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Original Article

Immunology

Intestinal lamina propria macrophages upregulate interleukin-10 mRNA in response to signals from commensal bacteria recognized by MGL1/CD301a

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Abstract

Ligand-induced cellular signaling involved in interleukin 10 (IL-10) production by lamina propria macrophages (LPMs) during their interactions with commensal bacteria is not clearly understood. We previously showed, using mice lacking a C-type lectin MGL1/CD301a, that this molecule on colonic LPMs plays an important role in the induction of IL-10 upon interaction with commensal bacteria, *Streptococcus sp*. In the present report, we show that the physical engagement of MGL1/CD301a on LPMs with in-situ isolated *Streptococcus sp.* bacteria leads to IL-10 messenger RNA (mRNA) induction. Spleen tyrosine kinase (Syk), caspase recruitment domain 9 (CARD9) and extracellular signal-regulated kinase (ERK), but not NF-κB pathway, are shown to be indispensable for IL-10 mRNA induction after stimulation with heat-killed *Streptococcus sp*. Guanidine hydrochloride treatment of *Streptococcus sp.*, which is known to extract bacterial cell surface glycan-rich components, abolished bacterial binding to recombinant MGL1/CD301a. The extract contained materials which bound rMGL1 in ELISA and appeared to induce IL-10 mRNA expression in LPMs in vitro. Lectin blotting showed that the extract contained glycoproteins that are considered as putative ligands for MGL1. Some human commensal *Lactobacillus* species also induced IL-10 mRNA expression by colonic LPMs in vitro, which depends on the presence of MGL1/CD301a and CARD9. The present results are the first to show that MGL1/CD301a acts as a signal transducer during colonic host–microbe interactions.

Key words: gut–microbe interactions, interleukin 10, lamina propria macrophage, MGL1/CD301a, signal transduction

Introduction

The gastrointestinal tract is constantly exposed to environmental stimuli and harbors a complex ecosystem of bacterial species, particularly in the colon. Given the enormous number of residential and pathogenic bacteria, the immune response in the gut needs to be tightly regulated to keep homeostasis. Specialized subsets of dendritic cells (DCs) and macrophages are responsible for keeping the balance between eliciting immune responses to pathogens and keeping tolerance to food-borne antigens and commensal bacteria. Macrophages are present throughout the gut and are especially numerous in the lamina propria, where they constitutively release high levels of the anti-inflammatory cytokine interleukin-10 (IL-10) (Bain et al. 2013; Rivollier et al. 2012) in response to stimuli by the gut microenvironment. Exactly how IL-10 production in macrophages is elicited by the gut environment remains incompletely understood.

We have focused on the role of C-type lectins in gut homeostasis based on the assumption that these lectins can potentially recognize carbohydrate ligands on bacterial cell surfaces. Many C-type lectins are expressed on cells in the innate immune system. In the present study, we focused on macrophage galactose-type/calcium-type lectin 1 (MGL1/CD301a/Clec10a, hereafter called MGL1), which is uniquely expressed on a limited subset of macrophages and dendritic cells (Denda-Nagai et al. 2010). Mice have two distinct MGL genes, *Mgl1* and *Mgl2*, whereas rats and humans only have one copy (Higashi et al. 2002; Tsuiji et al. 2002). MGL seems to be expressed exclusively by cells having myeloid origins. Its closest homolog, asialoglycoprotein receptor (ASGPR), composed of a major and a minor subunit, ASGR1 and ASGR2, respectively, is predominantly expressed by hepatocytes, with some exceptions (Kanemaru et al. 2019). Human MGL/CD301/CLEC10A and mouse MGL2 recognize terminal *N*-acetylgalactosamine (GalNAc) residues (Oo-Puthinan et al. 2008; Suzuki et al. 1996). Mouse MGL1 has oligosaccharide specificity for Lewis X (Oo-Puthinan et al. 2008; Tsuiji et al. 2002). MGL1 has been shown to be involved in the dampening of autoimmune responses in various disease settings (Ilarregui et al. 2019; Kanemaru et al. 2019; Li et al. 2012; van Vliet et al. 2013; van Vliet et al. 2006). In the case of experimental inflammatory colitis, dextran sulfate sodium (DSS)-treated $Mgl1^{-/-}$ mice showed more severe inflammation than wild-type mice (Saba et al. 2009), leading us to speculate that MGL1 has some role in protecting the colon from inflammation. We then showed that MGL1 expressed by colonic lamina propria macrophages (LPMs) plays an anti-inflammatory role in murine experimental colitis by inducing IL-10 production by LPMs (Saba et al. 2009). However, it remains unclear whether commensal bacteria directly bind to the MGL1 molecule and which key molecules are part of the signaling cascade that leads to the induction of IL-10 expression after the recognition of colonic commensal bacteria by its extracellular domain.

Here, we examined the binding of in-situ isolated heat-treated *Streptococcus sp.* and four commonly found *Lactobacillus* species to recombinant MGL1 in vitro. We also investigated the signaling cascade that leads to the induction of IL-10 mRNA in colonic LPMs after the direct interaction of MGL1 on isolated colonic LPMs with these commensal bacteria. Our findings provide a clue that the induction of IL-10 mRNA expression in colonic LPMs occurs via MGL1, spleen tyrosine kinase (Syk), caspase recruitment domain-containing protein 9 (CARD9) and extracellular signal-regulated kinase (ERK). Induction of IL-10 mRNA expression was not observed in LPMs from $Mgl1^{-/-}$ or $Card9^{-/-}$ mice, indicating that MGL1 has a nonredundant function in LPMs and CARD9 is an essential downstream element. These results suggest that MGL1 plays a key role in commensal recognition and signal transduction, which leads to the suppression of inflammatory responses in colonic LPMs.

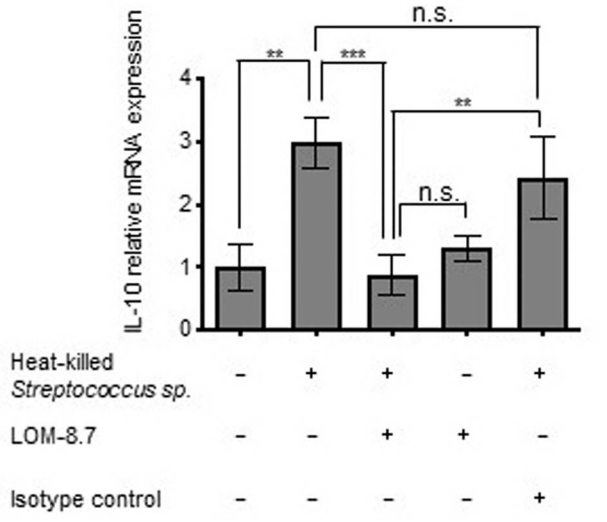

Fig. 1. Inhibition of IL-10 induction in colonic lamina propria macrophages (LPMs) by anti-MGL1 antibody. Isolated colonic LPMs were incubated with or without anti-MGL1 antibody (LOM-8.7) or isotype control antibody for 30 min. Then, antibody-treated and untreated cells were incubated with or without heat-killed *Streptococcus sp.* for 16 h. mRNA from these cells was isolated and the expression of IL-10 was measured by real-time reverse transcription polymerase chain reaction (RT-PCR). Expression levels were normalized to the expression of β-actin, and fold increase of cytokine expression was normalized to untreated cells. Data shown are mean ± SD. Data are representative of three independent experiments each conducted in triplicate. Tukey's multiple comparison test. $^{**}P < 0.01$, $^{***}P < 0.001$, n.s.; not significant.

Results

Inhibition of IL-10 induction by anti-MGL1 antibody

Previously, we showed that stimulation of colonic LPMs with heat-killed *Streptococcus sp.* induced IL-10 mRNA and protein expression in LPMs from wild-type mice, but not from $Mgl1^{-/-}$ mice (Saba et al. 2009). Based on this finding, we assessed whether the induction of IL-10 mRNA expression in colonic LPMs by heat-killed *Streptococcus sp.* occurred as a direct consequence of the interaction between the carbohydrate recognition domain of MGL1 and surface molecules of *Streptococcus sp.* No induction of IL-10 mRNA expression was observed when LPMs were pre-incubated with anti-MGL1 blocking antibody LOM-8.7 before stimulation with heat-killed *Streptococcus sp.* (Figure 1).

The role of Syk in the induction of IL-10 mRNA expression by heat-killed *Streptococcus sp.*

MGL1 has an amino acid sequence (YENL) in its cytoplasmic tail, which potentially functions as a hemi-immunoreceptor tyrosine-based activation motif (hemITAM). Syk was shown to associate with the hemITAM motif present in the cytoplasmic tail of the C-type lectin Dectin-1 (Rogers et al. 2005). To investigate the contribution of Syk in the induction of IL-10 expression, the phosphorylation of Syk in colonic LPMs from wild-type mice was measured at

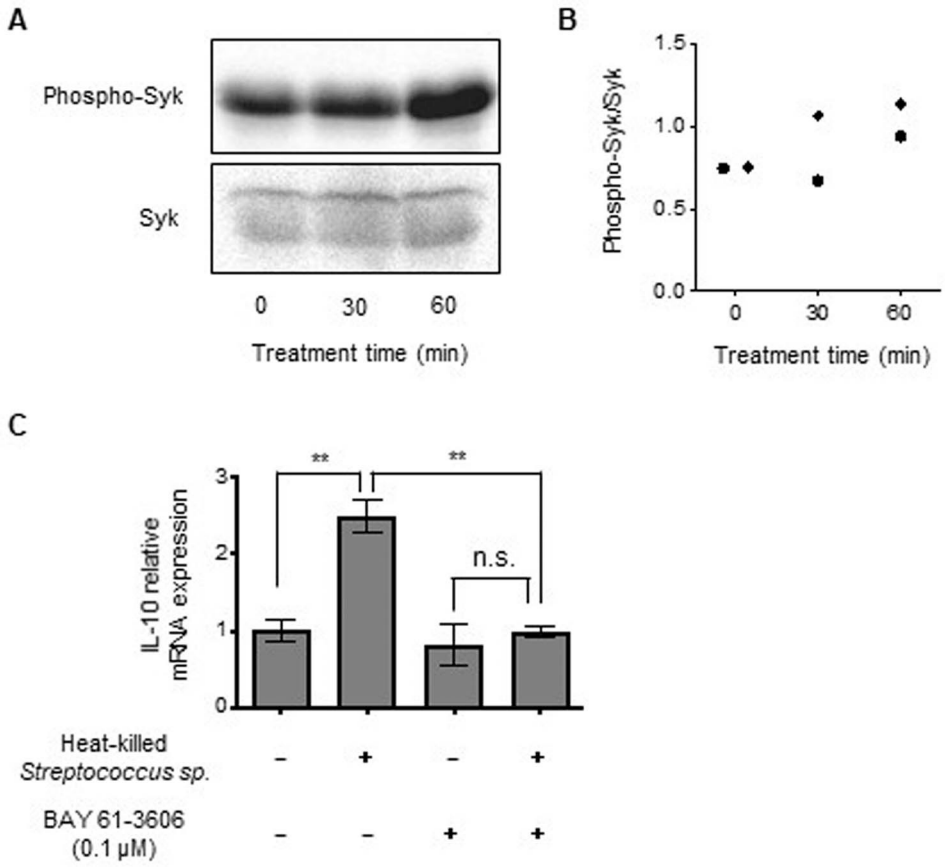

Fig. 2. The role of spleen tyrosine kinase (Syk) in IL-10 induction in isolated colonic lamina propria macrophages (LPMs) after incubation with heat-killed *Streptococcus sp.* (**A**) Isolated LPMs were incubated with heat-killed *Streptococcus sp.* for 0, 30 and 60 min. Lysates were extracted from these cells. Phospho-Syk and Syk were detected by immunoblotting. For Syk, two very close bands were observed. (**B**) Semi-quantitative analysis of the data shown in (A). Band intensities were measured with Image J software and the ratio of Phospho-Syk to Syk is shown. For Syk, the intensities of both bands were added and counted as Syk. (**C**) Isolated colonic LPMs were incubated with or without Syk inhibitor (0.1 μM BAY61–3606) for 30 min. Treated and untreated cells were incubated with or without heat-killed *Streptococcus sp.* for 16 h. mRNA from these cells was isolated and the expression of IL-10 was measured by real-time RT-PCR. Relative expression levels were normalized to the expression of β-actin, and fold increase of the cytokine expression was normalized to untreated cells. Data shown in (A) are representative of two independent experiments. In (B), data from two independent experiments are shown. Data shown in (C) are mean ± SD. Data are representative of two independent experiments each conducted in triplicates. Tukey's multiple comparison test. $^{**}P < 0.01$, n.s.; not significant.

0, 30 and 60 min after stimulation with heat-killed *Streptococcus sp*. Syk protein appeared as two very close bands in western blot and both bands were used for protein quantification. As a result, phosphorylation of Syk was detectable at 60 min (Figure 2A and B).

To verify that Syk is involved in the induction of IL-10 mRNA expression by heat-killed *Streptococcus sp*., BAY61–3606, a highly selective inhibitor of Syk kinase, was added to the co-culture of colonic LPMs and heat-killed *Streptococcus sp*. from wild-type mice, and then IL-10 mRNA expression was measured. After treatment with BAY61–3606, induction of IL-10 mRNA expression was not observed (Figure 2C).

MGL1 and CARD9-dependent IL-10 induction

Downstream signaling from C-type lectin receptors such as Dectin-1 through Syk is mediated by the adaptor protein CARD9 (Gross et al. 2006; Hara et al. 2007). To determine whether the signaling from MGL1 that induces IL-10 mRNA expression in colonic LPMs depends on MGL1 and is coupled through CARD9, we utilized colonic LPMs from *Mgl1*$^{-/-}$ and *Card9*$^{-/-}$ mice stimulated with heat-killed *Streptococcus sp*. The expression level of MGL1 in *Card9*$^{-/-}$ LPMs was similar to that of wild-type mice (data not shown). As we previously observed (Saba et al. 2009), induction of IL-10 mRNA expression was present in wild-type mice (Figure 3A), but absent in colonic LPMs from *Mgl1*$^{-/-}$ mice (Figure 3B). In addition, no induction of IL-10 mRNA was seen in *Card9*$^{-/-}$ mice (Figure 3C). Next, to investigate the functional involvement of toll-like receptor (TLR) signaling in IL-10 induction, we stimulated colonic LPMs from *Myd88*$^{-/-}$ mice with heat-killed *Streptococcus sp*. The expression level of MGL1 on *Myd88*$^{-/-}$ LPMs was similar to that of wild-type mice (data not shown). Induction of IL-10 mRNA expression was observed in colonic LPMs from *Myd88*$^{-/-}$ mice stimulated with *Streptococcus sp*. ($P = 0.066$), although it did not reach statistical significance (Figure 3D).

Heat-killed *Streptococcus sp.* activates MAPK pathway but not NF-κB pathway in colonic LPMs

There is a possibility that NF-κB pathway and/or MAPK pathway are utilized for the induction of IL-10 mRNA expression in colonic LPMs after the direct recognition between MGL1 on colonic LPMs and heat-killed *Streptococcus sp*. (Hara and Saito 2009). To identify

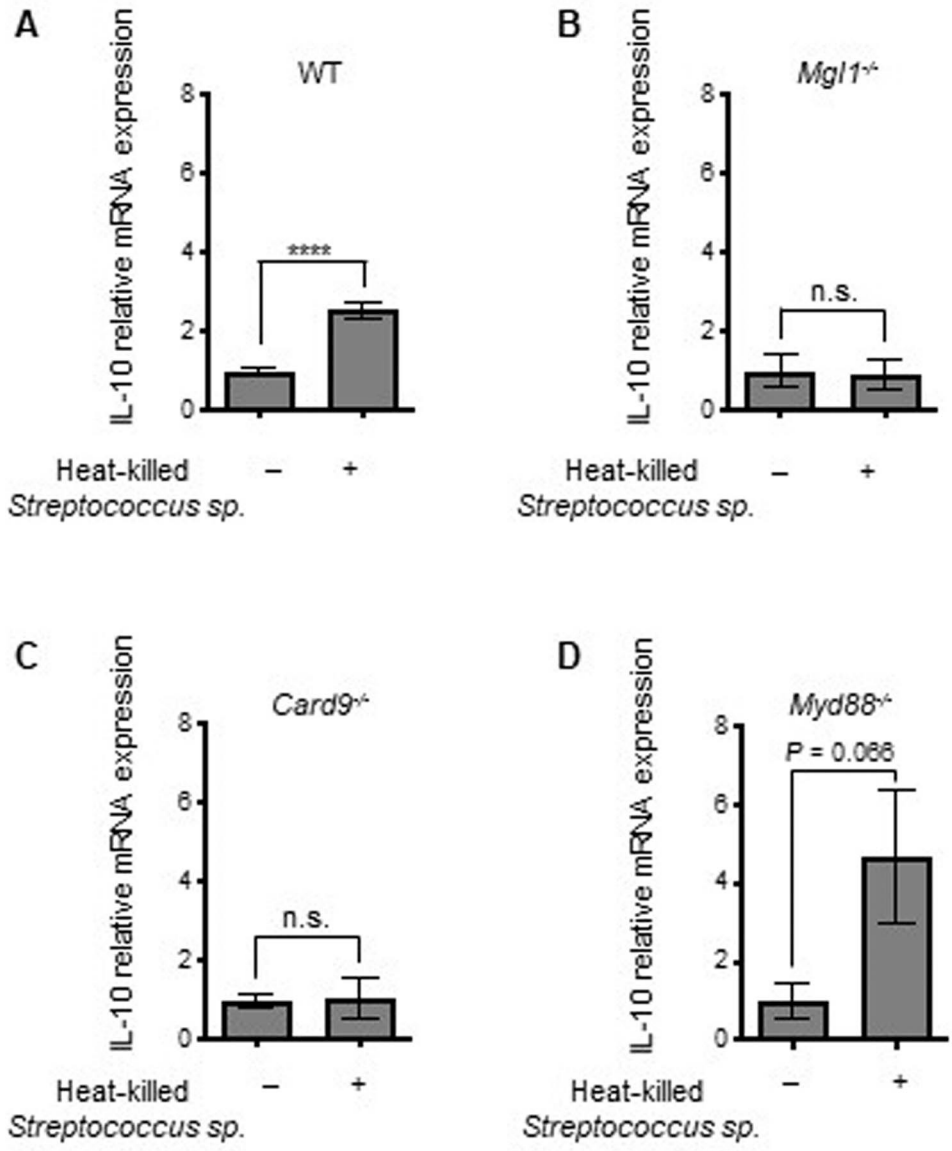

Fig. 3. Expression of IL-10 in colonic lamina propria macrophages (LPMs) after incubation with heat-killed *Streptococcus sp.* Isolated colonic LPMs from (**A**) wild-type mice, (**B**) *Mgl1*$^{-/-}$ mice, (**C**) *Card9*$^{-/-}$ mice and (**D**) *Myd88*$^{-/-}$ mice were incubated with or without heat-killed *Streptococcus sp.* for 16 h. mRNA from these cells was isolated and the expression of IL-10 was measured by real-time RT-PCR. Relative expression levels were normalized to the expression of β-actin, and fold increase of IL-10 expression was normalized to untreated cells. Data shown are mean ± SEM of $N = 8$ (wild-type), $N = 4$ (*Mgl1*$^{-/-}$), $N = 4$ (*Card9*$^{-/-}$) and $N = 3$ (*Myd88*$^{-/-}$) experiments, each conducted in triplicates or duplicates. Welch's *t*-test. $^{****}P < 0.0001$; n.s. not significant.

the molecular mechanism that accounts for the induction of IL-10 mRNA expression, we analyzed the protein expression of MAPKs and NF-κB in colonic LPMs stimulated with heat-killed *Streptococcus sp*. As a result, phosphorylated total ERK (ERK 1/2), as normalized to αTubulin, apparently increased at 30 min and again at 60 min (Figure 4A and C). However, no apparent change was observed in the phosphorylation of p38 and the degradation of IκB-α (Figure 4A) or in the ratio of p38/αTubulin (Figure 4B) and IκB-α/αTubulin (Figure 4D) between time points. Next, to investigate the relationship between ERK phosphorylation and the induction of IL-10 mRNA expression, we used PD98059 to inhibit the phosphorylation of ERK. The induction of IL-10 mRNA expression in colonic LPMs was completely inhibited by PD98059 (Figure 4E).

Identification of bacterial ligands that trigger the MGL1-IL-10 axis

We have made attempts to identify the ligand(s) by extracting surface proteins from in-situ isolated *Streptococcus sp.* using GuHCl. Our data show that GuHCl treatment of *Streptococcus sp.* abolished the binding to recombinant MGL1 in a plate-based assay (Figure 5A). In addition, we show by enzyme-linked immunosorbent assay (ELISA) that rMGL1 binds to plate-coated *Streptococcus sp.* GuHCl cell surface extract and that this binding is inhibited by pre-incubation with galactose and ethylenediaminetetraacetic acid (EDTA) but not mannose (Figure 5B). When LPMs were stimulated in vitro, GuHCl extract-supplemented medium induced IL-10 mRNA expression in wild-type mice; however, no statistically significant IL-10 mRNA increase was observed in *Mgl1*$^{-/-}$, *Card9*$^{-/-}$, or *Myd88*$^{-/-}$ mice

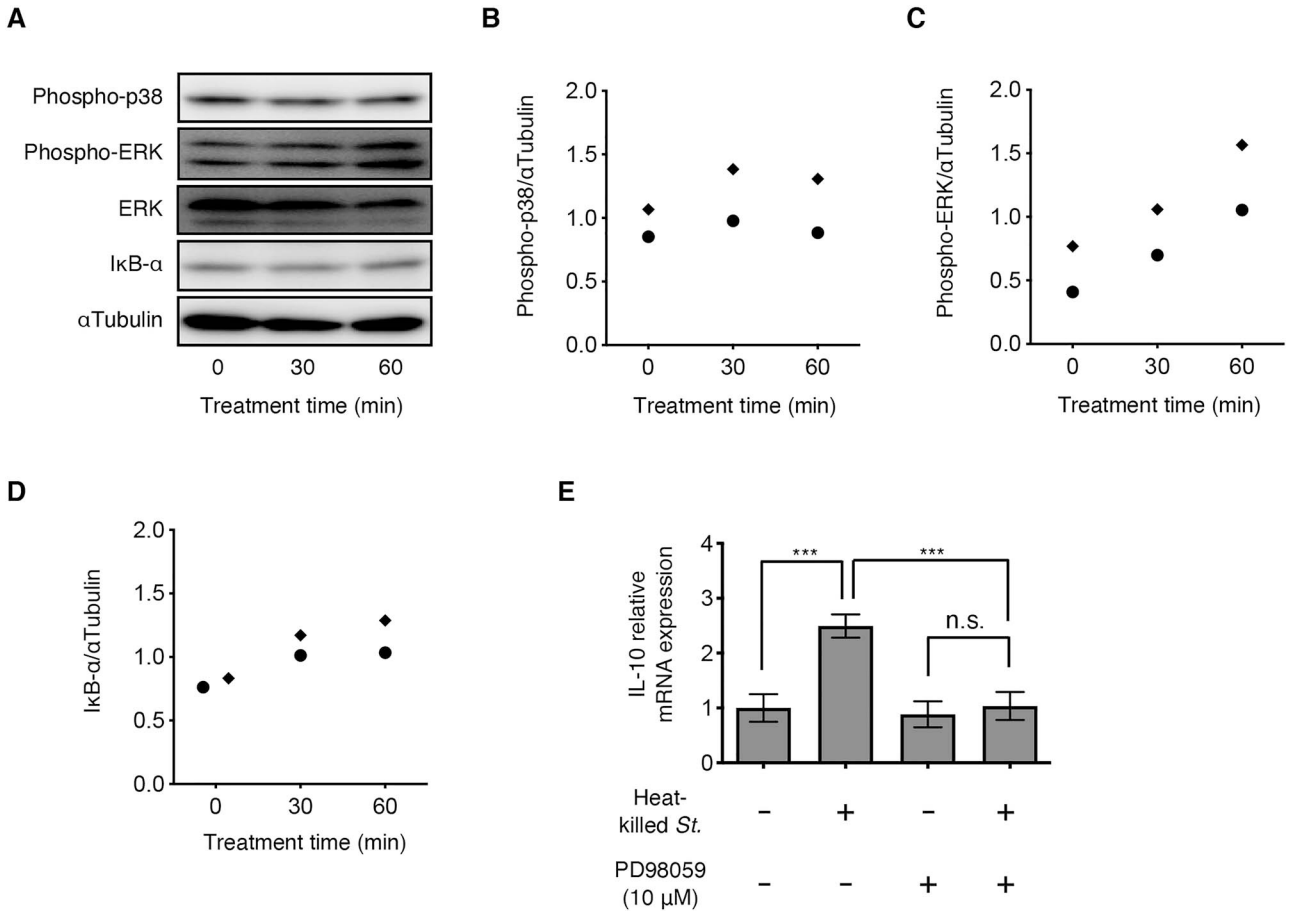

Fig. 4. Contribution of mitogen-activated protein kinase (MAPK)s and nuclear transcription factor kappa B (NF-κB) in the IL-10 induction pathway elicited by the binding of in situ isolated *Streptococcus sp.* to colonic LPMs. (**A**) Isolated colonic lamina propria macrophages (LPMs) were incubated with heat-killed *Streptococcus sp.* for 0, 30 and 60 min. Cell lysates were extracted and Phospho- extracellular signal-regulated kinase (ERK), ERK, Phospho-p38, inhibitor of kappa B (IκB)-α, and αTubulin were detected by immunoblotting. Data are representative of two independent experiments. (**B**) Semi-quantitative analysis of the data shown in (A). Phospho-p38 and αTubulin band intensities were measured by Image J software and the ratio is shown. (**C**) Semi-quantitative analysis of the data shown in (A). Phospho-ERK and αTubulin band intensities were measured by Image J software and the ratio is shown. (**D**) Semi-quantitative analysis of the data shown in (A). IκB-α and αTubulin band intensities were measured by Image J software and the ratio is shown. In (B), (C), and (D), data from two independent experiments are shown. (**E**) Isolated colonic LPMs were incubated with or without ERK inhibitor (10 μM PD98059) for 30 min. Treated and untreated cells were incubated with or without heat-killed *Streptococcus sp.* for 16 h. mRNA from these cells was isolated and the expression of IL-10 was measured by real-time RT-PCR. Relative expression levels were normalized to the expression of β-actin, and fold increase of cytokine expression was normalized to untreated cells. Data shown in (E) are mean ± SD. Data are representative of two independent experiments each conducted in triplicate. Tukey's multiple comparison test. $^{***}P < 0.001$, n.s.; not significant.

(Figure 5C). When nontreated bacterial bodies, GuHCl-treated bacterial bodies and the extract (devoid of bacteria) obtained after GuHCl treatment of bacterial bodies were probed with recombinant MGL1 in lectin blot, we observed bands of similar molecular size in the nontreated sample and in the extract, while those bands were very weak in the bacterial sample that had undergone GuHCl treatment (Figure 5D). Finally, when the GuHCl extract was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and blotted with various lectins, bands of similar size were observed with rMGL1, PNA, VVA and PHA-L4 (all specific for galactose and/or GalNAc as monosaccharide), while no such bands were observed with WGA (specific for *N*-acetyl glucosamine (GlcNAc) as monosaccharide) or ConA (specific for mannose as monosaccharide) (Figure 5E). These results suggest that ligands capable of binding to MGL1 and inducing IL-10 mRNA expression were extracted by GuHCl treatment and that the GuHCl extract contained glycoproteins having galactose or GalNAc.

Binding of *Lactobacillus* species to recombinant MGL1 (rMGL1) and subsequent IL-10 induction in colonic LPMs

Next, we investigated whether the induction of IL-10 is specific to *Streptococcus sp.* or not. For this, we utilized a panel of commonly found commensal *Lactobacillus* species. To investigate if *Lactobacillus* binds to rMGL1, we performed a plate-based binding assay using immobilized rMGL1 and heat-killed *Lactobacillus* bacteria. We found that *Lactobacillus casei, L. gasseri, L. rhamnosus* and *L. delbrueckii subsp. bulgaricus* bound to immobilized rMGL1 to different extents. *L. gasseri* and *L. delbrueckii* showed strong binding,

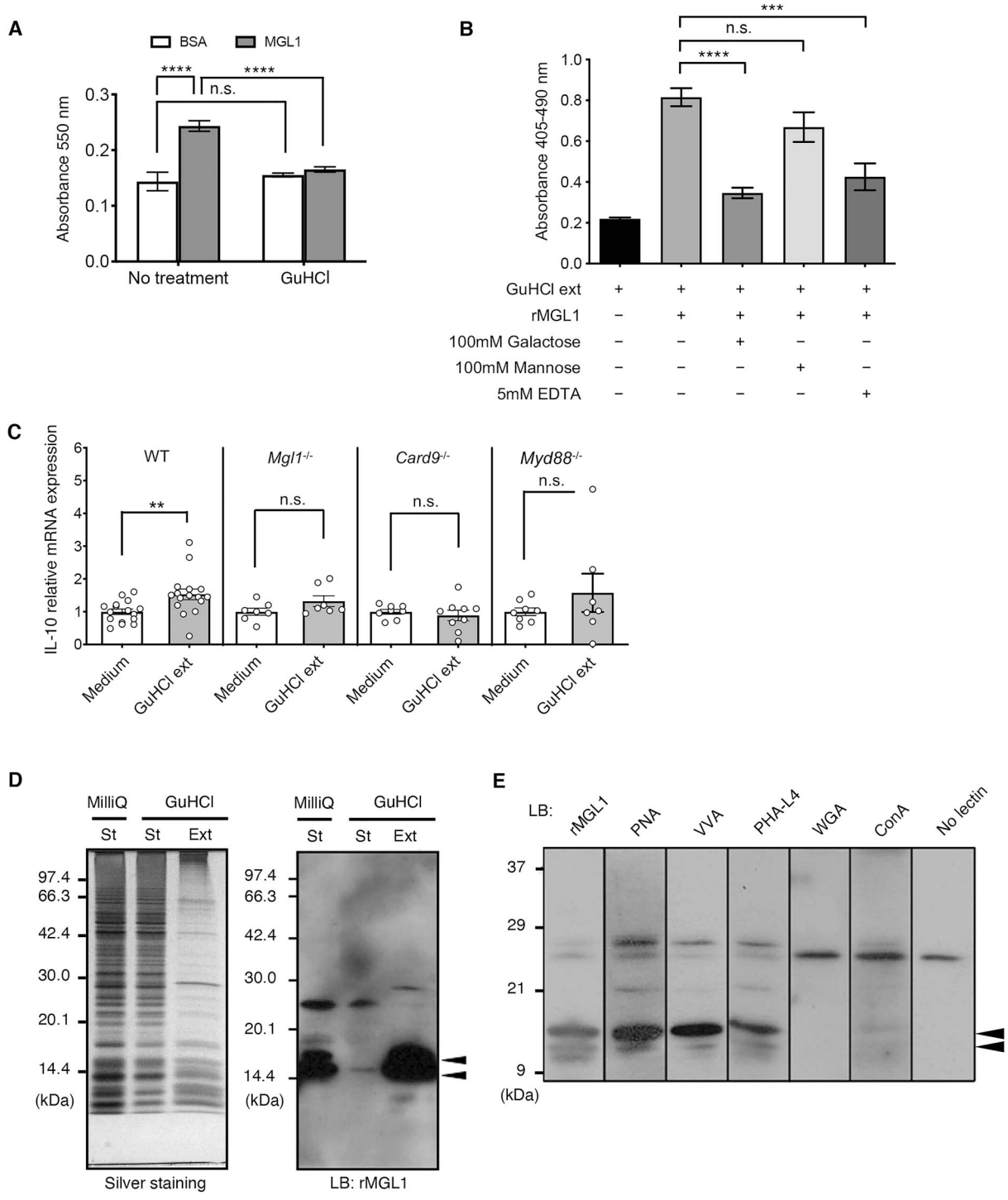

Fig. 5. Identification of MGL1 ligand(s) on the cell surface of *Streptococcus sp.* Cell surface glycoprotein components extracted by guanidine hydrochloride (GuHCl) recognize recombinant MGL1 (rMGL1) and induce IL-10 mRNA expression in colonic lamina propria macrophages (LPMs). (**A**) Binding of heat-killed *Streptococcus sp.* to rMGL1 is abolished when bacteria are treated with GuHCl. Plate-based assay. Shown are mean ± SD from one out of three independent experiments. Tukey's multiple comparison test. (**B**) rMGL1 binds to plate-coated GuHCl extract from *Streptococcus sp.* This binding is inhibited by pre-incubation of rMGL1 with 100 mM galactose and 5 mM EDTA, but not with 100 mM mannose. Data shown are means ± SEM of two independent experiments conducted in quadruplicate (rMGL1; 100 mM galactose; 100 mM mannose) and duplicate (GuHCl ext; 5 mM EDTA). Tukey's multiple comparison test. (**C**) GuHCl-extracted cell surface components from *Streptococcus sp.* (GuHCl ext) induce IL-10 mRNA expression in wild-type colonic lamina propria macrophages. Shown are individual replicates of $N = 6$ (wild-type), $N = 3$ ($Mgl1^{-/-}$), $N = 3$ ($Card9^{-/-}$) and $N = 3$ ($Myd88^{-/-}$) experiments, each conducted in duplicate or triplicate. Welch's t-test. (**D**) GuHCl-extracted cell surface components from *Streptococcus sp.* recognized rMGL1 in Western blot. Left panel: SDS-PAGE followed by silver staining showing the band patterns of MilliQ-treated *Streptococcus sp.* bacterial bodies (St), GuHCl-treated *Streptococcus sp.* bacterial bodies, and the extract obtained after GuHCl treatment of *Streptococcus sp.* (devoid of bacterial bodies) (Ext). Right panel: lectin blotting with recombinant MGL1 (rMGL1) after SDS-PAGE of MilliQ-treated S*treptococcus sp.* bacterial bodies, GuHCl-treated *Streptococcus sp.* bacterial bodies, and the extract obtained after GuHCl treatment of

while *L. casei* bound weakly and *L. rhamnosus* very weakly. Binding of *Lactobacillus* bacteria to rMGL1 was significantly reduced to negative control levels by pre-incubation of rMGL1 with galactose, suggesting the involvement of the MGL1 carbohydrate recognition domain in the interaction (Figure 6A).

To determine whether there is a correlation between the binding of *Lactobacillus* species to MGL1 and the induction of IL-10 expression, IL-10 mRNA expression in colonic LPMs of wild-type mice, *Mgl1*$^{-/-}$ mice and *Card9*$^{-/-}$ mice stimulated with *Lactobacillus* species was measured. In wild-type mice, stimulation with *L. casei, L. gasseri* and *L. delbrueckii subsp. bulgaricus* led to an induction of IL-10 mRNA expression in colonic LPMs, although in the case of *L. delbrueckii subsp. bulgaricus* it did not reach statistical significance (Figure 6B). Stimulation with *L. rhamnosus* did not show any IL-10 mRNA induction (Figure 6B). These findings are in agreement with the results from the binding assay in Figure 6A. In *Mgl1*$^{-/-}$ and in *Card9*$^{-/-}$ mice, no induction of IL-10 mRNA expression was observed after stimulation with any of the *Lactobacillus* species. (Figure 6B).

Discussion

Based on our previous findings showing that heat-killed *Streptococcus sp.* induced an increase in IL-10 expression by MGL1-positive LPMs, we here extend these findings by reporting that the downstream mediators Syk, CARD9 and ERK are involved in IL-10 mRNA induction and that this signaling cascade is initiated by the direct binding of *Streptococcus sp.* cell surface ligand(s) to MGL1. These results suggest that the amino acid sequence, YENL, in the cytoplasmic tail of MGL1 might function as a hemITAM motif in our experimental system, but further study is needed to prove it. In another experimental system, we previously revealed that the tyrosine within the MGL1 hemITAM motif is phosphorylated and then induces the recruitment of Syk and Src homology region 2 domain-containing phosphatase-1 (SHP-1) after stimulation with house dust mite allergen in bone marrow-derived macrophages (Kanemaru et al. 2019).

Until now, recognition through some C-type lectin receptors such as Dectin-1 was known to induce a Syk and CARD9-dependent cytokine production in macrophages and DCs, and it was generally thought that the downstream signaling pathway had two arms, one mainly involving NF-κB and another one possibly involving MAPKs (Hara and Saito 2009). It was also known that CARD9 is crucial for TLR-MyD88-dependent cytokine induction by regulating MAPK activation (Hara and Saito 2009). In contrast, here we have shown that downstream signaling elicited by the interaction of commensal bacteria and MGL1 expressed by LPMs depends on Syk, CARD9 and ERK, but is independent of NF-κB activation. Our results are supported by another study showing that CARD9 is dispensable for NF-κB activation and mediates ERK activation induced by the interaction of Dectin-1 with its ligands, such as curdlan or *Candida albicans* (Jia et al. 2014). Furthermore, we observed a similar tendency for *Streptococcus sp.*- and GuHCl-induced IL-10 mRNA expression in *Myd88*$^{-/-}$ LPMs, suggesting that MyD88 does not play a critical role in this IL-10 induction pathway. In previous studies, induction of various cytokines was dependent on TLR signaling, but IL-10 induction was MyD88-independent (Gross et al. 2006; Rogers et al. 2005). Furthermore, Gu et al. recently reported that ligation of DC-ASGPR, an isoform of human MGL expressed on human DCs (Valladeau et al. 2001), resulted in Syk and MAPK activation, but not NF-κB activation, for IL-10 induction (Gu et al. 2019). Since human MGL does not have a hemITAM motif in the cytoplasmic tail, it is not the exactly same pathway, but there may be a commonality in mouse and human MGL for IL-10 induction after ligand binding. There is still no consensus about what might be the human counterpart of MGL1. Based on the high amino acid sequence similarity of their C-type lectin domains and the hemITAM motif in their cytosolic tail domains, ASGPR (ASGR1) might be the closest possible human counterpart (Kanemaru et al. 2019). However, human MGL without hemITAM motif can still transduce signals induced by anti-MGL specific antibody or carbohydrate ligand-carrying antigens (Napoletano et al. 2012; van Vliet et al. 2013). The possibility of ligand cross-recognition between mouse and human MGL is high because the carbohydrate specificities of mouse and human MGL overlap.

In contrast to other studies, which used macrophages or DCs induced from bone marrow cells in vitro, we have used tissue-resident, in-situ isolated LPMs in the present report. It remains to be assessed whether the signaling cascades found in bone marrow-derived macrophages or DCs induced in vitro are actually operational in tissue-residing macrophages and DCs. Based on the findings in our experimental system, we propose that the activation process leading to IL-10 production in LPMs is unique to the colonic microenvironment and may have something to do with the specific roles of these cells in the colonic lamina propria where they are faced with a large commensal bacteria load. Colonic macrophages have been shown to be hyporesponsive to TLR ligand stimulation regarding cytokine production and expression of costimulatory molecules (Hirotani et al. 2005), a mechanism that is presumed to help prevent chronic intestinal inflammation. At the same time, colonic macrophages have been found to constitutively secrete copious amounts of IL-10 in response to stimulation by the commensal microbiota (Ilarregui et al. 2019; Rivollier et al. 2012). Because of the scarcity of LPMs that can be obtained from mice, we have conducted preliminary experiments in which we stimulated thioglycollate-induced peritoneal macrophages and macrophage colony stimulating factor-induced bone marrow-derived macrophages with *Streptococcus sp*. However, we found that in these experimental systems there is no difference in IL-10 mRNA induction after stimulation with *Streptococcus sp.* between wild-type and *Mgl1*$^{-/-}$ cells. Our preliminary results using thioglycollate-induced peritoneal macrophages obtained from *Mgl*1 and *Myd88* double knockout mice suggest that both MGL1 and MyD88 are required for IL-10 induction. These findings also support our notion that the signaling pathway in LPMs is unique.

Our present data show that in-situ isolated *Streptococcus sp.* and three out of four commonly found *Lactobacillus* species induce IL-10 expression in LPMs. It is tempting to speculate that commensal bacteria have acquired the capacity to induce IL-10 production in LPMs, a system that may have developed to sustain their long-term niche in the gut.

Streptococcus sp. (devoid of bacterial bodies). Arrowheads indicate two major bands indicative of *Streptococcus sp.* cell surface ligand(s) recognized by rMGL1. (**E**) Lectin blotting of *Streptococcus sp.* cell surface components extracted by GuHCl. Arrowheads indicate two major bands indicative of *Streptococcus sp.* cell surface ligands recognized by MGL1. PNA; peanut (*Arachis hypogaea)* agglutinin, VVA; *Vicia villosa* agglutinin, PHA-L4; *Phaseolus vulgaris* agglutinin-L4, WGA; wheat germ (*Triticum vulgaris)* agglutinin, ConA; Concanavalin A. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, n.s.; not significant.

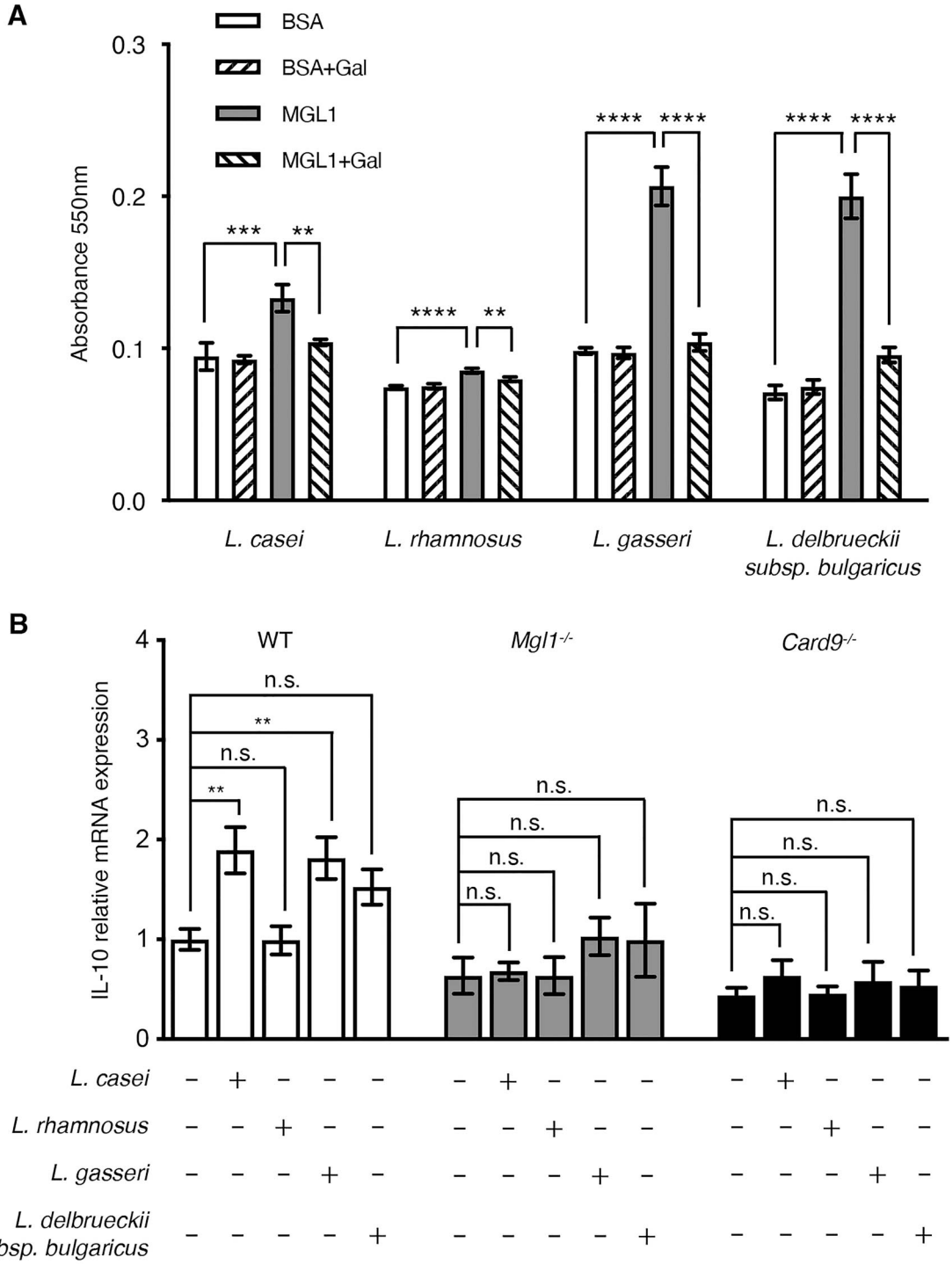

Fig. 6. Binding of recombinant MGL1 (rMGL1) to *Lactobacillus* species and *Lactobacillus* species induced IL-10 mRNA expression in LPMs. (**A**). Binding of recombinant MGL1 (rMGL1) to *Lactobacillus* species. Heat-killed *Lactobacillus* bacteria were applied to microtiter plates with immobilized rMGL1 or bovine serum albumin (BSA). Inhibition of binding was carried out by pre-incubation of immobilized rMGL1 or BSA with 100 mmol/L galactose (Gal) at room temperature. Bound bacteria were detected with crystal violet and quantified by measuring absorbance at 550 nm. Data shown are means ± SD of triplicates and are representative of three independent experiments. Tukey's multiple comparison test. (**B**) Expression of IL-10 mRNA in colonic lamina propria macrophages (LPMs) after incubation with heat-killed *Lactobacillus* species. Isolated colonic LPMs from wild-type mice, *Mgl1*$^{-/-}$, and *Card9*$^{-/-}$ mice were incubated with or without heat-killed *Lactobacillus* bacteria for 16 h. mRNA from these cells was isolated and the expression of IL-10 was measured by real-time RT-PCR. Relative expression levels were normalized to the expression of β-actin, and fold increase of IL-10 expression was normalized to untreated cells. Data shown are mean ± SEM of $N = 3$ (wild-type), $N = 2$ (*Mgl1*$^{-/-}$) and $N = 2$ (*Card9*$^{-/-}$) independent experiments conducted in triplicate. Dunnett's multiple comparison test. $^{**}P < 0.01$; $^{***}P < 0.001$, $^{****}P < 0.0001$; n.s. not significant.

We have previously shown that in the case of house dust mite-induced atopic dermatitis, MGL1 expressed by skin macrophages is involved in the dampening of the immune response and signals through Syk. In the case of skin macrophages, MGL1 functions by downregulating the production of TLR4-induced inflammatory cytokines, while IL-10 production in the same cells remains unaffected (Kanemaru et al. 2019). Although MGL1 is also expressed on DCs in the skin, DCs do not seem to engage with this immunosuppression (Kanemaru et al. 2019). In macrophages residing in the colonic microenvironment, MGL1 exerts unique signaling pathways leading to suppression of inflammation without interfering with TLR-mediated pathways. Such unique signaling pathways may provide keys to understand the cell and tissue-specific roles of MGL1.

In the future, it will be important to elucidate the bacterial surface molecules that serve as the ligands for MGL1 in various microenvironments. Gram-positive bacterial cell surface proteins potentially capable of interacting with MGL1 might be S-layer proteins, exopolysaccharides or cell wall-associated proteins (Sengupta et al. 2013). We have started to work in this direction by separating in-situ isolated *Streptococcus sp.* bacteria into two fractions, a cell surface component fraction and a remainder fraction. Our experiments have shown that the cell surface component fraction obtainable by GuHCl treatment contains surface proteins recognized by MGL1 and that this recognition can be abolished by pre-incubation of rMGL1 with galactose and EDTA but not with mannose, suggesting that this recognition requires the carbohydrate recognition domain of MGL1. Further, in lectin blotting analysis of the cell surface component fraction strong binding at the migration distance of interest (around 15 kDa) was observed with PNA, VVA-B4 and PHA-L4, indicating the possibility that the ligand contains terminal galactose or GalNAc. In contrast, no binding at this migration distance was seen with WGA or ConA, indicating that terminal GlcNAc, GlcNAc repeats or mannose are probably not present in the ligand. Overall, our data suggest that the ligand(s), which bind to MGL1, seem to be glycoproteins containing galactose or GalNAc at its nonreducing terminus. Finally, our data show that heat-inactivated in situ isolated *Streptococcus sp.* and the GuHCl cell surface extract of in situ isolated *Streptococcus sp.* induced IL-10 mRNA expression in isolated LPMs from wild-type mice and tended to increase IL-10 mRNA expression in *Myd88*$^{-/-}$ mice, but not in *Mgl1*$^{-/-}$ or *Card9*$^{-/-}$ mice in vitro. We think these data clearly show that as yet unidentified protein(s) on the surface of in situ isolated *Streptococcus sp.* interact with MGL1 to induce IL-10 mRNA expression in LPMs. We have recently shown that MGL1 expressed by skin macrophages recognizes mucin-like ligands present in house dust mite components (Kanemaru et al. 2019). In the case of *Lactobacillus* species, it has been reported that *L. casei* and *L. delbrueckii subsp. bulgaricus* have no S-layer proteins (Hynonen and Palva 2013). Similarly, *Streptococcus thermophilus* also does not express S-layer proteins (Klotz and Barrangou 2018). These reports lead us to speculate that the ligand which binds to rMGL1 is not an S-layer protein. Ultimately, knock-out strains lacking specific bacterial surface molecules should be important tools in MGL1 ligand identification.

In conclusion, we have demonstrated that MGL1 on in-situ colonic LPMs directly interacts with commensal bacteria present in the mouse colon. We have shown that Syk, CARD9 and ERK are indispensable for IL-10 induction in wild-type mice. In addition, we have also shown that potential bacterial ligands for MGL1 are present in the cell surface extract of *Streptococcus sp.* In the future, elucidation of the surface molecules and ligands on commensal bacteria responsible for the interaction with MGL1 will provide new insights into this anti-inflammatory mechanism and open up new avenues for therapeutic intervention of inflammatory bowel diseases.

Materials and methods

Antibodies

Anti-MGL1 antibody LOM-8.7 was previously generated in our laboratory (Kimura et al. 1995). The following antibodies were purchased: anti-Phospho-Syk (Tyr525/526, Cell Signaling Technology, Danvers, MA, USA), anti-Syk (Cell Signaling Technology), fluorescein isothiocyanate-conjugated anti-CD11b (Biolegend, San Diego, CA, USA), phycoerythrin-conjugated anti-F4/80 (eBioscience, San Diego, CA, USA). The following antibodies were a kind gift from Profs. Ichijo Hidenori (The University of Tokyo, Tokyo, Japan) and Atsushi Matsuzawa (Tohoku University, Sendai, Japan), anti-Phospho-p38 MAPK (Thr180/Tyr182, Cell Signaling Technology), anti-IκB-α (H-4, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-αTubulin (B-7, Santa Cruz Biotechnology), anti-Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204, clone E10, Cell Signaling Technology), anti-ERK 2 (C-14, Santa Cruz Biotechnology).

Preparation of heat-killed *Streptococcus sp.* and *Lactobacillus* species

Streptococcus sp. were isolated from mesenteric lymph nodes of DSS-treated mice (obtained on day 7) and a single bacterial colony was cultured and heat-treated as previously described (Saba et al. 2009). *Streptococcus sp.* were considered to be a commensal strain according to the results of 16S ribosomal RNA sequencing and BLAST search (Supplementary Figure S1). *L. casei* (ATCC 334T), *L. rhamnosus* (ATCC 7469T), *L. gasseri* (DSM 20243 T) and *L. delbrueckii subsp. bulgaricus* (ATCC 11842T) were cultured in lactobacilli-MRS broth (Difco, Franklin Lakes, NY, USA) for 20 h at 37°C, collected by centrifugation, suspended in MilliQ water, heat-treated at 100°C for 30 min, and lyophilized. Heat-killed *Lactobacillus* species were a kind gift from Dr. Kan Shida (Yakult Central Institute, Tokyo, Japan).

Mice. *Mgl1*$^{-/-}$ mice were originally obtained from Dr. Stephen M. Hedrick's laboratory (Onami et al. 2002) and back crossed into C57BL/6J strain. *Card9*$^{-/-}$ mice were previously generated (Hara et al. 2007). *Myd88*$^{-/-}$ mice were provided by Dr. Shizuo Akira (Adachi et al. 1998). C57BL/6J mice were purchased from Japan SLC (Hamamatsu, Japan). All mice were maintained under specific pathogen-free conditions at the Graduate School of Pharmaceutical Sciences of The University of Tokyo.

Isolation, flow cytometry and cell sorting of LPMs

Lamina propria mononuclear cells were isolated and stained as previously described (Saba et al. 2009). F4/80^{high}, CD11b^{high} cells were sorted as LPMs by FACSAria (BD, Franklin Lakes, NJ, USA) and analyzed with FlowJo software (Tree Star, Ashland, OR, USA).

Stimulation of LPMs with heat-killed bacteria

Isolated sorted LPMs were cultured in RPMI-1640 medium. For incubation with heat-killed bacteria, *Streptococcus sp.* or *Lactobacillus* species were added to the culture at a concentration of 10 μg/mL for 16 h at 37°C. Based on the knowledge that 1 mg of *Lactobacillus casei* corresponds to 1×10^9 bacteria, we estimate that the

bacteria-to-cell ratio ranged from 30:1 to 60:1 in our experimental system. For blocking MGL1 on LPMs in vitro, LOM-8.7 mAb or rat IgG2a isotype control antibody was added into the cell culture at a concentration of 50 μg/mL, and incubated for 30 min at 4°C before the addition of heat-killed bacteria. For inhibition of Syk, sorted LPMs were incubated with BAY61-3606 (0.1 μM, Calbiochem, San Diego, CA, USA), a highly selective inhibitor of Syk kinase, for 30 min on ice before the addition of heat-killed bacteria. For the inhibition of ERK, sorted LPMs were incubated with PD98059 (10 μM, Merck, Darmstadt, Germany), a potent and selective inhibitor of MAP kinase kinases (MEK1 and MEK2), for 30 min on ice before the addition of heat-killed bacteria.

Western blotting

Cells were rinsed twice with cold phosphate-buffered saline (PBS), and then lysed in lysis buffer (50 mM Tris–HCl, pH 7.6, 150 mM NaCl, 1% NP40, 0.1% SDS, 0.5% deoxycholate, 1 mM phenylmethylsulfonyl fluoride). Cell lysates were separated on 12% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. Non-specific binding was blocked with 3% bovine serum albumin (BSA) in Tris-buffered saline (TBS) containing 0.1% Tween 20 (TBST) for 1 h at room temperature. After incubation with primary antibodies (Phospho-p38, Phospho-ERK, ERK, Phospho-Syk, Syk, IκB-α and αTubulin), membranes were washed three times with PBS containing 0.1% Tween 20 (PBST) and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h. After washing three times with PBST, protein bands were detected with enhanced chemiluminescent (ECL) detection reagent. Protein expression levels were normalized to the band intensity of Syk and/or αTubulin as an internal control.

Binding of heat-treated bacteria to recombinant MGL1

Recombinant mouse MGL1 (rMGL1) was prepared as previously described (Tsuiji et al. 2002). Binding of heat-killed bacteria to immobilized recombinant MGL1 was measured as previously described (Saba et al. 2009) using immobilized BSA as a negative control.

Extraction of *Streptococcus sp.* cell surface proteins

Cultured bacteria were collected and heat-killed at 100°C for 20 min. Heat-killed bacteria were suspended in 2 M guanidine hydrochloride (GuHCl), incubated at 37°C for 2 h. After centrifugation, the supernatant was dialyzed against Ca^{2+}, Mg^{2+} containing TBS and the resulting preparation was called "guanidine hydrochloride extract (GuHCl extract)." The remaining pellet, containing bacterial bodies devoid of cell surface components, was washed three times with MilliQ water. For stimulation of LPMs, 10 μg/mL of the GuHCl extract were used.

Binding of rMGL1 to plate-bound GuHCl extract

Streptococcus sp. GuHCl extract (10 μg/ml) was coated to 96-well plates (Greiner) at 4°C overnight. Plates were washed with Ca^{2+}-, Mg^{2+}-containing PBST and then blocked with 3% BSA in Ca^{2+}-, Mg^{2+}-containing PBST. Biotinylated rMGL1 diluted to 10 μg/mL in 3% BSA/Ca^{2+}-, Mg^{2+}-containing PBST was added to the plate and incubated for 2 h at room temperature. After washing with Ca^{2+}, Mg^{2+}-containing PBST, HRP-Streptavidin (Invitrogen, 1:5000) was added to the plate and incubated for 1 h at room temperature. For color development, 1 mM 2,2′-azino-bis (3-ethyobenzthiazoline-6-sulfonic acid) diammonium salt in 0.1 M citric acid buffer, pH 4.3, containing 0.34% hydrogen peroxide was added and absorbance was measured at 405 and 490 nm. For binding inhibition, rMGL1 was pre-incubated with 100 mM galactose, 100 mM mannose or 5 mM EDTA for 2 h at room temperature before addition to the plate.

Lectin blotting

Heat-killed bacteria or GuHCl extracts were boiled in sample buffer containing 2-mercaptoethanol. Each sample was separated on a 15% SDS-PAGE gel, and transferred to Immobilon-P PVDF membrane (Millipore). After blocking with 3% BSA/TBST, the membranes were incubated with 3 μg/mL biotinylated rMGL1 or 5 μg/mL biotinylated peanut (*Arachis hypogaea)* agglutinin (PNA, VECTOR, Burlingame, CA), *Vicia villosa* agglutinin (VVA, VECTOR), *Phaseolus vulgaris* agglutinin-L4 (PHA-L4, Seikagaku, Tokyo, Japan), wheat germ (*Triticum vulgaris)* agglutinin (WGA, VECTOR) or Concanavalin A (ConA, Seikagaku) at 4°C overnight. After washing, bound lectins were detected by HRP-streptavidin for 1 h at room temperature and visualized with ECL detection reagent.

Ethical approval

All animal experiments were approved by the Animal Care and Use Committee of the Graduate School of Pharmaceutical Sciences of The University of Tokyo.

Statistics

Depending on the nature of the experiment, the data are presented either as individual points or as mean ± SD or SEM. Data were analyzed using GraphPad Prism 8 software, and statistical significance was evaluated by Welch's *t*-test or multiple comparison test where appropriate. *P*-values of <0.05 were considered statistically significant.

Supplementary data

Supplementary data for this article is available at *Glycobiology* online.

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Conflict of interest statement

All authors have no conflict of interest to report.

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Abbreviations

ASGPR, asialoglycoprotein receptor; BSA, bovine serum albumin; CARD9, caspase recruitment domain-containing protein 9; ConA, Concanavalin A; DC, dendritic cell; DSS, dextran sulfate sodium; EDTA, ethylenediaminetetraacetic acid; ERK, extracellular signal-regulated kinase; GuHCl, guanidine hydrochloride; hemITAM, hemi-immunoreceptor tyrosine-based activation motif; HRP, horseradish peroxidase; IκB, inhibitor of kappa B; IL-10, interleukin 10; LPM, lamina propria macrophage; MAPK, mitogen-activated protein kinase; MGL, macrophage galactose-type calcium-type lectin; MyD88, myeloid differentiation primary response 88; NF-κB, nuclear transcription factor kappa B; PBS, phosphate-buffered saline; PHA-L4, *Phaseolus vulgaris* agglutinin-L4; PNA, peanut (*Arachis hypogaea*) agglutinin; Syk, spleen tyrosine kinase; TLR, toll-like receptor; TBS, Tris-buffered saline; VVA, *Vicia villosa* agglutinin; WGA, wheat germ (*Triticum vulgaris*) agglutinin

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