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MARCO Is Required for TLR2- and Nod2-Mediated Responses to *Streptococcus pneumoniae* and Clearance of Pneumococcal Colonization in the Murine Nasopharynx

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***Streptococcus pneumoniae* is a common human pathogen that accounts for >1 million deaths every year. Colonization of the nasopharynx by *S. pneumoniae* precedes pulmonary and other invasive diseases and, therefore, is a promising target for intervention. Because the receptors scavenger receptor A (SRA), macrophage receptor with collagenous structure (MARCO), and mannose receptor (MR) have been identified as nonopsonic receptors for *S. pneumoniae* in the lung, we used scavenger receptor knockout mice to study the roles of these receptors in the clearance of *S. pneumoniae* from the nasopharynx. MARCO$^{-/-}$, but not SRA$^{-/-}$ or MR$^{-/-}$, mice had significantly impaired clearance of *S. pneumoniae* from the nasopharynx. In addition to impairment in bacterial clearance, MARCO$^{-/-}$ mice had abrogated cytokine production and cellular recruitment to the nasopharynx following colonization. Furthermore, macrophages from MARCO$^{-/-}$ mice were deficient in cytokine and chemokine production, including type I IFNs, in response to *S. pneumoniae*. MARCO was required for maximal TLR2- and nucleotide-binding oligomerization domain–containing (Nod)2-dependent NF-κB activation and signaling that ultimately resulted in clearance. Thus, MARCO is an important component of anti-*S. pneumoniae* responses in the murine nasopharynx during colonization. *The Journal of Immunology*, 2013, 190: 250–258.**

Streptococcus pneumoniae (the pneumococcus) is one of the most prevalent human pathogens and causes >1 million deaths each year, most of which are young children (1). The bulk of these deaths occurs as the result of pneumococcal pneumonia, in which bacteria spread from their preferred niche, the nasopharynx, to the lungs. Pneumococci are armed with a plethora of colonization factors that allow them to establish a carrier state in the nasopharynx of ∼10% of the adult population at any given time, with higher rates in children <5 y of age (2). Colonization events are sequential, and each lasts days to weeks before clearance. Despite the use of antibiotics and the introduction of polysaccharide-based vaccines, antibiotic resistance and serotype replacement have resulted in continued challenges in the management of this pathogen (3–6).

The initial immune response to pneumococcal colonization is characterized by a brisk, yet ineffective, neutrophil response (7). The resolution of the carrier state appears to require the subsequent recruitment of monocytes from the blood into the nasal interstitial spaces, where they differentiate into macrophages, which are then thought to phagocytose and destroy the bacteria. These monocytes are attracted by the chemokine CCL2, which is produced in response to the recognition of bacterial peptidoglycan by nucleotide-binding oligomerization domain–containing (Nod)2 (8). Signaling through Nod2 also drives the expression of type I IFNs, which contributes to clearance (9). Additionally, the influx of monocytes is affected by TLR2, although the mechanism of this interaction has not been fully elucidated (7). These innate pathways involved in clearance of pneumococcal colonization all require prior recognition of the pneumococcus by resident cells, yet this initial interaction remains elusive.

It also was shown that the pneumococcus, through the expression of its capsular polysaccharide and other mechanisms, is able to avoid opsonization by Abs (10, 11), as well as complement components (12–14). Although it is not known which macrophage receptors recognize the pneumococcus in the low-opsonic environment of the nasopharynx, previous studies showed that mannose receptor (MR) (15), SIGNR1 (16), and the class A scavenger receptors (SRs), known as scavenger receptor A (SRA) (17) and macrophage receptor with collagenous structure (MARCO) (18), are capable of binding to *S. pneumoniae* via nonopsonic mechanisms.

The class A SRs are known for their broad ligand specificity and phagocytic functions (19). They were also shown to directly and indirectly modulate TLR and NLR signaling (20, 21). MARCO is constitutively expressed only on specific subsets of macrophages (22); however, its expression can also be induced on macrophages at sites of inflammation as a result of bacterial infection, thus reinforcing the notion that it is important in immune defense. In contrast, SRA is constitutively expressed on most, if not all, macrophages, as well as other cells, such as endothelial cells. MARCO and

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Abbreviations used in this article: GFP-VSV, GFP-expressing vesicular stomatitis virus; MΦ, BioGel-elicited macrophage; MARCO, macrophage receptor with collagenous structure; MDP, muramyl dipeptide; MOI, multiplicity of infection; MR, mannose receptor; Nod, nucleotide-binding oligomerization domain–containing receptor; SR, scavenger receptor; SRA, scavenger receptor A; URT, upper respiratory tract; WT, wild-type.

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SRA were shown to directly bind and phagocytose *S. pneumoniae* in a murine pneumonia model; however, the bacterial ligands were not identified (23, 24). The role of SRs in colonization is not known and is the focus of this study.

In the current study, we show that MARCO is a key component in the macrophage response to *S. pneumoniae* during colonization. We demonstrate that MARCO is involved in the timely clearance of pneumococcal colonization from the nasopharynx of mice by augmenting the production of proinflammatory cytokines and chemokines. In vitro analyses confirm MARCO's role in the TLR2- and Nod2-signaling pathways that lead to the production of these soluble mediators. To our knowledge, we also show, for the first time, a connection between SR activity and the production of type I IFNs in response to extracellular bacteria.

Materials and Methods

Mice

C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, MA) and The Jackson Laboratory (Bar Harbor, ME) at 6–8 wk of age. Original MARCO$^{-/-}$ and SRA$^{-/-}$ mice were generously provided by the laboratory of Professor Siamon Gordon (University of Oxford, Oxford, U.K.) and bred at the McMaster Central Animal Facility. MR$^{-/-}$ mice were from University of Oxford, C3$^{-/-}$ mice by John Lambris (University of Pennsylvania), and MAC1$^{-/-}$ mice were purchased from The Jackson Laboratory. All animals were used at either 9–11 wk of age or when they reached 20 g in weight, whichever occurred first. Wild-type (WT) mice were sex matched to the knockout groups. All procedures were performed in accordance with the McMaster Animal Research Ethics Board guidelines and Institutional Animal Care and Use Committee protocols at the University of Pennsylvania.

Bacterial strains and culture conditions

S. pneumoniae strain P1121 (a clinical isolate, serotype 23F) (25) was used for all assays and inoculations. TIGR4 (a clinical isolate, serotype 4) was also used for colonization studies of SR knockout mice. Bacteria were propagated in tryptic soy broth (Life Technologies) at 37˚C and 5% CO_2 until cultures reached log phase, OD_{600} between 0.45 and 0.50. In vitro and ex vivo experiments were performed with lysozyme-digested bacteria, because this enzyme is abundant on the mucosa and during macrophage processing. Lysozyme treatment does not lyse the pneumococcus, but it releases peptidoglycan fragments that promote innate-immune signaling (8). Lysozyme-digested pneumococcus was prepared as follows: bacteria were heat-killed by incubation at 65˚C for 10 min, followed by incubation for 18 h in the presence of 0.5 μg/ml recombinant human lysozyme (Cedarlane, Burlington, ON, Canada) at room temperature, with vortexing every few hours.

Murine model of pneumococcal colonization

Pneumococcus was grown to log phase, concentrated 10-fold in PBS, and stored on ice. Unanesthetized mice were inoculated intranasally with 10 μl bacterial suspension containing ~1×10^7 CFU. At the time indicated, mice were sacrificed, the trachea was cannulated, and 200 μl PBS was instilled. Lavage fluid was collected from the nares, serially diluted in PBS, and plated on tryptic soy plates containing 5% sheep's blood and neomycin (5 μg/ml for TIGR4 and 10 μg/ml for P1121). Colonies were counted after overnight incubation at 37˚C and 5% CO_2.

Macrophage culture and stimulation

All macrophages were cultured in a humidified environment at 37˚C with 5% CO_2 in RPMI 1640 supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), and 10 mM L-glutamine. For assays involving stimulation of cultured cells, lysozyme-digested P1121 was added in RPMI 1640 containing 1% FCS and L-glutamine. To study recruited or "elicited" macrophages, BioGel elicitation was performed. Mice were injected i.p. with 1 ml 2% (w/v) BioGel P100 45–90-μm-diameter microbeads (Bio-Rad). Peritoneal lavages were performed 5 d later with 10 ml ice-cold PBS. Cells were then washed once with RPMI 1640 and resuspended in RPMI 1640 supplemented with 10% FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), and 10 mM L-glutamine and incubated in 24-well tissue culture plates at a concentration of 5×10^5 cells/well in 1 ml medium. Cells were allowed to adhere for 2 h and then nonadherent cells were removed by washing with warm media, and adherent cells were incubated at 37˚C with 5% CO_2 overnight. The following day, cells were stimulated with 20 μg/ml recombinant murine IFN-γ (PeproTech) for 24 h to upregulate Nod2 expression. Elicited macrophages were then stimulated with lysozyme-digested *S. pneumoniae* at a multiplicity of infection (MOI) of 25, 10 ng/ml LPS (Sigma) plus 5 μg/ml muramyl dipeptide (MDP; Sigma), or media alone for 16 h. Supernatants were collected and either used immediately for cytokine ELISA or stored at −80˚C for later use.

RNA extraction and RT-PCR

Total RNA was extracted from nasal lavages collected with RLT Lysis Buffer (QIAGEN) containing 0.7% 2-ME. An RNA extraction Micro kit (Ambion) was used following the manufacturer's directions. cDNA was synthesized using MMLV reverse transcriptase (Invitrogen), following the manufacturer's directions. Quantitative real-time PCR was performed on cDNA with SYBR green (Promega), following the manufacturer's directions, and results were compared with a GAPDH control gene. The primers used for each gene can be found in Table I.

Immunofluorescent staining

Nasal lavage fluid was applied to ColorFrost Plus Microscope Glass Slides using the Shandon Cytospin 3 cytocentrifuge (Thermo Scientific) at 230 × *g* for 10 min. The cytospin preparations were air-dried briefly before fixing in acetone. Samples were blocked with 10% donkey serum before the addition of primary Ab. Signal was detected with Cy2- or Cy3-conjugated species-specific secondary Ab (Jackson ImmunoResearch, West Grove, PA) incubated at 1:500 dilution in block for 2 h at room temperature. After washing with PBS followed by distilled H_2O, sections were counterstained with DAPI (Molecular Probes, Invitrogen, Carlsbad, CA) diluted 1:10,000 in distilled H_2O. All image analysis was carried out using iVision-Mac (BioVision Technologies, Exton, PA).

For positive control of SR staining, spleens were dissected from naive mice and fresh-frozen in Tissue-Tek OCT embedding medium (Miles) in a Tissue-Tek Cryomold. Five-micrometer-thick sections were cut and stored at −80˚C. Tissue sections were stained as above.

Flow cytometry

Nasal lavage samples were stained with the following fluorescent Abs: Ly6C:FITC, Ly6G:PE, F4/80:allophycocyanin, and CD45:Pacific Blue (eBioscience). Staining was completed by incubating cells with the fluorophore-conjugated Abs after blocking with the 2.4G2 Ab (eBioscience) at 4˚C. Cells were then fixed with 1% paraformaldehyde and assayed with a BD LSRII flow cytometer the following day. Data were gathered using FACSDiva software (BD) and analyzed using FlowJo software (TreeStar).

Cytokine ELISA

ELISAs for TNF-α, IL-1β, CCL2, and IL-6 were performed as per the manufacturer's directions (eBioscience). Plates were read on a Safire plate reader within 20 min of the addition of H_2SO_4.

IFN bioassay

Elicited macrophages were prepared as described above and stimulated with lysozyme-digested P1121 at an MOI of 25 for 24 h. Supernatants were removed from the macrophages, serially diluted, and added to a confluent monolayer of L929 cells overnight at 37˚C and 5% CO_2. IFN-α was used as a concentration standard. The next day, the medium was removed and replaced with 30 μl 1×10^5 PFU/ml a GFP-expressing vesicular stomatitis virus (GFP-VSV) in serum-free media for 24 h, as described (26). If the supernatants contained any type I IFN (i.e., if the macrophages produced IFN in response to the bacterial stimulation) then the virus would not be able to replicate and no fluorescence would be seen. The fluorescence signal given off by GFP was measured the next day using a Typhoon Trio variable mode imager and quantified using ImageQuant software (ImageMaster).

NF-κB luciferase assay

Human TLR2, CD14, TLR9, and NF-κB luciferase plasmids were provided by Dr. Cynthia Leifer (Cornell University, Ithaca, NY), human Nod2 plasmid was provided by Dr. Dana Philpott (University of Toronto, Toronto, ON, Canada), and β-galactosidase plasmid was provided by Dr. Brian Lichty (McMaster University). All plasmids were amplified by *Escherichia coli* DH5-α cells and purified using a HiPure Plasmid Filter Midiprep Kit (Invitrogen).

Low-passage ($n \leq 4$) HEK293T cells were seeded at 5×10^5 cells/well in 3 ml DMEM/well in a six-well plate overnight. HEK293T cells were transfected with NF-κB luciferase (100 ng), β-galactosidase (100 ng), and optimal combinations of human MARCO (300 ng), TLR2 (30 ng), CD14 (30 ng), and Nod2 (50 ng). The total amount of DNA was brought to 1 μg by transfecting empty pcDNA3.1 vector. Transfections were performed

using GeneJuice transfection reagent, as per the manufacturer's instructions (Novagen). At 5 h posttransfection, serum-free DMEM media was replaced with complete DMEM media. Twenty-four hours later, transfected cells were stimulated with lysozyme-digested P1121 (MOI 25) in 3 ml serum-free DMEM media. After 48 h, the lysates were collected using Reporter Lysis Buffer (Agilent) and were analyzed for luciferase (Agilent) and β-galactosidase (Clontech) activity using a luminometer (Turner Biosystems).

Cell association/internalization assay

BioGel-elicited macrophages (MΦs) or HEK293T cells were suspended in 1 ml HBSS at a concentration of 1×10^6 cells/ml. Live P1121 was added at an MOI of 10, and the solution was mixed on a nutating mixer at 37°C for 1 h. The cells were then separated from unbound bacteria by centrifuging at 1500 rpm for 5 min. To measure cell association, cells were washed once in HBSS and then lysed in H_2O. Serial dilutions were performed in H_2O and plated on sheep's blood agar supplemented with 10 μg/ml neomycin. Colonies were counted the next day. To measure uptake directly, extracellular bacteria were removed by adding 25 μg/ml gentamicin for 10 min at 37°C. Cells were then washed with HBSS and lysed in H_2O.

Statistics

Statistical analyses were carried out using the unpaired Student *t* test (GraphPad), except where indicated. Results were considered statistically significant if $p \leq 0.05$.

Results

Clearance of S. pneumoniae *colonization does not require opsonins*

Although Abs have been shown to be dispensable for pneumococcal clearance in the nasopharynx (7, 10), it is not clear whether other opsonins, such as complement, play a role in the clearance of pneumococcal colonization. To address this, mice lacking complement receptor 3 (also known as MAC-1) or complement component 3 (C3) were colonized intranasally with *S. pneumoniae* isolate P1121. The bacterial burden in the nasopharynx was measured in WT, MAC-$1^{-/-}$, and $C3^{-/-}$ mice at 21 d postinoculation (Fig. 1A). The clearance of *S. pneumoniae* was not significantly different among the three genotypes, demonstrating that complement opsonization is not necessary for the clearance of pneumococcal colonization.

MARCO and SRA were shown to be opsonin-independent phagocytic receptors important in innate-immune pneumococcal surveillance (17, 18). MR was also shown to bind to pneumococcal polysaccharides (27). To determine the importance of these receptors in the clearance of P1121 from the nasopharyngeal passage, we inoculated mice with 10^7 CFU intranasally and assessed bacterial numbers at various time points postinoculation. We compared pneumococcal CFU in the nasal lavages of WT, MARCO$^{-/-}$, SRA$^{-/-}$, and MR$^{-/-}$ mice. MARCO$^{-/-}$ mice, but not SRA$^{-/-}$ or MR$^{-/-}$ mice, were significantly impaired in clearing the bacteria beginning at day 14, with an even greater deficit seen at day 21 postinoculation, at which point clearance was completed in WT mice (Fig. 1B, 1C). The role of MARCO in the clearance of pneumococcal colonization was confirmed using TIGR4, an isolate that expresses a different capsular polysaccharide (Fig. 1D), thus demonstrating that the effect of MARCO is likely conserved across pneumococcal serotypes.

RNA transcripts of SIGNR1, an additional nonopsonic receptor that has been implicated in antipneumococcal immune responses (16), were undetectable in colonized nasal lavages (data not shown), effectively ruling it out as an important receptor in our model.

Expression of class A SRs in the nasopharynx

To determine the cell population expressing MARCO in the upper respiratory tract (URT), nasal lavages were analyzed for MARCO transcripts at 30 min, as well as days 3 and 7, when the effector monocytes/macrophages reach a maximum level in the URT. All primers used for mRNA transcript analysis can be found in Table I. CD45 transcript increased at days 3 and 7, correlating with the influx of effector cells (Fig. 2A). However, although MARCO transcript was detected in nasal lavages at all time points, the amount of transcript did not increase (Fig. 2A). This suggests that MARCO is present on resident cells in the nasopharynx but not the recruited effector cells. To further confirm this, we stained cytospin preparations of nasal lavages from colonized mice at the same time points. Although SRs CD68 and MR were both detected on recruited lumenal cells, MARCO was not detected on this population (Fig. 2B).

Cellular recruitment to the URT is hindered in MARCO$^{-/-}$ mice

It was shown that effector cells are recruited to the nasopharynx after pneumococcal colonization and are required for clearance (7). Thus, we examined whether MARCO$^{-/-}$ mice were deficient in

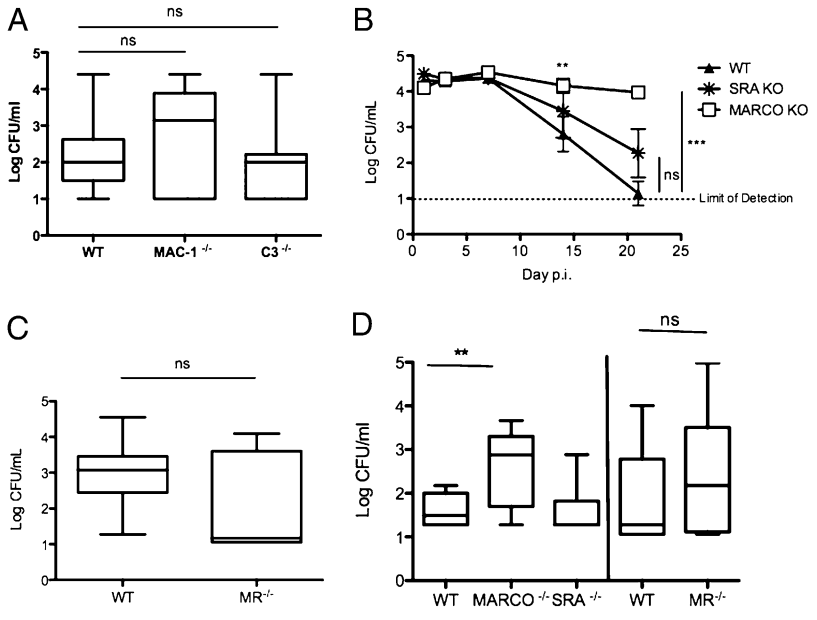

FIGURE 1. Clearance of pneumococcal colonization is complement independent and MARCO dependent. Mice were inoculated intranasally with *S. pneumoniae*, and colonization was assessed at the indicated time points. (**A**) WT, $C3^{-/-}$, and MAC-$1^{-/-}$ mice were colonized with P1121 for 21 d ($n \geq 10$/group), and bacterial burden in nasal lavages was determined. (**B**) Bacterial burden in nasal lavages was determined for WT, MARCO$^{-/-}$, and SRA$^{-/-}$ mice colonized with P1121 for 1, 3, 7, 14, and 21 d postinoculation (p.i.) ($n \geq 6$/group). Data are mean ± SEM. (**C**) WT and MR$^{-/-}$ mice were colonized with P1121 for 21 d ($n \geq 7$/group), and bacterial burden in nasal lavages was determined. (**D**) WT, MARCO$^{-/-}$, SRA$^{-/-}$, and MR$^{-/-}$ mice were colonized with TIGR4 for 21 d ($n \geq 7$/group), and bacterial burden was determined in nasal lavages. Box-and-whisker plots indicate high and low values, median, and interquartile ranges. $^{**}p \leq 0.005$, $^{***}p \leq 0.001$, WT versus MARCO$^{-/-}$ mice, by Mann–Whitney *U* test. ns, Not significant.

Table I. RT-PCR primer sequences

Gene	Sense Primer (5′–3′)	Antisense Primer (5′–3′)
marco	GGCACCAAGGGAGACAAA	TCCCTTCATGCCCATGTC
cd45	CAGAGCATTCCACGGGTATT	GGACCCTGCATCTCCATTTA
ccl2	GTCTGTGCTGACCCCAAGAAG	TGGTTCCGATCCAGGTTTTTA
il6	ATACCACTTCACAAGTCGGAGGC	CTCCAGAAGACCAGAGGAAATTTTC
tnfa	CAAAGGGAGAGTGGTCAGGT	ATTGCACCTCAGGGAAGAGT
il1b	GCCTCGTGCTGTCGGACCCATA	GATCCACACTCTCCAGCTGCAGG
ifnb	GCACTGGGTGGAATGAGACT	AGTGGAGAGCAGTTGAGGACA
gapdh	TGTGTCCGTCGTGGATCTGA	CCTGCTTCACCACCTTCTTGA

neutrophil, macrophage, and monocyte recruitment to the nasopharynx during colonization. We used a highly sensitive flow cytometry assay to quantitate the low numbers of cells recruited to the murine nasopharynx during pneumococcal colonization. Neutrophil ($Ly6G^+Ly6C^-$) counts remain high in the WT lavages until day 21, when the majority of bacteria have been cleared, whereas neutrophil levels remain low in $MARCO^{-/-}$ mice. Monocyte ($Ly6C^+Ly6G^-$) recruitment also remains at basal levels throughout colonization in $MARCO^{-/-}$ mice, whereas WT mice show robust levels of monocytes at day 14. Interestingly, there is an influx of recruited macrophages ($F4/80^+$) by day 14 postinoculation in WT mice, whereas this recruitment is delayed in $MARCO^{-/-}$ mice, possibly accounting for the lack of bacterial clearance in these mice at this time point (Fig. 3C). In summary, $MARCO^{-/-}$ mice have delayed recruitment of leukocytes in response to pneumococcal colonization.

MARCO enhances S. pneumoniae*–induced chemokine and cytokine responses*

During the course of pneumococcal colonization, macrophage-mediated detection of bacteria or bacterial components drives an inflammatory response that is required for clearance. It was shown that class A SRs are able to modulate cytokine production (20). To determine whether MARCO contributes to the production of inflammatory cytokines, we examined the expression of TNF-α, IL-1β, and IL-6 mRNA in the nasopharynx of WT and $MARCO^{-/-}$ mice during the course of colonization (Fig. 4A), as well as cytokine production resulting from stimulation of MΦs with *S. pneumoniae* ex vivo (Fig. 4B). $MARCO^{-/-}$ mice colonized with P1121 exhibited significantly delayed TNF-α, IL-6, and IL-1β mRNA transcription compared with WT mice throughout colonization, with the largest differences seen at day 7. Correspondingly, MΦs from $MARCO^{-/-}$ mice produced significantly less of all three cytokines compared with WT MΦs when stimulated with bacteria ex vivo. In fact, in most cases, MARCO-deficient macrophages did not produce any cytokines when stimulated with *S. pneumoniae* compared with stimulation with media alone.

The macrophage-chemotactic protein CCL2 (also known as MCP-1) was shown to be of primary importance for the recruitment of monocytes/macrophages to the nasopharynx during *S. pneumoniae* colonization (8). We investigated CCL2 expression at the RNA level in nasal lavages during colonization of WT and $MARCO^{-/-}$ mice (Fig. 4A). $MARCO^{-/-}$ mice colonized with P1121 had significantly delayed CCL2 transcription. At days 1 and 3, there were no significant differences in CCL2 transcription; however, by day 7 there was a significant reduction in CCL2 transcription in the $MARCO^{-/-}$ mice compared with WT mice. When MΦs from $MARCO^{-/-}$ mice were stimulated ex vivo with

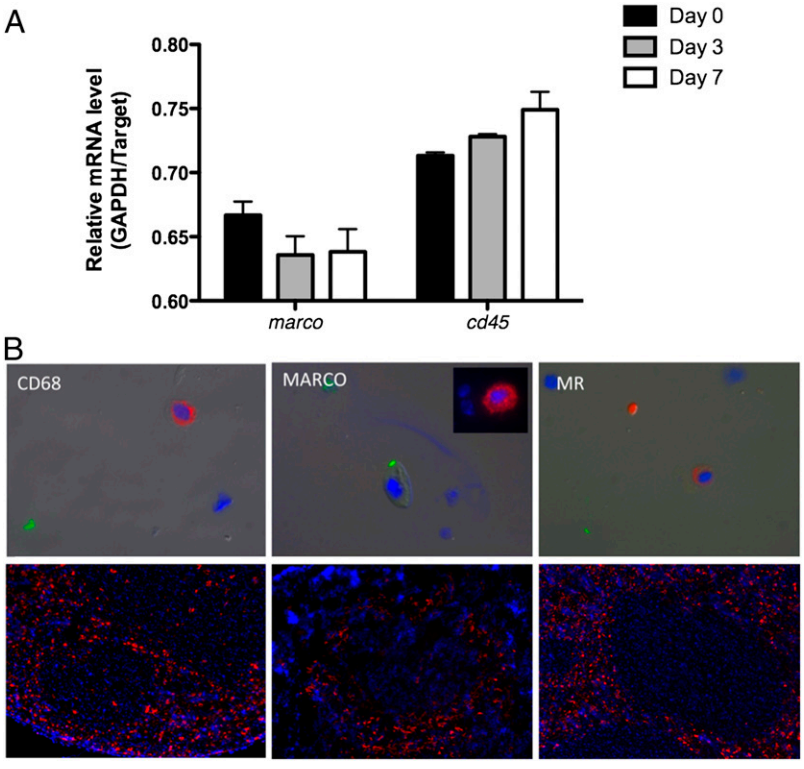

FIGURE 2. MARCO is not expressed on the effector cells recruited to the nasopharynx. (**A**) Quantitative RT-PCR on RNA lysis buffer nasal lavages at 30 min, 3 d, and 7 d postinoculation with P1121. Bars represent relative mRNA levels (GAPDH/Target) ($n \geq 3$). (**B**) Immunofluorescent staining of cytospin preparations from nasal lavages (*upper panels*). Representative images from day 3 (original magnification ×400). *Inset* in MARCO panel demonstrates positive staining in alveolar macrophages (original magnification ×400). Fresh-frozen spleen sections stained as positive controls for SR Abs (*lower panels*, original magnification ×100).

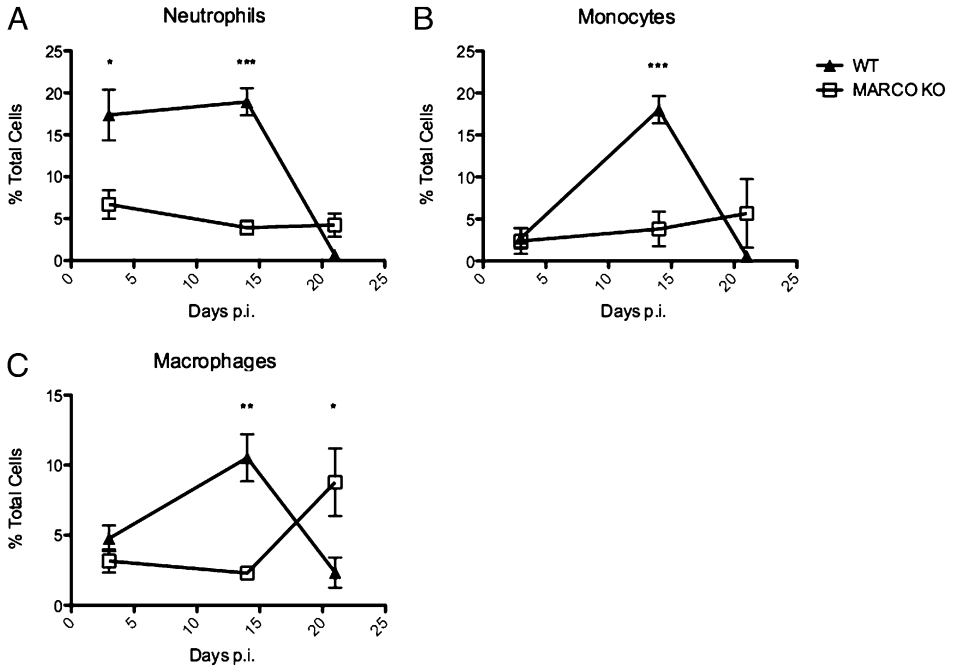

FIGURE 3. Recruitment of leukocytes to the nasopharynx is impaired in MARCO$^{-/-}$ mice. WT and MARCO$^{-/-}$ mice were colonized with *S. pneumoniae* for 3, 14, and 21 d postinoculation (p.i.). Nasal lavages were stained and analyzed for cell surface markers by flow cytometry. These markers were used to determine the number of neutrophils (Ly6G^{+}Ly6C^{-}) (**A**), monocytes (Ly6C^{+}Ly6G^{-}) (**B**), and macrophages (F4/80^{+}) (**C**) in each population ($n \geq 5$/group). Data are mean percentage of total cells ± SEM. *$p \leq 0.05$, **$p \leq 0.005$, ***$p \leq 0.001$.

S. pneumoniae, the level of CCL2 production did not exceed background levels; however, their WT counterparts produced a robust CCL2 response (Fig. 4B). This reduction in CCL2 production by macrophages was consistent with the diminished macrophage influx seen in MARCO$^{-/-}$ mice.

Together, these results demonstrate that MARCO-mediated recognition drives the inflammatory response during pneumococcal colonization.

MARCO modulates type I IFN production in macrophages

We recently showed that pneumococcal colonization of the nasopharynx leads to an increase in type I IFN production (9). To test whether MARCO was involved in type I IFN production, we first analyzed nasal lavages from WT and MARCO$^{-/-}$ mice for IFN-β mRNA content. MARCO$^{-/-}$ mice had significantly less IFN-β mRNA than did their WT counterparts at days 1, 3, and 7 postinoculation (Fig. 5A). We then stimulated MΦs with *S. pneumoniae* for 24 h, after which the cell supernatants were used in a standard bioassay to measure type I IFN production. Supernatants from WT and MARCO$^{-/-}$ MΦs stimulated with P1121 were transferred to L929 cells that were subsequently infected with a GFP-VSV. Cell supernatants from WT MΦs treated with *S. pneumoniae* protected L929 cells from GFP-VSV infection, indicating pneumococcal IFN stimulation, as measured by low levels of viral GFP fluorescence. Conversely, supernatants from MARCO$^{-/-}$ MΦs yielded less protection from viral infection, indicating that they produced significantly less type I IFN (Fig. 5B). Thus, MARCO has a role in the production of type I IFNs in response to pneumococcal stimulation.

Type I IFN signaling was shown to require uptake of the bacteria and subsequent intracellular signaling (28). To determine whether MARCO's role in type I IFN production was related to its capacity to bind and internalize the bacteria, we performed a bacterial cell–association assay with WT and MARCO$^{-/-}$ MΦs (Fig. 5C). Upon stimulation with live P1121 for 1 h, bacterial association was reduced by ~50% in MARCO-deficient MΦs

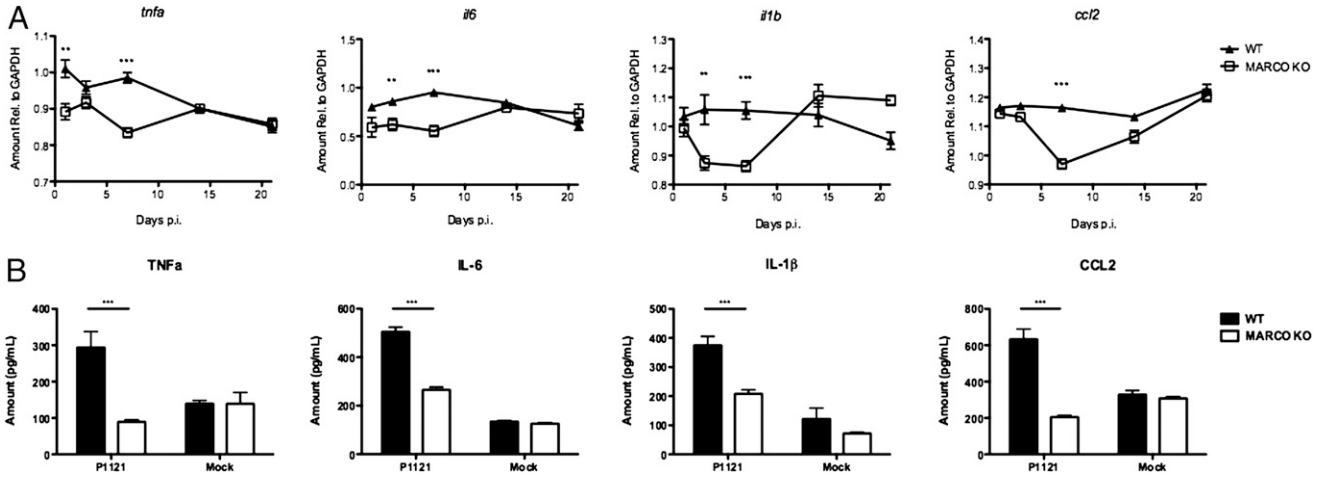

FIGURE 4. MARCO enhances the production of cytokines and chemokines. (**A**) WT and MARCO$^{-/-}$ mice were inoculated intranasally with *S. pneumoniae* and sacrificed at days 1, 3, 7, 14, and 21 postinoculation (p.i.). RNA was isolated by intratracheal nasal lavage. cDNA was analyzed by semiquantitative RT-PCR ($n \geq 3$/time point). (**B**) MΦs were isolated from WT and MARCO$^{-/-}$ mice and stimulated with lysozyme-digested *S. pneumoniae* preparations and controls ex vivo. Cytokine production was measured by ELISA ($n \geq 6$/group). Data are mean ± SEM. *$p \leq 0.05$, **$p \leq 0.005$, ***$p \leq 0.001$.

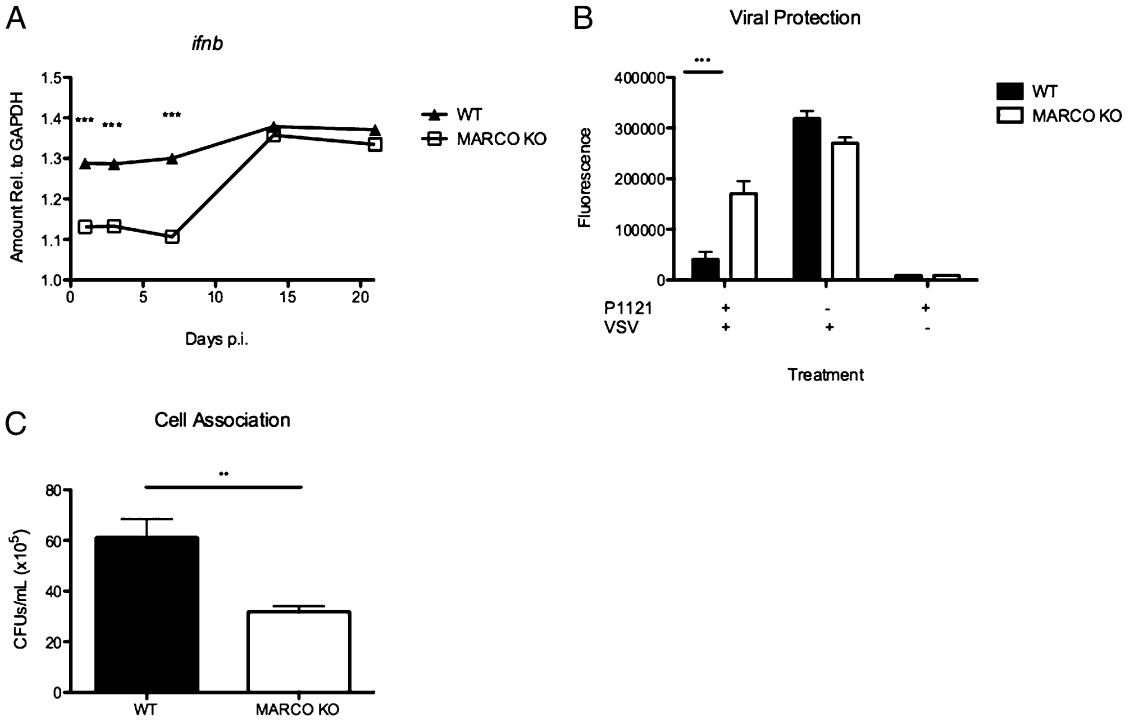

FIGURE 5. MARCO modulates the production of type I IFNs. (**A**) IFN-β mRNA from nasal lavages of colonized mice. RNA from WT and MARCO$^{-/-}$ mice was examined by RT-PCR. (**B**) MΦs from WT and MARCO$^{-/-}$ mice were isolated and stimulated with lysozyme-digested *S. pneumoniae*. Resulting supernatants were then added to L929 cells in culture. VSV-GFP was used to infect L929 cells, and cell infection was measured by GFP fluorescence. (**C**) Bacterial association with MΦs from WT and MARCO$^{-/-}$ mice was measured by cellular lysis after 1 h of stimulation. GFP fluorescence data, indicating viral infection, are shown as mean fluorescence units across replicates ($n = 3$) ± SEM. $^{**}p \leq 0.005$, $^{***}p \leq 0.001$.

compared with WT MΦs, providing us with a possible mechanism for MARCO's role in type I IFN production.

MARCO contributes to TLR- and NLR-signaling pathways

Although MARCO itself has no known signaling capacity, it was shown to enhance TLR signaling in response to certain bacteria or their components (29). Therefore, we hypothesized that MARCO may be required for bacterial recognition by pattern recognition receptors, such as TLR2 and Nod2, to enhance *S. pneumoniae*–specific responses. We used an NF-κB luciferase assay to discern MARCO's role in the signaling capacities of these receptors. Cells transfected with MARCO and Nod2 demonstrated significantly more NF-κB activation upon stimulation with P1121 than did cells transfected with Nod2 alone (Fig. 6A). The same was true for cells transfected with MARCO, TLR2, and CD14 versus those transfected with just TLR2 and CD14 (Fig. 6B). Interestingly, cells transfected with TLR2, CD14, and SRA showed a decrease in NF-κB activation compared with cells transfected with TLR2 and CD14 alone. Cells transfected with MARCO or CD14 alone did not show any NF-κB activation, nor did MARCO enhance NF-κB activation upon stimulation with the TLR2 ligand Pam3CSK4 or the Nod2 ligand MDP (data not shown). In addition, when this assay was performed using plasmids expressing TLR4 or TLR9, MARCO had no effect on NF-κB activation (data not shown).

To determine whether the forced expression of MARCO by the HEK293T cells led to an increased ability of the cells to bind and internalize the bacteria, we performed a bacterial cell–association assay with HEK293T cells transfected with a MARCO-expressing plasmid or an empty vector. When these cells were incubated with live P1121 for 1 h, there was no significant difference in total cell association (i.e., binding and uptake) of the bacteria (Fig. 6C). However, when all extracellular bacteria were eliminated by the addition of gentamicin, the MARCO-expressing cells were better able to internalize the bacteria (Fig. 6D), although the total bacterial numbers were quite low for both sets of cells. Together, these results demonstrate that MARCO enhances some, but not all, TLR and NLR responses to pneumococci, which may be due to its phagocytic capabilities.

Discussion

Because nasopharyngeal colonization precedes pneumococcal disease, it is an attractive therapeutic target; thus, it is important to understand host defense at this site, which requires interactions between the effector cells and the bacteria. It was reported that macrophages recruited to the nasal mucosa are important effector cells in the clearance of colonization and that they are able to recognize the bacteria without the aid of opsonizing Abs (10). In this study, we showed that clearance of pneumococcal colonization does not require complement C3 or its cognate receptor, supporting the idea that macrophages are able to act without the aid of opsonins (Fig. 1A). Although this had been shown in the lung, we are now able to extend the role of nonopsonic receptors in pneumococcal clearance from the nasopharynx.

MARCO, MR, and SRA were shown to be important for clearance of pneumococci in the lungs and the CNS (15, 17, 18, 27). This led us to investigate their role in clearance from the nasopharynx. Interestingly, the SRs appear to play distinct roles in upper versus lower respiratory tract clearance. In the lungs, MARCO and SRA play redundant roles in the recognition, uptake, and subsequent clearance of pneumococci. We can only hypothesize about the importance of this redundancy, although it is likely rooted in the need to overcome the abundance of virulence factors expressed by the pneumococcus in the environment of the lung. Both MARCO and SRA, which are constitutively expressed on alveolar macrophages, were shown to directly recognize the bacteria and trigger their engulfment by these cells (17, 23). MR

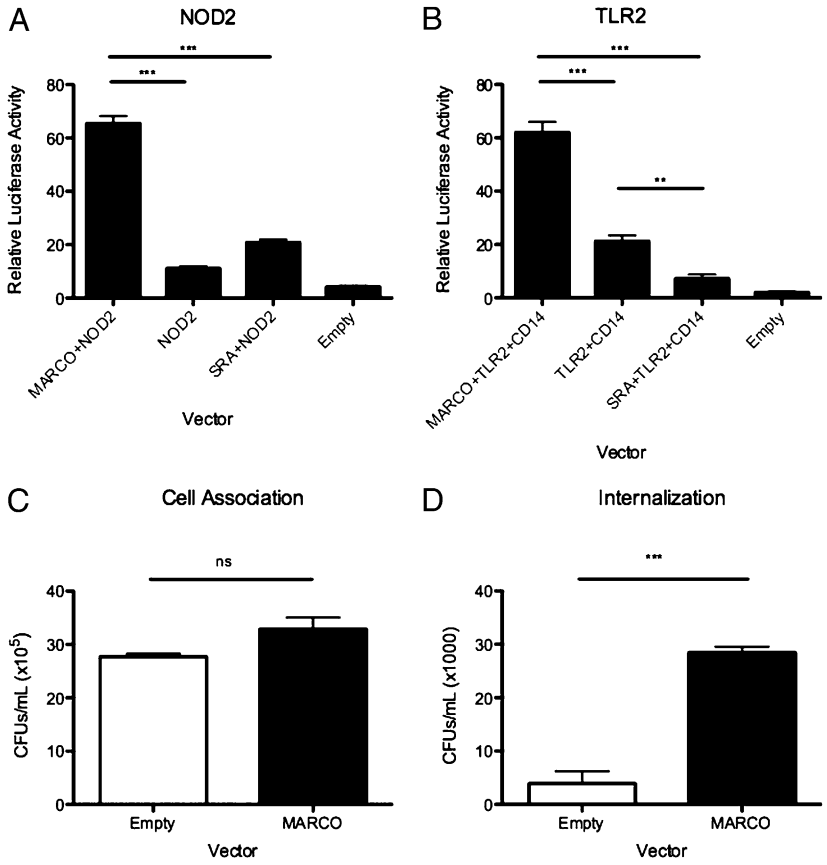

FIGURE 6. MARCO affects Nod2 and TLR2 responses to the pneumococcus. HEK293T cells were cotransfected with various combinations of plasmids expressing Nod2, SRA, and MARCO or empty vector (**A**) or TLR2, CD14, SRA, and MARCO or empty vector (**B**). 24 h posttransfection, cells were infected with lysozyme-digested *S. pneumoniae* at an MOI of 25. Twenty-four hours postinfection, luciferase activity was measured ($n \geq 6$/group). This assay was normalized for transfection efficiency by dividing the luciferase activity by the β-galactosidase activity. Average of three independent experiments ± SEM. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, one-way ANOVA with the Bonferroni posttest. Bacterial association with HEK293T cells transfected with MARCO plasmid or empty vector was measured by cell lysis before (**C**) or after (**D**) the addition of gentamicin to kill extracellular bacteria. $^{***}p \leq 0.001$. ns, Not significant.

was also shown to directly recognize pneumococci (27). Conversely, despite MARCO, MR, and SRA (data not shown) being expressed in the nasopharynx, only MARCO enhances clearance of pneumococcal colonization from the nasopharynx (Fig. 1). Also, although MARCO is required for efficient clearance of nasal colonization, we did not find it on the recruited effector cells in WT mice. This leads us to believe that MARCO's role may be linked to resident cells in the nasopharyngeal mucosa, which function in immune surveillance (Fig. 2). These cells likely act as indirect mediators of the immune response to *S. pneumoniae* by contributing to the complex cytokine and chemokine milieu at the site of colonization. The receptor(s) on the effector cells responsible for recognizing the pneumococcus remains unknown. These differences in the role of SRs could explain why pneumococci are able to asymptomatically colonize the nasopharynx but induce a violent inflammatory state once they gain access to the lungs.

The chemokine CCL2 was shown to be vital to the recruitment of monocytes/macrophages to the nasopharynx during pneumococcal colonization in a TLR2- and Nod2-dependent manner (8). We showed a significant deficiency in the transcription of CCL2 mRNA in the nasopharynx of MARCO$^{-/-}$ mice at early time points in colonization compared with WT mice (Fig. 4A). We also showed that CCL2 production by macrophages from MARCO$^{-/-}$ mice in response to pneumococcal stimulation is severely impaired (Fig. 4B). This defect in CCL2 production is likely responsible for the impaired recruitment of myeloid cells, and especially macrophages, to the nasopharynx throughout colonization (Fig. 3). The increased recruitment of macrophages to the nasopharynx in WT mice correlated with decreased bacterial load, which began between days 7 and 14 postinoculation (Fig. 1B). Neutrophil recruitment was also impaired in MARCO$^{-/-}$ mice; however, prior studies showed that, although these cells are robustly recruited early during colonization, they are not sufficient to clear the pneumococcus (7). It is important to note that the macrophages recruited to the nasopharynx were CD11c^{-}MHCII^{-} and CD11b^{hi} (data not shown) and, therefore, were more similar to recently recruited monocytes in the process of differentiation to macrophages than they were to alveolar macrophages.

A consequence of pneumococcal activation of TLR2 and Nod2 is a robust proinflammatory response. In accordance with this, we showed that MARCO is important in the pathway leading to Nod2- and TLR2-dependent NF-κB activation, which leads to the production of proinflammatory cytokines and chemokines. Nasal lavages from MARCO$^{-/-}$ mice had significantly lower levels of TNF-α, IL-1β, and IL-6 mRNA at day 7 postinoculation compared with WT mice (Fig. 4A). This time point coincides with the beginning of bacterial clearance in WT mice (Fig. 1B). Also, MΦs from MARCO$^{-/-}$ mice were unable to produce these proteins following stimulation with pneumococci (Fig. 4B). This is consistent with other studies demonstrating MARCO's contribution to the proinflammatory response by macrophages when stimulated with other pathogenic organisms (18, 23, 29, 30). Although it is unclear whether this MARCO-dependent production of proinflammatory cytokines is a TLR2- or a Nod2-specific phenomenon, we have provided evidence that MARCO enhances both TLR2- and Nod2-mediated NF-κB activation. Both of these receptors were shown to be important in pneumococcal clearance in previous studies (8, 31). We hypothesize that either TLR2 or Nod2, or both, is the primary signaling receptor involved in the production of TNF-α, IL-1β, and IL-6 and that MARCO is responsible for enhancing this response during colonization. Interestingly, we showed that MARCO is also involved in the production of type I IFNs by MΦs. Two studies showed a protective effect for IFN-β in pneumococcal disease (32, 33), whereas another showed the opposite in the setting of concurrent influenza A infection (9). Thus, the production of type I IFNs during pneumococcal colonization

has profound effects on the disease outcome and is linked to MARCO-mediated signaling.

The mechanism by which MARCO enhances TLR2 and Nod2 signaling has yet to be elucidated. MARCO appears to be essential for, and possibly upstream of, Nod2 and TLR2 signaling in response to the pneumococcus but not to monomeric ligands, such as MDP and Pam_3CSK_4 (data not shown). Likewise, many studies showed that Nod2- and some TLR2-signaling pathways require internalization of ligands (34, 35). Our data showing an increased capacity of MARCO-expressing cells to internalize the bacteria (Figs. 5C, 6D) provide evidence that MARCO can increase signaling by intracellular receptors, such as Nod2, by facilitating the transition of the bacteria into the cell. This is especially evident in the NF-κB luciferase assays in which HEK293T cells not expressing MARCO were almost completely unable to internalize live pneumococci. However, because overall binding of the bacteria to HEK293T cells is not significantly affected by the expression of MARCO, we do not believe that MARCO's role in TLR2 signaling is confined to its binding and uptake capacities. Also consistent with the hypothesis that MARCO lies upstream of TLR2 and Nod2, the degree to which the bacteria were able to persist in $MARCO^{-/-}$ mice was greater than shown in previous studies on $Nod2^{-/-}$ (8) and $TLR2^{-/-}$ (10) mice. Previous studies, as well as our work, showed an inhibitory effect of SRA on TLR2 signaling (Fig. 6) (18, 23, 29, 30). A recent report presented a mechanism for TLR4 inhibition by SRA, which involves direct contact between SRA and the signaling machinery of TLR4 (21). It is possible that a similar mechanism occurs with MARCO, where it can directly bind the TLR2-signaling machinery but, in this case, to enhance signaling.

The production of type I IFNs in response to extracellular bacteria is also reliant on the ability of the cell to internalize bacterial ligands via endocytosis (28). Therefore, it is probable that MARCO's role in type I IFN production is linked to its ability to internalize the bacteria, as shown in Fig. 5C. This does not preclude roles for other molecules formerly established in other laboratories. For example, it is possible that the uptake of live bacteria into a cell could lead to pneumolysin-dependent rupture of endosomes, leading to bacterial ligands reaching the cytoplasm to be sensed by cytosolic or transmembrane receptors (as proposed in Refs. 32, 36).

In summary, we showed that MARCO is vital to the clearance of *S. pneumoniae* colonization from the murine nasopharynx. This is due to its role in nonopsonic recognition of the bacteria, which leads to increased Nod2- and TLR2-dependent chemokine and cytokine production and, ultimately, the recruitment of effector monocytes/macrophages. To the best of our knowledge, this is the first demonstration of MARCO-mediated collaboration with pattern recognition receptor signaling contributing to the clearance of the pneumococcus. Our hope is that targeting Ags to the MARCO-mediated response will provide us with novel, serotype-independent vaccination strategies against the pneumococcus.

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Disclosures

The authors have no financial conflicts of interest.

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