5×10^4) cultured in the presence of sTLR2 (*A* and *B*) or Fc-sTLR2 (*C*) containing serum-free culture supernatants, or those from mock-transfected cells or the control fusion protein Fc-CD46. The cells were stimulated for 5 h with the indicated amounts of the synthetic bacterial lipopeptide Pam$_3$Cys. In control experiments (*B*), cells were stimulated with LPS. *D*, PBMC (2×10^5 cells) were stimulated with the indicated concentrations of Pam$_3$Cys in the absence or presence of 2% serum depleted of sTLR2 or mock depleted (anti-plexin-C1-treated serum). *Upper right inset*, Shows the extent of the sTLR2 depletion (92%) of the serum used in the experiment shown here. Cytokines were tested by ELISA. Results are means ± SD of triplicate cultures of one experiment representative of three (*A*, *B*, and *D*) or four (*C*). *C*, The differences in cytokine release between mock and Fc-sTLR2 cultures were compared using Student's *t* test: *, $p < 0.005$; **, $p < 0.001$; ***, $p < 0.0001$. *D*, The difference in IL-8 levels between PBMC cultured in mock- and sTLR2-depleted serum was significant: *, $p < 0.005$; **, $p < 0.001$.

(data not shown). Notably, Triton X-114 detergent partitioning of the total cellular proteins demonstrated an enrichment of the 83- and 70-kDa TLR2 polypeptides in the aqueous phase, indicating that these were soluble proteins (Fig. 4*C*, *right inset*). The 110-kDa TLR2 band was found enriched in the detergent phase, consistent with this being the membrane-associated receptor. The 66-kDa TLR2 band was barely detectable following detergent partitioning, most likely due to its initial lower intensity. These findings indicated that monocytes contain a substantial intracellular pool of sTLR2 as well as the full-length TLR2 molecule.

sTLR2 and Fc-sTLR2 inhibit lipopeptide-induced cell activation

To evaluate the functional consequences of the existence of sTLR2, we tested the Pam$_3$Cys bacterial lipopeptide-induced IL-8

and TNF-α production by Mono Mac-6 monocytes that had been stimulated in the presence of sTLR2-containing serum-free culture supernatants or those from mock-transfected cells (Fig. 5). Two sources of sTLR2 were used: CHO-TLR2 transfectants and Fc-sTLR2-producing HEK-293 cells. The Fc-sTLR2 fusion protein consisted of the full extracellular domain of human TLR2. In this way, we secured the consistent release of a single sTLR2 molecular species (see *Materials and Methods*) similar to 83-kDa sTLR2, the highest M_r sTLR2 detected in plasma and monocyte culture supernatants. In the presence of sTLR2, IL-8 and TNF-α production induced by different Pam$_3$Cys concentrations was consistently lower (Fig. 5*A*). Notably, sTLR2 did not affect cell stimulation induced by LPS (Fig. 5*B*). Lower levels of IL-8 and TNF-α were also found in the culture supernatants of cells stimulated with Pam$_3$Cys in the presence of Fc-sTLR2, but not in the presence of the irrelevant fusion protein Fc-CD46 (Fig. 5*C*).

To test the biological significance of the existence of sTLR2 forms capable of modulating cell activation, we examined the sensitivity of PBMC to stimulation by bacterial lipopeptide in the presence of serum depleted of sTLR2. Fig. 5*D* shows that reducing the amount of sTLR2 in serum increased significantly cell sensitivity to Pam$_3$Cys.

Detection of sTLR2 polypeptides in human breast milk

To further evaluate the biological relevance of the existence of sTLR2 forms, we tested for their presence in human breast milk.
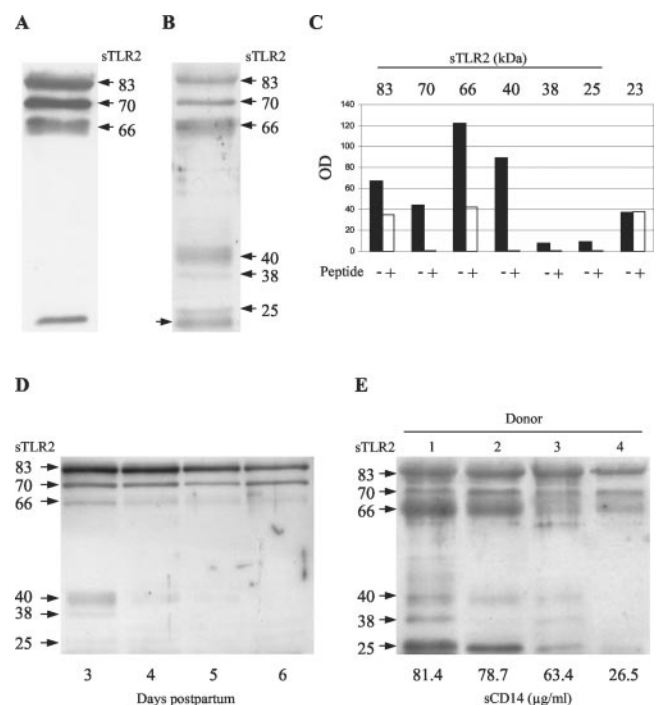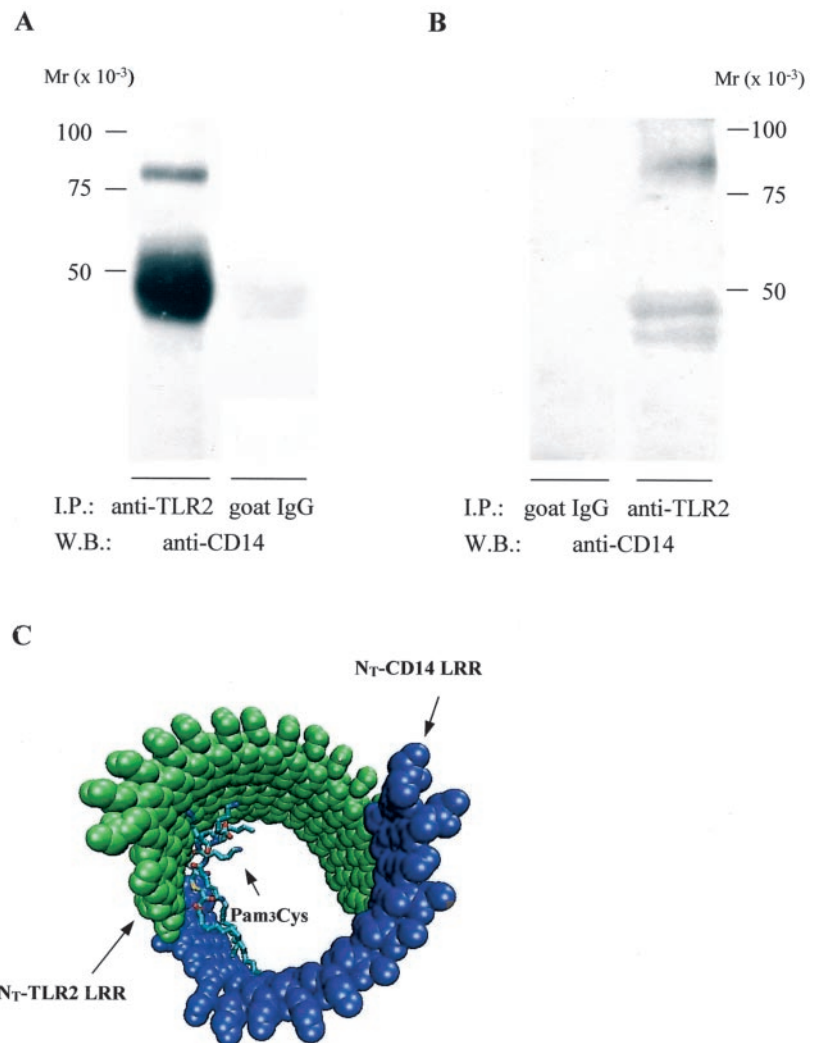

FIGURE 6. Detection of sTLR2 in human breast milk. *A* and *B*, Representative sTLR2 polypeptide patterns in milk samples ($n = 32$) by Western blot using the anti-human TLR2-specific Ab IMG-410 (*A*) or sc8689 (*B*). sc8689 Ab recognizes an N-terminal peptide of TLR2. Arrows indicate the molecular mass (kDa) of the sTLR2 polypeptides. In *B*, arrow at the *bottom left* points at a nonspecific polypeptide band (~23 kDa), as indicated in *C* by the lack of peptide competition. *C*, Densitometric scanning analysis of Western blots of milk samples by using the anti-TLR2 Ab sc8689 preincubated (+) or not (−) with the peptide used for immunization. The peptide competition shown is representative of four. *D*, Declining levels of sTLR2 polypeptides in milk samples over time postpartum as evaluated by Western blotting. Results are from one donor representative of three. *E*, Correlation of sTLR2 levels (sc8689 Ab) with the sCD14 concentration in the same milk sample. Results shown are from samples of four different donors.

A

Mr (x 10^{-3})

100 —

75 —

50 —

FIGURE 7. sTLR2 interacts with sCD14. sTLR2 was immunoprecipitated from human breast milk (*A*) or plasma (*B*) samples with the anti-TLR2 Ab sc8689 (goat). The immunoprecipitates were analyzed by Western blotting with a rabbit anti-CD14 Ab. Results are representative of two obtained by using milk and plasma samples from two different donors. *C,* Molecular modeling of the interaction between the LRR domains of TLR2 (green) and those of CD14 (blue) as predicted by computational molecular docking experiments. A cylindrical supramolecular arrangement was deduced as the best assembly mode. Only the interior, hydrophobic part of the complex is shown. Within this predicted central hydrophobic pocket, bacterial lipopeptide may dock near to the N terminus (N$_T$) end of the TLR2 LRR domains, stabilizing further a putative sTLR2-sCD14 interaction. The docking experiments indicate a C terminus-N terminus assembly of the two molecules.

I.P.: anti-TLR2 goat IgG
W.B.: anti-CD14

B

Mr (x 10^{-3})

—100

— 75

— 50

I.P.: goat IgG anti-TLR2
W.B.: anti-CD14

C

N$_T$-CD14 LRR

Pam3Cys

N$_T$-TLR2 LRR

We reasoned that, due to the expression of TLR4 and TLR2 by fetal enterocytes and the latter's hypersensitivity to LPS (36, 37) as well as the high levels of sCD14 in milk (16, 17), the milk sCD14-mediated microbial recognition in the neonatal gut must be regulated to avoid excessive local inflammation.

Western blot analysis of human breast milk samples (*n* = 32) using the anti-TLR2 Ab IMG-410, IMG-319, and TLR2PM showed in each case the three high M_r sTLR2 polypeptide bands of 83, 70, and 66 kDa (Fig. 6*A*, IMG-410 shown). The two anti-N terminus TLR2 peptide Abs sc8689 and TLR2p allowed the detection of the lower M_r polypeptides of ~40, 38, and 25 kDa (Fig. 6*B*, sc8689 shown). The specificity of the detection was confirmed by performing peptide competition experiments by immunoblotting (Fig. 6*C*). An additional ~23-kDa band (Fig. 6*B*) did not show competition (Fig. 6*C*), indicating the nonspecific nature of this band. The amount of sTLR2 in milk, although variable between donors, decreased over time postpartum (Fig. 6*D*), and mirrored the level of sCD14 in the same sample (Fig. 6*E*).

Interaction of sTLR2 and sCD14: milk and plasma coimmunoprecipitations and computational molecular docking studies

We asked whether sTLR2 interacts with sCD14 in milk and plasma. The analysis of milk sTLR2 immunoprecipitates by Western blotting using an anti-CD14-specific Ab, revealed the presence of the previously reported typical ~48-kDa milk sCD14 polypep-

tide band (16), as well as an as yet nonidentified ~80-kDa structure (Fig. 7*A*). Similarly, the typical plasma sCD14 polypeptide pattern under nonreducing electrophoretic conditions (48–50 kDa) (13) was detected in the sTLR2 immunoprecipitates from plasma samples (Fig. 7*B*). These results indicated an interaction between the natural forms of sTLR2 and sCD14. Moreover, it suggested that the ~48-kDa biotinylated protein coprecipitated with biotinylated sTLR2 from monocyte culture supernatants and shown in Fig. 2*B* may correspond to sCD14, because we previously demonstrated that one of the two sCD14 forms, sCD14α (48 kDa), may originate by conversion of cell membrane CD14 (14).

An insight into the way sTLR2 and sCD14 interact was obtained by performing computational molecular docking studies. The results of the simulated interaction of the LRR domains of TLR2 and CD14 indicated that sTLR2 and sCD14 may form a stable heterodimer (Fig. 7*C*). The model predicts that the divalent cations Ca^{2+} and Mg^{2+} are critical to the stable formation of such dimers. Furthermore, the deduced quaternary structure of the heterodimers, based on the arrangements of the LRR domains, indicates as the best assembly mode a cylindrical supramolecular structure, thus predicting the existence of a central, hydrophobic pocket in which bacterial lipopeptides or lipopolysaccharides may dock, stabilizing further the putative sTLR2-sCD14 interaction. Notably, the docking experiments showed that Pam$_3$Cys can dock near to the C terminus of the CD14 LRRs and in the vicinity of the N terminus of the TLR2 LRRs.

Discussion

In this study, we present experimental evidence demonstrating the existence of a natural soluble form of one of the innate immune receptors critically involved in the recognition of, and response to, a broad range of microbial components, namely TLR2. We also demonstrate that sTLR2 is capable of modulating cell activation by bacterial lipopeptide. Notably, we show that sTLR2 occurs naturally in the first source of nutrients for the newborn, human breast milk, as well as in normal human plasma, and that depletion of sTLR2 from serum increases cell sensitivity to lipopeptide. Furthermore, we demonstrate that sTLR2 release is modulated in vitro and in vivo by cell activation and mycobacterial infection, respectively, and that sTLR2 and the TLR coreceptor sCD14 may interact in their natural milieu.

Up to six sTLR2 polypeptides were detected in human milk, plasma, and the monocyte culture supernatants. Interestingly, two anti-TLR2 Abs capable of detecting the six sTLR2 polypeptides recognize peptides located at the N terminus of the TLR2 protein, thus indicating that processing of TLR2 proceeds from the C to the N terminus. Estimations based on the published amino acid sequence of human TLR2 indicate a molecular mass for the extracellular domain of ~64 kDa, with N-glycosylation of the four acceptor sequences increasing the M_r up to ~84,000. We therefore speculate that the 83-kDa sTLR2 polypeptide may correspond to the full extracellular domain, and that the lower M_r sTLR2 polypeptides may originate by further proteolytic processing of the 83-kDa molecular species. The level of each sTLR2 polypeptide may reflect a different processing of the TLR2 protein in the cell type source of sTLR2, as well as a different stability ($t_{1/2}$) of each soluble molecular species. These factors may explain the presence of 83- and 70-kDa sTLR2 in milk and their very low levels or absence in plasma. It is possible that the milk 83- and 70-kDa molecular species originate in the mammary gland epithelial cells, which we found to produce 83- and 70-kDa sTLR2 (our unpublished observations). By contrast, the abundance of 66-kDa sTLR2 in plasma may reflect the fact that this is the main soluble TLR2 polypeptide released by the blood monocytes (see Fig. 1B), which are most likely to be the main source of plasma sTLR2. At present, we cannot, however, exclude the possibility that the adipocytes surrounding the mammary ducts and alveoli also contribute to the pool of milk sTLR2, because their capacity to express TLR2 has been demonstrated (38). Similarly, plasma sTLR2 may originate not only from monocytes, but from the contribution of additional blood leukocytes, neutrophils in particular, because they express TLR2, albeit less than monocytes (39).

Monensin, an antibiotic known to interfere with intracellular processing, affected the release of sTLR2, thus suggesting that processing of TLR2 is an intracellular event. This finding, together with the detection of biotinylated 83-, 70-, and 66-kDa sTLR2 in the culture supernatant of cell surface-labeled monocytes (Fig. 2), and the existence of a substantial intracellular pool of sTLR2 (Fig. 4), is consistent with a mechanism of sTLR2 production involving: 1) endocytosis of the cell surface receptor, 2) its conversion into sTLR2 by processing in an internal acidic compartment, and 3) the subsequent release of sTLR2 by exocytosis, or 4) its retention in an intracellular pool. A similar mechanism has been proposed for the conversion of cell surface CD14 into sCD14 (14).

Cell activation resulted in the rapid down-modulation of cell surface TLR2 and the similarly fast release of sTLR2. Indeed, we have detected increased release of sTLR2 after just 10 min of monocyte stimulation by lipopeptide (our unpublished observations). This response to cell activation may serve two purposes that are not mutually exclusive: 1) to terminate or reduce the ligand-induced triggering of the cell surface receptor through its down-modulation, thus avoiding an excessive proinflammatory response, as was reported for TLR4 (40); 2) to release a soluble receptor that may have an intrinsic biological function, as has been reported for a number of other soluble receptors (19, 20). The presence of sTLR2 in human plasma and breast milk prompted us to test the latter possibility.

In contrast to the up-modulation of sTLR2 following cell activation in vitro, in vivo exposure to *Mycobacterium tuberculosis*, a microorganism recognized at least in part by TLR2 as well as TLR4, resulted in a marked down-modulation of serum sTLR2 (Fig. 1E). Further experimentation will be needed to determine the mechanism underlying this down-modulation of sTLR2. Nevertheless, in view of these findings, it will be of interest to explore the prognostic value of variations of serum sTLR2 concentrations in tuberculosis as well as in other infectious diseases.

The functional relevance of the existence of sTLR2 was supported by the inhibitory effect of sTLR2 and the TLR2 extracellular domain on cytokine production by lipopeptide-stimulated monocytes. The use of cell transfectants expressing the Fc-sTLR2 fusion protein not only enabled the testing of the activity of a single sTLR2 molecular species, but also secured dimerization of the soluble receptor. It is possible that sTLR2 dimerizes for an efficient modulatory activity, because the capacity of cell surface TLR2 to homodimerize or heterodimerize has been demonstrated (33, 41, 42). The physiological significance of the modulatory capacity of sTLR2 was indicated by the observation (Fig. 5D) that PBMC sensitivity to lipopeptide increases when the amount of sTLR2 in serum decreases. This finding suggests that serum sTLR2 may contribute to the regulation, and thus the efficiency, of the innate immune response to microbial pathogens.

The mechanism underlying the modulatory effect of sTLR2 reported in this study remains to be elucidated. However, it is possible that sTLR2 exerts its inhibitory activity by interacting with sCD14, as demonstrated in this work, and/or with cell membrane-bound CD14, thus interfering with the CD14-mediated triggering of cell membrane TLR2 by lipopeptide. Alternatively, sTLR2 may homodimerize with cell surface TLR2 or act as a decoy receptor by binding to the microbial components recognized by TLR2, thus reducing the efficiency of TLR2 signaling. We have considered the possibility that the inhibitory effect of sTLR2 was due to the induction of cellular apoptosis, as demonstrated for cell surface TLR2 (43). However, our preliminary results do not support this possibility. Interestingly, a splice variant of the mouse TLR4 mRNA, which coded for a putative partially secreted 20-kDa protein, has recently been described (44). Introduction of this splice variant mRNA in a mouse macrophage cell line rendered these cells less sensitive to LPS. It remains to be established, however, whether this putative soluble TLR4 protein is naturally expressed and spontaneously released by normal mouse macrophages. Clarification of the exact molecular mechanism by which TLRs recognize bacterial products will help us to understand the inhibitory activity of sTLR2 shown in this study.

The biological relevance of the presence of sTLR2 in human plasma and milk was further supported by the coimmunoprecipitation experiments with milk and plasma samples and the computational molecular docking studies of the TLR2-CD14 interaction, both indicating that the naturally expressed sTLR2 has the capacity to interact in solution with sCD14. This was in agreement with a previous report demonstrating the binding of human rsCD14 to the extracellular domain of human rTLR2 coated to microtiter wells (45). It is conceivable that breast milk provides the neonate not

only with the critical coreceptor sCD14, but also with a TLR signaling regulator, sTLR2. In this way, an excessive local inflammation of the neonatal gut following bacterial colonization would be avoided. Future studies will address this possibility.

Nevertheless, the capacity of sTLR2 to interact with CD14 and inhibit cell activation defines sTLR2 as a modulator of TLR2 signaling. This may explain the maintenance of a substantial intracellular pool of sTLR2 as reported in this study, because it would guarantee an almost immediate regulation of cell activation through the rapid release of the preformed modulator. This modulatory capacity of sTLR2 may lead to the design of new therapeutics for the prevention and/or treatment of severe bacterial-induced pathological condition, including septic shock.

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