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

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Running title: **Chicken TLR2 subfamily**

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Summary

Human TLR2 subfamily recognizes bacterial lipoproteins (BLP) and peptidoglycan (PGN). According to the genome information, chicken has structural orthologs of TLRs 1 and 2, in addition to TLRs3, 4, 5 and 7. Chicken has two additional TLRs, TLR15 and TLR21, which orthologs human lacks. The chicken (ch)TLR1 and 2 genes are individually duplicated to encode for two different proteins, chTLRs, 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- κ B 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, 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- κ B 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- κ B. 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 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.

Key words: chicken, Toll-like receptor 2, peptidoglycan, bacterial lipoprotein, pattern-recognition

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 TIR structures and is identified across fish to human (2,3). This subfamily of TLRs appears to have advanced as a pattern recognition receptor in conformity with principles of evolution. We had identified chTLR2 type1 and chTLR2 type2 earlier than 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- κ B 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, although the cDNA sequences were somewhat different from 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 human, 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 (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- κ B leading to 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 chTLR1 (1-1 or 1-2) participated in full activation of the chTLR2 pathway. TLR2 subfamily members may have evolved to form hetero-multimers for sensing various microbial patterns by their combinations on the cell surface.

Materials and Methods

Cells, bacteria, and reagents

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

Two strains of *Mycobacterium avium* (*M. avium*) were purchased from American Tissue Culture Collection (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 μ l) were then incubated with HEK293 cells (10^5) expressing single or double chTLR2 members (see Fig. 6).

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, Carisbad CA). Reverse transcription reaction was carried out with M-MLV Reverse transcriptase (Promega, Madison WI). To amplify the full length ORF of the chicken TLR1 cDNAs, we used error free Taq polymerase, Pyrobest (TAKARA BIO INC. Otsu Shiga). The cDNA clones were sequenced on ABI 310 sequencer. We chose the clone that does not have PCR error for following analyses. The cDNA sequences of ORF were deposited to DNA data bank of Japan (DDBJ). 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 (11). pFLAG-chTLRs (N-terminal labeled) were made for surface FACS analysis using a pCMV vector by the addition of the FLAG sequence at the end of the signal sequence as reported (6). pFLAG-huTLR3 were provided as described previously (6).

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

Luciferase assay

HEK293 cells were transiently transfected with 2 μ g 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 μ g/ml Pam3CSK4 or 10 μ g/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 instruction.

Immunoprecipitation and Western blotting

HEK293 cells were transiently transfected with 3 μ g of Flag-tagged huTLR3, chTLR1-1, chTLR1-2, chTLR2-1, chTLR2-2, or vector only, together with 6 μ g 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, Indianapolis, IN). The lysates were precleared for 1 h with protein G-Sepharose and immunoprecipitated with 2 μ g of anti-Flag M2 Ab or 2 μ g 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 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)

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 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 x 60 magnification under a FLUOVIEW (Olympus, Tokyo). Images were captured using the attached computer software, FLUOVIEW.

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 open reading frames (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 phylogenetic 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/>).

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 LRR domains exhibited relative divergence. Particularly, chTLR1-2 had a short N-terminal region of the LRRs compared to 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).

Human and mouse have the TLR2 subfamily consisting of TLR1, TLR2, TLR6 and TLR10. Natural ligand of TLR10 has not been identified. The 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 closely 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 clarified 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. 33-85% of the transfected cells 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 microscope, 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 on 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 (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 to the control. Under similar conditions chTLRs were barely coprecipitated with huTLR3 when huTLR3 FLAG was used as 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.

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 peptidoglycan (PGN) 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 bacterial lipoproteins (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). *Mycobacterium avium* (*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- κ B 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 lipopeptides are recognized by TLR2 in a TLR1- and TLR6-independent manner in human (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 if in mammals TLRs other than TLR1 and TLR6 pair with TLR2 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 unknown. 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 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- κ B, 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- κ B 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.

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Figure legends

Fig. 1. TLR2 subfamily proteins in the chicken. Panel 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. Panel 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 (>800) are not indicated. Asterisk indicated trichotomy. hu; human, mo; mouse, ch; chicken, fu; fugu.

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. Left two panels are negative (vector only) and positive (transfected with huTLR2) controls.

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 (panels A and B). Human TLR3 with C-terminal FLAG was used as control (panel 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.

Fig. 4. Immunoprecipitation of chTLRs. HA-tagged chTLR1-1 (panel A) and chTLR1-2 (panel 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.

Fig. 5. Pattern-recognition profiles of chTLR2 subfamily. Panel A: Single expression of chTLR2 members does not confer NF- κ B activation. HEK293 cells expressing the indicated chTLR2 members and pELAM plasmid (for NF- κ B) were stimulated with PGN, Malp-2, pam3 or just sterilized PBS. ALL represents HEK cells transfected with all four plasmids. At timed interval (typically 24 h), cells were harvested for reporter assay. Luciferase activities are shown as the mean relative stimulation \pm SD. Panel 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 assay as in panel A. Panel C: chTLR2-1 and chTLR1-2 cooperatively recognize PGN. HEK293 cells expressing chTLR2 member combinations and pELAM were prepared as in panel B. Cells were stimulated with PGN and assayed as in panel 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 μ l of $A_{600} = 0.4$) at 37 $^{\circ}$ C for 24 h (see the Methods section). 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 the experiments were performed and similar results to this figure were obtained.

Fig.1.A

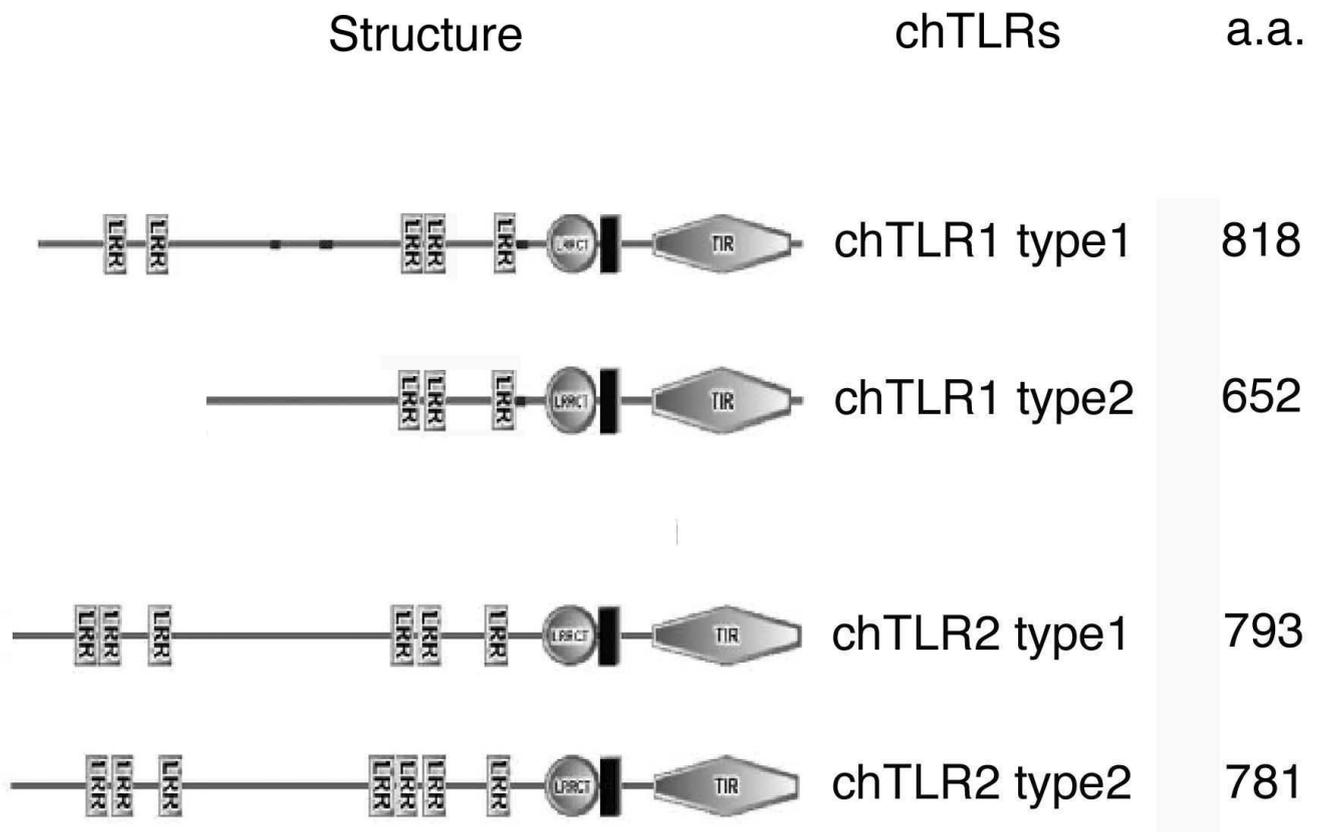


Fig.1B

Whole	chTLR1.2	huTLR1	Whole	chTLR2.2	huTLR2
chTLR1.1	56 %	50 %	chTLR2.1	81 %	52 %
chTLR1.2		54 %	chTLR2.2		53 %

LRR	chTLR1.2	huTLR1	LRR	chTLR2.2	huTLR1
chTLR1.1	45 %	41 %	chTLR2.1	76 %	45 %
chTLR1.2		44 %	chTLR2.2		46 %

TIR	chTLR1.2	huTLR1	TIR	chTLR2.2	huTLR1
chTLR1.1	96 %	83 %	chTLR2.1	100 %	81 %
chTLR1.2		83 %	chTLR2.2		81 %

Fig.1 C

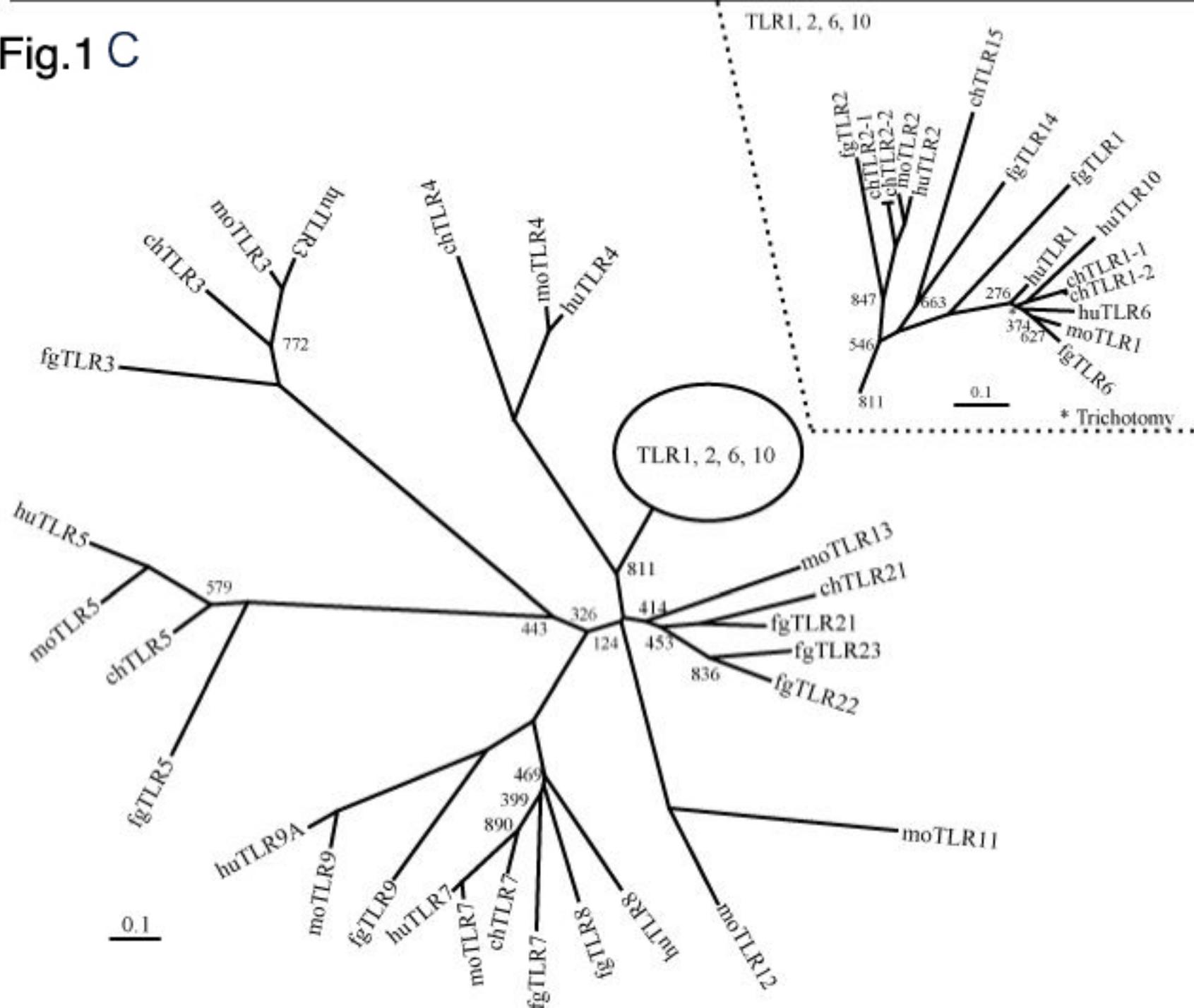


Fig.2

Cell surface

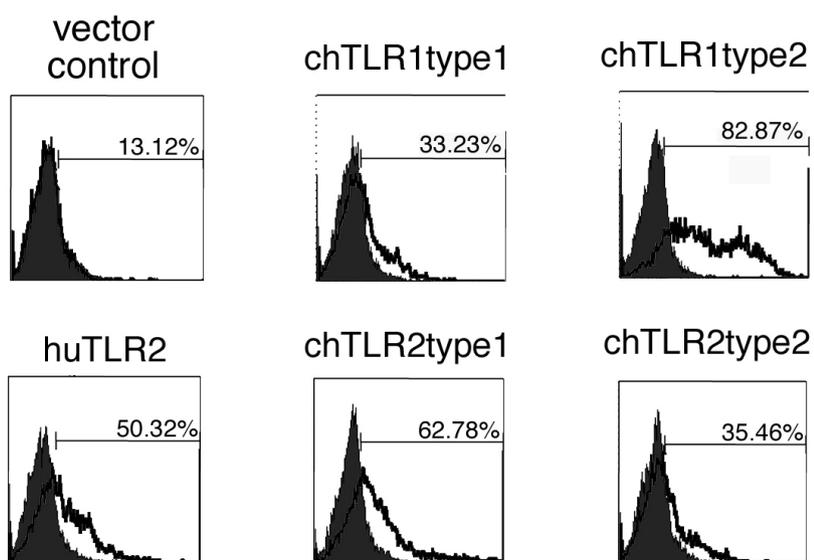


Fig.3

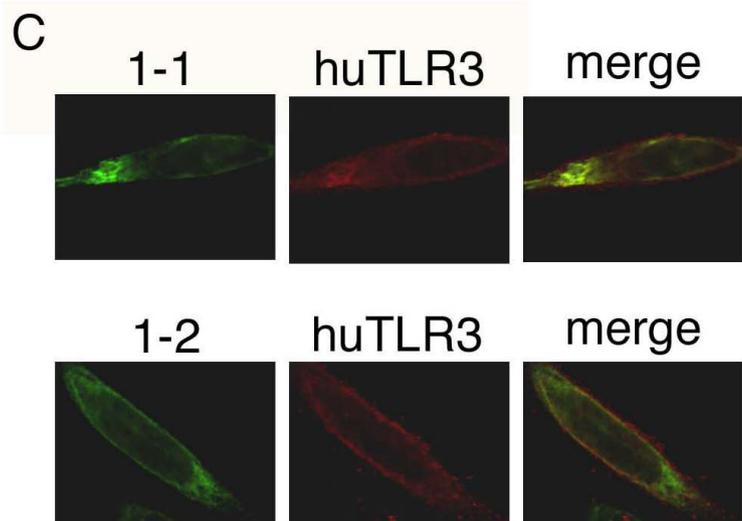
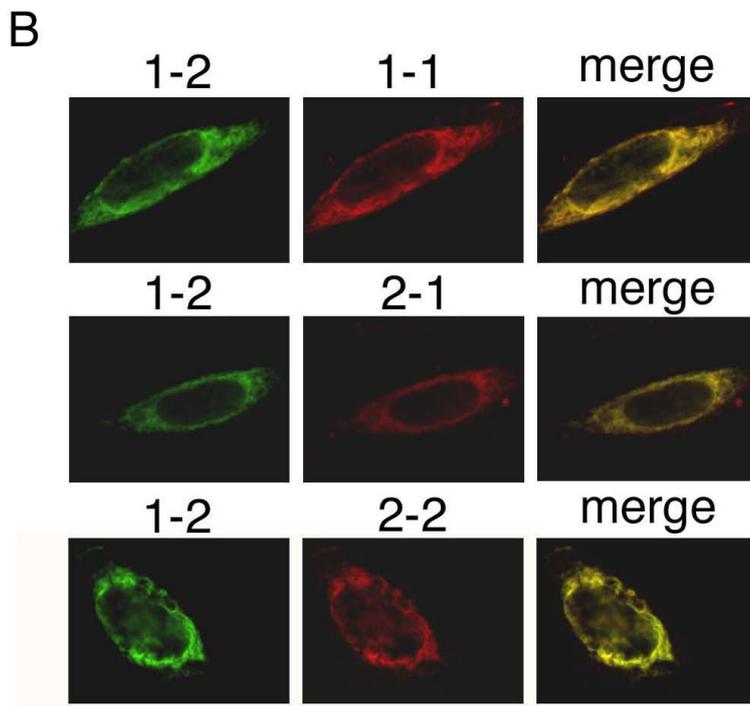
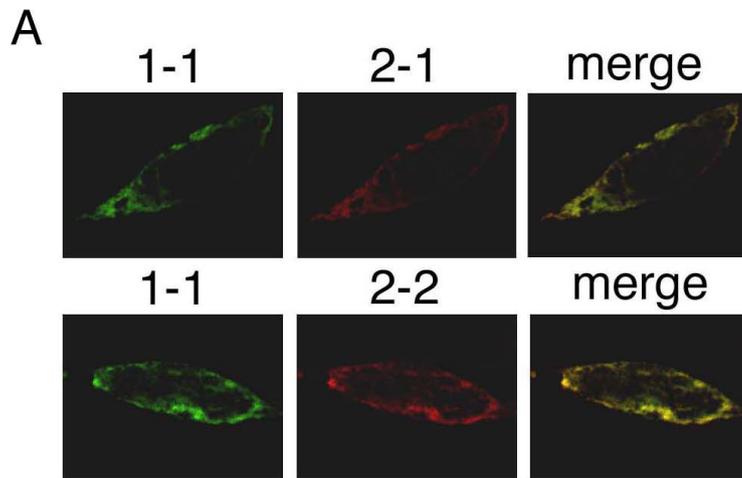
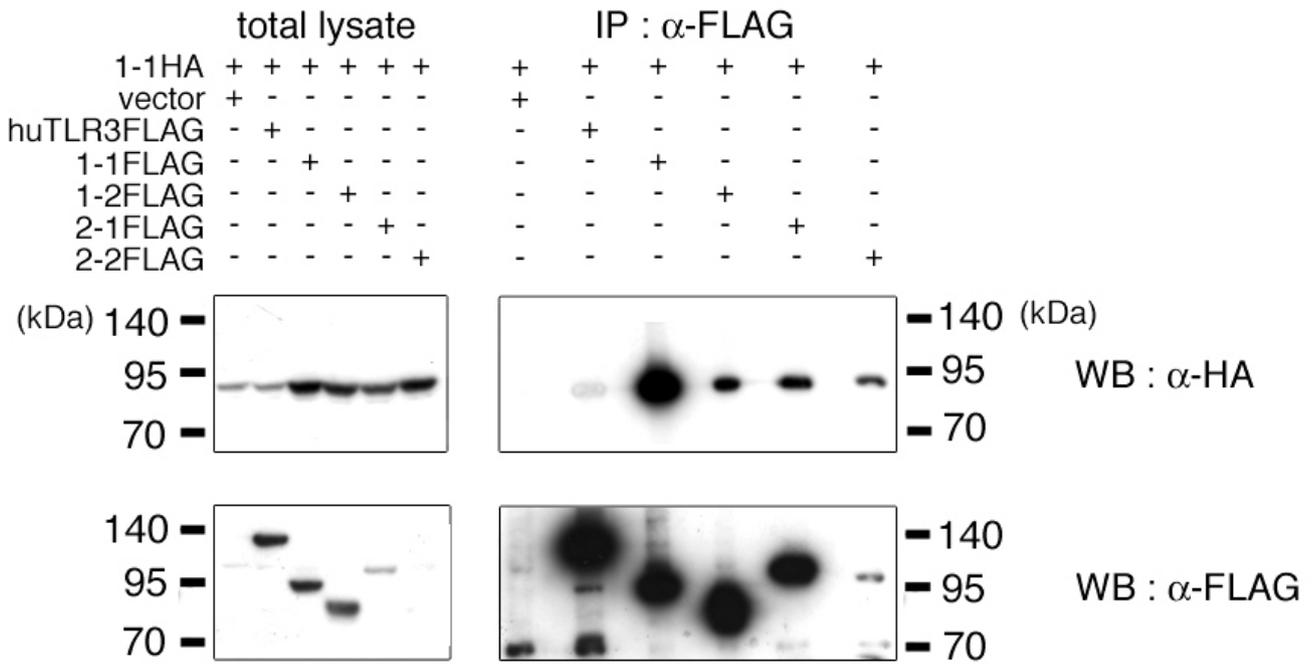


Fig.4

A



B

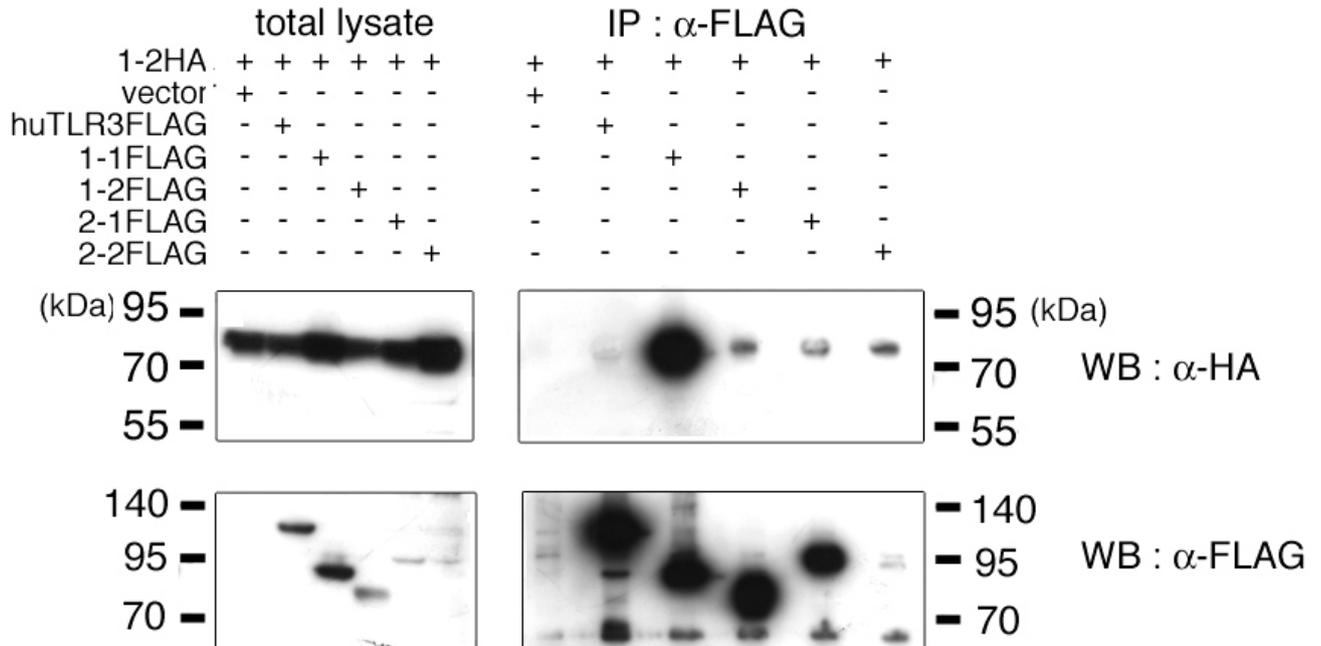
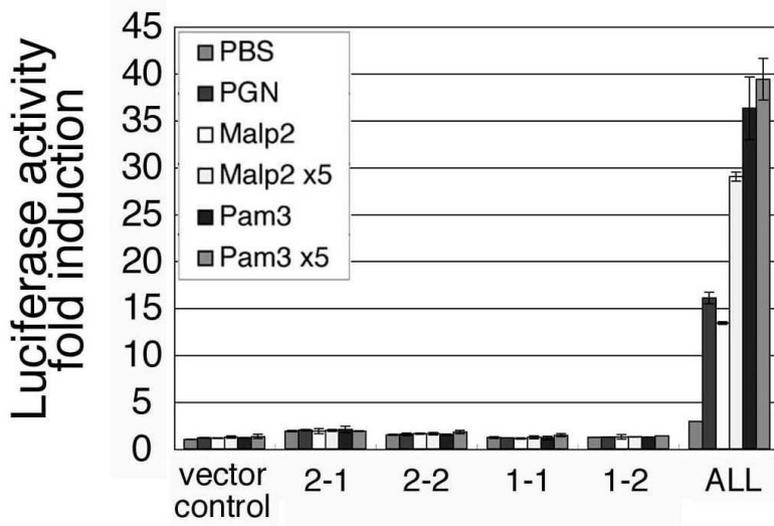
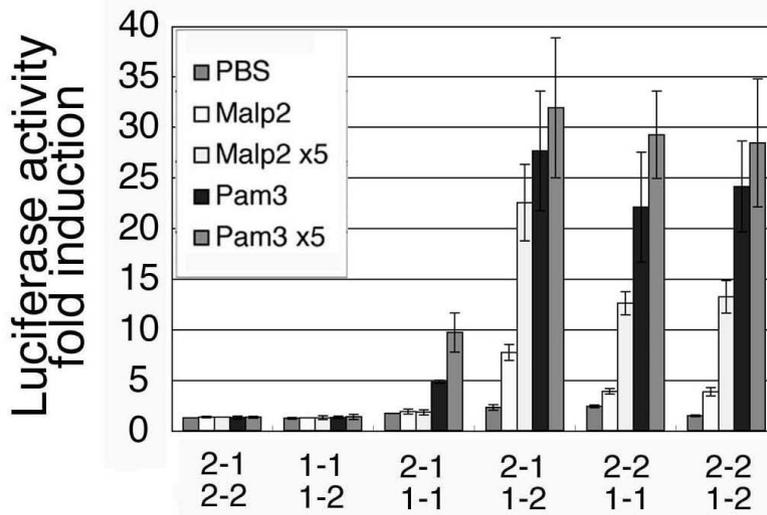


Fig.5

A



B



C

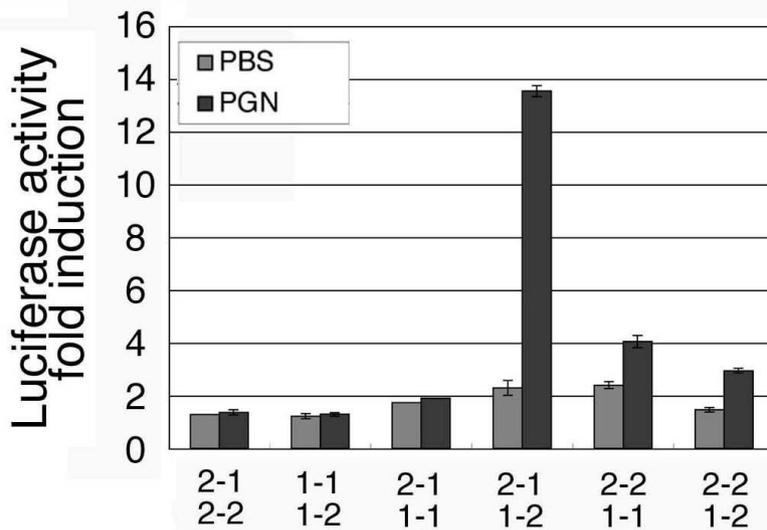


Fig.6

