研究成果の刊行に関する一覧表

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雑誌

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Combinational recognition of bacterial lipoproteins and peptidoglycan by chicken Toll-like receptor 2 subfamily

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Abstract
Human Toll-like receptor 2 (TLR2) subfamily recognizes bacterial lipoproteins (BLP) and peptidoglycan (PGN). According to the genome information, chicken has structural orthologs of TLRs1 and 2, in addition to TLRs3, 4, 5 and 7. Chicken has two additional TLRs, TLR15 and TLR21, whose orthologs human lacks. The chicken (ch)TLR1 and 2 genes are individually duplicated to encode for four different proteins, chTLR1-1, 1-2, 2-1 and 2-2, of the TLR2 subfamily. Here we investigated the functional profile of these TLR2 subfamily proteins of chicken. By NF-κB reporter assay using HEK293 cells, we found that chTLR2-1 and chTLR1-2 cooperatively signal the presence of PGN. A combination of chTLR2-1 and chTLR1-2 also most efficiently recognized diacylated BLP, macrophage-activating lipopeptide 2 kDa (Malp-2), while the combination of chTLR2-1 and chTLR1-1 failed to recognize Malp-2. All combinations, however, recognized triacylated BLP, Pam3. Consistent with these results, human TLR2-stimulating mycobacteria preparations, BCG-cell wall and cell lysate of *Mycobacterium avium*, induced activation of NF-κB in cells expressing chTLR2-1 and 1-2 and to lesser extents, cells with chTLR2-2 and either of chTLR1. Strikingly, expression of either of these alone did not activate the reporter for NF-κB. These chTLRs are likely to have the combination functional feature as in the human TLR2 subfamily. Confocal and immunoprecipitation analyses of human cell transfectants showed that they

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cluster on the cell surface by a physical molecular association, causing all of them to merge and coprecipitate. These results suggest that chTLR2 subfamily members discriminate between their ligands by combinational events.

1. Introduction

The Toll-like receptor (TLR) family proteins participate in detecting microbial pattern molecules [1]. So far, 10 members of the human TLR family have been discovered. Most of their structural orthologues are found in the genomes of vertebrate species. TLR2 subfamily contains multiple proteins with similar Toll/interleukin 1 receptor (TIR) homology domain structures and is identified across fish to humans [2,3]. This subfamily of TLRs appears to have advanced as a pattern recognition receptor in conformity with the principles of evolution. We had identified chTLR2 type1 and chTLR2 type2 before the chicken genome information was published [4]. Since chicken TLR2 consisted of duplicated isoforms with high similarity, we previously assigned their functional profiles according to contemporary knowledge [4]. They responded to mycoplasmal diacylated lipoprotein, namely the macrophage-activating lipopeptide 2 kDa (Malp-2) leading to NF-κB activation [5,6], though the response was very weak [4]. Recently, Yilmaz et al. [7] have found from bioinformatics analysis of the chicken genome that chicken has a TLR2 subfamily consisting of chTLR1 type1 (1-1), chTLR1 type2 (1-2), chTLR2 type1 (2-1) and chTLR2 type2 (2-2). These four TLR2-related cDNAs were identified by molecular cloning in our laboratory, although the cDNA sequences were somewhat different form those expected from the genome. They are ubiquitously distributed in chicken organs [7]. Further bioinformatics studies suggested the presence of additional chTLRs, TLR15 and TLR21, which might function in association with TLR2 [8].

In humans, the TLR2 subfamily consists of TLR1, TLR2, TLR6 and TLR10 mapped in different loci [3,8]. TLR2 recognizes a variety of microbial components, such as peptidoglycan (PGN), triacylated bacterial lipoproteins (BLPs) (Pam3), mycoplasmal diacylated lipoprotein (Malp-2), and GPI anchors [9] and a thiol-disulfide oxidoreductase-related protein Tc52 from *Trypanosoma cruzi* [10]. Human TLRs tend to form homo- or hetero-dimers, which further specifies their ligand recognition properties [11,12]. Notably, TLR6 preferentially recognizes diacyl lipopeptide such as Malp-2 in cooperation with TLR2 [6,11,12]. TLR1 together with TLR2 fastidiously recognizes triacyl lipopeptide Pam3 [6,11,13]. TLR2 alone weakly recognizes these BLPs and is sufficient for full recognition of PGN [6,11]. Hence, human TLR2 is a functional core for the recognition of BLPs and PGN. In combination or alone, human TLR2 activates NF-κB leading to the activation of immune cells.

These observations allow us to postulate that the chicken TLR2 subfamily members act co-operatively to recognize BLPs or PGN similar to that in human TLR2. As reported previously [4], chicken TLR2 alone or even chTLR2-1 and chTLR2-2 inefficiently signaled the presence of PGN or BLP. In this investigation, we have defined the combinations that are essential for recognition of diacylated BLP, triacylated BLP and PGN by chTLRs. Either of the chTLR1's (1-1 or 1-2) participated in full activation of the chTLR2 pathway. TLR2 subfamily members may have evolved to form heteromultimers for sensing various microbial patterns by their combinations on the cell surface.

2. Materials and methods

2.1. Cells, bacteria and reagents

Human embryonic kidney (HEK) 293 and HeLa cells were purchased from American Tissue Culture Collection (ATCC) (Bethesda, MD). HEK293 cells were cultured in RPMI-1640 containing 10% FCS and HeLa cells in MEM (Nissui, Tokyo Japan) containing 10% FCS as described previously [11]. A synthetic *N*-palmitoyl-*S*-dipalmitoylglyceryl Cys-Ser-(Lys)4 (Pam3CSK4) was purchased from Roche Diagnostics (Indianapolis, IN) and Malp-2 [14] was prepared as described previously [6,11]. PGN (*Staphylococcus aureus*) was purchased from Fluka Chemie, Tokyo. Mycobacterial PGN was purified from BCG cell-wall skeleton as described previously [15].

Two strains of *Mycobacterium avium* were purchased from ATCC (ATCC35718 and 15769). Cells ($A_{600} = 0.4$) were heated in a water bath at 98 °C for 10 min. Cells were sonicated and aliquots (50 μl) were then incubated with HEK293 cells (10^5) expressing single or double chTLR2 members (see Fig. 6).

2.2. Expression vectors and FACS analysis

Molecular cloning of chicken TLR2 type1 (AB050005) and type2 (AB046533) were described previously [4]. These two genes were tandemly arranged in chromosome 4q1.1 [4]. Chicken total RNA was extracted from chicken kidney tissue with TRIZOL (Invitrogen, Carlsbad, CA). Reverse transcription reaction was carried out with M-MLV reverse transcriptase (Promega, Madison, WI). To amplify the full-length open reading frame (ORF) of the chicken TLR1 cDNAs, we used error-free Taq polymerase, Pyrobest (TAKARA BIO INC. Otsu Shiga). The cDNA clones were sequenced on an ABI 310 sequencer. We chose the clone that did not have PCR error for the following analyses. The cDNA sequences of ORF were deposited to the DNA data bank of Japan. The accession numbers are AB109401 (chTLR1 type1) and AB290903 (chTLR1 type2), which are localized proximal to each other in a micro-chromosome [7]. ChTLR1-1 and chTLR1-2 tagged with hemagglutinin (HA) at the C-terminus were generated by PCR and ligated into the *XhoI-NotI* site of the expression plasmid pME18S. pFLAG-chTLRs (C-terminal labeled) were made for confocal analysis using pME18S as described previously [11]. pFLAG-chTLRs (N-terminal labeled) were made for surface fluorescence-activated cell sorting (FACS) analysis using a pCMV vector by the addition of the FLAG

sequence at the end of the signal sequence as reported previously [6]. pFLAG-huTLR3 was provided as described previously [6].

The plasmids (4 μg) were transfected with HEK293 cells and 24 h later cells were treated with anti-FLAG Ab and secondary Abs, and analyzed by FACS [11].

2.3. Luciferase assay

HEK293 cells were transiently transfected with 2 μg of the indicated vectors along with a pELAM luciferase reporter plasmid [11] and a pRL-TK (Promega, Madison, WI) using Lipofectamine 2000 (Invitrogen, San Diego, CA). pRL-TK was used for normalization of transfection efficiency. Similarly, HEK cells were then transfected with plasmids with chTLRs. Twenty-four hours after transfection, the cells were stimulated with 100 nM/ml Malp-2, 10 μg/ml Pam3CSK4 or 10 μg/ml PGN for 8 h. Then the cells were lysed and luciferase activity was measured using the dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions.

2.4. Immunoprecipitation and western blotting

HEK293 cells were transiently transfected with 3 μg of FLAG-tagged huTLR3, chTLR1-1, chTLR1-2, chTLR2-1, chTLR2-2 or vector only, together with 6 μg of HA-tagged chTLR1-1 as indicated. After 36 h, the cells were lysed in the lysis buffer containing 1.0% Nonidet P-40, 150 mM NaCl, 20 mM Tris–HCl (pH 7.5), 5 mM EDTA and a protease inhibitor mixture tablet, Complete (Roche Diagnostics). The lysates were precleared for 1 h with protein G-Sepharose and immunoprecipitated with 2 μg of anti-FLAG M2 Ab or 2 μg of anti-HA 12CA5 Ab and protein G-Sepharose for 12 h. The beads were washed with the lysis buffer four times and immunoprecipitated proteins were eluted with SDS-PAGE sample buffer, separated on SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. HA-tagged chTLR1-1 and 1-2 were detected with anti-HA Ab (Roche Diagnostics) and HRP-labeled anti-mouse Ig Ab. FLAG-tagged proteins were identified with HRP-conjugated anti-FLAG M2 Ab. Then the bound Abs were detected by the ECL system (DuPont, Boston, MA).

2.5. Confocal microscopy analysis

HeLa cells transfected with indicated plasmids were used in this study. The adherent cells were fixed for 30 min with 3% formaldehyde in phosphate-buffered saline (PBS) and permeabilized with 0.5% saponin in 1% BSA/PBS for 30 min, and then washed four times with PBS. Cells were stained with rabbit anti-HA polyclonal Ab or mouse anti-FLAG monoclonal Ab (mAb) (5 μg) for 1 h at room temperature in PBS and the cells were washed twice with the above buffer. To see the HA-tagged chTLRs, we treated the cells with Alexa 488-conjugated goat anti-rabbit IgG (Molecular Probes) for 30 min. To see the FLAG-tagged chTLRs, we treated the cells with Alexa 568-conjugated goat anti-mouse IgG (Molecular Probes) for 30 min. This method facilitated discrimination of two tags of TLRs as reported previously [11]. The stained cells were visualized at × 60 magnification under a FLUOVIEW (Olympus, Tokyo). Images were captured using the attached computer software, FLUOVIEW.

2.6. Gene analysis

Assembling and editing of the determined DNA sequences were performed with ATGC and GENETYX-MAC version 12.1 software (Software Development, Tokyo). The sequences of the predicted ORFs or the TIR domains were compared with other sequences in a homology search by the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/). TLR members of *Homo sapiens*, *Mus musculus*, *Gallus gallus*, *Xenopus tropicalis* and *Takifugu rubripes* were identified in the database (http://www.ncbi.gov/Genbank/). Alignment of the amino acid sequences and unrooted phylogenic analysis of TLRs were performed by Clustal W program (http://www.ddbj.nig.ac.jp/search/clustalw-j.html). Functional domains of the proteins were predicted by SMART program (http://smart.embl-heidelberg.de/).

3. Results and discussion

The domain structures of chTLR1 type1 (1-1), chTLR1 type2 (1-2), chTLR2 type1 (2-1) and chTLR2 type2 (2-2) are shown in Fig. 1A. Their TIRs are highly homologous (identities >75%) to each other while the leucine-rich repeat (LRR) domains exhibited relative divergence. Particularly, chTLR1-2 had a short N-terminal region of the LRRs compared with chTLR1-1. Gene tree bootstrap probability analysis suggests that chTLR2 type1 and type2 cluster in the TLR2 subgroup and chTLR1 type1 and type2 cluster in the TLR1 subgroup (Fig. 1B). The TIR domain of huTLR1 was >80% homologous to those of chTLR1 and chTLR2. Divergence of type1 and type2 in either chTLR1 or TLR2 genes appears to have occurred after the separation of birds and mammals from a common ancestor (Fig. 1C).

Humans and mice have the TLR2 subfamily consisting of TLR1, TLR2, TLR6 and TLR10. The natural ligand of TLR10 has not been identified. Swine, dog and bovine TLRs of the TLR2 subfamily are similar to those of human and mouse, although their structural orthologues may not reflect their functions [8]. In these mammals, TLR1, TLR6 and TLR10 are mapped close together on one chromosome and TLR2 is mapped to another chromosome [8]. In contrast, fish and xenopus have unique TLR2 subfamilies of TLR1, TLR2 and TLR14 [2,8,16]. Even the lamprey appears to have the TLR2 subfamily [17]. This study is aiming at clarification of the functional correspondence of the human TLR2 family [18] to chicken TLR1 (1-1 and 1-2) and TLR2 (2-1 and 2-2). Chicken-specific TLR15 [19] and putative TLR6/10 [20] might join in this subfamily, although their functional properties remain unknown. Species-dependent differential properties of TLR members in the TLR2 subfamily suggest that each species has experienced different microbial environments. An alternative hypothesis is that different species have simply evolved different solutions to similar microbial challenges.

The protein expression levels of chTLR1 and chTLR2 (N-terminal tagged with FLAG) in transfected human cell line HEK293 were analyzed by flow cytometry using anti-FLAG Ab. Of the transfected cells, 33–85% were TLR-positive according to FLAG, suggesting the presence of chTLR1 and

chTLR2 on the cell surface (Fig. 2). Staining these cells with anti-FLAG Ab after permeabilization resulted in further shifts by FACS, suggesting that there are small intracellular pools of these chTLRs in transfected cells (data not shown). The expression profile of huTLR2 (positive control) resembled that of chTLR1-1, chTLR2-1 and chTLR2-2 (Fig. 2).

By confocal microscopy, TLR1-1, TLR1-2, TLR2-1 and TLR2-2 were colocalized on the cell surface and partly in the cytoplasm as well (Fig. 3). The results of the chTLR2 members are largely consistent with those obtained with human TLR1, TLR2 and TLR6 by confocal analysis using specific monoclonal Abs [11]. Next, we examined whether these chTLRs physically associate with other chTLRs. For this, HEK293 cells were cotransfected with FLAG-tagged chTLRs and HA-tagged chTLR1-1. Total cell lysates contained comparable amounts of proteins (left panels of Fig. 4), although the expression efficacy was relatively low in FLAG-tagged chTLR2-1 and 2-2. Immunoprecipitation of FLAG-tagged chTLRs with anti-FLAG Ab resulted in coprecipitation of HA-tagged chTLR1-1 (Fig. 4A) or HA-tagged chTLR1-2 (Fig. 4B). This indicated the physical association of chTLR1-1 or chTLR1-2 with other chTLRs in HEK293 transfectants. The blots probed with anti-FLAG Ab suggested that almost all members of proteins were precipitated with anti-FLAG Ab

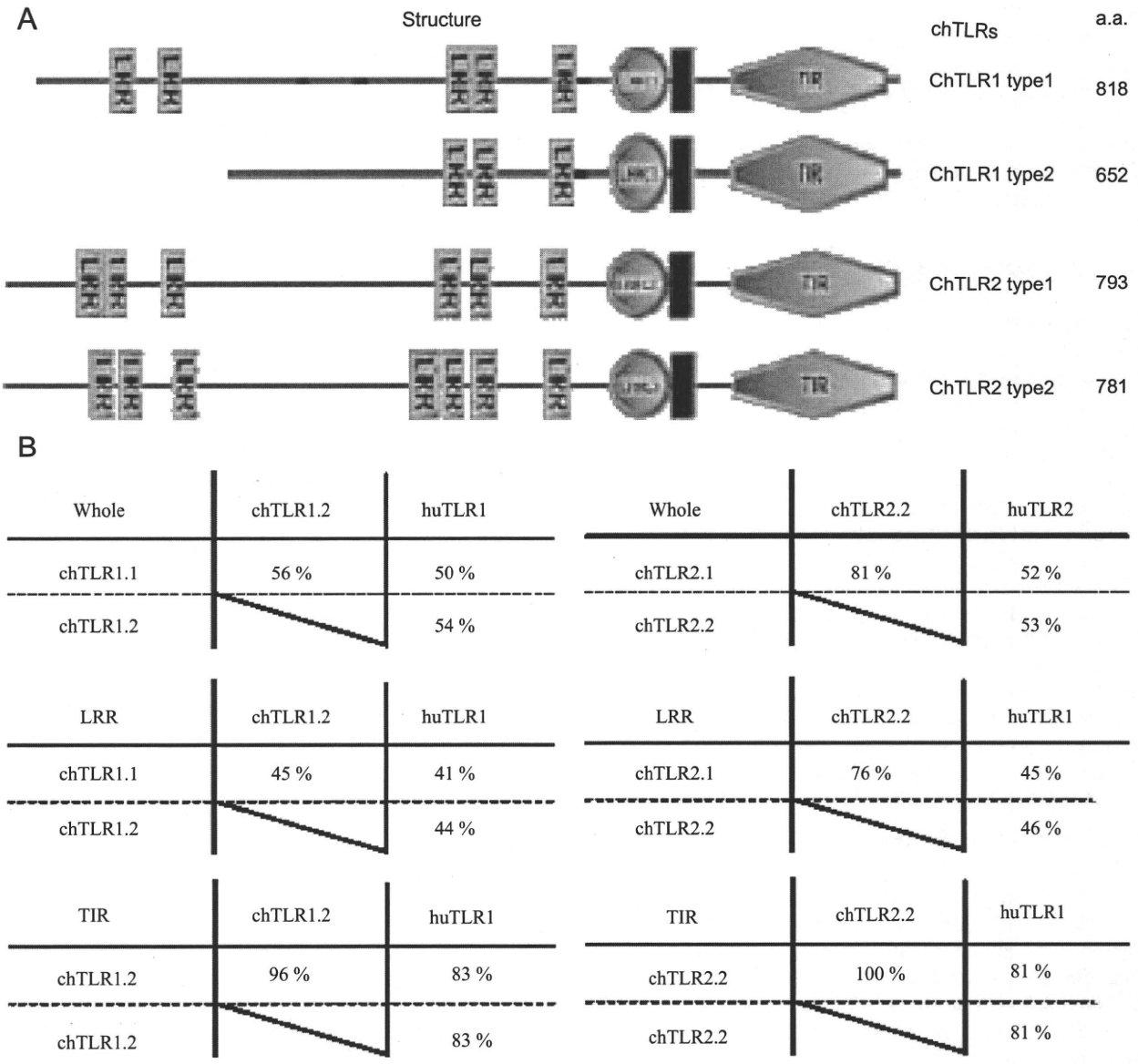

Fig. 1 TLR2 subfamily proteins in the chicken. (A) Depiction of motif structures of chicken TLRs. ChTLR1 type1 (1-1), chTLR1 type2 (1-2), chTLR2 type1 (2-1) and chTLR2 type2 (2-2). N-terminal vertical gray bars represent LRRs and vertical black bars represent transmembrane domains. LRR-CT (the leucine-rich repeat at the C-terminus) is shown by circles. TIRs are shown to the right. Their numbers of amino acids (a.a.) are indicated. (B) Percent homology between chicken TLRs and human TLRs. Amino acid sequences of the LRR and TIR regions are separately compared in the bottom two tables. Panel (C) Unrooted phylogenetic tree of TLRs in vertebrates. The tree was constructed through the Clustal W program. The relationships were calculated on the basis of the amino acid sequences of the TIR domains. Bootstrap values (>900) are not indicated. Asterisk indicated trichotomy. hu; human, mo; mouse, ch; chicken, fu; fugu.

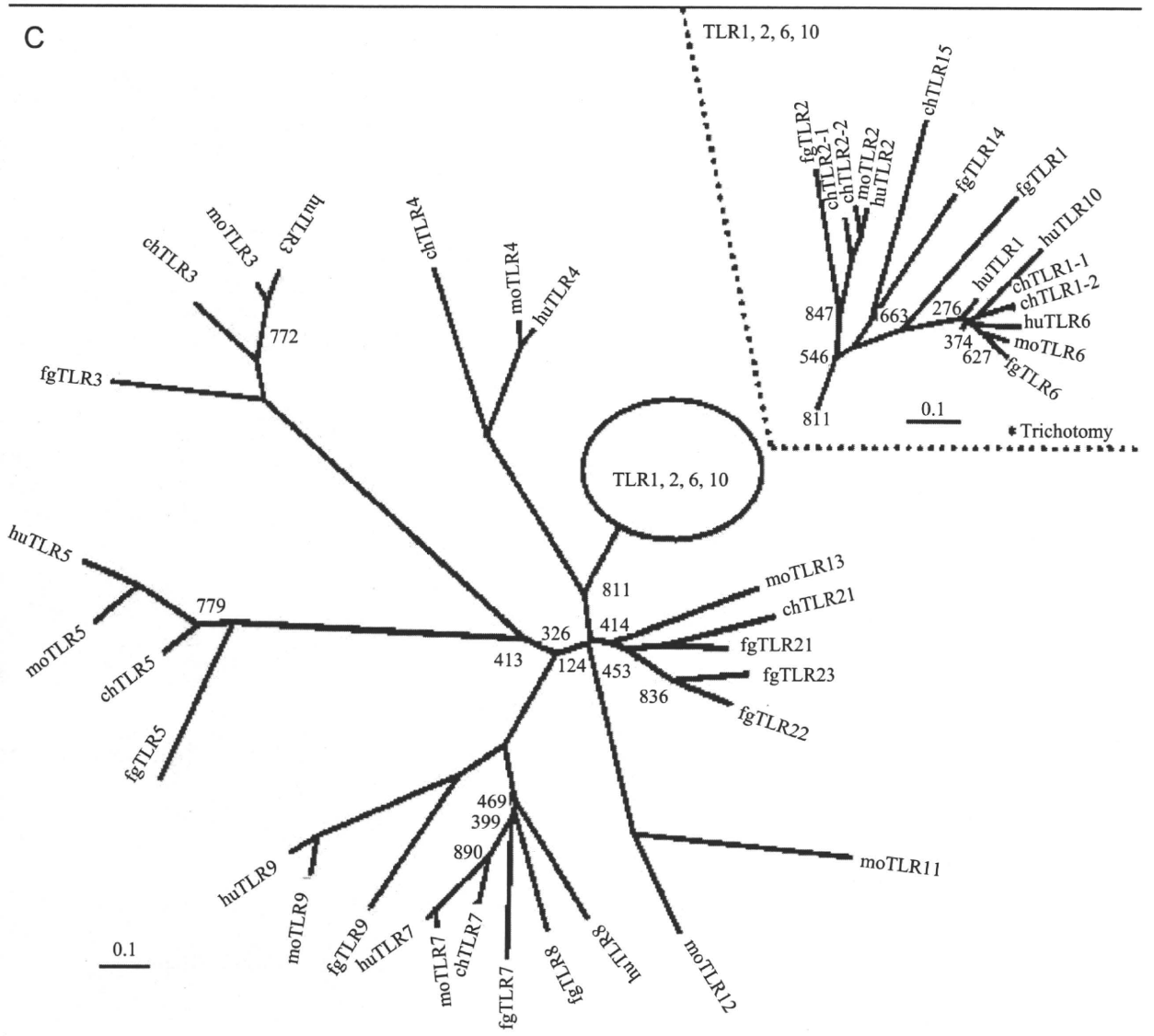

Fig. 1 (*Continued*)

(lower right panels of Fig. 4), although the amounts of the precipitates bound to chTLR1-1 and chTLR1-2 were somewhat variable among the chTLRs tested compared with the control. Under similar conditions, chTLRs were barely coprecipitated with huTLR3 when huTLR3 FLAG was used as a negative control (right panels of Fig. 4). The strongest HA-signal was detected with FLAG-tagged chTLR1-1 proteins (lanes 3 in the right panels of Fig. 4A and B). We surmised that chTLR1-1-FLAG nonspecifically interacts with anti-HA Ab since in both panels A and B the strong signal was observed only in the lane with chTLR1-1-FLAG. The results suggest that all four chTLR2-related proteins assemble in the same plasma membrane compartments of HEK cells. Homophilic adhesion predominantly occurred in the chTLR molecular complex. Stimulation with Pam3 did not affect the extent of association between chTLR2 and chTLR1 (data not shown). Taken together with the confocal analysis (Fig. 3), these results suggest that chTLR2 and chTLR1 associate with each other in a ligand-independent manner in HEK293 cells.

A reporter assay was employed to determine the functional profile of each chTLR (Fig. 5). We first confirmed that the chTLRs function in the human system using human HEK293 cells as reported previously [4]. A single TLR member of the chTLR2 subfamily alone had no ability to induce luciferase activity in response to PGN, Malp-2 or Pam3. The results were confirmed with chimera molecules consisting of extracellular chicken TLRs and intracellular human TLR2 (data not shown). Expression of all four chTLRs activated reporters in response to the stimuli (Fig. 5A). Thus, various combinations of these chTLRs are apparently required to recognize PGN and lipoproteins.

We then provided HEK cells expressing two sets of chTLRs as shown in Fig. 5B and C. A diacylated lipopeptide Malp-2 was mainly recognized by the set of chTLR2-1 and chTLR1-2. Recognition by chTLR2-2 together with either chTLR1-1 or chTLR1-2 was observed to a lesser extent. These three combinations also recognized Pam3 to a variable level. The combination of chTLR2-1 and chTLR1-1 responded weakly to Pam3. On the other hand, staphylococcal PGN

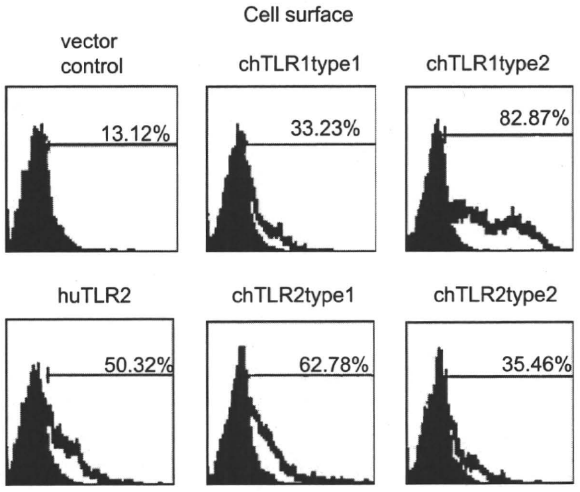

Fig. 2 Expression profiles of chTLR1 and chTLR2. Chicken TLR2 members with N-terminal FLAG were expressed on HEK293 cells and detected by Ab against FLAG. % Positive cells were indicated in the insets assuming that the cells positioned in the gate and above the control are positive. The two left panels are negative (vector only) and positive (transfected with huTLR2) controls.

was exclusively recognized by the combination of chTLR2-1 and chTLR1-2 (Fig. 5C). chTLR2-1 and chTLR1-2 also exclusively responded to another PGN of mycobacteria origin [15] (data not shown). Thus, the chTLR2 subfamily recognizes BLP and PGN in distinct combinations.

We next investigated whether surface-expressed chTLRs sense whole bacteria. Mycobacteria possess lipoproteins and PGN and exhibit agonistic function toward TLR2 [15,21]. *M. avium* infects even humans with compromised immune states such as AIDS [22], where human TLR2 may be involved. Human TLR2 plays an essential role in promoting immune responses against mycobacterial infections [21]. Glycopeptidolipids (GPLs) are highly expressed on *M. avium* and GPLs activated mouse macrophages in a TLR2- and MyD88-dependent manner [15,23]. Although there are some discrepancies between in vitro and in vivo data, MyD88 pathways participate in cellular responses induced by *M. avium* [24]. Heat-inactivated mycobacteria were incubated with HEK cells expressing C-terminal-tagged chTLR2 members (Fig. 6). In the chicken TLR system, we found that the combination of chTLR1-2 and chTLR2-1 most efficiently induces signal for NF-κB activation in response to *M. avium* (Fig. 6). To a lesser extent, other combinations of chTLR2-2 and either of chTLR1-1 or 1-2 responded to *M. avium*. Untagged chTLR2 members gave rise to results similar to those obtained with the C-terminal tagged proteins, but N-terminal tagging resulted in the disruption of the chTLR2 function (data not shown). The results infer that PGN as well as GPLs participates in chicken TLR activation, the case being similar to that of BCG cell wall, the active center of which is BCG-PGN and presumably muramyl dipeptide [15,18].

Mammalian TLR2 recognizes meningococcal porin, PorB, through direct binding, and the PorB-induced cell activation is mediated by a TLR2/TLR1 complex [25]. In addition, several reports documented that some distinct properties of

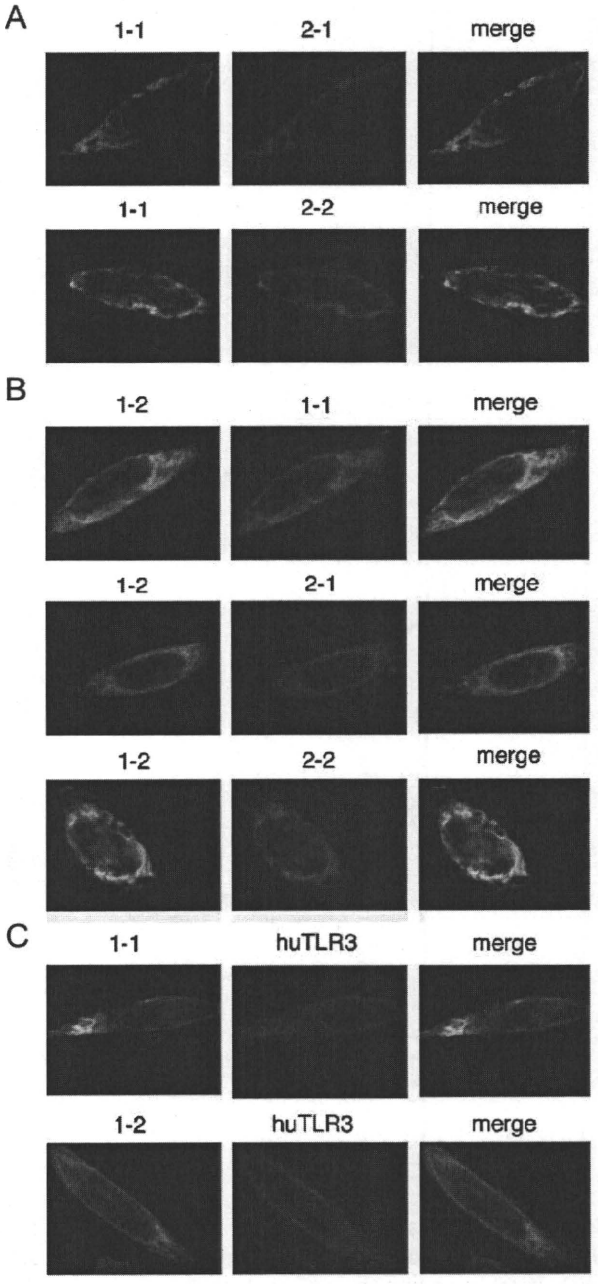

Fig. 3 Colocalization of chTLR1 and chTLR2. Confocal analysis was performed using HeLa cells expressing indicated chTLR1-1 or chTLR1-2 with C-terminal HA, and chTLR2-1 and chTLR2-2 with C-terminal FLAG (A and B). Human TLR3 with C-terminal FLAG was used as control (C). Cells were labeled with anti-FLAG mAb (mouse) and stained with Alexa568-conjugated goat anti-mouse IgG (red), and then with anti-HA polyclonal Ab (rabbit) and stained with Alexa-488-conjugated goat anti-rabbit IgG (green). Cells were analyzed on FLOVIEW.

lipopeptides are recognized by TLR2 in a TLR1- and TLR6-independent manner in humans [13,26]. TLR2 appears to be sufficient to signal the presence of PGN of mycobacteria and Gram-positive bacteria in mammals [10,27,28]. In addition, the response to PGN is not renounced in either TLR1- or TLR6-deficient mice [15,26]. It is still unclear whether in mammals, TLRs other than TLR1 and TLR6 pair with TLR2

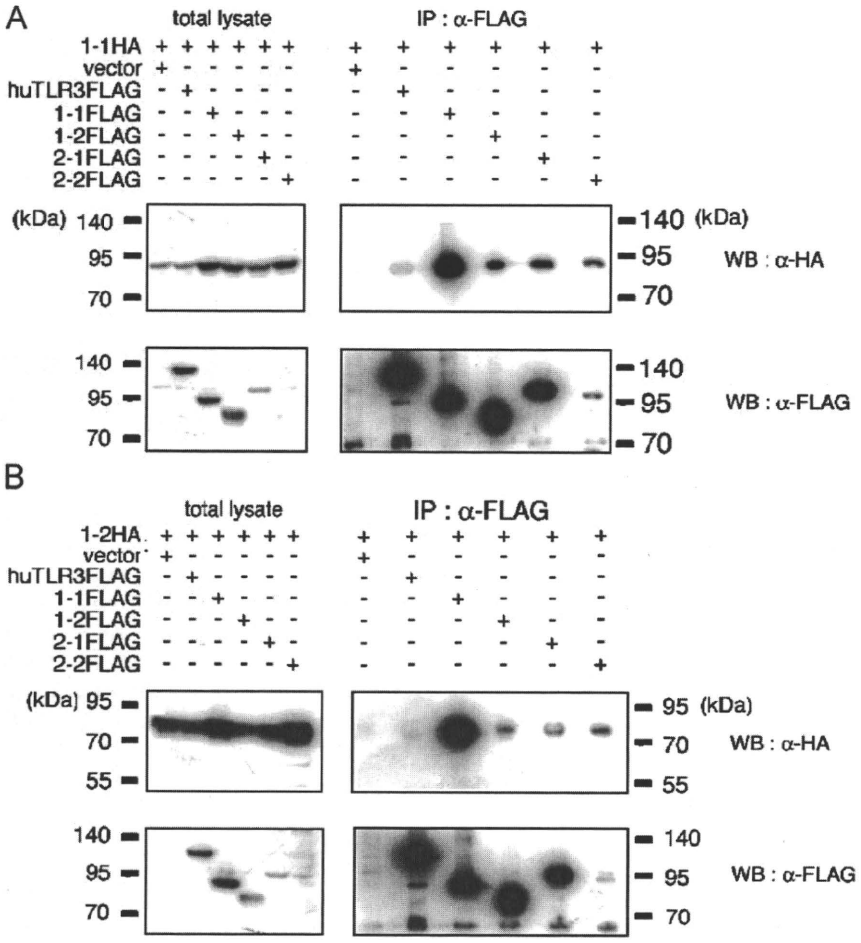

Fig. 4 Immunoprecipitation of chTLRs. HA-tagged chTLR1-1 (A) and chTLR1-2 (B) were expressed in HEK293 cells together with FLAG-tagged chTLR members as indicated. Human TLR3 was used as a control. Cells were lysed and the lysates directly resolved on SDS-PAGE followed by blotting (left panels in A and B). The blots were probed with anti-HA Ab (upper panels) or anti-FLAG Ab (lower panels). In the lysates, the FLAG-labeled proteins were immunoprecipitated with anti-FLAG Ab. The precipitates were resolved on SDS-PAGE followed by blotting. The blots were probed with anti-HA Ab (upper panel) or anti-FLAG Ab (lower panel). The upper right panels reflect the amounts of HA-tagged proteins (chTLR1-1 or 1-2) coprecipitated with FLAG-tagged proteins.

to recognize PGN or TLR2 alone is sufficient to detect it. In this context, this study is the first to reveal that in chicken TLR2-1 has to be coupled with chTLR1-2 for the recognition of PGN. Functional studies of chTLRs near the gene tree of the chTLR2 subfamily (including chTLR15, chTLR6/10, etc.) may give us a clue to explore additional role of chTLRs.

However, the component where these chTLR2 members exist as a large receptor complex is still unaddressed. It was reported that human TLR1, 2 and 6 naturally form a complex on the human monocyte membranes [11]. Human TLR4 and CD14 may reside in a certain lipid raft in the membrane [29]. Further studies will be required to identify the exact competent components that recruit the receptor complex. Although the structural basis for the requirement of heterodimeric composition of chTLRs remains unknown, the information of the functional properties of chicken TLRs would be adaptable to comparative investigation of the specific role of the vertebrate TLR2 subfamily members.

Chicken heterophils and monocytes are actually activated through chTLR signaling [20,30]. Cytokines, chemokines and nitric oxide are induced via chTLRs in response to their agonists [20,30,31]. These findings imply that chTLR2 [30], chTLR15 [19], chTLR4 [20], chTLR5 [32], chTLR7 [33] and chTLR3 [34] are involved in leukocyte activation in chicken. Investigations pursuing chTLR-specific agonists/antagonists may give us a new strategy to design adjuvants and immune therapies for chicken infectious diseases in which BLP and PGN are involved as pathogenesis. The data will be useful for zoonosis control of pathogens permitting interspecies infection between birds and primates.

In summary, the present study provides evidence that four proteins of the chTLR2 subfamily recognize diacylated BLP, triacylated BLP and PGN by distinct combinations. Chicken TLR2, either type 1 or type 2, alone exhibits minimal ability to activate NF-κB, unlike human TLR2 [3]. In particular, only one combination with chTLR2-1 and chTLR1-2 predominantly recognizes PGN. Finally, expression of all four members of the chTLR2 subfamily results in NF-κB activation in response to BLP or PGN. They cooperatively function to recognize BLP and PGN by forming a molecular complex on the membrane. Specific antibodies against these chTLRs will facilitate analysis of actual localization of chTLRs on chicken cells/tissues.

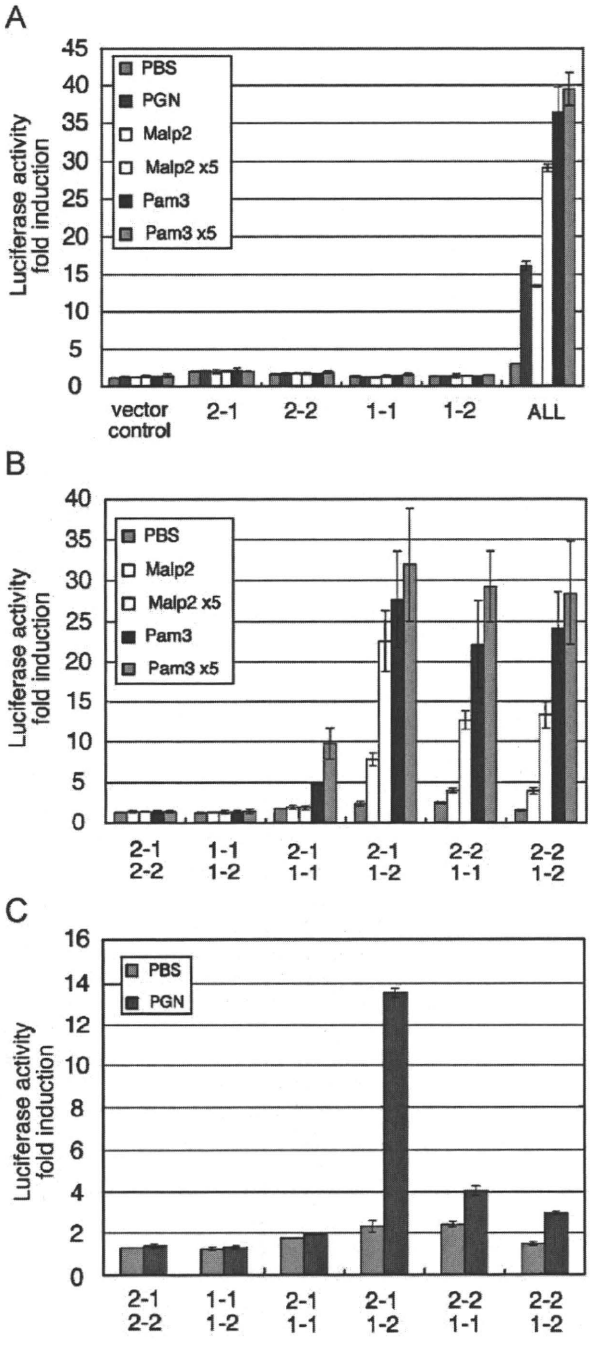

Fig. 5 Pattern-recognition profiles of chTLR2 subfamily. (A) Single expression of chTLR2 members does not confer NF-κB activation. HEK293 cells expressing indicated chTLR2 members and pELAM plasmid (for NF-κB) were stimulated with PGN, Malp-2, Pam3 or just sterilized PBS. ALL represents HEK cells transfected with all four plasmids. At timed intervals (typically 24 h), cells were harvested for reporter assays. Luciferase activities are shown as the mean relative stimulation $\pm$SD. (B) chTLR2 and chTLR1 recognize lipopeptides. HEK293 cells were transfected with the indicated combinations of plasmids of chTLR2 members and pELAM plasmid. Cells were stimulated with Malp-2, Pam3 or just sterilized PBS and harvested for reporter assays as in panel (A). (C) chTLR2-1 and chTLR1-2 cooperatively recognize PGN. HEK293 cells expressing chTLR2 member combinations and pELAM were prepared as in (B). Cells were stimulated with PGN and assayed as in (B). Typical one of the three experiments is shown in all panels.

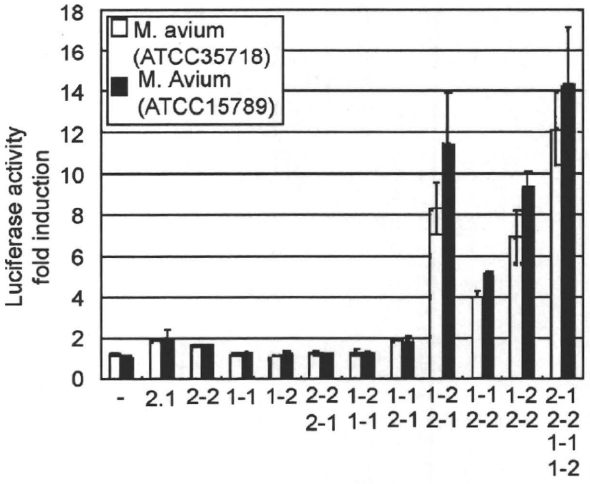

Fig. 6 ChTLR2 subfamily members sense *M. avium*. HEK293 cells with a pELAM luciferase reporter plasmid and expressing chTLR2 members (indicated in the figure) (10^5 cells) were incubated with sonicated *M. avium* (50 μl of $A_{600} = 0.4$) at 37 °C for 24 h (see Section 2). HEK cells were then harvested and luciferase activity was determined as in Fig. 5. Two bars represent two different strains of *M. avium*. Two additional sets of experiments were performed and similar results to this figure were obtained.

Acknowledgments

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The Proinflammatory Cytokine Response to *Chlamydia trachomatis* Elementary Bodies in Human Macrophages Is Partly Mediated by a Lipoprotein, the Macrophage Infectivity Potentiator, through TLR2/TLR1/TLR6 and CD14[1]

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Chlamydiae components and signaling pathway(s) responsible for the production of proinflammatory cytokines by human monocytes/macrophages are not clearly identified. To this aim, *Chlamydia trachomatis*-inactivated elementary bodies (EB) as well as the following seven individual Ags were tested for their ability to induce the production of proinflammatory cytokines by human monocytes/macrophages and THP-1 cells: purified LPS, recombinant heat shock protein (rhsp)70, rhsp60, rhsp10, recombinant polypeptide encoded by open reading frame 3 of the plasmid (rpgp3), recombinant macrophage infectivity potentiator (rMip), and recombinant outer membrane protein 2 (rOmp2). Aside from EB, rMip displayed the highest ability to induce release of IL-1β, TNF-α, IL-6, and IL-8. rMip proinflammatory activity could not be attributed to *Escherichia coli* LPS contamination as determined by the *Limulus* Amoebocyte lysate assay, insensitivity to polymyxin B (50 μg/ml), and different serum requirement. We have recently demonstrated that Mip is a "classical" bacterial lipoprotein, exposed at the surface of EB. The proinflammatory activity of EB was significantly attenuated in the presence of polyclonal Ab to rMip. Native Mip was able to induce TNF-α and IL-8 secretion, whereas a nonlipidated C20A rMip variant was not. Proinflammatory activity of rMip was unaffected by heat or proteinase K treatments but was greatly reduced by treatment with lipases, supporting a role of lipid modification in this process. Stimulating pathways appeared to involve TLR2/TLR1/TLR6 with the help of CD14 but not TLR4. These data support a role of Mip lipoprotein in pathogenesis of *C. trachomatis*-induced inflammatory responses. *The Journal of Immunology,* 2008, 180: 1158–1168.

Chlamydiae are important human pathogens due to the wide repertoire of important diseases that they cause (1–5). These microorganisms infect primary epithelial cells with subsequent attraction of monocytes/macrophages (6, 7). They frequently cause chronic inflammatory diseases characterized by the presence of a high number of mononuclear cells (8) that are involved in the pathogenesis by inducing mediators of inflammation. However, few data have been published about chlamydiae components involved in inflammatory response, and results are still debated. Chlamydial LPS (9, 10) and heat shock protein (hsp)[3]60 (11, 12) were involved in some reports but not in others (13–15). In signaling pathway, whole bacteria or *Chlamydia* hsp60 have been shown to induce TLR-mediated activation, but the signaling receptor differed among studies from both TLR2 and TLR4 (16), only TLR4 (17, 18), only TLR2 (14), to largely TLR2 and to a minor extent TLR4 (19). Except LPS and hsp60, chlamydiae components responsible for these effects were unidentified.

To identify chlamydial component(s) able to induce production of proinflammatory cytokines by human monocytes/macrophages, inactivated elementary bodies (EB), one of the two forms presented by chlamydiae in their biphasic developmental cycle, as well as seven individual chlamydial Ags: purified LPS, recombinant hsp70 (rhsp70), rhsp60, rhsp10, recombinant polypeptide encoded by open reading frame 3 of the plasmid (rpgp3), recombinant macrophage infectivity potentiator (rMip), and recombinant outer membrane protein 2 (rOmp2) were carefully purified and tested. Aside from EB, rMip displayed the highest proinflammatory activity, stimulating the synthesis of IL-1β, TNF-α, IL-6, and IL-8. Mip was recently shown to have lipid modification similar to that of other procaryotic lipoproteins and to be exposed at the surface of EB (20). The proinflammatory activity of EB was significantly attenuated in the presence of polyclonal Ab to rMip. Failure of stimulation with a nonlipidated C20A rMip variant as well as after lipase treatment of rMip showed that the proinflammatory activity was dependent upon lipid modification. Use of blocking mAb and human embryonic kidney (HEK)-293 transfected cells

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[3] Abbreviations used in this paper: EB, elementary body; hsp, heat shock protein; pgp3, polypeptide encoded by open reading frame 3 of the plasmid; Mip, macrophage infectivity potentiator; Omp2, outer membrane protein 2; WT, wild type; HEK, human embryonic kidney.

revealed that TLR2/TLR1/TLR6 and CD14 but not TLR4 were involved in mediating these effects.

Materials and Methods

Bacteria and Ags

EB of *Chlamydia trachomatis* LGV2 strain 434 (inactivated by a photochemical treatment affecting bacterial genomes) were either purchased from Biodesign International (Milan Analytica) or prepared according to the method of Boleti et al. (21). *Chlamydia* LPS was extracted according to Rund et al. (22). LPS from *Escherichia coli* serotype O55:B5 and *Salmonella typhimurium* were purchased from Sigma-Aldrich (Fluka Chemie) and repurified (23) to rule out protein and lipoprotein contamination. Cloning, expression in *E. coli*, and purification of six different recombinant proteins from *C. trachomatis* (rhsp70, rhsp60, rhsp10, rpgp3, rMip, and rOmp2) were previously described (24, 25). To rule out LPS and LPS-associated molecule contamination (26–28), all recombinant proteins were subsequently treated by polymyxin B-agarose (Sigma-Aldrich) and had <10 endotoxin units per milligram, according to the *Limulus* Amoebocyte Lysate chromogenic assay (BioWhittaker Cambrex), which is an amount that did not stimulate proinflammatory cytokine production by itself. Preparations of native Mip and C20A rMip variant were previously described (20). Protein concentrations were determined by using a microbicinchoninic acid protein assay kit (Pierce, Perbio Science). Racemic Pam_3CSK_4 (Pam_3-Cys-Ser-Lys_4-OH) and Pam_2CSK_4 (Pam_2-Cys-Ser-Lys_4-OH) used as synthetic triacylated and diacylated control lipopeptides, respectively, were obtained from EMC Microcollections.

Abs and antiserum preparation

Anti-CD14 (clone MY4, IgG2b) was purchased from Coulter Clone (Beckman Coulter), anti-TLR1 (clone GD2.F4, IgG1) from Hycult Biotechnology (Biocoba), anti-TLR2 and anti-TLR4 (clone TL2.1 and HTA125, respectively, IgG2a) from ImmunoKontact (AMS Biotechnology). Anti-TLR6 (clone 127, IgG1) was previously described (29). Isotype-matched mouse IgG1 control was purchased from Southern Biotechnology Associates and IgG2a, and IgG2b from Coulter Clone. Preparation of IgG fraction from rMip antiserum was previously described (20).

Human monocyte/macrophage culture

PBMC from healthy blood donors were isolated by density gradient centrifugation with Ficoll-Hypaque (Amersham Biosciences). Monocytes/macrophages were separated by aggregation, gradient of FBS (Invitrogen Life Technologies), and rosetting (30, 31). Monocyte purity consisted of ≥90% $CD14^+$ cells, ≤1% $CD3^+$ cells, and ≤1% $CD19^+$ cells as assessed by flow cytometry. The enriched monocytes were cultured in 24-well plates (10^5 cells/1 ml per well) in RPMI 1640 containing 2 mM GlutaMAX I, supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin, and heat-inactivated (30 min at 56°C) endotoxin-free 10% (v/v) FBS (Invitrogen Life Technologies). After 48 h (or indicated time periods) stimulation at 37°C with indicated stimuli, cultures were centrifuged at 400 × *g* for 10 min at 4°C and cell-free supernatants were collected and stored at −70°C until cytokine measurements.

THP-1 cell culture

Cells of the human promyelomonocytic cell line THP-1 (32) were purchased from American Type Culture Collection and were grown in RPMI 1640 medium as described above. For monocytic differentiation, they were seeded in 24-well flat-bottom tissue culture plates at a density of 2.5×10^5 cells/1 ml per well and allowed to adhere and differentiate 48 h at 37°C in presence of 10 nM PMA (Sigma-Aldrich). After repeated washing with RPMI 1640, PMA-differentiated THP-1 cells were stimulated at 37°C with indicated stimuli. Cell-free supernatants were harvested after 4 h (or indicated time periods) of incubation and kept at −70°C until cytokine measurements.

Native Mip blocking experiments

PMA-differentiated THP-1 cells were pretreated at 37°C for 1 h with 50 μg/ml human IgG to block Fc receptors and prevent subsequent nonspecific binding of IgG and then stimulated with inactivated *C. trachomatis* EB (5×10^6/ml) in presence of rabbit polyclonal anti-rMip IgG or preimmune IgG (0, 40, or 80 μg/ml). Cell-free supernatants were harvested after 4 h of incubation and kept at −70°C until cytokine measurements.

Heat, proteinase K, alkaline, and lipase treatments of rMip

Heat sensitivity was determined by heating rMip at 100°C for 3 h and proteinase K sensitivity by incubating rMip with 100 μg/ml proteinase K (Promega) in 100 mM Tris-HCl (pH 8.0) at 37°C for 2 h, followed by addition of 200 μM PMSF. Alkaline hydrolysis was performed according to Muhlradt and Frisch (33). Briefly, rMip solution was adjusted to pH 13.0 with sodium hydroxide, incubated at 37°C for 1 h, and neutralized with HEPES before assay. Lipase sensitivity was determined with two glycerol ester hydrolases (E.C. 3.1.1.3.) having different side chain specificity (34). Either 1000 U/ml pig pancreas lipase (type VI-S lipase; Sigma-Aldrich) or 10,000 U/ml *Rhizopus arrhizus* lipase (type XI lipase; Sigma-Aldrich) were added to rMip at 37°C for 16 h, in 50 mM HEPES buffer (pH 7.5), 10 mM $CaCl_2$, followed by heating at 100°C before assay (35). As controls, rMip was incubated in buffer with no sodium hydroxide or enzymes and PMA-differentiated THP-1 cells were incubated with buffer and enzymes in the absence of rMip. Cell-free supernatants were harvested after 4 h of incubation and kept at −70°C until cytokine measurements.

CD14, TLR1, TLR2, TLR4, and TLR6 blocking experiments

Blocking experiments were performed after 1 h pretreatment of PMA-differentiated THP-1 cells with 50 μg/ml human IgG to block Fc receptors and prevent subsequent nonspecific binding of blocking Ab or nonimmune isotype controls. Cells were next incubated at 37°C for 1 h with blocking Ab (5 μg/ml) before stimulation with either 1 μg/ml rMip, inactivated *C. trachomatis* EB (5×10^6/ml), 0.01 μg/ml lipopeptides (Pam_3CSK_4 or Pam_2CSK_4), or 1 μg/ml *E. coli* LPS. Cell-free supernatants were harvested after 4 h of incubation and kept at −70°C until cytokine measurements.

Response of TLR/CD14 cell lines

HEK-293 cells stably transfected with either the empty plasmid (293-Null) or human TLR1/2, TLR2/6, or TLR2/CD14 genes were purchased from InvivoGen (LabForce) and maintained in DMEM (Invitrogen Life Technologies) supplemented with 4.5 g/L glucose, 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 10 μg/ml blasticidin S (InvivoGen) for 293-Null, 293-hTLR1/2, and 293-hTLR2/6 and with 50 μg/ml HygroGold (InvivoGen) for 293-hTLR2/CD14. For stimulation experiments, stable transfected cells were seeded into individual wells of a 48-well tissue culture plate at a concentration of 3×10^5 cells in 300 μl of complete medium and allowed to adhere overnight. The following day, fresh medium was added, and the cells were stimulated with either 1 μg/ml rMip, inactivated *C. trachomatis* EB (5×10^6/ml), 1 or 0.01 μg/ml lipopeptides (Pam_3CSK_4 or Pam_2CSK_4), or 1 μg/ml *E. coli* LPS for 24 h. Culture supernatants were collected, and IL-8 content was analyzed. Results were expressed in terms of fold increase over the IL-8 levels of unstimulated cells.

Cytokine measurements

Extracellular release of IL-1β, TNF-α, IL-6, and IL-8 was determined by a sandwich ELISA technique using the DuoSet ELISA Development Systems (R&D Systems), according to the manufacturer's instructions. The ELISA detection limits were 2 pg/ml for all tested cytokines. When the distributions in cytokine production were not normal, results were expressed as median and interquartile range.

Statistical analysis

Statistical analysis was performed using the SPSS statistical software (for Macintosh, v.10). Kruskal-Wallis and Mann-Whitney *U* tests were used to compare the levels of inflammatory cytokines produced by human monocytes/macrophages in response to various microbial components. A comparison between two groups was made only when the Kruskal-Wallis test yields statistically significant results. Statistical analysis for PMA-differentiated THP-1 cell and HEK assays were performed using a Student's *t* test. Differences were considered significant at $p < 0.05$.

Results

Inactivated C. trachomatis *EB and rMip elicited proinflammatory cytokine production by human monocytes/macrophages and PMA-differentiated THP-1 cells*

The pathology of *C. trachomatis* infection seems to be related to chronic inflammation, characterized by the dominating presence of macrophages in injured tissues. We have therefore tested the ability of inactivated EB and seven chlamydial components (LPS, rhsp70, rhsp60, rhsp10, rpgp3, rMip, and rOmp2) to stimulate the production of various proinflammatory cytokines by healthy blood donor monocytes/macrophages. Inactivated EB as well as rMip induced the release of IL-1β, TNF-α, IL-6, and IL-8 in contrast to

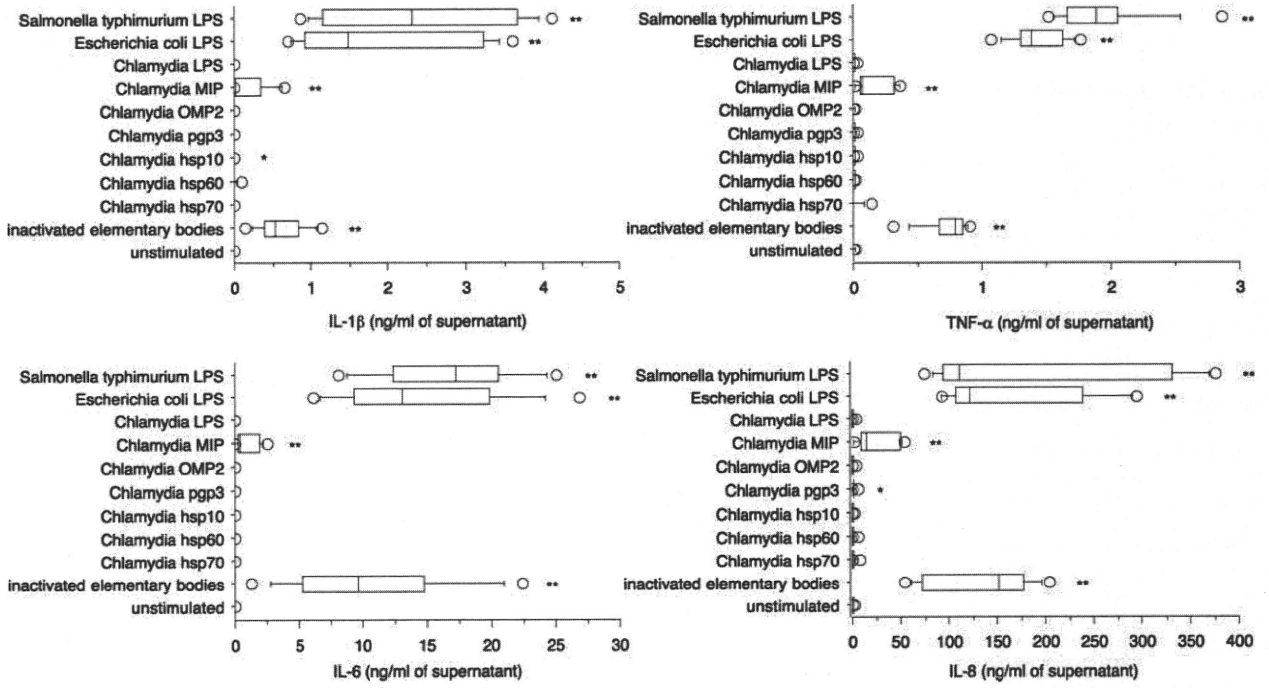

FIGURE 1. Cytokine productions by human monocytes/macrophages in response to various bacterial Ags. Human monocytes macrophages (10^5 cells/1 ml per well) were stimulated by inactivated *C. trachomatis* EB (5×10^6/ml), rhsp70, rhsp60, rhsp10, rpgp3, rOmp2, rMip, LPS, *E. coli* LPS or *S. typhimurium* LPS at 1 μg/ml. After 48 h of culture, supernatants were collected and their content in IL-1β, IL-6, IL-8, and TNF-α were analyzed by ELISA. Results were obtained from three different cultures performed in triplicates. Horizontal bar within boxes shows the median, boxes show the interquartile range, and vertical bar shows the 95% confidence interval (values above and below these levels were plotted separately). *, $p < 0.05$; **, $p < 0.005$ determined by comparison with unstimulated cells using Mann-Whitney U test.

other chlamydial Ags that did not display any consistent stimulatory effects. Both *E. coli* and *S. typhimurium* LPS, used as positive controls, highly induced the release of proinflammatory cytokines, in contrast to chlamydial LPS, as previously reported (10, 15, 36, 37) (Fig. 1). To further define the ability of rMip to stimulate the synthesis of proinflammatory cytokines, dose-response and time course experiments were performed. A dose-dependent stimulation was observed in presence of increasing concentrations of rMip (0.005–5 μg/ml) (Fig. 2). The release of TNF-α was time-dependent with maximal levels being reached after 8 h of culture (Fig. 3). Our data agree with previous findings showing that monocytes/macrophages produced TNF-α shortly after stimulation with lipoproteins (38, 39).

As PMA-differentiated THP-1 cells are resembling to tissue macrophages and are commonly used as models for human monocytes/macrophages (40), they were also tested for their ability to

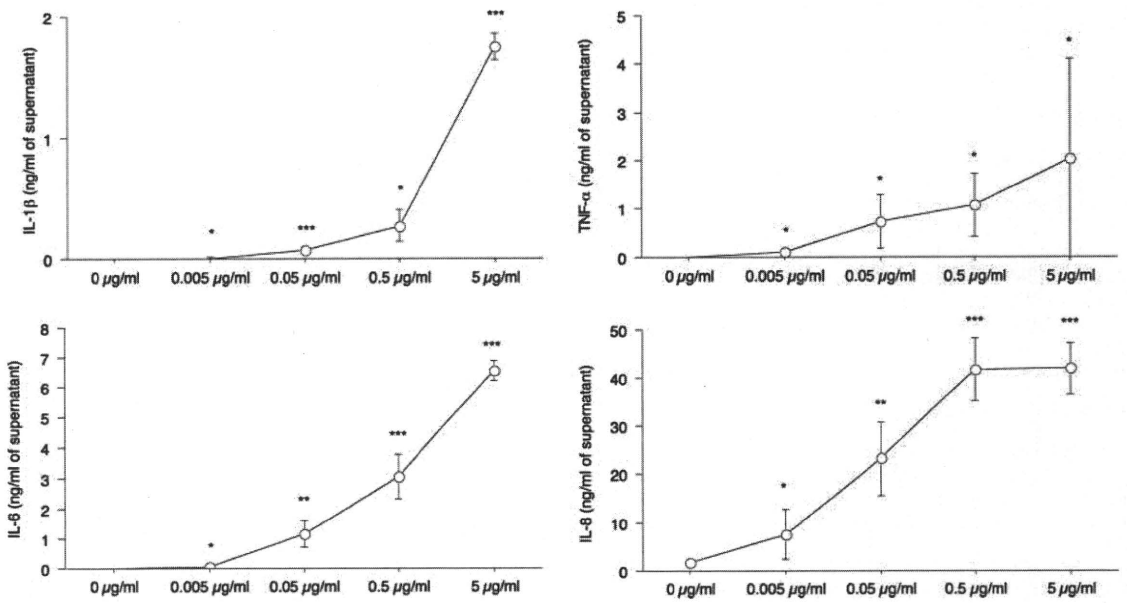

FIGURE 2. Cytokine productions by human monocytes/macrophages in response to increasing concentrations of rMip. Each value represents the mean ± SD of triplicates from two experiments. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0001$ determined by comparison with unstimulated cells using Mann-Whitney U test.

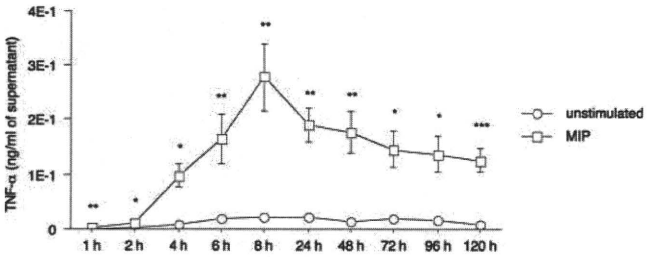

FIGURE 3. Kinetics of TNF-α production by human monocytes/macrophages in response to rMip (1 μg/ml). Each value represents the mean ± SD of triplicates from three experiments. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0001$ determined by comparison with unstimulated, cells at the same time point, using Mann-Whitney U test.

produce cytokines in presence of the bacterial components. As already observed with human monocytes/macrophages, inactivated EB as well as rMip were the most effective stimuli in triggering the synthesis of IL-1β, TNF-α, IL-6, and IL-8. Hsp60 triggered a lower but significant synthesis of IL-1β, TNF-α, IL-6, and IL-8. Hsp70 triggered a very low but significant synthesis of IL-6, and pgp3 and *Chlamydia* LPS stimulated a low IL-8 production, whereas *E. coli* and *S. typhimurium* LPS strongly stimulated the synthesis of all the cytokines (Fig. 4). Because rMip was the only chlamydial Ag that consistently stimulated THP-1 cells, dose-response and time course experiments were performed with rMip. At concentrations ranging from 0.001 to 10 μg/ml, rMip elicited a dose-dependent increase in IL-1β, TNF-α, and IL-8 production with maximal response at 10 μg/ml. The production of cytokines was significantly stimulated when cells were incubated with 1 μg/ml rMip (Fig. 5). When comparing Figs. 2 and 5, the proinflammatory activity of rMip was weaker in PMA-differentiated THP-1 cells than in primary human monocytes/macrophages. This difference might be due to different levels of TLRs or CD14 expression at the surface of the cells. In PMA-differentiated THP-1 cells, significant stimulation of TNF-α release was already detected within 2 h and increased rapidly thereafter. At 24 h, TNF-α levels reached peak values and then declined gradually (Fig. 6).

Because the response of human monocytes/macrophages varied from one donor to another, the proinflammatory activity of rMip was further studied on PMA-differentiated THP-1 cells to obtain more homogeneous and reproducible data. Based on above results and for practical reasons, the effect of rMip was further examined at 1 μg/ml concentration and at the shortest time point (4 h) required to obtain a significant production of TNF-α.

E. coli *LPS contamination is not involved in the production of TNF-α mediated by rMip*

Despite the fact that no endotoxin was detected by the *Limulus* Amoebocyte lysate assay in the highly purified preparation of rMip, the possibility that its proinflammatory activity could be attributed to *E. coli* LPS contamination was further investigated by testing rMip sensitivity to polymyxin B (50 μg/ml). The ability of rMip to induce TNF-α production by PMA-differentiated THP-1 cells was unaffected by the presence of polymyxin B whereas highly purified *E. coli* LPS was unable to induce TNF-α production in presence of polymyxin B. In addition, rMip and LPS differed in their serum requirement: in absence of serum, TNF-α production induced by rMip was significantly increased whereas *E. coli* LPS was devoid of effect as already reported (41–44) (Fig. 7).

Anti-rMip polyclonal IgG partly inhibit the production of TNF-α mediated by C. trachomatis *EB*

The probable involvement of native Mip in initiation of chlamydial infections has been demonstrated by Lundemose et al. (45) who

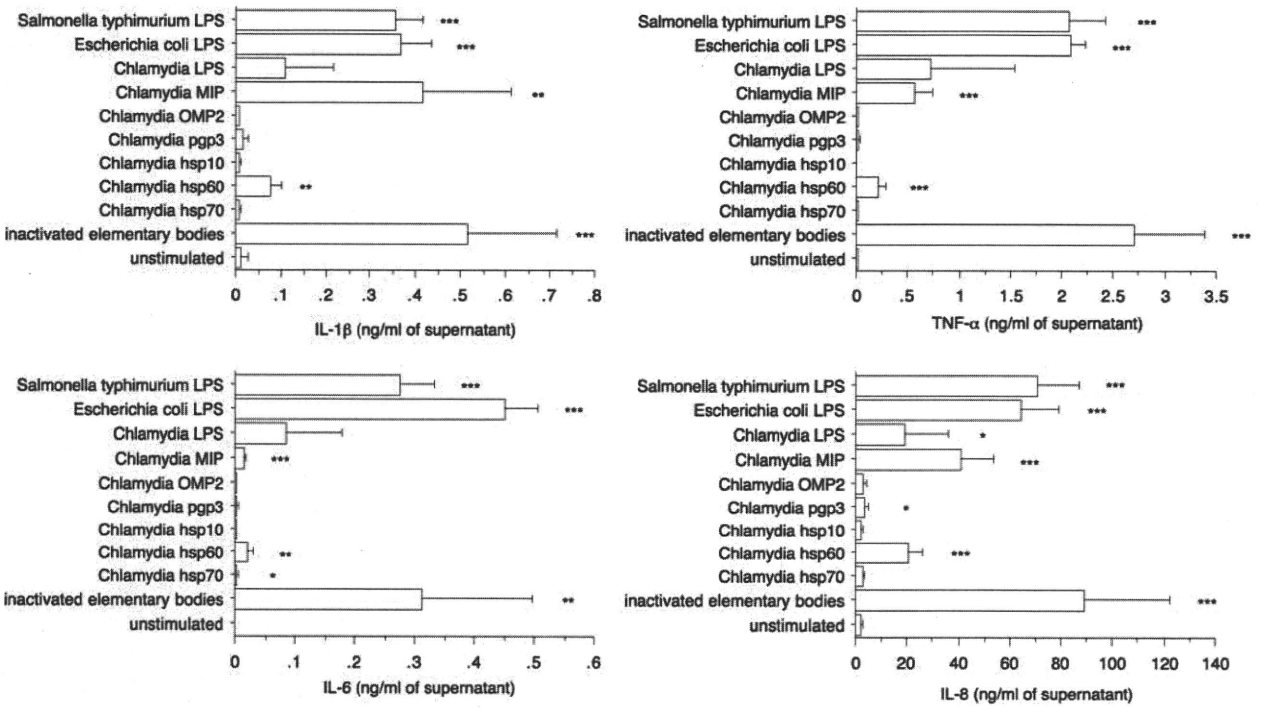

FIGURE 4. Cytokine productions by PMA-differentiated THP-1 cells in response to various bacterial Ags. THP-1 cells (2.5×10^5 cells/1 ml per well) were cultured with 10 nM PMA for 48 h and then stimulated by inactivated *C. trachomatis* EB (5×10^6/ml), rhsp70, rhsp60, rhsp10, rpgp3, rOmp2, rMip, LPS, *E. coli* LPS, or *S. typhimurium* LPS at 1 μg/ml. After 48 h of culture, supernatants were collected and their content in IL-1β, IL-6, IL-8, and TNF-α were analyzed by ELISA. Results were obtained from two different cultures performed in triplicates. Each value represents the mean ± SEM. *, $p < 0.05$; **, $p < 0.005$; ***, $p < 0.0001$ determined by comparison with unstimulated cells using Student's t test.

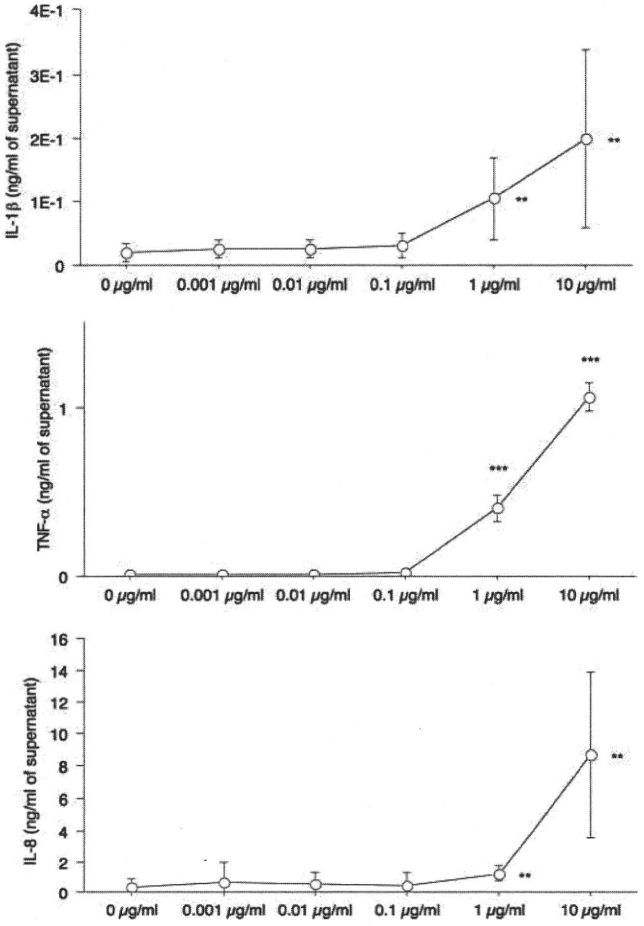

FIGURE 5. Cytokine productions by PMA-differentiated THP-1 cells after 4 h stimulation with increasing concentrations of rMip. Each value represents the mean ± SD of triplicates from three experiments. **, $p < 0.005$; ***, $p < 0.0001$ determined by comparison with unstimulated cells using Student's t test.

observed a neutralization of the organism in cell culture in presence of anti-Mip Ab. To investigate whether the proinflammatory activity of *C. trachomatis* EB could be attributed to the presence of native Mip exposed at the EB surface (20), experiments were conducted in presence and in absence of rabbit polyclonal IgG anti-rMip. The presence of 80 μg/ml IgG anti-rMip led to significant inhibition of TNF-α release (33%, $p = 0.014$) when compared with TNF-α release in presence of preimmune rabbit IgG (Fig. 8). These results show that native Mip exposed at the EB surface

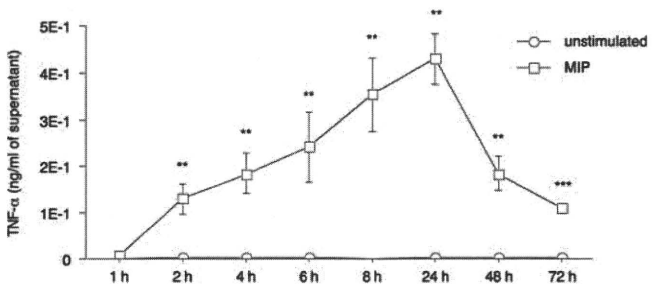

FIGURE 6. Kinetics of TNF-α production by PMA-differentiated THP-1 cells in response to rMip (1 μg/ml). Each value represents the mean ± SD of triplicates from one representative experiment. **, $p < 0.005$; ***, $p < 0.0001$ determined by comparison with unstimulated cells, at the same time point, using Student's t test.

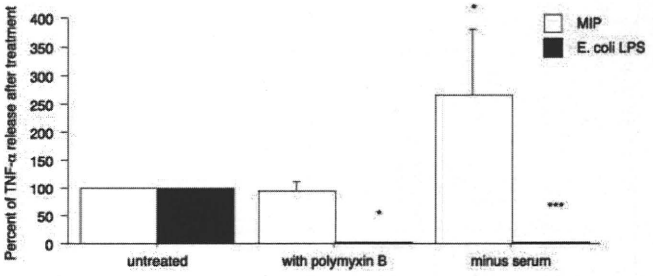

FIGURE 7. Effect of polymyxin B and FBS on the ability of rMip and *E. coli* LPS to induce TNF-α production by PMA-differentiated THP-1 cells. Cells (2.5×10^5 cells/1 ml per well) were cultured with 10 nM PMA for 48 h and then stimulated by rMip or *E. coli* LPS (1 μg/ml) in the presence or absence of 50 μg/ml polymyxin B sulfate, and in the presence or absence of 10% FBS. After 4 h stimulation, supernatants were collected and their content in TNF-α were analyzed by ELISA. Each value represents the mean ± SD of triplicates from two experiments for polymyxin assay and from four experiments for FBS assay. *, $p < 0.05$; ***, $p < 0.0001$ determined by comparison with medium alone using Student's t test.

contributes to induce TNF-α production when PMA-differentiated THP-1 cells are cultured in presence of *C. trachomatis* EB.

Native Mip but not C20A rMip variant elicited proinflammatory cytokine production

To ascertain that wild-type (WT) rMip activity featured the same stimulatory properties than native Mip, this lipoprotein was purified from *C. trachomatis* EB by immunoprecipitation and tested for its ability to induce the production of TNF-α by PMA-differentiated THP-1 cells. In addition, to assess the importance of lipid modification, proinflammatory activity of nonlipidated C20A rMip variant was also tested. As shown in Fig. 9, native Mip was able to induce release of TNF-α and IL-8, in a similar amount than WT rMip. In contrast, no cytokine production was observed when THP-1 cells were cultured in presence of C20A rMip variant suggesting that lipidation plays a major role in proinflammatory activity of native and WT rMip.

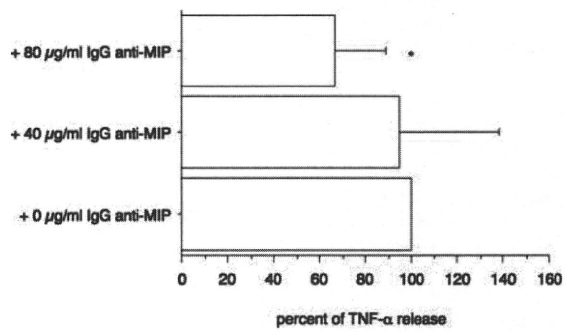

FIGURE 8. Effect of increasing concentrations of anti-rMip polyclonal IgG upon the production of TNF-α mediated by *C. trachomatis* EB. THP-1 cells (2.5×10^5 cells/1 ml per well) were cultured with 10 nM PMA for 48 h, preincubated for 1 h with 50 μg/ml human IgG to block Fc receptors to prevent nonspecific binding of blocking Ab or preimmune IgG, and then stimulated by inactivated *C. trachomatis* EB (5×10^6/ml). Stimulation was performed in presence of 0, 40, or 80 μg/ml of either rabbit polyclonal IgG anti-rMip or preimmune rabbit IgG. After 4 h of stimulation, supernatants were collected and their content in TNF-α were analyzed by ELISA. Results were obtained from two different cultures performed in triplicates. Each value represents mean ± SEM. *, $p < 0.05$ determined by comparison with EB activation in presence of preimmune rabbit IgG.

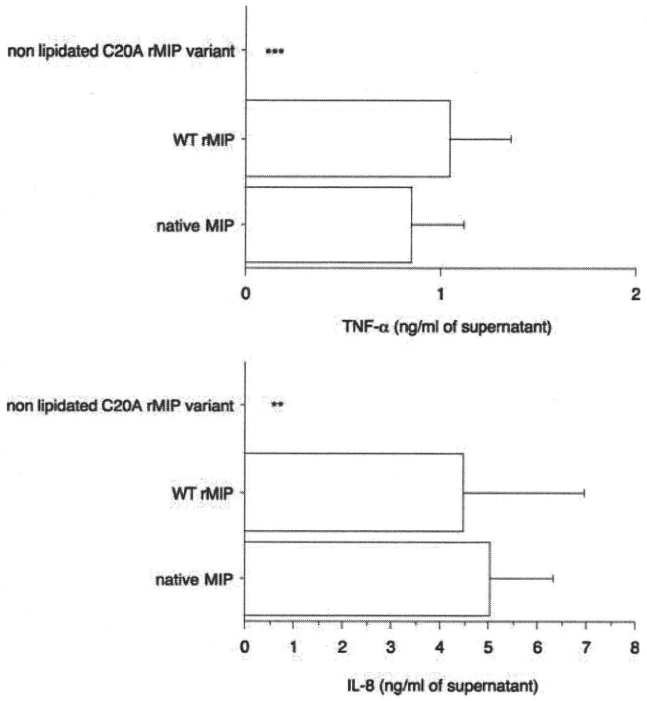

FIGURE 9. Cytokine productions by PMA-differentiated THP-1 cells in response to various Mip preparations. THP-1 cells (2.5×10^5 cells/1 ml per well) were cultured with 10 nM PMA for 48 h and then stimulated by native Mip, rMip, or nonlipidated C20A rMip variant at 1 μg/ml. After 4 h of stimulation, supernatants were collected and their content in IL-8 and TNF-α were analyzed by ELISA. Results were obtained from two different cultures performed in triplicates. Each value represents mean $\pm$ SEM. **, $p < 0.005$; ***, $p < 0.0001$ determined by comparison with native Mip stimulated cells.

Production of proinflammatory cytokines induced by rMip is lipid dependent

Because rMip appeared to have the same proinflammatory activity that native Mip and because it is not feasible to purify adequate amount of native Mip from *C. trachomatis* for analysis, WT rMip, previously shown to be lipidated as native Mip (20), was used for further investigation. To determine the biochemical nature of rMip proinflammatory activity, experiments were conducted to examine the possible involvement of protein and lipid parts of rMip. To determine the importance of the total protein part, attempts to destroy rMip activity by heat, and proteinase K were tested. Heat treatment was unsuccessful, indicating that protein's native conformation is not essential for rMip stimulatory activity. Digestion with proteinase K resulted in loss of the 27.6- and 32-kDa bands (20) (data not shown) but did not affect proinflammatory activity. This result suggests that rMip activity may still reside in resulting lipopeptides after proteolytic digestion, as was already reported for a macrophage-activating lipopeptide from *Mycoplasma fermentans* (46). The involvement of the lipopeptide moiety was confirmed by three different treatments releasing ester-linked fatty acids. Alkaline hydrolysis completely abolished rMip activity and treatment with lipases from two different sources led to significant losses of TNF-α release (76% with pig pancreas and 78% with *R. arrhizus* lipases, $p < 0.05$) (Fig. 10). No significant difference was observed between untreated and mock-treated PMA-differentiated THP-1 cells (data not shown). The substitution of rMip with fatty acids in an ester linkage, alkali-labile (47), appears therefore to be crucial for its proinflammatory activity, as reported for other lipoproteins or lipopeptides (33, 48–51). These data confirm that

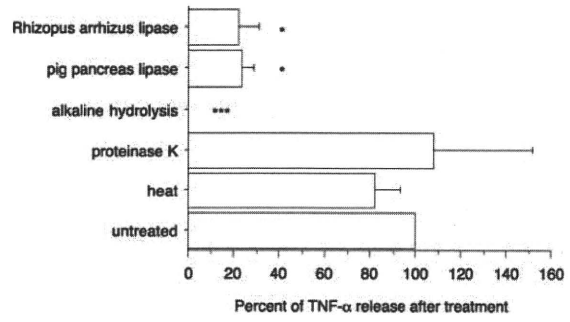

FIGURE 10. Effect of heat, proteinase K, alkaline hydrolysis, and lipase treatments of rMip on TNF-α production by PMA-differentiated THP-1 cells. Cells were stimulated by untreated or treated rMip (1 μg/ml). rMip was either heated at 100°C during 3 h, or incubated at 37°C for 2 h with proteinase K (100 μg/ml), or treated for 1 h with sodium hydroxide (pH 13) before HEPES neutralization (33), or incubated at 37°C for 16 h with pig pancreas or *Rhizopus arrhizus* lipases. Digests were either treated by 200 μM PMSF or heated at 100°C before assay. Other conditions were described above. Each value represents the mean $\pm$ SD of triplicates from two experiments. *, $p < 0.05$; ***, $p < 0.0001$ determined by comparison with untreated rMip using Student's t test.

lipid modification of rMip is essential for its ability to stimulate production of proinflammatory cytokines by PMA-differentiated THP-1 cells.

CD14, TLR1, TLR2, and TLR6 but not TLR4 are involved in rMip activation of THP-1 cells

If rMip is able to induce proinflammatory cytokines in human monocytes/macrophages, the specific host receptors that mediate its activation is unknown. As TLRs and CD14 are usually involved in bacterial lipoprotein recognition (52), experiments were conducted in the presence and in the absence of function-blocking mAbs against CD14, TLR1, TLR2, TLR4, and TLR6. These receptors are expressed on cell surface of both human monocytes and monocytic cell line THP-1 (29, 53, 54). The pretreatment of THP-1 cells with MY4, an Ab blocking the mCD14-part of the LPS receptor (55), led to significant inhibition ($p < 0.05$) of rMip-mediated TNF-α production, indicating that rMip binds to mCD14 at the LPS-binding site as already observed for spirochaetal lipoproteins (53, 56, 57). The pretreatment of THP-1 cells with TL2.1, a specific blocker of extracellular human TLR2 (58) or GD2.F4, a mAb blocking TLR1 (59), led to significant inhibitions ($p < 0.05$) of rMip-mediated TNF-α production, whereas no significant effect was observed neither in presence of HTA125, an anti-TLR4 blocking mAb (60, 61) nor in presence of the clone 127, an anti-TLR6 blocking mAb (29). The combination of anti-TLR2 with either anti-TLR6 or -CD14 led to clear inhibitions ($p < 0.005$), and the combination of anti-TLR2 with anti-TLR1 to an almost complete inhibition ($p < 0.0001$) of TNF-α secretion, as shown in Fig. 11*A*. When inactivated *C. trachomatis* EB were used as stimulant, the combination of anti-TLR2 with anti-CD14, anti-TLR1, or anti-TLR6 induced significant inhibitions ($p < 0.05$ to 0.005), whereas the combination of anti-TLR4 with anti-CD14 provoked no significant reduction of EB-mediated TNF-α synthesis, as shown in Fig. 11*B*. Because TLR1 and TLR2 have been shown to be required for recognition of triacylated lipopeptides, such as Pam_3CSK_4 (62–64) whereas TLR6 and TLR2 were required for recognition of diacylated lipopeptides such as Pam_2CSK_4 but not for recognition of triacylated lipopeptides (63, 65), these two prototypic synthetic lipopeptides were used as controls and tested in the same conditions. When Pam_3CSK_4 was used as stimulant, the pretreatment of THP-1 cells with the combination