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**Inflammatory lipoproteins purified from a toxigenic and arthritogenic strain of
Mycoplasma arthritidis are dependent on TLR2 and CD14.**

Running title; TLR2 and CD14 dependent mycoplasmal lipoproteins.

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Abstract

Mycoplasma arthritidis is a naturally-occurring murine pathogen and the disease model has been used extensively to understand inflammatory mechanisms. Recently, Triton X-114 extracts of a virulent strain of *M. arthritidis* were found to be more potent in activating macrophages than were those from an avirulent strain suggesting a role in disease. Here, octyl glucoside extraction of cells was used to identify four distinct bioactive moieties with molecular masses of ~ 41, 37, 34 and 17 kDa. Their bioactivities were resistant to proteinase K but were destroyed by alkaline hydrolysis and oxidation. As for MALP-2, all were dependent upon TLR2 but unlike MALP-2, they were also dependent upon CD14. The *M. arthritidis* lipoproteins exhibited IR absorbances at 2900 and 1662 cm⁻¹ similar to those seen in Pam3-Cys-Ser-(Lys)₄. Edman degradation failed to reveal N-terminal sequences suggesting that they were blocked and therefore might be tri-acylated. However, mass spectrometry of fragments revealed that the 41kDa moiety had similarity with the recently described MlpD, lipoprotein of *M. arthritidis* and possessed an ability to bind to serum apolipoprotein A-1.

Introduction

Mycoplasmas, or Mollicutes, are the smallest self-replicating prokaryotes and are common parasites of humans, animals, and plants. *M. arthritidis* infections in rodents serve as animal models for a chronic arthritis that closely resembles human rheumatoid arthritis (RA), necrotizing fasciitis-like syndrome, and acute toxic shock syndrome (6). Virulence factors include a superantigen (SAg), the *M. arthritidis* mitogen (MAM, (2) which utilizes Toll-like receptor (TLR)2 and TLR4 (14), two membrane lipoproteins, Maa1 (32) and Maa2 (34), and bacteriophage MAV1 (28). MAM is a potent activator of T-cells, B-cells, and macrophages (2). Although MAM can induce arthritis when directly injected into rat joints, systemic administration into rodents is without significant clinical effects. However, major changes in immune function do occur after intravenous injection of MAM (15) and MAM can trigger and exacerbate autoimmune collagen-induced arthritis (3). The inability of MAM to cause overt clinical disease by itself, supports the notion that other virulence factors participate in the inflammatory effects of whole organisms.

We recently reported that Triton X-114 (TX-114) extracts of whole cells of a virulent strain of *M. arthritidis* contained components that activated macrophages through TLR2 and caused dendritic cell maturation as evidenced by upregulation of surface expression of class II MHC, CD40, B7-1 and B7-2 (4). These activities were not due to contamination with MAM or lipopolysaccharide (LPS) and were significantly less potent in extracts from an avirulent strain of *M. arthritidis*, suggesting an association with disease. Other mycoplasmas have also been shown to possess macrophage-activating components,

1 including MALP-2 ((16) a lipopeptide derivative of MALP-404 from *M. fermentan*
2 fibroblast- and macrophage-stimulating lipopeptide FSL-1 (23) from *M. salivarium*, and a
3 partially purified 29 kDa lipoprotein from *M. hominis* (19). The role of these potential
4 inflammatory molecules in clinical disease remains to be defined in part because of the
5 absence of a suitable animal model. However, such a model is available for *M. arthritidis*
6 (2, 5); prior to initiating these studies the first goal would be to purify and characterize the
7 bioactive components of this organism. In the present study we purify four macrophage-
8 activating components from *M. arthritidis* the activities all of which are TLR2- and CD14-
9 dependent. We also show that one, OGex A (see below), which has binding avidity for
10 serum apolipoprotein A-1 (9), is related to the MlpD, MlpE, and MlpF lipoproteins of
11 unknown function, recently described by Washburn et al. (31, 35).

14 **Materials and Methods**

15 **Organism and culture conditions.** *Mycoplasma arthritidis* 158p10p9 (8) was
16 grown in modified Edward medium (7) consisting of PPLO broth supplemented with 15%
17 (vol/vol) heat inactivated horse serum, 1.5% yeast extract (vol/vol, Invitrogen Corp),
18 0.25% (wt/vol) L-arginine HCl, 0.001% (wt/vol) NAD, and 500 U of penicillin G per ml.
19 To adapt the organisms to a serum-free medium, the serum content was gradually reduced
20 to 0% and was replaced with 3% (wt/vol) BSA and 0.4% (vol/vol) of a 250 x cholesterol
21 lipid concentrate (Invitrogen) as based on that previously (30). Cultures of *M. arthritidis*
22 grew up to 2×10^8 CFU/ml after 48-72 h incubation in serum-free broth, i.e.~ ten-fold less

1 than in Edward medium. The organisms were harvested by centrifugation at 27,000 x g for
2 30 minutes, washed three times with normal saline (NS), concentrated 100-fold and frozen
3 at -70 °C until use.

4 **Chemicals, enzymes and antibodies.** Pam3-Cys-Ser-(Lys)4 (Pam3CSK4), a
5 synthetic bacterial lipopeptide analogue from *E. coli* (1); MALP-2, a 2-kDa synthetic
6 lipopeptide from *M. fermentans*, and LPS from *E. coli* R515 were purchased from Alexis
7 Biochemicals (San Diego, CA). Endotoxin-free NS was from Baxter Healthcare (Dearfield,
8 IL). Polymyxin B, n-octyl- β -glucopyranoside (OG), and proteinase K, PK-506 (PK) were
9 from Sigma, St. Louis, MO. Monoclonal antibodies (mAb) against the Maa1 or Maa2
10 lipoproteins from *M. arthritidis* were prepared as described (29). HRP-conjugated anti-
11 mouse IgG was from eBioscience (San Diego, CA). Fluorescence-conjugated anti-human
12 CD25 mAb (clone M-A251) and purified rat anti-mouse CD14 mAb (clone 4C1/CD14)
13 were from BD biosciences.

14 Mouse antisera were prepared against five recombinant *M. arthritidis* lipoproteins,
15 MlpA, MlpC, MlpD, MlpE, and MlpF (31, 35). Mlp genes, minus their signal peptide-
16 encoding regions, were amplified by PCR with primers placing restriction sites at 5' and 3'
17 ends. PCR primer sequences for Mlps are listed in Table 1. Amplification conditions were
18 as described (34). Amplicons were inserted into the appropriate pRSET vectors
19 (Invitrogen); recombinant proteins were expressed in *Escherichia coli* BL21 (DE3)pLysS
20 and purified as described (Invitrogen). Protein emulsions in incomplete Freund's adjuvant,
21 were injected subcutaneously at ~50 μ g into each of five male BALB/c mice. Mice were

1 boosted two weeks later by intraperitoneal injection of 50 µg in H₂O. Antibody was
2 monitored by ELISA. Pooled sera were collected from each group.

3 **Mice.** Female C57BL/6 and C57BL/6 CD14 (-/-) KO mice were purchased from
4 Jackson Laboratory (Bar Harbor, ME); C57BL/6 TLR2 (-/-) KO mice were from Dr.
5 Thomas Hawn (University of Washington School of Medicine, Seattle, WA) courtesy of Dr.
6 Shizuo Akira (Osaka University, Japan). All KO mice were bred in the animal care facility
7 of the University of Utah under specific-pathogen-free conditions in compliance with the
8 Animal Welfare Act, and were used at 8 to 12 weeks of age. BALB/c mice for production
9 of anti-Mlp antisera were purchased from Harlan Sprague-Dawley (Indianapolis, IN) and
10 housed in the University of South Dakota animal care facility.

11 **Cell culturing for cytokine induction and ELISA.** Mouse peritoneal adherent
12 cells and mouse macrophage RAW 264.7 cell lines were prepared as described (15).
13 Levels of TNF-α, IL-6 and/or IL12p40 in culture supernatants were determined by using
14 ELISA kits purchased from eBioscience or BD Biosciences. For each experiment, triplicate
15 cell suspensions were treated with each agonist or control prior to ELISA and results were
16 expressed as means +/- SD. CHO cells stably transfected with murine CD14 and ELAM-
17 CD25 and with TLR2 were provided by Dr. D. Golenbock (University of Massachusetts
18 Medical Center, Worcester, MA) courtesy of Dr. Janis Weiss (University of Utah Health
19 Sciences Center, UT) and were used as described (14).

20
21 **Isolation and Purification of the bioactive components.** The lipoprotein fraction
22 was extracted from *M. arthritidis* by OG as previously described (23, 24). Briefly, OG-

1 extracted (lipo)proteins (OGex) were separated on a 10% SDS-PAGE gel and were
2 transferred to a 0.45- μ m-pore-size cellulose nitrate membrane (Bio-Rad) which was cut
3 into 2-mm strips, that were each dissolved in 1 ml dimethylsulfoxide. The (lipo)protein-
4 coated particles, formed by dropwise addition of 3 ml 0.05 M sodium carbonate buffer
5 (pH 9.6) to the dissolved membrane, were washed three times with NS, and tested for
6 ability to induce RAW 264.7 cells to secrete TNF. SDS-PAGE of extracted strips was
7 repeated and the gel was stained with zinc (Bio-Rad); the bioactive (lipo)protein bands
8 were excised and the gel slices eluted by Electro-Eluter (Bio-Rad). Resulting lipoproteins
9 were precipitated and washed with ice-cold acetone; purity was confirmed by SDS-PAGE
10 stained with silver.

11 **Characterization of lipoproteins.** The effect of proteolytic digestion on bioactivity was
12 determined by incubating lipoproteins at 1 μ g/ml with 5 μ g/ml proteinase K (PK-5056,
13 Sigma; PK) for 1 hr at 37°C followed by 100°C for 15 min to inactivate the enzyme. The
14 susceptibility of lipoproteins to alkaline hydrolysis indicating acyl groups and to oxidation
15 indicating presence of thioesters was performed as described (4). Infra-red (IR) absorbance
16 spectra to identify bioactive groups, was performed on dried fractions of each lipoprotein
17 in KBr pellets using a Bruker, Model IFS 88 spectrometer. Flow cytometry to determine
18 TLR-2 expression on transfected CHO cells exposed to agonists was as previously
19 described (4).

20 Partial amino acid sequences were examined by Edman degradation using lipoproteins
21 blotted onto Immobilon PVDF membranes that were analyzed by an ABI Procise sequencer
22 (Applied Biosystems, Foster City, CA) or by mass spectrometry of tryptic digests using an

ion-trap mass spectrometer (LCQ-Decca; Thermo/Finnigan Corp., Mountain View, CA).
DNA sequencing of genes encoding Mlps was performed by the Iowa State University
DNA Synthesis and Sequencing Facility.

Western and dot blot analyses were performed with polyvinylidene difluoride
(PVDF) membrane (Millipore, Bedford, MA), HRP-conjugated anti-mouse IgG
(eBioscience) and chemiluminescent substrate (Pierce).

RESULTS AND DISCUSSION

Isolation and purification of bioactive lipoproteins from *M. arthritidis*

The OG lipoprotein fraction (OGex) from washed cells of the virulent 158p10p9 strain of
M. arthritidis induced TNF α production by murine RAW 264.7 macrophages in a dose-
dependent manner, with a concentration of 0.2 to 1.0 μ g/ml being optimal (Fig. 1A). The
activity in the extracts was not due to contamination with endotoxin or the MAM SAg as
determined by methods previously described (4).

SDS-PAGE of OGex revealed the presence of seven major bands (Fig. 1B). When the
gel was blotted to a cellulose nitrate membrane and strips therefrom extracted, the TNF α -
inducing bioactivity was found in two major areas corresponding to approximately 42 to
32 kDa and 20 to 14 kDa, the latter comprising the dye front. (Fig 1B). Bands occurring in

1 the regions of activity were identified by negative zinc staining and were extracted by
2 electro-elution. Repeat SDS gels and extraction revealed the presence of 4 homogeneous
3 components that corresponded to mol masses of ~ 41, 37, 34 and 17 kDa (Fig 1C) which
4 we designated as OGex A, B, C and D, respectively. All four purified lipoproteins activated
5 RAW.264.7 cells in a dose-dependent manner with activity levels being similar (Fig 1 D).

7 **Characterization of *M. arthritidis* lipoproteins**

8 Before characterizing these moieties in more detail, we first tested whether they might
9 be related to the Maa1 and Maa2 *M. arthritidis* lipoprotein adhesins, virulence factors for
10 disease caused by *M. arthritidis* (33) . We concluded that there was no relationship since
11 the molecular masses of Maa1 and Maa2 were markedly greater (86 and 56 kDa,
12 respectively), and Western blotting of OGex and each of OGex A, B, C, and D with
13 specific polyclonal anti-sera to Maa1 and Maa2 provided no evidence of cross-reactivity
14 (data not shown). Also, peptide fragments of OGex lipoproteins did not match adhesin
15 sequences in the Mascot data base (see below).

17 Since a number of studies have shown that the bioactivities of lipoproteins derived from
18 mycoplasmas or other bacteria, (10, 16, 23) are borne on small lipopeptides, we treated the
19 *M. arthritidis* lipoproteins with PK or NS as a control. The protein bands of all moieties
20 were totally digested by PK (Fig. 2A) and the all of the bioactivity now migrated to the dye
21 front on blotted and extracted gels (Fig 2A). Importantly PK treatment did not decrease the

1 bioactivity of the lipoproteins as compared with that seen for NS-treated lipoproteins (Fig
2 2B).

3
4 To confirm that the active moieties were associated with lipids, we demonstrated that all
5 four purified components were susceptible to alkaline hydrolysis indicating a role for fatty
6 acid acyl groups (Fig 2C), and were also susceptible to oxidation with H₂O₂ indicating
7 involvement of thioesters (Fig 2D). The presence of these bioactive lipid groups was
8 confirmed by IR spectrometry by showing that as for the bioactive synthetic Pam3CSK4
9 lipopeptide based on the bioactive region of a lipoprotein derived from *E. coli*, the spectra
10 of OGex A, B, C, and D exhibited absorbances at ~1,700 cm⁻¹ and ~2,900 cm⁻¹ (Fig 3),
11 again suggesting the presence of acyl chains and thioester bonds, respectively, that are
12 characteristic of microbial lipoproteins (10). Interestingly, the overall profiles of the four
13 lipoproteins were quite similar although not identical.

14
15 **Macrophage activation by *M. arthritidis* lipoproteins is dependent on TLR2 and CD14**
16 **co-receptors.**

17
18 A wide range of bioactive bacterial lipoproteins are known to be dependent upon TLRs
19 (11, 13, 25) and preliminary findings also suggested that the bioactivity of TX114 extracts
20 of *M. arthritidis* might be also dependent upon TLR2 (4). To determine TLR usage by the
21 purified lipoproteins of *M. arthritidis*, we tested their ability to up-regulate CD25 on CHO
22 cells transfected with TLR2 and CD14, which act as an indicator of cell activation of NF-

κB through TLR. CD25 expression on these CHO cells was up-regulated by MALP-2 as well as all OGex moieties indicating activation through TLR2 (Fig 4). We also demonstrated that whereas peritoneal macrophages from wild-type C57BL/6 mice produced high levels of TNFα, those from C57BL/6 KO mice failed to induce significant amounts (P< 0.0005). In contrast, the levels of TNFα induced by LPS, which utilizes TLR4, were not significantly different (P> 0.9) in macrophages from either TLR2+/+ or TLR2-/- mice (Fig. 5A).

Toll-like receptors are now known to be key molecules in innate immunity, recognizing pathogen-associated molecular patterns on microbial as well as endogenous agonists (13) (26). Microbial lipoproteins such as those from *Trepanema pallidum*, OSPA from *Borrelia burgdorferi* (22), possibly a lipoprotein of *M. penetrans* (24), and Pam₃CSK₄ from *E. coli* (1) are triacylated. The triacylated lipoproteins possess two acyl fatty acids attached to the cysteine residue at the N-terminus of the molecule through a sulfhydryl group and the third added to the free N-terminus by acyl transferase during cleavage of the mature molecule from the signal peptide (20). In the case of MALP-2 from *M. fermentans* (16), and probably also the FSL lipopeptide from *M. salivarium* (23), the third acyl group is missing, due to absence of acyl transferase. Characteristically, triacylated lipoproteins use TLR1 with TLR2 and diacylated lipoproteins use TLR6 with TLR2 although recent studies indicate that the N-terminal cysteine peptide region can also influence TLR usage (18) (27). In addition, at least one other co-receptor may be required for agonist recognition since triacylated lipoproteins and LPS also require CD14 (12), (17, 22). The requirement for

CD14 by diacylated lipoproteins has been more controversial (17, 21; Muhlradt, personal communication).

In view of the above, several approaches were used to determine the comparative usage of CD14 by the OGex lipoproteins and MALP-2 using LPS as a positive control for CD14 usage. First, we examined the effect of anti-CD14 antibody on TNF α induction by each of the agonists. In a preliminary dose/response experiment, we established that 10 μ g/ml of anti-CD14 antibody was the lowest concentration that reduced TNF α levels induced by OGex to background levels. Anti-CD14 antibody alone failed to influence spontaneous TNF α release by cells in the absence of stimulants. However, antibody to CD14 which, as expected, completely inhibited TNF α production by murine RAW 264.7 in response to LPS ($P < 0.0005$), also significantly inhibited ($P < 0.0005$) TNF α production by the OGex A, B, C, and D lipoproteins. In contrast, the response to the MALP-2 lipopeptide was not significantly inhibited ($P > 0.05$) by antibody to CD14 (Fig. 5B), even when doses as low as 0.25ng/ml of MALP-2 were used (data not shown). To confirm this observation, we compared TNF α production from peritoneal macrophages isolated from C57BL/6 wild-type mice versus those from C57BL/6 CD14 KO mice (Fig 5C). Absence of CD14 virtually abolished the ability of the *M. arthritidis* lipoproteins ($P < 0.02$) and LPS ($P < 0.05$) to induce TNF α in resident peritoneal macrophages, whereas the response to MALP-2 was not decreased by absence of CD14 ($P > 0.05$). We conclude that cytokine induction by OGex lipoproteins is CD14-dependent and thus quite unlike the MALP-2 di-acylated lipopeptide which, in our hands, does not require CD14.

Relationship of OGex A, B, C and D with other lipoproteins. Edman degradation to derive N-terminal sequences of OGex A, B, C, and D failed suggesting that the N-terminal cysteine was blocked and thus, likely tri-acylated. This finding supports our results described above indicating that the OGex lipoproteins require CD-14. Trypsin digests of OGex A, B, C and D were also analyzed by mass spectrometry for internal fragment sequences that matched proteins in the MASCOT data base (Fig. 6). Two fragments were found in OGex A (LLELNILK and LLELNDLK) that were identical to two sequences in *M. arthritidis* MlpD and, except for a single amino acid, in MlpE, formerly L-Rep, (31). Similar sequences were subsequently found in the newly identified lipoprotein, MlpF (Fig. 6). These three Mlps share extensive DNA and amino acid sequence homology and contain two domains of tandem, nonidentical repeats. The OGexA-like sequences are located near the beginning of repeat units in the second domain. Although we obtained some information on peptide fragment sequences from OGex B, C and D, their amino acid sequences did not match any in the MASCOT databases.

To confirm the relatedness between OGex A and the Mlp lipoproteins, OGex A, B, C and D were blotted onto PVDF membranes and tested for their reactivity to anti-MlpA, -C, -D, -E, and -F antibody and to pre-immune sera. OGex A reacted with anti-MlpD, -MlpE and -MlpF but OGex B, C, and D not react and neither did the pre-immune sera. The results indicate that OGex A is closely related to the MlpD, MlpE, and MlpF lipoproteins of *M. arthritidis*, but that MlpA, MlpB, and MlpC might be distinct.

The bioactive lipid moieties of microbial lipoproteins are characteristically borne on the N-terminal cysteine residue of the molecule. An analysis of the sequences of the mature

MlpD, MlpE, and MlpF lipoproteins revealed that all possessed only one cysteine residue and that the adjacent region, which in MlpD was “CDNTNKKLEEPKKE”, was highly conserved with MlpE and MlpF. We therefore propose that this region likely represents the bioactive lipopeptide contained in OGex A. Interestingly, although MlpA, MlpB, and MlpC share less sequence with the other Mlps they all bear N-terminal cysteine regions which are very similar to those in MlpD, MlpE and MlpF. This raises the possibility that all of the Mlp family of lipoproteins may have bioactivity mediated by a common lipopeptide. Inasmuch as MlpA, -B, and -C exhibit molecular masses similar to OGex D, B and C, we cannot exclude the possibility that they may in fact be related despite our preliminary failure to detect sequence similarity by mass spectrometry.

Concluding remarks.

These studies have established that *M. arthritidis* possesses multiple macrophage-activating lipoproteins that appear to be related to the Mlp family of lipoproteins (31). Furthermore, the OGex moieties appear to be quite distinct from the MALP-404 lipoprotein of *M. fermentans* and its synthetic lipopeptide derivative, MALP-2, in that they are likely tri-acylated and have a requirement for CD14 whereas the MALP molecules, are di-acylated and, are independent of CD14 for cytokine induction. Although the function of the *M. arthritidis* Mlp lipoproteins was recently considered to be unknown (31), the present study suggests that they have inflammatory potential and thus might play a role in disease mediated by this organism. Preliminary studies have indeed shown an association with the potency of the lipoproteins and organism virulence (4). Of importance is their dependency

1 on TLR2, a molecule that is instrumental in *M. arthritidis* -mediated inflammatory disease
2 (14, 15). In this regard it should be re-emphasized that the *M. arthritidis* SAg MAM can
3 also not only regulate the immune system through TLR2, but also through TLR4 (14). The
4 additional finding that MAM engagement of TLR4 can down-regulate inflammatory
5 disease mediated by *M. arthritidis* (Cole and Mu, unpublished observations) suggests that
6 both MAM and the OGex lipoproteins play a significant role in disease pathogenesis.
7 Furthermore, concurrent work has demonstrated that OGex A lipopeptides can bind to
8 apolipoprotein A-1, a component of HDL cholesterol, also suggests a mechanism whereby
9 microbial agonists might render host lipoproteins inflammatory, a process which in this
10 case may contribute to atherosclerosis (9).

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Table 1. PCR primers for *mlp* gene amplification.

primer	sequence (5'-3')^a	Mlp reference or accession number
<i>mlpA-f</i>	CTC <i>ggatcc</i> GCGATAATACCGAGAAAAAGCC	unpublished AF154924 (OrfA)
<i>mlpA-r</i>	CAG <i>gaattc</i> TTTCTTGCTAAAGAACCTTG	
<i>mlpC-f</i>	CAG <i>ggatcc</i> GATAATACTGATAAAAAACTAG	(31)
<i>mlpC-r</i>	CTG <i>gaattc</i> CATTGCTATTTTGTCTGTTTCTCC	
<i>mlpD-f</i>	CGC <i>cagctg</i> ATAATACTAATAAAAACTAGAAGAAC C	(31)
<i>mlpD-r</i>	CTATTTGG <i>aagctt</i> CTATAGC	
<i>mlpEF-f</i> ^b	CGG <i>ctgcag</i> ATAATACATATAAAAAAC C	AY544183
<i>mlpE-r</i>	GGC <i>ggtacc</i> CTATTTTGAAGCTTCTTCGGC	
<i>mlpF-r</i>	GGC <i>gaattc</i> TTATTGTTTAAATTCTTTAAC	DQ451673

^a Restriction sites added by PCR (except for *mlpD-r*) for cloning into expression vectors are italicized and in lower case. The *Hind*III site in *mlpD-r* occurs naturally at the 3' end of *mlpD*.

^b The same forward primer was used for both *mlpE* and *mlpF*.

Figure legends

Figure 1. Purification of bioactive components from *M. arthritidis*. The following purification data are representative of repeated OG extractions on two separate batches of organisms. **(A)** Dose dependency of OGex induction of TNF- α in murine RAW 264.7 cells. **(B)** SDS-PAGE of OGex. Lane 1, protein standards; lane 2, OGex. Gel was blotted to cellulose nitrate, 2mm strips were excised, dissolved in DMSO, precipitated, and the (lipo)protein-coated particles, tested for TNF- α production in culture supernatants of RAW cells. **(C)** SDS-PAGE of purified lipoproteins stained with silver. Lane 1, protein standards; lane 2, OGex A; lane 3, OGex B; lane 4, OGex C; lane 5, OGex D. **(D)** Dose response of TNF- α production by purified OGex A, B, C and D in RAW 264.7 cells.

Figure 2. Properties of OGex lipoproteins. **(A)** SDS-PAGE of OGex. Lane 1, protein standards; **lane 2**, OGex treated with PK. PK-treated OGex were blotted to cellulose nitrate strips extracted and tested for activity which migrated to the dye front. **(B)** OGex were incubated with NS or with PK and after heating to 100C for 15 min were assayed for ability to induce TNF α in RAW cell cultures. **(C)** Effect of alkaline hydrolysis on the activity of 50ng /ml of OGex A, B, C and D as indicated by the ability to stimulate TNF α production by RAW 264.7 cells. **(D)** Effect of H₂O₂ treatment on the bioactivity of OGex A, B, C and D. Tests for lipid groups in Figs (C) and (D) were assayed on three separate cell suspensions stimulated with each of the treated OGex lipoproteins. Mean results +/- SD are shown.

Figure 3. IR spectra of Pam3CSK4, OGex A, B, C and D. White arrow shows the signal about 2900 cm^{-1} , suggesting the presence of fatty acid acyl chains. Black arrow shows the signal about 1700 cm^{-1} , suggesting the presence of ester bonds.

Figure 4. Flow cytometric analysis of CHO/TLR2 cells. The cells were stained with FITC-labeled anti-human CD25 mAb and analysed by flow cytometry for the expression of the CD25 transgene, an indicator of TLR activation. The activation of cells was expressed by mean fluorescence intensity (MFI). Thin lines, no Ab; broken lines, isotype control Ab; thick lines, anti-human CD25 mAb.

Figure 5. (A) TNF- α production by peritoneal macrophages of C57BL/6 mice or C57BL/6 TLR2KO mice stimulated with OGex A, B, C, D, LPS or NS. The concentration of OGex A, B, C, D and LPS were 100 ng/ml. Peritoneal adherent cells were stimulated for 18 h with inducers, and the amounts of TNF- α in cell culture supernatants were tested by ELISA. The data is representative of 2 experiments using 4-5 mice in each. The experiment was repeated twice. **(B)** RAW 264.7 cells were pre-incubated with NS, isotype control Ab or anti-mouse CD14 mAb (10 $\mu\text{g/ml}$) for 1 h. Then, they were stimulated with LPS (10 ng/ml), MALP-2, OGex A, B, C or D (20 ng/ml) for 18 h. **(C)** Peritoneal macrophages from C57BL/6 or C57BL/6 CD14 KO mice were stimulated with 50 ng/ml LPS, MALP-2 or OGex A, B, C, or D and culture supernatants tested for the presence of TNF α . Representative data from two similar experiments is shown using 4-5 mice in each.

Figure 6. Amino acid sequence comparisons between OGexA fragments and Mlps D, E, and F. Amino acid residues identical to one or both of the OGexA peptides are shaded black.

Figure 7. Dot blot analysis of OGex A, B, C and D. Recombinant Mlp (rMlp) A, C, D, E, F lipoprotein, OGex A, B, C and D were placed on a PVDF membrane and detected by anti Mlp anti serum. **(A)** Schema of protein Spotting patterns. **(B)** PVDF membranes of dot blot analysis. Antigens were detected by anti Mlp anti serum. The amount of protein was 2µg/dot, respectively. The experiment was conducted 3 times using different antigen concentrations but with similar results.

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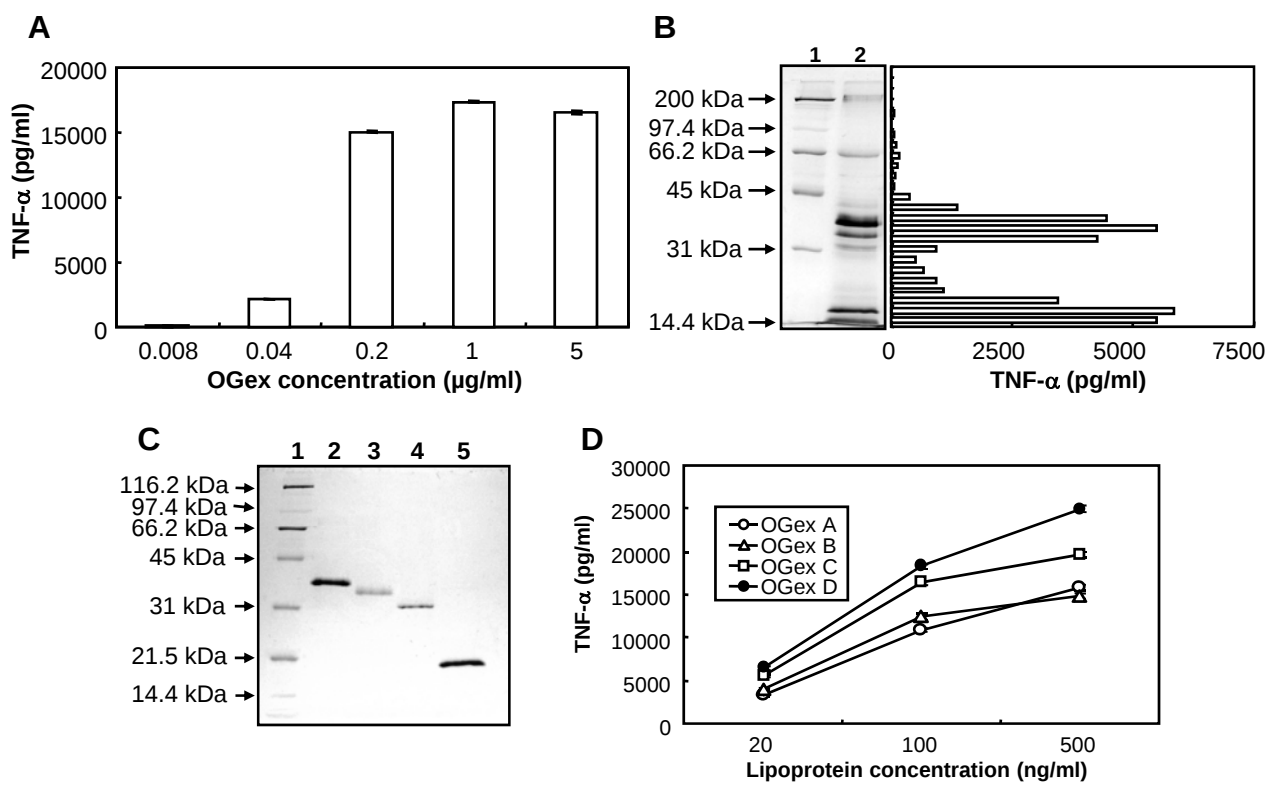


Fig. 1

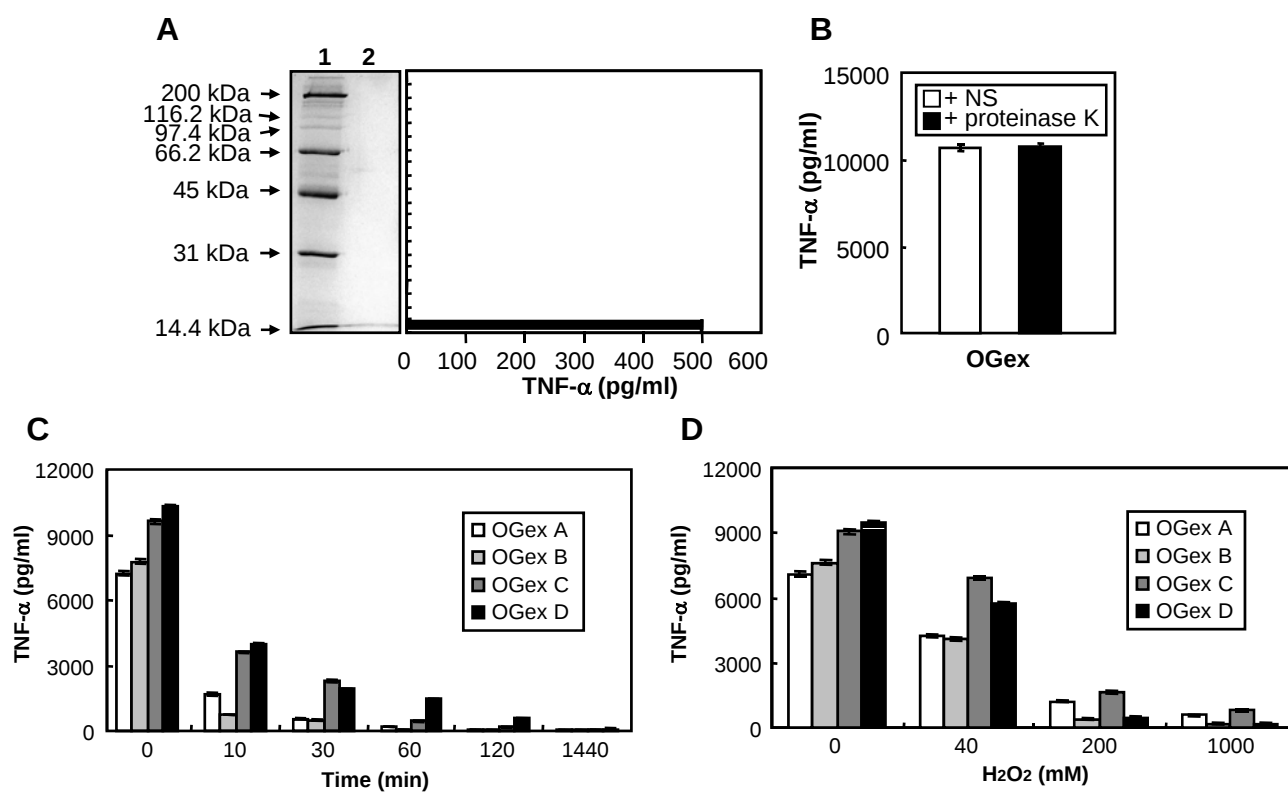


Fig. 2

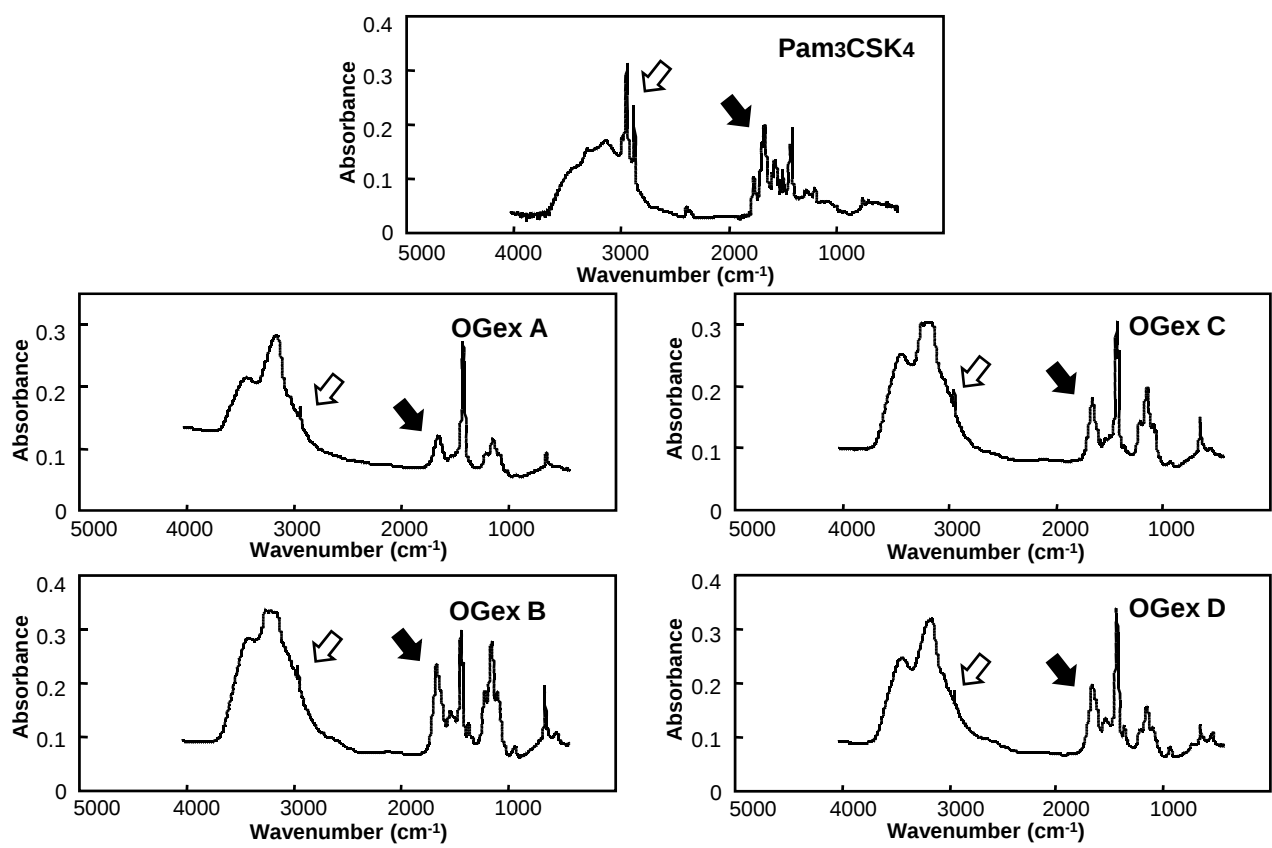


Fig. 3

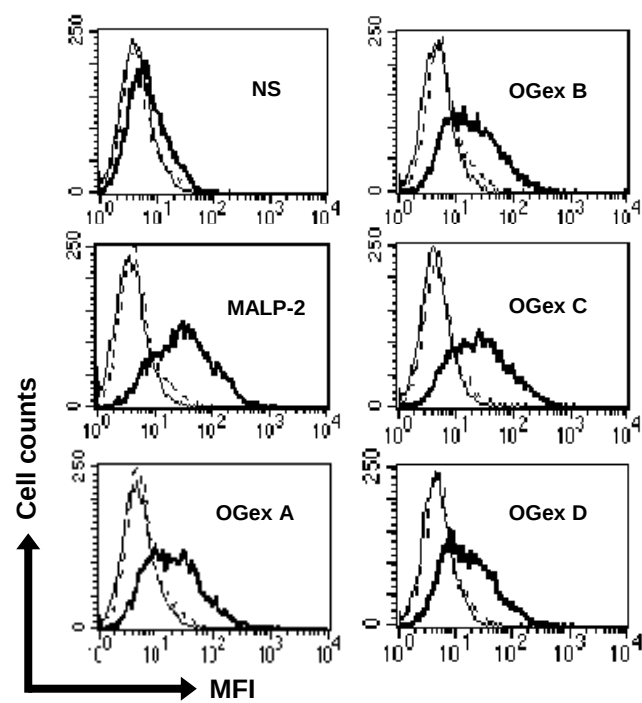


Fig. 4

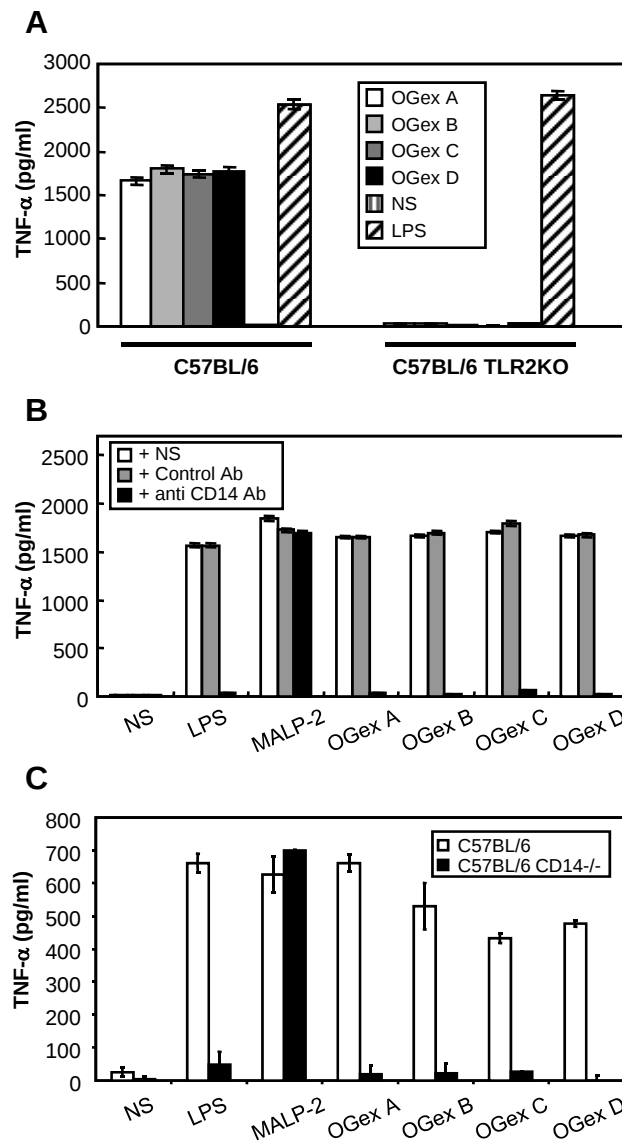


Fig. 5

MlpD repeat 1:	EERLLELNILKKEIAKK
MlpD repeat 2:	EKRLLLELNDLKKEIAKK
MlpE repeat 1:	EERLLELN ^S LKKEITKK
MlpE repeat 2:	ERKLLEINDLKKEIAKK
MlpF repeat 1:	EERLLELN ^K LKKEITKK
MlpF repeat 2:	EERLLELN ^K LKKEITKK
OGexA 1 st peptide:	LLELNDLK
OGexA 2 nd peptide:	LLELNILK

Fig. 6

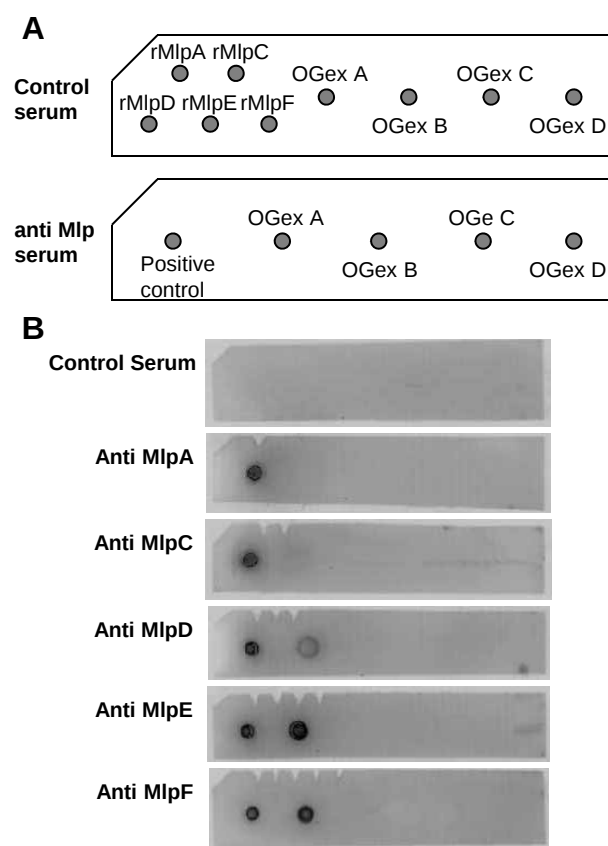


Fig. 7