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The TLR9 signaling pathway regulates MR1-mediated bacterial antigen presentation in B cells

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Abbreviations: Ag, antigen; B-LCL, Human B lymphoblastoid cell line; BFA, brefeldin A; CpG-A, class A CpG; CpG-B, class B CpG; MAIT, mucosal-associated invariant T cells; IRF7, Interferon-regulatory factor 7; NC, negative control; ODN, oligodeoxynucleotide; PAMP,

pathogen-associated molecular pattern; pDC, plasmacytoid dendritic cell; shRNA, small-hairpin RNA; TLR, toll-like receptor; TRAF3, tumor-necrosis factor (TNF)-receptor-associated factor 3.

Summary

Mucosal-associated invariant T (MAIT) cells are conserved T cells that express a semi-invariant TCR (Vα7.2 in humans and Vα19 in mice). The development of MAIT cells requires the antigen (Ag) presenting MHC-related protein 1 (MR1), as well as commensal bacteria. The mechanisms that regulate the functional expression of MR1 molecules and their loading with bacterial Ag in APCs are largely unknown. We have found that treating B cells with the TLR9 agonist CpG increases MR1 surface expression. Interestingly, activation of TLR9 by CpG-A (but not CpG-B) enhances MR1 surface expression. This is only limited to B cells and not other types of cells such as monocytes, T or NK cells. Knocking-down TLR9 expression by shRNA reduces MR1 surface expression and MR1-mediated bacterial Ag presentation. CpG-A triggers early endosomal TLR9 activation, whereas CpG-B is responsible for late endosomal/lysosomal activation of TLR9. Consistently, blocking ER to Golgi protein transport, rather than lysosomal acidification, suppressed MR1 Ag presentation. Overall, our results indicate that early endosomal TLR9 activation is important for MR1-mediated bacterial Ag presentation.

Introduction

Mucosal-associated invariant T (MAIT) cells are conserved T cells that express a semi-invariant TCR (Vα7.2 in humans and Vα19 in mice). The development of MAIT cells requires the Ag presenting MHC class I-related protein 1 (MR1), as well as commensal bacteria, because germ-free or MR1-deficient animals do not develop MAIT cells [1]. Human MAIT cells are abundant in the gut lamina propria, lung and liver [1]. Similar to another subpopulation of innate T cells, CD1d-restricted natural killer T (NKT) cells, MAIT cells have the unique capacity to bridge

innate and adaptive immunity. MAIT cells can respond to bacteria-infected APCs and rapidly secrete pro-inflammatory cytokines such as IFN-γ and TNF-α, as well as Granzymes A and B [1]. The most studied and only known function of MAIT cells is their anti-microbial activity [2, 3]. Reduced MAIT cell levels have been reported in different pathologic conditions, such as obesity, bacterial and viral infections, as well as autoimmune diseases [4-10].

In addition to MAIT cell development, MR1 is also required for MAIT cell activation [11, 12]. It was recently discovered that bacterial vitamin B-derived metabolites are Ags for MAIT cells [13, 14]. B cells were the first identified (and are the most prominent) APCs for MAIT cells [11], although other cell types, such as thymocytes, monocytes and epithelial cells can also activate MAIT cells [15-17]. MR1 is ubiquitously expressed, but is mostly localized in the cytosol. Thus, B cells normally express few MR1 molecules on the cell surface; however, upon a bacterial infection, MR1 translocates to the cell surface with loaded Ag and activates MAIT cells via interacting with the invariant TCR [12, 18].

Toll-like receptors (TLRs), a type of pattern-recognition receptors, are germline-encoded proteins that recognize conserved molecules of foreign pathogens, also called pathogen-associated molecular patterns (PAMPs). TLRs are essential for host defense against infections [19]. For example, upon a bacterial infection, TLR-expressing cells can recognize the bacterial PAMPs and lead to the secretion of Type I interferon, TNF-α, IL-12 and other cytokines [20]. In addition, the activation of TLRs can modify endogenous lipid Ag synthesis in APCs and impact CD1d-mediated Ag presentation to NKT cells [21, 22]. However, MAIT cell activation is different from that of NKT cells, in that it requires Ags such as microbial vitamin B-derived metabolites

[13]. The role of TLRs in MR1-mediated bacterial Ag presentation to MAIT cells has not been extensively studied. With B cells being essential for MAIT cell development in the periphery [11], in the current study, we explored the role of TLRs in regulating MR1-mediated bacterial Ag presentation in B cells.

Material and methods

Cells and reagents

An EBV-transformed human B lymphoblastoid cell line (B-LCL) from de-identified donated human blood was generated (and kindly provided) by Dr. Janice Blum (Indiana University, Indianapolis, IN). THP-1, a human monocytic leukemia cell line, was a kind gift from Dr. Louis Pelus (Indiana University, Indianapolis, IN). A panel of lentiviral short hairpin RNAs (shRNA) targeting TLR9 (Sigma-Aldrich, St. Louis, MO) were used to generate individual lines of TLR9-deficient B-LCL cells. To generate MAIT cells, human PBMCs were isolated from de-identified buffy-coats from healthy blood donors (Indiana Blood Center, Indianapolis, IN) by density gradient centrifugation using Ficoll-Hypaque (GE Healthcare, Piscataway, NJ). $V\alpha7.2^+$ $CD161^+$ T (MAIT) cells were sorted by flow cytometry. MAIT cells were expanded by stimulation with irradiated allogeneic human PBMC in the presence of paraformaldehyde-fixed *E. coli* (DH5α, MOI=300) and recombinant human IL-2 (10 ng/ml; Peprotech, Rocky Hill, NJ). The enrichment and expansion of MAIT cells after 21 days in culture are shown in Supplemental Fig. 1. All cells were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. A human TLR 1-9 agonist kit containing stock solutions of Pam3CSK4 (TLR1/2, 100 µg/ml), HKLM (TLR2, 10^{10} cells/ml), Poly(I:C)-HMW (TLR3, 1 mg/ml), LPS (TLR4, 100 µg/ml), Flagellin (TLR5, 100 µg/ml), FSL1

(TLR6/2, 100 µg/ml), Imiquimod (TLR7, 100 µg/ml), ssRNA40 (TLR8, 100 µg/ml) and ODN2006 (TLR9, 500 µM), was purchased from InvivoGen (San Diego, CA). Unmethylated CpG oligodeoxynucleotides ODN2216 (CpG-A, 500 µM), ODN2006 (CpG-B, 500 µM), ODN TTAGGG (TLR9 antagonist, 500 µM) and controls were also purchased from InvivoGen. 6-formylpterin (6-FP) was purchased from Cayman Chemical Co. (Ann Arbor, MI). Conditioned medium (CM) is media from CpG-treated B-LCL cells and was concentrated using an ultra-centrifugal filter (MW=10K cut-off; Millipore, Billerica, MA). PE/Cy7-conjugated anti-human CD19, BD Horizon™ V450-conjugated anti-human CD16, AlexaFluro700-conjugated anti-human CD14, AlexaFluro488-conjugated anti-human CD3, Brefeldin A and Monesin were purchased from BD Biosciences (San Jose, CA). Chloroquine and bafilomycin A1 were from Sigma-Aldrich.

Flow cytometry

B-LCL cells were treated with paraformaldehyde-fixed *E. coli* (MOI=300) or the TLR9 agonist CpG (5 µM) overnight as indicated, followed by staining with an allophycocyanin (APC)-conjugated anti-MR1 mAb (Clone 26.5; Biolegend, San Diego, CA). For total MR1 staining, cells were fixed with paraformaldehyde and permeabilized with 0.1% saponin. Cells were then stained with APC-conjugated anti-MR1. For human PBMCs, cells were treated with fixed *E. coli* (MOI=300) or TLR9 agonists overnight. The cells were stained with PE/Cy7-conjugated anti-CD19, BD Horizon™ V450-conjugated anti-CD16, AlexaFluro700-conjugated anti-CD14, AlexaFluro488-conjugated anti-CD3 and APC-conjugated anti-MR1 Abs. The cells were analyzed by flow cytometry as previously described [23].

Western blotting

Cell lysates were analyzed by immunoblotting as described previously [24]. TLR9-, TRAF3- and IRF7-specific Abs (Cell Signaling Technology, Danvers, MA) were used to detect TLR9, TRAF3 and IRF7 expression, respectively. The membrane was stripped and re-probed with a GAPDH-specific Ab (Cell Signaling Technology) as a control for sample loading. Protein expression was quantified using ImageJ software (1.46v; National Institutes of Health, Bethesda, MD).

MAIT cell activation assay

B-LCL cells were co-cultured with MAIT cells in the presence of fixed *E. coli* (MOI=300) or CpG-A (5 μM) for 72 h. For drug treatments, B-LCL cells were treated with the specific inhibitors as indicated, in the presence of fixed *E. coli* overnight. The cells were then washed, fixed and co-cultured with MAIT cells for 72 h. For shRNA-expressing B-LCL cells, the cells were treated with fixed *E. coli* overnight. The cells were then fixed and co-cultured with MAIT cells as described above. MAIT cell activation was measured by IFN-γ production into the supernatants and detected by ELISA.

Statistical analysis

Graphs were generated and analyzed by an unpaired two-tailed Student's *t*-test or one-way ANOVA using Prism software (version 5.00 for Windows; GraphPad, San Diego, CA). The error bars in the bar graphs show the standard deviation (SD) from the mean. A P value < 0.05 was considered significant.

Results

TLR9 activation increases MR1 surface expression in B cells

Previous work has shown that a bacterial infection of B cells increases MR1 surface expression [12]. Because bacteria contain many PAMPs that may activate host immune cells, we assessed whether the activation of TLRs is responsible for this upregulation of MR1 surface expression. We observed that only the TLR9 agonist ODN 2216 at high concentrations (i.e., 1:100; 5 μM) profoundly increased MR1 surface expression on B-LCL cells (Fig. 1A, 1B). The upregulation of surface MR1 was even higher when B-LCL cells were treated with a 50 μM of ODN 2216 (data not shown), suggesting a dose-dependent response. This MR1 upregulation by ODN 2216 was similar to that induced by bacteria (Fig. 1B). Our data suggest that TLR9 activation increases MR1 surface expression in B cells. Although fixed *E. coli* or CpG treatment increased surface MR1, total MR1 levels were not altered (Fig. 1C, 1D). This observation suggests that TLR9 activation promotes the intracellular translocation of MR1 to the cell surface. In line with this idea, the upregulation of MR1 surface expression by CpG ODN2216 was blocked by a TLR9 antagonist (ODN TTAGGG), but not by control ODN (Fig. 1E). These data demonstrate TLR9 specificity in the induction of MR1 cell surface expression. In addition, when treated with CpG ODN2216 or fixed *E. coli*, primary $CD19^+$ B cells from human blood also showed increased MR1 surface expression (Fig. 1F). Altogether, these results suggest that the activation of TLR9 enhances MR1 surface expression in B cells.

CpG-A (but not CpG-B) induces MR1 surface expression in B cells

Unlike CpG ODN2216, another TLR9 CpG agonist, ODN2006, did not induce MR1 surface expression in B-LCL cells (Fig. 1A). CpG DNA is categorized into three major classes based on structure and sequence motif: A, B and C [25]. Class A CpG (CpG-A) contains a central palindromic phosphodiester CpG sequence and a phosphorothioate-modified 3' poly-G tail. CpG-A is better at activating NK and plasmacytoid dendritic cells (pDCs), but is a poor activator of B cells [25]. Class B CpG (CpG-B) has a nuclease-resistant phosphorothioate backbone and is better at stimulating B cells. Class C CpG is a combination of Class A and B [25]. Interestingly, we found that ODN2216 (CpG-A), but not ODN2006 (CpG-B), increased MR1 surface expression in B-LCL cells (Fig. 2A) or primary B cells (Fig. 2B, 2C). Both CpG-A and CpG-B stimulated IL-6 production from human PBMCs (Supplemental Fig. 2), confirming the bioactivity of both reagents. Therefore, we conclude that CpG-A, although better known as an activator of NK and pDCs, also increases MR1 surface expression on B cells.

TLR9 agonists have different effects on MR1 surface expression in other cell types

To determine how TLR9 activation impacts MR1 expression in other cell types, we treated human PBMCs with CpG-A, -B and fixed *E. coli*. We found that neither CpG-A nor CpG-B enhanced MR1 surface expression on T ($CD3^+$) cells, although adding fixed *E. coli* did (Fig. 3A). Although CpG-A at a high concentration (5 μM) significantly enhanced MR1 surface expression on NK ($CD16^+$) cells, the increase was less than 1.5 fold (Fig. 3B; bar graph). CpG-B treatment caused no change in MR1 surface expression on NK cells (Fig. 3B). Interestingly, on the other hand, both CpG-A and CpG-B increased MR1 surface expression on monocytes ($CD14^+$) in a dose-dependent manner (Fig. 3C). To further test the effect of CpG-A and -B on

MR1 surface expression in monocytes, we utilized the monocytic cell line, THP-1. THP-1 cells express a higher level of surface MR1 compared to monocytes derived from PBMCs (Fig. 3D). We found that TLR4 and TLR5 agonists increased MR1 surface expression on THP-1 cells (Fig. 3F). In contrast, although CpG-A and –B increased MR1 surface expression on monocytes, they both reduced MR1 surface expression in THP-1 cells (Fig. 3E). These data suggest that THP-1 cells are different from primary monocytes in responding to activation by TLR9 agonists. However, it is important to mention that treatment with fixed *E. coli* enhanced MR1 surface expression in B cells, THP-1 cells and all other PBMC-derived blood cells we tested (Fig. 1B, 1F, 3A-3C, 3E). These results demonstrate that TLR9 may be the prominent TLR that regulates the cell surface expression of MR1 on B cells; other TLR agonists can impact MR1 surface expression in other cell types.

TLR9 is required for MR1 surface expression in B-LCL cells

It was previously reported that B cells are essential for MAIT cell development in the periphery [11]. Thus, we focused our study on B cells. To confirm that TLR9 is necessary for MR1 surface expression in B cells, we used a small hairpin RNA (shRNA) approach to specifically knock down TLR9 expression in B-LCL cells. Reduced TLR9 expression was verified by Western blot analysis (Fig. 4A and 4B). When these TLR9 shRNA cells were treated with fixed *E. coli* or CpG-A, reduced surface MR1 was observed in these cells as compared to cells expressing scrambled shRNA (NC; Fig. 4C, 4D and 4E). It is well-known that B-LCL cells produce many cytokines and chemokines upon activation [26]. To determine whether any soluble factors from CpG-activated B-LCL cells, rather than TLR9 activation itself, upregulate MR1 surface expression, B-LCL cells were cultured in conditioned medium from CpG-treated B-LCL cells.

We found that conditioned medium did not rescue reduced MR1 surface expression in TLR9-deficient cells (Fig. 4F). These results demonstrate that TLR9 is directly responsible for the upregulation of MR1 surface expression.

Reduced bacterial Ag presentation in TLR9-deficient B cells

It has been previously shown that bacterial Ags are essential for MR1-mediated stimulation of MAIT cells [2]. To determine whether the enhanced MR1 surface expression induced by TLR9 activation is sufficient to stimulate MAIT cells, B-LCL cells were cultured with MAIT cells in the presence of CpG-A. Adding CpG-A to B-LCL cells did not activate MAIT cells; on the other hand, treating B-LCL cells with fixed *E. coli* activated MAIT cells in an MR1-dependent manner (Fig. 5A). These results confirm the necessity of a bacteria-derived Ag for MAIT cell activation [13]. To determine the role for TLR9 in MR1-mediated bacterial Ag presentation, B-LCL cells in which TLR9 was knocked down by shRNA were used as APCs. There are several factors other than Ag presentation that may impact MAIT cell activation, such as different growth rates in APCs or cytokines (i.e., IL-12 and IL-18) from activated APCs. To eliminate these potential factors, APCs were fixed in paraformaldehyde after being treated with fixed *E. coli* overnight, and then co-cultured with MAIT cells. We found that B-LCL cells in which TLR9 was knocked down were significantly impaired in their ability to stimulate MAIT cells (Fig. 5B and Supplemental Fig. 3). Therefore, we conclude that TLR9 is important for MR1-mediated Ag presentation.

Early endosomal signaling of TLR9 by CpG-A enhances MR1 surface expression and Ag presentation

Previous studies have demonstrated that CpG-A and CpG-B induce distinct TLR9 downstream signaling pathways in pDCs [27]. CpG-A is less stable and usually aggregates and interacts with TLR9 in early endosomes [28]. This interaction triggers the activation of MyD88 and other downstream mediators, such as TRAF3, IRF7, and type I IFN production [27]. In contrast, CpG-B is more stable and transported to late endosomes/lysosomes, which results in the activation of different downstream signaling molecules in the TLR9 signaling pathway, such as NF-κB and MAPK; this leads to proinflammatory cytokine production [27]. Because CpG-A (but not –B) increased MR1 surface expression in B cells (Fig. 2), we hypothesized that early endosomal signaling of the TLR9 pathway by CpG-A is important for the observed increase in MR1 surface expression. To test this hypothesis, TRAF3 and IRF7, two downstream mediators of the early endosomal TLR9 signaling pathway induced by CpG-A [27], were knocked-down in B-LCL cells using lentiviral shRNA constructs. Reduced TRAF3 or IRF7 expression in B-LCL cells was confirmed by Western blot analysis (Supplemental Fig. 4A and 4B). These cells, when treated with CpG-A, showed reduced MR1 surface expression as compared to control cells (Fig. 6A, 6B). These results provide further strong support that early endosomal TLR9 signaling is important for MR1 surface expression.

Importantly, blocking lysosomal function with the lysosomal inhibitors bafilomycin and chloroquine, had little impact on MR1 surface expression (Fig. 6C) or MR1-mediated Ag presentation (Fig. 6D). This is consistent with our observation that CpG-B, which signals through late endosomes/lysosomes [27], does not enhance MR1 surface expression in B cells. In contrast, the Golgi inhibitors brefeldin A (BFA) and monesin, impaired both MR1 surface

expression and MR1-mediated Ag presentation (Fig. 6C, 6E; Supplemental Fig. 5). All of these pharmacological inhibitors were used at concentrations that exhibited little effect on cell viability (data not shown). Overall, these data support the importance of early endosomes, but not late endosomes or lysosomes, in bacterial Ag presentation by MR1. Therefore, we conclude that early endosomal signaling of TLR9 by CpG-A enhances MR1 surface expression and Ag presentation.

Discussion

In the current study, we demonstrate that activation of TLR9 regulates MR1 surface expression and bacterial Ag presentation in B cells (Fig. 7). It has been previously shown that the activation of TLRs in APCs enhances MAIT cell activation in a TCR-independent manner [17, 29]. The Lantz group has shown that bone marrow-derived dendritic cells from MyD88 knock-out mice are less able to activate MAIT cells [2], suggesting TLRs are likely to be important for MR1-mediated Ag presentation. Our data suggest that bacteria not only provide bacterial Ags, but also PAMPs that activate MAIT cells. Indeed, Chen et al., have demonstrated that co-stimulatory signals, such as TLR ligands, are as essential as microbial Ag in MAIT cell activation and accumulation *in vivo* [30]. It has been demonstrated by several laboratories that endocytic trafficking is important for MR1-mediated Ag presentation [12, 31-33]. To the best of our knowledge, our study is the first to directly show that the early endosomal TLR9 signaling pathway is important for Ag presentation by MR1. Consistent with our data, two groups have recently shown that brefeldin A suppressed the functional expression of MR1 in their individual experimental systems [32, 33]. The SNARE proteins Stx18 and VAMP4, as well as the small GTPase Rab6, were identified as important

regulators of MR1-dependent Ag presentation [33]. Future investigations will focus on how TLR9 signaling influences the function of SNARE proteins and intracellular trafficking of MR1.

Our finding that the TLR9 agonist CpG-A enhances MR1 surface expression was a surprise, because CpG-A is not considered a potent activator of B cells. It is well-known that CpG-A is a strong activator of pDCs and NK cells, whereas CpG-B activates B cells [25]. CpG-A has been shown to induce a modest response in B cells, but only at a high concentration [34]. This is consistent with our observation that CpG-A induces the upregulation of MR1 at high concentrations (e.g., 5 μM; Fig. 1). The fact that CpG-A stimulates B cells only at much higher concentrations, as compared to CpG-B, is likely due to the instability of CpG-A and a paucity of CpG-A-binding cell surface receptors (e.g., CXCL16) on B cells [34, 35]. On the other hand, previous studies have suggested that high concentrations of CpG-B inhibit B cell activation [36]. We also observed reduced IL-6 production from PBMCs when stimulated with high concentrations of CpG-B (Supplemental Fig. 2). However, at none of the concentrations we tested, was CpG-B able to induce MR1 surface expression (Fig. 3C). Therefore, our data suggest that CpG-A can activate B cells at high concentrations and its function is different from CpG-B.

We found that TLR9 activation increases MR1 surface expression in some types of cells (e.g., monocytes and B cells) but has a minimal impact in others (e.g., T and NK cells). TLR4 and TLR5 agonists enhance MR1 surface expression in THP-1, but not B-LCL cells (Fig. 1A, 3F). This may be explained by differential expression of TLRs in these cells. Ussher et al., reported that activation of several TLRs by TLR-specific agonists enhances MR1-mediated Ag presentation [31]. They also found almost no increase in surface MR1 expression after treatment with fixed *E. coli* in B-LCL or THP-1 cells. They also showed low baseline MR1 surface expression in THP-1 cells [31]. The differences in MR1 surface expression between our two studies

may simply be due to the MR1-specific reagents used. For example, Ussher et al., used an anti-MR1 antibody conjugated with AlexaFluro488, a weaker fluorochrome compared to allophycocyanin, which is what we used; this would impact the level of detectable MR1 on cell surface by flow cytometry. Additionally, others have reported that a bacterial infection of B cells increases MR1 surface expression [12].

McWilliam et al., have recently shown that MR1 molecules are localized in the ER at steady state and traffic to the plasma membrane; however, this only occurs after being loaded with bacterial Ag [32]. Our studies suggest a different mechanism for MR1 surface expression in the absence of a bacterial Ag. This TLR9-dependent upregulation of MR1 is likely to be different from bacterial Ag-mediated MR1 surface expression, because we only observed MR1 upregulation in B cells and monocytes (Fig. 1 and Fig. 3C). The bacteria-induced MR1 upregulation is common among all cells analyzed to date [32]. Interestingly, treatment of fixed *E. coli* increases MR1 surface expression in almost all cell types we tested (Fig. 1, Fig. 3 and data not shown). Thus, the increase of MR1 surface expression by fixed *E. coli* may be due to it being loaded with bacterial Ags, rather than by the activation of TLRs. It will be interesting to determine whether these two different surface expression mechanisms influence each other in bacterial-infected APCs.

We have demonstrated that the TLR9 agonist CpG-A increases MR1 surface expression in B cells. Reduced MR1 surface expression in TLR9-deficient B-LCL cells further confirmed the importance of TLR9 signaling in regulating MR1 function (Fig. 4). In fixed *E. coli*-treated TLR9-deficient B-LCL cells, although there was a significantly reduced ability to activate MAIT

cells, they could still stimulate a very high level of IFN-γ production by MAIT cells (Fig. 5B). One may question whether other factors, such as IL-12 and IL-18 from APCs, contributed to the MAIT cell activation in this case. This is highly unlikely, because the APCs were fixed and would not be expected to produce any cytokines. The observed MAIT cell activation might be due to the presence of other intact TLRs in B cells, although activation of these TLRs does not increase MR1 surface expression. An alternative explanation could be that the residual TLR9 in shRNA cells was sufficient to stimulate MR1-mediated Ag presentation at a high level. It is worthwhile to point out that adding pure bacterial Ags to APC/MAIT cell co-cultures can stimulate MAIT cell activation [14]. This suggests that TLR activation is not required for MR1-mediated bacterial Ag presentation; rather, we think it helps facilitate this response.

Even if MR1 is present at low levels on the cell surface, if Ag is available in excess, it would be very likely sufficient for the activation of MAIT cells [14, 37]. However, it could also be argued that high levels of Ag-loaded MR1 might "mask" the importance of TLR activation in the regulation of MR1-mediated bacterial Ag presentation. In support of this idea, Ussher et al. (2016) showed that, although MAIT cells incubated with THP-1 or B-LCL cells in the presence of *E.coli* culture supernatants showed little activation, when MR1-overexpressing APCs were used, MAIT cell stimulation was significantly enhanced. In contrast, in the presence of intact *E.coli*, APCs with or without MR1 overexpression comparably stimulated MAIT cell activation [31]. Their work suggests the importance of TLR activation when MR1 expression is low. Therefore, we speculate that primary signaling from Ag-loaded MR1 is essential for MAIT cell activation. That being said, secondary signaling from TLRs is also important in enhancing MAIT cell activation, especially when MR1 and/or bacterial Ags are at relatively lower levels.

The potential of bacterial DNA to activate TLR9 is dependent on the individual dinucleotide [CG] content [38]. Interestingly, those bacterial species that are more able to stimulate TLR9, such as *P. aeruginosa* or *K. pneumoniae*, activate MAIT cells at a low multiplicities of infection (MOI=1-10). On the other hand, a few other bacterial strains, including *S. aureus* and *S. epidermidis*, have a minimal ability to activate cells via TLR9. Thus, they activate MAIT at an MOI of 100 or above [2, 38]. This is consistent with our data showing that TLR9 is important for MAIT cell activation. *E. faecalis* is also a poor activator of MAIT cells, likely due to lacking the bacterial Ag riboflavin synthesis genes [39]. Interestingly, *E. faecalis* also lacks the ability to activate TLR9 [2, 38]. *E. faecalis*, when mixed with *E. coli* supernatants that contain bacterial Ags, still cannot induce an efficient MAIT cell response [31]. The importance of TLR9 activation in MR1-mediated Ag presentation may provide an alternative explanation for the inability of *E. faecalis* to activate MAIT cells, even in the presence of bacterial Ag. Our work supports the hypothesis that certain bacterial species in the gut microbiota favor MAIT cell function, because they not only provide bacterial Ags, but also activate TLR pathways that enhance MAIT cell activation. A recent study has found that TLR agonists (including CpG) provide the necessary co-stimulatory function to drive MAIT cell accumulation when administered *in vivo*, together with a synthetic MR1 Ag [30]. Thus, we conclude that TLR9 is critical for MR1-mediated bacterial Ag presentation.

MAIT cells have the ability to clear bacterial infections [40]. B cells are required for MAIT cell expansion in the periphery [41]. Our data suggest that TLR9 regulates MR1-mediated bacterial Ag presentation in B cells. This further supports the importance of B cells in MAIT cell development and host immune response to bacterial infections. Indeed, patients with a B cell deficiency, such as X-linked agammaglobulinemia, do experience recurrent infections [42]. This

would certainly be consistent with these patients lacking MAIT cells, as patients with X-linked lymphoproliferative (XLP) syndrome do have low numbers of MAIT (and NKT) cells [43]. Our study indicates a unique function of B cells in sensing bacterial PAMPs and activating MAIT cells.

In conclusion, we have shown that TLR9 activation is essential for MR1-mediated bacterial Ag presentation and it does so by regulating MR1 intracellular trafficking (summarized in Fig. 7). Future work will focus on how the TLR9 signaling pathway regulates the endocytic trafficking of MR1 and bacterial Ag processing/presentation. Our studies provide insightful information for potentially developing CpG-A as an adjuvant for MAIT cell-based immunotherapy.

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Author contributions

JL and RRB were responsible for study design, collaborated in performance of the experiments, analysis of data and the writing of the manuscript. JL performed the experiments.

Disclosures

There are no financial conflicts of interest by either of the authors.

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Figure legends

Figure 1. TLR9 activation increases MR1 surface expression in B cells. (A) B-LCL cells were treated overnight with a human TLR 1-9 agonist kit and ODN2216 (TLR9), using 10-fold serial dilutions of the stock solutions as indicated in the Material and Methods section. Fixed *E.coli* (MOI 300) and 6-FP (50 µM) were included as positive controls. The cells were stained with an APC-conjugated anti-MR1 Ab and analyzed by flow cytometry. Data from three independent experiments are summarized and the relative mean fluorescent intensity (MFI, Vehicle=1) is shown. Data from agonist-treated cells were compared to vehicle-treated cells. *, $p<0.05$. (B) B-LCL cells were treated with fixed *E. coli*, the TLR9 agonist CpG ODN2216 or control ODN overnight. The cells were stained with an APC-conjugated anti-MR1 Ab. (C) To determine whether CpG increases the level of total cellular MR1 , the cells were fixed, permeabilized and stained with APC-conjugated anti-MR1. (D) B-LCL cells were treated overnight with fixed *E.*

coli and stained for surface or total MR1 expression. (E) B-LCL cells were treated overnight with the TLR9 agonist CpG ODN2216 alone, or in the presence of the TLR9 antagonist ODN TTAGGG. The cells were stained with an APC-conjugated anti-MR1. (F) Human PBMCs were treated with fixed *E. coli* or the TLR9 agonist CpG ODN2216 overnight. The cells were stained with a PE-Cy7-conjugated anti-CD19 and APC-conjugated anti-MR1 mAb. MR1 surface expression on $CD19^+$ PBMCs is shown. The data shown are representative of at least three independent experiments.

Figure 2. CpG-A (but not CpG-B) induces MR1 surface expression in B cells. (A) B-LCL cells were treated CpG-A (ODN2216) or CpG-B (ODN2006) overnight. The cells were stained with an APC-conjugated anti-MR1 Ab. (B) Human PBMCs were treated CpG-A or CpG-B overnight. The cells were stained with a PE/Cy7-conjugated anti-CD19 and APC-conjugated anti-MR1 mAb. The mean MFI of MR1 surface expression on $CD19^+$-gated PBMCs from three different donors is shown in (C). The data shown are representative of at least three independent experiments. *, $p<0.05$.

Figure 3. TLR9 agonists have different effects on MR1 surface expression in other cell types. (A, B, C) Human PBMCs were treated with 5 μM of CpG-A or CpG-B, or fixed *E. coli* (MOI=100) overnight (solid line). Vehicle-treated PBMCs were used as controls (grey filled histogram). The cells were co-stained with an APC-conjugated anti-MR1 mAb, AlexaFluro488-conjugated anti-human CD3, V450-conjugated anti-human CD16 or AlexaFluro700-conjugated anti-human CD14 mAbs. MR1 expression on T cells ($CD3^+$-gated, A), NK cells ($CD16^+$-gated, B) and monocytes ($CD14^+$-gated, C) is shown. The bar graphs quantify MR1's MFI on each cell

type when treated with various concentrations of CpG DNA or fixed *E. coli* (MOI=100) as indicated. The MFI of MR1 surface expression on T cells, NK cells and monocytes from three different healthy donors is displayed relative to vehicle-treated cells (vehicle=1). (D) Human PBMCs and THP-1 cells were stained with an AlexaFluro700-conjugated anti-CD14 and APC-conjugated anti-MR1 mAb. MR1 expression on $CD14^+$-gated cells is shown. (E) Human PBMCs and THP-1 cells were treated with CpG-A, -B or fixed *E. coli* overnight. The cells were stained with an AlexaFluro700-conjugated anti-CD14 and APC-conjugated anti-MR1 mAb. The MFI of MR1 expression is shown. (F) THP-1 cells were treated with a human TLR 1-9 agonist kit and ODN2216 (TLR9), using 10-fold serial dilutions of the stock solutions as indicated. The cells were stained with an APC-conjugated anti-MR1 mAb. The data shown are representative of at least two independent experiments. *, $p<0.05$; ***, $p<0.001$.

Figure 4. TLR9 is required for MR1 surface expression. (A) Western blots to confirm reduced TLR9 in B-LCL cells expressing TLR9 shRNA or a negative control (NC). The bands were quantified using ImageJ and the data shown in (B). (C) TLR9-specific shRNA and NC cells were treated with CpG-A overnight. Surface expression of MR1 is shown in the histograms. The relative MFI (NC=1) of MR1 is shown in (D). (E) TLR9 shRNA and NC cells were treated with fixed *E. coli* overnight. Surface expression of MR1 is displayed relative to the control (NC=1). (F) TLR9 shRNA and NC cells were treated with either 2× concentrated CpG-A conditioned medium (CM) or CpG-A overnight. The cells were stained with an APC-conjugated anti-MR1 Ab. The relative MFI of MR1 compared to the control is shown (NC=1). The data are representative of at least two independent experiments.

Figure 5. Reduced bacterial Ag presentation in TLR9-deficient cells. (A) Human Vα7.2$^+$ CD161$^+$ T (MAIT) cells isolated from human PBMCs were sorted by flow cytometry. The cells were co-cultured with B-LCL cells in the presence or absence of CpG or fixed *E. coli* for 3 days. An MR1-specific or isotype control Ab was added to block MAIT cell activation. Supernatants were harvested and IFN-γ measured by ELISA. (B) B-LCL cells expressing NC and TLR9 shRNA were treated with fixed *E. coli* overnight. The cells were fixed and co-cultured with MAIT cells for 3 days. MAIT cell activation was measured by IFN-γ production into the supernatants. Each bar is the mean of triplicate samples ± SD. *, $p<0.05$; **, $p<0.01$. The data are representative of at least three independent experiments.

Figure 6. Early endosomal TLR9 signaling by CpG-A enhances MR1 surface expression and Ag presentation. (A) TRAF3 shRNA and NC cells were treated with CpG-A overnight. The cells were stained with an APC-conjugated anti-MR1 mAb and analyzed by flow cytometry. (B) IRF7 shRNA and NC cells were treated with CpG-A overnight. The cells were stained with an APC-conjugated anti-MR1 mAb for MR1 cell surface expression analysis by flow cytometry. (C) B-LCL cells were treated with fixed *E. coli* in the presence of the indicated concentrations of bafilomycin A, chloroquine or brefeldin A overnight. The cells were stained with an APC-conjugated anti-MR1 mAb and surface MR1 was analyzed by flow cytometry. The relative MFI of MR1 expression compared to the control is shown (vehicle=100). (D) B-LCL cells were treated with bafilomycin A1 (Baf) or chloroquine (Chl) overnight, fixed and co-cultured with MAIT cells in the presence of fixed *E. coli* for 3 days. (E) B-LCL cells were treated with brefeldin A (BFA) or monensin (Mon) overnight, fixed, and co-cultured with MAIT cells in the presence of fixed *E. coli* for 3 days. Activation of MAIT cells was measured by IFN-γ

production into the supernatants. Each bar is the mean of duplicate samples ± SD. **, $p<0.01$. Representative data from three independent experiments are shown.

Figure 7. Illustration showing early endosomal TLR9-dependent signaling in the control of MR1-mediated bacterial Ag presentation in APCs. Upon a bacterial infection, activation of the TLR9 early endosomal signaling pathway enhances the endocytic trafficking of MR1 to the cell surface and thereby regulates MR1-mediated bacterial Ag presentation. EE: early endosomes; ER: endoplasmic reticulum.

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Fig. 1

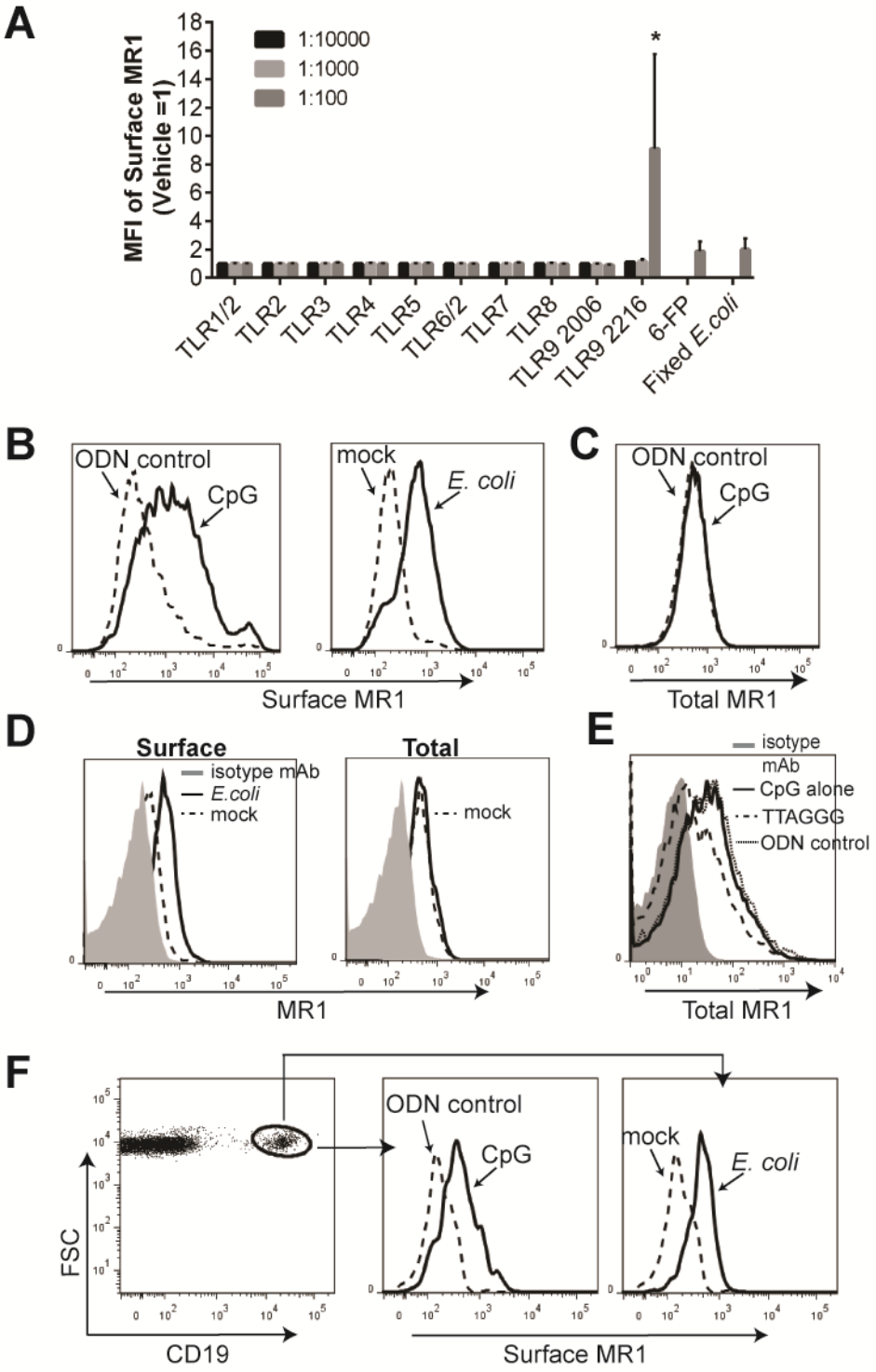

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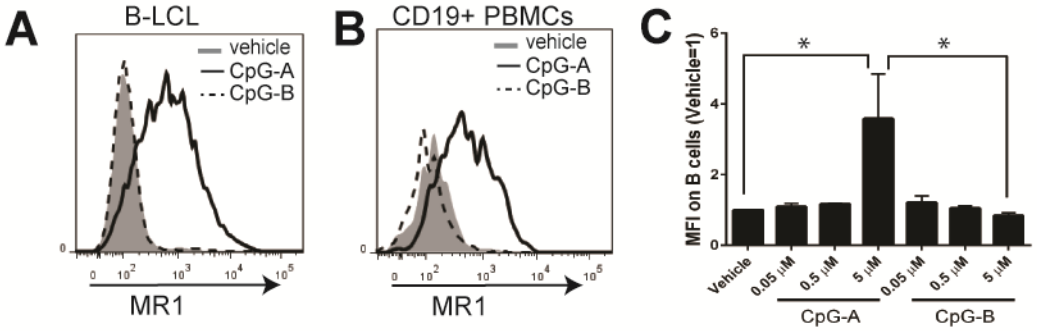
Fig. 2



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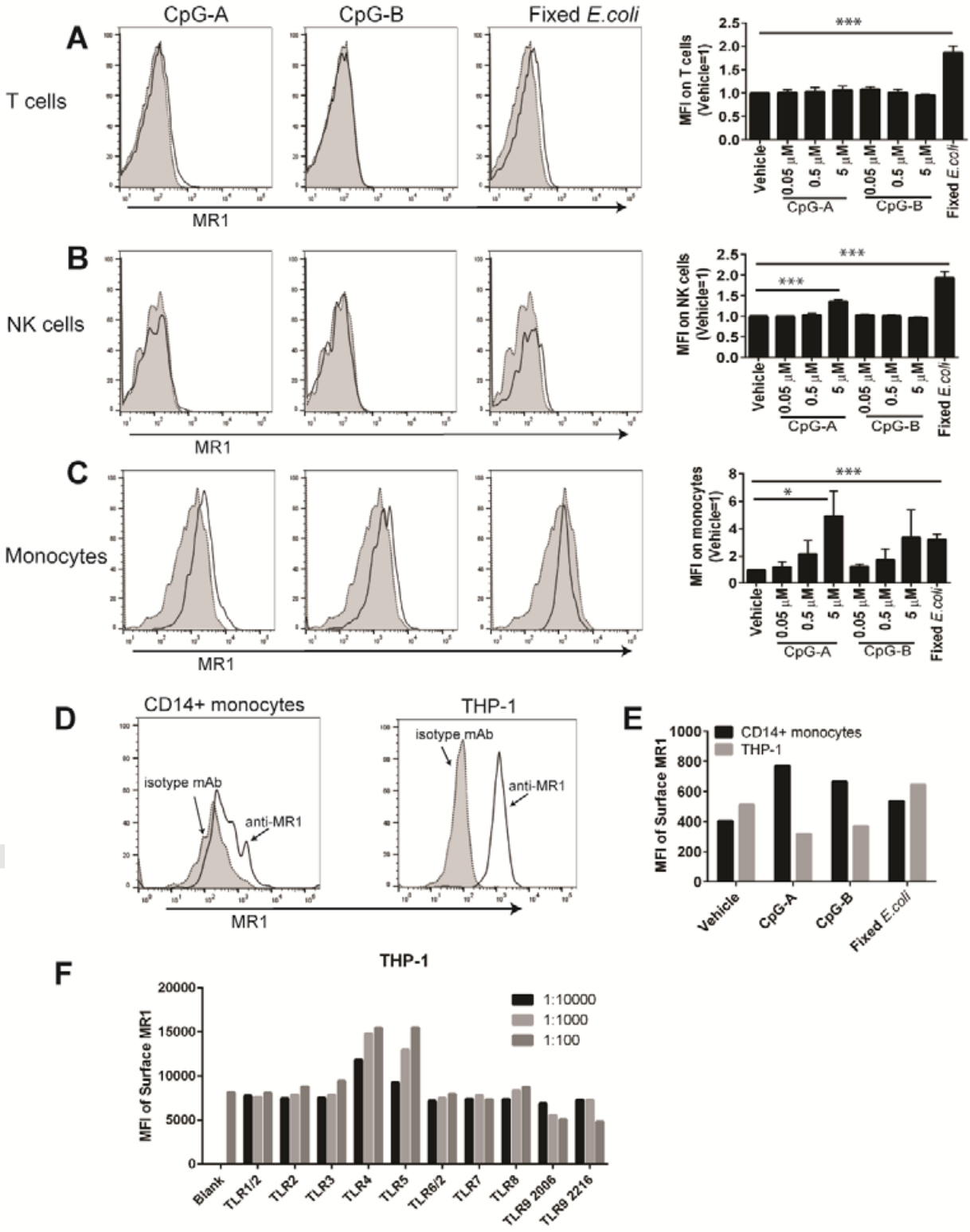

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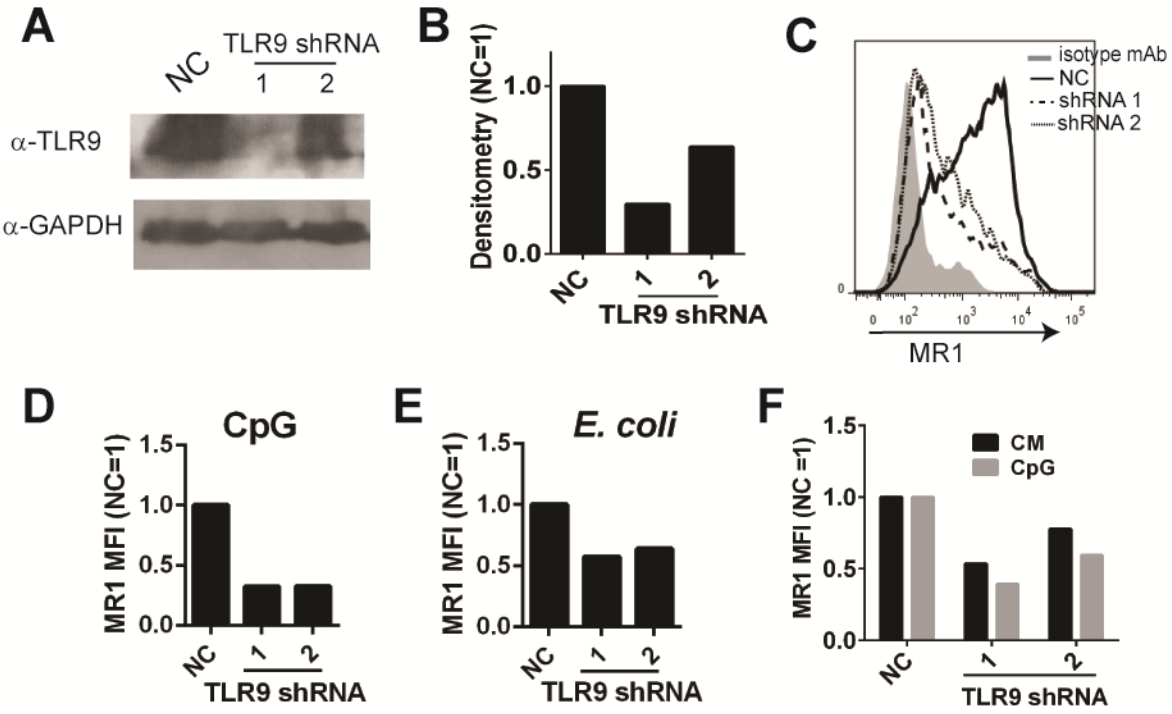

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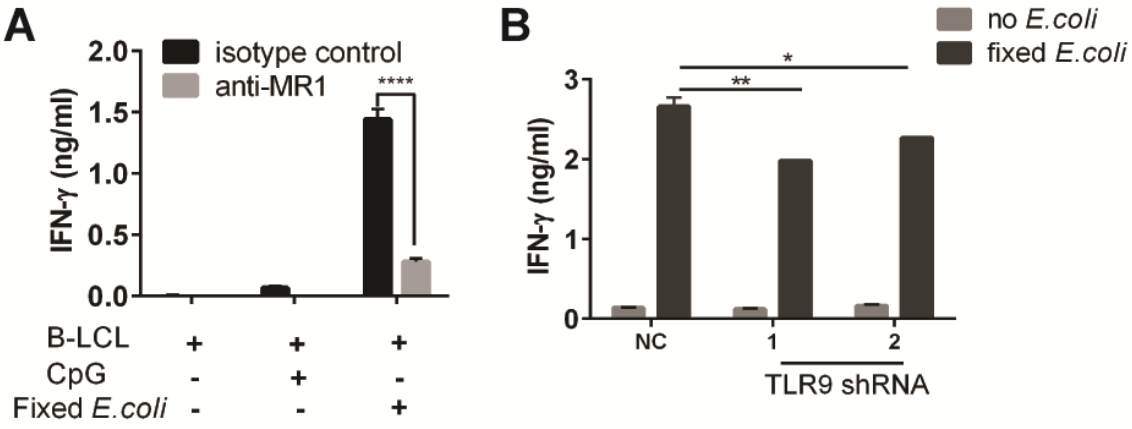

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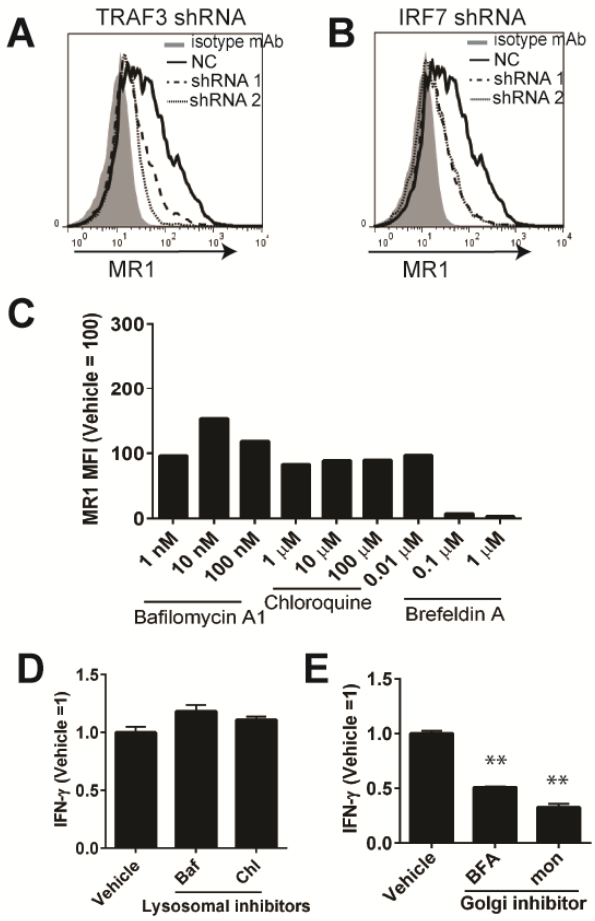

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Fig. 7